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ORIGINAL ARTICLE

Gabapentin Attenuates Oxidative Stress and Apoptosis in the Diabetic Rat Retina

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Abstract

Neurodegeneration in diabetic retina has been widely considered as initiating factor that may lead to vascular damage, the classical hallmark of diabetic retinopathy. Diabetes induced altered glutamate metabolism in the retina, especially through glutamate excitotoxicity might play a major role in the neurodegeneration. Increased level of branched chain amino acids (BCAAs) measured in diabetic retina might cause an increase in the neurotoxic level of glutamate by transamination of citric acid cycle intermediates. In order to analyze the transamination of BCAAs and their influence on neurodegenerative factors, we treated streptozotocin-induced diabetic rats with gabapentin, a leucine analogue and an inhibitor of branched chain amino transferase (BCATc). Interestingly, gabapentin lowered the retinal level of BCAAs in diabetic rats. Furthermore, gabapentin treatments ameliorated the reduced antioxidant glutathione level and increased malondialdehyde (MDA), the marker of lipid peroxidation in diabetic rat retinas. In addition, gabapentin also reduced the expression of proapoptotic caspase-3, a marker of apoptosis and increased anti-apoptotic marker Bcl-2 in diabetic retinas. Thus, these results suggest that gabapentin stimulates glutamate disposal, and ameliorates apoptosis and oxidative stress in diabetic rat retina. The influence of gabapentin may be due to its capacity to increase the ratio of BCKA to BCAA which in turn would reduce glutamate excitotoxicity in diabetic retina.

Keywords Gabapentin · Glutamate · Neurodegeneration · Retina · Diabetes

Introduction

Diabetic retinopathy is a severe complication of diabetes that is characterized by vision loss and blindness in patients with diabetes. Alterations in retinal vasculature and neovascularization are known classical hallmark of diabetic retinopathy. Over the last two decades, early neurodegeneration in diabetic retina has been widely considered as initiating factor that may lead to vascular damage (Feng et al. 2009; Moran et al. 2016). A growing body of evidence suggest that the neuronal component of the retina is compromised, particularly glial cells are activated and retinal ganglion cells die early in diabetic retinopathy (Antonetti et al. 2006; Barber et al. 1998; Ola et al. 2012). This is well supported by numerous functional studies of diabetic retina using electroretinogram, dark adaptation, contrast sensitivity, and color vision tests, indicating that neuronal function is compromised before the onset of vascular lesions both in humans and animal models of diabetes (Bresnick and Palta 1987; Bearse Jr et al. 2004; Bengtsson et al. 2005). The exact reason(s) of neuronal cell damage early in diabetic retina is not known. However, several studies reported increased levels of neurodegenerative metabolites and altered levels of neurotrophins in the diabetic retina (Ola and Alhomida 2014; Gardner and Davila 2017). Glutamate is one of the major excitotoxic metabolites found to be increased in the vitreous and retina of diabetic patients, and also in animal models of diabetes which may implicate neuronal damage in the retina (Ishikawa et al. 1995; Sucher et al. 1997; Ambati et al. 1997). This is evident from an elegant study of Kusari et al. (2007) which reported that blockade of the excitatory glutamate receptors of postsynaptic neurons could improve neuronal function and viability of diabetic retinal neurons.

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Various possible mechanism(s) for deregulated glutamate metabolism in the diabetic retina have been suggested that may cause neurodegeneration. For instance, diabetes alters enzymes and transporters, responsible for glutamate uptake and disposal within the retina. This alteration in glutamate metabolism especially within Muller cells may cause an increase in synaptic glutamate levels in the retina leading to glutamate excitotoxicity and neuronal cell death (Lieth et al. 2000; Li and Puro 2002). In addition, few studies reported an excess of branched chain amino acids (BCAAs) valine, leucine, and isoleucine in the serum and retinas of diabetic rats (Frayser and Buse 1978; Gowda et al. 2011) that might cause an increase in the level of glutamate by transamination of α-ketoglutarate within glial cells just as they do in cultured brain astrocytes (McKenna et al. 1998; Hutson et al. 1998; Ola et al. 2011). To produce glutamate from citric acid cycle intermediates, a source of nitrogen is needed which is provided by excess BCAAs using either of the two isoforms of branched-chain aminotransferase (BCAT), mitochondrial branched-chain aminotransferase (BCATm) expressed in Muller cells and a cytosolic BCAT isoform (BCATc) only expressed in the neuronal cytosol (Hutson et al. 1998). In addition, the increased level of BCAAs may interfere with glutamate clearance from synaptic space resulting in excitotoxicity to post-synaptic neurons (Hutson et al. 1998; Sweatt et al. 2004). As a result of glutamate excitotoxicity, two major events seem to play an important role in the death of neurons: the increase in the production of free radicals and the induction of the apoptotic cascade which may trigger pathological mechanisms leading to neuronal death (Lipton and Rosenberg 1994; Niizuma et al. 2009).

