Effect of Memantine on Neuroretinal Function and Retinal Vascular Changes of Streptozotocin-Induced Diabetic Rats

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PURPOSE. To test whether chronic memantine (MEM) treatment improves retinal function and prevents neurodegeneration and vascular changes in the retinas of streptozotocin (STZ)-induced diabetic rats.

METHODS. Based on basal body weight and blood glucose, Brown Norway (BN) rats were divided into three groups. One group of rats was treated with vehicle (VEH), and the other two groups were treated with 65 mg/kg STZ. After 7 days, VEH-treated rats were treated further with a second VEH, and STZ-treated diabetic rats were treated either with the second VEH or with MEM (10 mg/kg daily) for another 3 to 4 weeks using mini-osmotic pumps. At end of the study, electroretinogram findings, retinal ganglion cell (RGC) count, vitreoretinal vascular endothelial growth factor (VEGF) protein levels, and blood-retinal barrier (BRB) breakdown of the animals were measured.

RESULTS. Within 4 to 5 weeks of STZ treatment, the diabetic rats demonstrated significantly less retinal function and fewer RGCs than VEH-treated nondiabetic rats. The diabetic animals also had significantly elevated VEGF protein levels in retina and vitreous fluid and BRB breakdown compared with control nondiabetic rats. Chronic MEM treatment significantly improved retinal function and protected RGC loss in STZ-induced diabetic rats. MEM treatment also significantly decreased elevated vitreoretinal VEGF protein levels and retinal BRB leakage in the diabetic rats. This effect of MEM was not seen in nondiabetic rats.

CONCLUSIONS. These results indicate that MEM could be useful for the treatment of ocular diseases, including diabetic retinopathy with neurodegeneration, elevated vitreoretinal VEGF protein levels, and increased BRB breakdown. In addition to the neuroprotective effect of this compound, MEM can reduce vascular changes seen in diabetic retinas. These data are the first to identify the vasculoprotective effect of MEM. (Invest Ophthalmol Vis Sci. 2007;48:5152–5159) DOI: 10.1167/iovs.07-0427

Diabetic retinopathy (DR) is the most common complication of diabetes; it affects more than 90% of persons with diabetes and progresses to legal blindness in approximately 5%. DR has long been considered a microvascular disease, and the blood-retinal barrier (BRB) breakdown is a hallmark of this disease.1 However, DR has also recently been viewed as a neurodegenerative disease of the retina. Much evidence suggests that changes in the functional molecules and viability of neurons in the retina occur early after the onset of diabetes, preceding the vascular complications in humans and experimental animals.2 In terms of an effective pharmacologic treatment for this diabetic complication, inhibition of hyperglycemia by insulin has been found to inhibit retinal neurodegeneration in diabetic rats,3 but this has not yet been assessed in humans with diabetes.

The most widely accepted animal model for the evaluation of retinal complications in diabetes is the streptozotocin-induced diabetic rat. The retinal lesions observed in the diabetic rats resemble the initial process of diabetic retinopathy that occurs in humans.3 Thickening of the basement membrane with increased glucose concentrations is always observed in all species and represents the first lesion during the retinopathic process that culminates in the formation of new vessels.4,5 In addition, other vascular changes, including microaneurysms, decrease in pericyte number, increased vascular permeability, breakdown of the BRB, and early growth factor changes that are characteristics of background/nonproliferative diabetic retinopathy, are also observed in STZ-treated diabetic rats.6–8 It has also been shown using STZ-treated rats that significantly more neuronal cells undergo apoptosis in retinas of diabetic rats than in control animals.9,10 Others have observed loss of the axonal fibers in diabetic rat retinas.11,12 Electroretinography performed in diabetic rats has detected reduced electroretinographic (ERG) responses as early as 2 weeks after the onset of diabetes.12 Thus, STZ-induced diabetic rats exhibit retinal dysfunction, degeneration, and vascular alterations characteristics of early diabetic retinopathy.

