Autonomic neuropathy in streptozotocin diabetic rats: effect of acetyl-L-carnitine

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Abstract

The present study was designed to characterize cardiac autonomic neuropathy in streptozotocin-induced (45 mg/kg i.v.) diabetic rat by analysis of heart rate variability (HRV), and to assess, in this model, the effects of treatment with acetyl-L-carnitine (ALC). Heart rate was reduced in diabetic rats (332 ± 22 vs. 411 ± 35 beat per min; \( P < 0.0001 \)). This bradycardia was partly reversed with ALC (369 ± 52 beat per min; \( P < 0.05 \) vs. untreated). Both time- and frequency-domain parameters of HRV were significantly reduced in diabetic rats. The reduction of spectral power was around 50% at high frequencies and about 70% at low frequencies, suggesting a decrease of parasympathetic activity. Low/high frequency ratio was significantly decreased in diabetic rats suggesting decreased sympathetic tone, while nonlinear analysis indicated a reduction of the chaotic complexity of heart rate dynamics in diabetic rats. Standard deviation of heart rate in ALC-treated rats was significantly higher than in untreated diabetic rats (\( P < 0.0001 \)). ALC counteracts the reduction of the power spectrum observed in diabetic animals (\( P < 0.0005 \)) normalizing the spectra profile. ALC restored chaotic complexity of heart rate dynamics. These results on the whole indicate that both sympathetic and parasympathetic cardiac tone were reduced significantly in diabetic rats and that ALC treatment prevents the development of autonomic neuropathy in streptozotocin-induced diabetes in rats. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Diabetes; Autonomic nervous system; Heart rate variability; Lipid metabolism

1. Introduction

A common complication of diabetes mellitus is peripheral neuropathy [1,2] that can affect both the somatic and the autonomic nervous systems and is characterized by decreased nerve conduction velocity, abnormal cardiovascular reflex, vascular dysfunction etc.; progressive neuroanatomical changes affect both neuronal structures, the endoneural vasculature and supporting connective tissue elements [3–6]. Loss of autonomic reflex control of the cardiovascular system can be particularly devastating to patients, resulting in decreased exercise tolerance, orthostatic hypoten-
sion, painless myocardial infarction, and sudden death [1,2,7]; diabetic patients with abnormal cardiovascular reflexes have a higher incidence of mortality than those with normal autonomic reflex function [8].

Carnitine and its short-chain esters facilitate transport of long-chain fatty acids across the inner mitochondrial membrane for β-oxidation, thereby promoting energy availability and preventing toxic accumulation of long-chain fatty acids [9]. L-carnitine [10] and acetyl-L-carnitine (ALC) [11] levels are decreased in sciatic nerve in streptozotocin diabetic rats, and ALC treatment corrects electoretinographic abnormalities, slows nerve conduction velocity [10–12], and increases vascular permeability in ocular tissue and peripheral nerve [9].

The present studies were therefore designed to characterize cardiac autonomic neuropathy in streptozotocin-induced diabetic rats and to assess the effects of intervention with ALC on functional abnormalities characterizing autonomic neuropathy. Both the effect of streptozotocin diabetes on autonomic nervous system function and the eventual protective action of ALC were assessed by means of time- and frequency-domain analyses of heart rate variability (HRV). Furthermore, the degree of complexity of the tachograms was assessed by means of recurrence quantification analysis (RQA).

RQA is a relatively new non-linear analytical technique used in fields ranging from theoretical physics [13] to molecular biology [14] and particularly relevant to our study because it is able to give both a reliable representation of heart rate regulation in rats [15,16] and to offer reliable indices of diabetes-induced autonomic dysfunction in humans [17].

2. Methods

2.1. Animals

Experiments were performed on 27 male Wistar rats (Charles River, Calco, Lecco, Italy) weighing between 250 and 280 g and housed in individual cages with free access to tap water and standard rat food. Twenty rats were made diabetic by a single administration of streptozotocin (STZ) (Sigma Chemical, St. Louis, MO, USA) 45 mg/kg i.v. dissolved in citrate buffer (pH 4.5) and injected within 5 min in rats fasted overnight; Seven rats were injected with citrate buffer and served as controls. Plasma glucose concentration was measured weekly by means of Glucometer (Glucometer Elite, Bayer Divisione Diagnostici, Milan, Italy).

A week after STZ administration, diabetic rats were divided at random into two groups, untreated (STZ n = 10), and ALC-treated (STZ–ALC n = 10) in which ALC (33 mg/ml) (Sigma-Tau Pomezia, Rome, Italy) was administered at a dose of 100 mg/kg (dissolved in physiological solution) SC every day for 5 weeks. Untreated diabetic rats received an equal volume of vehicle.