Gabapentin (Neurontin) is a leucine analogue and a specific inhibitor of BCATc (Goto et al. 2005), which has been used in the treatment of partial epilepsy and neuropathic pain (Rossi et al. 2013). Previously, our group studied the effect of gabapentin in slowing down the formation of glutamate and glutamine from bicarbonate while increasing the appearance of α-ketoglutarate and other citric acid cycle intermediates in the retinas and in cultured brain astrocytes (Hutson et al. 1998; Lieth et al. 2001; Hutson et al. 2001; Xu et al. 2004). Gabapentin may influence a decrease in the level of BCAAs by inhibiting BCATc in the diabetic retinas, which in turn may lower glutamate synthesis and increase rates of glutamate oxidation as shown in the schematic diagram of Fig. 1. Thus, gabapentin might be a potential therapeutic drug in ameliorating oxidative stress and apoptosis in diabetic retina by inhibiting glutamate excitotoxicity (Kim et al. 2009). Till date, no study has examined the new role of gabapentin that is associated with neuroprotection in diabetic retina. In this study, we employed 2-week oral treatments of gabapentin to diabetic rats in order to analyze the beneficial effects on altering glutamate metabolism, which might be protective toward oxidative stress and apoptosis in the retina.

Methods

Animals and Treatments

Sprague-Dawley rats were used for all experiments. Rats were housed under a 12:12-h light-dark cycle and allowed free access to food and water. Twelve-week-old rats, weighing 250–300 g, were received from Experimental Animal Care Center (King Saud University, Riyadh, Saudi Arabia). Diabetes was induced by single intraperitoneal injection of streptozotocin (STZ) (Sigma, St. Louis, MO, USA) at a dose of 65 mg/kg body weight, dissolved in 0.1 mol/L citrate-buffered solution (pH 4.5). Three days after STZ injection, blood glucose was measured and blood glucose > 250 mg/dL was considered diabetic and included in the study. After 5 weeks of diabetes, rats were randomly divided into four groups as follows: group 1: control rats treated with vehicle (C); group 2: control rats treated with gabapentin (C + G); group 3: diabetic rats treated with vehicle (D); and group 4: diabetic rats treated with gabapentin (D + G). Gabapentin (Cat No: PHR1049; Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) diluted in sterile physiological saline was given orally (300 mg/kg/daily) by gavage for 2 weeks to the diabetic rats. At the end of treatments, animals were fasted overnight, then anesthetized with ketamine-xylazine (53 mg ketamine, 5.3 mg xylazine/kg) and fasting blood samples were collected and serum separated and stored. Retinas were dissected and isolated immediately, rinsed in ice-cold saline, and kept at − 70 °C until analysis. All experimental procedures and protocols including anesthesia were in accordance with the Association for Research in Vision and Ophthalmology (ARVO) guidelines to the Care and Use of Experimental Animals. The experimental animal protocol used for the current study has been approved by the Experimental Animal Care Center Review Board, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia.

Measurements of BCAAs and BCKAs in Retina

The retina of gabapentin treated and untreated diabetic and non-diabetic rats were lysed in 50 mM HEPES buffer, pH 7.5 containing protease inhibitor mixtures and sonicated. The extract was centrifuged at 15,000×*g* for 20 min, and the supernatant was assayed as described previously (Gowda et al. 2011). The mass amount of branched chain ketoacids (BCKAs) namely α-keto-isovaleric acid (KIV), α-keto-isocaproic acid (KIC), and α-keto-β-methylvaleric acid (KMV) were quantitated in a similar way by measuring the change in nicotinamide adenine dinucleotide (NADH) (fluorometrically) (Gowda et al. 2011). In this assay, the sample is added to a cuvette with buffer containing Hepes, $MgCl_2$, EDTA, aspartate, glutamate, aspartate aminotransferase

