Niaspan inhibits diabetic retinopathy-induced vascular inflammation by downregulating the tumor necrosis factor-α pathway

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Abstract. Diabetic retinopathy (DR) is a serious microvascular complication of diabetes and a major cause of blindness in the developing world. Early DR is characterized by vascular neuroinflammation, cell apoptosis and breakdown of the blood-retinal barrier (BRB). However, optimal treatment options and associated mechanisms remain unclear. Niaspan, which is widely used in the prevention and treatment of hyperlipidemia-associated diseases, has been reported to inhibit inflammation. However, the effects of Niaspan and the mechanisms underlying the anti-inflammatory effects of Niaspan on DR have yet to be reported. The present study aimed to investigate the anti-inflammatory effects and mechanisms of Niaspan in a rat model of DR. Rats with DR exhibited a significant increase in BRB breakdown, retinal apoptosis, and tumor necrosis factor-α (TNF-α) and nuclear factor-κB (NF-κB) expression. In addition, the expression levels of inducible nitric oxide synthase (iNOS) and intercellular cell adhesion molecule-1 (ICAM-1) were increased in the retinas of DR rats compared with in the normal control group. In conclusion, treatment with Niaspan significantly improved clinical and histopathological outcomes; decreased the expression levels of TNF-α, NF-κB, iNOS and ICAM-1; and decreased apoptosis and BRB breakdown, as compared with in the retinas of DR rats. The present study is the first, to the best of our knowledge, to demonstrate that Niaspan treatment ameliorates DR by inhibiting inflammation, and also suggests that the TNF-α pathway may contribute to the beneficial effects of Niaspan treatment.

Introduction

Diabetic retinopathy (DR) is a serious microvascular complication of diabetes and a major cause of blindness, which usually affects individuals between 30 and 70 years old (1). In a previous study, neuroinflammation has been suggested as an early event in the pathogenesis of DR (2). Diabetes affects the entire neurovascular unit of the retina, with gradual neurodegeneration, gliosis, neuroinflammation, vascular abnormalities including plasma leakage, compromised vascular blood-retinal barrier (BRB), edema, angiogenesis, and eventual fibrosis, all of which occur at increasing frequency (3). However, the treatment options for DR remain limited and are often associated with adverse effects; therefore, patients with diabetes have a high risk of eventual blindness. There is an emerging requirement to develop novel therapeutic approaches for this devastating disease.

Niacin (vitamin B3 or nicotinic acid) is the most effective medication for the treatment of the atherosclerosis in current clinical use, which increases high-density lipoprotein levels, and substantially lowers total cholesterol and triglyceride levels (4). Niaspan is a prolonged release formulation of niacin, which is safe to use in patients with diabetes (5). It has previously been reported that prolonged niacin treatment may exert anti-inflammatory effects (6). Furthermore, niacin has been revealed to inhibit vascular inflammation by downregulating the nuclear factor-κB (NF-κB) signaling pathway (7). However, the anti-inflammatory effects of Niaspan on DR have yet to be elucidated.

The present study aimed to examine the anti-inflammatory effects of Niaspan on streptozotocin (STZ)-induced DR. The results demonstrated that administration of Niaspan .3 months after the induction of diabetes significantly improved functional outcome, and inhibited vascular inflammation in the retina.

Materials and methods

Animals. Adult Male Wistar rats (age, 7 weeks; weight, 225-250 g) were purchased from the Academy of Military Medical Science (Beijing, China). The rats were housed in specific pathogen-free conditions (temperature 22±2°C,
light/dark cycle 12/12 h) with ad libitum access to food and water. All procedures involving rats were approved by the Laboratory Animal Care and Use Committee of Tianjin Medical University (Tianjin, China), and conformed to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research (8).

Diabetes induction and treatment. Diabetes was induced via injection of STZ (45 mg/kg; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) into the tail vein of Wistar rats. Fasting blood glucose levels were determined using a glucose analyzer 6 days after STZ injection; rats with fasting blood glucose levels >16.7 mmol/l were identified as diabetic and were used in the present study (9). Niaspan (China Resources Pharmaceutical Group Co., Ltd., Beijing, China) was dissolved in water, and 40 mg/kg/day was administered following STZ injection (the 7th day following STZ injection). A total of 90 rats were divided into the following groups: i) Normal control group (control group; n=30); ii) DR model group without Niaspan treatment (DR group; n=30); and iii) DR model group treated with Niaspan (Niaspan group; n=30).