Glutamate is the major excitatory neurotransmitter in the retina and is involved in neurotransmission from photoreceptors to bipolar cells and from bipolar cells to ganglion cells. However, elevated glutamate level is implicated in neurodegeneration.13 Vitreoretinal glutamate levels are elevated in patients with diabetes and in experimental diabetes.14–16 Diabetic-induced dysfunction of the glutamate transporter in retinal Müller cells is responsible for elevated retinal glutamate levels.17 Expression of the N-methyl-D-aspartate (NMDA)-type glutamate receptors is also upregulated in the diabetic retina.18 Experimental diabetes in rats and diabetes mellitus in humans are accompanied by increased apoptosis of retinal neural cells.9 Excessive retinal glutamate and NMDA receptor activity in diabetes most probably increases intracellular Ca++ concentration, which sets in motion the cascade leading to eventual cell death.19

Increased calcium concentration by elevated glutamate has also been shown to stimulate protein kinase C (PKC), which is responsible for the upregulation of retinal vascular endothelial growth factor (VEGF) proteins.20 VEGF is one of the most potent inducers of vascular permeability and is a powerful mitogen for endothelial cells. Recent evidence has suggested that VEGF may play a role in the pathogenesis of neovascularization, including proliferative diabetic retinopathy (PDR) and...

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age-related macular degeneration (AMD), and in the increase of vascular permeability that characterizes early stages of diabetic retinopathy, tumors, wound healing, and inflammatory conditions. Various stimuli associated with diabetic retinopathy have been reported to increase the vascular expression of VEGF, including hypoxia, an elevated glucose concentration, activation of PKC, and angiotensin II. Recent studies have also detected increased expression of VEGF receptors in the diabetic retina. Increased VEGF levels have been reported in the retina, aqueous humor, and vitreous fluid of patients with diabetic macular edema and retinopathy. VEGF is produced by retinal pigment epithelium cells, ganglion cells, Müller cells, pericytes, and smooth muscle cells of human retina and choroid. VEGF may act directly on endothelial cell tight junctions to decrease their protein content or to increase their phosphorylation, and either or both of these effects may increase paracellular permeability. It is thought that therapeutic maneuvers that suppress VEGF production or activity should be able to prevent the earlier stages of diabetic retinopathy and thus inhibit the development or progression of diabetic macular edema and proliferative diabetic retinopathy.

It is possible that neuronal cell apoptosis and elevated VEGF protein levels seen early in the diabetic retina is caused by increased NMDA receptor activity and could be attenuated by blocking the overactivity of the receptor. To test this hypothesis, we measured electroretinogram findings, RGC count, vitreoretinal VEGF protein levels, and retinal BRB breakdown of STZ-treated diabetic rats after long-term treatment with vehicle (VEH) or MEM, and results were compared with control nondiabetic rats. MEM is an NMDA receptor channel blocker and acts as an uncompetitive antagonist. The compound has been shown to be neuroprotective in models of ischemia of the CNS and retina. It is used for the treatment of Parkinson disease and vascular dementia and has recently been approved for the treatment of moderate to severe Alzheimer disease.

Materials and Methods

Diabetic Animals and Drug Treatment

Male Brown Norway (BN) rats (Charles River Laboratories, Inc., Wilmington, MA), each weighing 250 to 300 g, were acclimated to the animal research facilities at Allergan for at least 1 week before experiments were initiated. All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with the Allergan Institutional Animal Care and Use Committee guidelines. Animals were housed and maintained on a normal diet.

After acclimation, BN rats were weighed, and tail-snip baseline blood glucose was determined using a blood glucose monitoring system (One Touch Ultra Blood Glucose Monitoring; Lifescan, Milpitas, CA). Based on blood glucose and body weight, the animals were divided into three groups so that distributions of body weight and blood glucose level were similar among the groups. With the use of one-time intravenous injection, one group of animals was treated with VEH (citrate buffer, pH 4.5), and the other two groups were treated with 65 mg/kg STZ. To verify whether STZ-treated animals developed hyperglycemia (blood glucose level greater than 250 mg/dL), tail-snip glucose levels of the rats were again determined after 3 days, as described. Drug treatment was initiated after 7 days, when the diabetic rats showed significantly elevated retinal VEGF protein levels and BRB leakage (not shown) and were stabilized after the effects of toxin injection. VEH-treated rats were treated further with second VEH (distilled water), and STZ-treated diabetic rats were treated with second VEH (distilled water) or MEM (10 mg/kg daily) for 5 to 4 weeks. Water or MEM was administered continuously with a mini-osmotic pump (model 2ML2, 5 μL/h; Alzet Osmotic Pumps, Cupertino, CA), which was inserted subcutaneously in the backs of the animals.