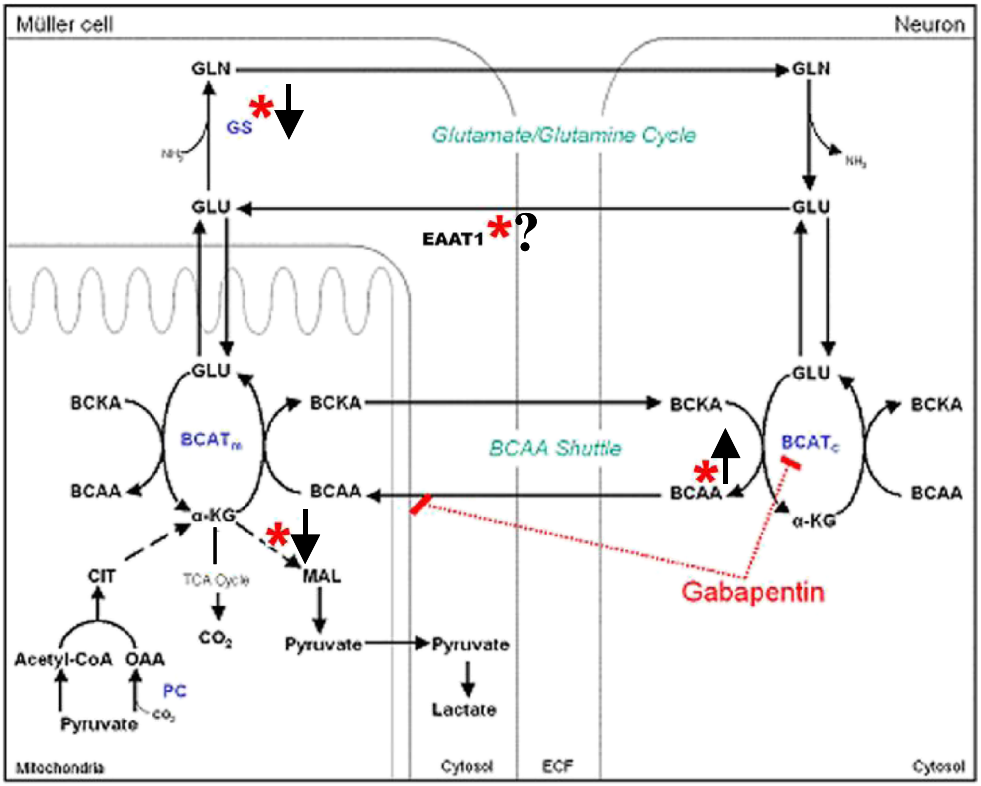

Fig. 1 Schematic diagram of glutamate metabolism and inhibition of BCAAs by gabapentin in neural tissues. The scheme highlights the influence of gabapentin on glutamate transamination with branched-chain keto acids in neurons and the inhibition of the transport of branched-chain amino acid into Müller cells of the product of that transamination. Abbreviations are as follows: *GLN* glutamine, *GLU* glutamate, *MAL* malic acid, *CIT* citrate, *α-KG* α-ketoglutarate, *OAA* oxaloacetate, *BCAA* branched-chain amino acid, *BCKA* branched-chain keto acid, *GS* glutamine synthetase, *PC* pyruvate carboxylase, *BCATm* mitochondrial isoform of branched-chain aminotransferase, *BCATc* cytosolic isoform of branched-chain aminotransferase, *EAAT1* Müller cell Na^+-linked glutamate transporter. Asterisks indicate proposed sites of diabetes effects; arrows indicate direction of effects

(AspAT), malate dehydrogenase (MDH), and NADH. Fluorescence is measured before and after addition of BCATc. The change in NADH fluorescence provides a measure of BCKA. Fluorescence changes are measured using a custom made fluorometer built by C&L instruments, Inc. Hummelstown, USA. Retinal BCAAs leucine, isoleucine, and valine were derivatized with phenylisothiocynate, and then separated and quantified by reverse phase high-performance liquid chromatography (Hariharan et al. 1993).

Glutathione Assay

The total glutathione (GSH) levels were measured in the retina of gabapentin treated and untreated diabetic and non-diabetic rats using the method described by Sedlak and Lindsay (1968) with slight modification. Retinal homogenate was prepared in the 50 mM HEPES lysis buffer, pH 7.4, containing 100 mM NaCl, 1% triton X-100, 0.2% sodium dodecyl sulfate (SDS), and protease inhibitor cocktail. The homogenate was centrifuged at 15,000×g for 20 min, and the supernatant separated for GSH assay. Retinal supernatant was deproteinized by adding an equal volume of metaphosphoric acid (2.5%, *w*/*v*). The mixture was centrifuged at 12,000 g for 5 min and supernatant collected. In the 50 μL supernatant, 5 μL of 4 M triethanolamine per 100 μL was added and assay was performed. To this mixture, 100 μL of 0.01 M Ellman's reagent (5,50-dithiobis-(2-nitro-benzoic acid)) (DTNB) was added. The absorbance of the clear supernatants was recorded to measure the concentration of GSH using spectrophotometer at 412 nm within 5 min. A standard curve of GSH was prepared from 0 to 10 μM.

Estimation of Malondialdehyde Levels

The lipid peroxidation product malondialdehyde (MDA) levels were measured in the retina using a commercially available assay kit (ZeptoMetrix Co., Buffalo, NY, USA). Retinal homogenate was prepared by applying short burst of ultrasonication in the 10 mM HEPES lysis buffer, pH 7.4, containing 100 mM NaCl, 1% triton X-100, 0.2% sodium dodecyl sulfate (SDS), and protease inhibitor cocktail. The homogenates were then centrifuged at 15,000 g for 20 min at 4 °C. Following the centrifugation, supernatants were separated and collected for MDA quantification. Then, 100 μL of the supernatant was mixed with 2.5 mL of reaction buffer provided in the kit. The mixture was then heated at 95 °C for 60 min. After cooling and centrifugation, the MDA-TBA adducts formed by the reaction of MDA and TBA under high temperature and acidic conditions were measured calorimetrically at 532 nm. A standard curve ranging from 0 to 100 nmol/mL MDA content was used. The protein concentrations in each sample were estimated using Lowry method (Lowry et al. 1951).

Measurement of Reactive Oxygen Species Generation Under In Vivo Condition in the Rat Retina

In this study, we have adapted a new technique for reactive oxygen species (ROS) measurement in the retinas of 5 weeks diabetic, diabetic treated with gabapentin, and age-matched control rats under in vivo conditions. The measurement of ROS is based on a fluorogenic marker 5-(and 6-) chloromethyl-2,7-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA), a chloromethyl derivative of H_2DCFDA (from Molecular Probes). The fluorogenic marker passively diffuses into cells and after subsequent cleavage of ester bonds, the dye are trapped inside the cell due to negative charge carried by carboxy-H_2DCFDA. Subsequent oxidation of some dye yields fluorescent adducts which provides a measure of ROS.

