Peroxynitrite Mediates Retinal Neurodegeneration by Inhibiting Nerve Growth Factor Survival Signaling in Experimental and Human Diabetes

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OBJECTIVE—Recently we have shown that diabetes-induced retinal neurodegeneration positively correlates with oxidative stress and peroxynitrite. Studies also show that peroxynitrite impairs nerve growth factor (NGF) survival signaling in sensory neurons. However, the causal role of peroxynitrite and the impact of tyrosine nitration on diabetes-induced retinal neurodegeneration and NGF survival signaling have not been elucidated.

RESEARCH DESIGN AND METHODS—Expression of NGF and its receptors was examined in retinas from human and streptozotocin-induced diabetic rats and retinal ganglion cells (RGCs). Diabetic animals were treated with FeTPPS (15 mg · kg^{-1} · day^{-1} ip), which catalytically decomposes peroxynitrite to nitrate. After 4 weeks of diabetes, retinal cell death was determined by TUNEL assay. Lipid peroxidation and nitrotyrosine were determined using MDA assay, immunofluorescence, and Slot-Blot analysis. Expression of NGF and its receptors was determined by enzyme-linked immunosorbent assay (ELISA), real-time PCR, immunoprecipitation, and Western blot analyses.

RESULTS—Analyses of retinal neuronal death and NGF showed ninefold and twofold increases, respectively, in diabetic retinas compared with controls. Diabetes also induced increases in lipid peroxidation, nitrotyrosine, and the pro-apoptotic $p75^{NTR}$ receptor in human and rat retinas. These effects were associated with tyrosine nitration of the pro-survival TrkA receptor, resulting in diminished phosphorylation of TrkA and its downstream target, Akt. Furthermore, peroxynitrite induced neuronal death, TrkA nitration, and activation of p38 mitogen-activated protein kinase (MAPK) in RGCs, even in the presence of exogenous NGF. FeTPPS prevented tyrosine nitration, restored NGF survival signal, and prevented neuronal death in vitro and in vivo.

CONCLUSIONS—Together, these data suggest that diabetes-induced peroxynitrite impairs NGF neuronal survival by nitrating TrkA receptor and enhancing $p75^{NTR}$ expression. ***Diabetes*** **57: 889–898, 2008**

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4-HNE, 4-hydroxynonenal; bFGF, basic fibroblast growth factor; DR, diabetic retinopathy; ELISA, enzyme-linked immunosorbent assay; MAPK, mitogen-activated protein kinase; NGF, nerve growth factor; PI3K, phosphatidylinositol 3-kinase; RGC, retinal ganglion cells; ROD, relative optical density; PN, peroxynitrite; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; VEGF, vascular endothelial growth factor.

Diabetic retinopathy (DR), the leading cause of blindness in working-age adults, has enormous public health implications: the number of people worldwide at risk of developing vision loss from diabetes is predicted to double over the next 25 years (1). Chronic hyperglycemia, with subsequent dysfunction of the retinal vasculature and neural network, is thought to be the origin of DR (2,3). The loss of vision has been linked to macular edema and vitreoretinal neovascularization secondary to vascular dysfunction (2,4). However, histopathological and functional studies have emphasized the early loss of neurons in human and experimental models of DR (5–10). Studies have shown that retinal ganglion cells (RGCs) in particular are early targets of diabetic insult before vascular changes can be detected in DR (6,7). Neurotrophins, including nerve growth factor (NGF), are not only important regulators of retinal development, but also play a key role in regeneration of neural circuits in the visual system in retinal degenerative diseases (11). NGF signals by binding tyrosine kinase receptor, TrkA, and the low-affinity neurotrophin receptor, $p75^{NTR}$. NGF binding to TrkA activates the phosphatidylinositol 3-kinase (PI3K)/Akt cascade resulting in neuronal survival signal (12). P75NTR, a member of the tumor necrosis factor (TNF) receptor family, is a bifunctional receptor that can facilitate TrkA-mediated survival or activate the pro-apoptotic p38 mitogen-activated protein kinase (MAPK) signal in neurons lacking TrkA expression or activity (rev. in 13,14). Paradoxically, while diabetes accelerates neuronal death, it has also been reported to stimulate local and systemic NGF levels in experimental and human diabetes (15–19).

Despite the increased nitric oxide formation under diabetic conditions, the increase in superoxide anion in the neural retina leads to decreased bioavailability of nitric oxide and thus peroxynitrite formation (20). Previously, we have shown that an increase in peroxynitrite, as indicated by tyrosine nitration, correlates with accelerated retinal endothelial cell death, breakdown of the brain-retinal barrier, and accelerated neuronal cell death in models of experimental diabetes and neurotoxicity (10,21–23). These studies suggest a key role of peroxynitrite in mediating different aspects of DR. However, the causal role of peroxynitrite in diabetes-induced neurodegeneration or the potential neuroprotective effects of the peroxynitrite decomposition catalyst, FeTTPS, have not been elucidated. Studies in neuronal cells suggest the potential role of peroxynitrite to inhibit the NGF/TrkA signal by nitrating tyrosine residues on the TrkA receptor, thus preventing them from becoming phosphorylated by NGF

(24,25). The aim of this study is to investigate the causal role of peroxynitrite in mediating diabetes-induced neuronal death and to elucidate the molecular mechanism by which tyrosine nitration alters the NGF pro-survival pathway in the diabetic retina and RGC.

RESEARCH DESIGN AND METHODS

Postmortem eye specimens. Human eyes obtained from The Georgia Eye Bank (Atlanta, GA) had the following selection criteria: >50 years old, either insulin-requiring diabetes or no diabetes, and no life-support measures. The eyes were enucleated an average of 6.71 ± 0.84 h after death. Retinas were preserved by immediate freezing, followed by homogenization and analysis of protein expression using biochemical assays as described below.

Experimental animals. All procedures with animals were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the VA Medical Center Animal Care and Use Committee. Male Sprague-Dawley rats (~250 g body weight) were randomly assigned to control, treated-control, diabetic, or treated-diabetic groups. Three sets of animals were prepared (totaling 62 rats) to study the effects of 4 weeks of experimental diabetes.

Diabetes was induced by intravenous tail-vein injection of streptozotocin (65 mg/kg). After 48 h, diabetic status was determined by urine detection of glucose. Diabetes was confirmed with blood-glucose levels >350 mg/dl. The animals were treated with the peroxynitrite decomposition catalyst, FeTPPS [5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron (III)] (Calbiochem), via intraperitoneal injections at 15 mg/kg, daily for 14 days. FeTPPS exhibits minimal superoxide dismutase (SOD) mimetic activity, does not complex with nitric oxide, and catalytically isomerizes peroxynitrite to nitrate. Treatment dose was selected based on previous studies showing protective effects at 10 mg/kg ip (26). After 4 weeks of diabetes, animals were killed and eyes were enucleated for analyses.

Evaluation of neural cell death. Retinal cell death was determined by TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) assay, using immunoperoxidase staining (ApopTag-Peroxidase; Chemicon). For total quantifiable calculation of cell death, whole, flat-mounted samples were processed as described previously (6,10) with a minor modification. Endogenous peroxidase quenching was skipped, as this has been noted to cause DNA breaks and lower TdT-enzyme activity (27). Samples were stained with 3-amino-9-ethylcarbazole (Sigma) following the manufacturer's instructions. The total number of TUNEL-positive cells was counted using light-microscopy. Appropriate negative and positive controls were used to assess proper reactivity. In addition, the histological distribution of cell death was analyzed using 10-μm optimum cutting temperature–frozen eye sections stained with TUNEL fluorescence (ApopTag Fluoroscein) as described previously (23) or labeled with caspase-3 antibody (Cell Signaling). Müller cells were labeled using CRALBP antibody (ABR). RGCs were labeled using antibodies for Thy-1 (BD Biosciences) or NeuN (Millipore).

