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ARTICLE

NRF2 plays a protective role in diabetic retinopathy in mice

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Abstract
Aims/hypothesis Although much is known about the pathophysiological processes contributing to diabetic retinopathy (DR), the role of protective pathways has received less attention. The transcription factor nuclear factor erythroid-2-related factor 2 (also known as NFE2L2 or NRF2) is an important regulator of oxidative stress and also has anti-inflammatory effects. The objective of this study was to explore the potential role of NRF2 as a protective mechanism in DR.
Methods Retinal expression of NRF2 was investigated in human donor and mouse eyes by immunohistochemistry. The effect of NRF2 modulation on oxidative stress was studied in the human Müller cell line MIO-M1. Non-diabetic and streptozotocin-induced diabetic wild-type and *Nrf2* knockout mice were evaluated for multiple DR endpoints.
Results NRF2 was expressed prominently in Müller glial cells and astrocytes in both human and mouse retinas. In cultured MIO-M1 cells, NRF2 inhibition significantly decreased antioxidant gene expression and exacerbated *tert*-butyl hydroperoxide- and hydrogen peroxide-induced oxidative stress. NRF2 activation strongly increased NRF2 target gene expression and suppressed oxidant-induced reactive oxygen species. Diabetic mice exhibited retinal NRF2 activation, indicated by nuclear translocation. Superoxide levels were significantly increased by diabetes in *Nrf2* knockout mice as compared with wild-type mice. Diabetic *Nrf2* knockout mice exhibited a reduction in retinal glutathione and an increase in TNF-α protein compared with wild-type mice. *Nrf2* knockout mice exhibited early onset of blood–retina barrier dysfunction and exacerbation of neuronal dysfunction in diabetes.
Conclusions/interpretation These results indicate that NRF2 is an important protective factor regulating the progression of DR and suggest enhancement of the NRF2 pathway as a potential therapeutic strategy.

Keywords Diabetic retinopathy · Inflammation · Müller glial cells · Neuronal dysfunction · NF-E2-related factor-2 · Reactive oxygen species · Transcription factor · Vascular permeability

Abbreviations

BRB	Blood–retina barrier
CS	Contrast sensitivity
DCF	Dichlorofluorescein
DR	Diabetic retinopathy
DTNB	5,5′-Dithio-*bis*-2-(nitrobenzoic acid)
GCL	Ganglion cell layer
GFAP	Glial fibrillary acidic protein
GSH	Glutathione
H_2O_2	Hydrogen peroxide
INL	Inner nuclear layer

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KEAP1	Kelch-like ECH-associated protein 1
NRF2	Nuclear factor erythroid-2-related factor 2
ONL	Outer nuclear layer
qPCR	Quantitative PCR
ROS	Reactive oxygen species
siRNA	Small interference RNA
SF	Spatial frequency
STZ	Streptozotocin
TBH	*tert*-Butyl hydroperoxide
TNB	5-Thio-2-nitrobenzoic acid
VEGF	Vascular endothelial growth factor

Introduction

Diabetic retinopathy (DR) is the leading cause of blindness in working-age adults worldwide [1]. With an estimated prevalence of 93 million people, including 28 million with vision-threatening retinopathy, DR may become the leading cause of visual impairment in the world [2]. The pathogenesis of DR is multi-faceted, including oxidative stress [3], pro-inflammatory changes [4, 5] and advanced glycation end products [6]. These processes lead to dysfunction of multiple retinal cell types, including the vasculature and neuroretina [4, 5, 7, 8].

Research into DR has largely focused on the identification of pathogenic molecules such as vascular endothelial growth factor (VEGF). There is a growing awareness of the importance of identifying protective pathways, the activity of which could be augmented in patients with DR. It is increasingly appreciated that diabetic vascular complications result from an imbalance between causal (harmful) factors and protective factors [9, 10]. This suggests that new therapeutic strategies for DR, including both vascular and neuronal protection, should focus on enhancing the action of endogenous protective mechanisms in addition to inhibiting pathogenic mechanisms [9, 10].

The involvement of oxidative stress and pro-inflammatory changes in DR leads us to consider nuclear factor erythroid-2-related factor 2 (also known as NFE2L2 or NRF2), since it regulates both processes. NRF2 is a transcription factor that protects cells from endogenous and exogenous stresses [11, 12]. NRF2 is considered to be part of the most important cellular pathway protecting against oxidative stress [11, 12], and it also has an important role as a negative regulator of inflammation, attenuating inflammation-associated pathogenesis in multiple disease conditions [13–15]. Overall, NRF2 has been implicated as an important protective factor, especially in disease processes in which reactive oxygen species (ROS) and inflammation play a critical role.

Importantly, evidence is accumulating regarding a potential protective role for NRF2 in the retina [16, 17]. We found NRF2 to have a cytoprotective role for both neurons and vasculature following retinal ischaemia–reperfusion injury [17]. Intriguingly, NRF2 has been found to be protective in the context of diabetic disease, especially diabetic nephropathy. *Nrf2* knockout mice ($Nrf2^{-/-}$) displayed significantly greater renal injury compared with wild-type mice ($Nrf2^{+/+}$) in a streptozotocin (STZ)-induced model of diabetes [18]. Therapeutic activation of NRF2 had beneficial effects against diabetic kidney disease in rodents [19]. In this study we investigated whether NRF2 might have a beneficial role in suppressing the progression of DR. Using a loss of function strategy with genetic ablation of NRF2, we found evidence supporting the involvement of NRF2 in a protective mechanism against multiple endpoints that model DR in mice.

