Epicatechin blocks pro-nerve growth factor (proNGF)-mediated retinal neurodegeneration via inhibition of p75 neurotrophin receptor proNGF expression in a rat model of diabetes

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Received: 23 June 2010 / Accepted: 25 October 2010 / Published online: 7 December 2010
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Abstract

Aims/hypothesis Accumulation of pro-nerve growth factor (NGF), the pro form of NGF, has been detected in neurodegenerative diseases. However, the role of proNGF in the diabetic retina and the molecular mechanisms by which proNGF causes retinal neurodegeneration remain unknown. The aim of this study was to elucidate the role of proNGF in neuroglial activation and to examine the neuroprotective effects of epicatechin, a selective inhibitor of tyrosine nitration, in an experimental rat model of diabetes.

Methods Expression of proNGF and its receptors was examined in retinas from streptozotocin-induced diabetic rats, and in retinal Müller and retinal ganglion cells (RGCs). RGC death was assessed by TUNEL and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays in diabetic retinas and cell culture. Nitrotyrosine was determined using Slot-blot. Activation of the tyrosine kinase A (TrkA) receptor and p38 mitogen-activated protein kinase (p38MAPK) was assessed by western blot.

Electronic supplementary material The online version of this article (doi:10.1007/s00125-010-1994-3) contains supplementary material, which is available to authorised users.

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Results Diabetes-induced peroxynitrite impaired phosphorylation of TrkA-Y490 via tyrosine nitration, activated glial cells and increased expression of proNGF and its receptor, p75 neurotrophin receptor (p75^{NTR}), in vivo and in Müller cells. These effects were associated with activation of p38MAPK, cleaved poly-(ADP-ribose) polymerase and RGC death. Treatment of diabetic animals with epicatechin (100 mg kg⁻¹ day⁻¹, orally) blocked these effects and restored neuronal survival. Co-cultures of RGCs with conditioned medium of activated Müller cells significantly reduced RGC viability (44%). Silencing expression of p75^{NTR} by use of small interfering RNA protected against high glucose- and proNGF-induced apoptosis in RGC cultures. **Conclusions/interpretation** Diabetes-induced peroxynitrite stimulates p75^{NTR} and proNGF expression in Müller cells. It also impairs TrkA receptor phosphorylation and activates the p75^{NTR} apoptotic pathway in RGCs, leading to neuronal cell death. These effects were blocked by epicatechin, a safe dietary supplement, suggesting its potential therapeutic use in diabetic patients.

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Keywords Diabetes · Epicatechin · Neuroprotection · Peroxynitrite · p75^{NTR} · proNGF

Abbreviations

GFAP	Glial fibrillary acidic protein
GSH	Free glutathione
GSSG	Oxidised glutathione
MAPK	Mitogen-activated protein kinase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NGF	Nerve growth factor
p75 ^{NTR}	p75 neurotrophin receptor
PARP	Poly-(ADP-ribose) polymerase
RGC	Retinal ganglion cell
rMC-1	Retinal Müller glial cell line
ROD	Relative optical density
siRNA	Small interfering RNA
TrkA	Tyrosine kinase A

Introduction

Diabetic retinopathy, a progressive and potentially devastating vascular-neurodegenerative disease, is the leading cause of blindness in working-age adults in the USA [1]. Current therapeutic options, which include photocoagulation and vitrectomy, are invasive and limited by considerable side effects, as reviewed by members of this author team [2]. Therefore, there is a great need for new non-invasive therapies to prevent diabetic retinopathy. Retinal neurodegeneration is a critical component of diabetic retinopathy and has been linked to impairment of visual function due to cell death of the inner retinal and ganglion cells [3–7].

A growing body of evidence supports the role of oxidative stress and in particular peroxynitrite in activating glia and secreting growth factors as part of a defence mechanism. We and others have demonstrated increases in expression of nerve growth factor (NGF) in experimental models of diabetes and in clinical diabetes [7–9]. Recent findings by our group showed that diabetes-induced peroxynitrite formation impairs cleavage and maturation of NGF, leading to accumulation of its precursor ‘proNGF’ at the expense of the mature NGF levels, both in experimental models and in clinical diabetes [10]. Accumulation of proNGF after injury has been detected in several neurodegenerative diseases, such as Alzheimer’s, [11, 12]. However the role of proNGF in the diabetic retina and the specific molecular mechanism regulating proNGF production and its subsequent effects on retinal neurodegeneration are not fully understood. While mature NGF mediates neuronal cell survival through binding of tyrosine kinase A (TrkA) and p75 neurotrophin receptors (p75^{NTR}), reviews by

others have established that proNGF can promote neuronal apoptosis because of its high affinity to p75^{NTR} [13, 14]. A recent study demonstrated that the outcome of proNGF signalling, i.e. neurotrophic or apoptotic, can be dependent upon relative levels of its receptors [15, 16]. Therefore, we examined the expression of proNGF and its receptors TrkA and p75^{NTR} in the diabetic retina and isolated retinal cells cultured in high glucose.