We made a stock solution (2.16 mM in DMSO) of CM-H_2DCFDA just before each experiment and 3 μL from the stock was injected intravitreally into the both eye cavities of anesthetized rats. After 6 h, the dye-injected rats were anesthetized and the whole retina dissected and then immersed in 300 μL of 50 mM HEPES buffer, pH 7.4 containing 0.1% SDS. Retinas were sonicated, centrifuged, and 100 μL supernatant assayed fluorometrically using excitation and emission wavelengths of 485 and 538 nm respectively with a plate reader (SpectraMax Plus; Molecular Devices, Sunnyvale, CA).

Measurement of Caspase-3 Activity in Retinas

Rats retinas were excised immediately and sonicated on ice in 100 μL of lysis buffer (25 mmol/l HEPES (pH 7.5), 5 mmol/l $MgCl_2$, 5 mmol/l EDTA, 5 mmol/l dithiothreitol, 1% Nonidet P-40) that contained one tablet per 10 mL of complete EDTA-free protease inhibitor cocktail (Roche). The sonication was followed by 20-min incubation at 4 °C and centrifugation at 15,000 *g* at 4 °C. Caspase-3 activity was measured in the supernatants, with enzyme activity normalized to protein content as measured by a Bio-Rad protein assay (Bio-Rad, Hercules, CA). The Fluorometric CaspACE Assay System was purchased from Promega (Madison, WI) and used according to the manufacturer's directions for the 96-well plate assay format with minor modifications (incubations were performed at 37 °C instead of 30 °C). Duplicate assays were performed for each sample, with blanks and negative controls. A 7-amino-4-methyl coumarin (AMC) standard curve was run with each group of unknowns. Fluorescence was measured using a SpectraMAX Gemini XS (Molecular Devices, Sunnyvale, CA) fluorescence plate reader at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Caspase-3 enzyme activity is expressed as picomoles of AMC liberated per milligram of protein per minute.

Western Blot Analysis

Western blot was used for the expression of proapoptotic and anti-apoptotic proteins expression in the retina of control, diabetic, and gabapentin-treated diabetic rats. We analyzed the expression of Bcl-2, and caspase-3 proteins. First, we made retinal tissues homogenate by ultrasonication in the 10 mM HEPES buffer (pH 7.4), containing 100 mM NaCl, 1 mM Na_3VO_4, 10 mM sodium pyrophosphate, 10 mM NaF, 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100, 0.2% SDS, and a protease inhibitor cocktail. Samples were centrifuged at 15,000 g for 15 min in cooling centrifuge and supernatants collected and the protein concentrations estimated. Protein samples were boiled in Laemmli sample buffer for 5 min, and equal amount of proteins (50 μg/well) were separated on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. After transferring the proteins, the membranes were blocked for 90 min at room temperature with 5% non-fat milk made in Tris-buffered saline containing 0.1% Tween-20 (TBS-T). The membranes were incubated overnight at 4 °C with anti-Bcl-2 and anti-caspase-3 (1 μg/mL; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) primary antibodies. After overnight incubation with primary antibodies, membranes were washed three times with TBS-T (5 min each) and then incubated with their respective secondary horseradish peroxidase-conjugated antibodies (1:2000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at room temperature for 90 min. Membranes were then washed four times with TBS-T for 5 min each, and the immunoreactivity of bands was visualized on a LI-COR C-Digit Blot Scanner from Biosciences, Lincoln, NE, USA, using enhanced chemiluminescence (Western blotting luminol reagents (1:1), Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Protein bands were quantified by densitometry analysis using Image-Lab 2.0.1 software (Bio-Rad Laboratories Inc., Hercules, CA, USA). For internal control, membranes were washed and incubated with a mouse monoclonal β-actin antibody (1:2000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and all the steps were followed as described above.

Statistical Analysis

All statistical analysis was conducted using Statistical Package for the Social Sciences Version 12 (SPSS 12.0, Chicago, IL, USA). All data are reported as means ± SEM. Analysis of groups were determined using one-way analysis of variance (ANOVA) with Mann-Whitney test for analysis of pre- and post-treatment measurements between diabetes, control, and gabapentin-treated groups. Statistical significance was accepted when $p < 0.05$.

Results

Gabapentin Reduced the Level of Branched Chain Amino Acids in the Diabetic Rat Retinas

The three branched chain amino acids (leucine, isoleucine, and valine) were quantitated separately from each group of control, diabetic, and gabapentin-treated diabetic rats. But all the three amino acids were pooled together from each group of rats to include in branched chain amino acids (BCAAs) as shown in the Table 1. Similarly, branched chain keto acids (KIV, KIC, and KMV) were also measured separately but added together from each group of rats termed as BCKAs. The quantification of the amounts of BCAAs in the retinas revealed a significant increase in BCAAs in diabetic retinas compared to age matched controls (36.89 ± 6.59 vs. 21.36 ± 2.38 nmol/mg proteins; $p < 0.01$), while the amount of BCKAs were similar in control and diabetic retinas. However, in the gabapentin-treated diabetic rats, a significant decrease in BCAAs were observed compared to untreated diabetic rats (25.66 ± 4.34 vs. 36.89 ± 6.59 nmol/mg protein; $p < 0.05$). The levels of BCKAs were slightly but not significantly increased in gabapentin treated diabetic retina compared to untreated diabetic rats.

