

Treadmill exercise ameliorates apoptotic cell death in the retinas of diabetic rats

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Abstract. Apoptotic neuronal cell death in the retina is a hallmark of diabetic retinopathy. Exercise has been recommended for the alleviation of symptoms in patients with diabetes. In the present study, the effect of treadmill exercise on apoptosis in the retinas of diabetic rats was investigated. Diabetes was induced by intraperitoneal injection of streptozotocin. The rats in the exercise groups ran on a treadmill for 30 min/day, 5 times a week, over the course of 6 weeks. In this study, the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay, immunohistochemistry staining of caspase-3 and western blot analysis for Bax, Bcl-2 and phosphorylated protein kinase B (p-Akt) in the retinas of diabetic rats were performed. The results demonstrated that the number of TUNEL- and caspase-3-positive cells was increased in the retinas of diabetic rats, whereas treadmill exercise decreased these numbers. In addition, the expression of the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2 was enhanced in the retinas of diabetic rats. Treadmill exercise suppressed Bax and enhanced Bcl-2 levels. The expression of the cell survival factor, p-Akt, was decreased in the retinas of diabetic rats and treadmill exercise increased the expression of p-Akt. The results of the present study demonstrated that treadmill exercise ameliorated diabetes-induced apoptosis in retinal cells by enhancing p-Akt levels in the retina. Treadmill exercise represents an effective strategy to delay or prevent the onset of ocular complications in diabetic patients.

Introduction

Diabetes mellitus (DM) is a metabolic disease associated with hyperglycemia, which is caused by absolute or relative

insulin deficiency and resistance (1). In addition to the condition, numerous secondary complications are associated with DM. Of these, diabetic retinopathy is one of the most serious complications caused by DM.

Apoptosis, also known as programmed cell death, is a form of cell death that occurs during several pathological processes in multicellular organisms and contributes to cell replacement, tissue remodeling and the removal of damaged cells under normal conditions. However, inappropriate apoptosis is also implicated in a number of neurodegenerative diseases (2-4). Apoptosis is a well-known hallmark associated with the underlying mechanisms of diabetic retinopathy (5-7). Hyperglycemia has been demonstrated to induce neuronal cell death through an apoptotic pathway in diabetic retinas (8,9). Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining detects DNA fragmentation which is characteristic of apoptotic cell death (2,3). Caspases are cysteine proteases which regulate apoptotic cell death in a variety of cells, including neurons (4). In particular, caspase-3, an executioner caspase, functions as a downstream death signal and activates other caspases. Previous studies have reported that DM-induced apoptosis occurs through caspase-dependent pathways, including caspase-3 (6,10). In addition, hyperglycemia-induced apoptotic cell death in the retina is triggered by caspase-3 activation (8,9,11).

The Bcl-2 family of proteins function as critical regulators in the pathways of apoptosis, inhibiting or promoting cell death. Bax forms a high molecular weight oligomer in the mitochondrial membrane enabling the release of cytochrome c, which initiates apoptosis (12,13). Podestà *et al* (14) demonstrated that Bax expression was enhanced in diabetic retinas and its increased expression contributed to pericyte loss and the development of vascular complications in diabetic retinopathy. By contrast, Bcl-2 inhibits apoptosis by suppressing Bax (15).

Protein kinase B (Akt) is a main effector in the phosphoinositide 3-kinase (PI3K) signaling pathway. Increased Akt activity blocks the mitochondrial apoptotic pathway by phosphorylating members of the Bcl-2 family via the inactivation of pro-apoptotic members, including Bad or by directly inhibiting the activation of caspase-9 (16,17). Akt is important for a number of cellular processes, including cell survival, metabolism, growth, proliferation and mobility (18). In addition,

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tion, Akt is known to protect against apoptotic neuronal cell death by targeting the activity of several transcription factors implicated in the regulation of cell survival (19,20).

Exercise has been recommended for the alleviation of symptoms in patients with diabetes (3,21-23). Regular physical exercise is known to be effective in the prevention and delay of non-insulin-dependent diabetes onset, increasing insulin sensitivity and ameliorating glucose metabolism (24). Exercise is known to relieve a number of symptoms of DM; however, the effect of exercise on diabetes-induced apoptotic retinal cell death in association with Akt expression has not yet been clarified. In the present study, the effects of treadmill exercise on apoptosis and Akt expression were investigated in the retinas of diabetic rats.