Our previous studies had shown that excessive peroxynitrite formation, as indicated by increases in nitrotyrosine formation, positively correlated with accelerated vascular cell death, blood–retina barrier breakdown and neuronal cell death in models of diabetes, and with ischaemic retinopathy and retinal neurotoxicity [6, 7, 10, 17–22]. Of particular note, our recent study demonstrated a specific role of peroxynitrite in impairing the survival receptor TrkA via tyrosine nitration and upregulation of the death receptor p75^{NTR}, leading to neuronal death in experimental models and in clinical diabetes [7]. These findings prompted us to examine the neuroprotective effects of epicatechin, a green tea constituent and selective inhibitor of tyrosine nitration, in a streptozotocin-induced animal model of diabetes. The current study also elucidates the role of p75^{NTR} in inducing proNGF expression in retinal Müller cells and in mediating retinal ganglion cell (RGC) death in response to proNGF and high glucose.

Methods

Animal preparation

All procedures with animals were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the Charlie Norwood VA Medical Center Animal Care and Use Committee. Male Sprague–Dawley rats ($n=66$; ~250 g body weight; Harlan Laboratories, Indianapolis, IN, USA) were randomly assigned to control, treated control, diabetic or treated diabetic groups. Diabetes was induced by intravenous injection of streptozotocin (60 mg/kg) dissolved in 0.01 mol/l sodium citrate buffer, pH 4.5. Detection of glucose in the urine of injected animals and blood glucose levels >13.9 mmol/l were used as markers of diabetes. One week later, treated groups received oral gavages of 100 mg kg⁻¹ day⁻¹ of epicatechin in PBS for the entire study. Control and diabetic animals received oral gavages of PBS only. After 4 weeks of diabetes, animals were killed and eyes enucleated for analyses.

Determination of nitrotyrosine

Slot-blot analysis was used as described previously [7, 21], with 30 µg of retinal homogenate from rat samples being

immobilised on to a nitrocellulose membrane. After blocking, membranes were reacted with antibodies against nitrotyrosine (Calbiochem, San Diego, CA, USA) and the optical density of various samples compared with that of controls.

Evaluation of neural cell death in rat retina

TUNEL assay was performed to detect retinal cell death by immunoperoxidase staining (ApopTag-peroxidase) in whole-mount retina as described previously by our group [7]. Formalin-fixed retinas were flat-mounted, dehydrated in ethanol, defatted by xylenes and rehydrated. After permeabilisation, TUNEL-horseradish peroxidase staining with 3-amino-9-ethylcarbazole was performed following the manufacturer's instructions. The total number of TUNEL-horseradish peroxidase-positive cells was counted in each retina using light microscopy. TUNEL was also performed in 10 μm optical coherence tomography-frozen eye sections using the ApopTAG in situ cell death detection kit (TUNEL-FITC) as described previously [7, 20].

Determination of glial activation and immune-localisation studies

Optical coherence tomography-frozen sections (10 μm) of eyes were fixed using 2% (vol./vol.) paraformaldehyde in PBS and reacted with monoclonal anti-glial fibrillary acidic protein (GFAP) antibody (for glial cell activation; Affinity BioReagents, Rockford, IL, USA), polyclonal anti-proNGF (Alomone Labs, Jerusalem, Israel) or polyclonal anti-p75^{NTR} (Millipore, Billerica, MA, USA), followed by Texas red or Oregon green-conjugated goat anti-mouse or goat anti-rabbit antibodies (Invitrogen, Carlsbad, CA, USA). Data (three fields/retina, $n=6$ in each group) were analysed using a microscope (AxioObserver.Z1; Zeiss, Germany) and Axio-software to quantify the density of immunostaining.

Retinal protein extraction and western blot analysis

Retinas were isolated and homogenised in RIPA buffer as described previously [7]. Samples (50 μg protein) were separated by SDS-PAGE and electroblotted to nitrocellulose membrane. Antibodies for proNGF (Alomone), p75^{NTR} (Millipore), phospho-p38 mitogen-activated protein kinase (p38MAPK; Cell Signaling Technology, Danvers, MA, USA), nitrotyrosine (Calbiochem), cleaved poly-(ADP-ribose) polymerase (PARP; BD Bioscience Pharmingen, San Diego, CA, USA), TrkA (Chemicon International, CA, USA) and phospho-TrkA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used. Membranes were re-probed with β -actin (Sigma-Aldrich, St Louis, MO,

USA) to confirm equal loading. The primary antibody was detected using a horseradish peroxidase-conjugated sheep anti-rabbit antibody (GE-Healthcare) and enhanced chemiluminescence. The films were scanned and the band intensity was quantified using densitometry software (alphEaseFC) and expressed as relative optical density (ROD).