Histological and immunohistochemical analyses. Rats were anesthetized via injection of chloral hydrate (concentration: 10%; 600 mg/kg) into the tail vein of Wistar rats in the third month following Niaspan treatment. Then the eyes were removed and were fixed in 4% paraformaldehyde with phosphate-buffered saline (PBS; pH 7.4) for 2 h at 4°C. The eyes were then dehydrated in a graded alcohol series and embedded in paraffin. The paraffin-embedded tissues were cut into 5 µm sections. Subsequently, the sections were stained with hematoxylin and eosin (H&E) by fluorescence microscope (Leica DMI4000B; Leica Microsystems GmbH, Dürren, Germany). For immunohistochemical analysis, sections (5 µm) were prepared from paraffin-embedded tissues and were incubated overnight at 4°C with antibodies against tumor necrosis factor-α (TNF-α; polyclonal rabbit anti-rat; cat. no. 74120; 1:100; GeneTex, Inc., Irvine, CA, USA). The sections were then stained with biotinylated anti-rabbit immunoglobulin G secondary antibody (cat. no. BA-1000; 1:200; Vector Laboratories, Inc., Burlingame, CA, USA) for 2 h (room temperature) followed by incubation with horseradish peroxidase streptavidin (cat. no. SA-5704; Vector Laboratories, Inc., Burlingame, CA, USA) for 2 h (room temperature) followed by incubation with horseradish peroxidase streptavidin (cat. no. SA-5704; Vector Laboratories, Inc., Burlingame, CA, USA). Retinal cell numbers in the ganglion cell layer (GCL) were counted in the region within a fixed 100-µm column.

Western blotting. Western blotting was performed using standard methods. Retinal protein was extracted using a radio-immunoprecipitation assay buffer (Beijing Zhongshan Golden Bridge Biotechnology; OriGene Technologies, Inc., Rockville, MD, USA) and was quantified using a protein assay (Bradford Protein Assay; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equal amounts of protein (800 µmol/l) were separated by 8-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked in 5% skim milk for 2 h (room temperature) and were incubated with antibodies against TNF-α (polyclonal rabbit anti-rat; cat. no. 74120; 1:1,000; GeneTex, Inc.), NF-κB (polyclonal rabbit anti-rat; cat. no. 54672; 1:1,000; GeneTex, Inc.), inducible nitric oxide synthase (iNOS; polyclonal rabbit anti-rat; cat. no. ab15323; 1:500; Abcam, Cambridge, UK) and intercellular adhesion molecule-1 (ICAM-1; polyclonal rabbit anti-rat; cat. no. 16174-1-AP; 1:1,000; Proteintech Group, Inc., Rosemont, IL, USA) overnight at 4°C. Subsequently, the membranes were washed in 0.1% TBS-Tween-20 and incubated with anti-rabbit IgG secondary antibody (cat. no. ZDR-5306; 1:5,000, Beijing Zhongshan Golden Bridge Biotechnology; OriGene Technologies, Inc.) at room temperature for 1 h. Monoclonal mouse anti-β-actin (cat. no. TA-09; 1:1,000; Zhongshan Golden Bridge Biotechnology Co., Ltd.) was used as an internal reference. Finally, the blots were scanned with a ChemiDoc™ MP system (Bio-Rad Laboratories, Inc.) and the bands were semi-quantified using ImageJ 1.51 software (National Institutes of Health, Bethesda, MA, USA).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from retinas (taken from 3 rats for each group) using TRIzol® reagent (cat. no. 15596; Thermo Fisher Scientific, Inc., Waltham, MA, USA). RNA was reverse transcribed into cDNA using the TransScript First-Strand cDNA Synthesis SuperMix (cat. no. AT301; TransGen Biotech Co., Ltd., Beijing, China). The primer sequences were as follows: β-actin, forward 5'-AGC CATGTCAGTACGCTACCC-3', reverse 5'-ACCCCTCATTGAGGACAGAC-3'; NF-κB, forward 5'-TGGAGCTTTGGTTGTAGA-3', reverse 5'-TTATGGCTGAGGTCGTC-3'; iNOS, forward 5'-TATCTGACACATACTTACC-3', reverse 5'-TCTGGTGAACACTCTGATT-3'; and ICAM-1, forward 5'-GGCTCTCAGTGTGTA-3' and reverse 5'-AAC CCCATCCAGGCTCA-3'. The relative mRNA expression levels of NF-κB, iNOS and ICAM-1 were determined by RT-qPCR with TransStart Top Green qPCR SuperMix (cat. no. AQ131; TransGen Biotech Co., Ltd.). β-actin mRNA was used as an internal control. All procedures were performed according to the manufacturers' protocols. The relative mRNA expression levels were determined using the 2-ΔΔCq method (10).