Briefly, rats were anesthetized by isoflurane inhalation (5% induction and 2%–3% maintenance by nose cone). An area of approximately 2 × 3 inches on the back of the rat was shaved, rinsed with saline solution, cleaned with antiseptic soap solution, and wiped with 70% ethanol. A single 1-inch incision was made perpendicularly to the long axis of the animal in the skin covering the lumbar region of the back. With the use of blunt scissors, a subcutaneous pocket was made toward the head of the animal. The sterile osmotic pump filled with 2 mL water or MEM (25–27 μg/μL) was placed into the subcutaneous pocket, and the incision was closed with up to four surgical clips. Every 2 weeks, the pumps were replaced. To ensure that the pumps worked, plasma levels of MEM were determined at different times after insertion of the pumps (results not shown). After 3 to 4 weeks of treatment, ERG, RGC, and VEGF protein levels in retina and vitreous fluid and retinal BRB breakdown were measured in different groups of rats, as described. In some experiments, nondiabetic BN rats were treated with VEH (distilled water) or MEM (10 mg/kg daily) for 4 weeks. At end of the study, retinal and vitreous fluid VEGF protein levels were determined.

ERG Methodology

Bilaterial flash electroretinograms were recorded in BN rats using the electrophysiology system (Espion; DiagnoSYS Systems Inc., Kissimmee, FL). Briefly, both eyes were dilated with a drop of 1% tropicamide and 10% phenylephrine and were dark adapted for more than 15 minutes. Animals were sedated with 50 mg/kg ketamine and 5 mg/kg xylazine intravenously and placed on a heating platform built to slide into the Ganzfeld stimulator. The corneas were anesthetized with proparacaine. A noninvasive platinum wire loop electrode moisturized with artificial tears (Celluvisc; Allergan, Irvine, CA) was placed on the cornea, and a single 25-gauge stainless steel needle was placed subcutaneously on top of the nose as a reference electrode. Retinas were stimulated using 1 cd · s/m² flash for an average of 10 tracings at 0.1 Hz. Recording filter was set at 300 Hz. ERG responses were analyzed using graphics software (Labview [National Instruments, Austin, TX]; Excel [Microsoft, Redmond, WA]). The b-wave amplitude was measured from the trough of the a-wave to the peak of the b-wave, and the a-wave was measured as the difference in amplitude between recording at onset and the trough of the negative deflection. Recordings were taken before (baseline) and 4 weeks after STZ treatment.

RGC Count

Retinal ganglion cell (RGC) number was determined by retrogradely labeling the RGCs with dextran tetramethyl rhodamine (DTMR, 3000 MW; Molecular Probes, Eugene, OR). At the end of the experimental period, rats were deeply anesthetized intravenously with ketamine (50 mg/kg) and xylazine (5 mg/kg). The optic nerve was exposed, and DTMR crystals were then applied to an incision site of the optic nerve 2 to 3 mm from the globe. Animals were killed 24 hours later. Eyes were fixed with 4% paraformaldehyde, and retinas were flattened. Under fluorescence microscopy, fluorescently labeled ganglion cells were counted in 12 regions in the four quadrants of each retina approximately 1 mm and 2 to 3 mm from the edge of the optic disc. Seven or eight retinas from each group of BN rats were evaluated.

Analysis of Retinal and Vitreous Fluid VEGF Protein Levels

VEGF protein levels in retina and vitreous fluid were determined as described previously. Briefly, rats were killed by CO₂. Retinas and vitreous fluid were rapidly isolated and frozen in liquid nitrogen. One retina from each rat was used for VEGF protein assay. Vitreous fluid from two eyes of each rat was pooled for each VEGF protein assay. Retinal samples from the rats were sonicated at 4 using a homogenizer (Branson Sonifier 150; Branson Ultrasonics, Danbury, CT) in 200 μL phosphate-buffered saline (PBS) containing a protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). The lysate was centrifuged at 12,000g for 10 minutes (4°C). Supernatant and vitreous fluid were evaluated for VEGF protein levels using an ELISA kit accord-
ing to the manufacturer’s instructions (R&D Systems Inc., Minneapolis, MN). Total protein was determined by the Bradford method (protein assay reagent kit; Bio-Rad Laboratories, Hercules, CA). Retinal VEGF levels were normalized to total protein.

