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Protective Effects of *L*-Carnitine on Myocardium of Experimentally Diabetic Rats with Additional Ischaemia and Reperfusion

Rick Schneider[1], **Heinz Löster**[2], **Wolfram Aust**[2], **Steffen Craatz**[1], **Klaus Welt**[1], and **Günther Fitzl**[1,*]

[1] Institute of Anatomy, Department of Medicine, University of Leipzig, D-04103 Leipzig, Germany
[2] Institute of Laboratory Medicine, Clinical Chemistry and Molecular Diagnostics, Department of Medicine, University of Leipzig, D-04103 Leipzig, Germany

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Summary. We investigated the protective effects of *L*-carnitine against damage to the heart caused by diabetes-induced alterations and additional ischaemia and reperfusion in diabetic BB/OK rats using histological techniques, morphometry, biochemical parameters of oxidative stress, and SOD expression. The results revealed that diabetes-induced morphological changes were partly improved or nearly prevented by substitution of *L*-carnitine, which also seemed to improve the reduced tolerance of diabetic myocardium towards ischaemia/reperfusion with respect to morphological parameters. Immunohistochemical and biochemical parameters of oxidative stress such as SOD protein expression as well as SOD and GPx activity indicate increased free oxygen radical level in the ischaemic/reperfused diabetic myocardium, which is clearly decreased by *L*-carnitine treatment. We suggest that *L*-carnitine may be an adequate "causal" agent in the protection of myocardial alterations in diabetes with additional ischaemia and reperfusion, as it stabilizes mitochondrial and cellular function and acts through its antioxidative or radical scavenging potential. Further investigations are necessary to determine an approach towards adjuvant treatment of diabetic myocardial complications using *L*-carnitine.

Keywords. *L*-Carnitine; Myocardium; Diabetes; Ischaemia; Reperfusion.

Introduction

Diabetes mellitus is known to result in cardiac abnormalities in both clinical and experimental settings. The increased incidence and mortality of ischaemic events (myocardial infarction) has been partly explained by the presence of diabetic

* Corresponding author. E-mail: fitzlg@medizin.uni-leipzig.de

cardiomyopathy. Diabetic cardiomyopathy results in functional disorders associated with structural alterations such as myocardial hypertrophy, fibrosis, and lipid deposits. Permanently increased oxidative stress, resulting from carbohydrate and lipid metabolism impairment, plays a key role in the development of specific heart muscle disease. In diabetes, glucose utilization is insignificant, and energy production is shifted almost exclusively toward β-oxidation of free fatty acids [1]. The importance of carnitine as the requisite carrier of long-chain fatty acids across the mitochondrial membrane in the pathogenesis of diabetes associated complications in type I diabetes has been demonstrated evidence, that myocardial carnitine deficiency is associated with cardiomyopathy and biventricular hypertrophy [2–4].

Although the longevity of diabetic patients was enhanced by adequate insulin therapy, late complications of chronic diabetics cannot be completely avoided. Consequently, attempts have been made to find additive therapeutic possibilities. Studies in the literature have demonstrated that *L*-carnitine and its derivatives, namely propionylcarnitine, protect cardiac metabolism and function in diabetes mellitus and additional ischaemic heart disease by scavenging for intracellular superoxide radicals, stabilizing the mitochondrial metabolism and *ATP* production, and reducing DNA damage [5–10]. In contrast to functional and biochemical studies, only little is known about the influence of *L*-carnitine on diabetes-induced morphological alterations [11, 12].

The aim of this study was to demonstrate the beneficial effects of *L*-carnitine on the myocardium in spontaneously diabetic rats and diabetic rats additionally exposed to ischaemia with reperfusion as a model of ischaemic emergency in diabetics using morphological, immunohistochemical, and biochemical techniques.

Results and Discussion

The Protective Effects of L-Carnitine against Diabetes

Histological and Morphometric Findings

After 6 months of diabetes, our spontaneously diabetic rats had developed severe alterations in the myocardial structure of both ventricles with respect to the primary components of myocardial architecture. Diabetes caused pathological alterations of myocardium – areas of sporadic necrosis and severely damaged heart muscle cells involving myocytolysis as well as areas with compensative hypertrophic myocytes and evident extension of interstitial connective tissue with pronounced fibrosis (elevation of fibroblast nuclei), along with diminishing and irregular profiles as well as arrangement of microvessels (Figs. 1 and 2).

Staining for toluidine blue revealed considerable mast-cell accumulation in diabetic myocardium observed mainly around blood vessels and within fibrotic areas. Staining with Sudan black also revealed an accumulation of intracellular lipid granules in diabetic cardiomyocytes, but no evidence of extracellular lipid storage (Fig. 3).

