



# Salutary effect of fenofibrate on type 1 diabetic retinopathy via inhibiting oxidative stress–mediated Wnt/ $\beta$ -catenin pathway activation

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## Abstract

Fenofibrate has been shown to have therapeutic effects on diabetic retinopathy (DR). Our previous studies demonstrated that the oxidative stress–activated Wnt/ $\beta$ -catenin pathway plays a pathogenic role in diabetic complications. In the present study, we evaluate the effect and mechanism of fenofibrate on regulating the oxidative stress–activated Wnt/ $\beta$ -catenin pathway by using the genetic type 1 diabetes model of *C57BL/6J-Ins2<sup>Akita</sup>* mice and high glucose (HG)–treated ARPE-19. Our results demonstrated that retinal phosphorylation of LRP6 and nuclear  $\beta$ -catenin were increased in *C57BL/6J-Ins2<sup>Akita</sup>* mice suggesting activation of Wnt/ $\beta$ -catenin signaling. Meanwhile, *C57BL/6J-Ins2<sup>Akita</sup>* showed upregulation of oxidant enzyme Nox4 and Nox2 and downregulation of antioxidant enzyme SOD1 and SOD2. All these alterations were reversed in *C57BL/6J-Ins2<sup>Akita</sup>* mice with fenofibrate treatment. Moreover, fenofibrate significantly ameliorated diabetes-induced retinal vascular leakage in *C57BL/6J-Ins2<sup>Akita</sup>* mice. In cultured ARPE-19, fenofibrate decreased HG-induced Nox2 and Nox4 upregulation, attenuated SOD1 and SOD2 downregulation and inhibited LRP6 phosphorylation. Moreover, activation of Wnt/ $\beta$ -catenin by Wnt3a conditional medium (WCM) reduced SOD1 and SOD2 and did not affect Nox2 and Nox4. Fenofibrate suppressed WCM-induced LRP6 phosphorylation and reversed SOD downregulation. Importantly, Nox4 overexpression directly phosphorylated LRP6 in ARPE19; conversely, Nox4 knockdown suppressed HG-induced LRP6 phosphorylation. Taken together, Nox-mediated oxidative stress contributes to Wnt/ $\beta$ -catenin activation in DR. Fenofibrate ameliorated DR through coordinate attenuation of oxidative stress and blockade of Wnt/ $\beta$ -catenin signaling.

**Keywords** Diabetic retinopathy · NADPH oxidase · Superoxide dismutase · Wnt · Oxidative stress

## Introduction

Diabetic retinopathy (DR) is a common cause of visual impairment and one of the major neurovascular complications of both type 1 and type 2 diabetes mellitus. The overall preva-

lence of DR has been estimated at approximately 30% of diabetic patients, with the risk factors including the duration of diabetes, increased HbA1c, high blood pressure and hyperlipidemia. Clinical trials have demonstrated that intensive control of hyperglycemia and hypertension reduced the incidence and progression of DR (1998; Diabetes et al. 1993). A commonly used clinical treatment of DR is retinal laser coagulation and vitrectomy. Recently, intravitreal injection of anti-VEGF agent has proven to reduce retinal neovascularization and attenuate diabetic macular edema. However, there are also drawbacks of retinal laser coagulation with the loss of peripheral visual fields and blurred vision at night. Anti-VEGF agent requires multiple injections, which brings a heavy economic burden, especially in developing countries. In addition, repetitive intraocular injections will increase the risk of infection. A systemic drug treatment for DR is desired.

Fenofibrate is a PPAR- $\alpha$  agonist and originally developed to treat dyslipidemia. It is known to effectively reduce both low-density lipoprotein (LDL) and very low-density

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lipoprotein (VLDL), while elevate high-density lipoprotein (HDL) levels. Intriguingly, FIELD (Fenofibrate Intervention and Event Lowering in Diabetes) study and ACCORD (Action to Control Cardiovascular Risk in Diabetes)-Eye Study demonstrated that fenofibrate significantly slowed the progression of DR (Chew et al. 2014; Keech et al. 2007). However, the protecting mechanisms of fenofibrate on DR remain elusive.