2.2. ECG Recording

At the end of the treatment period, rats were anaesthetized with 50-mg/kg i.p. pentobarbital sodium (Sigma Chemical), and through a left thoracotomy a pair of stainless steel bipolar electrodes were implanted; the leads were tunneled subcutaneously to the neck and soldered to contact pins. Pins were fixed to the skull by dental cement and the incision was closed with sutures. Rats were allowed 5 days to recover from surgery and to become acquainted with the experimental environment, in individual cages in which the animals were unrestricted.

On the sixth day and after a 60-min period of stabilization, a 5 min ECG (BM 613 ECG amplifier, Biomedica Mangoni, Pisa, Italy) was recorded as previously described [18]. Briefly, the recordings were digitally sampled by an analogue-to-digital converter (BM IDAS Programmable Acquisition System, Biomedica Mangoni) at 1000 Hz and R–R intervals were computerized and stored on a hard disk through an appropriate software (BM IDAS-ECG, Biomedica Mangoni). Premature beats and artifacts were manually identified and edited by linear interpolation [19]. Such events were less than 1% of all the analyzed beats.
2.3. Heart rate variability analysis

Analyses were performed on a time-series of 1200 R–R intervals. HRV was analyzed in time-domain by calculation of the standard deviation (S.D.), and in frequency-domain using fast Fourier Transform of R–R intervals time-series. The spectra were integrated into three frequency bands defined as very low (VLF = 0.025–0.199 Hz), low (LF = 0.20–0.59 Hz), and high (HF = 0.60–2.5 Hz) frequency. This HF range was used because our animals always presented respiratory frequencies ranging within this band. Furthermore, R–R intervals were investigated by means of nonlinear analysis using recurrence quantification analysis.

2.4. Recurrence quantification analysis of HR

Recurrence Quantification Analysis (RQA) is a relatively new non-linear signal analysis technique especially suited for short, non-stationary series. RQA is based on the computation of the Euclidean distance matrix (DM) between the rows (epochs) of the embedding matrix (EM), corresponding to the multivariate data matrix having as variables the studied series plus M-lagged copies corresponding to the series itself progressively shifted at a constant delay. The DM has been shown to keep track of the entire information content (in terms of autocorrelation structure) embedded in the original signal [20] without constraints linked to the particular nature of the signal at hand (linearity, stationarity, etc.). The DM undergoes a first filter by simply darkening the pixels, located at specific (i, j) coordinates corresponding to distance values between the i-th and j-th segments lower than a pre-defined radius [21].

The features of the distance function (i.e. the Euclidean distance) make the plot symmetric (DMi, j = DMj, i) and with a darkened main diagonal corresponding to the identity line (DMi, j = 0 when j = i). The darkened (recurrent) points single out recurrences within the series and the plot can be considered as a global picture of the autocorrelation structure [21]. In addition to the global impression given by the graphic appearance of the plot, the indices developed by Webber and Zbilut [21] allow for a quantitative description of the recurrence structure of the plot. The indices constituting the quantitative description of the recurrence plots and thus RQA, the most relevant in studying R–R intervals are:

1. %REC (percent recurrence): fraction of the plot filled by recurrent points. It corresponds to the fraction of recurrent points over all the possible pairs of segments or, equivalently, to the fraction of coupled distances below the chosen radius among all the computed distances.
2. %DET (percent determinism): this is the percentage of subsequent recurrent points that form diagonal line structures, of two points or more, in the distance matrix.
3. Lmax (maximal line): this index is simply the length (in terms of consecutive points) of the longest recurrent lines in the plot, and is inversely related to the largest positive Lyapunov exponent [22]. The Lyapunov exponent measures the sensitivity of the studied dynamics to the initial conditions, so from a physiological point of view, can be interpreted as a measure of the ‘sensitivity to change’ of the autonomic system.

To analyze R–R intervals by RQA, the following parameters were selected: the delay of reconstruction was chosen as 1, the embedding dimension (ED) was taken as 15 dimensional Euclidean spaces, and, for each analysis, a radius value of 10 and a threshold line length of 10 were used.

2.5. Statistical analysis

Results are expressed as mean ± S.D. Student’s t-test to compare means was used. A P value of 0.05 or lower was considered as statistically significant.