Retinal protein extraction. Individual rat retinas or equivalent-size human retinas were homogenized in a Mini-Bead beater with treated Ottawa sand in 300 μl of RIPA (radioimmunoprecipitation assay) buffer (Upstate).

Determination of lipid peroxidation. Amount of lipid peroxides present in human and rat retinas was determined by methods previously described by our group (10,22). Briefly, retinal homogenate was incubated with SDS, acetic acid, and thiobarbituric acid at 95°C for 1 h. 1,1,3,3-tetraethoxypropane was used as a standard. Lipid peroxides were expressed as millimoles malondialdehyde (MDA) normalized to milligrams of retinal protein.

Slot-blot analysis was used to measure oxidative and nitrative stress markers: 4-hydroxynonenal (4-HNE) adducts and nitrotyrosine, respectively. As described previously (22), 30 μg of retinal homogenate from human or rat samples was immobilized onto a nitrocellulose membrane. After blocking, membranes were reacted with antibodies against 4-HNE (Alpha Diagnostics International) or nitrotyrosine (Calbiochem), and the optical density of various samples were compared with that of controls.

Immunolocalization of nitrotyrosine, a biomarker for peroxynitrite. Retinal cross-sections were processed as described previously (22). Sections were stained with nitrotyrosine (Calbiochem, CA). Images were collected from three fields per retina (n = 6/group) using an AxioObserver.Z1 Microscope (Zeiss, Germany) and morphometric software to quantify the intensity of nitrotyrosine signal.

Determination of NGF. NGF levels in plasma of various animal groups were measured according to the manufacturer's instructions using an ELISA kit (Chemicon). Plasma samples were diluted 1:2 and incubated with anti-NGF antibody followed by peroxidase conjugate antibody. The amount of NGF was determined from a standard curve, and the absorbance was measured at 450 nm.

Retinal mRNA NGF expression. Retinal mRNA was prepared according to the manufacturer's instructions using a Promega kit. The One-Step qRT-PCR Invitrogen kit was used to amplify 10 ng retinal mRNA from each sample. A pair of rat-specific NGF primers was synthesized using accession no. M36589 and was used to amplify a 121-bp DNA fragment forward-primer 5′-8 ACAT TCCGGAGCCAACTCTACCAA and reverse-primer 5′ AGCATCCTGCTTTCT GACCAGTCT (corresponding to nucleotides 13841–505). The rat 18-S (accession no. X01117) was used to amplify sequence position 881-1110 and as an internal marker for each sample (forward-primer CGCGGTTCTATTTTGT TGGT and reverse primer AGTCGGCATCGTTTATGGTC). Quantitative PCR was performed using a Realplex Mastercycler (Eppendorf). NGF expression was normalized to the 18S level in each sample and expressed as relative expression to normal controls.

Western blot and immunoprecipitation. Retinal protein homogenate of 60 μg was separated on SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and incubated with specific antibodies (TrkA from Chemicon; phospho-TrkA-Y490, Akt, phospho-Akt, phospho-p38 MAPK, and p38 MAPK from Cell Signaling; $p75^{NTR}$ and nitrotyrosine from Upstate; and NGF and phosphotyrosine from Santa Cruz). Membranes were reprobed with β-actin (Sigma) to confirm equal loading. The primary antibody was detected using a horseradish peroxidase–conjugated antibody and enhanced chemiluminescence (Amersham BioSciences). The films were subsequently scanned, and band intensity was quantified using densitometry software (Alpha Innotech) and expressed as relative optical density (ROD). For immunoprecipitation, 200 μg of retinal protein was diluted with RIPA buffer and incubated with 1 μg of anti-TrkA antibody. Samples were rocked at 4°C for 2 h followed by agarose beads and rocked at 4°C overnight. Final extract was boiled, processed, and analyzed as described above. Membrane was probed with nitrotyrosine or phosphotyrosine antibodies.

RGCs were kindly gifted by Dr. Agrawal (Fort Worth, TX). Cell culture medium was purchased from Mediatech, peroxynitrite from Upstate, and NGF from Santa Cruz. The immortalized cells were grown to confluence, switched to serum-free medium, and cultured overnight. Dose-response studies showed that (100 μmol/l) peroxynitrite was the optimal dose to induce apoptosis without affecting cell number. Cells were treated with peroxynitrite (100 μmol/l) in the presence or absence of FeTPPS (2.5 μmol/l). For some experiments, cells were stimulated with NGF. Stock concentrations of peroxynitrite were freshly prepared in 0.1N NaOH. An equal amount of NaOH or decomposed peroxynitrite was used for control experiments. Neither of these had an effect on the analyzed parameters.

Determination of caspase-3 activity and TUNEL-positive cells in RGCs. Caspase-3 activity was determined as previously described (21) using a kit from R&D Systems (Minneapolis, MN) according to the manufacturer's recommendations. Cell lysate was incubated with caspase-3 fluorogenic substrate (DEVD-AFC) for 2 h at 37°C. The fluorescence was measured with Synergy-2 plate-reader (BioTek Instruments) at 400 nm excitation and 505 nm emission.

Additionally, RGC death was confirmed using TUNEL fluorescence (ApopTag Fluoroscein) and counterstained with DAPI. The total number of TUNEL-positive cells was counted in various groups.

Data analysis. The results are expressed as means ± SD. Differences among experimental groups were evaluated by analysis of variance, and the significance of differences between groups was assessed by the post hoc test (Fisher's protected least significant difference) when indicated. Significance was defined as $P < 0.05$.

RESULTS

Diabetes increases oxidative stress and tyrosine nitration in rat and human retinas. We and others have reported increases in peroxynitrite formation as indicated by nitrotyrosine in diabetic rat retinas. However, tyrosine nitration has not been examined previously in the human retina. Therefore, we examined postmortem human retinas from donors with and without diabetes. The clinical details of each donor are summarized in Table 1. As shown in Fig. 1*A*, slot-blot analysis of human retinal homogenate showed a significant 1.7-fold increase of total tyrosine nitration in diabetic compared with nondiabetic controls ($P < 0.02$).

Similarly, retinas from diabetic rats (4 weeks) showed an ~1.7-fold increase ($P < 0.01$) of tyrosine nitration compared with controls (Fig. 1*B*). Immunolabeling of nitrotyrosine in retinal sections showed prominent stain-

TABLE 1
Clinical characteristics of eye donors

Sample	Age (years)	Sex	Diabetes duration (years)	Retinal pathology	Cause of death
1	62	M	—	None	Prostate CA
2	53	M	—	None	Colon CA
3	78	F	—	None	CVA
4	36	M	—	None	MVC
5	46	M	—	None	ACI
6	67	F	8	No known retinopathy	CHF
7	50	M	8	No known retinopathy	Cardiac arrest
8	74	F	20	No known retinopathy	Other
9	55	M	1	No known retinopathy	Cardiac arrest

Postmortem human retinas were homogenized and used for Western blot, slot blot, and immunoprecipitation analysis. ACI, acute coronary insufficiency; CA, cancer; CHF, congestive heart failure; CVA, cerebrovascular accident; MVC, motor vehicle collision.

ing within the nerve fiber layer of diabetic rat retinas (Fig. 1*C*).