The Wnt signaling pathway is involved in regulating retinal vascular development (Drenser 2016). Mutations of Wnt cascade components are associated with inherited retinal vascular dysplasia such as familial exudative vitreoretinopathy and Norrie's disease (Warden et al. 2007). The Wnt signaling mainly branches into two distinct pathways: canonical and non-canonical. The canonical Wnt pathway or Wnt/ $\beta$ -catenin pathway is initiated when Wnt ligands bind with the seven-transmembrane domain frizzled (Fz) receptor and form a complex with a single-transmembrane domain LDL receptor-related protein 5/6 (LRP5/6). The extracellular signals were transduced into the cytoplasm by translocation of Dishevelled (Dvl) to frizzled co-receptors and subsequent recruitment and degradation of Axin by LRP5/6. The "destruction complex" composed by Axin, adenomatous polyposis coli (APC) and glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) were disassembled. The phosphorylation and degradation of  $\beta$ -catenin are attenuated. Stabilized  $\beta$ -catenin accumulates in the cytoplasm, translocates into the nucleus and associates with transcription factor T cell factor (TCF)/lymphoid enhancer factor (LEF) to activate target gene expression. Emerging evidence indicates that Wnt signaling activity is regulated by multiple post-transcriptional modifications such as phosphorylation, ubiquitination and glycosylation (Gao et al. 2014). Phosphorylation is one of the most efficient and well-characterized modifications in regulating cellular signal transduction. Many components of the Wnt/ $\beta$ -catenin pathway are regulated by phosphorylation including Fz, LRP6, Dvl and "destruction complex" members. Phosphorylation of LRP6 serves as a critical step for initiating Wnt/ $\beta$ -catenin cascade. Our group first reported that activation of Wnt/ $\beta$ -catenin signaling was implicated in and contributed to the development of DR (Chen et al. 2009).

Hyperglycemia-induced oxidative stress contributes to Wnt/ $\beta$ -catenin pathway activation and renal dysfunction in diabetic kidney (Zhou et al. 2012). Oxidative stress is characterized by the excessive generation of reactive oxygen species (ROS). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase or Nox, the major source of ROS production in the retinal microvasculature, neuroglial cells and circulating leukocytes, is closely associated with retinal vascular leakage, neuroinflammation and neovascularization in DR or ischemic retinopathy (Deliyanti and Wilkinson-Berka 2015; Li et al. 2015; Saito et al. 2007; Wilkinson-Berka et al. 2014). The Nox family consists of seven members (Nox1–Nox5,

Duox1 and Duox2) that transfer electrons across the biological membrane to generate superoxide. Among multiple isoforms, Nox2 and Nox4 have been shown to play a central role in retinal oxidative damage during the development of DR (Li et al. 2010; Rojas et al. 2013). Superoxide dismutase (SOD) is one of the major antioxidants against ROS. Three distinct isoforms of SOD have been identified (SOD1–SOD3). Loss of SOD activity increased ROS production and exaggerated retinal oxidative damage (Hashizume et al. 2008; Justilien et al. 2007).

Our previous study demonstrated that fenofibrate attenuated retinal inflammation and microvascular dysfunction in genetic diabetes model of *C57BL/6J-Ins2<sup>Akita</sup>* mice and streptozotocin-induced diabetic mice (Chen et al. 2013; Ding et al. 2014). In addition, fenofibrate has been shown to ameliorate oxidative stress-mediated renal injuries in diabetic nephropathy (Cheng et al. 2016a; Cheng et al. 2016b). Thus, we speculated that fenofibrate might also exert an anti-oxidative effect on diabetic retinas by regulating expression of oxidant and antioxidant enzymes. Meanwhile, inhibition of Wnt/ $\beta$ -catenin signaling protected retinal cells from diabetes-induced injuries. In the present study, we explore whether fenofibrate directly affects oxidative stress-elicited Wnt/ $\beta$ -catenin activation in retinas of *C57BL/6J-Ins2<sup>Akita</sup>* mice.

## Materials and methods

### Animal models

Genetic type 1 diabetes model of *C57BL/6J-Ins2<sup>Akita</sup>* and littermate control mice were purchased from Jackson Laboratory (Bar Harbor, MI). Blood glucose and body weight were monitored. At the age of 12 weeks, *C57BL/6J-Ins2<sup>Akita</sup>* and littermate control mice were fed 0.1% fenofibrate (LabDiet, Fort Worth, TX) in their diet for 4 weeks. All experiments were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of Animals in Ophthalmic and Vision Research and University of Oklahoma Health Sciences Center (OUHSC) guideline for Animal in research.