**Measurement of BRB Breakdown Using Evans Blue**

BRB breakdown was quantitated as described earlier using Evans blue dye, which noncovalently binds to plasma albumin in the bloodstream. Briefly, Evans blue dye was dissolved in normal saline (45 mg/mL). Then, under deep anesthesia, the dye (45 mg/kg) was injected through the jugular vein of each rat. Blood (200 μL) was withdrawn from the iliac artery 2 minutes after Evans blue injection and then every 30 minutes for up to 120 minutes. After the dye circulated for 120 minutes, the chest cavity was opened, and the left heart ventricle was cannulated. Each rat was perfused with 0.05 M citrate buffer, pH 3.5 (37°C), for 2 minutes at 66 mL/min to clear the dye. Immediately after perfusion, the eyes were enucleated and the retinas were carefully dissected under an operating microscope. Evans blue in the retina and blood samples was detected as described earlier.

**Calculations**

Data, expressed as mean ± SEM, were compiled on a spreadsheet (Excel; Microsoft) and statistically analyzed (JMP; SAS Institute, Cary, NC). Comparisons between groups were made using analysis of variance with the Tukey-Kramer HSD procedure to test for differences among all three pairwise comparisons to control the overall alpha (significance) level. By default, this test simplifies to a two-tailed, two-sample equal variance (homoscedastic) Student’s t-test for the end points where only two groups were compared. Significance values were set at \( P < 0.05 \) (*), \( P < 0.01 \) (**), and \( P < 0.001 \) (***/****).

**RESULTS**

**Effect of Chronic Treatment of MEM on Body Weight and Blood Glucose of Diabetic Rats**

To determine effect of MEM on body weight and blood glucose of STZ-treated diabetic rats, animals were divided, based on similar distributions of body weight and blood glucose. One group of animals was treated with VEH, and the other two groups were treated with STZ. Seven days after VEH or STZ treatment, VEH-treated animals were further treated with a second vehicle (VEH), and STZ-treated animals were treated with a second vehicle (STZ) or memantine (MEM) for another 21 days using mini-osmotic pumps. Four weeks after the end of the study, body weight and blood glucose levels of the animals were measured. Body weight of VEH-treated BN rats increased modestly but not significantly—from 323 ± 11 g to 349 ± 16 g—with 4 weeks of treatment. Compared with VEH-treated rats (VEH, 349 ± 16 g), there was a significant decrease after 4 weeks in body weight of animals treated with STZ and then either VEH (STZ, 266 ± 5 g; \( P < 0.01 \) vs. VEH) or memantine (MEM, 265 ± 13 g; \( P < 0.01 \) vs. VEH). However, there was no difference in body weight between STZ- and MEM-treated groups after 4 weeks of the toxin treatment. As shown in Figure 1, STZ-treated animals exhibited significantly higher blood glucose levels (STZ, 534 ± 23 mg/dL or MEM, 550 ± 21 mg/dL, \( P < 0.001 \)) after 4 weeks than VEH-treated animals (VEH, 104 ± 6 mg/dL), but there was no significant difference in blood glucose levels between the STZ- and MEM-treated groups (Fig. 1). In conclusion, the STZ-induced diabetic rats had significantly higher blood glucose levels and lower body weight than the VEH-treated nondiabetic rats after 4 weeks. MEM treatment for 3 days did not affect blood glucose levels or body weight of the STZ-treated rats.

**Effect of MEM on Retinal Function of STZ-Induced Diabetic Rats**

To determine the effect of MEM on retinal function, BN rats were divided into three groups and treated with VEH, STZ, or STZ and MEM, as described. ERGs were measured at baseline and 4 weeks after STZ treatment. Amplitudes of a- or b-waves at 1 cd·s/m² were similar among the three groups at baseline (Fig. 2). Compared with baseline, there was also no alteration in amplitudes of ERG a- or b-waves in BN rats treated with VEH for 4 weeks. However, there was a significant decrease in amplitudes of a- or b-waves in STZ-induced diabetic rats compared with VEH-treated animals (a-wave: STZ = 267 ± 8 μV, \( P < 0.001 \) vs. VEH; b-wave: STZ = 806 ± 27 μV, \( P < 0.001 \) vs. VEH). Long-term treatment with MEM for 3 weeks (1 week after STZ) significantly improved amplitudes of a- and b-waves in STZ-treated diabetic rats (a-wave: MEM = 305 ± 9 μV, \( P < 0.05 \) vs. STZ; b-wave: MEM = 1019 ± 18 μV, \( P < 0.01 \) vs. STZ). Thus, MEM significantly compromised ERGs of the diabetic animals, and long-term MEM administration significantly improved ERG abnormalities in these animals.