Methods

Human tissue Human donor eyes were fixed in 10% neutral buffered formalin for at least 24 h before paraffin embedding and subsequent sectioning. Unstained sections from human donor eyes were obtained from the Wilmer Eye Institute Ophthalmic Pathology laboratory with Institutional Review Board approval. All eyes were fixed within 18 h postmortem.

Immunohistochemistry Deparaffinised sections were boiled in 1× target retrieval solution (Dako, Carpinteria, CA, USA) for 20 min. Anti-NRF2 antibody (No. 2178-1; Epitomics, Burlingame, CA, USA) and isotype- and concentration-matched IgG control (ISO-2178; Epitomics) were used for incubation at 4°C overnight. Secondary biotinylated goat anti-rabbit IgG and ABC reagent (Vector Labs, Burlingame, CA, USA) were applied to the sections. Vector blue AP substrate kit III (Vector Labs) was used to stain the sections. Sections were also counter-stained with nuclear fast red (Vector Labs).

Cell culture, small interference RNA (siRNA) transfection, quantitative PCR and western blot analysis The human Müller glial cell line MIO-M1 [20] was cultured in DMEM plus 10% FBS and 1% GlutaMAX-I supplement (Invitrogen, Grand Island, NY, USA). Sub-confluent MIO-M1 cells were transfected with negative control No. 2 siRNA, *NRF2* siRNA (s9493) and *KEAP1* siRNA (s18983) (Applied Biosystems, Foster city, CA, USA) using Lipofectamine 2000 for 48 h. Total RNA was isolated using the RNeasy mini kit (Qiagen, Valencia, CA, USA) and single-stranded cDNA was synthesised using MMLV Reverse Transcriptase (Invitrogen). Quantitative PCR (qPCR) was performed using the QuantiTect SYBR Green PCR Kit (Qiagen) with a StepOnePlus real-time PCR system (Applied Biosystems). The qPCR primers were as follows: NQO1: (5′-CAGCTCACCGAGAGCCTAGT-3′) and (5′-ACCACCTCCCATCCTTTCTT-3′); GCLC: 5′-AC CATCATCAATGGGAAGGA-3′) and (5′-GCGATAAA

CTCCCTCATCCA-3′); HO-1: (5′-ATGACACCAA GGACCAGAGC-3′) and (5′-GTGTAAGGACC CATCGGAGA-3′); β-actin: (5′-AGAAAATCTGGC ACCACACC-3′) and (5′-GGGGTGTTGAAGGTCTCAAA-3′). For western blot analysis, anti-NRF2 (Epitomics), anti-NQO1 (Cell Signaling Technology, Danvers, MA, USA), anti-HO-1 (Enzo Life Science International, Farmingdale, NY, USA) and anti-β-actin antibodies (Cell Signaling Technology) were used. For analysis of NRF2 nuclear translocation, nuclear extracts from mouse retinas were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (ThermoFisher Scientific, Waltham, MA, USA). Rabbit monoclonal NRF2 antibody (Cell Signaling Technology) and Lamin B antibody (Santa Cruz Biotechnology, Dallas, Texas, USA) were used. The band intensity was quantified using the Image J program (version 1.47, NIH, http://imagej.nih.gov/ij/).

Dichlorofluorescein assay ROS production was quantified by the dichlorofluorescein (DCF) assay. Forty-eight hours after siRNA transfection, MIO-M1 cells were treated with or without different doses of *tert*-butyl hydroperoxide (TBH, Sigma, St Louis, MO, USA) and hydrogen peroxide (H_2O_2, Sigma) for 1 h. CM-H2DCFDA (10 μmol/l, Invitrogen) was then added and the cells were incubated for 30 min. DCF fluorescence was measured with a spectrophotometer (BMG Labtech, Durham, NC, USA).

Animals and treatments $Nrf2^{-/-}$ and $Nrf2^{+/+}$ mice on a C57BL/6 background [21, 22] were used for all experiments. Experimental diabetes was induced in 8-week-old male mice by intraperitoneal injection of STZ (45 mg/kg body weight in 10 mmol/l of citrate buffer, pH 4.5) for 5 consecutive days, as described [23]. Mice were considered diabetic when the blood glucose level was higher than 13.89 mmol/l. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University School of Medicine and conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Immunofluorescence analysis of mouse retinal frozen cryosections Cryosections (10 μm) of mouse eyes were fixed in 4% paraformaldehyde. Anti-NRF2 (R&D Systems, Minneapolis, MN, USA), anti-glial fibrillary acidic protein (GFAP) (ThermoFisher Scientific) and anti-Vimentin (Sigma) antibodies were used. Alexa fluor 488-, Alexa fluor 594- (Invitrogen) or Cy3- (Jackson ImmunoResearch, West Grove, PA, USA) conjugated IgG were used as secondary antibodies. DAPI (Invitrogen) was used to stain nuclei. Photographs were taken with a Zeiss LSM 710 confocal microscope (Carl Zeiss Microscopy, Thornwood, NY, USA).