### Immunohistochemistry

Mice were humanly euthanized. Eye balls were harvested and briefly fixed in 4% paraformaldehyde. After removing cornea and lens, the eyecups were post-fixed in 4% paraformaldehyde for 30 min and immersed in gradient sucrose solution. Eyecups were embed in Optimal Cutting Temperature Compound (OCT; Sakura Finetek, Torrance, CA) and sectioned with Leica Cryostat. Sections were blocked with blocking buffer for 1 h and then incubated with the following primary antibodies for overnight: anti-Nox4 (Santa Cruz

Biotechnology, Santa Cruz, CA), anti-Nox2 (Santa Cruz), anti-SOD1 (Abcam), anti-SOD2 (Stressgen, Victoria, BC), anti-3-nitrotyrosine (3-NT, Millipore, Billerica, MA), anti-albumin (Bethyl Laboratories, Montgomery, TX) and anti-CD31 (BD Pharmingen, San Diego, CA). After intensive rinsing, sections were incubated with FITC- or Cy3-conjugated secondary antibodies (Invitrogen, Carlsbad, CA) for 1 h. The slides were coverslipped with VECTASHIELD mounting medium with DAPI (Vector Laboratories, Burlingame, CA) and examined under fluorescence microscope (Zeiss, Germany). The representative images were taken with the same exposure time.

### Retinal nuclear protein fraction

Mouse retinas were harvested and thoroughly homogenized by mortar and pestle in liquid nitrogen. Retinal nuclear extracts were prepared by using Nuclear extraction kit (Active Motif, Carlsbad, CA) following the manufacturer's instructions.

### Retinal oxidative stress detection

Generation of retinal ROS was analyzed by fluorometric assay with 2',7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCF-DA, Invitrogen) probe as described previously (Li et al. 2010). Briefly, eyeballs were embed with OCT. Retinal cryosections were obtained and then incubated with 5 μM H<sub>2</sub>DCF-DA dissolved in HBSS for 30 min at 37 °C under dark. The retinal sections were washed with PBS and nuclei were counterstained with DAPI. The representative images were quickly captured with fluorescence microscope (Zeiss) to avoid photo-oxidation.

### Retinal vascular leakage assay

Retinal vascular permeability was measured by quantifying fluorescein isothiocyanate (FITC)-dextran leakage from the retinal vasculature as described previously (Chen et al. 2012). Deeply anesthetized mice were injected with FITC-dextran (4kD, 50 mg/mL in PBS, 100 μL, Sigma-Aldrich) via the caudal vein. After circulation for 15 min, blood was withdrawn from the right ventricle and serum was collected by centrifugation. PBS was perfused into the left ventricle to thoroughly remove the FITC-dextran from the retinal vessels. The retinas were dissected, weighted and homogenized in equal amount of water. Fluorescence of retinal extracts and plasma was measured using a Spectrofluorometer (Perkin Elmer, Waltham, MA) with excitation wavelength at 485 nm and emission wavelength at 535 nm. Retinal vascular permeability was calculated using the following equation: [retinal FITC – dextran (μg)/retinal weight (g)]/[serum FITC-dextran concentration (μg/μL) × circulating time (h)].

### Cell culture

ARPE-19, a cell line derived from human retinal pigment epithelium, was obtained from ATCC (Manassas, VA) and maintained in DMEM with 10% FBS. L-cells and L-cells expressing Wnt3a were obtained from ATCC and maintained in DMEM with 10% FBS and 0.4 mg/ml G418 (Invitrogen). The conditioned medium from L-cells (LCM) and the L-cells expressing Wnt3a (WCM) were collected following ATCC guidelines. Fenofibrate, D-glucose and L-glucose were all purchased from Sigma-Aldrich.

### Western blot analysis

Mouse retinas and ARPE-19 cells were lysed in RIPA buffer containing protease inhibitors. Twenty-five micrograms of total protein were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. After blocking with 5% non-fatty milk in TBST, the membranes were incubated overnight with the following antibodies anti-phospho-LRP6 (Cell Signaling, Danvers, MA), anti-LRP6 (generated in Dr. Ma's lab), anti-β-catenin (Abcam), anti-Nox4 and anti-Nox2 (both from Santa Cruz), SOD1 (Abcam) and SOD2 (Stressgen). After washing with TBST, membranes were incubated with secondary antibodies for 1 h. The same membranes were re-blotted with anti-β-actin antibody (Sigma-Aldrich) or anti-Histone H3 (Cell Signaling) as loading controls.

### Real-time RT-PCR

Total RNA was isolated from ARPE-19 cells with the RNeasy mRNA extraction kit (Qiagen, Valencia, CA) following the manufacturer's instructions. One microgram of total RNA was used for cDNA synthesis with iScript cDNA synthesis Kit (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Real-time PCR was performed with SYBR Green PCR master (Bio-Rad). Primer sequences for real-time RT-PCR were as follows: Nox2: forward: 5'-CCAG TGAAGATGTGTTTCAGCT-3', reverse: 5'-GCAC AGCCAGTAGAAGTAGAT-3' (Ago et al. 2004); Nox4: forward: 5'-AGTCAAACAGATGGGATA-3', reverse: 5'-TGTC CCATATGAGTTGTT-3' (Ago, Kitazono, Ooboshi, Iyama, Han, Takada, Wakisaka, Ibayashi, Utsumi and Iida 2004); SOD1: forward: 5'-AGGGCATCATCAATTTTCGAGC-3', reverse: 5'-GCCCCACCGTGTCTTCTGGA-3'; SOD2: forward: 5'-AACCTCAGCCCTAACGGTG-3', reverse: 5'-AGCA GCAATTTGTAAGTGTCCC-3'.