Tissue culture studies

Retinal ganglion cells We used RGC-5, a cell line kindly donated by N. Agarwal (Department of Cell Biology, UT Health Science Center, Fort Worth, TX, USA) and previously characterised [23]. Cells were grown to confluence in complete medium (DMEM with 6% [vol./vol.] FBS and 10% [vol./vol.] penicillin/streptomycin) then switched to normal-glucose medium (5 mmol/l) or high-glucose medium (25 mmol/l) in the presence or absence of epicatechin (100 $\mu\text{mol/l}$) for 3 days.

Co-culture studies Cultures of a transformed retinal Müller glial cell line (rMC-1), which has been previously characterised [24], were obtained from V. Sarthy (Department of Ophthalmology, Chicago, IL, USA) and grown to 80% confluence. Cells were maintained in high glucose and normal glucose for 72 h in the presence or absence of epicatechin (100 $\mu\text{mol/l}$). Condition media of rMC-1 were concentrated using filter devices (Amicon-Ultracentrifugal 10K MWCO; Millipore) and used to determine proNGF and p75^{NTR} levels using western blot; they were also used for co-culture studies with RGCs.

MTT assay

Viability of RGC-5 cells was determined by incubating cells for 4 h at 37°C with 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in PBS. MTT, a yellow dye, is reduced to purple formazan in living cells, which was dissolved in acid isopropanol (1:9 of 1 N HCl/isopropanol). Optical density was measured at 540 and 690 nm using microplate reader (Bio-Tek Instruments, VT, USA).

Silencing of p75^{NTR} expression using small interfering RNA

RGCs (2×10^6) were transfected using electroporation and a cell line kit (basic Nucleofector; Lonza, Switzerland) according to the manufacturer's protocol. Both the SMARTpool of small interfering RNA (siRNA) specific for p75^{NTR} (gene also known as *Ngfr*) and its scrambled form were obtained commercially from Dharmacon (Lafayette, CO, USA). Pilot

experiments were performed to optimise levels of $p75^{NTR}$ siRNA. Results showed that 200 nmol/l $p75^{NTR}$ siRNA inhibited its mRNA expression by 76%. Transfection of RGCs with siRNA or a scrambled form (200 nmol/l) was performed together with 2 μ g pmax green fluorescent protein (in Lonza kit) to assess the transfection efficiency after electroporation by Nucleofector. The transfected cells were cultured for 16 h in six-well plates with complete medium containing 10% (vol./vol.) FBS. Transfection efficiency was monitored by fluorescence microscope 16 h after transfection by calculating the percentage of green fluorescent protein-producing cells per total number of cells (Electronic supplementary material [ESM] Fig. 1). Three independent cultures were used for each condition. The medium was then changed and cells switched to high glucose or normal glucose for 3 days, followed by treatment with proNGF (50 ng/ml) for 24 h.

Determination of TUNEL-positive cells in RGCs

RGC death was determined using TUNEL fluorescence (ApopTag-Fluorescein) counterstained with DAPI. The total number of TUNEL-positive cells was counted and expressed as percentage of TUNEL-positive cells per total number of cells in various groups.

Data analysis

The results are expressed as mean $\pm$ SEM. Differences between experimental groups were evaluated by ANOVA 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

Epicatechin treatment does not alter body weight or blood glucose levels

As shown in Table 1, streptozotocin-injected animals had significant increases of blood glucose level compared with control rats. Treatment with epicatechin had no significant effect on body weight or blood glucose levels in diabetic rats or in treated controls.

Epicatechin prevents diabetes-induced neuronal cell death

Quantitative analysis of TUNEL-labelled cells in whole flat-mounted retinas showed an approximately fivefold increase in the frequency of retinal cell death in diabetic retinas compared with controls (Fig. 1a). Treatment of

diabetic animals with epicatechin reduced the number of TUNEL-positive cells ($p\leq 0.05$) to control level, but it did not affect the control rats. Previous studies have shown that after 4 weeks of diabetes, neurons and RGCs in particular are most vulnerable to cell death [3, 7]. In agreement with this, TUNEL staining of retina sections showed that diabetes increased cell death in inner retina layers and ganglion cell layers, an effect that was blocked in diabetic animals by treatment with epicatechin (Fig. 1b).

Epicatechin blocks diabetes-induced glial activation and proNGF expression in rat retina

We next evaluated the protective effects of epicatechin on modulating glial activation and on expression of proNGF. As shown in Fig. 2a, diabetes induced glial activation as indicated in the filaments of Müller cells by prominent GFAP immunoreactivity extending from the nerve fibre layer and inner plexiform layer into the outer nuclear layer of retina, as compared with controls. Recent studies have identified Müller cells as a major site of proNGF expression in the retina [10, 25]. Consistent with this, diabetic retinas showed prominent immunolocalisation of proNGF in Müller cells and inner retinal layers, as indicated by prominent colocalisation of proNGF and GFAP, a marker of Muller activation (Fig. 2b, c). Western blot analysis showed a 1.8-fold increase in proNGF levels in diabetic rat retinas compared with controls (Fig. 2d). Treatment of diabetic animals with epicatechin (100 mg kg⁻¹ day⁻¹, orally) prevented glial activation and proNGF production. We next evaluated the expression and activity levels of proNGF receptors TrkA and $p75^{NTR}$.

