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ARTICLE

Loss of X-box binding protein 1 in Müller cells augments retinal inflammation in a mouse model of diabetes

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Abstract

Aims/hypothesis Müller glia (MG) are major sources of retinal cytokines, and their activation is closely linked to retinal inflammation and vascular leakage in diabetic retinopathy. Previously, we demonstrated that X-box binding protein 1 (XBP1), a transcription factor activated by endoplasmic reticulum (ER) stress in diabetic retinopathy, is involved in regulation of inflammation in retinal endothelial cells. Now, we have explored the role of *XBP1* and ER stress in the regulation of MG-derived proinflammatory factors, and their influence on vascular permeability in diabetic retinopathy.

Methods MG-specific conditional *Xbp1* knockout ($Xbp1^{Müller-/-}$) mice were generated by crossing *Xbp1* flox/flox mice with Müller–Cre transgenic mice. Diabetes was modelled by induction with streptozotocin, and retinal vascular permeability was measured with FITC-conjugated dextran 2 months after induction. Primary Müller cells were isolated from $Xbp1^{Müller-/-}$ and $Xbp1^{Müller+/+}$ mice and exposed to hypoxia and high levels of glucose. Levels of ER-stress and inflammatory factors were examined by real-time PCR, western blotting or immunohistochemistry.

Results $Xbp1^{Müller-/-}$ mice exhibited normal retinal development and retinal function and expressed similar levels of ER-stress and inflammatory genes to $Xbp1^{Müller+/+}$ littermates. In diabetes-inducing conditions, compared with $Xbp1^{Müller+/+}$ mice, $Xbp1^{Müller-/-}$ mice had higher mRNA levels of retinal *Vegf* (also known as *Vegfa*) and *Tnf-α* (also known as *Tnf*) and ER-stress marker genes *Grp78* (also known as *Hspa5*), *Atf4*, *Chop* (also known as *Ddit3*) and *Atf6* and higher protein levels of vascular endothelial growth factor (VEGF), TNF-α, phospho-c-Jun N-terminal kinase (JNK), 78 kDa glucose-regulated protein (GRP78), phospho-eukaryotic translation initiation factor (eIF)2α and activating transcription factor (ATF)6. Retinal vascular permeability was significantly higher in diabetic $Xbp1^{Müller-/-}$ mice than in diabetic $Xbp1^{Müller+/+}$ mice ($p < 0.01$). Results obtained in vitro with primary Müller cells isolated from $Xbp1^{Müller-/-}$ mice confirmed higher expression levels of inflammatory and ER-stress markers (but not GRP78) than in *cells from* $Xbp1^{Müller+/+}$ mice. Moreover, *XBP1-deficient Müller cells* were more susceptible to high-glucose- or hypoxia-induced ER stress and inflammation than *cells from* $Xbp1^{Müller+/+}$ mice. Inhibition of ER stress with chemical chaperones suppressed hypoxia-induced VEGF and TNF-α production in *XBP1*-deficient *Müller cells*.

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Research in context

What is already known about this subject?

- Diabetes induces dysfunction of the endoplasmic reticulum (ER), resulting in ER stress in retinal cells, including Müller glia (MG)
- MG have a crucial role in regulation of proinflammatory factors in the retina, and among these factors, MG-derived vascular endothelial growth factor (VEGF) is a major contributor to retinal inflammation and vascular hyperpermeability
- X-box binding protein 1 (XBP1) is a central regulator of the unfolded protein response that acts to maintain ER homeostasis and eliminate ER stress

What is the key question?

- What is the influence of ER stress, induced by deletion of *Xbp1* in MG, on retinal inflammation and vascular leakage in diabetic retinopathy?

What are the new findings?

- In vivo, *Xbp1* deletion in MG exacerbates diabetes-induced upregulation of retinal VEGF and TNF-α, ER stress and vascular permeability
- In vitro, XBP1-deficient Müller cells are more susceptible to high-glucose- or hypoxia-induced ER stress and inflammation than wild-type cells
- ER-stress inhibition via chemical chaperones suppresses hypoxia-induced production of VEGF and TNF-α in XBP1-deficient Müller cells

How might this impact on clinical practice in the foreseeable future?

- Suppression of ER stress is a potential approach for prevention and treatment of diabetic retinopathy

Conclusions/interpretation Our results have revealed an important role of XBP1 and ER stress in MG-driven retinal inflammation, and suggest that targeting ER stress may represent a promising approach for the prevention and treatment of diabetic retinopathy.

Keywords Diabetic retinopathy · ER stress · Inflammation · Müller cell · X-box binding protein 1

Abbreviations

ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
eIF2α	Eukaryotic translation initiation factor 2α
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
ERG	Electroretinography
GRP78	78 kDa glucose-regulated protein
HIF1-α	Hypoxia-inducible transcription factor 1α
ICAM-1	Intercellular adhesion molecule 1
IRE1α	Inositol-requiring enzyme 1α
JNK	c-Jun N-terminal kinase
MG	Müller glia
PBA	4-Phenyl butyric acid
TMAO	Trimethylamine N-oxide
UPR	Unfolded protein response
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
XBP1	X-box binding protein 1

Introduction

Diabetic retinopathy is a leading cause of acquired blindness in individuals ≥40 years old, and places a substantial burden on public-health resources worldwide [1, 2]. Although the precise mechanisms involved in diabetic retinopathy remain elusive, dysregulated production of cytokines from stressed retinal cells is a key factor contributing to neurovascular injury [3–6]. Among these cytokines, vascular endothelial growth factor (VEGF) and tumour necrosis factor-α (TNF-α) have been extensively studied in relation to diabetic retinopathy. VEGF is a major angiogenic factor with a critical role in maintenance of endothelial cell function, but excessive production of VEGF disrupts the blood–retina barrier and stimulates endothelial cell growth, resulting in vascular leakage and pathological neovascularisation in diabetic retinopathy (reviewed in [7]). Anti-VEGF therapies have been developed, and are effective for reduction of diabetic macular

oedema and improvement of visual outcomes in individuals with diabetes [8]. In addition to VEGF, a number of other proinflammatory cytokines, such as TNF-α and intercellular adhesion molecule 1 (ICAM-1), are upregulated at the early stage of diabetic retinopathy [9], and high levels of proinflammatory cytokines are observed in the vitreous of individuals with proliferative diabetic retinopathy [10] and in the retinas of animal models of diabetes [11]. Ablation of TNF-α suppresses leucostasis and reduces vascular permeability in diabetic retinopathy [12], suggesting that dysregulation of cytokine production contributes to pathogenesis.

Diabetes induces dysfunction of the endoplasmic reticulum (ER), resulting in ER stress and retinal inflammation [4, 11, 13–15]. In response to ER stress, cells initiate an adaptive signalling pathway known as the unfolded protein response (UPR) to facilitate protein folding and restore ER homeostasis. In normal cells, activation of the UPR eliminates ER stress through upregulation of ER chaperones, promotion of ER-associated degradation (ERAD) and modulation of the protein translation rate. Failure of this process has detrimental effects on cell activities and triggers cell death [16, 17]. X-box binding protein 1 (XBP1) is a major transcription factor that regulates ER chaperones and ERAD. XBP1 overexpression suppresses NF-κB activation and reduces adhesion-molecule expression in endothelial cells [4]. Conversely, loss of XBP1 leads to heightened ER stress and increases inflammation related to diabetic retinopathy [4] and Alzheimer's disease [18]. Furthermore, inhibition of ER stress reduces production of inflammatory cytokines in various tissues and alleviates retinal vascular leakage in animal models of diabetes [3, 19], suggesting an important role of ER stress and XBP1 in the regulation of retinal inflammation and diabetic complications.