Materials and methods

Animals. Thirty-two male Sprague-Dawley rats weighing 200 ± 10 g (7 weeks old) were used in this experiment. The rats were housed under controlled temperature ($20 \pm 2^\circ\text{C}$) and lighting conditions (07:00-19:00 h), with food and water made available *ad libitum* throughout the experiment. Experimental procedures were performed in accordance with the animal care guidelines of the National Institutes of Health and the Korean Academy of Medical Sciences. The animals were randomly divided into four groups (each group, $n=4$): Control, control and exercise, streptozotocin (STZ)-induced diabetes and STZ-induced diabetes and exercise. Blood glucose levels were measured following fasting at 0, 2, 4 and 6 weeks.

Induction of diabetes. To induce diabetes in the experimental animals, a single intraperitoneal (i.p.) injection of STZ (50 mg/kg, dissolved in 0.01 M citrate buffer at pH 4.5; Sigma-Aldrich, St. Louis, MO, USA) was administered to each animal as described previously (3). Changes in body weight and blood glucose levels were determined 2 days following STZ injection using a blood glucose tester (Arkray Inc., Kyoto, Japan). Animals with blood glucose levels of ≥ 300 mg/dl were used as subjects in the diabetic groups.

Treadmill exercise. Rats in the exercise groups ran on the treadmill for 30 min/day, five times a week, over 6 weeks. Exercise consisted of running at a speed of 3 m/min for the first 5 min, 5 m/min for the next 5 min and then 8 m/min for the last 20 min, with a 0° incline.

Tissue preparation. Animals were anesthetized using Zoletil 50 (10 mg/kg, i.p.; Vibac Laboratories, Carros, France), transcardially perfused with 50 mM phosphate-buffered saline and fixed with a freshly prepared solution consisting of 4% paraformaldehyde in a 100 mM phosphate buffer (pH 7.4). The retinas were dissected, postfixed in the same fixative overnight and then transferred into a 30% sucrose solution for cryoprotection. Coronal sections of $20\text{-}\mu\text{m}$ thickness were generated using a freezing microtome (Leica Biosystems GmbH, Nussloch, Germany).

TUNEL staining. DNA fragmentation was visualized by TUNEL staining performed using an *In Situ* Cell Death Detection kit (Roche Diagnostics GmbH, Mannheim, Germany) according to

the manufacturer's instructions (2). The retinas were suspended in 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA and incubated at 55°C for 30 min. Next, the sections were incubated with proteinase K (100 $\mu\text{g}/\text{ml}$), rinsed, incubated in 3% H_2O_2 , permeabilized with 0.5% Triton X-100, rinsed again and incubated in a TUNEL reaction mixture. The sections were rinsed and visualized using Converter-POD with 0.03% 3,3'-diaminobenzidine (DAB) and then finally mounted onto gelatin-coated slides. The slides were air-dried overnight at room temperature and coverslips were mounted using Permount (Fisher Scientific, Fair Lawn, NJ, USA).

Caspase-3 immunohistochemistry. Caspase-3 immunohistochemistry was performed as described previously (2). Sections from each retina were incubated overnight with a mouse anti-caspase-3 antibody (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and then for a further 1 h with a biotinylated mouse secondary antibody (1:200; Vector Laboratories, Burlingame, CA, USA). A bounded secondary antibody was then amplified with a Vector Elite ABC kit (1:100; Vector Laboratories). Antibody-biotin-avidin-peroxidase complexes were visualized using 0.03% DAB and the sections were mounted onto gelatin-coated slides. The slides were air-dried overnight at room temperature and the coverslips were mounted using Permount.

Western blot analysis. Western blot analysis was performed as described previously (2). For the western blot analysis, retinal tissues were lysed in a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% deoxycholic acid, 1% Nonidet P-40, 0.1% SDS, 1 mM PMSF and 100 mg/ml leupeptin. Protein content was measured using a Bio-Rad colorimetric protein assay kit (Bio-Rad, Hercules, CA, USA). The protein was separated on SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane. Mouse anti-Bax and anti-Bcl-2 (both 1:1,000; Santa Cruz Biotechnology, Inc.) and rabbit anti-Akt and anti-phospho-Akt antibodies (both 1:1,000; Cell Signaling Technology Inc., Beverly, MA, USA) were used as primary antibodies. Horseradish peroxidase-conjugated anti-mouse antibody against Bax and Bcl-2 (1:3,000) and horseradish peroxidase-conjugated anti-rabbit antibody against Akt and phospho-Akt (1:5,000; both Vector Laboratories) were used as the secondary antibodies. Band detection was performed using the enhanced chemiluminescence detection kit (Santa Cruz Biotechnology, Inc.).

