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Research Article

Attenuation of diabetic retinopathy by the molecular chaperone-inducer amino acid analogue canavanine in streptozotocin-diabetic rats

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Abstract. The effect of canavanine treatment on the electroretinograms of healthy and streptozotocin-diabetic rats was studied. The characteristic amplitudes of the a-wave, W_2 and W_3 oscillatory potentials were markedly diminished in the 2-week streptozotocin-diabetic rats compared with those of the control rats. In contrast, the amplitudes of all the responses of the canavanine-pretreated streptozotocin-diabetic rats were practically indistinguishable from those of the control animals. Our results prompt further investigations for the use of amino acid analogues and other inducers of molecular chaperones in easing the chronic consequences of diabetes such as retinopathy.

Key words. Molecular chaperone; heat shock protein; Hsp70; canavanine; retina; retinopathy; electroretinography.

Molecular chaperones [heat shock (Hsp), glucose-regulated or stress proteins] are among the most abundant and conserved proteins of living organisms, and probably played a major role in the evolution of modern enzymes [1]. Their major function is to maintain the conformational stability of other proteins in the cell and to direct the proteins to their final destination inside the cell by helping them to reach or maintain the necessary conformation [2]. Since the number of misfolded proteins increases if the cell experiences stress, the help of molecular chaperones is essential to survive the various types of stresses experienced in disease states such as diabetes. In accordance with this assumption, recent developments in stress research have linked these proteins to the aetiology and treatment of several human diseases [3, 4].

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Despite the close link between changes in extracellular glucose level and regulation of the synthesis of molecular chaperones such as glucose-regulated proteins [2–4], only a few reports have explored the changes in synthesis and the function of chaperones in diabetes [5–7]. Recent reports established the chaperone coinducer Bimoclomol as a potent compound that induces acceleration of diabetic wound healing and slowing of diabetic neuropathy [8, 9]. Based on these results, as a continuation of our initial studies to characterize changes in molecular chaperones in diabetic animals [10, 11], the present work analyses the effect of a well-known inducer of molecular chaperones, canavanine [12–17], on diabetic retinopathy.

Diabetic retinopathy is one of the leading causes of blindness in middle age. Electroretinographic abnormalities have been recognized as sensitive early indicators of diabetic retinopathy. Diabetes reduces the

amplitude and increases the latency of oscillatory potentials. From the five commonly measured oscillatory potentials (W_1–W_5) the proximal, mostly GABA-ergic W_2, and the more distal, mostly glycine-sensitive W_3, are especially good markers of diabetic retinal damage together with the change in a-wave on the gross electroretinogram [18–22].

Here we report that pretreatment with the molecular chaperone inducer canavanine attenuates diabetes-induced changes in rat electroretinograms. This finding may indicate the potential benefit of molecular chaperone inducers in easing the chronic consequences of diabetes.

Materials and methods

Animals. Male Wistar rats (LATI, Hungary) weighing 280–300 g were treated with daily subcutaneous (s.c.) injections of 350 mg/kg of canavanine (CAN, Sigma, St. Louis, MO, USA) for 5 days and/or with injection of 50 mg/kg of streptozotocin (STZ, Sigma) in the following experimental groups (five animals each):

- c: control group with vehicle injections instead of STZ or CAN treatments
- 2d: 2 weeks diabetes after STZ treatment
- 4d: 4 weeks diabetes after STZ treatment
- 2d + i: 2 weeks diabetes with insulin injections in the 2nd week to normalize blood sugar levels
- ca: 5 days CAN treatment, no STZ
- ca + 2d: 5 days CAN treatment followed by STZ injection, 2-week development of diabetes after STZ
- ca + 2d + i: 5 days CAN treatment followed by STZ injection, 2-week development of diabetes after STZ with insulin injections in the 2nd week to normalize blood sugar levels.

STZ and insulin treatments were performed as described earlier [23, 24]. In insulin-replacement studies ultralente insulin (Novo, Copenhagen, Denmark) was administered daily to the diabetic animals. Insulin was given subcutaneously in an individual dose (range: 4.1–8.5 IU) to normalize the blood glucose level of the respective experimental animal. The dose of canavanine was optimized in pilot experiments by monitoring the induction of retinal Hsp70 content. Animal experiments were performed in accordance with the guidelines for scientific experiments on animals issued by the Hungarian Council of Medical Sciences and the Senate of Semmelweis University.