In mammalian retina, Müller glia (MG) constitute the major glial cell population and span the entire retinal thickness [20]. During infection and stress conditions such as diabetes, MG are activated and secrete a diverse array of cytokines, thereby regulating the inflammatory responses of retinal cells [21, 22]. In addition, MG have a crucial role in regulation of pro-angiogenic factors [23], among which MG-derived VEGF is a major contributor to retinal inflammation, vascular leakage and pathological angiogenesis in diabetic retinopathy [24]. Deletion of *Vegf* (also known as *Vegfa*) in MG in mice drastically reduces diabetes-induced upregulation of retinal ICAM-1, TNF-α and NF-κB [25]. Understanding the mechanisms of regulation of MG-derived proinflammatory and pro-angiogenic cytokines, in particular VEGF, is important for identification of new targets for the treatment of retinal inflammation and for development of new therapies for diabetic retinopathy. In the present study, we investigated the role of ER stress and XBP1 in the regulation of MG-derived production of inflammatory cytokines, with a special focus on VEGF, under normal and diabetic conditions in conditional-knockout mice in which the *Xbp1* gene was specifically deleted in MG.

Methods

Animals MG-specific *Xbp1*-knockout ($Xbp1^{\text{Müller}-/-}$) mice on a C57BL/6J background were generated by crossing 'floxed' *Xbp1* mice harbouring loxP sites around exon 2 of *Xbp1* [26] with MG-specific-Cre transgenic mice, which express Cre recombinase in Müller cells [25], at University of Oklahoma Health Sciences Centre (OUHSC, Oklahoma City, OK, USA). Cre-recombinase negative, *Xbp1* flox/flox ($Xbp1^{\text{Müller}+/+}$) littermates were used as wild-type controls in all experiments. The care, use and treatment of all animals in this study were in strict agreement with the Statement for the Use of Animals in Ophthalmic and Vision Research from the Association for Research in Vision and Ophthalmology, and with the guidelines set forth by the University at Buffalo and OUHSC. To induce diabetes, 8-week-old male mice were randomly assigned to receive five consecutive i. p. injections of streptozotocin (50 mg/kg body weight/day; Sigma-Aldrich, St Louis, MO, USA, dissolved in 0.01 mol/l citrate buffer, pH 4.5) or vehicle (0.01 mol/l citrate buffer, pH 4.5) as a control. Subsequently, 8 weeks after streptozotocin injection, the mice were humanely euthanised, and their eyes were harvested for analyses.

Primary Müller cell culture and characterisation Mice 7–10 days old were used for primary Müller cell cultures following a protocol described elsewhere [25]. Passage three cells were seeded onto glass coverslips pre-coated with poly-L-lysine and laminin (Sigma-Aldrich), and grown overnight. Cells were then fixed with cold acetone and immunostained by incubation with a mouse antibody to glutamine synthetase (1:100 dilution; Millipore, Billerica, MA, USA) overnight at 4°C, followed by a Cy3-conjugated secondary antibody. Fluorescence was visualised with an AX70 microscope (Olympus, Tokyo, Japan).

Mouse retina flat-mounting and blood vessel staining Mouse globes were enucleated and the retinas were carefully dissected out. The retinas were blocked with blocking buffer (1× PBS with 3% (vol./vol.) normal donkey serum and 0.3% (vol./vol.) Triton X-100) and then incubated with isolectin GS-IB4 (1:200; Invitrogen, Carlsbad, CA, USA) for 48 h at 4°C. Retinas were then washed with 1× PBS and flat-mounted onto microscope slides for confocal laser scanning microscopy.

Electroretinography Mouse retinal function was assessed by electroretinography (ERG) in wild-type mice with an Espion E2 System (Diagnosys, Lowell, MA, USA). Briefly, the mice were dark-adapted overnight, and their pupils were dilated with 1% atropine and 1% cyclopentolate prior to ERG. After anaesthesia was administered, two platinum recording electrodes were positioned on the cornea, with a reference electrode in the mouth and a ground electrode on the tail. Two flash intensities, 1000 and 2000 cd/m^2, were used. The amplitudes of the a-wave and b-wave of scotopic and photopic ERGs were recorded and averaged for comparison of retinal function [27].

Quantitative real-time RT-PCR Total RNA was extracted from mouse retinas using TRIzol (Invitrogen) and reverse-transcribed into cDNA using random hexamers and a SuperScript III First Strand Synthesis System (Invitrogen). Quantitative real-time RT-PCR was performed using SYBR Green PCR Master Mix (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's protocol, and primers as listed in ESM Table 1. For each gene examined, samples were run in triplicate, and the median C_t value was normalised against the C_t for 18S rRNA to generate a $\log_2$ transformed value for gene-expression levels.

Western blot analysis Retinas and cells were lysed in radioimmune-precipitation-assay buffer with a protease-inhibitor mixture, phenylmethylsulfonyl fluoride and sodium orthovanadate (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein mixtures (25 μg per well) were resolved by SDS-PAGE and transferred to membranes, which were probed with mouse anti-TNF-α, anti-VEGF, anti-phospho-JNK (phosphorylated c-Jun N-terminal kinase; p-JNK) and anti-HIF1-α (hypoxia-inducible transcription factor 1α) antibodies, rabbit anti-ATF4 (activating transcription factor 4; these antibodies were obtained from Santa Cruz Biotechnology and used at 1:200 dilution), rabbit anti-GRP78 (78 kDa glucose-regulated protein; Abcam, Cambridge, MA, USA; 1:2000), anti-phospho-eIF2α (phosphorylated eukaryotic translation initiation factor 2α; p-eIF2α; Cell Signaling, Technology, Danvers, MA, USA; 1:1000) and anti-ATF6 (Abcam; 1:1000). Each membrane was stripped and reblotted with a mouse anti-β-actin antibody (Abcam; 1:10,000), to demonstrate similar levels of the loading control β-actin in each lane. Antibodies were validated using positive and negative controls.

Retinal vascular permeability assay Retinal vascular permeability was quantified as previously described [11]. Mice were deeply anaesthetised and intraventricularly injected with FITC-conjugated dextran (4.4 kDa; Sigma) at 50 mg/kg body weight. After 15 min, the chest cavity was opened, a blood sample was collected from the ventricle, and the vessels were perfused with PBS (500 ml/kg body weight). The retinas were carefully dissected, weighed and homogenised, and FITC–dextran in the retinal homogenate was extracted by centrifugation. Fluorescence in the retina and plasma samples was measured with a spectrofluorometer with excitation at 485 nm and emission at 538 nm, and permeability was calculated according to the following equation:

$$\frac{\text{Retinal FITC–dextran (μg)/retinal weight (g)}}{\text{Plasma FITC–dextran concentration (μg/μl)} \times \text{circulation time (h)}}$$

Immunohistochemistry for cryosections and cultured primary Müller cells Mouse globes were fixed with 4% (wt/vol.) paraformaldehyde, and sections were obtained with a cryostat. Cultured primary Müller cells were fixed with cold acetone. Retinal sections were immunostained by incubation with rabbit anti-GRP78 (1:600), rabbit anti-p-eIF2α (1:100), mouse anti-VEGF (1:100), mouse anti-TNF-α (Santa Cruz Biotechnology; 1:100), rabbit anti-ATF6 (Abcam; 1:100) or mouse anti-glutamine synthetase (EMD Millipore, 1:100) antibodies overnight at 4°C, followed by incubation with Cy3-conjugated or biotinylated secondary antibodies at room temperature for 90 min. Antibodies were validated using positive and negative controls. Fluorescence was visualised with an Olympus AX70 microscope. Relative fluorescence intensity of VEGF or TNF-α in Müller cells was quantified, blind to treatment or genotype, in five random areas on each image using NIH ImageJ software and was normalised to the total cell number. Data from three images per group were used for statistical analysis.