Quantitative analysis of the entire retina image showed a 1.8-fold increase ($P < 0.0005$) in the diabetic retinas compared with controls (Fig. 1*D*). Treatment of diabetic animals with the peroxynitrite decomposition catalyst, FeTPPS (15 mg · kg^{-1} · day^{-1}), reduced formation of nitrotyrosine to near control levels.

Due to its high content of polyunsaturated fatty acid, the neuronal retina is particularly susceptible to oxidative insult (28). Our results showed that diabetes increased oxidative stress,s as indicated by 1.6-fold increase in lipid peroxidation in diabetic human retinas (Fig. 1*E*) and an ~1.7- fold increase in diabetic rat retinas (Fig. 1*F*). This effect was significantly reduced ($P < 0.008$) by treatment with FeTPPS. Lipid peroxide formation was not altered by FeTPPS alone in treated controls. Furthermore, 4-HNE adducts, the most cytotoxic aldehyde formed during lipid-peroxidation, which can covalently modify protein residues and lead to functional impairment, were assessed using an antibody for 4-HNE (29). As shown in Fig. 1*G*, slot-blot analysis of human retinal homogenate showed a significant 1.4-fold increase of 4-HNE in diabetic compared with nondiabetic controls ($P < 0.03$). Similarly, retinas from diabetic rats showed an ~1.5-fold increase ($P < 0.01$) of 4-HNE compared with controls (Fig. 1*H*). Treatment with FeTPPS reduced the levels of 4-HNE to control levels.

Neutralizing peroxynitrite does not alter metabolic conditions in experimental diabetes. As shown in Table 2), there was no significant difference between the beginning and end weights of treated and control animal groups. Treatment with FeTPPS had no significant effect on altering blood glucose levels of treated control or diabetic rats.

Neutralizing peroxynitrite prevents neuronal cell death in the diabetic retina. Accelerated neuronal death has been well documented in human and experimental diabetes (6,10,30), which positively correlates with increases in oxidative stress and peroxynitrite formation. Yet, the causal role of peroxynitrite in mediating neuronal death has not been elucidated. Quantitative analysis of TUNEL-labeled cells in whole flat-mounted retinas showed a more than ninefold increase in the frequency of retinal cell death in the diabetic retinas (Fig. 2*A* and *B*). Treatment with FeTPPS blocked the number of TUNEL-positive cells ($P < 0.008$) to near control levels. Treatment with FeTPPS did not alter TUNEL results in the treated controls (data not shown). Our studies using B4-isolectin showed that TUNEL-positive nuclei did not colocalize with the endothelial marker (data not shown), suggesting that the majority of cells undergoing death were not vascular. We further confirmed that the cell death was neuronal rather than vascular by staining frozen sections using TUNEL-FITC (fluorescein isothiocyanate) and the specific RGC marker, Thy-1. As shown in Fig. 2*C*, TUNEL-positive nuclei (green) were frequently detected in RGCs (red) of diabetic retinas. Further immuno-colocalization studies were performed to confirm diabetes-induced apoptosis in the RGC layer using antibodies for the apoptotic marker caspase-3 (green) and the RGC marker, Thy-1, (red) or neuronal marker, NeuN (red). Diabetic retinas showed enhanced caspase-3 expression within RGCs, as indicated by colocalization (orange) with NeuN (Fig. 2*D*) or with the Thy-1 (Fig. 2*E*), compared with controls.

Treatment of diabetic animals with FeTPPS reduced caspase-3 expression back to control levels. In addition, our studies using the specific glial Müller cell marker (CRALBP) showed that TUNEL-positive nuclei did not colocalize with glial cells (Fig. 2*F*).

Diabetes increases serum and retinal NGF. Increases in levels of NGF have been previously reported in experimental and human diabetes (15–19). ELISA results demonstrated a twofold increase in plasma NGF levels of diabetic animals compared with controls (Fig. 3*A*), which decreased significantly after treatment with FeTPPS ($P < 0.04$). In parallel, real-time PCR analysis of rat retina showed a significant approximate twofold increase ($P < 0.009$) in NGF mRNA level of diabetic rats compared with controls. Treatment of diabetic animals with FeTPPS reduced NGF mRNA levels to 1.25-fold (Fig. 3*B*). These results suggest that diabetes increases NGF locally and systemically.

Diabetes causes peroxynitrite-mediated tyrosine nitration of TrkA in experimental and human diabetes. NGF mediates its survival signal by binding TrkA and $p75^{NTR}$. As shown in Fig. 4*A*, Western blot analysis of TrkA expression showed no significant difference ($P < 0.29$) between the various animal groups. Similarly, TrkA expression was not altered between diabetic and nondiabetic humans (Fig. 4*D*). Previous studies show that TrkA receptor has been tyrosine nitrated and inhibited in response to peroxynitrite (25). Together, these findings suggest that diabetes-induced peroxynitrite formation might cause posttranslational modification by nitrating tyrosine residues on TrkA receptor and inactivating the NGF/TrkA survival signal. As shown in Fig. 4*B* and *D*, retinas from diabetic humans and diabetic rats showed a ~1.7-fold and a 2-fold increase, respectively, in tyrosine nitration of