Electroretinography. In electroretinographic experiments the techniques and protocols of the 1994 updated standards of the International Society for Clinical Electrophysiology of Vision (ISCEV) were followed [25] with certain modifications essential for measurements in rats [26]. Rats were adapted to the dark for minimum of 3 h before the experiment due to the slow regeneration of rhodopsin in the rat retina [27]. Dark-adapted animals were anaesthetized with pentobarbital, intraperitoneally 60 mg/kg. The intact eye was anaesthetized by local anaestheticum ophtalmicum Humacain 0.4% eye drop containing 40 mg of oxibuprocaine · Cl, 1 mg of benzalconyl · Cl and 175 mg of boric acid in 10 ml of aqueous solution. The pupil was dilated with 0.5% Humapent eye drops containing 50 mg of cyclopentolate · Cl and 1 mg of benzalconyl · Cl in 10 ml of aqueous solution.

During the experiment the animals were kept in a conditioned, electrostatically shielded box [26]. Electroretinograms were recorded by means of nonpolarizable Clark electrodes (type E-205, Clark Electromedical Instruments, Pangbourne, UK). The recording electrode was mounted in a Plexiglas 'one-point-touch' tube and placed on the corneal margin, just above the sulcus sclerae of the nasal canthus. The corneal surface was protected during the experiment with a nonirritating and nonallergic ionic conducting solution (Oculogutta viscosa, Formula Normalis VI, Hungary). The reference electrode was placed at the contrary (temporal) canthus, below the eyelid, just below the sclera rim, in contact with the conjunctiva. This 'bipolar' electrode configuration was extremely stable electrically. The bipolar offset potential was about 100 $\mu V \pm 10$ μV/h.

Oscillatory potentials were stimulated by 5 to 10 flashes of white light (with a duration of 74 μs at a light intensity of 3 cd/m^2, resulting in a retinal illumination of 6500 lux at a colour temperature corresponding to 7000 K) in 15-s intervals. Due to the different initial level of retinal adaptation [28], the first electroretinogram was not included in the averaging process. For some of the light flash series a 4-lux 660-nm red background illumination was also applied. Background illumination increased the adaptation of retinas and decreased the height of b-wave of the gross electroretinogram, thus significantly increasing the signal-to-noise ratio of W_2 and W_3 oscillatory potentials [21].

Electroretinograms were recorded with stable offset potential compensation and with 10-kHz high-pass filters using DC amplification. In some of the light flash series a 10-Hz low-pass filter was also applied. The 10-Hz low-pass filter was highly effective in filtering out most of the b-waves (with an approximate frequency of 15 Hz), thus further increasing the signal-to-noise ratio of W_2 and W_3 oscillatory potentials. Combination of the 10-Hz low-pass filter with the DC amplification also eliminated the slow baseline drift, which sometimes makes evaluation of the electroretinograms very difficult. In several experiments both the red background illumination and the 10-Hz low-end filter were

applied to improve the signal-to-noise ratio of the W_2 and W_3 oscillatory potentials.

Latency times were calculated by measuring the time between the onset of the light impulse and the peak of a-wave, or of the respective oscillatory potential. The amplitudes of the oscillatory potentials were measured from the oscillatory potential nadir to the adjacent peak and also from the linear fit contour of the b-wave to the peak of the adjacent oscillatory potential. Amplitude values gained by the two methods showed similar changes in all the experimental groups tested.

Western blot. After electroretinography, the animals were sacrificed and their retinas were dissected. Retinal extracts were prepared in an isolation buffer of 20 mM Hepes, pH 7.4, 25 mM NaCl, 2 mM dithiothreitol, 2 mM phenylmethylsulphonyl fluoride and 1 mM EGTA. The protein concentration of the extracts was determined by the method of Bradford [29]. Samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (in the presence of 10 mM EDTA in Laemmli buffer) after DNase I (Sigma) treatment (in the presence of an additional 3 mM $MgCl_2$) to prevent gel formation of sample DNA, as described previously [30].