Statistical analysis Results are expressed as the mean ± SD of at least three independent experiments. Statistical significance among three or more groups was determined by one-way ANOVA with Bonferroni's or Tukey's multiple-comparison test. Statistical significance between two groups was determined by unpaired Student's *t* test. Probability values of $p < 0.05$ were considered to indicate a significant difference.

Results

XBP1 deficiency in Müller cells does not influence retinal structure and function Multiple analyses revealed no significant differences in retinal structure and function between adult Müller-*Xbp1* knockout (*Xbp1*$^{\text{Müller}-/-}$) and age-matched wild-type (*Xbp1*$^{\text{Müller}+/+}$) mice. Histochemical staining identified no visible structural abnormalities (such as retinal folding, granulations or necrosis) in *Xbp1*$^{\text{Müller}-/-}$ mice, and no obvious differences in the thickness of the whole retina, outer

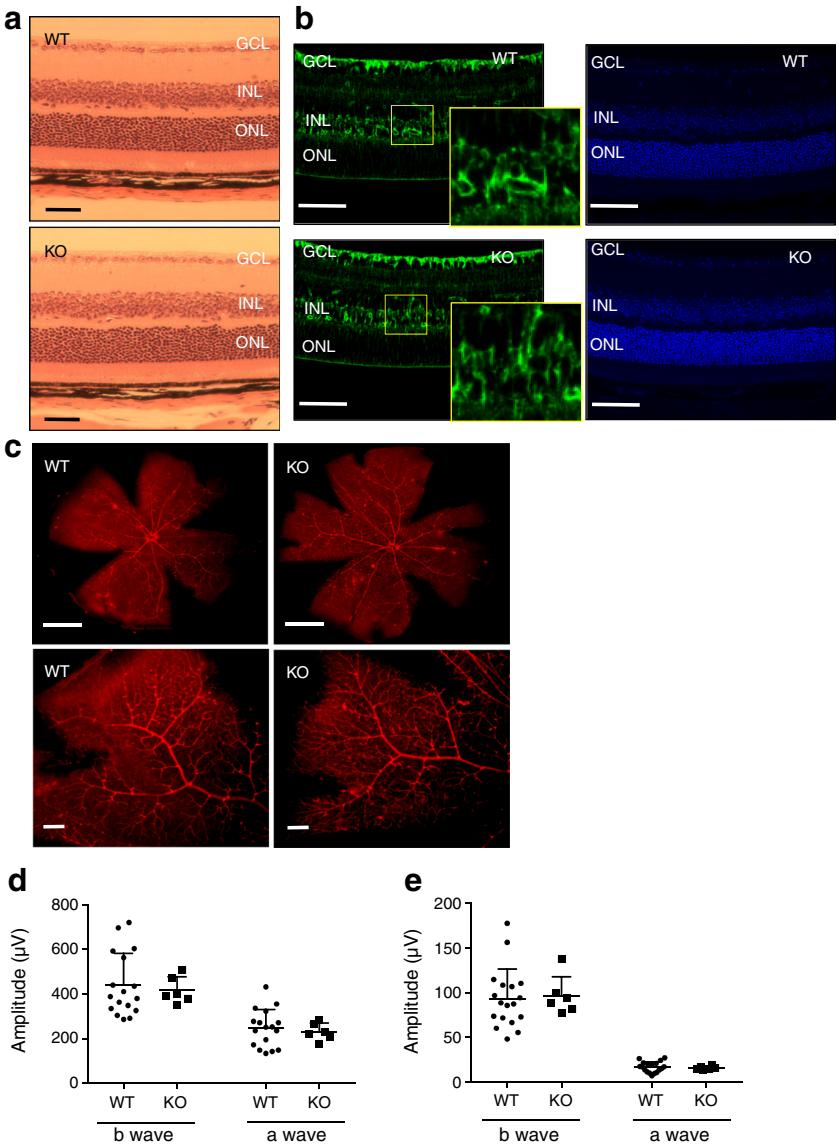


Fig. 1 XBP1 deficiency in Müller cells does not alter retinal neurovascular development and function. (**a**) Representative images of retinal sections with H&E staining from 1-month-old $Xbp1^{\text{Müller+/+}}$ (WT) or $Xbp1^{\text{Müller−/−}}$ (KO) mice. Scale bars, 50 μm. (**b**) Representative retinal sections with immunofluorescence staining for glutamine synthetase (green) and nuclear counterstaining with DAPI (blue), to identify retinal layers. Scale bars, 50 μm. (**c**) Retinal whole-mounts with isolectin B4 staining (red) showing no gross abnormality in the retinal vasculature in adult KO mice. Scale bars, 1000 μm for the upper panels and 200 μm for lower panels. (**d**) Scotopic ERG and (**e**) photopic ERG recordings showing no detectable differences in retinal function between KO and WT mice (analysed by Student's *t* test). Results are expressed as the mean ± SD, $n = 17$ eyes in WT mice and $n = 6$ eyes in KO mice. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer

nuclear layer or inner nuclear layer compared with wild-type mice (Fig. 1a and ESM Fig. 1a). Immunollabeling of the MG marker glutamine synthetase demonstrated no visible differences in MG morphology or numbers (Fig. 1b and ESM Fig. 1b). Additionally, isolectin staining of retinal flat-mounts revealed normal vascular development in $Xbp1^{\text{Müller−/−}}$ mice (Fig. 1c). Functionally, ERG recordings from wild-type and $Xbp1^{\text{Müller−/−}}$ retinas were indistinguishable (Fig. 1d, e and ESM Fig. 1c). From these analyses, we concluded that the $Xbp1^{\text{Müller−/−}}$ retina develops and functions similarly to the retina in wild-type mice.

Enhanced retinal inflammation, ER stress and vascular permeability in diabetic $Xbp1^{\text{Müller−/−}}$ mice Diabetes was modelled by i. p. injection of streptozotocin, and no significant differences were observed in blood glucose and body weight between diabetic $Xbp1^{\text{Müller−/−}}$ and diabetic $Xbp1^{\text{Müller+/+}}$ mice (Table 1). Compared with nondiabetic $Xbp1^{\text{Müller+/+}}$ mice, retinal vascular permeability was significantly (1.5-fold; $p < 0.01$) higher in $Xbp1^{\text{Müller+/+}}$ mice 8 weeks after diabetes onset (Fig. 2a).

Table 1 Characteristics of experimental animals

Group	*n*	Body weight (g)	Blood glucose (mmol/l)
WT NDM	7	28.7 ± 2.1	7.6 ± 1.7
KO NDM	7	30.9 ± 2.0	6.8 ± 0.9
WT DM	7	23.8 ± 2.3**	26.5 ± 1.3**
KO DM	7	23.5 ± 1.9††	27.2 ± 1.5††

Data are shown as mean ± SD

**$p < 0.01$ vs WT NDM, ††$p < 0.01$ vs KO NDM; one-way ANOVA with Bonferroni post hoc test

DM, diabetes induced; KO, $Xbp1^{\text{Müller−/−}}$; NDM, diabetes not induced; WT, $Xbp1^{\text{Müller+/+}}$