L-Carnitine-protected diabetic myocardium showed less extensive effects with respect to necrotic areas, enlargements in the interstitium, lysis and hypertrophy of

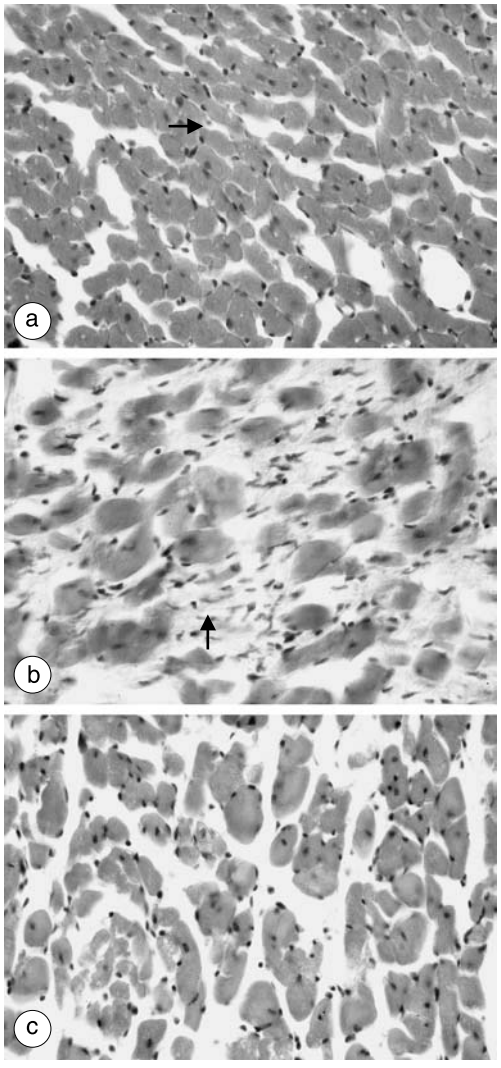

Fig. 1. Haematoxylin-eosin-stained sections of rat myocardium (×375); a: control, normal appearance of heart muscle (→); b: diabetic rat, myocytolysis and lightening of cytoplasm (↑); c: *L*-carnitine-protected diabetic rat, smaller myocytolysis and lightening of cytoplasm than in b

cardiomyocytes, rarefication of microvasculature, accumulation of mast cells, and elevation of intracellular lipids (Figs. 1–3).

Measurements of cardiomyocyte diameter confirmed the qualitative findings (Table 1).

The diabetes-induced increase in myocyte diameter (Dmyo) was significantly less pronounced in *L*-carnitine-treated animals. The number of myocyte cross-sections (NAmyo) and capillaries (NAcap), severely decreased in the unprotected myocardium, was less decreased in the protected animals. Diabetes-induced dilatation of the capillary diameter (Dcap) as well as the increased intercapillary distance (ICD) – the distance between capillary and mitochondria of myocyte – was somewhat less expressed in the *L*-carnitine group. The number of mast cells (NAmast),

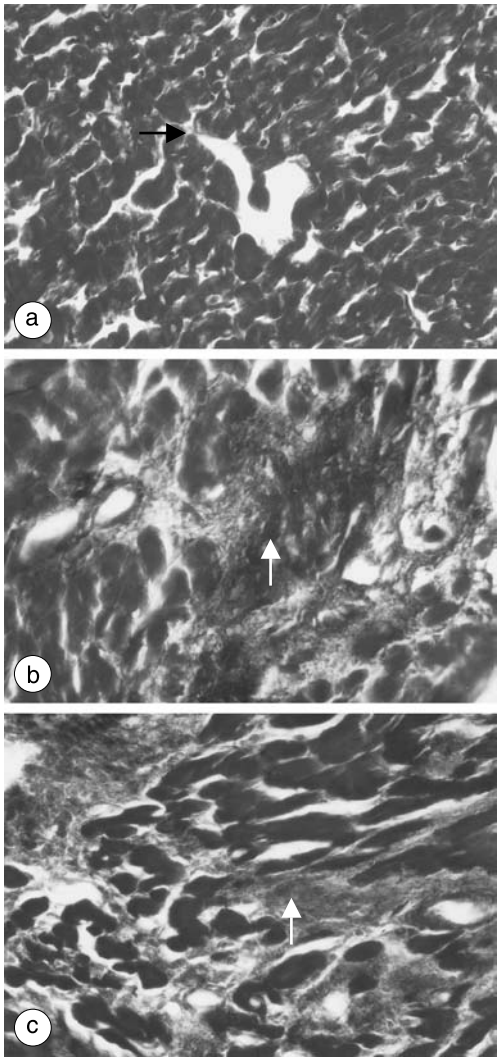

Fig. 2. Azan-stained sections of rat myocardium (×375); a: control, normal appearance of heart muscle cells and connective tissue (→); b: diabetic rat, massively increased amount of perivascular and interstitial connective tissue (collagen deposits) (↑); c: *L*-carnitine-protected diabetic rat, smaller deposits of connective tissue than in b (↑)

however, was significantly less increased in the protected than in the unprotected group.

Morphometric analysis revealed no significant differences between groups with or without ischaemia/reperfusion at light-microscopic level.