3. Results

3.1. General observation

Blood glucose before interventions was around 4.4 mmol/l and was similar in all three groups.
One week after STZ injection blood glucose increased in both groups of diabetic rats (16.5 ± 2.3 and 16.8 ± 3.0 mmol/l in STZ and STZ-ALC groups) and further increased after 6 weeks reaching 26.2 ± 2.3 and 26.8 ± 2.2 mmol/l, respectively. Corresponding values in control rats ranged between 4.4 and 5.5 mmol/l during the 6 weeks.

Control animals gained body weight from 262 ± 15 to 431 ± 15 g, whereas both groups of diabetic animals slightly increased body weight throughout the 6 weeks (from 279 ± 17 to 306 ± 30 g and from 270 ± 9 to 315 ± 33 g, respectively in STZ and STZ-ALC groups).

Heart rate was 411 ± 35 beat per min in control rats, but decreased dramatically in diabetic rats (332 ± 22 beat per min; \( P < 0.0001 \) vs. controls). Bradycardia was partly reversed by ALC treatment (369 ± 52 beat per min; \( P < 0.05 \) vs. controls and \( P < 0.05 \) vs. STZ).

### 3.2. Heart rate variability

HRV characteristics of the three groups of animals are shown in Tables 1 and 2. Consequent to the reduction of heart rate, R–R intervals were significantly increased in diabetic rats (181 ± 12 vs. 147 ± 12 ms controls; \( P < 0.0001 \)). Standard deviation of R–R intervals in STZ rats was significantly reduced with respect to controls (5.0 ± 1.2 vs. 8.2 ± 0.8 ms \( P < 0.0001 \)).

Fig. 1 displays two examples of tachogram and spectral power of R–R intervals, one from a control and the other from a diabetic rat; as in the examples spectral power is markedly reduced in diabetic rats, the total integrated area of the Fourier spectra (TP) being 1.07 ± 0.27 ms² in controls and 0.50 ± 0.21 ms² in the diabetic animals (\( P < 0.0001 \)).

This marked reduction was of about 50% in VLF and HF bands, but more pronounced in the LF band (about 70%), which corresponds to parasympathetic activity (Table 1). Moreover, LF/HF ratio in STZ rats was significantly decreased with respect to control animals suggesting a decrease of sympathetic tone (Table 1).

The parameters considered in the RQ analysis (Table 2), namely %REC, %DET and Lmax were significantly higher in diabetic rats compared with controls (5.9 ± 6.5, 49 ± 16 and 90 ± 74, respectively; \( P < 0.05 \); \( P < 0.005 \) and \( P < 0.05 \) vs. controls), indicating a reduction of the chaotic complexity of HR dynamics. The R–R intervals are more ‘repetitive’ and less flexible, suggesting an imbalance of autonomic cardiac control.

### 3.3. ALC effect on HRV

Parallel with the reduction of the observed bradycardia, the reduction of R–R intervals was

<table>
<thead>
<tr>
<th>( n )</th>
<th>Controls</th>
<th>STZ</th>
<th>STZ-ALC</th>
</tr>
</thead>
<tbody>
<tr>
<td>R–R (ms)</td>
<td>147 ± 12</td>
<td>181 ± 12 d</td>
<td>165 ± 21 a, A</td>
</tr>
<tr>
<td>R–R S.D. (ms)</td>
<td>8.2 ± 0.8</td>
<td>5.0 ± 1.2 d</td>
<td>7.3 ± 0.8 a, D</td>
</tr>
<tr>
<td>TP</td>
<td>1.07 ± 0.27</td>
<td>0.50 ± 0.21 d</td>
<td>1.11 ± 0.45 D</td>
</tr>
<tr>
<td>VLF</td>
<td>0.65 ± 0.24</td>
<td>0.33 ± 0.18 b</td>
<td>0.62 ± 0.33 A</td>
</tr>
<tr>
<td>LF</td>
<td>0.21 ± 0.10</td>
<td>0.06 ± 0.03 c</td>
<td>0.26 ± 0.21 C</td>
</tr>
<tr>
<td>HF</td>
<td>0.21 ± 0.12</td>
<td>0.11 ± 0.05 a</td>
<td>0.24 ± 0.09 B</td>
</tr>
<tr>
<td>LF/HF</td>
<td>1.09 ± 0.37</td>
<td>0.57 ± 0.24 b</td>
<td>1.14 ± 0.65 A</td>
</tr>
</tbody>
</table>

Mean ± S.D. TP, total area under the Fourier spectrum; VLF, area under Fourier spectrum at very low frequency (0.025–0.05 Hz); LF, area under Fourier spectrum at low frequency (0.05–0.15 Hz); HF, area under Fourier spectrum at high frequency (0.15–0.4 Hz); \( a = P < 0.05 \); \( b = P < 0.005 \); \( c = P < 0.001 \); \( d = P < 0.0001 \) vs. controls. \( A = P < 0.05 \); \( B = P < 0.01 \); \( C = P < 0.005 \); \( D = P < 0.001 \) vs. STZ.