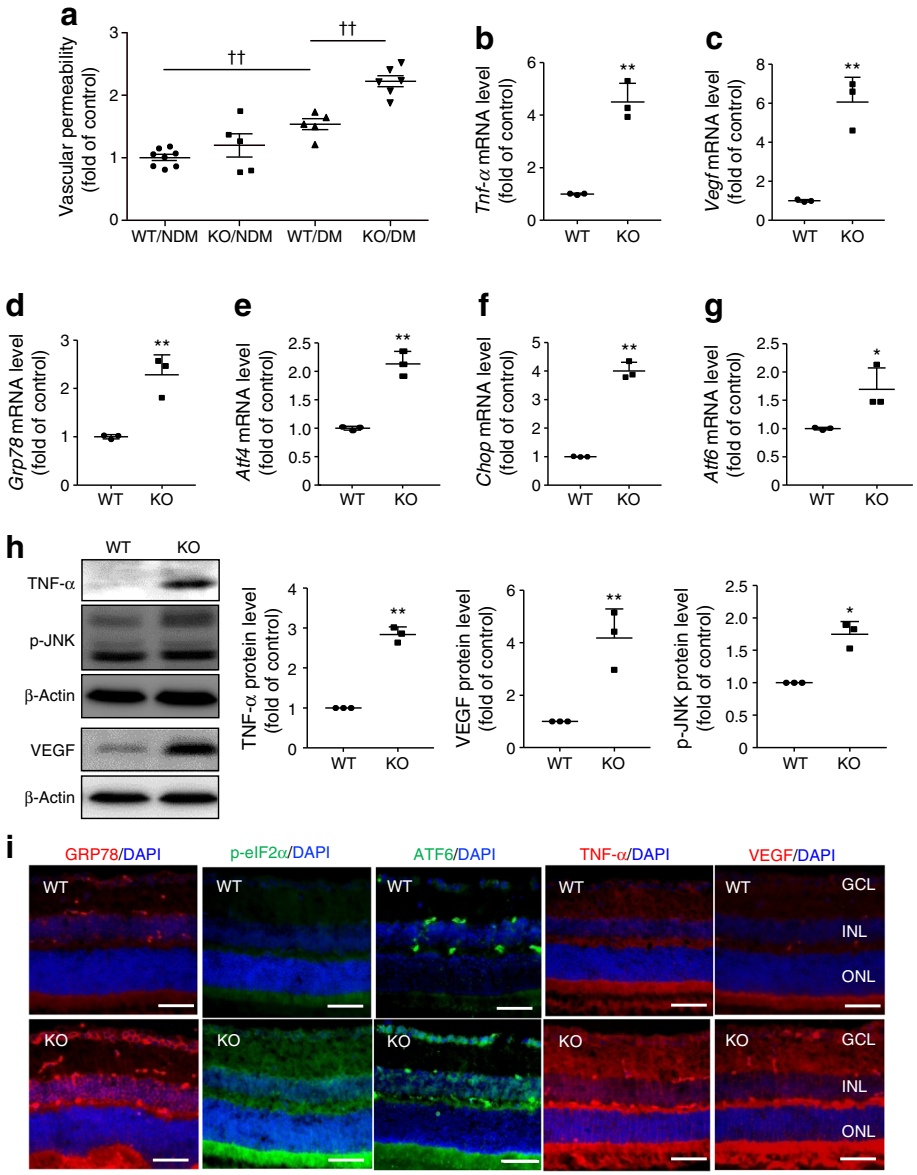


Fig. 2 Increased retinal vascular permeability, ER stress and inflammation in diabetic *Xbp1*$^{\text{Müller}-/-}$ mice. Adult *Xbp1*$^{\text{Müller}+/+}$ mice (WT) and *Xbp1*$^{\text{Müller}-/-}$ (KO) mice were examined 8 weeks after induction of diabetes with streptozotocin treatment (DM), or no induction (NDM). (**a**) Retinal vascular permeability was measured with FITC–dextran; $n = 8$ mice in WT/NDM group, $n = 5$ in KO/NDM group, $n = 5$ in WT/DM group and $n = 6$ in KO/DM group; $^{\dagger\dagger}p < 0.01$ by one-way ANOVA with Bonferroni comparison test. (**b–i**) Diabetes was induced in all mice. (**b–g**) mRNA levels of genes encoding retinal ER-stress markers and inflammatory cytokines were measured by real-time RT-PCR; $n = 3$ mice per group. (**h**) Protein levels of TNF-α, VEGF and p-JNK were examined by western blot analysis and quantified by densitometry, with β-actin as the loading control; $n = 3$ mice per group. (**i**) Protein-expression levels of ER-stress markers, TNF-α and VEGF were examined by immunofluorescence staining in retinal sections. Scale bars, 50 μm. All results are shown as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$ vs WT by Student's *t* test. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer

Vascular permeability was significantly (1.4-fold; $p < 0.01$) higher in diabetic *Xbp1*$^{\text{Müller}-/-}$ mice than in diabetic *Xbp1*$^{\text{Müller}+/+}$ mice at 8 weeks, and more than twofold higher than in nondiabetic *Xbp1*$^{\text{Müller}+/+}$ mice (Fig. 2a). Vascular permeability was not significantly different between nondiabetic *Xbp1*$^{\text{Müller}-/-}$ and nondiabetic *Xbp1*$^{\text{Müller}+/+}$ mice (Fig. 2a). These results suggest that XBP1 deficiency in MG exacerbates diabetes-induced retinal vascular hyperpermeability.

To determine whether the observed change in vascular permeability was associated with inflammation and ER stress, we measured gene-expression and protein levels of major inflammatory factors and ER-stress markers in retinas from diabetic *Xbp1*$^{\text{Müller}-/-}$ and *Xbp1*$^{\text{Müller}+/+}$ mice. Expression of *Vegf* in diabetic *Xbp1*$^{\text{Müller}-/-}$ retina was approximately sixfold higher than that in diabetic *Xbp1*$^{\text{Müller}+/+}$ retina, and expression of *Tnf-α* in diabetic *Xbp1*$^{\text{Müller}-/-}$ retina was ~4.5-fold higher than in diabetic *Xbp1*$^{\text{Müller}+/+}$ retina (Fig. 2b–g). Expression levels of ER-stress-associated genes (*Grp78*, *Atf4*, *Chop* and *Atf6*) were also significantly higher in diabetic *Xbp1*$^{\text{Müller}-/-}$ retina than in diabetic *Xbp1*$^{\text{Müller}+/+}$ retina. Notably, there were no significant differences in expression levels of ER-stress and inflammatory genes between nondiabetic *Xbp1*$^{\text{Müller}+/+}$ and nondiabetic *Xbp1*$^{\text{Müller}-/-}$ retinas (ESM Fig. 2). Western blot analysis confirmed that there were significantly higher levels of VEGF, TNF-α and p-JNK in diabetic *Xbp1*$^{\text{Müller}-/-}$ retina than in diabetic *Xbp1*$^{\text{Müller}+/+}$ retina (Fig. 2h). The higher levels of ER stress and inflammation in the diabetic *Xbp1*$^{\text{Müller}-/-}$ retina were further validated by immunohistochemical labelling of GRP78, p-eIF2α, ATF6, TNF-α and VEGF on retinal cryosections (Fig. 2i). High levels of

GRP78, ATF6 and TNF-α were observed co-localised with MG of diabetic *Xbp1*$^{\text{Müller}-/-}$ and *Xbp1*$^{\text{Müller}+/+}$ retinas (ESM Fig. 3). Notably, higher levels of ATF6 localisation to the nuclei of Müller cells was observed in diabetic *Xbp1*$^{\text{Müller}-/-}$ retinas than in *Xbp1*$^{\text{Müller}+/+}$ retinas (ESM Fig. 3b). Altogether, these results indicate that, with diabetic conditions, XBP1 deficiency in MG results in increased retinal ER stress and inflammation, leading to exacerbated vascular leakage.