Immunohistochemical Findings

The interstitial collagen fibre type distribution showed a diabetes-induced, massively amplified expression of fibril-forming collagen types I and III (predominantly perivascular) and microfibrillar collagen VI in both ventricles of the untreated diabetic heart. The treated diabetic group only showed a moderate

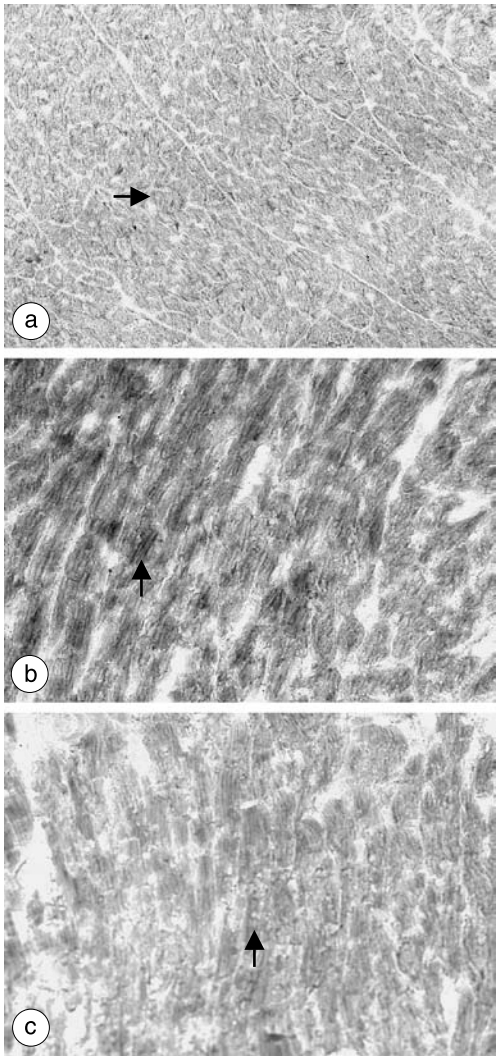

Fig. 3. Sudan black-stained sections of rat myocardium (×375); a: control, discreet spots of intracellular lipid drops (→); b: diabetic rat, accumulation of intracellular lipid drops (↑); c: *L*-carnitine-protected diabetic rat, less accumulation of lipid drops than in b (↑)

increase in certain fibre types at different locations. The semiquantitatively evaluated high-grade fibrosis in the diabetic rats seemed to be lowered by the effect of *L*-carnitine (Fig. 4).

Electron Microscopic Findings

Electron-microscopic inspection also confirmed the protective effects of *L*-carnitine against diabetic cardiomyopathy with altered cardiomyocytes and areas of degeneration and misalignment at ultrastructural level (Fig. 5). In the protected diabetic group, the ultrastructure of most organelles seemed to be somewhat better than in the unprotected diabetic group. Irregularities in sarcomeres with loss of

Table 1. Morphometrical parameters of myocardium in control, *L*-carnitine-protected, and unprotected diabetic rats (mean ± SD) ($^{*} = p \leq 0.5$, $^{**} = p \leq 0.01$)

	Control	Diabetes	Diabetes + *L*-carnitine
		**	*
Mean diameter of cardiomyocytes (Dmyo) (μm)	10.4 ± 0.6	12.6 ± 1.0	11.0 ± 0.8
		**	
Number of myocyte cross sections (NAmyo) (mm^{-2})	4826 ± 129	3787 ± 161	3928 ± 131
		**	
Mean diameter of capillaries (Dcap) (μm)	4.8 ± 0.3	6.1 ± 0.4	5.9 ± 0.3
		**	*
Number of capillaries (NAcap) (mm^{-2})	3587 ± 93	2496 ± 93	2656 ± 137
		**	
Intercapillary distance (ICD) (μm)	13.1 ± 0.3	15.3 ± 0.9	15.2 ± 0.4
		**	**
Number of mast cells (NAmast) (mm^{-2})	23.6 ± 5.5	49.8 ± 8.7	32.1 ± 6.7

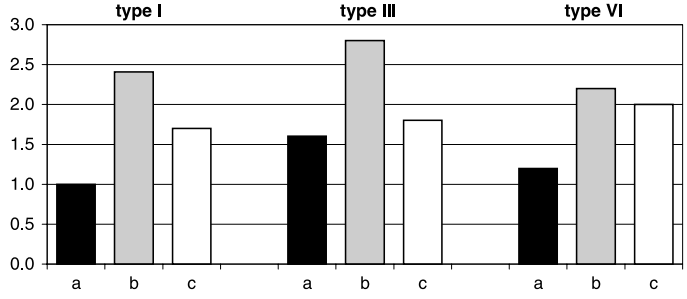

Fig. 4. Semi-quantitative determined strength and volume in collagen expression of types I, III, and VI (score 1–3); a: control; b: diabetes; c: diabetes + *L*-carnitine

texture, contraction ribbons, dilated elements of sarcoplasmic reticulum (SR), and swollen mitochondria with partial loss of structural integrity appeared to be decreased in cardiomyocytes from protected rats. Accumulation of glycogen granules, partly sequestered by membranes and lipid droplets, partly related to mitochondria, was somewhat less increased. The frequency of diabetes-induced lipofuscin granules appeared to be lower in the protected animals.