Epicatechin blocks diabetes-induced tyrosine nitration and restores TrkA phosphorylation

As shown in Fig. 3a, Slot blot analysis of nitrotyrosine showed a 1.7-fold increase in diabetic rat retinas compared with controls. Treatment with the selective tyrosine nitration inhibitor, epicatechin (100 mg kg⁻¹ day⁻¹, orally) blocked this effect in diabetic rats, but did not affect nitrotyrosine level in control animals. Tyrosine nitration of TrkA was assessed by immunoprecipitation of retinal lysate with TrkA, followed by immunoblotting with anti-nitrotyrosine. Western blot analysis showed a ~2.2-fold increase in tyrosine nitration of TrkA. Assessment of TrkA phosphorylation at tyrosine residue 490 (PY-490) showed a ~45% decrease in diabetic retinas compared with controls. Co-treatment of diabetic animals with epicatechin prevented tyrosine nitration and restored phosphorylation of TrkA in diabetic animals, but did not affect controls (Fig. 3b, c). In addition, analysis of the ratio of oxidised

Table 1 Effects of streptozotocin-induced diabetes on body weight and blood glucose levels in rat groups as indicated

Group	<i>n</i>	Start weight (g)	End weight (g)	Blood glucose (mmol/l)
Control	18	250.2±4.26	273.4±10.72	10.9±1.1
Diabetes	18	249.8±5.58	234.6±7.38	27±1.05*
Control + epicatechin	12	242.2±6.26	281.4±9.51	10.5±1.27
Diabetes + epicatechin	18	251.5±5.17	226.5±4.8	30.4±0.69*

Data are mean ± SE

Epicatechin was given orally at 100 mg kg⁻¹ day⁻¹; animals were made diabetic by a single streptozotocin injection (60 mg/kg) in freshly prepared 10 mmol/l sodium citrate buffer, pH 4.5

*Significant difference vs other groups at *p*<0.05

glutathione (GSSG)/free glutathione (GSH) in blood was used to measure the systemic antioxidant defence. Our results showed that diabetes significantly decreased the antioxidant defence as indicated by a twofold increase in GSSG/GSH ratio (0.62) compared with controls (0.36). Treatment of diabetic rats with epicatechin maintained the high GSSG/GSH ratio (0.52), suggesting that epicatechin has minimal effects as a general antioxidant. Our previous studies using epicatechin had demonstrated selective inhibition of peroxynitrite-mediated tyrosine nitration, but not of thiol oxidation [17, 21, 26].

Epicatechin blocks diabetes-induced p75^{NTR} expression in rat retina

Western blot analysis showed an approximately twofold increase in p75^{NTR} in diabetic rat retinas compared with controls (Fig. 4a). In the retina, p75^{NTR} is expressed mainly in Müller cells and RGCs [27–29]. Immunolocalisation of p75^{NTR} showed increased levels in Müller cells and inner retinal layers in diabetic rat retinas compared with controls (Fig. 4b). Epicatechin blocked diabetes-induced changes, but did not affect controls. While upregulation of p75^{NTR} expression has been previously linked to neuronal death,

it can induce expression of proinflammatory mediators in Müller cells. Therefore, we next evaluated the effects of high glucose on p75^{NTR} expression in Müller cells and RGCs.

Epicatechin blocks high glucose-induced expression of p75^{NTR} and proNGF in Müller cells

Glial rMC-1 cells were cultured for 72 h in different media as follows: normal glucose (5 mmol/l glucose), high glucose (25 mmol/l glucose) and high glucose+100 μmol/l epicatechin. Cells were lysed to detect p75^{NTR} expression while condition media were collected, concentrated and used to determine proNGF levels. Western blot analysis showed that high glucose induced p75^{NTR} production (2.3-fold) and an approximately 2.2-fold increase in secreted proNGF levels compared with normal glucose controls (Fig. 5a, b). Co-treatment with epicatechin (100 μmol/l) significantly reduced p75^{NTR} levels and blocked proNGF production in Müller cultures maintained in high glucose, but not in normal glucose cultures. We next examined the detrimental effects of secreted proNGF on adjacent retinal neurons by using a co-culture model of RGCs with condition media from high glucose-activated glial Müller cells to