Lucigenin assay Superoxide anion in the retina was quantified by lucigenin assay as described [24]. Fresh retinas were placed in 0.2 ml Krebs/HEPES buffer and incubated in the dark at 37°C under 5% CO_2 for 10 min. Lucigenin (Sigma) was added to a final concentration of 0.5 mmol/l and photon emission was measured over 10 s with a luminometer three times (Analytical Luminescence Laboratory, San Diego, CA, USA). Retinas were then sonicated in 200 μl RIPA lysis buffer (Sigma). Protein concentration was measured by DC protein assay (BioRad, Hercules, CA, USA) and used to normalise the final superoxide level.

Glutathione assay Retinas were sonicated in 0.5 mmol/l PB buffer (pH 6.8 and 1 mmol/l EDTA). The samples were cleared by centrifugation at 10,000*g* for 15 min. Retinal protein extract (18 μg) was deproteinised and assayed with a glutathione (GSH) assay kit (Cayman Chemical, Ann Arbor, Michigan, USA). 5,5′-Dithio-*bis*-2-(nitrobenzoic acid (DTNB) was added to the extract to react with GSH and produce a yellow-coloured 5-thio-2-nitrobenzoic acid (TNB). After incubation for 25 min, the absorbance of TNB was measured at 405 nm using a microplate reader (Epoch BioTek, Winooski, VT, USA).

ELISA for TNF-α Retinas were sonicated in 0.1% Triton X-100 in PBS supplemented with a mixture of protease inhibitors (Invitrogen). The samples were cleared of debris by centrifugation at 12,000*g* for 10 min and then assessed for protein concentration. TNF-α levels were measured using an ELISA kit (R&D Systems). The TNF-α concentration was calculated from a standard curve and corrected for total protein concentration.

Blood–retina barrier assay The blood–retina barrier (BRB) assay was performed as described [25, 26]. Mice were given an intraperitoneal injection of 37,000 Bq/g body weight of [^{3}H]mannitol. One hour after injection, mice were sedated and retinas rapidly removed and dissected to free the lens, vitreous and retinal-pigment epithelium. Retinal dry weight was measured. One millilitre of NCSII solubilising solution (GE Healthcare, Piscataway, NJ, USA) was added to each sample, and the samples were incubated overnight at 50°C. Solubilised tissue was brought to room temperature and decolourised with 20% benzoyl peroxide in toluene at 50°C. After re-equilibration to room temperature, 5 ml of scintillation fluid cytoscint ES (ThermoFisher Scientific) and 30 μl of glacial acetic acid were added. Samples were stored for several hours in darkness at 4°C to eliminate chemoluminescence. Radioactivity was counted with a scintillation counter (LS 6500 Liquid Scintillation Counter; Beckman-Coulter, Indianapolis, IN, USA). The cpm of retina tissue from $Nrf2^{-/-}$ and $Nrf2^{+/+}$ groups were measured and normalised to the dry weight of each retina.

Behavioural assessment of visual thresholds in mice A virtual optomotor system [27] (Optomotry™; Cerebral Mechanics, White Plains, NY, USA) was used to assess visual function in diabetic and non-diabetic mice. The apparatus has four inward-facing LCD panels arranged in a square around an elevated testing platform (7 cm diameter). A moving vertical sine wave grating is displayed on the monitors, creating an illusion of a virtual cylinder rotating at 12°/s around the testing platform. A video camera mounted to the top lid of the apparatus reports to a computer program, enabling the experimenter to score the test animal's optokinetic reflex response to the moving grating.

The spatial frequency (SF) threshold and contrast sensitivity (CS) were assessed in awake, freely moving mice. When mice were able to see the moving target, their head and upper body tracked the grating pattern. SF (cycles/degree) was always measured at maximum contrast (100%) by using a staircase method beginning with a minimum preset frequency of 0.072 cycles/degree. The contrast threshold (%) was measured at an SF of 0.092 cycles/degree. CS threshold was calculated as the reciprocal of the CS score. The SF threshold and CS were identified as the highest values that still elicited a response in the mouse. Twenty-three mice were used for visual threshold testing. Both SF and CS were averaged over three trials on consecutive days for each mouse.

Retinal thickness and cell count in ganglion cell layer Cross-sections cut through the optic nerve were prepared and stained with hematoxylin and eosin for retinal morphology examination. Three cryosections from each retina and six locations in the retina from each cryosection (three locations on each side of the optic nerve) were quantified with respect to nuclei in the ganglion cell layer (GCL) (excluding nuclei of blood vessels) and the thickness of the inner nuclear layer (INL) and outer nuclear layer (ONL), under 400× magnification with Zeiss AxioVision software (AxioVS40 V4.7.2.0, Carl Zeiss) [28].

Statistical analysis Data were presented as means ± SD, and were analysed by using non-parametric Mann–Whitney test for in vivo studies and two-tailed Student's t test for cell culture studies. A value of $p < 0.05$ was considered statistically significant.

Results

NRF2 is expressed in human retinas We first investigated the expression and localisation of NRF2 in human retinas. Immunohistochemical staining for NRF2 was performed using human ocular sections. Retinas showed prominent NRF2 immunoreactivity in Müller cells spanning the retina. There was also prominent immunoreactivity in the nerve fibre layer and GCL in the cells that were morphologically consistent with astrocytes, microglia and ganglion cells (Fig. 1a). Staining was also observed in vascular endothelial cells, although relatively weak (see electronic supplementary material [ESM] Fig. 1). Normal control IgG did not give any positive staining (Fig. 1b). Immunostaining of NRF2 in mouse retina showed co-localisation of NRF2 with Vimentin (Müller glia marker) (Fig. 1c) and GFAP (astrocyte marker) (Fig. 1d), indicating that NRF2 was strongly expressed in retinal glial cells. NRF2 showed similar localisation in diabetic retinas and non-diabetic control retinas (data not shown).