Measurement of BRB breakdown using Evans blue. 2% Evans blue dye (Sigma-Aldrich; Merck Millipore) in saline was administered via the tail vein of rats (n=4/group) as a BRB permeability tracer 2 h prior to sacrifice. Rats were sacrificed via injection of chloral hydrate (concentration: 10%; 600 mg/kg) into the tail vein. For 10 sec at a dose of 45 mg/kg, and after the dye had circulated for 120 min, the eyes were immediately fixed in 4% paraformaldehyde for 2 h. Subsequently, the anterior segments were removed and the retinas were dissected and washed in cold PBS. The retinas were then spread on glass slides, vitreous side up, and mounted with mounting medium. Images were captured using a confocal scanning laser imaging system fitted with krypton-argon lasers (FV1000; Olympus Corporation, Tokyo, Japan).
Quantitative evaluation of Evans blue dye extravasation.

Evans blue dye (Sigma-Aldrich; Merck Millipore) was dissolved in normal saline (30 mg/ml), and was injected into the tail vein of rats (n=3/group) for 10 sec at a dose of 45 mg/kg. After the dye had circulated for 120 min, the chest cavity was opened and the left heart ventricle was cannulated. Each rat was perfused with PBS (37˚C) for 2 min to clear the dye, ensuring the physiological pressure was maintained at 120 mmHg. Immediately after perfusion, the eyes were enucleated and retinas were carefully dissected. The weight of each retina was measured after thorough drying in a Speed-Vac. Albumin leakage into the retinal tissue was estimated via the measurement of extravasated Evans blue dye. Evans blue was extracted by incubating each retina in 0.3 ml formamide for 18 h at 70˚C. The extract was filtered through a 30,000 MW filter at a speed of 300 x g for 45 min at 4˚C. The absorbance of the filtrate was measured using a spectrophotometer at 620 and 740 nm, the absorption maximum for Evans blue in formamide. Calculations were based on the external standards dissolved in the same solvent. The concentration of dye in the extracts was calculated from a standard curve of Evans blue in formamide and normalized to the dry retinal weight and the time-averaged concentration of Evans Blue in the plasma.

Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL). Apoptosis was examined by TUNEL assay. TUNEL-positive nuclei in the GCL of the retina were counted. Briefly, following 8 min fixation with ice-cold acetone solution, cryopreserved tissue sections were washed three times with PBS. The sections were incubated with 1 ml blocking buffer (3% normal goat serum (Sigma-Aldrich; Merck Millipore) in PBS for 1 h at room temperature. Following incubation, the sections were washed in a permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. After washing, the sections were incubated in 50 µl TUNEL reaction mixture (cat. no. 12156792910; Roche Diagnostics GmbH, Mannheim, Germany) for 60 min at 37˚C in the dark. Subsequently, the sections were counterstained with 4',6-diamidino-2-phenylindole. The sections were washed and observed under a fluorescence microscope (Olympus Corporation). The retinal cell numbers in the GCL were counted in the region within a 1-mm column.

Statistical analysis. Data are presented as the mean ± standard deviation (each experiment was repeated 3 times) and were analyzed by SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Results were analyzed by one-way analysis of variance followed by a least significant difference procedure. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of Niaspan treatment on DR. To determine whether diabetes induces DR and whether Niaspan treatment regulates DR recovery, H&E staining was performed. As presented in Fig. 1, non-diabetic rats exhibited normal retinas; all cell
layers of the retina were clear and neatly arranged. In the DR group, cells were disorganized after diabetic modeling. Obvious inflammatory cell infiltration in the GCL, hemorrhage, and neovascularization in the inner nuclear layer (INL) were observed. In the Niaspan-treated retina, retinal edema and hemorrhage were markedly attenuated, and the ganglionic layer was neatly arranged (Fig. 1A). Furthermore, cell number in the retinal GCL was significantly reduced in the DR group (P<0.05) compared with the control group. Treatment with Niaspan was able to significantly reverse the reduction in retinal cell numbers (P<0.05) compared with diabetic retinas (Fig. 1B).