Pilot experiments showed that chaperone levels were a linear function of the protein content of the extract in the range of 10 to 200 μg total protein analysed by Western blot technique [31] using antibodies against protein disulphide isomerase (poly- and monoclonal), Hsp70, hsc70, Hsp90 and Grp94 (SPA-890, -891, -810, -820, -830 and -850 antibodies from StressGen) and polyclonal rabbit antibodies against Hsp90 and Grp94 (a kind gift of Drs. Yoshihiko Miyata and Ichiro Yahara, Tokyo Metropolitan Institute of Medical Science) [32, 33]. In further Western blot studies 50 μg of total retinal protein was loaded per lane.

Statistical analysis. Data are expressed as mean $\pm$ SD. The mean values were compared by unpaired Student's t test and were also analysed applying the analysis of variance (ANOVA) test using the Origin 4.1 statistical software package. $P < 0.05$ was considered statistically significant.

Results

Expression of retinal chaperones after amino acid analogue treatment in healthy rats. A literature survey of the known stress-protein-inducer amino acid analogues showed that the most widely examined analogues were the arginine analogue canavanine and the proline analogue L-azetidine-2-carboxylic acid [12–17]. L-Azetidine-2-carboxylic acid treatment of experimental rats (range: 10 mg/day to 200 mg/day) showed no induction of any retinal stress proteins tested (data not shown). Similarly, canavanine treatment caused no significant induction of retinal protein disulphide isomerase, Hsp90 and Grp94 (fig. 1A and data not shown). In contrast to the other stress proteins, a significant (1.7-fold, $P < 0.005$) increase in retinal Hsp70 could be observed after canavanine treatment of control rats.

Canavanine pretreatment of STZ-diabetic rats and effects of canavanine on retinal chaperones of diabetic rats. Canavanine treatment alone had no effect on blood glucose (4.0 ± 0.4 mM vs. 3.8 ± 0.3 mM in control animals). Canavanine pretreatment reduced the blood glucose level of STZ-treated animals by 20% (18.8 ± 2.3 mM vs. 23.6 ± 1.7 mM, $P < 0.005$) and caused a delay in the 'secondary' effects of STZ diabetes (weight loss, skin lesions etc. from days 10 to 12 of diabetes in canavanine-pretreated animals vs. the same effects from day 7 of diabetes in the nonpretreated group).

Two-week STZ diabetes induced no significant change in the retinal levels of any of the heat shock proteins tested (fig. 1 and data not shown). The canavanine-induced, elevated levels of retinal Hsp70 persisted, albeit at a reduced efficiency, in the 2-week STZ-diabetic rats without (2d vs. ca + 2d, 1.2-fold increase, $P < 0.1$) or with insulin treatment (2d vs. ca + 2d + i, 1.4-fold increase, $P < 0.01$). Interestingly, there was a significant ($P < 0.025$) increase in Hsp70 in the insulin-treated STZ-diabetic animals compared with the control group.

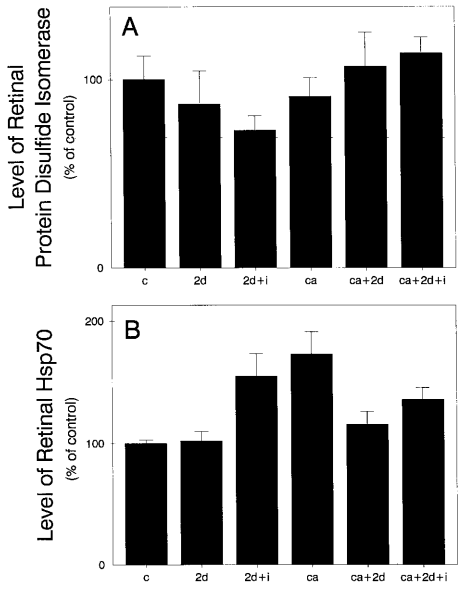

Figure 1. Changes in retinal molecular chaperone levels in control (c), 2-week STZ-diabetic (2d), insulin-treated 2-week diabetic (2d + i), canavanine-pretreated (ca), canavanine-pretreated 2-week STZ-diabetic (ca + 2d) and canavanine-pretreated, insulin-treated 2-week STZ-diabetic (ca + 2d + i) rats. (*A*) Changes in protein disulphide isomerase protein levels. (*B*) Changes in protein levels of the inducible 70-kDa heat shock protein Hsp70.