NRF2 has a protective effect in human Müller cells against oxidant-induced oxidative stress To determine whether NRF2 could be beneficial for retinal cells against oxidative stress, we used siRNA to modulate NRF2. We used an adult human Müller glial cell culture system because of the strong

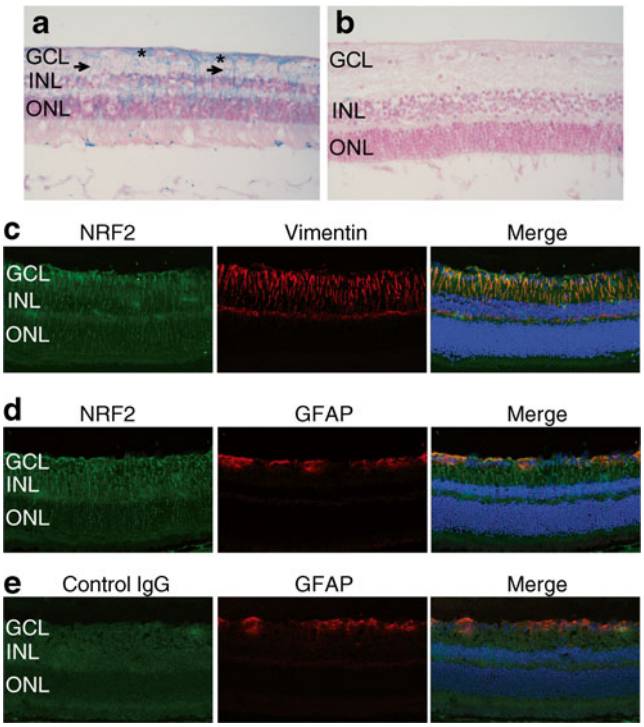

Fig. 1 Expression of NRF2 in human and mouse retinas. Paraffin-embedded human retinal sections were immunostained with NRF2 antibody (blue) and counter-stained with nuclear fast red. (**a**) Retina from donors showed NRF2 immunoreactivity in Müller cells spanning the retina. NRF2 was also expressed in the GCL. Arrows indicate Müller cell processes and asterisks indicate the nerve fibre layer. (**b**) Negative immunohistochemical staining in human retina with rabbit control IgG. The images are representative of eight donors and were taken at 20× objective. (**c**) Mouse retinal cryosections were analysed by immunofluorescence for NRF2 (green) and Vimentin (red). (**d**) Mouse retinal cryosections were analysed by immunofluorescence for NRF2 (green) and GFAP (red). (**e**) Immunofluorescence for control IgG (green). DAPI (blue) was used to stain nuclei in all retinal sections

expression of NRF2 in Müller cells (Fig. 1) and the important role of Müller cells in DR [29, 30]. Kelch-like ECH-associated protein 1 (KEAP1) is a major repressor of NRF2 that interacts with NRF2 and mediates its ubiquitination and proteolysis. We first confirmed that transfecting *NRF2* siRNA and *KEAP1* siRNA efficiently decreased and increased, respectively, NRF2 protein levels in MIO-M1 cells (Fig. 2b). *NRF2* siRNA significantly decreased mRNA levels of the NRF2 target genes *NQO1* and *GCLC* ($n=4$, $p<0.05$). KEAP1 knockdown significantly increased the expression of established NRF2-target antioxidant genes, including *NQO1*, *GCLC*, *GCLM* and *HO-1* (also known as *HMOX1*) from 1.6- to 3.4-fold ($n=4$, $p<0.01$) (Fig. 2a). Western blot analysis further confirmed elevation of NQO1 and HO-1 protein levels in *KEAP1* siRNA-transfected MIO-M1 cells (Fig. 2b). To evaluate whether NRF2 knockdown or activation, respectively, regulates oxidative stress, we treated MIO-M1 cells with two oxidants, TBH and H_2O_2. As shown in Fig. 2c, TBH (10–100 μmol/l) and H_2O_2 (50–200 μmol/l) significantly increased ROS in MIO-M1 cells compared with PBS control in a dose-dependent fashion. NRF2 knockdown exacerbated TBH- and H_2O_2-increased ROS (Fig. 2c). Conversely, NRF2 activation by KEAP1 knockdown significantly reduced both TBH and H_2O_2 induction of ROS.