Niaspan reduces DR-induced apoptosis of retinal cells in the GCL. Niaspan inhibits retinal cell apoptosis. Abundant numbers of TUNEL+ cells were detected in the GCL of diabetic retina (40.25±5.7373; P<0.05) compared with the control group. Conversely, fewer apoptotic cells were detected in the GCL of the Niaspan-treated group (19.75±6.0208; P<0.05) compared with the diabetic retina. No TUNEL+ cells were observed in the control group retinas (Fig. 2).

Niaspan prevents DR-induced BRB breakdown. DR-induced breakdown of the BRB was assessed by Evans blue extravasation from retinal vessels. As an initial approach, retinal blood vessel integrity was analyzed in flat mount retinas. Evans blue dye was shown to leak from the vessels to the surrounding tissue in DR rats. Niaspan treatment of diabetic rats was able to prevent this effect (Fig. 3A). Quantitative detection of Evans blue dye from the retinal tissue confirmed the results obtained by fluorescence microscopy. Diabetes increased BRB permeability in diabetic rats (29.71±1.3214 ng Evans blue/mg dry weight retina; P<0.05) compared with the control rats (12.5±0.91591 ng Evans blue/mg dry weight retina). Treatment with Niaspan significantly prevented BRB breakdown in diabetic rats (18.15±0.45211 ng Evans blue/mg dry weight retina; P<0.05) compared with untreated diabetic rats (Fig. 3B).

Niaspan reduces DR-induced TNF-α and NF-κB retinal expression. To determine whether treatment with Niaspan regulates TNF-α and NF-κB expression TNF-α and NF-κB expression levels were detected. As presented in Fig. 4, western blotting indicated that DR markedly increased the expression levels of TNF-α and NF-κB (Fig. 4A). A quantitative analysis revealed that there was a significant increase in TNF-α and NF-κB in diabetic rats (P<0.05) compared with the control rats. Treatment with Niaspan was able to significantly prevent the increase in TNF-α and NF-κB (P<0.05) compared with diabetic rats (Fig. 4B).

Immunohistochemistry indicated that treatment with Niaspan significantly decreased the expression levels of TNF-α (Fig. 5A). A quantitative analysis revealed that there was a significant increase in TNF-α expression in diabetic
Figure 3. BRB leakage analysis in a DR model. (A) Evans blue assay for BRB leakage (n=4/group). (B) Quantitative analysis of Evans blue exudation. The arrows indicate Evans blue extravasation from retinal vessels. *P<0.05 compared with nondiabetic retina in the CON group; #P<0.05 compared with diabetic retina in the DR group. CON, control; DR, diabetic retinopathy; NA, Niaspan; BRB, blood-retinal barrier.

Figure 4. Effects of Niaspan on the retinal expression levels of TNF-α and NF-κB in a rat model of DR. (A) Western blotting and (B) quantitative analysis of TNF-α and NF-κB expression in the retina (n=4/group). *P<0.05 compared with nondiabetic retina in the CON group; #P<0.05 compared with diabetic retina in the DR group. CON, control; DR, diabetic retinopathy; NA, Niaspan; TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor-κB.
rats (P<0.05) compared with control rats. Treatment with Niaspan was able to significantly prevent the increase in TNF-α (P<0.05) compared with in diabetic rats (Fig. 5B). PCR analysis detected a significant increase in NF-κB expression in diabetic rats (P<0.05) compared with control rats. Treatment with Niaspan was able to significantly prevent the increase in NF-κB expression (P<0.05) compared with in diabetic rats (Fig. 5C).

**Discussion**

The present study used the common animal model of STZ-induced DR; the results confirmed that STZ injection resulted in diabetes and significantly induced DR; however, long-term Niaspan treatment reduced the formation and development of DR, and inhibited the development of inflammation. This conclusion is based on several lines of experimental evidence. Initially, the present study indicated that treatment of diabetic rats with Niaspan markedly decreased hemorrhage, leukocyte infiltration and apoptosis in the GCL of the diabetic retina. STZ has previously been demonstrated to induce hyperglycemia and lead to the generation of oxidative stress (11), which is a typical characteristic of DR in rats, which may promote the destruction of endothelial integrity and breakdown of the BRB. These alterations in endothelial integrity were detected by cell apoptosis analysis and retinal vascular permeability assay. Thirdly, treatment of DR with Niaspan significantly decreased the expression of inflammatory mediators, including TNF-α, NF-κB, iNOS and ICAM-1 compared with in diabetic retinas.