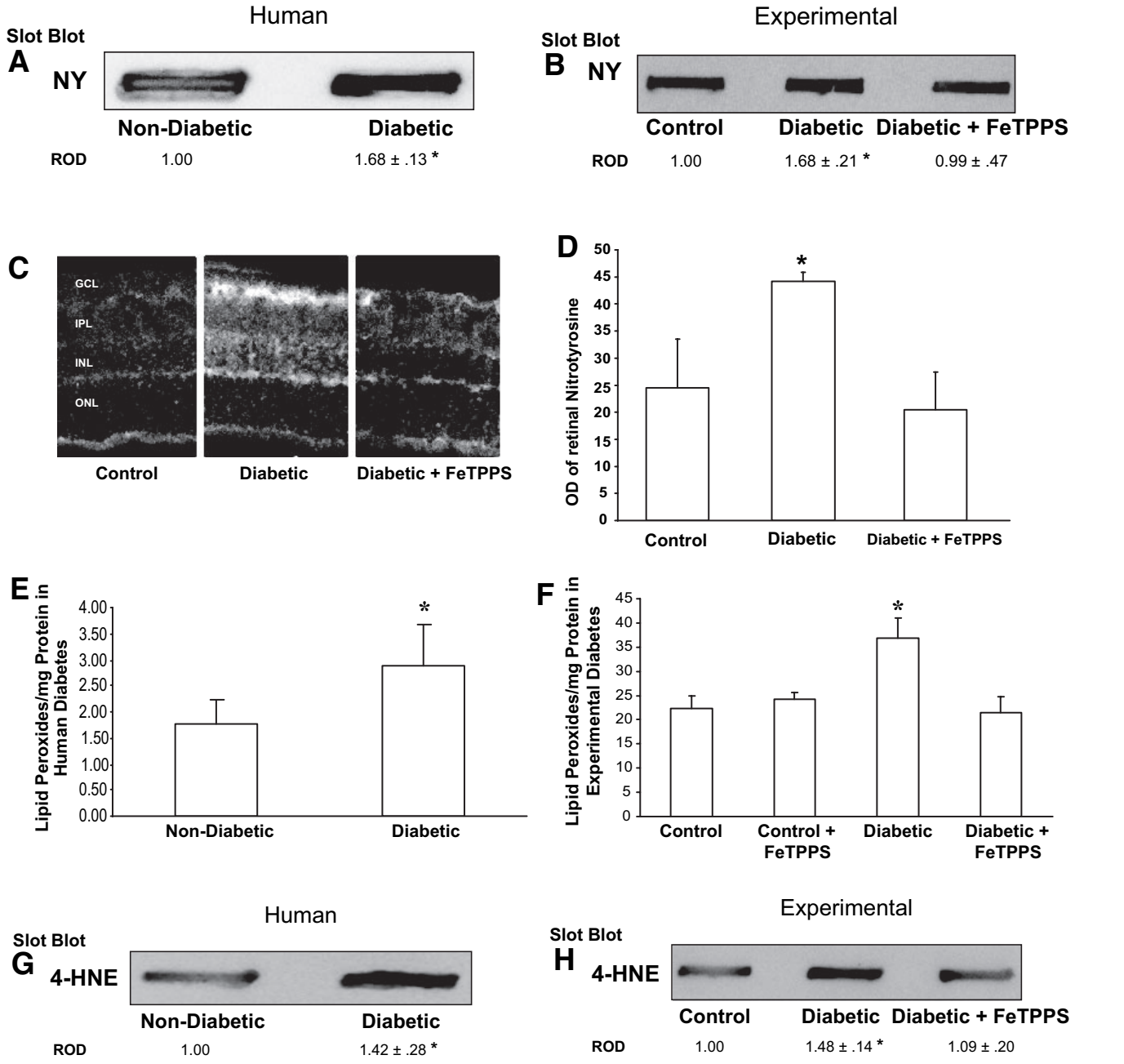

FIG. 1. Diabetes increases oxidative stress and nitrotyrosine formation in experimental and human diabetic retinas. *A*: Slot-blot analysis of human retinal homogenate showing ~1.7-fold increase in nitrotyrosine formation in diabetic retina ($n = 4$) compared with nondiabetic controls ($n = 5$).*$P < 0.02$. *B*: Slot-blot analysis of retinal homogenate showing an ~1.7-fold increase in nitrotyrosine formation in diabetic retina compared with controls. Treatment with the decomposition catalyst, FeTPPS (15 mg · kg^{-1} · day^{-1}), normalized tyrosine nitration to control levels. * $P < 0.01$. *C*: A representative image showing prominent immunolocalization of nitrotyrosine within ganglion cell layer (GCL) and inner nuclear layer (INL) and less distribution in inner plexiform layer (IPL) and outer uclear layer (ONL) in the diabetic rat retinas compared with control and FeTPPS-treated animals. *D*. Statistical analysis of the optical density of nitrotyrosine showing a 1.8-fold increase in diabetic retinas compared with control and FeTPPS-treated animals. *$P < 0.0005$. *E*: Assay for lipid peroxidation using TBARS showed an ~1.6-fold increase in diabetic retinas ($n = 6$) compared with nondiabetic controls ($n = 5$).* $P < 0.02$. *F*: Assay for lipid peroxidation using TBARS showed an ~1.5- fold increase in diabetic retinas compared with controls. Treatment with FeTPPS significantly decreased the amount of lipid peroxidation in diabetic animals but did not alter basal level in the treated controls. *$P < 0.008$. *G*: Slot-blot analysis of human retinal homogenate showing an ~1.4-fold increase in 4-HNE adduct formation in diabetic retina ($n = 6$) compared with nondiabetic controls ($n = 5$).* $P < 0.02$. *H*: Slot-blot analysis of retinal homogenate showing an ~1.5-fold increase in 4-HNE adduct formation in diabetic retina compared with controls. Treatment with the decomposition catalyst, FeTPPS (15 mg · kg^{-1} · day^{-1}), reduced tyrosine nitration to control levels. *$P < 0.01$. Data are means ± SD of six animals per group.

the TrkA receptor compared with controls. We further confirmed the inhibitory effect of tyrosine nitration on TrkA by measuring tyrosine phosphorylation of the receptor. We observed a decreased global phosphorylation of the TrkA receptor in retinas from human diabetic subjects (Fig. 4*E*) and diabetic rats compared with controls (data not shown). We next evaluated the activation of TrkA at its Y490 residue, as it is responsible for triggering the PI3K/Akt neuronal survival pathway. Western blot analysis of phospho-TrkAY490 showed impaired phosphorylation in diabetic rat retinas (Fig. 4*C*). Treatment of diabetic rats with FeTPPS completely

TABLE 2
Effects of STZ-induced diabetes (4 weeks) on body weight and blood glucose levels in control, diabetic, diabetic treated with FeTPPS, and control treated with FeTPPS animals

Animal group	*n*	Start weight (g)	End weight (g)	Blood glucose (mg/dl)
Control	18	235.4 ± 6.9	382.6 ± 19.0	116.3 ± 11.8
Control + FeTPPS	5	285.3 ± 2.9	402.7 ± 13.1	124.7 ± 11.3
Diabetic	21	253.5 ± 4.3	284.5 ± 13.3	460.9 ± 13.9
Diabetic + FeTPPS	18	253.4 ± 4.4	301.2 ± 13.8	467.1 ± 12.0

Data and means ± SE. Animals were made diabetic by a single STZ injection (60 mg/kg) in freshly prepared 10 mmol/l sodium citrate buffer, pH 4.5.

prevented TrkA tyrosine nitration and restored TrkA-Y490 phosphorylation.

Diabetes increases p75^{NTR} expression in experimental and human diabetes. P75NTR is a bifunctional receptor that can mediate both neuronal survival and death. Muller and retinal ganglion cells (RGCs) are the only cells in the retina that express the NGF p75^{NTR} receptor. Western blot analysis showed a 1.75-fold increase in diabetic human (Fig. 5*A*) and a 2-fold increase in diabetic rat retinas compared with controls (Fig. 5*B*). Treatment of diabetic rats with FeTPPS maintained the level of p75^{NTR} in the diabetic retina, but did not alter the level in treated controls (Fig. 5*B*). NGF/ p75^{NTR} activates the pro-apoptotic p38 MAPK in neuronal cells. As shown in Fig. 5*C*, Western blot analysis showed significant activation of p38 MAPK in diabetic rat retinas comparedwitho controls, which was decreased by treatment with FeTPPS.