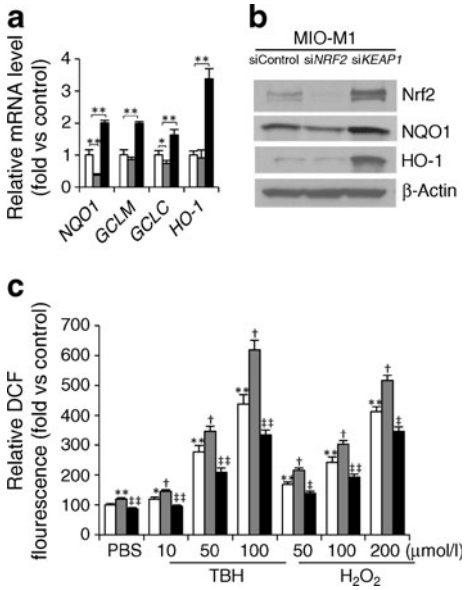

Fig. 2 NRF2 protects against oxidant-induced ROS increase in human Müller cells. (**a**) Relative expression of NRF2-responsive antioxidant genes in MIO-M1 cells after 48 h of *NRF2* siRNA and *KEAP1* siRNA transfection. White bars, control siRNA; grey bars, *NRF2* siRNA; black bars, *KEAP1* siRNA. $n=4$; $^{*}p<0.05$, $^{**}p<0.01$. (**b**) Western blot analysis of NRF2, NQO1 and HO-1 expression after 48 h of *NRF2* siRNA and *KEAP1* siRNA transfection. (**c**) The effect of knocking down NRF2 or KEAP1 on different oxidant-induced ROS increase in MIO-M1 cells. Forty-eight hours after siRNA transfection, cells were challenged by different doses of TBH or H_2O_2 for 1 h. ROS levels were determined by DCF assay. White bars, control siRNA; grey bars, *NRF2* siRNA; black bars, *KEAP1* siRNA. $n=4$; $^{*}p<0.05$ and $^{**}p<0.01$ vs control siRNA with PBS treatment; $^{\dagger}p<0.05$, $^{\ddagger}p<0.05$ and $^{\ddagger\ddagger}p<0.01$ vs control siRNA with respective treatment

Body weight and blood glucose level of STZ-induced diabetic and control mice The protective role of NRF2 in cultured Müller glial cells encouraged us to investigate whether NRF2 is involved in a protective pathway in a mouse model of DR, using wild-type and $Nrf2^{-/-}$ mice. After 8 weeks of diabetes, diabetic mice from both wild-type and $Nrf2^{-/-}$ groups weighed significantly less than their respective control groups ($p<0.001$). Blood glucose levels in the diabetic groups of mice were significantly higher than in their respective control groups ($p<0.001$). The weight and blood glucose levels were not significantly different when comparing wild-type mice and $Nrf2^{-/-}$ mice, under both diabetic and non-diabetic conditions (Table 1).

Nrf2 was activated in the retinas of STZ-induced diabetic mice Nuclear translocation of NRF2 [11] was evaluated to determine whether NRF2 was activated or inactivated during diabetes. There was an approximately twofold ($n=6$, $p<0.01$) increase of nuclear NRF2 in wild-type diabetic mice compared with non-diabetic controls (Fig. 3) after 8 weeks of diabetes, which indicated that NRF2 was activated during diabetes. We observed no difference in levels of total retinal NRF2 protein between diabetic and non-diabetic mice at 8 weeks of diabetes (data not shown).

NRF2 deficiency increased retinal ROS levels in STZ-induced diabetic mice To implicate NRF2 as being part of a protective mechanism in DR, we used a loss of function strategy with genetic ablation of NRF2 in mice. We first investigated the role of NRF2 in regulating diabetes-induced oxidative stress, as measured by superoxide generation. Retinas were extracted from non-diabetic and diabetic mice and superoxide levels were measured by lucigenin assay [24]. Retinal superoxide was significantly increased in diabetic $Nrf2^{-/-}$ mice compared with wild-type mice after 5 weeks of diabetes (Fig. 4).

Table 1 Body weight and blood glucose level in non-diabetic and diabetic wild-type ($Nrf2^{+/+}$) and *Nrf2* knockout mice ($Nrf2^{-/-}$) after 8 weeks of diabetes

Group	Body weight (g)	Blood glucose (mmol/l)
$Nrf2^{+/+}$ non-diabetic ($n=12$)	28.47±3.22	9.64±1.56
$Nrf2^{+/+}$ diabetic ($n=13$)	24.28±1.97*	37.59±5.09*
$Nrf2^{-/-}$ non-diabetic ($n=11$)	29.73±1.76	11.79±3.88
$Nrf2^{-/-}$ diabetic ($n=8$)	24.10±1.92*	33.81±8.89*

Data are means ± SD

*$p<0.001$ compared with the respective $Nrf2^{+/+}$ or $Nrf2^{-/-}$ non-diabetic group

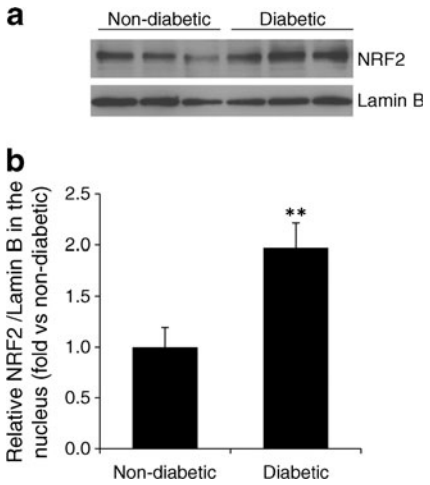

Fig. 3 Nuclear translocation of NRF2 in diabetic mouse retinas. (**a**) Western blot analysis of NRF2 with retina nuclear extract from wild-type mice after 8 weeks of diabetes. Lamin B was used as a loading control. (**b**). Quantitative analysis of nuclear NRF2 protein level. The intensity of NRF2 is presented relative to Lamin B in the same sample and normalised to non-diabetic control retinas. $n=6$ for each group; $^{**}p<0.01$

GSH is a vital antioxidant that regulates cellular redox status, and NRF2 plays a key role in regulating cellular GSH homeostasis [11]. Retinas were extracted from diabetic mice for measurement of GSH levels [31]. There was no significant difference in GSH levels in non-diabetic control groups between wild-type and *Nrf2*$^{-/-}$ mice (Fig. 5). Retinal GSH levels were significantly lower in diabetic *Nrf2*$^{-/-}$ mice compared with wild-type diabetic mice ($p<0.05$) (Fig. 5).