### Detection of intracellular reactive oxygen species

Intracellular reactive oxygen species was measured by H<sub>2</sub>DCF-DA assay as described previously (Liu et al. 2013). The generated fluorescence intensity was determined by a

spectrofluorometer (Perkin Elmer) with the excitation wavelength at 485 nm and emission wavelength at 535 nm.

### Adenoviral infection and RNAi transfection

Adenoviral vectors expressing Nox4 (Ad-Nox4) or  $\beta$ -galactosidase (Ad-LacZ) were amplified in 293 cells and tittered with Adeno-X™ rapid titer kit (Clontech Laboratories, Mountain View, CA) as described previously (Cheng et al. 2016a). Knockdown of Nox4 was achieved by transfection with the Nox4 siRNA (Santa Cruz) using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions.

### Statistical analysis

Data were shown as mean  $\pm$  S.D. Statistical analysis was performed with two-tailed Student's *t* tests for comparing difference between two groups and one-way analysis of variance (ANOVA) with Bonferroni's post hoc tests for the comparison of difference among four groups. A *p* value less than 0.05 was considered as statistical significance.

## Results

### Activation of Wnt/ $\beta$ -catenin signaling and imbalance between Nox and SOD in retinas of diabetic *C57BL/6J-Ins2<sup>Akita</sup>* mice

First, we evaluated activation of the Wnt/ $\beta$ -catenin pathway in retinas of *C57BL/6J-Ins2<sup>Akita</sup>* mice, which is a commonly used genetic model of type 1 diabetes. As shown in Fig. 1(a, a'), *C57BL/6J-Ins2<sup>Akita</sup>* mouse retinas displayed increased phosphorylated and total LRP6, when compared with non-diabetic littermate controls. It is known that activation of Wnt/ $\beta$ -catenin receptors resulted in dephosphorylation, stabilization and nuclear distribution of  $\beta$ -catenin. Consistently, increased nuclear  $\beta$ -catenin levels were found in the retinas of *C57BL/6J-Ins2<sup>Akita</sup>* mice (Fig. 1b, b'), which further suggested activation of Wnt/ $\beta$ -catenin signaling in diabetic retinas. Our previous studies demonstrated that oxidative stress can trigger activation of Wnt/ $\beta$ -catenin signaling. Nox are enzymes that catalyze the production of reactive oxygen species (ROS). However, SOD are responsible for decomposition of ROS. We next compared expression of Nox and SOD in the retinas of *C57BL/6J-Ins2<sup>Akita</sup>* mice and their non-diabetic littermate controls. We found that Nox4 expression, predominantly co-immunostained with retinal endothelial marker CD31, was significantly increased in *C57BL/6J-Ins2<sup>Akita</sup>* retinas (Fig. 1c, c', d, d'''). In addition, Nox2 expression was also increased in retinas of *C57BL/6J-Ins2<sup>Akita</sup>* mice as shown