Fig. 1 Epicatechin prevents diabetes-induced neuronal cell death. **a** Quantification of TUNEL staining of flat-mounted retinas, showing a fivefold increase in cell death in diabetic retinas compared with normal control retinas (*n*=4–6). **b** TUNEL staining of retinal sections showing increased cell death in inner retinal layers of diabetic rats as compared

with controls (magnification ×200). Treatment with epicatechin (Epi; 100 mg kg⁻¹ day⁻¹, orally) blocked these effects in diabetic rats, but did not affect control rats. C+Epi, control plus epicatechin; D+Epi, diabetes plus epicatechin; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer

Fig. 2 Epicatechin (Epi) blocks diabetes-induced glial activation and proNGF expression in rat retina. **a** In diabetic animal retinas, the end feet of the Müller cells showed abundant GFAP immunofluorescence (red) and the radial processes stained intensely throughout the inner and the outer retina (magnification $\times 200$). **b** Representative images of rat retinal sections stained with anti-proNGF and showing increased proNGF expression in Müller cells and the inner retinal layer as compared with control animals ($400\times$ magnification). **c** Representative image of diabetic rat retinal section stained with anti-proNGF (green)

and GFAP (red), showing colocalisation of proNGF within activated Müller cells (magnification $\times 400$). **d** Western blot showing 1.8-fold increases, as quantified by ROD, in proNGF expression in diabetic rat retina as compared with normal control rats. Treatment with epicatechin ($100 \text{ mg kg}^{-1} \text{ day}^{-1}$, orally) blocked these effects in diabetic rats, but did not affect control rats ($n=6$). $*p<0.05$ vs other groups. C+Epi, control plus epicatechin; D+Epi, diabetes plus epicatechin; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer

mimic the in vivo setting. After 48 h treatment of RGCs with conditioned medium from various groups, viability assay was performed using MTT. RGC viability decreased to 44% of the basal level in response to conditioned medium from high glucose-treated rMC1 cultures compared with normal glucose-treated cultures. Co-treating high glucose rMC-1 cultures with epicatechin did not significantly affect RGC viability (Fig. 5c). These results suggest that high glucose-induced expression of proNGF expression involve upregulation of $p75^{\text{NTR}}$ in Müller cells and that the neuroprotective action of epicatechin is partially due to blocking expression of $p75^{\text{NTR}}$ and proNGF.

Epicatechin blocks diabetes-induced $p75^{\text{NTR}}$ apoptotic pathway in rat retina and RGC culture

The above data indicate that while diabetes-induced peroxynitrite nitrates and impairs the survival receptor TrkA, it stimulates expression of the death receptor $p75^{\text{NTR}}$ and proNGF. Therefore, we evaluated the effects of high glucose and proNGF on the expression of $p75^{\text{NTR}}$ and its downstream target p38MAPK in RGC cultures. Western blot analysis showed that high glucose upregulated $p75^{\text{NTR}}$ production (1.8-fold) and p38MAPK phosphorylation (1.7-fold) compared with normal glucose. Exogenous

Fig. 3 Epicatechin (Epi) blocks diabetes-induced tyrosine nitration and restores TrkA phosphorylation in rat retina. **a** Slot blot analysis showing significant increases in nitrotyrosine (NY) formation in diabetic rat retinas compared with normal control rats ($n=6$). Immunoprecipitation with TrkA followed by blotting with antibodies against nitrotyrosine (NY) (**b**) and phospho-TrkA (P-Y490) (**c**) showed 2.2-fold increases in TrkA nitration (**b**) and 45% decreases in tyrosine phosphorylation (**c**) in diabetic rats compared with controls ($n=5-6$). Treatment with epicatechin ($100 \text{ mg kg}^{-1} \text{ day}^{-1}$, orally) blocked these effects in diabetic rats, but did not affect control rats. * $p<0.05$ vs other groups. C+Epi, control plus epicatechin; D+Epi, diabetes plus epicatechin

treatment of RGCs with proNGF (50 ng/ml) increased basal levels of $p75^{\text{NTR}}$ expression and activation of p38MAPK (~ 1.7) in normal glucose and augmented high glucose effects (~ 2.2 -fold). Co-treatment with epicatechin ($100 \mu\text{mol/l}$) blocked these effects (Fig. 6a, b). In

parallel, activation of the pro-apoptotic pathway was evident by a 1.8-fold and 2.2-fold increase in phosphorylation of p38MAPK and expression of cleaved PARP, respectively, in diabetic retinas as compared with controls (Fig. 6c, d). These effects were blocked by epicatechin ($100 \text{ mg kg}^{-1} \text{ day}^{-1}$, orally) in diabetic, but not in control animals.