### Table 1

Time- and frequency-domain parameters of HRV in control, streptozotocin (STZ) diabetic and diabetic acetyl-l-carnitine treated (STZ-ALC) rats

<table>
<thead>
<tr>
<th>( n )</th>
<th>Controls</th>
<th>STZ</th>
<th>STZ-ALC</th>
</tr>
</thead>
<tbody>
<tr>
<td>%REC</td>
<td>0.34 ± 0.36</td>
<td>5.9 ± 6.5 a</td>
<td>0.49 ± 0.82 B</td>
</tr>
<tr>
<td>%DET</td>
<td>24 ± 11</td>
<td>49 ± 16 b</td>
<td>23 ± 10 C</td>
</tr>
<tr>
<td>Lmax</td>
<td>34 ± 18</td>
<td>90 ± 74 a</td>
<td>33 ± 20 A</td>
</tr>
</tbody>
</table>

Mean ± S.D. %REC, percentage of recurrence; %DET, percentage of determinism; Lmax, length index. \( a = P < 0.05 \); \( b = P < 0.005 \); \( A = P < 0.05 \); \( B = P < 0.01 \); \( C = P < 0.001 \) vs. STZ.
less pronounced in STZ–ALC than in STZ rats (165 ± 21 ms, \( P < 0.05 \) vs. STZ and \( P < 0.05 \) vs. controls) (Table 1).

The time-domain index of HRV considered, the S.D. of \( R-R \)-intervals, was reduced in STZ–ALC rats compared with controls (7.3 ± 0.8 ms; \( P < 0.05 \)) but was significantly higher than that of STZ rats (\( P < 0.001 \)) (Table 1). Frequency-domain analysis shows that ALC treatment counteracts the reduction of the TP spectrum observed in diabetic rats (1.11 ± 0.45; \( P < 0.001 \) vs. STZ). This last effect was noted at all frequencies considered (VLF, 0.62 ± 0.33; LF, 0.26 ± 0.21; HF, 0.24 ± 0.09; respectively \( P < 0.05 \), \( P < 0.005 \), and \( P < 0.01 \) vs. STZ) thus normalizing the spectral profile of treated diabetic rats.

RQ analysis of \( R-R \) intervals of diabetic rats treated with ALC shows a reduction of three parameters considered in comparison with STZ (%REC, 0.49 ± 0.82; %DET, 23 ± 10 and Lmax, 33 ± 20; respectively, \( P > 0.01 \), \( P < 0.001 \) and \( P < 0.05 \) (Table 2) thus restoring the chaotic complexity of HR dynamics observed with RQA.

In contrast to cardiac autonomic neuropathy in humans in which tachycardia is a common finding, we found that in the STZ experimental model, diabetes is associated with bradycardia. These results are in agreement with the data from other laboratories [23,24] that have also demonstrated a resting bradycardia in this animal model. The mechanisms underlying this diabetes-associ-

4. Discussion

Results of this study demonstrate that STZ-induced diabetes is associated with bradycardia and with an alteration of cardiac autonomic nervous system control and that acetyl-L-carnitine treatment counteracts these alterations. Cardiac autonomic nervous alteration in this model of diabetes involves both sympathetic and parasympathetic branches and was highlighted by the reduction of heart rate variability, in time- and in frequency-domain and by a reduction of the chaotic complexity of HR dynamics observed with RQA.
ated bradycardia are not known. Studies in isolated cardiac preparations indicate that STZ-induced bradycardia is associated with a depression in the basal spontaneous pacemaker rate [24,25]. This decline in spontaneous rate is not affected by atropine [25], indicating that it is not mediated by endogenous neurotransmitter release. Thus, the reduced rate may reflect changes in electrophysiological properties of the sinoatrial node.

The bradycardia observed in diabetic rats could also be mediated, in part, by alterations in autonomic nervous system control, as was suggested by the observation in other experiments performed on the increase of heart rate when diabetic rats were anaesthetized with Nembutal (from 323 ± 17 beat per min in conscious rats to 357 ± 35 beat per min in anaesthetized rats; P < 0.05). For example, a decline in sympathetic tone would diminish heart rate.