Gabapentin Increased the Level of Glutathione and Reduced Lipid Peroxidation in Diabetic Rat Retinas

The level of glutathione (GSH) and malondialdehyde (MDA) were measured with and without gabapentin treatment in the retina of diabetic rats as a measure of oxidative stress. GSH is the endogenous antioxidant, and MDA is usually a standard marker for lipid peroxidation. The level of GSH significantly decreased to almost 40% ($p < 0.01$) in diabetic retinas as compared to controls. However, gabapentin treatments to diabetic rats augmented the retinal GSH almost to control level, and significantly increased as compared to non-treated diabetic rats (65.2 ± 6.5 vs. 45.3 ± 4.5 nmol/μg protein; $p < 0.05$) (Fig. 2a). Whereas, the level of MDA increased to almost twofold in diabetic retinas compared to controls. Gabapentin treatments to diabetic rats significantly reduced the retinal levels of MDA as compared to non-treated diabetic rats (45.5 ± 5.2 vs.75.3 ± 7.4 nmol/mg protein; $p < 0.05$) (Fig. 2b). Thus, diabetes significantly elevated oxidative stress

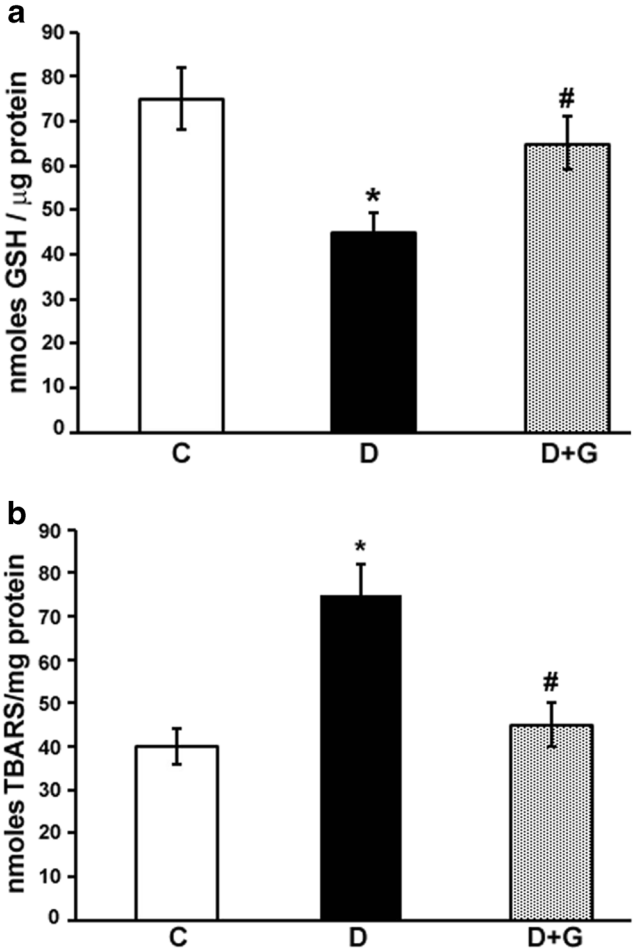

Fig. 2 Effects of gabapentin on glutathione (GSH) and thiobarbituric acid reactive substances (TBARS) levels in the retina of diabetic and non-diabetic animals. **a**, **b** Represents measurements of GSH and TBARS respectively. Values are expressed as means ± SEM (standard error of mean); $n = 6$/group. Diabetic (D) significantly different from control (C) (*$p < 0.01$); and D + G significantly different from D (#$p < 0.05$). C represents control, D as diabetic, and D + G as diabetic rats treated with gabapentin

Table 1 The effect of gabapentin on retinal BCAA and BCKA

Conditions	Branched chain amino acids (BCAAs) nmol/mg protein	Branched chain keto acids (BCKAs) nmol/mg protein
Control (C)	21.36 ± 2.38*	0.65 ± 0.13
Diabetic (D)	36.89 ± 6.59	0.60 ± 0.08
Diabetic + gabapentin (D + G)	25.66 ± 4.34**	0.71 ± 0.09

Branched chain amino acids (BCAAs) and branched chain keto acids (BCKAs) were measured in retinal extracts from control and 5-week diabetic rats, treated or not treated with gabapentin (300 mg/kg/day for 2 weeks). BCKAs were measured fluorometrically and BCAAs were measured by HPLC. *$p < 0.01$ C vs. D; **$p < 0.05$ D vs. G

in rat retinas; however, gabapentin treatment reduced their level by increasing the level of antioxidant, GSH, and reducing the lipid peroxidation in the retinas of diabetic rats similar to those observed in non-diabetic controls.

Reactive Oxygen Species Generation Under In Vivo Condition in the Rat Retina

We have successfully adapted the technique for reactive oxygen species (ROS) measurement under in vivo conditions in rat retinas. This adaptation used carboxy-H_2DCFDA, a chemically reduced acetylated form of fluorescein derivative as a cell permeant indicator for ROS. The oxidation by ROS makes the dye fluorescent (Jakubowski and Bartosz 2000). First, we standardized this technique and optimized the time period for the injected dye into the vitreous to diffuse into the retina. We found that 6 h of incubation time after injection a small amount of the dye (3 μL, 2.16 mM) was sufficient to obtain maximum diffusion into the retinal cells as measured by maximum florescence in the retina. We injected the dye into the eye cavity of anesthetized control, diabetic, and gabapentin-treated diabetic rats. Data obtained using this procedure is shown in Fig. 3. ROS levels were increased to almost 55% in the diabetic retinas compared to control rats ($p < 0.05$). In the gabapentin-treated diabetic rats, ROS levels declined to near control levels as measured by dye fluorescence. Thus, we developed successfully the in vivo technique to analyze the oxidative stress in the rat retinas. Oxidative stress determination using this in vivo technique seems to be comparable to results obtained by the analysis of the markers of oxidative stress determined using in vitro techniques.