The protective effects of *L*-carnitine were also visible in microangiopathic and fibrotic changes of diabetic myocardium. The structural integrity of myocardial microvessels (true capillaries, postcapillary venules) seemed to be somewhat better in the protected than in the unprotected diabetic group. In many capillaries, the diabetes-induced thickening of basement membrane and the increased deposition of small fibre bundles in the interstitium and around vessels appeared to be less pronounced in the *L*-carnitine-protected animals.

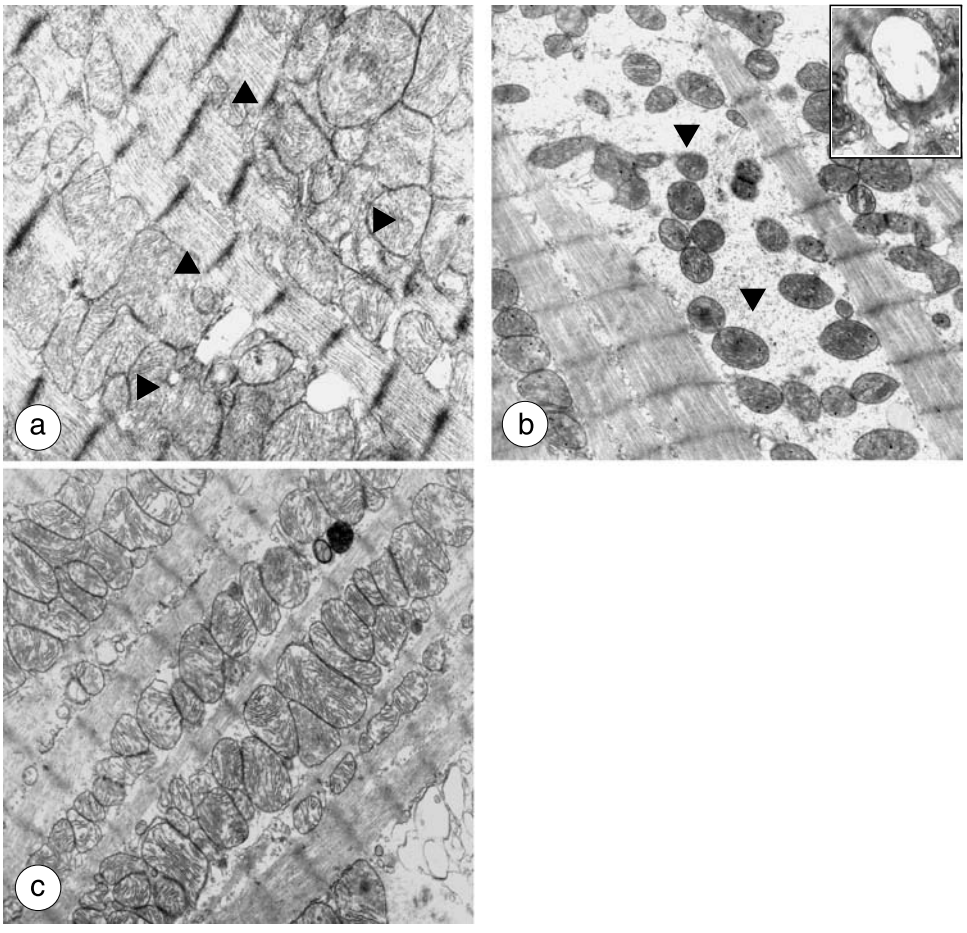

Fig. 5. Electronmicroscopic images of rat cardiomyocytes (×40000); a: healthy control animal with regular intracellular structure (sarcomeres (▲), mitochondria (▶)); b: diabetic BB rat, structural alterations (loss and irregularities of sarcomeres (▼) and mitochondria, glycogen distributed between the myofibrils), inset: amplified structural loss of mitochondria under additional ischaemia/reperfusion; c: *L*-carnitine-protected diabetic rat after ischaemia/reperfusion, the structure appears better preserved than in b

Discussion

The importance of carnitine has been demonstrated in clinical and experimental studies showing myocardial carnitine deficiency associated with impairment of mitochondrial fatty acid oxidation. This may be a metabolic mechanism in the pathogenesis of diabetic cardiomyopathy [3] and biventricular hypertrophy [8], and can be prevented to an extent by *L*-carnitine administration [13]. *L*-Carnitine deficiency is partly caused by reduced gastrointestinal absorption in chronic diabetes and renal excretion of carnitine due to polyuria; also, esterified carnitine cannot be reabsorbed [4, 14]. Therefore, *L*-carnitine substitution appears useful.

In agreement with literature [15], our spontaneously diabetic rats with 6 months of insulin-dependent diabetes developed specific cardiomyopathy with architectural alterations of myocardial geometry regarding the primary components of

the cardiac tissue – cardiomyocytes, interstitial and microvascular elements that underwent reversible compensatory processes and developed chronic degenerative biventricular alterations at structural and ultrastructural level.