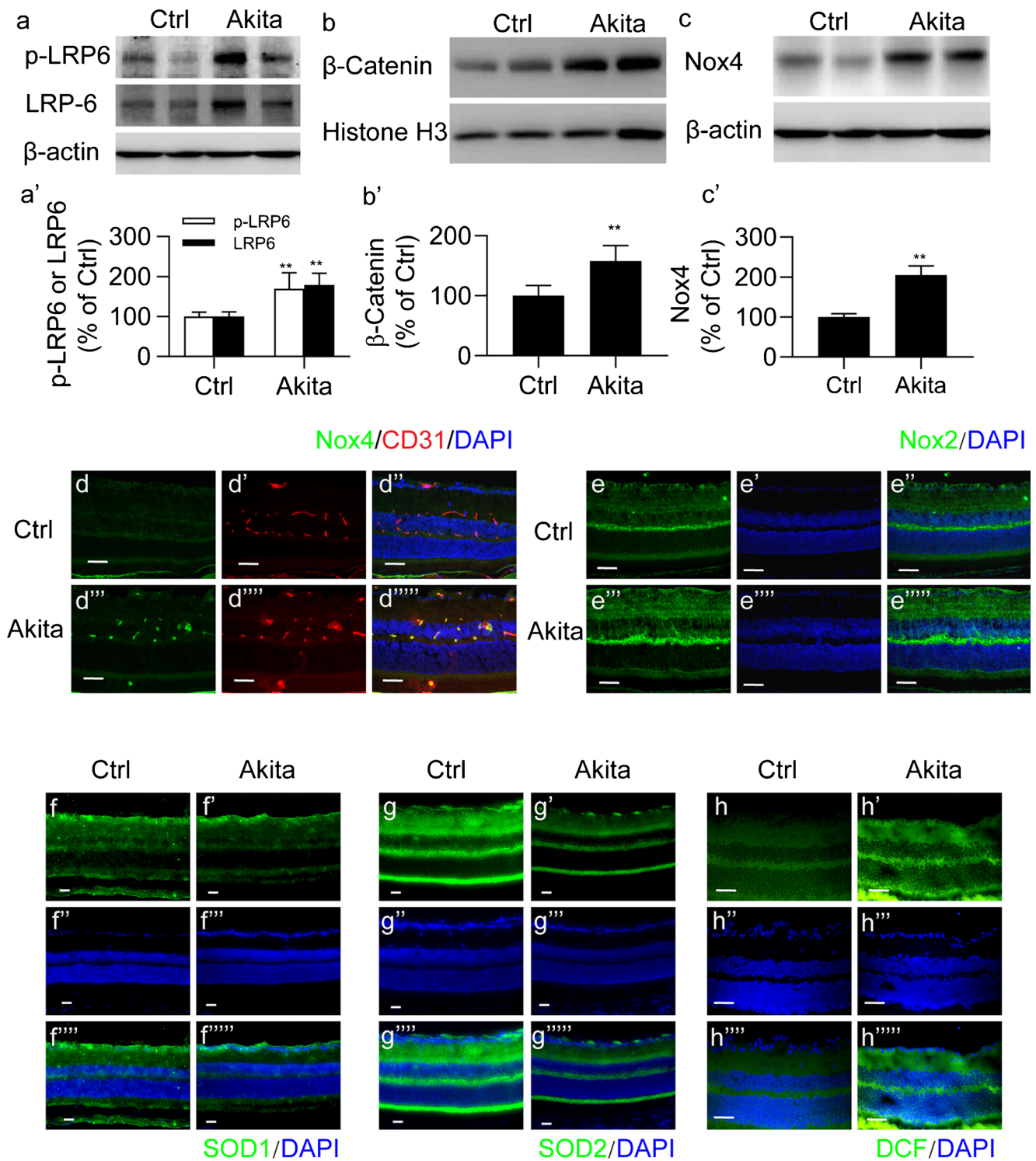
by immunofluorescence staining (Fig. 1e–e'''). In contrast, SOD1 and SOD2, two major antioxidant enzymes in the retinas, were significantly reduced in *C57BL/6J-Ins2<sup>Akita</sup>* mice (Fig. 1f–f''', g, g'''). Retinal ROS production was examined by an ROS-sensitive fluorogenic probe (H<sub>2</sub>DCF-DA). The result demonstrated that retinal ROS production indicated by DCF fluorescence intensity was markedly increased in *C57BL/6J-Ins2<sup>Akita</sup>* mice compared with littermate control mice (Fig. 1h–h'''). Collectively, upregulation of Nox and downregulation of SOD may attribute to increased ROS generation and compromised ROS clearance in diabetic retinas. The imbalance of oxidant enzymes and antioxidant enzymes may synergistically exaggerate retinal oxidative stress.

### Fenofibrate attenuated high glucose (HG)-induced activation of Wnt/ $\beta$ -catenin signaling and oxidative stress in ARPE-19 cells

Previous studies from our group reported that fenofibrate attenuated retinal inflammation and vascular leakage in rodent models of type 1 diabetes (Chen et al. 2013). Here, we determined whether fenofibrate exerted its protecting effect on diabetic retinopathy via inhibition of the Wnt/ $\beta$ -catenin pathway. ARPE-19 cells were cultured with 25 mM D-glucose (high glucose, HG) for 48 h and then treated with different concentrations of fenofibrate for another 16 h. Cells maintained in 25 mM L-glucose (LG) were used as an osmotic control. As shown in Fig. 2(a, a'), fenofibrate inhibited HG-induced LRP6 phosphorylation in a concentration-dependent manner. Hyperglycemia could increase retinal ROS generation during diabetes. We next determined fenofibrate's influence on HG-elicited ROS generation in ARPE-19 cells. ROS was significantly increased by HG, which was dose-dependently suppressed by fenofibrate treatment (Fig. 2b). Together with increased ROS generation, Nox2 and Nox4 expression was markedly upregulated by HG (Fig. 2c, c'). Meanwhile, SOD1 and SOD2 were downregulated by HG (Fig. 2d, d'). Fenofibrate treatment lowered HG-induced upregulation of Nox2 and Nox4 (Fig. 2c, c'). Similarly, HG increased mRNA levels of Nox2 and Nox4 in ARPE-19 cells, which were abolished by fenofibrate (Fig. 2e). In addition, fenofibrate treatment reversed HG-induced downregulation of SOD1 and SOD2 (Fig. 2d, d'). These data suggested that fenofibrate could ameliorate HG-induced ROS production via regulating cellular expression of Nox and SOD.