**Effect of MEM on RGC Count of STZ-Induced Diabetic Rats**

Neurodegeneration of the retina is a critical component of diabetic retinopathy, and significant loss of RGCs has been reported in STZ-induced diabetic rats. MEM may prevent RGC loss in diabetic animals because the compound has been shown to be neuroprotective in models of ischemia of the CNS and retina.

To address this issue, ganglion cells were counted in retinas of VEH-treated nondiabetic rats, STZ-induced diabetic rats, and diabetic rats after chronic treatment...
with MEM. BN rats were randomized to three groups and treated with VEH, STZ, or STZ and MEM, as described. At the end of the study, retinal and vitreal VEGF protein levels were measured. Compared with VEH-treated animals (VEH), VEGF protein levels were significantly increased in retinas and vitreous fluid of diabetic rats 5 weeks after treatment with STZ (retina: STZ = 719 ± 81 pg/mg protein, VEH = 482 ± 55 pg/mg protein; P < 0.05 vs. VEH; vitreous fluid: STZ = 259 ± 7 pg/mL, VEH = 157 ± 20 pg/mL, P < 0.001 vs. VEH; Fig. 4). However, treatment with MEM for 4 weeks (1 week after STZ) significantly improved RGC counts in diabetic animals (RGC counts: MEM = 3938 ± 107, STZ = 3395 ± 100, P < 0.05 vs. STZ; Fig. 3B). Thus, STZ treatment significantly decreased the number of RGCs in diabetic animals, and long-term treatment with MEM significantly improved cell numbers in these animals.

Effect of MEM on VEGF Protein Levels in Retina and Vitreous Fluid of STZ-Induced Diabetic Rats

To examine the effect of long-term treatment with MEM on retinal and vitreal VEGF proteins of STZ-treated diabetic rats, BN rats were randomly assigned to three treatment groups based on body weight and blood glucose. With the use of one-time intravenous injection, one group of animals was treated with VEH and two other groups were treated with 65 mg/kg STZ. Seven days after VEH or STZ treatment, VEH-treated rats were treated further with a second VEH (VEH), and STZ-treated diabetic rats were treated with a second VEH (STZ) or with MEM (10 mg/kg daily; MEM) for another 21 days using mini-osmotic pumps. At the end of the study, amplitudes of a- and b-waves were measured again. (A) **P < 0.01 and (B) ***P < 0.001 vs. VEH. (A) +++P < 0.05 and (B) +++P < 0.01 vs. STZ. n = 5 to 6.

FIGURE 2. ERG a- (A) and b- (B) waves of BN rats before (baseline) and after (4 weeks) treatment with VEH, STZ, or STZ and MEM. BN rats were randomly assigned to three treatment groups based on body weight and blood glucose. Before treatment, amplitudes of a- and b-waves were measured as described. With the use of one time intravenous injection, one group of animals was treated with VEH and two other groups were treated with 65 mg/kg STZ. Seven days after VEH or STZ treatment, VEH-treated rats were treated further with a second VEH (VEH), and STZ-treated diabetic rats were treated with a second VEH (STZ) or with MEM (10 mg/kg daily; MEM) for another 21 days using mini-osmotic pumps. At the end of the study, amplitudes of a- and b-waves were measured again. (A) **P < 0.01 and (B) ***P < 0.001 vs. VEH. (A) +++P < 0.05 and (B) +++P < 0.01 vs. STZ. n = 5 to 6.