We observed focal necrosis of cardiomyocytes as well as extensive myocytolysis; this was confirmed by the diminished number of cross-sections. In contrast, the increased mean diameter of the cardiomyocytes may be understood as simultaneous compensative hypertrophy in agreement with the literature [16].

Treatment with *L*-carnitine reduced some of the histopathological abnormalities in diabetic cardiomyocytes mentioned above as observed at light-microscopic level. Stabilization of mitochondrial function by *L*-carnitine maintains impaired regulating mechanisms in the metabolic, ionic, and osmotic milieu, and may counteract cell swelling und lysis of myocytes. Additionally, an influence by expression of cytokines, cytoskeleton-coding proteins, and proto-oncogenes, as well as vasoactive peptides may be discussed [17, 18].

At ultrastructural level, we observed degenerative alterations of cardiomyocytes with loss of cellular integrity in the untreated diabetic group. Besides swelling and disruption of mitochondria as well as dilatation of sarcoplasmatic reticulum (SR) elements, important changes were represented by a loss of contractile material, local oedema, and an increase in lipid droplets in agreement with other authors [19–21].

In accordance with the literature [22], we observed a considerable diabetes-induced increase in lipid droplets, which was clearly prevented by *L*-carnitine. This may be explained by direct interaction with the lipid metabolism. Lipid accumulation is caused by the disturbance in the glucose and fatty acid metabolism in diabetes, leading to high levels of free fatty acids. Toxic metabolites generated in these processes (acylcarnitine, long-chain acyl-CoA) and free oxygen radicals disturb subcellular membrane structures while altering membrane fluidity and molecular dynamics through their detergent-like effects, and also through impairing the Ca^{++}-K^{+}-*ATP*ase of the sarcolemma by lipid peroxidation and membrane phospholipids breakdown, which is prevented by propionyl *L*-carnitine. Hyperlipidemia leads to an altered fatty acid profile in membranes, which decreases glucose transporter activity. The elevated free fatty acids lead to increased citrate level, thus inhibiting phosphofructokinase [23], which explains the glycogen accumulation observed. *L*-Carnitine substitution improves the long-chain fatty acid metabolism by mediating its transport from cytosol into mitochondria while diminishing the inhibition of β-oxidation of fatty acids and energy production due to carnitine depletion, thus inhibiting toxic accumulations of long-chain fatty acids, fatty acid esters, and the resulting formation of free radicals. This may further counteract the disturbance in membrane lipid composition and function, involving alterations in protein kinase C (PKC), Ca^{++}-*ATP*ase of SR, Na^{+}/K^{+}-*ATP*ase, Na^{+}/Ca^{++}-exchange, and Ca^{++} pump activity in myocardial sarcolemma [24, 13, 25]. Derivatives of *L*-carnitine are known to diminish the intracellular Ca^{++} accumulation [26].

The diabetes-induced increase in lipofuscin granules may be a result of enhanced free radical generation, lipid peroxidation, and accumulation of these metabolic end-products in diabetes. We suggest a protection of cells from oxidative stress by at least two different mechanisms known for propionyl-*L*-carnitine:

1. Prevention of reactive oxygen species formation and therefore decrease in membrane and protein damage; 2. Increase in mitochondrial metabolic performance, enabling maintenance of higher adenine nucleotide pool and energy charge [9, 27].

The accumulation of the extracellular matrix (EZM) reflects the architectural changes in myocardial geometry as a part of the remodelling process in diabetes due to an apparently higher proportion of ground substance, proliferation of components of EZM, and/or simply oedema. The interstitial fibrosis would be responsible for delayed myocardial relaxation and wall stiffness [28].

The formation of diabetic end-products disturbs the balance between collagen formation and degradation leading to impaired collagen accumulation [29]. The severely disproportional biventricular increase in collagen fibre accumulation in our untreated diabetic rats could partly be prevented by *L*-carnitine, resulting in diminished perivascular, interstitial, and replacement fibrosis with lower fragmentation or degeneration of myocytes. A possible explanation for this *L*-carnitine effect may be the diminished activation of protein kinase C as one of the key steps in fibrosis formation.

One of the most striking changes observed in our study was the mast cell accumulation in the untreated myocardium observed mainly around blood vessels and within fibrotic areas. Although our knowledge is still fragmentary, perivascular mast cells appear to be involved in the diabetic heart disease and its remodelling processes in myocytes, interstitium, and microvessels [30]. Inflammatory effective cytokines and also direct non-immunologic stimuli such as Substance P are known to be able to trigger migration as well as activation and degranulation in mast cells [31]. The histamine liberated may act as an important vasoactive mediator and stimulus for collagen synthesis and activation of inflammatory cells. *L*-Carnitine-protected myocardium, however, showed a clearly diminished accumulation of mast cells that could lead to a reduction in the effects of histamine mentioned above.