Statistical analysis. Following staining, immunoreactive cells were counted in each of the retinal sections using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA). To compare the relative expression of proteins, detected bands were calculated densitometrically using Image-pro Plus software. The results are expressed as the mean \pm SEM. Data were analyzed by one-way analysis of variance followed by Duncan's post-hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effect of treadmill exercise on body weight and blood glucose level. At 0, 2, 4 and 6 weeks of the experiment, the average

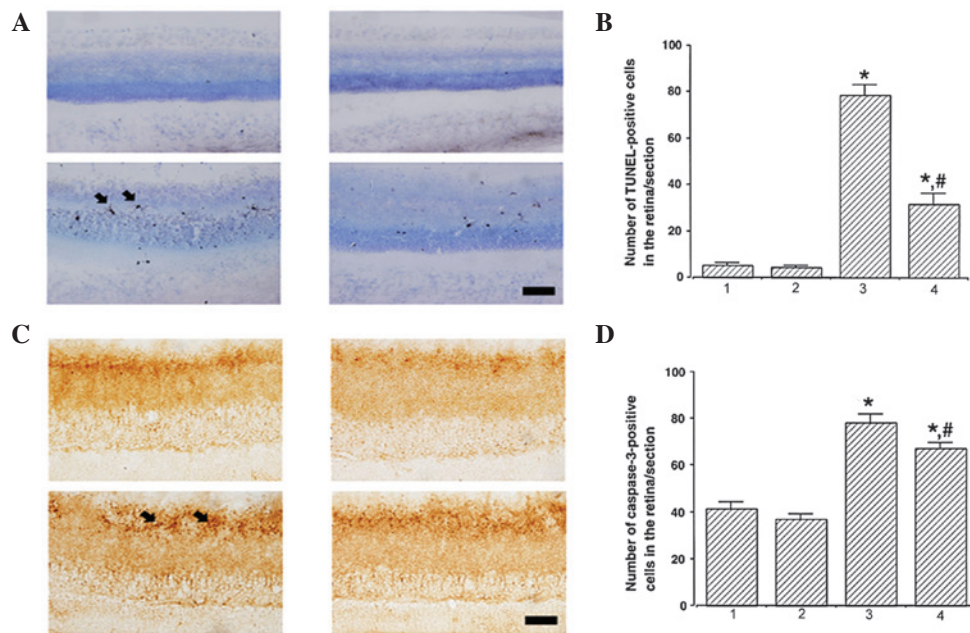


Figure 1. Effect of treadmill exercise on DNA fragmentation and caspase-3 expression in the retina. (A and B) Effect of treadmill exercise on DNA fragmentation in the retina. (A) Photomicrographs of TUNEL-positive cells in the retina (scale bar, 25 μ m) and (B) quantification of TUNEL-positive cells. (C and D) Effect of treadmill exercise on caspase-3 expression in the retina. (C) Photomicrographs of caspase-3-positive cells in the retina (scale bar, 25 μ m) and (D) quantification of caspase-3-positive cells. * P <0.05, vs. control; # P <0.05, vs. STZ-induced diabetes. 1, control; 2, control and exercise; 3, STZ-induced diabetes and 4, STZ-induced diabetes and exercise. Data are presented as the mean \pm SEM. TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; STZ, streptozotocin.

body weight was 265.33 ± 2.06 , 338.33 ± 12.37 , 387.33 ± 16.37 and 394.33 ± 16.72 g in the control group, 263.33 ± 2.82 , 322.50 ± 10.53 , 361.50 ± 12.19 and 374.00 ± 13.70 g in the control and exercise group, 264.00 ± 1.51 , 212.00 ± 5.98 , 213.00 ± 7.26 and 209.22 ± 8.77 g in the STZ-induced diabetes group and 265.20 ± 1.63 , 218.22 ± 3.88 , 223.25 ± 5.46 and 213.20 ± 13.77 g in the STZ-induced diabetes and exercise group, respectively. Loss of body weight was observed in diabetic rats (P <0.05) and reduced body weight was not restored following treadmill exercise in this group.