This might be explained by the ability of insulin to induce Hsp70 in cell culture studies [34] (fig. 1B).

Electroretinography of diabetic and canavanine-treated rats. A typical electroretinogram of control, 2-week STZ-diabetic and canavanine-pretreated 2-week STZ-diabetic animals is shown in figure 2. All the characteristic responses (a-wave, W_2 and W_3; see Kozak et al. [21]) are markedly diminished in the 2-week STZ-diabetic (2d) animals compared with those of the control rats (c). In contrast, the amplitudes of all the responses of the canavanine-pretreated STZ-diabetic rats (ca + 2d) are much less affected than those of the diabetic animals and are similar to those of the control animals. The only difference between the electroretinograms of the c and ca + 2d rats is a slight but definite delay of 3 to 5 ms in the onset and peak of the a-wave and characteristic oscillatory potentials.

A detailed analysis revealed that peak latencies of the a-wave and the oscillatory potentials W_2 and W_3 [21] did not show any significant changes in electroretinograms of STZ-diabetic rats vs. those of the control animals. In agreement with the data of the individual electroretinogram shown in figure 2, the only difference in the latency times was a 20–40% delay in all the responses of the canavanine-treated STZ-diabetic group (2.6-, 4.3- and 4.9-ms delay in peaks of a-wave, W_2 and W_3, respectively) which was statistically significant only in case of a-wave and W_2 peaks ($P < 0.013$, 0.046 and 0.13, respectively).

Analysis of electroretinograms was also made in 4-week STZ-diabetic rats. Surprisingly, these electroretinograms showed a significant improvement compared with those of the 2-week diabetic animals and were similar to the electroretinograms of control (healthy) rats. The 'spontaneous regeneration' of retinal activities of the 4-week diabetic animals was in spite of their constantly high blood glucose level (24.6 ± 3.5 mM) and was rather surprising. The exact reason for this finding is presently unknown. However, this behaviour may be a consequence of the smaller dose of streptozotocin (50 mg/kg instead of 60 mg/kg) used in our studies to avoid 'overstressing' the animals.

Statistical analysis of amplitudes of W_2 peaks shows similar changes in the qualitative assessment of the individual electroretinograms shown in figure 2. All the characteristic responses were markedly diminished in the 2-week STZ-diabetic (2d) animals compared with those of the control rats (c). Insulin treatment of diabetic animals restored the W_2-peak amplitude to the control level (2d + i). Canavanine pretreatment resulted in a significant improvement of the amplitudes of W_2 peaks of the electroretinograms of STZ-diabetic animals (cf. columns 2d and ca + 2d in fig. 3). The amplitudes of the W_2 peaks of the canavanine-pretreated rats (ca + 2d) were practically indistinguishable from those of the control animals (c, fig. 3). The diabetes-induced decreases and the canavanine-induced improvements of the electroretinograms are statistically significant, having P values in the range of 0.001 to 0.01. Insulin treatment of canavanine-pretreated diabetic animals did not cause a further improvement of the electrical responses of the retina (ca + 2d + i, fig. 3).

Analysis of a-wave amplitudes gives almost identical results to that of the W_2 oscillatory potentials (table 1). W_3 peaks show similar, albeit statistically less significant, changes (table 1). Insulin treatment of diabetic animals restored both a-wave and W_3 peak amplitudes

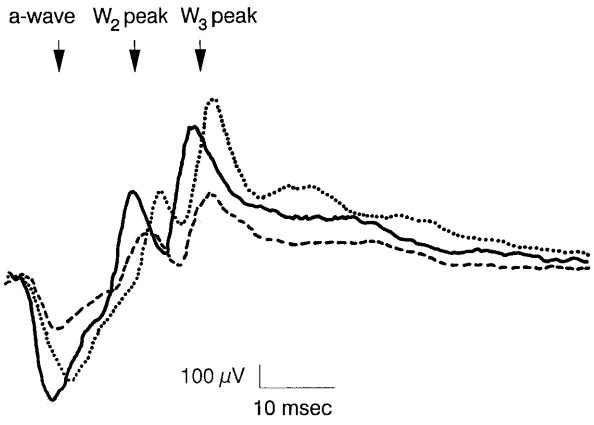

Figure 2. Representative electroretinograms of control (c, solid line), 2-week STZ-diabetic (2d, dashed line) and canavanine-pretreated 2-week STZ-diabetic (ca + 2d, dotted line) rats. The positions of the characteristic a-wave, W_2 and W_3 peaks are marked with arrows.