FIGURE 3. Neuroprotective effect of memantine on RGCs in STZ-induced diabetic rats. BN rats were randomly assigned to three treatment groups based on body weight and blood glucose. With the use of one-time intravenous injection, one group of animals was treated with VEH and two other groups were treated with 65 mg/kg STZ. Seven days after VEH or STZ treatment, VEH-treated rats were treated further with a second VEH (VEH), and STZ-treated diabetic rats were treated with a second VEH (STZ) or with MEM (10 mg/kg daily; MEM) for another 21 days using mini-osmotic pumps. RGCs were retrogradely labeled as described earlier with DTMR 24 hours before retina flatmount. (A) Retina flatmounts showing fluorescence-labeled RGCs from BN rats treated with VEH (A1), STZ (A2), and MEM (A3). This was a representative experiment independently conducted seven or eight times. (B) RGC counts in retinas from VEH, STZ, and MEM-treated rats. **P < 0.01 vs. VEH. +P < 0.05 vs. STZ. n = 7 to 8.
attenuated elevated VEGF protein levels in retina and vitreous fluid of STZ-treated animals (retina: STZ = 719 ± 81 pg/mg protein; MEM = 475 ± 26 pg/mg protein, \( P < 0.05 \) vs. STZ; vitreous fluid, STZ = 259 ± 7 pg/mL, MEM = 160 ± 14 pg/mL, \( P < 0.001 \) vs. STZ). Thus, STZ treatment significantly upregulated VEGF protein levels in retinas and vitreous fluid. Long-term treatment with MEM brought down elevated VEGF protein levels in retinas and vitreous fluid of STZ-treated animals similar to levels observed in VEH-treated nondiabetic controls.

**Effect of Chronic MEM Treatment on VEGF Protein Levels in Retina and Vitreous Fluid of Nondiabetic Rats**

To determine the effect of MEM on retinal and vitreous fluid VEGF protein levels in nondiabetic rats, BN rats were randomized to two groups based on basal glucose levels and body weight. One group of rats was treated with VEH (VEH-28D), and the other group was treated with 10 mg/kg MEM (MEM-28D) per day for 4 weeks using mini-osmotic pumps. At end of the study, animals were killed and retinas and vitreous fluids were collected for analysis of VEGF protein levels. As shown in Figure 5, there was no difference in retinal (A) or vitreous fluid (B) VEGF protein levels between VEH- and MEM-treated rats. These results suggest that, unlike STZ-treated rats, long-term treatment with MEM does not affect VEGF protein levels in the retinas and vitreous fluid of nondiabetic rats.

**Effect of MEM on Retinal BRB Leakage of STZ-Treated Diabetic Rats**

To examine the effect of long-term treatment of MEM on retinal BRB leakage of STZ-treated diabetic rats, BN rats were randomized to three groups and treated with VEH, STZ, or STZ and MEM, as described. Retinal BRB leakage was assayed at the end of the study. As shown in Figure 6, retinal permeability, similar to VEGF protein levels, significantly increased in STZ-treated rats within 5 weeks of treatment (STZ = 23 ± 3 µL plasma/g retina dry weight per hour, VEH = 9 ± 1 µL plasma/g retina dry weight per hour; \( P < 0.01 \) vs. VEH). However, treatment with MEM for 4 weeks (1 week after STZ treatment) significantly attenuated elevated BRB breakdown in the retinas of STZ-treated animals (STZ = 23 ± 3 µL plasma/g retina dry weight per hour, MEM = 12 ± 1 µL plasma/g retina dry weight per hour; \( P < 0.01 \) vs. STZ). Thus, STZ treatment significantly elevated retinal BRB breakdown in BN rats. Chronic MEM treatment reduced BRB breakdown in the retinas of STZ-treated animals to levels similar to those observed in VEH-treated nondiabetic controls.

**DISCUSSION**

BRB breakdown and the consequent retinal vascular hyperpermeability are among the early features of diabetic retinopathy. Increased retinal vascular permeability (RVP) results in the leakage of fluids, lipids, and plasma proteins from blood vessels to retinal tissue, which can further lead to macular edema, a major cause of vision loss in diabetic patients. The mechanisms underlying retinal vascular hyperpermeability are largely unclear. VEGF is referred to as vascular permeability factor based on its ability to induce vascular hyperpermeability. It has been shown that VEGF is one of the main mediators of increased RVP in ischemic and nonischemic retinal diseases. The upregulated expression of VEGF and
its receptors are associated with increased RVP in rats with STZ-induced diabetes. Recent studies also indicate that neurodegeneration of the retina is a critical component of diabetic retinopathy. It has been suggested that the gradual loss of neurons, which begins early in diabetes, may be a primary abnormality that gives rise to vascular changes. The present study provides the first experimental evidence that long-term treatment with MEM significantly improves retinal function and protects RGC loss in STZ-induced diabetic rats. Treatment with MEM also significantly reduces elevated VEGF protein levels in retina and vitreous fluid and BRB breakdown in the retinas of diabetic animals.