**Niaspan reduces DR-induced iNOS and ICAM-1 retinal expression.** To determine whether Niaspan treatment regulates iNOS and ICAM-1 target gene expression ICAM-1 and iNOS expression levels were detected. As presented in Fig. 6, western blotting indicated that Niaspan treatment markedly decreased the expression levels of ICAM-1 and iNOS (Fig. 6A). A quantitative analysis revealed that there was a significant increase in iNOS and ICAM-1 expression in diabetic rats (P<0.05) compared with in control rats. Treatment with Niaspan was able to significantly prevent the increase in iNOS and ICAM-1 expression (P<0.05) compared with in diabetic rats (Fig. 6B). PCR analysis detected a significant increase in iNOS and ICAM-1 expression in diabetic rats (P<0.05) compared with in control rats. Treatment with Niaspan was able to significantly prevent the increase in iNOS and ICAM-1 (P<0.05) expression compared with in diabetic rats (Fig. 6C).
death (15). As a consequence, Niaspan may mitigate cell apoptosis and BRB breakdown by downregulating inflammatory factors.

TNF-α is a major proinflammatory cytokine that is involved in numerous inflammatory pathologies, and is predominantly produced by macrophages (16). Increased levels of TNF-α have been detected in the vitreous of diabetic patients with proliferative DR (17) and in diabetic rat retinas (18). TNF-α is a potent mediator of leukostasis induced by vascular endothelial growth factor, interleukin-1 α, and platelet-activating factor in the retinal vasculature (19), and also mediates the cell death/apoptosis of retinal neurons and vascular endothelial cells in DR (13). The involvement of the inflammatory cytokine TNF-α in the apoptotic cell death of retinal endothelial cells during the early and late stages of DR in a rat model of STZ-induced diabetes has previously been investigated (18,20). The present study suggested that Niaspan markedly decreases TNF-α in the diabetic retina, which may contribute to the beneficial effects of Niaspan treatment.

Activation of NF-κB induces the expression of numerous inflammatory cytokines, including TNF-α (21), which are crucial factors in inflammation. However, TNF-α is not only induced by NF-κB, but is also a strong activator of NF-κB (22). In addition, inhibition of TNF-α may inhibit the activity of NF-κB (23), which is a widely expressed inducible transcription factor that is an important regulator of several genes involved in mammalian inflammatory and immune responses, proliferation and apoptosis (24). The present study demonstrated that Niaspan may significantly prevent the increase in TNF-α and NF-κB expression, which was induced by DR.
These findings suggested that NF-κB, under the regulation of TNF-α, may be associated with diabetes-induced inflammation in the retina.

Upregulation of iNOS has been detected in the retinas of experimental diabetic rodents and human patients in previous studies (25,26). In addition, iNOS serves an important role in leukostasis, apoptosis and BRB breakdown (27,28). Concurrently, white blood cells interact with, and bind to, ICAM-1 on the surface of endothelial cells in a multi-step process leading to adherence of the blood cells to the endothelial wall. Notably, suppression of ICAM-1 attenuates retinal leukostasis in animal models of DR (29). TNF-α regulates the expression of adhesion molecules, including ICAM-1, which is correlated with the increase in leukostasis and BRB breakdown in diabetic rat retinas (18). Furthermore, a previous study demonstrated that suppression of NF-κB (11 activation in the retinas of diabetic rats inhibited the expression of inflammatory mediators, including iNOS and ICAM-1, and capillary degeneration and pericyte loss in these animals (24).

In conclusion, the present study indicated that DR leads to the generation of important inflammatory cytokines, which may lead to endothelial cell apoptosis and BRB breakdown. These findings suggested that Niaspan-induced downregulation of TNF-α may contribute to amelioration of the inflammatory reaction in diabetic rats. Furthermore, TNF-α may induce reactive oxygen species formation, NF-κB activation and iNOS expression in inflammatory cells, and rapidly upregulate the expression of ICAM-1 at the endothelial surface (30). In accordance with these findings, the reduction of TNF-α may reduce apoptosis of endothelial cells and BRB breakdown. The results of the present study strongly indicated that Niaspan may be considered a potential therapeutic agent for the treatment of DR via inhibition of the inflammatory process.

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