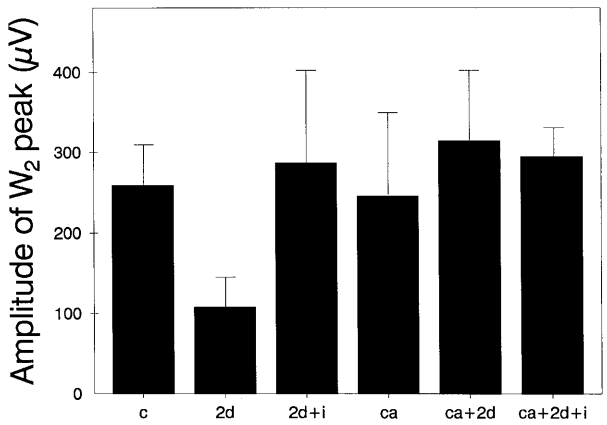

Figure 3. Peak amplitudes of W_2 oscillatory potentials in electroretinograms of control (c), 2-week STZ-diabetic (2d), insulin-treated 2-week diabetic (2d + i), canavanine-pretreated (ca), canavanine-pretreated 2-week STZ-diabetic (ca + 2d) and canavanine-pretreated, insulin-treated 2-week STZ-diabetic (ca + 2d + i) rats.

Table 1. Amplitudes of a-wave and W_3 oscillatory potentials in electroretinograms of control (c), 2-week STZ-diabetic (2d), insulin-treated 2-week diabetic (2d + i), canavanine-pretreated (ca), canavanine-pretreated 2-week STZ-diabetic (ca + 2d) and canavanine-pretreated, insulin-treated 2-week STZ-diabetic (ca + 2d + i) rats.

	a-wave (μV)	W_3 (μV)
c	319 ± 94*	292 ± 85†
2d	116 ± 54*‡	158 ± 89†§
2d + i	317 ± 73	349 ± 157
ca	264 ± 143	234 ± 148
ca + 2d	294 ± 85‡	501 ± 198§
ca + 2d + i	307 ± 25	455 ± 66

Values are means ± SD of three to five experiments from five animals in each group.
*Significant difference between a-wave amplitudes of control and STZ-diabetic rats, $P < 0.005$. †Significant difference between W_3 oscillatory potential amplitudes of control and STZ-diabetic rats, $P < 0.04$. ‡Significant difference between a-wave amplitudes of STZ-diabetic and canavanine-pretreated rats, $P < 0.01$. §Significant difference between W_3 oscillatory potential amplitudes of STZ-diabetic and canavanine-pretreated rats, $P < 0.02$.

to the control level. Interestingly, canavanine pretreatment improves the amplitudes of W_3 peaks of STZ-diabetic animals above the control level (increase of 70%, $P < 0.048$; table 1) and this 'overshoot' also appears if we compare the amplitudes of W_3 peaks of insulin-treated STZ-diabetic animals with those of canavanine-pretreated insulin-treated STZ-diabetic rats (increase of 30%, $P < 0.2$; table 1).

Discussion

Canavanine is a generally used inducer of stress proteins [12–17]. The arginine-analogue canavanine can be incorporated as a 'false amino acid' to the de novo synthesized proteins, including heat shock proteins themselves [35]. The altered conformation of canavanine-containing proteins provokes the 'quality control' mechanism of molecular chaperones and therefore accelerates protein turnover [36].