ProNGF mediates RGC death in a $p75^{\text{NTR}}$ -dependent way

We first examined the effects of high glucose on inducing RGC death using TUNEL assay. High glucose caused a threefold increase in the number of TUNEL-positive cells in RGC culture as compared with those cultured in normal glucose. Exogenous treatment with proNGF (50 ng/ml) increased the number of TUNEL-positive cells (2.7-fold) in normal glucose and augmented high glucose effects (3.6-fold). Co-treatment with epicatechin ($100 \mu\text{mol/l}$) blocked these effects (Fig. 7a). The causal role of $p75^{\text{NTR}}$ in mediating proNGF- and high glucose-induced RGC death was examined by silencing its expression using siRNA techniques. In scrambled $p75^{\text{NTR}}$ -treated cultures, high glucose increased the number of TUNEL-positive cells 2.8-fold compared with normal glucose. Exogenous proNGF treatment further increased the number of TUNEL-positive cells (2.3-fold) in normal glucose and augmented high glucose effects (3.2-fold). Silencing the expression of $p75^{\text{NTR}}$ using siRNA blocked the increase in the number of TUNEL-positive cells in normal and high glucose media (Fig. 7b).

Fig. 4 Epicatechin (Epi) blocks diabetes-induced $p75^{\text{NTR}}$ expression in rat retina. **a** Western blot showing a 1.95-fold increase in rat retinal lysate $p75^{\text{NTR}}$ expression in diabetic rat retinas compared with control rats ($n=5-6$). * $p<0.05$ vs other groups. **b** Representative images of rat retinal sections stained with anti $p75^{\text{NTR}}$ and showing increased proNGF expression in Müller cells and the inner retinal layer as

compared with control animals (magnification $\times 400$). Treatment with epicatechin ($100 \text{ mg kg}^{-1} \text{ day}^{-1}$, orally) blocked these effects in diabetic rats, but did not affect control rats. C+Epi, control plus epicatechin; D+Epi, diabetes plus epicatechin; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer

Fig. 5 Epicatechin blocks high glucose-induced expression of p75^{NTR} and proNGF in Müller cells. **a** Western blot showing 2.3-fold increase in p75^{NTR} expression in Müller cultures maintained in high glucose (HG) as compared with those maintained in normal glucose (NG). This increase was blocked by epicatechin (Epi) treatment (100 μ mol/l, $n=6$). **b** Western blot showing 2.2-fold increase in proNGF expression in Müller cultures maintained in HG as compared with those maintained in NG. This increase was blocked by epicatechin treatment (100 μ mol/l, $n=6$). * $p<0.05$ vs other groups. **c** Viability of RGC-5 cells treated for 72 h with different condition media from glial rMC-1 cells as follows: NG (5 mmol/l glucose), NG+100 μ mol/l epicatechin (NG+Epi), HG (25 mmol/l glucose) and HG+100 μ mol/l epicatechin (HG+Epi). RGC-5 viability decreased to 44% of the basal level in response to conditioned medium of HG treatment. RGC-5 viability was 85% of the basal level in response to epicatechin-treated HG condition medium ($n=6$)

Discussion

The main findings of the current study are that diabetes-induced peroxynitrite causes retinal neurodegeneration via multiple mechanisms including: (1) glial activation and upregulation of p75^{NTR} expression leading to increasing proNGF levels; (2) activation of the proapoptotic pathway p75^{NTR} and p38MAPK pathway in RGCs and (3) impairment of phosphorylation of the survival receptor TrkA-Y490 via tyrosine nitration. These effects were blunted by treatment of epicatechin, a safe and dietary supplement. To the best of our knowledge, our study demonstrates for the first time a dual role of p75^{NTR} in inducing proNGF expression and in mediating RGC death.

Epicatechin or polyphenolic flavonoid is one of several green tea constituents including epigallocatechin gallate, epigallocatechin, epicatechin gallate and epicatechin. The limited bioavailability of green tea extracts usually restricts their use as effective therapeutics. We used a repeated

dosing of epicatechin by oral gavage (100 mg kg⁻¹ day⁻¹), as it has proven successful in improving epicatechin bioavailability to cross blood–brain barrier (282%) in Sprague–Dawley rats compared with a single acute dose [30]. While the glucose-lowering effects of epigallocatechin gallate are well-documented in experimental models of diabetes at lower doses (25 mg kg⁻¹ day⁻¹) via its antioxidant properties [31, 32], little is known about the neuroprotective effects of epicatechin. Interestingly, our results demonstrated that treatment of diabetic animals with epicatechin did not affect blood glucose level or systemic antioxidant defence. In agreement with the above, we and others have previously shown that epicatechin is a selective inhibitor of tyrosine nitration, which does not affect the antioxidant defence [17, 21, 26, 33]. Our results here show that treatment with epicatechin (100 mg kg⁻¹ day⁻¹, orally) significantly reduced TUNEL-positive cells in retina flat mount and RGC cultures. Therefore, the protective effects of epicatechin in diabetic animals cannot be attributed to its ability to reduce blood glucose level or to the traditional antioxidant properties of green tea constituents. A growing body of evidence supports the role of oxidative stress and in particular peroxynitrite in activating glia to secrete growth factors such as proNGF. Our results showed significant increases in nitrotyrosine formation in diabetic rat retinas compared with controls. These results lend further support to previous findings showing enhanced peroxynitrite formation [6, 7, 10, 18, 34] and increases in NGF levels to compensate for neuronal injury [2, 8, 9]. Treatment of diabetic animals with epicatechin (100 mg kg⁻¹ day⁻¹, p.o.) blocked nitrotyrosine formation and proNGF expression.