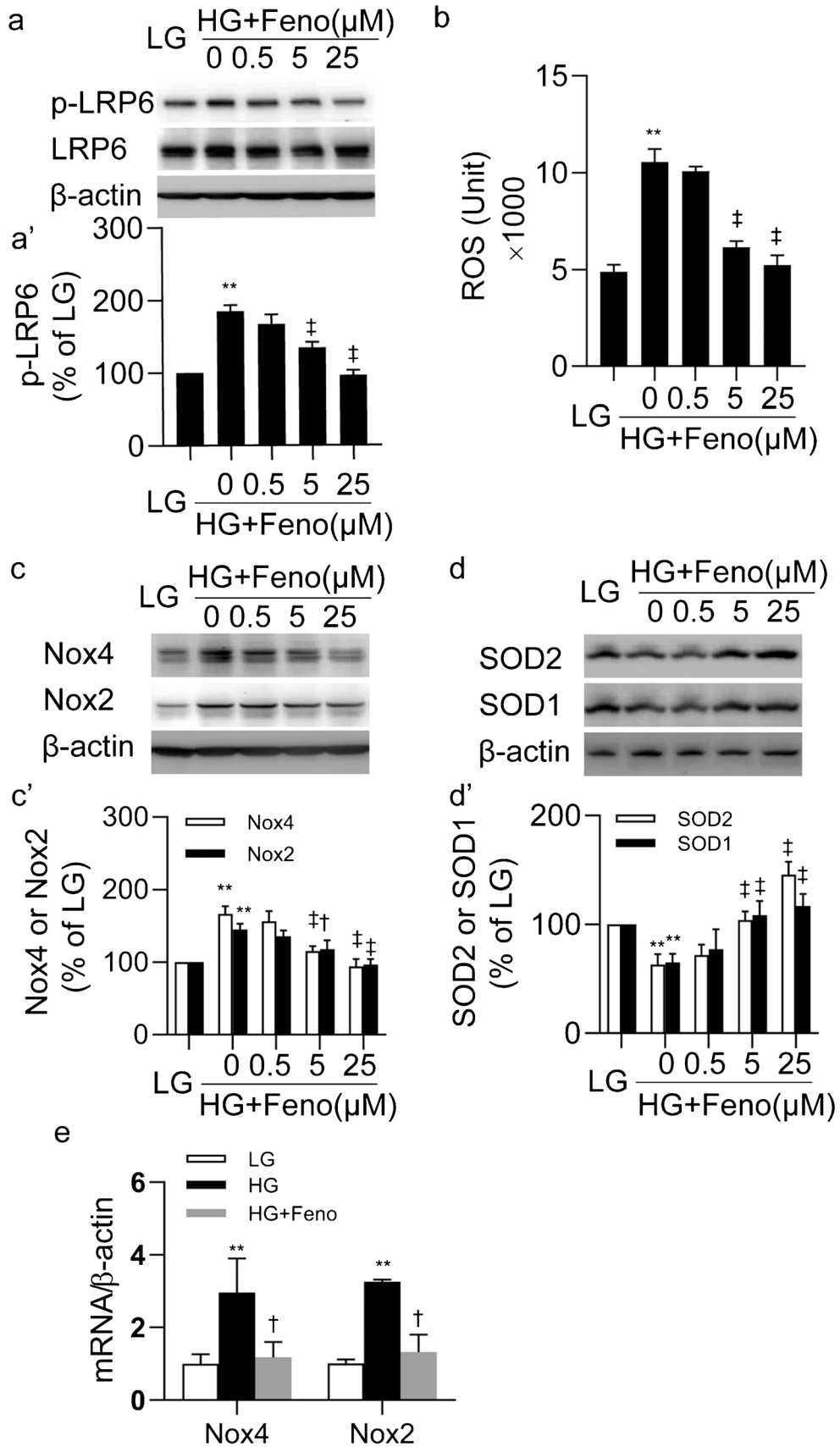
### Fenofibrate suppressed WCM-induced Wnt/ $\beta$ -catenin activation and ROS generation in ARPE-19 cells