Peroxynitrite induces apoptosis and activation of p38 MAPK in RGCs. To further confirm the role of peroxynitrite in altering the NGF survival signal leading to neuronal death, we examined the effects of exogenous peroxynitrite on NGF prosurvival function in RGCs. We chose this cell line as it has been characterized to express

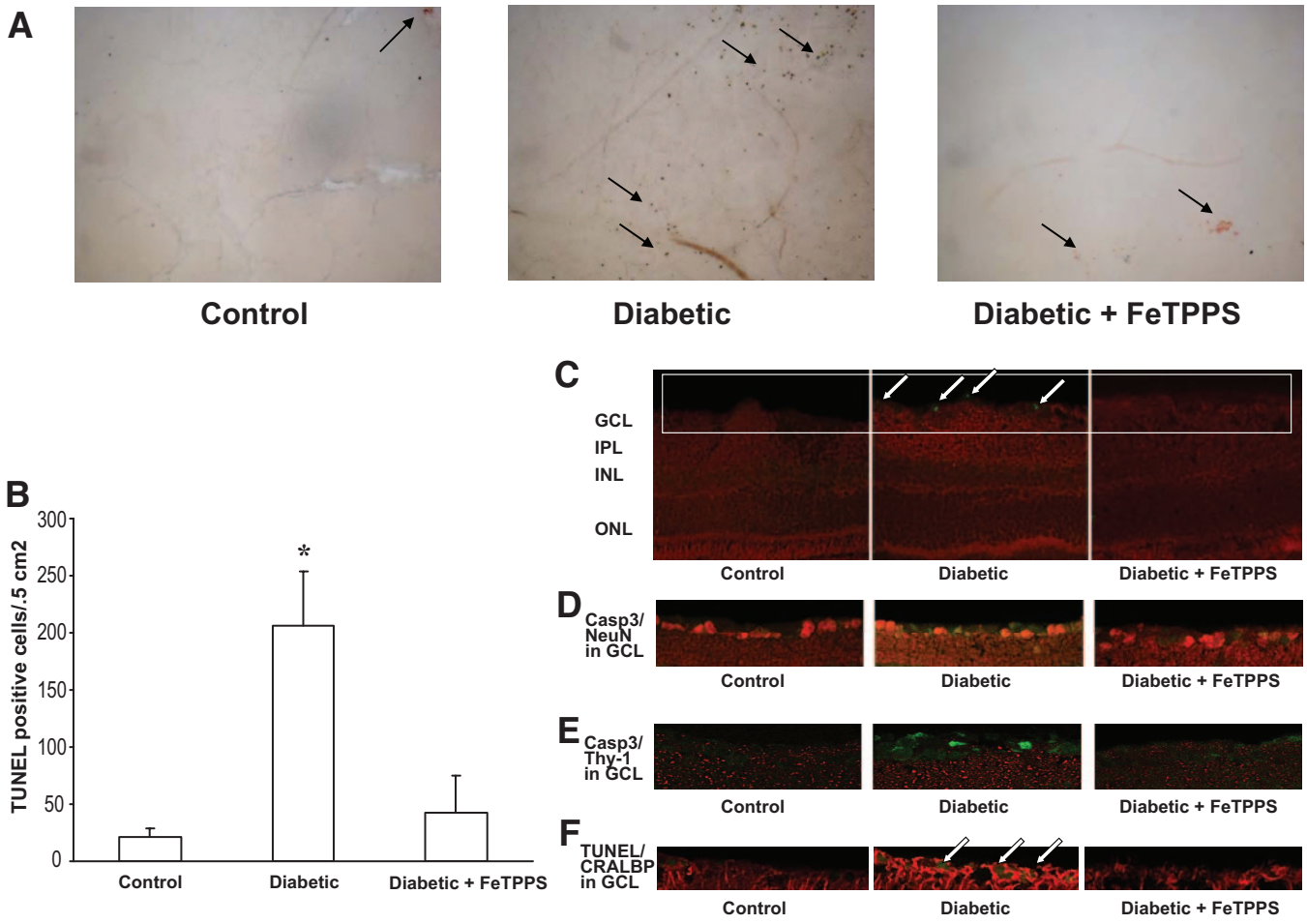

FIG. 2. FeTPPS prevents diabetes-induced neuronal cell death. *A*: Flat-mount retinas of diabetic animals showing frequent neuronal cell death (arrows) as detected by TUNEL assay. *B*: Statistical analysis showing a ninefold increase in the number of apoptotic cells in the diabetic retina that was blocked by treatment with FeTPPS (15 mg · kg^{-1} · day^{-1}). *$P < 0.008$. Data are means ± SD of six animals per group. *C*: Retinal sections showing colocalization of TUNEL-positive cells (green, white arrows) within the RGC layer labeled with Thy-1 (red). D: Enlarged window of retinal sections showing colocalization (orange) of the apoptotic marker caspase-3 (green) within the RGC layer labeled with NeuN (Red). *E*: Enlarged window of retinal sections showing localization of the apoptotic marker caspase-3 (green) within the RGC layer labeled with Thy-1 (Red). *F*: Enlarged window of TUNEL-positive cells (green) labeled with CRALP (red), the specific Müller cell marker, showing that Müller cells are not undergoing apoptosis after 4 weeks of diabetes. Instead, RGC are clearly TUNEL positive.

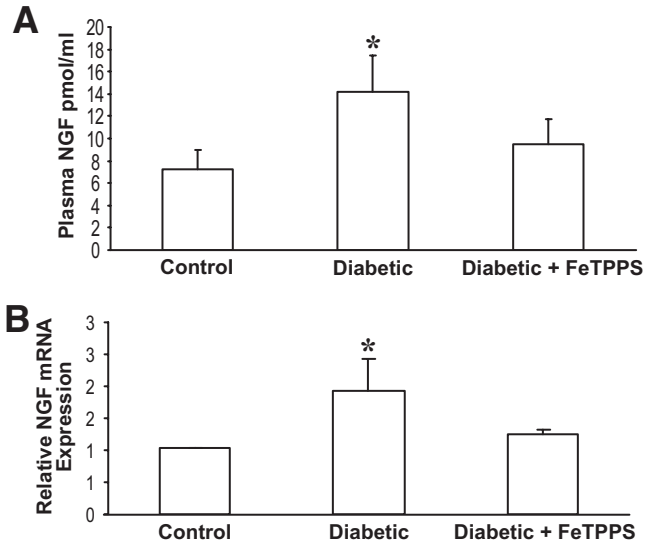

FIG. 3. Diabetes increases plasma and retinal NGF levels. *A*: Plasma levels of NGF assayed by ELISA showing a twofold increase in diabetic animals compared with controls. Treatment with FeTPPS (15 mg · kg^{-1} · day^{-1}) normalized the levels of NGF. The NGF level was calculated from a standard curve and corrected for protein concentration. *P < 0.04. *B*: Quantitative real-time PCR of NGF expression showed a twofold increase in mRNA levels of NGF in diabetic retinas compared with controls, and that treatment with FeTPPS (15 mg · kg^{-1} · day^{-1}) normalized the expression of NGF. *P < 0.0009. Data are means ± SD of six animals per group.

NGF, receptor TrkA, and receptor p75^{NTR} and can undergo apoptosis in response to serum deprivation or oxidative stress (31). As shown in Fig. 6*A*, peroxynitrite treatment (100 μmol/l) accelerated ganglion cell death as indicated by significant increases in caspase-3 activity compared with the control ($P < 0.01$). Peroxynitrite-induced cell death of RGCs was confirmed by a twofold increase in TUNEL-positive cells compared with the untreated control (Fig. 6*B*). In parallel, peroxynitrite induced p38 MAPK phosphorylation in RGCs compared with the control (Fig. 6*C*). Supplementation with NGF (50 ng/ml) did not rescue RGC cultures from peroxynitrite-induced apoptosis. However, supplementation with NGF in the presence of FeTPPS (2.5 μmol/l) blocked activation of p38 MAPK and restored the prosurvival effects of NGF on peroxynitrite-treated cultures (Fig. 6*A*–*C*).