Effect of NRF2 deficiency on diabetes-induced TNF-α expression TNF-α is thought to play an important role in DR, including BRB dysfunction [26, 32]. Since NRF2 is known to have an anti-inflammatory effect in the retina, including modulation of pro-inflammatory cytokines [16, 17], we investigated its potential effect on retinal levels of TNF-α in wild-type and *Nrf2*$^{-/-}$ mice. ELISA results showed that there was no difference in TNF-α in wild-type diabetic mice after 8 weeks of diabetes compared with wild-type controls. In contrast, there was a significantly greater amount of TNF-α protein in diabetic *Nrf2*$^{-/-}$ mice compared with their control group ($p<0.05$) (Fig. 6).

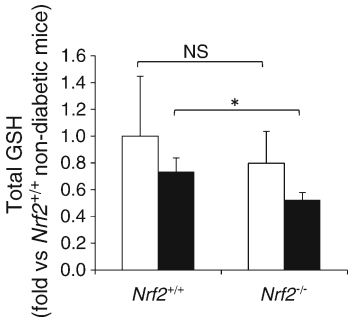

Fig. 5 NRF2-deficient diabetic mice exhibit lower retinal GSH levels compared with wild-type diabetic mice. GSH levels were measured in retinal protein lysates from wild-type and *Nrf2*$^{-/-}$ mice after 8 weeks of diabetes using a GSH assay kit. White bars, non-diabetic control mice; black bars, diabetic mice. $n=7$ for each group; $^{*}p<0.05$

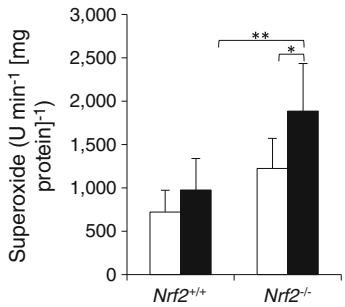

Fig. 4 NRF2-deficient mice exhibit increased oxidative stress in early diabetes. Fresh retinas were isolated from wild-type mice and *Nrf2*$^{-/-}$ mice after 5 weeks of diabetes. The level of retinal superoxide was determined by lucigenin assay. White bars, non-diabetic control mice; black bars, diabetic mice. Non-diabetic *Nrf2*$^{+/+}$ mice, $n=6$; diabetic *Nrf2*$^{+/+}$ mice, $n=7$; non-diabetic *Nrf2*$^{-/-}$ mice, $n=6$; diabetic *Nrf2*$^{-/-}$ mice, $n=5$; $^{*}p<0.05$, $^{**}p<0.01$

Effect of NRF2 deficiency on retinal vascular permeability in diabetic mice BRB breakdown is a major pathological change in DR, leading to retinal oedema and loss of vision. We investigated the effect of NRF2 deficiency on retinal vascular leakage in the STZ-induced diabetes model. We employed a protocol using [^{3}H]mannitol as a tracer [25, 26] to measure the amount of mannitol leaked by the retinal blood vessels into the surrounding retina. There was a significant increase in retinal vascular leakage in the *Nrf2*$^{-/-}$ mice after 8 weeks of diabetes as compared with wild-type diabetic mice ($p<0.05$) (Fig. 7).

NRF2 deficiency exacerbated diabetes-induced visual dysfunction in mice In addition to its effects on the retinal vasculature, diabetes is known to have adverse effects on neuronal function in the retina, including consequences on visual function [33]. In rodent models of diabetes, diabetes-

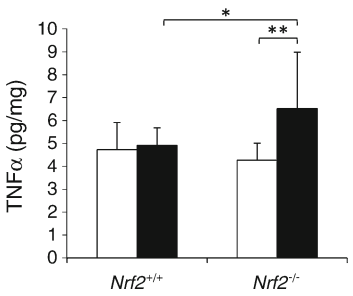

Fig. 6 NRF2-deficient mice exhibit increased retinal TNF-α protein levels in diabetes. After 8 weeks of diabetes, retinas were isolated and processed for TNF-α ELISA assay. White bars, non-diabetic control mice; black bars, diabetic mice. Non-diabetic *Nrf2*$^{+/+}$ mice, $n=10$; diabetic *Nrf2*$^{+/+}$ mice, $n=14$; non-diabetic *Nrf2*$^{-/-}$ mice, $n=10$, diabetic *Nrf2*$^{-/-}$ mice, $n=8$; $^{*}p<0.05$; $^{**}p<0.01$