The dilatation of capillaries in our untreated diabetic group as well as the intercapillary distance was slightly less reduced, and the number of capillaries was significantly less reduced by *L*-carnitine substitution. A possible explanation for this may be the induction by pharmacological agents [15] such as histamine, adenosine, and lactate, as well as passive dilation of microvessels. *Irat et al.* [32] suggested that the beneficial effects of *L*-carnitine treatment may partially improve vascular reactivity beyond its reduction of plasma lipids.

The Protective Effects of L-Carnitine against Ischaemia and Reperfusion Injury in the Diabetic Myocardium

Morphological Findings

Exposure of diabetic myocardium to additional ischaemia and subsequent reperfusion led to additional typical alterations in the ultrastructure of cardiomyocytes. Mitochondria showed progressive swelling with moderate clearing in the matrix, irregularities in structure, and disarrangement of cristae in the unprotected group that seemed to be less frequent in the protected animals (Fig. 5). *L*-Carnitine

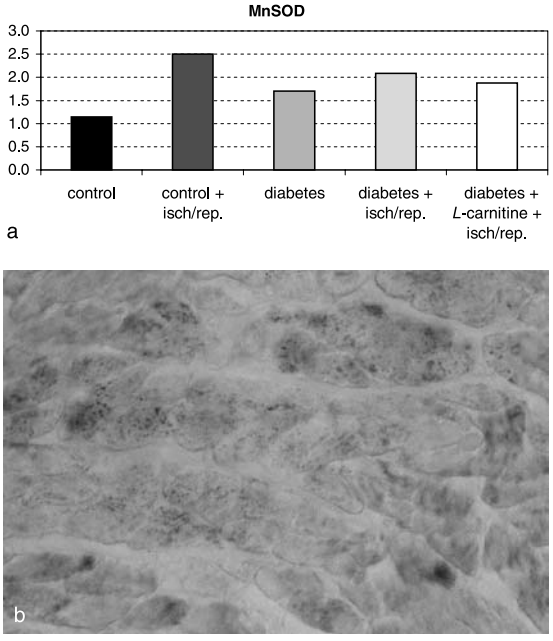

Fig. 6. Immunohistochemical demonstration of MnSOD protein expression in the myocardium of the experimental groups; a: semi-quantitative determined strength and volume of the immune reaction (score 1–3) (isch/rep.: ischaemia/reperfusion); b: immunostaining for MnSOD in myocardium of *L*-carnitine-protected diabetic rat under ischaemia and reperfusion (×375)

reduced the frequency of capillary endothelial oedema. Occasionally, the protected group's microvessels showed sparse formation of luminal blebs and protrusions. The alterations in mitochondrial integrity, dilatations in the SR, increase in amorphic substance, pericapillary debris, and cytoplasm vacuoles were generally less expressed by L-carnitine substitution, resulting in a weaker manifestation of this damage.

Immunohistochemical Findings

While additional ischaemia with reperfusion led to a moderate increase in MnSOD expression in the untreated diabetic heart, this mitochondrial enzyme's expression was clearly decreased in *L*-carnitine protected myocardium (Fig. 6). There was, however, only a slight decrease in CuZnSOD expression in the protected diabetic group after exposure to ischaemia/reperfusion injury compared to a slight diminishing of this cytoplasmatic enzyme in the unprotected ischaemic and reperfused diabetic heart.

Biochemical Results

After exposure to ischaemia and reperfusion, the slightly increased superoxide dismutase (SOD) activity in untreated diabetic hearts only showed a slightly

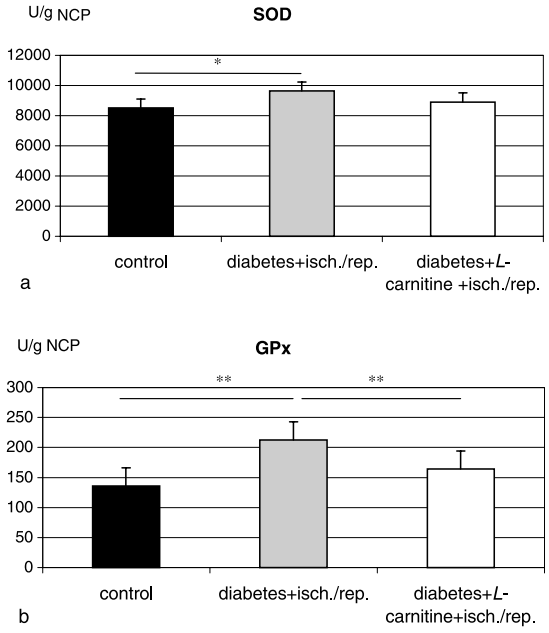

Fig. 7. Biochemically determined parameters of oxidative stress in myocardium of control, diabetic, and *L*-carnitine-protected diabetic rat; a: SOD-activity; b: GPx-activity (isch/rep.: ischaemia/reperfusion) ($^{*} = p \leq 0.05$, $^{**} = p \leq 0.01$)

diminishing effect after *L*-carnitine substitution. In contrast, GPx (glutathione peroxidase) activity of *L*-carnitine treated hearts was significantly lower in contrast to the severe rise in GPx activity in untreated diabetic hearts under experimental conditions (Fig. 7).