In our experiments canavanine proved to be a modest inducer of retinal Hsp70. On the other hand, canavanine treatment resulted in no significant induction of retinal protein disulphide isomerase, Hsp90 or Grp94, and L-azetidine-2-carboxylic acid showed no induction of any retinal stress proteins tested. These results are in agreement with the available data in the literature. Hsp90 is known to be expressed at very high constitutive levels in the retinal tissue, which prevents the observation of any further induction in total Hsp90 after heat shock [37]. Similarly, there was no detectable change in Hsp70 levels revealed by Western blot analysis after L-azetidine-2-carboxylic acid treatment of SV-40-transformed retinal pigment endothelium-derived cells [38]. Minor elevations of both Hsp70 and Hsp90 were only detectable in these cells using autoradiographic analysis of freshly synthesized ^{35}S-labelled proteins after heat shock or L-azetidine-2-carboxylic acid treatment [38, 39].

Canavanine pretreatment markedly improved all the characteristic values of the electrical response of the retina in STZ-diabetic rats. The improvement of W_2 oscillatory potential after canavanine pretreatment over that of the W_3 peak may indicate that GABAergic responses have a closer correlation with diabetic retinopathy than the status of the more distal, glycine-sensitive steps of visual stimulus transmission [20, 21]. The highest sensitivity of the W_2 oscillatory potential is in good agreement with the electroretinographic analysis of Sakai et al. [40] and Ishikawa et al. [41], and with the results of Ishikawa et al. [40] demonstrating increased GABA immunoreactivity in Muller cells of diabetic rat retinas but no changes in glycine immunoreactivity in the same area.

Individual values of a-wave or W_2 peak amplitudes show no significant correlation with the blood glucose or Hsp70 levels of the respective rats (data not shown), which indicates that neither the canavanine-induced slight improvement in blood glucose level nor a direct effect of canavanine on induction of Hsp70 can fully explain the dramatic improvement in the electroretinograms of canavanine-pretreated animals. Since plasma glucose levels of canavanine-treated diabetic rats were lower than those of untreated diabetic rats, and canavanine pretreatment caused a delay in secondary effects of diabetes such as body weight loss, the canavanine effect on the electroretinograms might be a consequence of a combined effect against the severity of diabetes. This combined effect probably incorporates both the canavanine-induced partial protection against the effect of streptozotocin and the protective effect of canavanine-induced Hsp70 on retinal tissue.

Besides being a heat shock protein-inducer amino acid analogue, canavanine is also a selective inhibitor of the inducible form of nitric oxide (NO) synthase [42], having a half-maximal inhibitory concentration for retinal NO synthase around 25 μM [43]. Guanidine analogues were shown to prevent diabetic vascular dysfunction via the inhibition of NO synthase and the formation of advanced glycation end products [44]. These mechanisms may be further important elements of canavanine-induced protection of retinal tissue. However, the significance of these effects is a bit questionable in our experiments, which measured electroretinograms 2 weeks after the end of the canavanine treatment.

Induced levels of retinal molecular chaperones might contribute to the reported improvement of diabetic electroretinograms by L-acetyl-carnitine [45], L-propionyl-carnitine [46], sorbinil [21, 45], beraprost sodium [47]

and by the simultaneous addition of aspirin and dipyridamole [48]. Our assumption is further substantiated by the fact that aspirin and other antiinflammatory agents such as cyclooxygenase and lipoxygenase inhibitors are known to enhance the induction of a great variety of molecular chaperones both in vitro and in vivo [49–51]. Canavanine pretreatment significantly improved the electrical responses of the diabetic retina. The improvement reached (or in some cases even surpassed) the control level at doses which are 5–10 times less than the reported lowest toxic levels [52, 53]. This small difference between the useful and toxic effects, however, makes the clinical use of canavanine as a drug to prevent diabetic retinopathy unlikely. Recently, the beneficial effects in diabetic wound healing and neuropathy of a molecular chaperone coinducer were reported [8, 9]. Our results are in agreement with the conclusions of these reports and prompt further investigations for the use of amino acid analogues other than canavanine and other nontoxic stress protein inducers in easing the chronic consequences of diabetes such as retinopathy.

Note added in proof. During the printing process of the present paper the study of Biro et al. [54] has been published, which describes the improvement of early changes in the electrical retinal responses of diabetic rats by Bimoclomol, a known chaperone-inducer drug-candidate. These effects of Bimoclomol support our findings and give a further example for the beneficial effects of chaperone inducers in prevention of chronic changes in diabetes.

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