We next investigated the possible mechanisms by which diabetes enhances proNGF levels, and the protective action of epicatechin. Our recent study demonstrated a critical role of peroxynitrite in inhibiting the protease matrix metalloproteinase-7, which cleaves proNGF into NGF leading to accumulation of proNGF [10]. Interestingly, epicatechin had minimal effects on cleavage of proNGF into NGF, suggesting its potential role in reducing proNGF expression. Previous studies have identified Müller cells as the main glia to secrete proNGF in the retina [10, 25]. In agreement with this, our results showed prominent glial Müller cell activation, which was evident from immunostaining of GFAP and accumulation of proNGF in the diabetic retina and in high glucose-cultured Müller cells. In light of the fact that p75^{NTR} is expressed in Müller cells and RGCs [27–29], our finding that the neuroprotective effect of epicatechin in vivo was associated with blocking the increases in expression of proNGF and p75^{NTR} suggests a paracrine effect of glial cells on neurons as target tissue. A co-culture model of RGCs with condition media from high glucose-treated glial rMC-1 showed an approximately 2.2-fold increase in proNGF levels and a 56% reduction in

Fig. 6 Epicatechin (Epi) blocks diabetes-induced p75^{NTR} apoptotic pathway in rat retina and RGC culture. **a** Western blot analysis showing 1.8-fold increase in p75^{NTR} in RGC cultures maintained in high glucose (HG) as compared with normal glucose (NG) medium. Exogenous treatment with proNGF (50 ng/ml; circled) increased p75^{NTR} expression by 1.7-fold in NG and by 2.3-fold in HG ($n=6$). **b** Western blot showing 1.7-fold increase in p38MAPK phosphorylation (pp38) in RGC cultures maintained in HG compared with NG medium. Exogenous treatment with proNGF (circled) increased p38MAPK phosphorylation by 1.8-fold in NG and by 2.2-fold in HG ($n=5-6$). Treatment of the Müller cell culture with 100 μ mol/l epica-

techin blocked p75^{NTR} expression in high glucose (HG+Epi) and did not affect normal glucose (NG+Epi) medium. **c** Western blot showed a 1.8-fold increase in p38MAPK phosphorylation in diabetic rat retinas compared with control rat retinas ($n=5-7$). **d** Western blot of cleaved PARP expression showing 2.2-fold increase in diabetic retinas compared with control retinas ($n=4-6$). Treatment with epicatechin (100 mg kg⁻¹ day⁻¹, orally) significantly reduced p38MAPK phosphorylation and cleaved PARP expression in diabetic rats but it did not affect control rats. * $p<0.05$ vs other groups. † $p<0.05$ vs HG+proNGF+Epi. C+Epi, control plus epicatechin; D+Epi, diabetes plus epicatechin

RGC viability compared with RGCs treated with normal glucose treatment conditioned medium. Treatment of Müller cultures with epicatechin blunted p75^{NTR} expression and proNGF levels, and did not significantly alter RGC viability (85% ~ of basal level). Together, these results suggest a dual role of enhanced p75^{NTR} expression in the retina, i.e. activation of proNGF expression in Müller cells and activation of neuronal death in RGCs. In support of this, several previous studies have shown that inhibiting p75^{NTR} on glial cells prevents apoptosis of photoreceptors and RGCs, and retinal degeneration via inhibition of proNGF or TNF- α [25, 35, 36]. However, we believe that our study is the first report demonstrating these effects in diabetes.

NGF and its precursor, proNGF, exert distinctive biological functions because of their different affinity to transmembrane receptors TrkA and p75^{NTR}, and the outcome, neurotrophic or apoptotic, will depend on expression and activity level of these receptors [15, 16]. Our previous study demonstrated that retinas from diabetic humans and rats showed no alteration in TrkA expression levels among various groups [7]. Therefore, we evaluated the post-translational modification of TrkA by determining its tyrosine nitration and phosphorylation. Our results showed a 2.2-fold increase in tyrosine nitration of TrkA and a 45% decrease in its phosphorylation site (Y490) in diabetic rat retinas compared with non-diabetic controls. Interestingly, tyrosine 490 is responsible for activating the phosphoinositide-3 kinase–Akt survival signal. These