Discussion

Ischaemia with reperfusion may implicate an increased oxygen and nutrient supply of the myocardium [33]. However, in addition to the increased oxidative stress in diabetic myocardium, ischaemia and reperfusion cause a burst of free oxygen radical generation [34]. The resulting cellular injury with further structural alterations dominates the beneficial effects of post-ischaemic recovery.

When additionally exposed to ischaemia/reperfusion, an increase in luminar protrusions and formation of luminar blebs in endothelial cells as well as progressive mitochondrial damage in myocytes can be observed in the diabetic heart. Based on the ultrastructural results, we assume that the reduced tolerance to ischaemia and reperfusion of the diabetic myocardium may be improved by *L*-carnitine substitution [35, 36], possibly caused by enhancement of fatty acid oxidation, thus preventing the accumulation of potentially harmful lipid intermediates and sources of free radicals during ischaemia/reperfusion [37]. As reported by *Bertelli et al.* [27], the derivative propionyl-*L*-carnitine seems to protect the endothelial membranes from peroxidation.

Alterations in antioxidant enzymes in the myocardium are understood to be a characteristic feature of the uncontrolled diabetic state, suggesting a connection between mitochondrial damage and changes in SOD status. In agreement with literature [19, 20, 38], we observed a moderate increase in MnSOD protein expression and a slight increase in total SOD activity in the untreated diabetic heart under ischaemia and reperfusion. This alteration may be a compensatory response to the increased oxidative stress and inducible stimuli such as cytokines. The slight decrease of CuZnSOD protein expression, however, may indicate structural alterations of the enzyme due to glycation and fragmentation of SOD by toxic free radicals [39]. Additionally, the rise in free radical formation under these conditions is reflected by a significant increase in GPx activity in our untreated diabetic hearts with ischaemia/reperfusion injury, in agreement with literature [40].

Our results show a clear decrease in MnSOD protein and a slight decrease in CuZnSOD protein and total SOD activity in *L*-carnitine-protected myocardium after exposure to ischaeamia and reperfusion injury. As reported by *Ronca et al.* [41], enzyme leakage after peroxidative injury in the presence of exogenous propionyl-*L*-carnitine was remarkably decreased, accounting for the beneficial and antiperoxidative effect of this substance on oxidative stress in ischaemia and reperfusion [7, 27]. We propose that the diminished immunostaining of enzyme protein as well as the depressed enzyme activity in the protected diabetic group may be due to decreased free radical production, synthesis-inducing stimuli, and enzyme synthesis rather than increased enzyme degradation. This theory is supported by the significantly diminished GPx activity after *L*-carnitine treatment. This may lead to downregulation in enzyme protein by diminishing free oxygen radicals through the reduced concentrations of toxic long-chain fatty acids and stabilization of mitochondrial function by *L*-carnitine. Other authors found a completely normalized oxidant/antioxidant state in diabetic rats treated with *L*-carnitine, possibly caused by its reduction with plasma lipids and resulting lipid peroxidation [32]. We cannot, however, exclude a loss of enzyme activity due to degradation and denaturation of enzyme protein as reported for intracellular acidification and activation of cellular proteases under ischaemia [42].

In conclusion, *L*-carnitine protects the myocardium from diabetes-induced alterations as well as additional ischaemia and reperfusion damage in most parameters investigated in agreement with benefits to heart function shown in clinical and experimental studies [8, 43, 44]. Since we know that a single hyperglycaemia-induced process of superoxide overproduction by the mitochondrial electron-transport chain seems to be the first and key event in the activation of all other pathways involved in the pathogenesis of diabetic complications [6], we suggest that *L*-carnitine may be an adequate ''causal'' agent in the protection of myocardial alterations in diabetes, stabilizing mitochondrial and cellular function and acting through its antioxidative or radical scavenging potential, as reported for carnitine analogues [9].

The possibility of a therapeutic approach in the treatment of diabetic myocardial complications by *L*-carnitine treatment requires further investigation – for example, a longer substitution period or combination with other protective antioxidants.

Experimental

Animals and Experimental Procedure

The experiments were approved by Leipzig's regional governing committee (*Regierungspräsidium* No. 10/00). Twenty-three 8–9-month-old male diabetic Biobreeding/Ottawa Karlsburg (BB/OK) and sixteen male non-diabetic BB/OK rats from *Gerhard Katsch* Institute of Diabetes Karlsburg, Germany kept separately under semisterile conditions were divided into 5 experimental groups.

Group I (Control): 10 Non-diabetic BB/OK rats without any treatment.

Group II (Control and additional ischaemia + reperfusion): Hearts from 6 non-diabetic BB/OK rats out of group I were exposed to ischaemia and reperfusion in a *Langendorff*-apparatus.

Group III (Diabetes): Manifestation of insulin dependent diabetes after 102 ± 31.6 days; weekly measurement of blood glucose levels. If plasma glucose was more than $4\,mg/cm^3$, nuchal subcutaneous insulin depot Linplant was installed. Five diabetic BB/OK rats were sacrificed after six months of diabetes.