Neutralizing peroxynitrite restores NGF prosurvival signal in RGCs. Similar to the in vivo results described earlier, treatment of RGC cells with peroxynitrite (100 μmol/l) resulted in significant tyrosine nitration of TrkA even in the presence of NGF (Fig. 7*A*). This effect was associated with impaired tyrosine phosphorylation of TrkA-Y490. Stimulation of RGCs with exogenous NGF (50 ng/ml) activated phospho-TrkA-Y490 in control but not in peroxynitrite-treated cells. Additionally, treatment with FeTPPS restored NGF-induced phospho-TrkA-Y490 phosphorylation in peroxynitrite-treated cells (Fig. 7*B*). We next evaluated the effects of peroxynitrite on NGF-induced activation of Akt, a downstream target of the PI3K pathway, which is activated by phospho-TrkA-Y490 in RGCs. NGF stimulated Akt phosphorylation, which was diminished by treatment with peroxynitrite. Neutralizing peroxynitrite with FeTPPS restored phosphorylation of Akt in response to NGF (Fig. 7*C*).

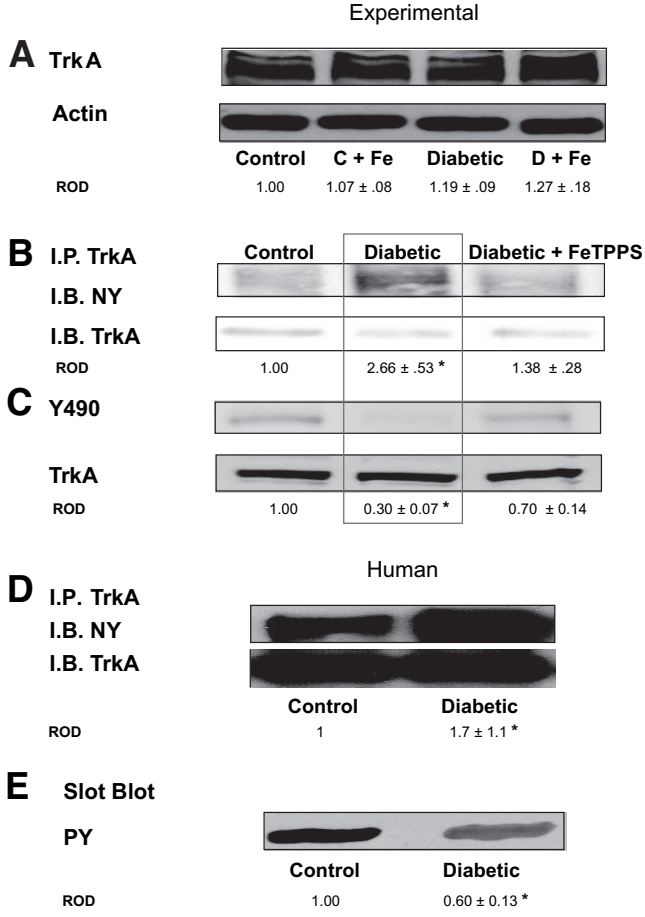

FIG. 4. Diabetes increases tyrosine nitration and decreases phosphorylation of TrkA. *A*: Western blot analysis showing that expression of the high-affinity NGF receptor, TrkA, was not significantly altered in the diabetic retina compared with other groups. Treatment with FeTPPS did not alter TrkA expression in the treated diabetic (D + Fe) or control animals (C + Fe). *B*: Diabetic rat retinas had twofold increases in tyrosine nitration of TrkA receptor as determined by immunoprecipitation (I.P.) of the TrkA and immunoblotting (I.B.) with the nitrotyrosine antibody. Equal loading was demonstrated by immunoblotting with TrkA antibody. Treatment with FeTPPS (15 mg · kg^{-1} · day^{-1}) completely prevented nitration of TrkA. *C*: Western blot analysis of phosphorylation of TrkA-Y490 showing diminished phosphorylation in diabetic rat retinas compared with controls that was restored by treatment with FeTPPS. Data are means ± SD of five animals per group. *P < 0.05. *D*: Diabetic human retinas showed 1.7-fold increases in tyrosine nitration of TrkA receptor as determined by immunoprecipitation of the TrkA and immunoblotting with the nitrotyrosine antibody. Equal loading was demonstrated by immunoblotting with TrkA antibody. *E*: Slot-blot analysis showing diminished global tyrosine phosphorylation in diabetic human retinas (n = 4) compared with nondiabetic controls (n = 5).

DISCUSSION

The present study documents novel data, suggesting that *1*) oxidative stress and peroxynitrite mediate diabetes-induced retinal neurodegeneration; *2*) peroxynitrite impairs the NGF prosurvival signal via tyrosine nitration; and *3*) treatment with FeTPPS, a selective peroxynitrite decomposition catalyst, restores the NGF survival signal and blocks retinal neuronal cell death.

To our knowledge, this is the first in vivo study to elucidate the mechanism by which peroxynitrite-mediated tyrosine nitration of the NGF/TrkA prosurvival pathway triggers neuronal cell death in experimental and human diabetes. Our results also suggest that treatments that target peroxynitrite formation or tyrosine nitration represent potentially effective therapeutics in attenuating reti-

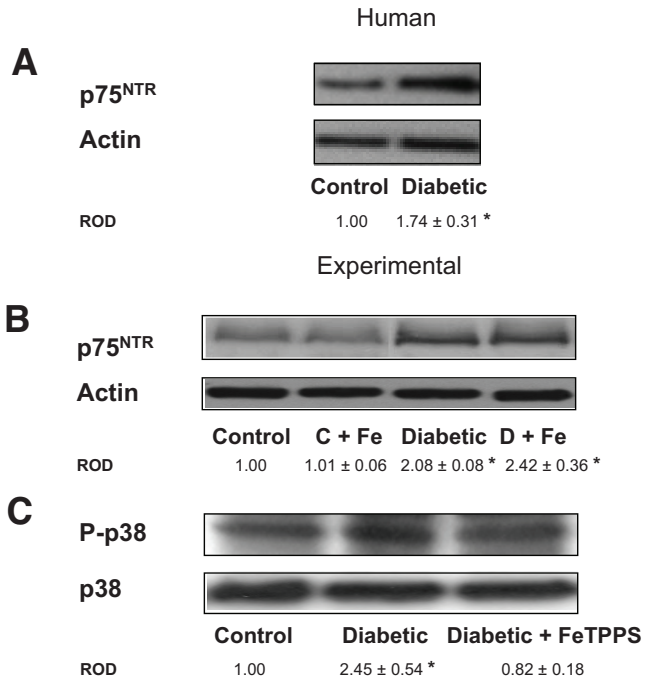

FIG. 5. Diabetes increases p75^{NTR} expression and activates p38 MAPK. *A*: Western blot analysis of p75^{NTR} expression showing a 1.75-fold increase in diabetic human retinas ($n = 4$) compared with nondiabetic controls ($n = 5$). *B*: Western blot analysis of p75^{NTR} expression showing a twofold increase in diabetic rat retinas compared with controls. Treatment with FeTPPS (15 mg · kg^{-1} · day^{-1}) maintained the levels in treated diabetic animals (D + Fe) but did not alter the basal level in the treated controls (C + Fe). *$P < 0.0004$. *C*: Western blot analysis showing a more than twofold increase in phosphorylation of p38 MAPK in the diabetic rat retinas compared with the controls. Treatment with FeTPPS normalized the levels of p38 MAPK phosphorylation (P-p38) back to control levels. Data are means ± SD of six animals per group.