To understand how the Wnt/ $\beta$ -catenin signaling pathway was directly involved in retinal oxidative stress, we treated ARPE-19 cells with WCM with different concentrations of



**Fig. 1** Activation of the Wnt/β-catenin signaling pathway, upregulation of oxidants and downregulation of antioxidants in retinas of *C57BL/6J-Ins2<sup>Akita</sup>* mice. (a–a') Phosphorylation of LRP-6 (p-LRP6) and total LRP6 were increased in the retinas of *C57BL/6J-Ins2<sup>Akita</sup>* mice compared to littermate controls. (b–b') Retinal nuclear of β-catenin was determined by Western blot analysis. (c–c') Nox4 and Nox2 were upregulated in the retinas of *C57BL/6J-Ins2<sup>Akita</sup>* mice compared to non-diabetic littermate controls. Expression of Nox4 was determined by Western blot analysis (c–c'). Retinal distribution of Nox4 (green) was examined by

immunofluorescence. CD31 (red) were used as an endothelial marker. Note that the major Nox4 immunosignal was prominently colocalized with CD31 (d–d'''). Expression of Nox2 was displayed by immunofluorescence (e–e'''). (f–g''') Downregulation of SOD1 (f–f''') and SOD2 (g–g''') in the retinas of *C57BL/6J-Ins2<sup>Akita</sup>* mice compared to littermate controls was indicated by immunofluorescence. (h–h''') Retinal ROS production was detected with H<sub>2</sub>DCF-DA fluorogenic probe. *n* = 6, \*\**p* < 0.01 vs Ctrl. Scale bar 50 μm



**Fig. 2** Fenofibrate inhibited HG-induced Wnt/ $\beta$ -catenin activation and oxidative stress in ARPE-19 cells. ARPE-19 cells were treated with HG (25 mM D-glucose) for 48 h followed by incubation with fenofibrate for 16 h. LG (25 mM L-glucose) was used as osmotic controls. (a–a') Fenofibrate inhibited HG-induced LRP6 phosphorylation in a dose-dependent manner. (b) Fenofibrate suppressed HG-induced ROS generation in ARPE-19 cells. (c–c') Fenofibrate downregulated HG-induced Nox2 and Nox4 expression in ARPE-19 cells. (d–d') Expression of SOD1 and SOD2 was decreased in APRE-19 cells treated with HG, which was reversed by fenofibrate. (e) Fenofibrate inhibited HG-induced Nox2 and Nox4 expression at the transcriptional level. Nox2 and Nox4 mRNAs were measured by real-time RT-PCR in ARPE-19 cells.  $n = 3$ ,  $**p < 0.01$  vs LG and  $\dagger p < 0.05$  or  $\ddagger p < 0.01$  vs HG

fenofibrate. We found that WCM induced robust phosphorylation of LRP6 (Fig. 3a, a'). Furthermore, WCM could also promote ROS generation in APRE-19 cells (Fig. 3b). Fenofibrate treatment significantly attenuated LRP6 phosphorylation and reduced ROS generation (Fig. 3a, a', b, b'). We next determined whether Wnt/ $\beta$ -catenin signaling regulated expression of Nox and SOD. We found that WCM does not alter Nox expression at neither protein nor mRNA levels (Fig. 3c, c', e). In contrast, WCM dramatically reduced SOD1 and SOD2 expression (Fig. 3d, d'). Fenofibrate reversed WCM-induced downregulation of SOD1 and SOD2 (Fig. 3d, d'). Moreover, WCM significantly decreased mRNA levels of SOD1 and SOD2 (Fig. 3e). Taken together, these results indicated that the Wnt/ $\beta$ -catenin signaling may regulate expression of SOD at the transcriptional level. Nox and their derived ROS may be upstream effectors that activate Wnt/ $\beta$ -catenin signaling during diabetes.

### Nox4 is essential for activation of Wnt/ $\beta$ -catenin signaling pathway in ARPE-19 cells

Nox4, one of the major NADPH oxidase isoforms, has been shown to be responsible for retinal vascular leakage and neovascularization of diabetic retinopathy (Li et al. 2010, 2015). Here, we evaluated whether Nox4 was required for Wnt/ $\beta$ -catenin pathway activation during diabetic condition. As shown in Fig. 4(a, a'), increase of Nox4 was achieved by infection of ARPE-19 cells with the wild-type Nox4 adenoviral vector (Ad-Nox4). Adenoviral vector with overexpression of  $\beta$ -galactosidase (Ad-LacZ) was used as a control. We found that overexpression of Nox4 significantly increased phosphorylation of LRP6 (Fig. 4b, b'). However, knockdown of Nox4 by specific siRNA significantly inhibited HG-induced LRP6 phosphorylation (Fig. 4c, c'). Nox4 knockdown efficiency was confirmed by real-time RT-PCR (Fig. 4d). Our results indicated that Nox4 played a causal role in activation of the Wnt/ $\beta$ -catenin signaling pathway.