effects were associated with a fivefold increase in neuronal cell death. Protein tyrosine nitration and subsequent loss of protein function have been well documented in response to peroxynitrite [17, 19, 21, 37–39]. The finding that blocking tyrosine nitration using epicatechin protects retinal neurons and inhibits the expression of p75^{NTR} supports a specific role of peroxynitrite in inducing neuronal death via upregulation of p75^{NTR} expression, which has been demonstrated previously in vitro and in vivo [40–42]. However, it is not completely understood how peroxynitrite increases the expression of p75^{NTR}. TrkA and p75^{NTR} are co-expressed throughout the nervous system and cross-talk between the two receptors showed a bidirectional relationship. Previous studies, in neuronal cells, showed that blocking TrkA expression or abrogating phosphorylation of TrkA (Y490) results in significant upregulation of p75^{NTR} expression [43, 44]. This effect was attributed to the fact that Y490 phosphorylation is necessary for the binding of an adaptor protein, Src homologous and collagen protein, and for downstream activation of Ras, leading to p75^{NTR} expression. In agreement with the above, our results show that diabetes-induced peroxynitrite formation abrogated Y490 phosphorylation at a site on TrkA via tyrosine nitration, and that this was reversed by epicatechin treatment. Other possible activators of p75^{NTR} expression include activation of protein kinase C [45], accumulation of glutamate, and activation of *N*-methyl-D-aspartate receptors [19], as well as diabetes-induced ischaemia [46]. Further

Fig. 7 ProNGF mediates RGC death in a manner that is dependent on $p75^{NTR}$. **a** Representative images showing TUNEL-positive cells in RGC culture. RGCs cultured in high glucose (HG) showed a threefold increase in TUNEL-positive cells as compared with normal glucose (NG). Exogenous treatment of RGCs with proNGF (50 ng/ml) as labelled caused a 2.7-fold increase in the number of TUNEL-positive cells in NG and augmented HG effects (3.8-fold). Co-treatment with epicatechin (Epi; 100 μ mol/l) blocked these effects ($n=3$). **b** Representative images showing TUNEL-positive cells in RGCs transfected with $p75^{NTR}$ siRNA and scrambled siRNA (Scr). In cultures treated with

scrambled RNA, HG caused a 2.8-fold increase in the number of TUNEL-positive cells as compared with culture in NG media. Silencing the expression of $p75^{NTR}$ using siRNA blocked the increase in the number of TUNEL-positive cells in HG. Exogenous treatment of scrambled siRNA-transfected cells with proNGF (50 ng/ml) increased the number of TUNEL-positive cells (2.3-fold) in NG and augmented HG effects (3.2-fold). Silencing the expression of $p75^{NTR}$ using siRNA blocked these effects in NG and HG media ($n=3$). * $p<0.05$ vs other groups. † $p<0.05$ vs HG+proNGF+Epi

studies are warranted to explore whether these upstream events share a common signalling cascade.

A critical role of $p75^{NTR}$, a member of the TNF superfamily, in mediating neuronal death has been docu-

mented in models of neurodegenerative diseases, including diabetes [47–49]. In agreement, our results showed that diabetes stimulates p38MAPK phosphorylation and cleaved PARP in vivo, and that high glucose stimulates $p75^{NTR}$ and

p38MAPK, and induces RGC death in vitro, all of which were blocked by epicatechin treatment, supporting a causal role of p75^{NTR} in mediation of RGC death. There is no commercial and reliable p75^{NTR} inhibitor; therefore, we used a molecular approach to confirm the causal role of p75^{NTR} in mediating RGC death. Silencing the expression of p75^{NTR} with siRNA completely blocked proNGF-induced or high glucose-induced RGC death compared with the scrambled form. Interestingly, our finding that exogenous proNGF exacerbated p75^{NTR}-mediated RGC death in basal and high glucose-treated RGC cultures suggest a vicious circle, where diabetes-induced initial neuronal injury will stimulate proNGF production, which will further damage RGCs. The notion that proNGF exacerbates RGC damage by enhancing p75^{NTR} expression is supported by a previous report showing a positive loop between increased level of neurotrophin and stimulation of p75^{NTR} expression in the target tissue [50]. Further assessment of retinal neuroglial dysfunction using electroretinogram in diabetic animals should provide valuable information that could be translated to humans.

In summary, diabetes-induced retinal neurodegeneration is an early and critical event that can be prevented using epicatechin via multiple mechanisms. Epicatechin inhibits tyrosine nitration and restores the TrkA survival signal, which is impaired in diabetic retina. As such, epicatechin inhibits upregulation of p75^{NTR} and hence proNGF levels in Müller cells, as well as activation of p75^{NTR} and p38MAPK pro-apoptotic signals in RGCs. The fact that epicatechin is a safe dietary supplement offers an additional advantage for its potential use as add-on therapy in diabetic patients.

Acknowledgements This work was supported by an American Heart Association Scientist Development Grant (0530170N to A. B. El-Remessy), the Juvenile Diabetes Research Foundation (grant 2-2008-149 to A. B. El-Remessy) and the University of Georgia Research Foundation (A. B. El-Remessy).

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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