Group IV (Diabetes and additional ischaemia + reperfusion): Hearts from 12 diabetic BB/OK rats taken from group III were exposed to ischaemia and reperfusion in a *Langendorff*-apparatus.

Group V (Diabetes with *L*-carnitine treatment and additional ischaemia + reperfusion): 6 Randomized rats taken from group III. After 5 months of diabetes, the rats were treated daily with 365 mg/kg b.w. of *L*-carnitine administered dissolved in drinking water (1 g *L*-carnitine per $100\,cm^3$ water). After 6 months of diabetes, the rats' hearts were exposed to ischaemia and reperfusion in the *Langendorff*-apparatus after one month's protection by *L*-carnitine.

Isolated Heart Perfusion (Langendorff Heart)

BB/OK rats from group II, IV, and V were anaesthetized with pentobarbital intraperitoneally. The hearts were excised, the aorta cannulated, and retrograde perfusion initiated at a pressure of 81 mm Hg using Tyrode solution saturated with 95% O_2 and 5% CO_2 gas at 37°C. In these experiments the hearts were perfused 30 min to allow functional stabilization, and then subjected to 35 min of 37°C global "no flow" ischaemia followed by 90 min reperfusion.

Tissue Processing

The animals were anaesthetized using pentobarbital. The heart was rapidly excised after thoracotomy, and tissue samples were taken from the left and right ventricle and processed in usual manner for histology, electron microscopy, and immunohistochemistry.

Histological Techniques

Paraffin sections were cut and stained in the usual manner with haematoxylin-eosin, *van Gieson*, azan, Sudan black, Sudan III, and toluidine blue.

Morphometric Analysis

Measurements were partly carried out using classic point-counting and intersection point-counting techniques and the SIS-image analysing system for the mean cardiomyocytes diameter in μm (Dmyo), number of cardiomyocyte cross-sections per mm^2 (NAmyo), and number of mast cells per mm^2 (NAmast).

The SIS image-analyzing system allowed the capillary diameter measurement in μm (Dcap), defined as the smallest diameter, and the number of capillaries per mm^2 (NAcap). The intercapillary distance in μm (ICD) was calculated. The results were statistically controlled using the t-test and *Wilcoxon* test.

Immunohistochemical Technique

Some serial sections were deparaffinized, rehydrated in descending alcohol cascade, treated with 3% H_2O_2 solution, rinsed in aqua dest, incubated in Tris buffer saline (TBS), and stored in serum protein

block serum-free (DAKO). The sections were stored overnight with the primary antibody at different dilutions ranging from 100 to 2000:1 at 4°C in a moist chamber rinsed in TBS, stored for one hour with the diluted secondary antibody at room temperature, rinsed in TBS, stored with the PAP complex (Rabbit or Mouse EnVision DAKO), diluted in Tris buffer or Avidin Biotinylated enzyme Complex (Vectastain ABC Kit, VACTOR LABORATORIES), and rinsed three times in TBS. Thereafter, the sections were developed for 1–5 min in DAB, rinsed in aqua dest, dehydrated in ascending alcohol cascade, and embedded in Canada balsam.

Primary antibodies:	Rabbit polyclonal antibody to MnSOD/CuZnSOD (Yamanashi Medical University, Japan)
	Rabbit anti-rat polyclonal antibody to collagen I/III (Chemikon)
	Rabbit anti-human polyclonal antibody to collagen IV (Chemikon)
	Rabbit anti-human polyclonal antibody to Factor VIII (DAKO)
Secondary antibodies:	Goat anti-rabbit IgG (Vector Lab)/goat anti-mouse IgG EnVision (DAKO)
Semiquantitative analysis:	A score of 3 grades was used for evaluation of immunostaining grade; the mean for each group was calculated.

Tissue Processing for Electron Microscopy

Tissue samples were taken from the left ventricle near the apex, minced into small blocks of about $1\,mm^3$, fixed in cold *Karnovsky*'s solution (buffered 2% glutardialdehyde, 2% paraformaldehyde, $pH = 7.4$), and processed for electron microscopy. Five tissue blocks per animal were embedded in Durcupan (FLUKA).

Semithin sections from each block were stained with toluidine blue to select interesting areas for electron microscopy. Ultrathin sections were made using the Ultracut E (Reichert-Jung); electron micrographs according to the requirements of morphology were taken using an EM 900 (Zeiss).

Biochemical Investigations of Parameters of Oxidative Stress

Superoxide dismutase (SOD): Cytosolic CuZnSOD and mitochondrial MnSOD activity was determined using an SOD assay kit of Bioxytech® SOD – 525™ of OXIS. The kit uses a proprietary reagent that undergoes alkaline autooxidation accelerated by SOD, yielding a chromophore with an absorption maximum at 525 nm.

Glutathioneperoxidase (GPx): The activity of the GPx was determined using an OxyScan™ cGPx – 340 assay. In this procedure, the reduction of oxidized glutathione by formation of $NADP^+$ of $NADPH/H^+$ indirectly reflects GPx activity as an absorption decrease in the spectrophotometer.

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