Enhanced inflammatory and UPR-related gene-expression and protein levels in XBP1-deficient Müller cells To determine if upregulation of ER stress and inflammation in X*bp1*$^{\text{Müller}-/-}$ retinas is derived from MG, we examined inflammation and UPR activation in primary MG cultures isolated from *Xbp1*$^{\text{Müller}-/-}$ and *Xbp1*$^{\text{Müller}+/+}$ mice. We confirmed the purity of the cell cultures by immunostaining for the MG-specific marker glutamine synthetase (Fig. 3a). Virtually every cell

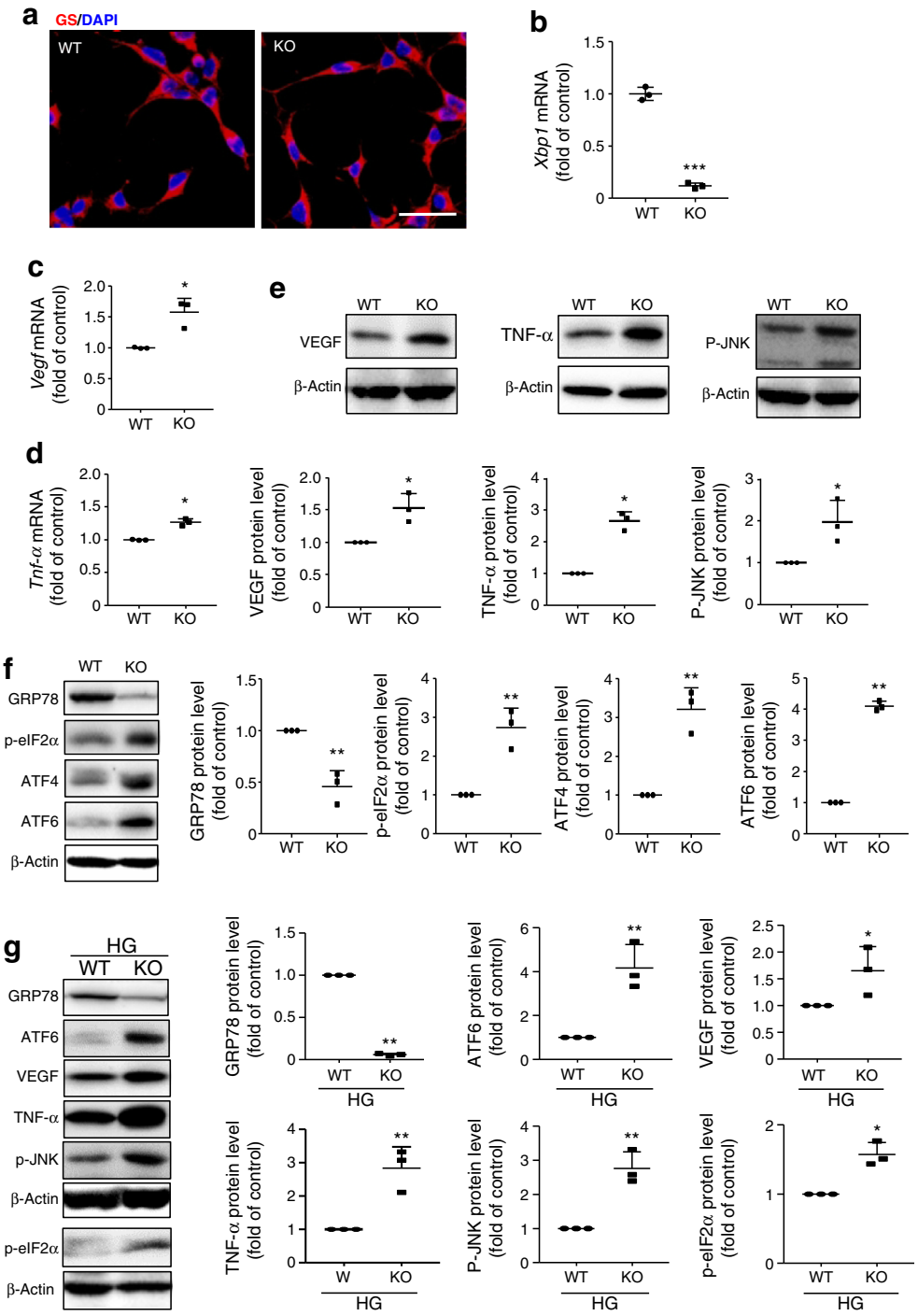


Fig. 3 Loss of XBP1 enhances expression of inflammatory factors and ER stress in Müller cells. (**a**) Primary retinal Müller cells (P1) isolated from *Xbp1*$^{\text{Müller}+/+}$ (WT) and *Xbp1*$^{\text{Müller}-/-}$ (KO) mice were stained for the Müller-cell marker glutamine synthetase (GS) (red), and nuclei were stained with DAPI (blue). (**b**) Knockout efficiency was evaluated by measuring expression of *Xbp1* mRNA by real-time RT-PCR ($n = 3$). (**c**, **d**) *Vegf* and *Tnf-α* mRNA levels were examined by real-time RT-PCR ($n = 3$). (**e**) Protein levels of VEGF, TNF-α and p-JNK were determined by western blot analysis and quantified by densitometry, with β-actin as the loading control ($n = 3$). (**f**) Levels of ER-stress-related proteins GRP78, p-eIF2α, ATF4 and ATF6 were determined by western blot analysis ($n = 3$). (**g**) Primary Müller cells were treated with high glucose (HG; 25 mmol/l) for up to 72 h. Expression levels of GRP78 and p-eIF2α were examined after 24 h of HG treatment, and levels of ATF6, VEGF, TNF-α and p-JNK were determined after 72 h of HG treatment, by western blot analysis ($n = 3$). All results are shown as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs WT, by Student's *t* test

was robustly positive for glutamine synthetase at this stage in the culture. Quantitative RT-PCR using primers designed specifically to the floxed exon (exon 2, ESM Table 1) confirmed that expression of *Xbp1* was very low in $Xbp1^{Müller-/-}$ MG (Fig. 3b). Compared with $Xbp1^{Müller+/+}$ MG, *Vegf* expression was 1.6-fold higher in $Xbp1^{Müller-/-}$ MG (Fig. 3c), and *Tnf-α* expression was 1.27-fold higher (Fig. 3d). Similarly, VEGF, TNF-α and p-JNK protein levels were all significantly higher in $Xbp1^{Müller-/-}$ MG than in $Xbp1^{Müller+/+}$ MG (Fig. 3e). Under the same culture conditions, the UPR proteins ATF6, ATF4 and p-eIF2α were upregulated in $Xbp1^{Müller-/-}$ MG, whereas the ER chaperone GRP78 was downregulated (Fig. 3f). These results indicate that, compared with wild-type cells, inflammation was upregulated and the UPR was activated in $Xbp1^{Müller-/-}$ MG.

XBP1 deficiency intensifies ER stress and inflammation in MG under high-glucose conditions To determine whether XBP1 deficiency exacerbates inflammation and the UPR in MG under diabetic conditions, we examined relevant markers after isolated MG were treated with high-glucose medium (containing 25 mmol/l glucose) for 72 h. ER-stress markers, including GRP78, ATF6 and p-eIF2α, as well as inflammatory proteins p-JNK, VEGF and TNF-α, were measured by western blot analysis. Compared with wild-type MG, XBP1-deficient MG exhibited significantly higher ATF6, p-eIF2α, p-JNK, VEGF and TNF-α protein levels with high-glucose treatment (Fig. 3g). By contrast, levels of the ER chaperone GRP78 were lower in $Xbp1^{Müller-/-}$ MG than in $Xbp1^{Müller+/+}$ MG (Fig. 3g).