Gabapentin Attenuated Apoptosis in the Diabetic Retina

To characterize gabapentin effects on apoptosis, we assessed the ability of systemic gabapentin treatment to decrease the activation of caspase-3 within the retina of diabetic rats. Retina lysates from gabapentin-treated or untreated diabetic rats, or treated or untreated control rats were assayed for caspase-3 activity as a measure of apoptosis. Caspase-3 activity increased almost to 32% within diabetic retina compared with control rats ($p < 0.05$) (Fig. 4). Gabapentin treatment significantly attenuated the increase in caspase-3 activity in the diabetic retina ($p < 0.003$). There was no statistical difference between the levels of caspase-3 activity in control animals, administered vehicle or administered gabapentin. These experiments demonstrate that treatment with gabapentin in diabetes can significantly reduce apoptosis within the retina.

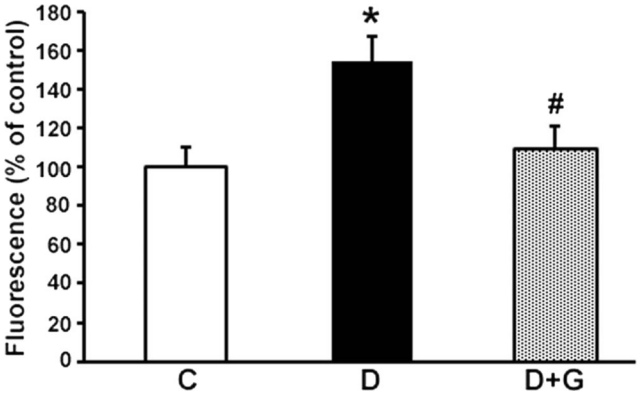

Fig. 3 The effect of gabapentin on ROS levels in the retinas of control, diabetic, and diabetic rats treated with gabapentin. Fluorescence from oxidized H_2DCFDA in the retina is proportional to ROS. Values are expressed as means ± SEM. *$p < 0.01$, control (C) vs. diabetic (D); #$p < 0.05$, diabetic + gabapentin (D + G) vs. diabetic (D) rats groups ($n = 7$)

Amelioration of the Retinal Expression of Bcl-2 and Caspase-3 in the Gabapentin-Treated Diabetic Rats

The expression of proapoptotic proteins, caspase-3, and anti-apoptotic protein Bcl-2 were examined in the retinas of control, diabetic, and gabapentin-treated diabetic rats by Western blot analysis (Fig. 5). Densitometry analyses of the bands show that expression levels of Bcl-2 reduced significantly in the diabetic retina compared to controls (100 ± 10.1 vs. 40 ± 5.3%; $p < 0.01$). However, the decreased level of Bcl-2 in diabetic retinas was significantly ameliorated when treated with gabapentin (40 ± 5.3 vs. 75 ± 8.2%; $p < 0.05$). Expression levels of proapoptotic caspase-3 increased significantly in the diabetic retinas as compared to controls ($p < 0.01$). Moreover, gabapentin administration to diabetic rats lowered the level of caspase-3 in the diabetic retina almost to their control levels ($p < 0.05$).

Discussion

The evidence that glutamate plays a role in neurodegeneration at an early stage of diabetic retinopathy is based in part on measurements of elevated glutamate in the vitreous and retina of patients with diabetes and in experimental diabetic rodents, besides altered glutamate metabolism observed in diabetic retinas (Ambati et al. 1997; Lieth et al. 2000; Li and Puro 2002; Gowda et al. 2011). Early onset of glutamate excitotoxicity and neurodegeneration in the retinas of diabetic rats has gained further credibility since an antagonist of *N*-methyl-D-aspartate (NMDA)-type glutamate receptor successfully combated the progression of retinal neurovascular degeneration in rats (Kusari et al. 2007). However, the exact