At 0, 2, 4 and 6 weeks of the experiment, the average blood glucose level was 91.83 ± 1.74 , 108.00 ± 2.91 , 115.33 ± 2.69 and 113.33 ± 2.11 mg/dl in the control group, 92.00 ± 1.21 , 103.00 ± 2.84 , 101.50 ± 2.54 and 110.66 ± 2.18 mg/dl in the control and exercise group, 332.50 ± 8.96 , 358.33 ± 14.69 , 498.89 ± 18.69 and 435.44 ± 19.65 mg/dl in the STZ-induced diabetes group and 337.90 ± 6.67 , 396.22 ± 20.44 , 459.87 ± 45.99 and 405.40 ± 11.56 mg/dl in the STZ-induced diabetes and exercise group, respectively. Blood glucose levels were observed to be significantly increased in the STZ-induced diabetic rats (P <0.05) and treadmill exercise did not exert any effect on the blood glucose levels in diabetic rats.

Effect of treadmill exercise on the numbers of TUNEL- and caspase-3-positive cells in the retinas of diabetic rats. Photomicrographs of TUNEL-positive cells in the retina are presented in Fig. 1A. The number of TUNEL-positive cells was 5.17 ± 0.98 , 4.50 ± 0.67 , 78.51 ± 4.74 and 31.50 ± 4.95 /section in the control, control and exercise, STZ-induced diabetes and STZ-induced diabetes and exercise groups, respectively (Fig. 1B). These results indicate that STZ-induced diabetes enhanced DNA fragmentation (P <0.05) and tread-

mill exercise significantly suppressed DNA fragmentation in diabetic retinas (P <0.05).

Photomicrographs of caspase-3-positive cells in the retina are presented in Fig. 1C. The number of caspase-3-positive cells was 41.17 ± 3.11 , 37.01 ± 2.29 , 78.33 ± 3.74 and 67.17 ± 2.69 /section in the control, control and exercise, STZ-induced diabetes and STZ-induced diabetes and exercise groups, respectively (Fig. 1D). These results indicate that STZ-induced diabetes enhanced caspase-3 expression (P <0.05) and treadmill exercise significantly suppressed caspase-3 expression in diabetic retinas (P <0.05).

Effect of treadmill exercise on the expression of Bax and Bcl-2 in the retinas of diabetic rats. When the level of Bax protein (24 kDa) in the control group was set at 1.00, the level of Bax was 1.18 ± 0.06 in the exercise group, 1.98 ± 0.16 in the STZ-induced diabetes group and 1.40 ± 0.12 in the STZ-induced diabetes and exercise group (Fig. 2A). These results demonstrate that STZ-induced diabetes enhanced Bax expression (P <0.05) and treadmill exercise significantly suppressed Bax expression in diabetic retinas (P <0.05).

When the level of Bcl-2 protein (26-29 kDa) was set at 1.00 in the control group, the level of Bcl-2 was 11.25 ± 2.92 in the control and exercise group, 8.35 ± 1.83 in the STZ-induced diabetes group and 19.04 ± 3.48 in the STZ-induced diabetes and exercise group (Fig. 2B). These results indicate that Bcl-2 expression was increased by STZ-induced diabetes and treadmill exercise markedly enhanced Bcl-2 expression in diabetic retinas (P <0.05).

Effect of treadmill exercise on the expression of p-Akt and Akt in the retinas of diabetic rats. Analysis of p-Akt and

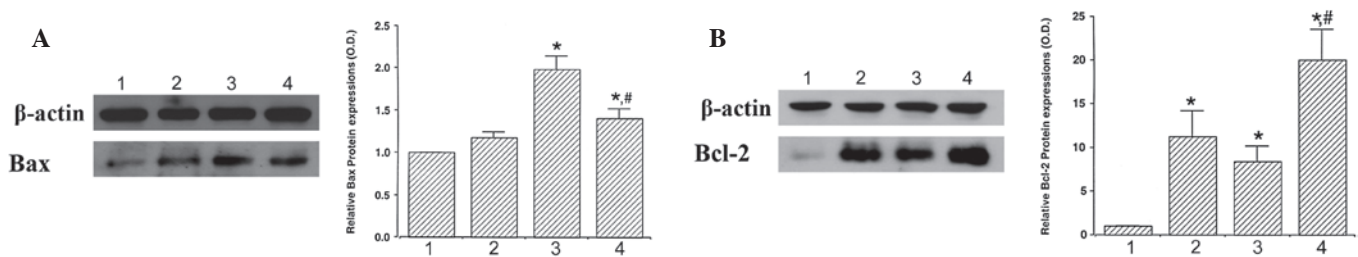


Figure 2. Effect of treadmill exercise on Bax and Bcl-2 expression in the retina. Expression of (A) Bax and (B) Bcl-2 in the retina following treadmill exercise. 1, control; 2, control and exercise; 3, STZ-induced diabetes and 4, STZ-induced diabetes and exercise. Data are presented as the mean \pm SEM. * $P < 0.05$, vs. control; # $P < 0.05$, vs. STZ-induced diabetes; STZ, streptozotocin.