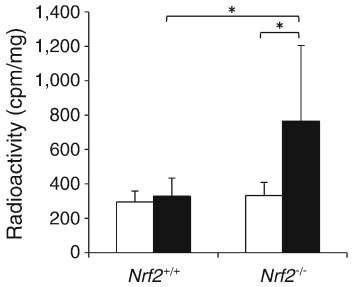

Fig. 7 NRF2-deficient mice exhibit earlier onset of BRB dysfunction in diabetes. BRB dysfunction was determined in $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice after 8 weeks of diabetes. Radioactivity per mg of retina was measured 1 h after intraperitoneal injection of [^{3}H]mannitol. White bars, non-diabetic control mice; black bars, diabetic mice. Non-diabetic $Nrf2^{+/+}$ mice, $n=4$; diabetic $Nrf2^{+/+}$ mice, $n=5$; non-diabetic $Nrf2^{-/-}$ mice, $n=4$; diabetic $Nrf2^{-/-}$ mice, $n=4$; $^*p<0.05$; $^{**}p<0.01$

induced neuroretinal dysfunction has been detected using tests of electrical activity [34, 35] as well as direct assessment of visual function by measurement of the optokinetic response [35]. Since NRF2 has neuroprotective effects in the retina [17], we analysed visual function in non-diabetic and diabetic $Nrf2^{-/-}$ and $Nrf2^{+/+}$ mice. We used a virtual optomotor system that allows precise quantification of both SF thresholds and CS [27]. After 8 weeks of diabetes, $Nrf2^{-/-}$ mice had significant visual deficits in both SF and CS as compared with age-matched non-diabetic $Nrf2^{-/-}$ controls ($p<0.05$) and diabetic wild-type mice ($p<0.01$; Fig. 8). Diabetic $Nrf2^{+/+}$ mice also had a significantly lower SF threshold compared with non-diabetic $Nrf2^{+/+}$controls ($p<0.05$; Fig. 8a). There was a trend toward reduction in CS in diabetic $Nrf2^{+/+}$ mice compared with non-diabetic $Nrf2^{+/+}$ controls, but this did not reach statistical significance (Fig. 8b).

Retinal morphology and integrity were examined in cryosections using haematoxylin and eosin staining, and no differences were found when comparing all four groups (ESM Fig. 2). There was no significant difference in the number of cells in the GCL between non-diabetic and diabetic $Nrf2^{+/+}$ mice (Table 2), or between diabetic $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice (Table 2). There were no significant differences in INL and ONL thickness between all four groups (Table 2). TUNEL and immunostaining of cleaved caspase 3 were also performed to detect any cell apoptosis in the retina. After 8 weeks of diabetes, we did not detect any observable apoptosis in $Nrf2^{+/+}$ or $Nrf2^{-/-}$ diabetic mice (ESM Fig. 3). Overall, this indicates a diabetes-induced neuronal dysfunction at this stage of diabetes, in the absence of overt retinal neuronal death.

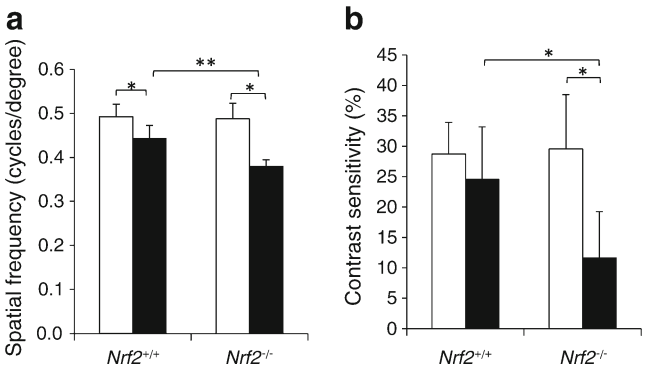

Fig. 8 NRF2-deficient mice have increased visual dysfunction induced by diabetes. After 8 weeks of diabetes, the SF (**a**) and CS (**b**) thresholds were obtained in wild-type and $Nrf2^{-/-}$ mice using Optomotry optokinetic testing. White bars, non-diabetic control mice; black bars, diabetic mice. Non-diabetic $Nrf2^{+/+}$ mice, $n=6$; diabetic $Nrf2^{+/+}$ mice, $n=6$; non-diabetic $Nrf2^{-/-}$ mice, $n=6$; diabetic $Nrf2^{-/-}$ mice, $n=4$; $^*p<0.05$ and $^{**}p<0.01$

Discussion

Although research into DR has largely focused on the identification of pathogenic molecules, there is a growing awareness of the importance of identifying protective pathways [9, 10]. This is highlighted by studies indicating protection from retinopathy in some individuals with very long-standing diabetes [36]. Our group reported a retinal cytoprotective role for NRF2 in ischaemia–reperfusion injury, in which it inhibited both oxidative stress and pro-inflammatory changes [17]. Since both these pathophysiological processes are thought to be critical contributors to DR, we were interested in investigating NRF2 as part of a potential protective mechanism in DR.

Although NRF2 has received significant attention in systemic settings, including the lung, there has been less investigation of NRF2 in the retina. We therefore first determined whether NRF2 is expressed in human retina. NRF2 immunohistochemistry disclosed staining in multiple cell types, including prominent staining in Müller glial cells. Although it is clear that multiple cell types play a role in DR, the Müller glial staining was intriguing given the increasing awareness of its role in DR [29, 30]. These previous studies indicate that the Müller glia may be an important driver in the progression of DR, including involvement in pro-inflammatory effects and production of TNF-α and VEGF. Our studies with cultured human Müller glial cells were therefore very encouraging in suggesting a protective role for NRF2 against

Table 2 Number of cells in GCL and retinal thickness of INL and ONL in non-diabetic and diabetic wild-type ($Nrf2^{+/+}$) and *Nrf2* knockout mice ($Nrf2^{-/-}$) after 8 weeks of diabetes