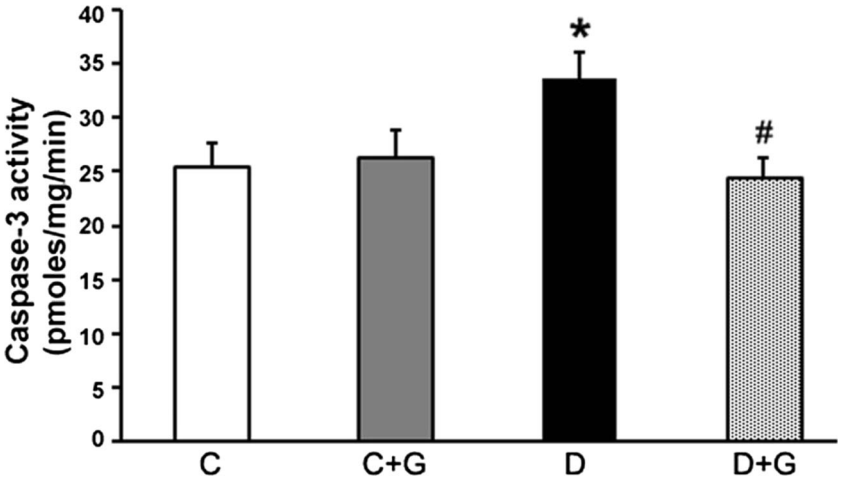

Fig. 4 Gabapentin attenuates the increase in retinal caspase-3 activity. Five-week diabetic, age-matched control rats were orally fed gabapentin (300 mg/kg/day) for 2 weeks. Protein lysates from isolated retinas were analyzed for caspase-3 activity. Data are means ± SEM. *$p < 0.003$ between control and diabetic rats. There was no statistical difference between control animals administered vehicle and diabetic animals administered gabapentin. #$p < 0.05$, significantly different from diabetic group

reason(s) for the increase in glutamate level in the diabetic retina is not known. Diabetes induced hyperglycemia is believed to be a potential factor in the increased level of glutamate in diabetic retina. Previously, our metabolic studies indicated that, despite having excess glucose in the diabetic retina, the retina oxidized less glucose to CO_2 and glutamate (Ola et al. 2006). Therefore, it is highly unlikely that hyperglycemia would escalate the glutamate level by increasing the flux through the citric acid cycle intermediates. Other possibilities of the increased level of glutamate might be due to an excess

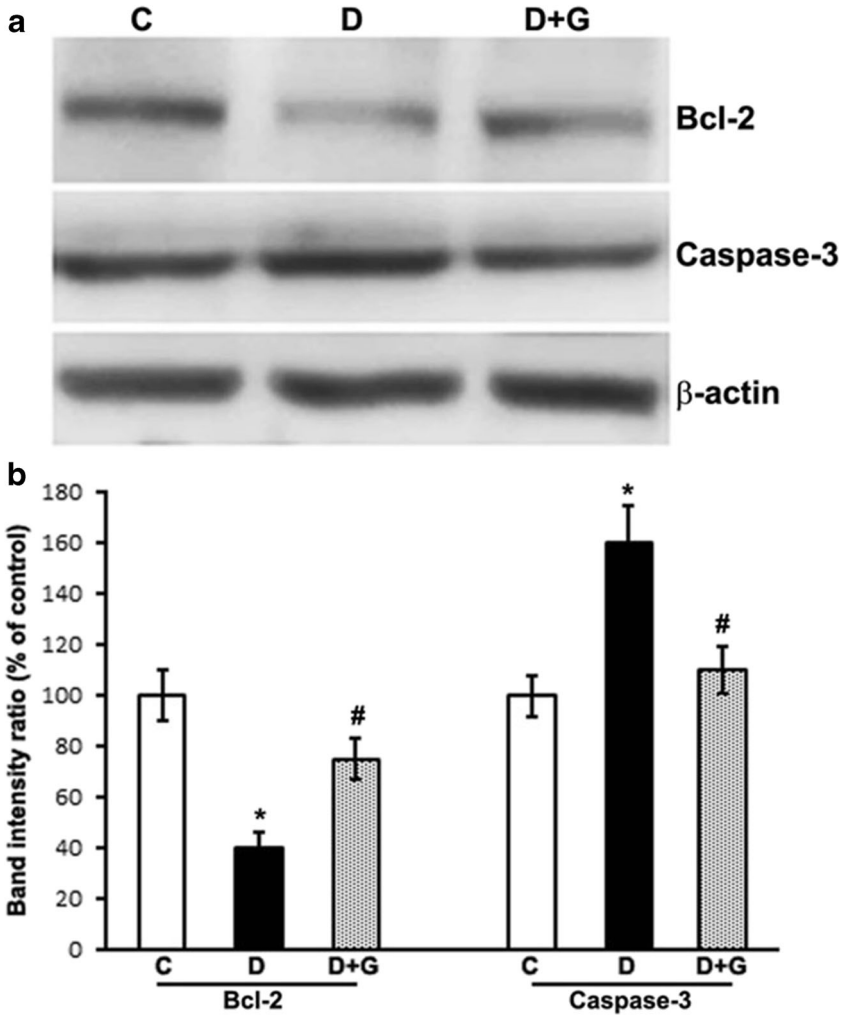

Fig. 5 Western blot analysis of the expression of Bcl-2 and capspase-3 in retinas from control, diabetic, and gabapentin-treated diabetic rats. The intensities of the bands were quantified by densitometry. Panel **a**, representative immunoblots of Bcl-2, caspase-3, and β-actin bands. Panel **b**, data presented as percent of control of band intensities ratios of those protein bands to β-actin. Values are means ± SEM for six determinations. *$p < 0.01$, significantly different from their controls; #$p < 0.05$, significantly different from diabetic. Immunoblotting experiments were repeated twice

level of BCAAs as measured in the serum and retinas of diabetic rats (Frayser and Buse 1978; Gowda et al. 2011). In agreement with previous studies, we also found an increased level of BCAAs in the diabetic retina which would likely favor the accumulation of high intra and extracellular glutamate. Thus, the increased level of glutamate may interfere with glutamate clearance from synaptic space, which in turn would further increase glutamate levels, causing glutamate excitotoxicity to postsynaptic neurons (Gowda et al. 2011).