Hypoxia induces ER stress and inflammation in MG Hypoxia can be induced by diabetes in retinal tissues, as a result of the extensive loss of capillaries, and is an important etiological factor for aberrant neovascularisation in advanced diabetic retinopathy, through induction of VEGF [28]. With exposure of cultured wild-type MG to hypoxia, p-eIF2α levels increased within 1 h and continued to increase for at least 4 h, to a level nearly 12 times that in nonhypoxic conditions (Fig. 4a). In parallel, spliced *Xbp1* (*Xbp1s*) and *Chop* mRNA levels increased significantly after 4 h of hypoxia (Fig. 4b, c). These results indicated that the UPR was evoked by hypoxia in wild-type MG. Notably, *Vegf* expression was also increased by hypoxia (Fig. 4d). To examine the role of XBP1 in hypoxia-induced ER stress and inflammation in MG, we exposed primary MG from $Xbp1^{Müller-/-}$ and $Xbp1^{Müller+/+}$ mice to hypoxia for 4 h and measured levels of ER-stress markers and inflammatory proteins by western blot analysis (Fig. 4e). With the exception of GRP78, each protein examined under normal (nonhypoxic) conditions was present at a higher level in $Xbp1^{Müller-/-}$ MG than in $Xbp1^{Müller+/+}$ MG. In both $Xbp1^{Müller+/+}$ and $Xbp1^{Müller-/-}$ MG, levels of all markers were higher in hypoxic conditions than in nonhypoxic conditions (Fig. 4e). With hypoxia, levels of all markers were higher in $Xbp1^{Müller-/-}$ MG than in $Xbp1^{Müller+/+}$ MG. Thus, loss of XBP1 may sensitise MG cells to hypoxia-induced ER stress and inflammation.

XBP1 links ER stress to hypoxia-induced inflammation in primary MG To further investigate whether ER stress has a role in MG inflammation, we pre-treated MG cells with the chemical chaperones 4-phenyl butyric acid (PBA; 0.5 and 1 mmol/l) or trimethylamine N-oxide (TMAO; 5 and 10 mmol/l) for 1 h prior to a 4 h exposure to hypoxia. Hypoxia alone resulted in upregulation of levels of ATF4, ATF6, p-eIF2α, VEGF and TNF-α (Fig. 5a and b). Hypoxic changes were largely abrogated by treatment with TMAO or PBA in both $Xbp1^{Müller+/+}$ and $Xbp1^{Müller-/-}$ MG (Fig. 5a, b). These results provided further evidence that XBP1 is critical to the inflammatory response in MG via UPR activation.

The suppression of hypoxia-induced upregulation of TNF-α and VEGF by chemical chaperones that we observed by western blotting was confirmed by immunohistochemical analysis. A 4 h hypoxia challenge resulted in dramatic increases in TNF-α and VEGF levels, especially in $Xbp1^{Müller-/-}$ MG (Fig. 6). No significant increase in fluorescence intensity in response to hypoxia was observed in MG pre-treated with either PBA or TMAO. These results indicated that hypoxia-induced inflammation is exacerbated by XBP1 deficiency and inhibited by UPR suppression.

Discussion

Here, we have presented the first evidence that retinal inflammation in diabetic retinopathy is directly linked to ER stress in MG. Upregulation of ER stress has previously been observed in the retina in animal models of both type 1 and type 2 diabetes, and in post-mortem retinal tissue of individuals with diabetic retinopathy [3, 13, 29, 30]. In immunofluorescence analysis, ER-stress markers are significantly upregulated in the inner retina and partially co-localised with MG [3, 30]. In vitro, ER stress can be induced in several types of retinal cells, including MG, by multiple etiological factors related to diabetic injury of retinal neurons and blood vessels, including high glucose, hypoxia, inflammatory cytokines and oxidative stress [3, 4, 13, 15, 29–31]. Among retinal cells, MG are the major type of glial cells that expand radially across almost the whole of the retina and surround its blood vessels. These cells also express and secrete a number of growth factors and cytokines, and have important roles in the regulation of retinal function and pathophysiology. At an early stage of diabetic retinopathy, MG are activated to increase production of pro-inflammatory cytokines, which probably contribute to the early changes in retinal blood flow, blood–retina-barrier integrity and vascular leakage [3, 25, 32]. Depletion of MG-derived VEGF production suppresses vascular leakage and retinal

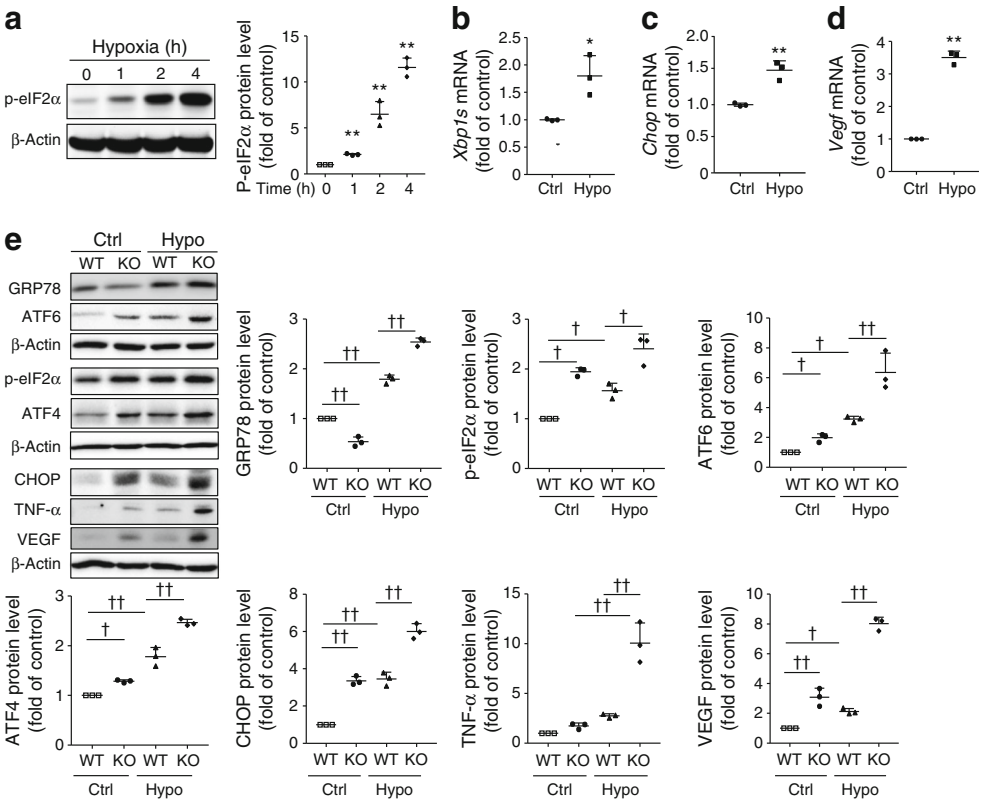
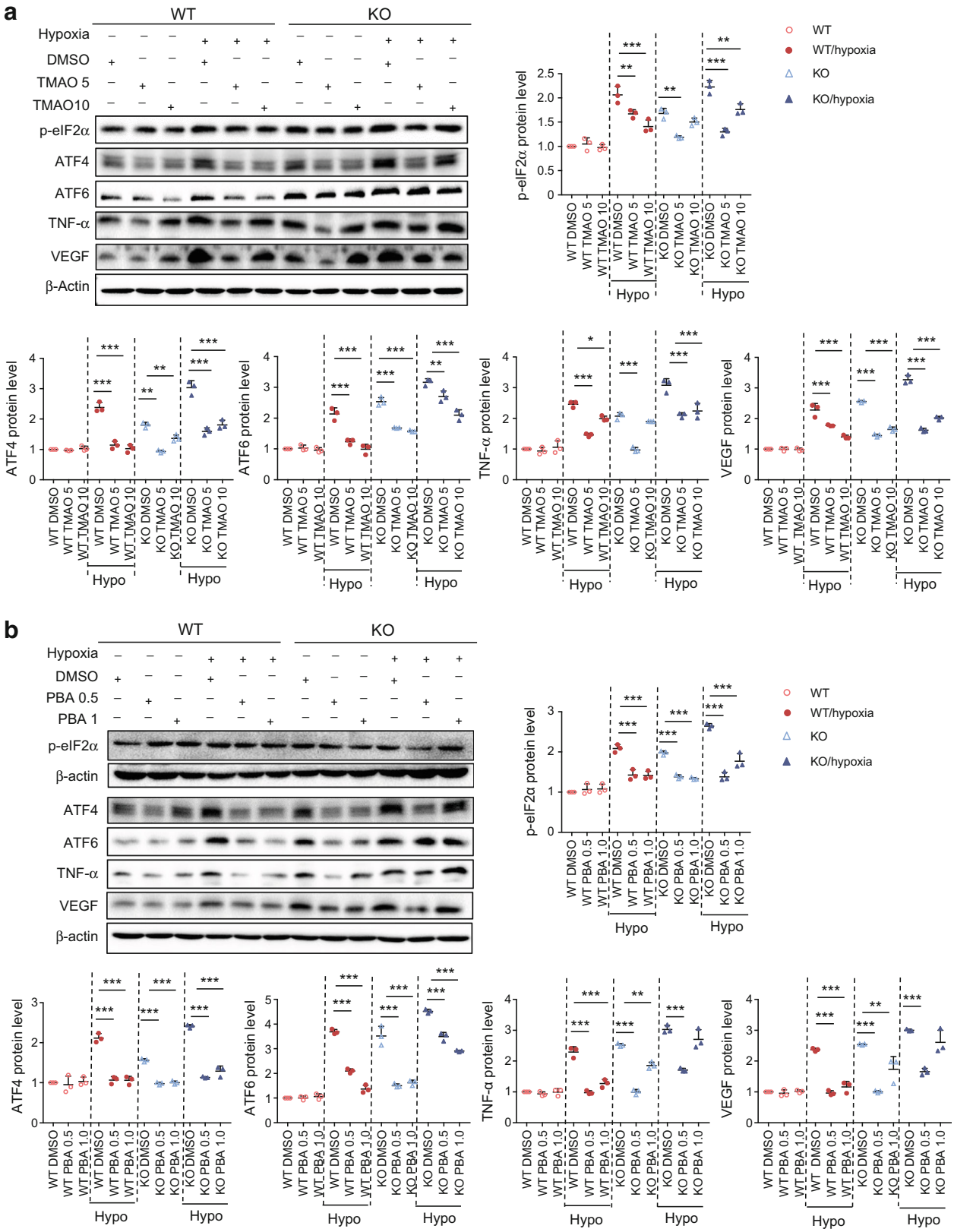