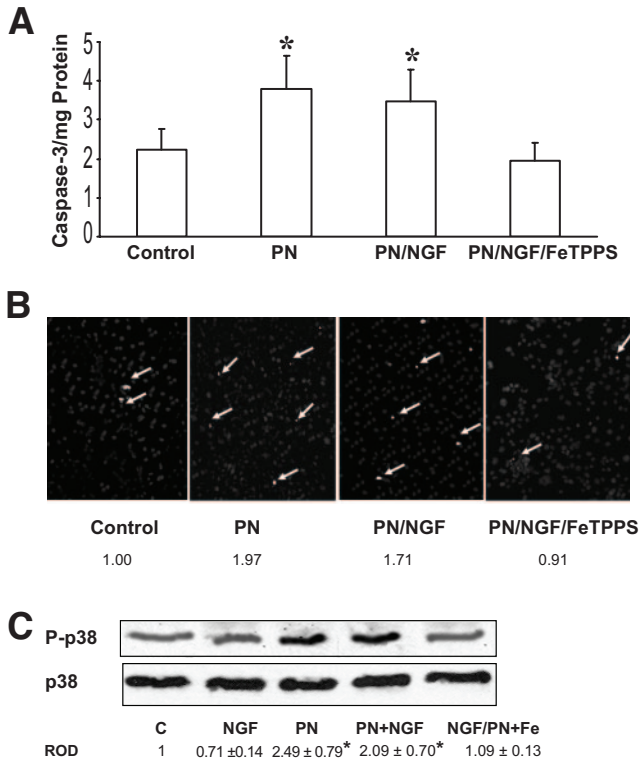

FIG. 6. Peroxynitrite induces apoptosis and activates p38 MAPK in RGCs. *A*: Peroxynitrite (100 μmol/l, PN) induced apoptosis as indicated by significant increases in caspase-3 activity even in the presence of NGF (50 ng/ml). Neutralizing peroxynitrite using FeTPPS (2.5 μmol/l) blocked caspase-3 activity and restored NGF prosurvival signal. *$P < 0.01$. *B*: Peroxynitrite (100 μmol/l, PN) induced neuronal death as indicated by significant increases in TUNEL-positive nuclei even in the presence of NGF (50 ng/ml). Neutralizing peroxynitrite using FeTPPS (2.5 μmol/l) reduced TUNEL-positive nuclei and restored NGF prosurvival signal. *C*: Western blot analysis showing that peroxynitrite (100 μmol/l) significantly stimulates p38 MAPK phosphorylation (P-p38) even in the presence of NGF (50 ng/ml). Neutralizing peroxynitrite using FeTPPS (2.5 μmol/l) reduced p38 MAPK activation to control level. *$P < 0.05$. Data are means ± SD of four per group.

nal neurodegenerative diseases. Accelerated retinal neuronal death has been well documented in both experimental and human diabetes (5,6,9,10,30,32). Increases in oxidative and nitrative stress have also been well documented in the diabetic retina (for review, see ref. 33). Previously, we have shown that an increase in peroxynitrite, as indicated by tyrosine nitration, correlates with accelerated retinal endothelial cell death, breakdown of the brain-retinal barrier, and accelerated neuronal cell death in models of experimental diabetes and neurotoxicity (10,21–23). These studies suggest a key role of peroxynitrite in mediating different aspects of DR. Yet, the causal role of peroxynitrite and tyrosine nitration in mediating retinal neurodegeneration has not been elucidated. Our studies showed, for the first time, that diabetes increases tyrosine nitration and lipid peroxidation in human diabetic retinas similar to the increases detected in diabetic rat retinas. The increase in tyrosine nitration and lipid peroxides were detected as early as 1 year of diabetes and were consistent after 20 years of diabetes in humans. Furthermore, these increases in tyrosine nitration and lipid peroxidation correlate with increases in neuronal death in diabetic rat retinas (Fig. 2) and with findings of previous studies in diabetic rats and humans (6,10). Our studies in diabetic rats showed that treatment with FeTPPS blocked diabetes-induced tyrosine nitration and prevented neuronal death. These results suggest a causal relationship between increased peroxynitrite and increased neuronal cell death within the diabetic retina. Decomposition catalysts, such as FeTPPS and FP15, isomerize peroxynitrite into nitrate (34) and have been proven effective in reducing peroxynitrite-mediated insults in models of ischemic retinopathy, DR, and neuropathy (21,35–38).

Neurotrophins, including NGF, are growth factors that are implicated in the development and survival of retinal neurons after axotomy or ischemia (11). Previous studies suggest that NGF administration could be beneficial for reducing the degeneration of NGF-responsive neurons (32,39,40). Paradoxically, several reports demonstrate that diabetes induces abnormal increased expression of NGF in ganglions and peripheral nerves of rats and skin biopsies and serum/tears of patients with diabetic neuropathy and retinopathy, respectively (15–19). Therefore, we evaluated NGF expression after 4 weeks of diabetes, where a ninefold increase of neuronal death was detected. Our results show a twofold increase in plasma and retinal NGF levels in diabetic rats, which decreased significantly after treatment with FeTPPS. These findings lend further support to previous reports showing that peroxynitrite increases NGF production in vitro and in vivo (41,42). These findings suggest an overproduction of NGF is induced by diabetic injury and that the increase in neurotrophin may represent a compensatory mechanism for impaired function of NGF-responsive neurons. In support of this con-

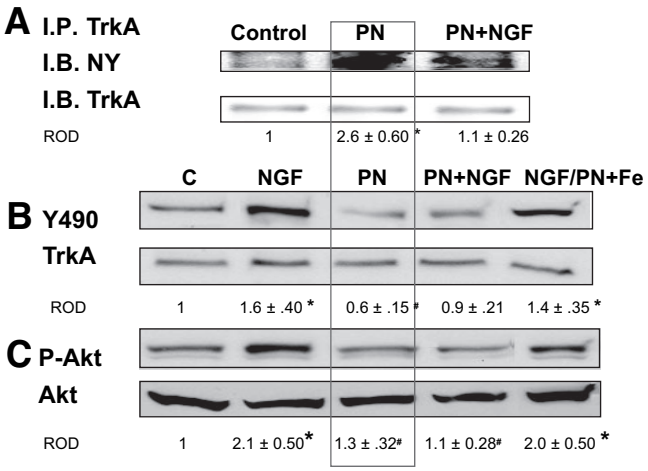

FIG. 7. FeTTPS blocks tyrosine nitration of TrkA receptor and restores TrkA-Y490 phosphorylation in RGCs. *A*: Peroxynitrite (PN) increased TrkA tyrosine nitration (more than twofold) as determined by immunoprecipitation (I.P.) of the TrkA and immunoblotting (I.B.) with the nitrotyrosine (NY) antibody. Equal loading was demonstrated by immunoblotting with TrkA antibody. Treatment of RGCs with NGF (50 ng/ml) maintained tyrosine nitration of TrkA. *B*: Western blot analysis showing that peroxynitrite (100 μmol/l) diminished NGF-induced TrkA phosphorylation at Y490 that was restored by treatment with FeTPPS (2.5 μmol/l). *C*: Western blot analysis showing that peroxynitrite (100 μmol/l) decreased NGF-induced Akt activation (P-Akt), a downstream target of TrkA survival signal. Treatment with FeTPPS restored Akt activity and NGF prosurvival signal. Data shown are means ± SD of four per group. *$P < 0.05$ compared with controls; #$P < 0.05$ compared with NGF-treated samples.