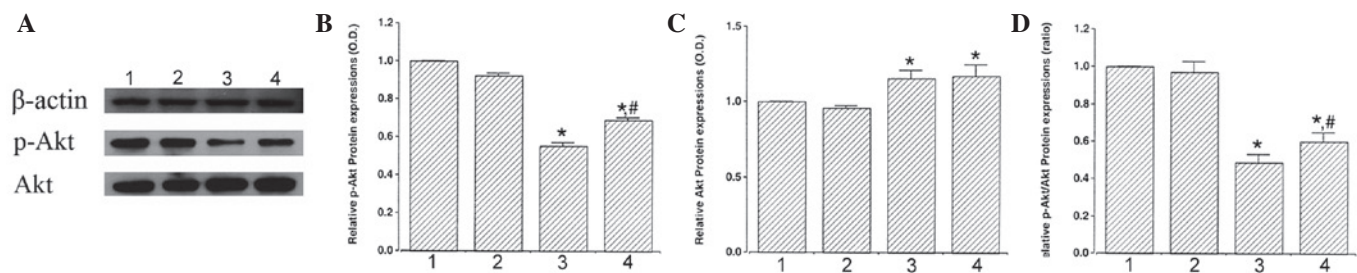


Figure 3. Effect of exercise on p-Akt and Akt protein expression in the retina. (A) Representative western blotting. Effect of treadmill exercise on (B) p-Akt, (C) Akt and (D) ratio of p-Akt/Akt in the retina. 1, control; 2, control and exercise; 3, STZ-induced diabetes and 4, STZ-induced diabetes and exercise. Data are presented as the mean \pm SEM. * $P < 0.05$, vs. control; # $P < 0.05$, vs. STZ-induced diabetes. Akt, protein kinase B; p-Akt, phosphorylated Akt; STZ, streptozotocin.

Akt expression was performed to estimate the relative levels of these proteins (Fig. 3A). When the expression of p-Akt (60 kDa) in the control group was set as 1.00, p-Akt level was 0.92 ± 0.01 in the control and exercise group, 0.56 ± 0.02 in the STZ-induced diabetes group and 0.69 ± 0.02 in the STZ-induced diabetes and exercise group (Fig. 3B). These results indicated that STZ-induced diabetes suppressed p-Akt expression in the retina ($P < 0.05$) and treadmill exercise significantly enhanced p-Akt expression in diabetic retinas ($P < 0.05$).

When the expression of Akt (60 kDa) in the control group was set as 1.00, the level of Akt level was 0.96 ± 0.01 in the control and exercise group, 1.16 ± 0.02 in the STZ-induced diabetes group and 1.17 ± 0.03 in the STZ-induced diabetes and exercise group (Fig. 3C). These results revealed that STZ-induced diabetes enhanced Akt expression ($P < 0.05$) and exercise was not found to significantly affect Akt expression in diabetic retinas.

When the ratio of p-Akt/Akt in the control group was set as 1.00, the ratio of p-Akt/Akt was 0.97 ± 0.03 in the control and exercise group, 0.49 ± 0.02 in the STZ-induced diabetes group and 0.60 ± 0.02 in the STZ-induced diabetes and exercise group (Fig. 3D). These results demonstrated that the ratio of p-Akt to Akt decreased in diabetic retinas ($P < 0.05$). However, treadmill exercise increased the ratio of p-Akt to Akt by enhancing the expression of the cell survival factor, p-Akt ($P < 0.05$).

Discussion

In the present study, STZ-induced DM led to reduced body weight and significantly increased blood glucose levels. Treadmill exercise for 6 weeks was not observed to restore lost body weight and did not exert an effect on blood glucose levels

in the diabetic rats. These observations are consistent with previous studies reporting that exercise exerts no significant effect on body weight and blood glucose levels in STZ-induced diabetic rats (3,25).