Group	Cell number in GCL	Retinal thickness (μm)	
		INL	ONL
$Nrf2^{+/+}$ non-diabetic ($n=6$)	12.66±1.00	25.74±1.91	53.23±2.41
$Nrf2^{+/+}$ diabetic ($n=5$)	13.12±1.11	25.36±2.44	53.58±5.06
$Nrf2^{-/-}$ non-diabetic ($n=5$)	13.87±0.77	25.23±1.85	55.86±3.01
$Nrf2^{-/-}$ diabetic ($n=5$)	13.05±1.43	24.11±3.53	53.47±7.46

Data are means ± SD

oxidative stress. To determine whether NRF2 is part of a protective mechanism in DR, we used a loss of function strategy with *Nrf2*$^{-/-}$ mice. Our studies indicate that NRF2 does indeed have a protective role in the retina with regard to retinal superoxide, GSH and TNF-α levels. The induction of oxidative stress is considered to be a key pathophysiological process in DR, and superoxide generation is thought to be especially critical [7, 37]. In addition, it is increasingly appreciated that pro-inflammatory processes contribute to DR [4, 5]. TNF-α is thought to be one of the important contributing cytokines [26, 32], with both microglia [38] and Müller cells [29] implicated as important sources in DR.

A crucial endpoint in DR is BRB dysfunction, which contributes to diabetic macular oedema, the most common cause of vision loss in diabetes [39]. There is strong evidence to suggest a role for both oxidative stress and pro-inflammatory cytokines in BRB dysfunction in DR [39, 40]. There is much support for the role of inflammatory cytokines, including TNF-α in producing BRB dysfunction in diabetes [39]. Our studies indicating the protective effect of NRF2 against oxidative stress and TNF-α expression therefore suggest that it might be protective against retinal vascular permeability in DR. Indeed, we found this to be the case, such that *Nrf2*$^{-/-}$ mice had significant BRB dysfunction at a time where BRB dysfunction was not yet evident in *Nrf2*$^{+/+}$ mice. This is reminiscent of other disease models such as cigarette smoke-induced emphysema, in which the onset of lung pathology occurred earlier in *Nrf2*$^{-/-}$ than in *Nrf2*$^{+/+}$ mice [41, 42], presumably because of the increased susceptibility of the *Nrf2*$^{-/-}$ mice. We should note that, in contrast to BRB dysfunction in early diabetes that has been reported in mice in other studies, often using Evans Blue as a tracer [35], we usually do not observe dysfunction until later in diabetes, at 3–6 months (data not shown).

We also examined the effect of NRF2 ablation on neuronal dysfunction. The pathogenesis of neuronal dysfunction in DR is not well understood. However, pro-inflammatory changes are a contributory cause of retinal neuronal apoptosis in DR [38], and treatment with an anti-inflammatory drug corrected retinal electrophysiological abnormalities in a rodent model of DR [43]. This suggests to us that NRF2 might also modulate neuronal dysfunction in DR. An approach for testing optomotor responses, specifically the virtual optomotor system, has emerged for assessing visual function in mice. This approach can ascertain two measures of spatial vision, acuity and CS [27]. We found that 8 weeks of diabetes resulted in a significant reduction of the SF threshold (Fig. 8a) in wild-type mice, similar to the findings of a study using diabetic rats [44]. There was a similar tendency toward a diabetes-induced reduction in CS in wild-type mice, although this did not reach statistical significance (Fig. 8b). Strikingly, we found that *Nrf2*$^{-/-}$ mice had a statistically significant reduction in both variables of visual function compared with wild-type mice. This indicates that NRF2 has an important protective role against neuronal dysfunction in the context of diabetes.

NRF2 has previously been demonstrated to have a protective role in DR [18]. *Nrf2*$^{-/-}$ mice had significantly greater renal injury compared with *Nrf2*$^{+/+}$ mice in the STZ-induced diabetes model [18]. Therapeutic activation of NRF2 reduced diabetic kidney disease in rodents. This suggests that NRF2 might be involved in a common protective mechanism for diabetic complications, making it an especially attractive pharmacological target, especially as NRF2 is readily amenable to pharmacological activation. Of note, a recent study in rats demonstrated that high glucose levels and diabetes impair NRF2 activity, with a resultant blunting of induction of the NRF2-responsive antioxidant gene *Gclc* [45]. This very interesting study suggests that individuals with DR might have suboptimal NRF2 activity, further highlighting the importance of pharmacological modulation of NRF2.

In summary, there is emerging appreciation of the importance of identifying endogenous protective factors in DR. Indeed, some protective molecules have already been identified, including manganese superoxide dismutase (MnSOD) [46] and pigment epithelium-derived factor (PEDF) [47]. Augmentation of these respective protective molecules inhibits DR and its pathological consequences [31, 47]. Our studies indicate that NRF2 is involved in an additional protective mechanism in the retina in diabetes. Pharmacological targeting of NRF2 may therefore be a promising strategy for protecting vascular and neuronal function in DR.

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Contribution statement ZX designed and performed the experiments, interpreted and analysed the data and wrote and edited the manuscript. YW, JG, HC, JKP, ES, HH, LW, CE, YD, TSK and AJB contributed to acquisition and analysis of the data and were involved in drafting the article. JTH, RT and SB contributed to analysis of the data and intellectual input, including ideas for development of the project and drafting the article. EJD conceived the study, designed the experimental plan, wrote the manuscript, supervised the entire work and edited the manuscript. All authors approved the final submitted version of the manuscript.

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