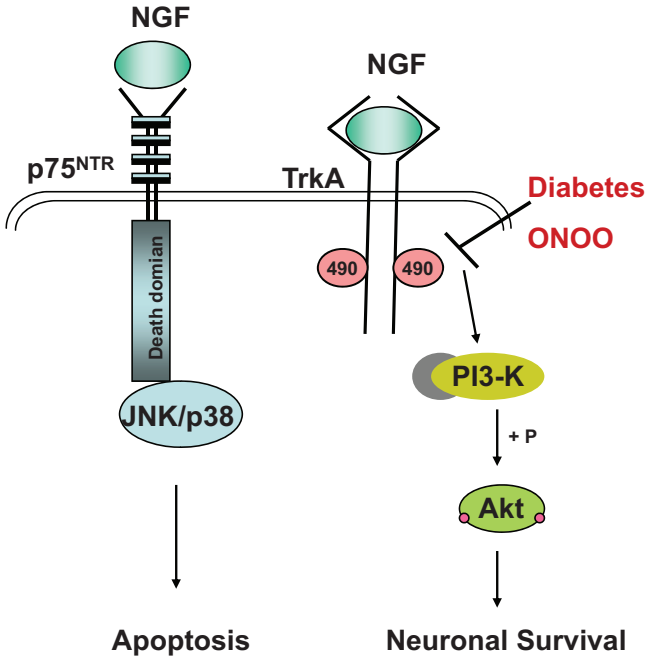

FIG. 8. Schematic diagram that summarizes the findings of the current study showing that diabetes-induced peroxynitrite inactivates the NGF/TrkA receptor prosurvival signal via tyrosine nitration. Instead, diabetes-induced peroxynitrite activates the NGF/p75^{NTR} proapoptotic signal leading to neuronal death. Treatments that target peroxynitrite formation or tyrosine nitration represent potentially effective therapeutics in attenuating retinal neurodegenerative diseases.

cept, our studies in RGCs showed that peroxynitrite induced apoptosis in these cells even in the presence of exogenous NGF (Fig. 6*A* and *B*). Neutralizing peroxynitrite using FeTPPS prevented RGC death and restored the NGF survival signal. NGF mediates its survival signal by binding high- and low-affinity receptors, TrkA and p75^{NTR}.

TrkA, which dimerizes upon ligand binding, leads to the NGF signals for growth and survival (43). Our studies in retinas from human and rats showed that diabetes did not alter TrkA expression levels among various groups. Therefore, we next evaluated the posttranslational modification of TrkA by determining tyrosine nitration and phosphorylation of TrkA. Our studies showed significant tyrosine nitration that resulted in diminished phosphorylation of TrkA in diabetic rat and human retinas compared with nondiabetic controls (Fig. 4). Furthermore, our studies in RGCs showed that peroxynitrite causes significant tyrosine nitration of the TrkA receptor even in the presence of exogenous NGF (Fig. 7*A*). Phosphorylation of TrkAY490 has been shown to activate PI3K/Akt and mediate NGF survival function (44,45).

Hence, we examined phosphorylation of TrkA-Y490 and its downstream target, Akt. Similar to the diabetic retina, peroxynitrite treatment caused diminished phosphorylation of TrkA-Y490and Akt in RGCs. Neutralizing peroxynitrite in diabetic rats and RGCs with FeTPPS blocked tyrosine nitration of TrkA and restored phosphorylation of TrkA-Y490 and its downstream target, Akt, suggesting a causal role of diabetes-induced peroxynitrite in inhibiting TrkA survival signal activation. Our findings lend further support of previous reports in other sensory neuronal cells, showing the potential role of peroxynitrite in inhibiting the NGF/TrkA signal by tyrosine residues on the TrkA receptor, thus preventing them from NGF phosphorylation (24,25). However, to our knowledge, our study is the first to elucidate the molecular mechanism by which tyrosine nitration of TrkA inactivates the NGF survival signal in experimental and human diabetes.

P75NTR, which, in the retina, is only expressed in Müller and RGCs, can coreceptor with TrkA, creating high-affinity binding sites for NGF and subsequent retrograde transport of the NGF signals for growth and survival (43). However, in the absence of TrkA, binding of NGF to p75^{NTR} results in apoptosis (13,46). Our results, showing an approximate twofold increase in p75^{NTR} expression in retinas from diabetic human and rats, lend further support to previous studies showing that diabetes upregulates p75NTR expression in rat retinas (32) and in sensory neurons (47).

Together, with the results showing tyrosine nitration and inhibition of the TrkA receptor, these findings provide compelling evidence that the NGF/TrkA survival signal is impaired in the diabetic retina and that the NGF/ p75^{NTR} pro-apoptotic signal is activated, leading to retinal neurodegeneration. The proposed mechanism is illustrated in Fig. 8.

P75NTR-mediated activation of the pro-apoptotic proteins p38 MAPK and caspase-3 underlies the neuronal cell loss. Therefore, we further evaluated activation of p38 MAPK in various animal groups and in peroxynitrite-treated RGCs. Our results showed that diabetes/peroxynitrite treatment stimulates p38 MAPK phosphorylation in the diabetic rat retina and in RGCs, respectively. p38 MAPK activation positively correlated with upregulation of p75^{NTR} expression and neuronal death both in vitro and in vivo. In agreement, activation of p38 MAPK has been reported in diabetic retinas (10,48) and in sensory neurons maintained under high-glucose conditions (49). Neutralizing peroxynitrite formation using FeTPPS reduced activation of p38 MAPK back to control levels (Fig. 5*B* and 7*B*).

Interestingly, our studies in diabetic animals showed that neutralizing peroxynitrite formation using FeTPPS maintained the increase in $p75^{NTR}$ expression but blocked the activation of p38 MAPK and neuronal death in the treated animals. Together with the results showing restoration of TrkA activation in the treated diabetic animals, these findings suggest that despite the upregulation of $p75^{NTR}$ expression, the downstream pro-apoptotic signal is not active. Thus, we believe that upregulated $p75^{NTR}$ expression may be enhancing the TrkA receptor affinity for NGF and promoting the cell survival signal.

Previous studies have suggested that deficiencies in growth factors, such as NGF and vascular endothelial growth factor (VEGF), may cause neurodegeneration (32,47,50). In contrast, we believe that the diabetic milieu present in retinopathy is associated with increases in these growth factors, as well as the increases in oxidative stress and peroxynitrite that interrupt survival signals and accelerate neural and vascular cell death. Our previous studies show that high-glucose or high-oxygen treatments stimulate peroxynitrite formation and accelerate apoptosis of retinal endothelial cells even in the presence of the VEGF or basic fibroblast growth factor (bFGF) (21,51). Furthermore, our studies show that tyrosine nitration of the p85 subunit of PI3K acts as a molecular switch between VEGF survival and death signals. The VEGF survival signal was restored by treatments that target peroxynitrite formation or block tyrosine nitration (21). Taken together, these findings along with the results of our current study provide evidence that understanding the biochemical changes and the events that control growth factor signaling under diabetic conditions could provide new effective therapeutic tools to combat the disease.

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