Observed differences between STZ-induced diabetes in rats and the clinical situation may result from species variation or from differences inherent in naturally occurring as compared with chemically-induced disease. The induction of diabetes in the rat results from a single insult which quickly destroys pancreatic β-cells and results in immediate and sustained hyperglycaemia; by contrast in humans the cause and time of onset of diabetes is usually unknown, and the time course of the rise in blood glucose less well defined. The animal model also differs from the clinical situations in that the hyperglycaemia in rats is not treated, and observed changes occur in the presence of continual and markedly elevated blood glucose; in contrast, patients are usually not left untreated for extended periods of time.

Diabetic rats showed a reduction of time-domain parameter of HRV thus suggesting a reduction of parasympathetic tone. Furthermore, fast Fourier analysis of R–R intervals showed a pronounced reduction of spectral power in VLF, LF and HF bands, and a reduction of LF/HF ratio in diabetic rats suggesting a reduction of both sympathetic and parasympathetic tone. Finally, nonlinear analysis of R–R intervals time series indicated on the whole a reduction of the chaotic complexity of HR dynamics in diabetic rats. The R–R intervals are more ‘repetitive’ and less flexible, suggesting an imbalance of autonomic cardiac control.

An analogous augmentation of RQA parameters was reported by Dabire et al. [15] in rats subjected to either parasympathetic blockade or ganglionic blockade, indicating that the augmented indices of RQA analysis of R–R intervals are related to a reduction of parasympathetic tone. More importantly, this result paralleled the results obtained in humans by Mestivier and collaborators, demonstrating that Lmax strongly correlates to the score of autonomic neuropathy diagnosed with the Ewing test [17].

In synthesis the results indicate that just as in Type 1 diabetes in humans [26,27], both sympathetic and parasympathetic tones with regard to the heart were reduced significantly in diabetic animals, in agreement with previous results reported by Hicks et al. in the same experimental model [23].

Furthermore, the results showed that ALC treatment restored autonomic nervous tone to control levels. Recently, carnitine deficiency in nerve has received considerable attention in regard of the pathogenesis of diabetic neuropathy [10,11,28]. However, the mechanism by which free carnitine content is diminished in diabetic nerves has not been well investigated. Ido et al. suggested that the decreased levels of carnitine in plasma and nerves would be mediated through increased carnitine excretion in the urine [10]. They also found that the urinary carnitine excretion was linked to the urine volume and plasma glucose concentration [10]. It has been reported that treatment with carnitine analogues can prevent delay in motor nerve conduction velocity, reduction of HRV, biochemical alteration and histopathological abnormalities [10,28–30]. Recently, preliminary findings of Turpeinen and collaborators demonstrated that treatment with ALC was able to prevent the progressive loss of myocardial sympathetic nervous function in people with diabetes [31].
Carnitine plays an important role in long-chain fatty acid metabolism by mediating its transport from cytosol into mitochondria. Therefore, carnitine depletion, which results in an inhibition of β-oxidation of fatty acids and energy production, causes the accumulation of long-chain fatty acids and fatty acid esters in nerve tissues of diabetic animals and may further disturb membrane lipid composition and functions, involving alterations in protein kinase C (PKC) and Na+/K+-ATPase activity [32]. On the other hand, myoinositol transport is thought to be regulated by PKC and/or Na+/K+-ATPase [33] and to be energy-dependent. Therefore, carnitine deficiency in diabetic nerves would mediate further myoinositol depletion through the decreased activity of PKC and/or Na+/K+-ATPase in addition to that induced by the polyol pathway. This hypothesis can be supported by the fact that it has been demonstrated that the nerve myo-inositol depletion in diabetic nerves was prevented by treatment with ALC [28].

A reduction in endoneurial blood flow in diabetic nerves and the preventive effect of ALC on this reduction has been reported [28]. One of the action mechanisms increasing nerve blood flow may be mediated through Ca++ homeostasis. An increased Ca++ concentration was reported in diabetic aorta, due to the decreased activity of Na+/K+-ATPase [34]. Therefore, ALC would decrease the Ca++ concentration by improving Na+/K+-ATPase activity, as previously reported [10,11]. In summary, the data suggest that STZ-induced diabetes in rats is associated with an autonomic neuropathy highlighted by the reduction of heart rate variability. Analysis of heart rate variability indicates that the autonomic neuropathy is characterized by a decrease in both sympathetic and parasympathetic cardiac tone. Treatment of diabetic rats with ALC was able to counteract most of the alteration of autonomic cardiac control observed; taking into account the possible differences between experimental and clinical diabetes, ALC may have potential value in preventing the progressive development of human autonomic neuropathy. The concordance, in terms of RQA parameters between human diabetic data and rats opens an intriguing perspective of direct translation of these data into clinical practice, given the non-invasive nature of ECG measures.

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References