We observed significant decreases in body weight and increases in blood glucose levels of STZ-treated rats. Long-term MEM treatment did not alter the body weight or blood glucose levels of the diabetic animals. Similar observations have been reported by Obrosova et al. for two antioxidants, DL-glutathione and N-acetyl-L-cysteine, in STZ-treated diabetic rats. These results argue against the role of MEM in peripheral metabolism.

The ERG is a functional test and is affected as soon as retinal function is disturbed. To assess the functional integrity of different retinal structures in control and diabetic rats, amplitudes of the ERG a- and b-waves were measured. The amplitudes of the a- and b-waves indicate photoreceptor function and the functional integrity of the inner nuclear layer, respectively. Compared with the control nondiabetic rats, ERG responses of STZ-induced diabetic rats were significantly compromised. Amplitudes of the b-waves in diabetic rats were more affected than the a-waves. These results indicate that the generators of the b-wave are relatively susceptible to the pathologic processes of early diabetes compared with the photoreceptors. The b-wave is considered to represent light-induced electrical activity in the ON-center bipolar cells and contributions of the Müller cells.

The impaired ERG observed in STZ-induced diabetic rats could be attributed to reduced light absorption by the opaque media because diabetic rats develop cataract at an early stage of the disease. However, we observed reductions in the ERG responses in diabetic rats as early as 2 weeks after STZ treatment. We noticed the first signs of cataract formation in the diabetic animals only 7 to 8 weeks after the toxin treatment (not shown). If light absorption by the cataract was the cause of reduced ERG responses, then a significant time-dependent reduction of the ERG responses was expected during the first 15 weeks of diabetes, when cataracts developed and became more opaque. However, we found that the amplitudes of the ERG a- and b-waves did not change significantly as long as 16 weeks of follow-up (not shown). Moreover, ERG responses in human patients were not significantly affected by cataractous eyes and, in some cases, were even increased in amplitude. Thus, the reductions of the ERG responses seen in the STZ-induced diabetic rats indicate deterioration of retinal function. This is consistent with previous observations of early reduction in retinal function resulting from diabetes. A loss of photoreceptor cells has also been reported in STZ-induced diabetic rats at an early stage of the disease.

In addition to compromised ERGs, we observed significant decreases in RGC counts in STZ-induced diabetic rats compared with nondiabetic control rats, consistent with previous observations of reduced numbers of RGCs in the retinas of diabetic rat and mouse. We noticed a 16% decrease in RGC counts in diabetic retinas after 4 weeks of STZ treatment. In a separate time-course study, we found 15% and 19% decreases in RGC counts in diabetic rats after 4 and 8 weeks, respectively, of STZ treatment (not shown). Similarly rapid decreases in RGC counts in diabetic animals have been reported by others using different methods to assess RGC number. For example, Zheng et al. observed a 20% loss of cells in the ganglion cell layer after 4 months of the induction of diabetes. Thus, the time-course study and results from other laboratories confirm our reported result of rapid loss in RGCs and indicate no significant progression of RGC loss from 4 to 16 weeks after STZ treatment. However, Barber et al. observed only a 10% decrease in RGCs in diabetic retinas after 7.5 months of STZ-induced diabetes. The smaller decrease in RGC number could be the result of insulin treatment, which may prevent RGC loss in diabetic animals.

Glutamate levels are elevated in the retinas and vitreous fluid of STZ-treated diabetic rats and diabetic patients. Glutamate is an important excitatory neurotransmitter in the retina. However, a great deal of evidence suggests that excessive stimulation by glutamate results in several types of neurodegenerative diseases. In the retina, elevated levels of vitreal glutamate have been reported in several pathologic conditions. The excitotoxicity of glutamate is the result of over-activation of NMDA-type glutamate receptor. Activation of NMDA receptors results in increases calcium permeability, which initiates calcium-dependent processes that lead to cell death. The presence of NMDA receptors in the RGCs makes them especially vulnerable to glutamate toxicity. Open-channel, uncompetitive blockade of the NMDA channel can prevent the excitotoxic action of glutamate.