Fig. 4 XBP1 deficiency exacerbates hypoxia-induced ER stress and inflammation in Müller cells. Primary mouse Müller cells were exposed to hypoxia for up to 4 h. (**a**) Protein levels of p-eIF2α were determined by western blot analysis and semi-quantified by densitometry ($n = 3$). (**b**–**d**) mRNA levels of spliced *Xbp1*, *Chop* and *Vegf* were examined by real-time RT-PCR after 4 h of hypoxia (Hypo) or normoxia (Ctrl) treatment ($n = 3$). (**e**) Müller cells from $Xbp1^{Müller+/+}$ (WT) and $Xbp1^{Müller-/-}$ (KO) mice were treated with hypoxia for 4 h. Protein levels of ER-stress markers, TNF-α and VEGF were determined by western blot analysis and quantified by densitometry, with β-actin as the loading control ($n = 3$). All results are shown as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$ vs baseline in (**a**), or vs Ctrl in (**b**–**d**), by Student's *t* test; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ for comparisons, by one-way ANOVA with Tukey's multiple comparisons test shown in (**e**)

inflammation in diabetic mice [25], implying a pivotal role of MG in the development of diabetic retinopathy. To determine whether ER stress in MG is implicated in retinal inflammation and diabetic retinopathy, we generated a conditional-knockout mouse line with MG-specific deletion of *Xbp1*, which encodes a conserved UPR factor that suppresses ER stress in eukaryotic cells. The mice were then used for an animal model of diabetes. Compared with their wild-type littermates, loss of *Xbp1* in these mice resulted in increased ER stress and enhanced VEGF production from MG, which in turn led to vascular hyperpermeability in the retinas of diabetic mice. Furthermore, the level of ER stress in vivo and in isolated MG in vitro determined the extent of inflammatory VEGF and TNF-α gene-expression levels, suggesting that induced ER stress leads to a heightened proinflammatory response of MG to diabetes, and that XBP1 has a critical role in inhibition of this response in diabetic retinopathy.

The transcription factor XBP1 was originally identified as a binding protein for X-box motifs in human MHC class II genes, and was later characterised as an effector of the UPR activated by inositol-requiring enzyme 1α (IRE1α). XBP1 has been extensively studied in the regulation of ER biogenesis and protein homeostasis [33]. XBP1 is indispensable for ER expansion and secretory cell development [34]. Global knockout of XBP1 leads to embryonic lethality because of severe liver hypoplasia and fetal anaemia [35]. Heterozygous *Xbp1* knockout mice exhibit higher levels of ER stress and insulin resistance than wild-type mice when exposed to diabetes-provoking conditions [36, 37]. Reduced expression or insufficient activation of the XBP1 pathway leads to enhanced ER stress and increased susceptibility to inflammation in the intestine, pancreas, liver and adipose tissue [26, 37, 38]. Conditional knockout of *Xbp1* in intestinal epithelial cells leads to spontaneous small-intestine inflammation and increases the susceptibility of mice to ER stress and lesions induced by inflammatory stimuli, recapitulating human inflammatory bowel disease [26]. Knockdown of XBP1 in retinal endothelial cells increases expression levels of cell adhesion molecules ICAM-1 and vascular cell adhesion molecule 1 (VCAM-1), whereas overexpression of spliced XBP1 or activation of XBP1 by preconditioning with ER stress attenuates ICAM-1 and VCAM-1 expression and leucocyte–endothelial-cell adhesion [4]. Furthermore, ER-stress preconditioning significantly reduces TNF-α-induced retinal leucostasis and vascular leakage in mouse retinas. Mechanistically, XBP1 suppresses ER-stress-related IRE1α activation and the downstream pathways mediated by JNK and NF-κB [4, 26]. These findings collectively suggest an important role for XBP1 in maintenance of ER homeostasis and suppression of the inflammatory response in cells and tissues including the retina. Our results have now confirmed and extended these ideas, demonstrating that XBP1



deficiency in MG resulted in elevation of ER stress and upregulation of expression of inflammatory VEGF and TNF-α. Blockade of ER stress by chemical chaperones TMAO and PBA successfully attenuated VEGF and TNF-α expression in *Xbp1*$^{\text{Müller}-/-}$ MG and in hypoxia-treated MG, suggesting that ER stress results in elevation

◀ **Fig. 5** Inhibition of ER stress by chemical chaperones reduces hypoxia-induced TNF-α and VEGF production in Müller cells. Müller cells from $Xbp1^{Müller+/+}$ (WT) and $Xbp1^{Müller-/-}$ (KO) mice were pre-treated with DMSO vehicle only, 5 mmol/l TMAO (TMAO 5) or 10 mmol/l TMAO (TMAO 10) (**a**) or with 0.5 mmol/l PBA (PBA 0.5) or 1.0 mmol/l PBA (PBA 1) (**b**) for 1 h and then exposed to hypoxia for an additional 4 h. Levels of ER-stress markers, TNF-α and VEGF were determined with western blot analysis and quantified by densitometry, with β-actin as the loading control ($n = 3$). All protein levels are expressed as fold of control and shown as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, by one-way ANOVA with Tukey's multiple comparisons test for comparisons shown

of inflammatory gene expression in conditions of XBP1-deficiency and hypoxia in MG.