Neuronal cell apoptosis in the retina is an important mechanism involved in the induction of ocular complications in diabetic retinopathy, glaucoma and ischemic injury (10,26,27). In the present study, the number of TUNEL- and caspase-3-positive cells in the retinas of STZ-induced diabetic rats were higher than those in the control. Increased neuronal apoptosis in the retina has been reported in experimental diabetic rats and in patients with diabetes (6,10). Elevated glucose concentration decreases cell viability (4) and Gao *et al* (7) demonstrated that the number of TUNEL-positive cells in the retina was increased in the diabetic rats. Treadmill exercise was found to significantly suppress the number of TUNEL- and caspase-3-positive cells in the retinas of STZ-induced diabetic rats. Abu El-Asrar *et al* (11) identified that inhibition of caspase-3 activity reduced DM-induced apoptotic cell death in retinas and apoptosis induced by hyperglycemia was effectively inhibited by pre-treatment with caspase-3 inhibitors (28). Treadmill exercise suppressed DNA fragmentation and caspase-3 expression (2,29). Zhang *et al* (29) reported that aerobic exercise training for 8 weeks significantly decreased the number of TUNEL-positive cells and attenuated caspase-3 activity in the heart following ischemia/reperfusion injury. The present observations reveal that treadmill exercise may ameliorate diabetes-induced apoptotic cell death in the retina.

In this study, a significant increase in the expression of the pro-apoptotic molecule, Bax, was observed in the retinas of diabetic rats. Bax is one of the main regulators of mitochondrial permeability during apoptosis (12,13). A significant increase in Bax expression in the retina led to retinal neuronal

cell apoptosis in the diabetic rats (30). In the present study, anti-apoptotic Bcl-2 expression in the retinas of STZ-induced diabetic rats was increased compared with the control. Bcl-2 is known to inhibit apoptosis by blocking the release of cytochrome c from the mitochondria and binding to pro-apoptotic molecules (31). Bcl-2 expression in the retinas was increased in diabetic rats with high glucose levels (7). Increased Bcl-2 expression in the retinas of diabetic rats is considered to represent a compensatory mechanism against hyperglycemia. Treadmill exercise was demonstrated to suppress Bax and increase Bcl-2 expression in diabetic retinas. The anti-apoptotic effects of exercise via the inhibition of Bax and increased Bcl-2 are well documented (2,25). In rats with spinal cord injuries, cycling exercise was found to significantly increase the mRNA expression of the anti-apoptotic marker Bcl-2 in the spinal cord and high levels of Bcl-2 mRNA were consistent with reduced expression of caspase-7 and -9 mRNA (25). Treadmill exercise suppressed the expression of Bax and increased Bcl-2 expression in the hippocampus following traumatic brain injury (2). In the present study, decreased Bax and increased Bcl-2 levels in diabetic retinas were observed following treadmill exercise and may prevent retinal cells from undergoing apoptosis.

The levels of p-Akt in retinas were decreased in the diabetic rats and treadmill exercise markedly enhanced p-Akt expression. The ratio of p-Akt to Akt was also increased following treadmill exercise through increased p-Akt expression. Activation of Akt by PI3K has been demonstrated to result in inhibition of apoptotic signals and promotion of cell survival signals (32). Phosphorylation of Akt inactivated the pro-apoptotic factors, Bad and procaspase-9, and increased resistance to apoptosis (33). Exercise increased Akt phosphorylation and reduced age-related insulin resistance of muscle protein metabolism (34). In diabetic rats, treadmill running activated Akt signaling and improved cognition and synaptic plasticity in aging rats (35). Increased phosphorylation levels of Akt by treadmill exercise also suppressed neuronal cell death in the transgenic mouse model of Alzheimer's disease (36). Wang *et al* (37) reported that diabetic retinopathy was the result of increased oxidative stress induced by chronic hyperglycemia and found that stimulation of Akt reduced oxidative stress. The present study indicates that the enhanced expression of p-Akt in retinas may contribute to the anti-apoptotic effect of treadmill exercise in diabetic rats.

The anti-apoptotic and ameliorating effects of treadmill exercise on neuropsychiatric disorders are well documented (2,36,38-40). In the present study, markers of apoptosis, including TUNEL- and caspase-3-positive cells, and Bax protein, were increased with decreased levels of p-Akt in the retinas of diabetic rats. Treadmill exercise inhibited these apoptotic markers with increased levels of Bcl-2 and p-Akt in the retinas of diabetic rats. The anti-apoptotic effect of treadmill exercise on diabetic retinas is hypothesized to be a result of the enhancing effect of treadmill exercise on the levels of p-Akt in the retina. Under normal conditions, treadmill exercise exerted no significant effect on these apoptotic markers in retinas. The present study demonstrated that treadmill exercise represents an effective strategy to delay or prevent the onset of ocular complications in patients with diabetes.

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