Elevated glutamate levels seen in the retinas and vitreous fluid in those with diabetes could be responsible for RGC loss and compromised ERGs. MEM significantly improved amplitudes of ERG a- and b-wave and protected RGC cell loss in STZ-induced diabetic rats, suggesting the expression of a role of glutamate excitotoxicity in the diabetic retina. It is likely that the antagonist interacts with NMDA receptors to reduce its interaction with glutamate and thus attenuates hyperactivity of the receptor. Type 3 serotonin (5-HT3) and nicotinic acetylcholine (ACH) receptors are involved in neurodegeneration and are present in the mammalian retina. MEM has been shown to be non-competitive inhibitor of these receptors. Thus, another possible mechanism of the neuroprotective effect of MEM in the retinas of STZ-induced diabetic rats could be the inhibition of retinal 5-HT3 and nicotinic ACh receptors, although elevated 5-HT and ACH levels have not been demonstrated in the diabetic retina.

In the present study, VEGF protein levels were measured in the retinas and vitreous fluid of diabetic rats after 5 weeks of diabetes.
STZ treatment and were found to be significantly higher than in age-matched VEH-treated nondiabetic controls. BRB breakdown also coincided with the increase in retinal and vitreal VEGF protein levels. These are consistent with previous observations of elevated retinal VEGF protein levels and BRB breakdown in STZ-induced diabetic rats at a very early stage of the disease.\(^{2,36,61}\) Previously, it has also been shown that VEGF causes early BRB breakdown in diabetes and that inhibition of the growth factor suppresses BRB breakdown in the superficial venules and capillaries of the inner retina, the principal site of vascular permeability in early diabetes.\(^{36}\) However, the mechanisms of increased vitreoretinal VEGF protein levels and retinal BRB leakage in early diabetes remain unknown. Increased RGC loss in diabetic animals could be responsible for the abnormal vitreoretinal VEGF and retinal BRB leakage. However, time-course studies did not concur with this possibility because significantly elevated retinal VEGF and BRB leakage was observed in STZ-treated diabetic animals within 1 week of toxin treatment (not shown), whereas significantly increased RGC loss was first noticed after 4 weeks of treatment. Thus, it is possible that, after interacting with its receptors, elevated retinal glutamate levels in diabetic animals\(^{14,16}\) might increase intracellular Ca\(^{2+}\) concentrations,\(^{19}\) which, in addition to causing cell death, stimulate PKC.\(^{52}\) Increased PKC activity might upregulate retinal VEGF proteins,\(^{20}\) leading to increased BRB leakage and elevated VEGF in vitreous fluid. However, elevated retinal glutamate could increase PKC activity and VEGF protein levels through some other mechanisms.\(^{63,64}\)

Surprisingly, we observed that long-term treatment with MEM significantly attenuated elevated VEGF protein levels in retinas and vitreous fluid and retinal BRB breakdown in STZ-induced diabetic rats but not in nondiabetic control rats. It is possible that MEM decreases retinal NMDA receptor activity by reducing its interaction with elevated glutamate seen in the retinas of diabetic animals.\(^{13,16}\) Compromised receptor activity through the attenuation of intracellular Ca\(^{2+}\) levels and PKC activity might reduce vitreoretinal VEGF protein levels and BRB breakdown in STZ-treated diabetic animals. Further studies are warranted to explore these exciting possibilities. The present study provides the first experimental evidence of the vasculo-protective effect of MEM and illustrates its well-established neuroprotective effect.

In summary, STZ treatment significantly reduced ERG a- and b-wave amplitudes and increased RGC loss in BN rats within 4 weeks of treatment. STZ-induced diabetic rats had significantly elevated vitreoretinal VEGF protein levels and experienced retinal BRB breakdown. Long-term treatment with MEM significantly improved amplitudes of ERG a- and b-waves and protected RGC loss in diabetic animals. MEM treatment also significantly reduced elevated VEGF protein levels in retina and vitreous fluid and retinal BRB breakdown of the diabetic animals. This effect of MEM is not seen in nondiabetic rats. MEM could be useful for the treatment of ocular diseases, including diabetic retinopathy, with neurodegeneration and elevated retinal and vitreous fluid VEGF protein levels. In addition to the neuroprotective effect of this compound, MEM can reduce the vascular changes seen in diabetic retinas.

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**References**