One of the potential strategies to attenuate the neurotoxic effects of glutamate is through inhibiting the synthesis and transport of glutamate, which otherwise may induce the glutamate excitotoxicity (Kusari et al. 2007; Ola et al. 2011). Earlier, few studies reported the beneficial effects of gabapentin in neuroprotection in chronic glutamate-mediated neural diseases and in the central nervous system of diabetic rats (Rothstein and Kuncl 1995; Baydas et al. 2005). Previously, we and others also reported that gabapentin lowers the synthesis of glutamate by transamination of BCAAs to BCKAs which in turn may increase oxidation of glutamate by the mitochondria (Ola et al. 2011; Hutson et al. 1998). Therefore, in this study, we employed gabapentin, a leucine analogue and specific inhibitor of BCATc, which may ameliorate altered glutamate synthesis and protect neurons. Indeed, when diabetic rats were treated with gabapentin, the increased level of BCAAs reduced to almost control level. However, only a slight increase in the level of BCKAs was observed in the gabapentin-treated diabetic rats compared to untreated rats. The increased level of BCKAs and lower level of BCAAs would favor the transamination reaction in the direction of glutamate oxidation which may reduce the pool size of glutamate. Thus, our results indicate that gabapentin decreased the ratio of BCAAs to BCKAs, either by lowering BCAAs or increasing BCKAs levels would favor in reducing the glutamate level in diabetic rats (Xu et al. 2004). These results are supported by ex vivo experiments when excised retinas were incubated in a physiological buffer with pyruvate and $^{14}CO_2$, gabapentin inhibited ^{14}C-glutamate synthesis (Lieth et al. 2001; Hutson et al. 2001). In another cellular study, Tönjes et al. 2013 investigated that gabapentin significantly lowered the BCAAs levels in glioblastomas. Moreover, addition of branched-chain keto acids to the medium surrounding excised retinas increased oxidation of glutamate to pyruvate, lactate, and CO_2 (Lieth et al. 2001; Hutson et al. 1998). Hence, our data suggest that gabapentin is likely to decrease the intracellular level of glutamate which in turn may lead to an increase in the clearance of extracellular glutamate to prevent glutamate excitotoxicity.

Diabetes induced increase in the level of oxidative stress has been recognized as a central factor in damaging the retina. However, the potential source of oxidative stress is still obscure especially early in diabetic retina (Ola et al. 2012). We speculate that glutamate excitotoxicity, due to increased levels of BCAAs, might implicate in the increase of oxidative stress leading to neuro-retinal damage in diabetic retinopathy. In agreement with previous studies, here we observed a low content of glutathione, an endogenous antioxidant, and an increased level of lipid peroxidation in diabetic rat retinas compared to normal retinas (Al-Dosari et al. 2017; Ola et al. 2015). However, gabapentin treatment to diabetic rats ameliorated the level of both glutathione and lipid peroxidation in retinas. In addition, we developed a new in vivo method to analyze the level of free radical generation in the rat retinas. In general, most of the researchers have been using dichlorofluorescin (DCF) derivatives for reactive oxygen species (ROS) detection by applying them to cultured cells or in vitro using frozen tissue. However, none have reported evidence of oxidative stress as we have reported here under in vivo conditions (as described in the method section). As expected, the level of ROS production was significantly higher in the diabetic retinas compared to non-diabetic rats. Diabetes may cause the release of extracellular glutamate, which activates NMDA receptors to induce a transmembrane ion imbalance, in particular a calcium influx, that in turn generates reactive oxygen species (Dong et al. 2006). These free radicals actively attack macromolecules within neurons and glial cells, resulting in structural and functional changes in proteins. Interestingly, treatment with gabapentin significantly reduced the level of ROS in the retina of diabetic rats compared to the control. The possible explanation for the effect of gabapentin on reduction of oxidative stress is through a decrease in glutamatergic neurotoxicity as reported in diabetic rat brain tissues (Baydas et al. 2005; Shimoyama et al. 2000). In addition, our findings in regard to the expression of apoptotic markers in diabetic rats are also in agreement with previous reports which show that oxidative insult may respond to neuronal cell death by increased apoptosis in diabetic retina (Barber et al. 1998; Ola et al. 2018). Indeed, we observed an increased level of capsase-3 and a lower level of anti-apoptotic Bcl-2 in the diabetic retina, but gabapentin ameliorated their levels to almost control level. Thus, these data provide the first indication that gabapentin regulates the expression of apoptotic markers, and thus a new role for gabapentin is associated with neuroprotection in the diabetic retina.

Despite of the numerous beneficial effects of gabapentin, several studies also reported adverse effects including dizziness, fatigue, drowsiness, weight gain, and peripheral edema (Parke-Davis 2009), blurred vision, and diplopia (Herranz et al. 2000). Recently, Kim et al. (2016) reported that oral gabapentin treatment caused macular edema and serous retinal detachment of macula in eyes as determined by dilated fundus examination and spectral domain optical coherence tomography. However, the precise mechanism of gabapentin action on macular edema needs to be investigated. Therefore, continued monitoring and clinical trials of the gabapentin are always warranted for the safer use in patients.

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