The protein kinase R-like endoplasmic reticulum kinase (PERK)–ATF4 pathway has a crucial role in the upregulation of VEGF expression in retinal endothelial cells and MG in vitro and in diabetic retinopathy in vivo [3, 11, 15]. As a transcription factor, ATF4 interacts with and enhances the expression/activation of multiple signalling molecules, including HIF1-α, signal transducer and activator of transcription 3, JNK, p38 and NF-κB, and is implicated in the regulation of VEGF and other inflammatory factors such as TNF-α and monocyte chemoattractant protein 1 [3, 11, 15]. In addition, ATF6, an ER-stress-inducible transcription factor, regulates VEGF expression in endothelial cells [39]. In the current study, compared with diabetic wild-type control mice, diabetic *Xbp1*-knockout mice exhibited significantly higher *Atf4* and *Atf6* mRNA (and ATF4 and ATF6 protein) expression levels in the retina and in isolated MG cells. Inhibition of ER stress by chemical chaperones significantly reduced ATF4 and ATF6 expression levels,

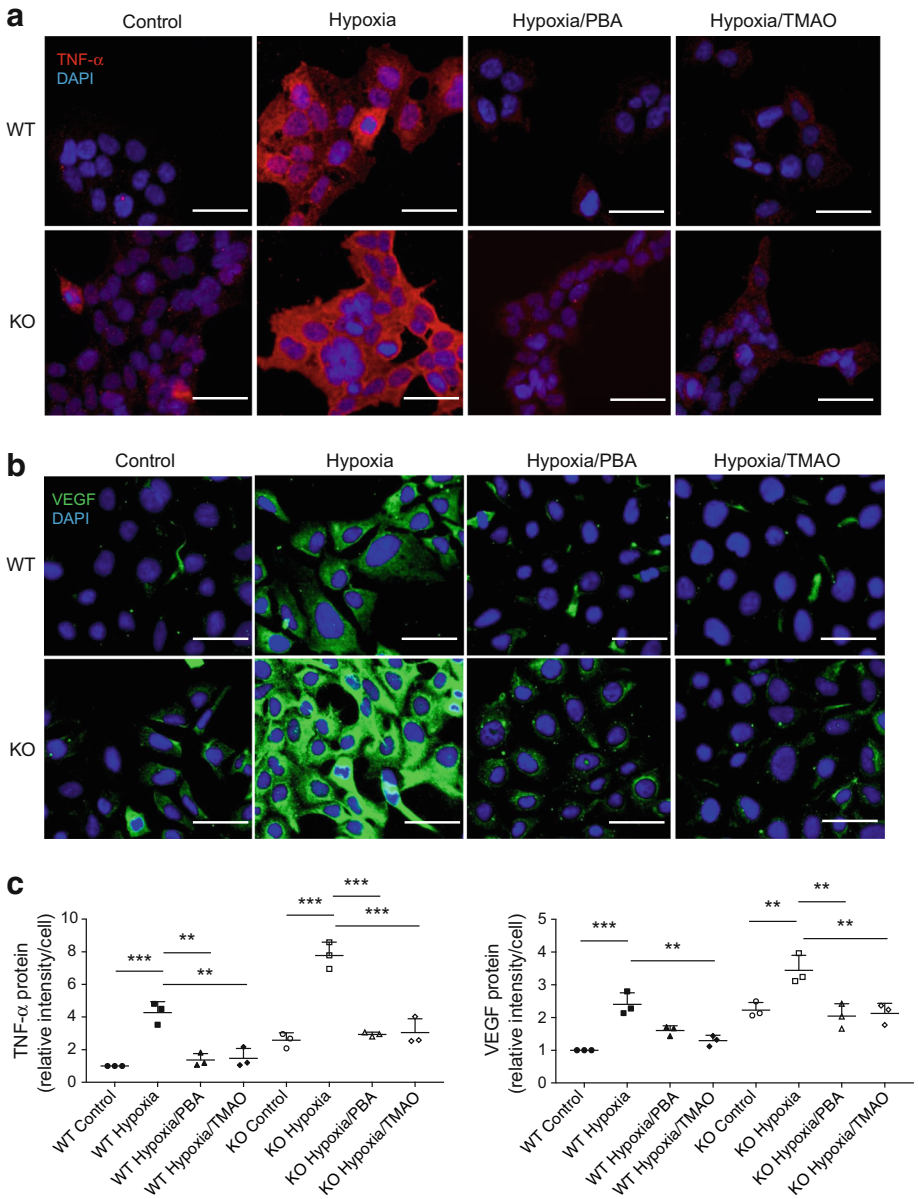


Fig. 6 Immunofluorescence study of TNF-α and VEGF levels in Müller cells. Müller cells from $Xbp1^{Müller+/+}$ (WT) and $Xbp1^{Müller-/-}$ (KO) mice were treated with 5 mmol/l TMAO or 0.5 mmol/l PBA for 1 h prior to exposure to hypoxia for 4 h. Protein levels of TNF-α and VEGF were examined by immunocytochemistry. Representative images from two independent experiments show increased TNF-α (**a**) and VEGF (**b**) levels in Müller cells after hypoxia, which were reduced by TMAO or PBA pre-treatment. The relative fluorescence intensity of VEGF or TNF-α was quantified by NIH ImageJ software and normalised to total cell numbers (**c**). Results are shown as mean ± SD; $n = 3$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, by one-way ANOVA with Tukey's multiple comparisons test for comparisons shown

suggesting that upregulation of these genes in XBP1-deficient MG may be attributed to ER stress. In contrast to the increases in most ER-stress markers, the levels of GRP78 (a major ER chaperone protein that facilitates protein folding and regulates the activation of the UPR) were reduced in XBP1-deficient MG under normal and high-glucose conditions. This result was probably because of the lack of XBP1, which functions as a major transcription factor for regulation of ER chaperones, including GRP78, $p58^{IPK}$, endoplasmic reticulum DNA J domain-containing protein 4 (ERDJ4), protein disulfide isomerase P5 (PDI-P5) and HEDJ, and proteins involved in ERAD, such as ERAD-associated E3 ubiquitin-protein ligase (HRD1), ER degradation-enhancing α-mannosidase-like protein 1 (EDEM1), Derlin-2 and Derlin-3 [40–42]. Notably, in contrast to these changes, hypoxia induced an increase in GRP78 in wild-type MG, and a greater increase in XBP1-deficient cells. Hypoxia also resulted in a significant increase in cleaved (active) ATF6 production in XBP1-deficient MG, and as ATF6 also binds to the ER-stress response element and transcriptionally regulates ER chaperone genes, including *Grp78* and genes encoding ERAD proteins [43], the upregulation of GRP78 is probably the result of the activation of ATF6 under hypoxic conditions.

In summary, our findings suggest that ER stress in MG is causatively linked to retinal inflammation and vascular leakage in diabetic retinopathy, and that XBP1 is important for maintenance of ER homeostasis and inflammatory factor production in MG. Although depletion of XBP1 does not result in gross abnormalities in neural retinal structure, retinal vasculature and retinal function evaluated by ERG, it remains unclear whether a lack of XBP1 would alter the MG secretome (which contains important neurotrophic factors, such as glial cell-line-derived neurotrophic factor and brain-derived neurotrophic factor) [44], leading to potentially harmful effects on retinal neuronal survival and function, particularly under disease and stress conditions. In addition, given the emerging role of MG as progenitor cells in the adult retina for neuronal differentiation and regeneration, it should be worth investigating whether a lack of XBP1 and altered ER homeostasis would influence the proliferation and differentiation abilities of MG.

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Data availability All data generated or analysed during this study are included in this published article. Some of the data were presented as an abstract at the 5th International Conference on Clinical & Experimental Ophthalmology in 2015.

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

Contribution statement JY, CC, TM and YW designed and performed the experiments, analysed the data and wrote the manuscript. YZL participated in the experiments, helped with data analysis and interpretation, and revised the manuscript. JJW and SXZ conceived and designed the study, analysed the data, and wrote and revised the manuscript. All authors reviewed and approved the final version of the manuscript. SXZ is the guarantor of this work.

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