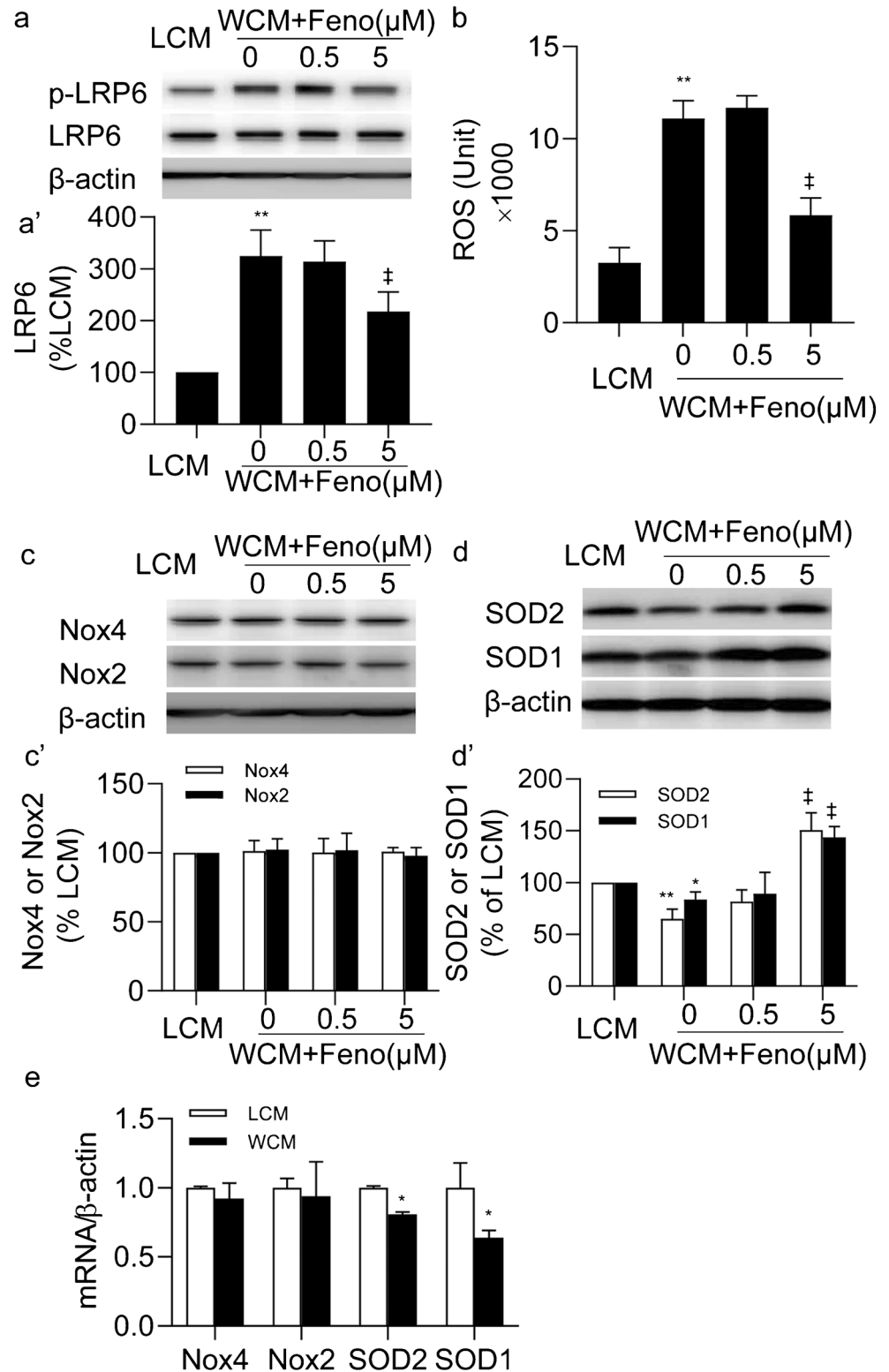
### Fenofibrate attenuated oxidative stress and blocked Wnt/ $\beta$ -catenin signaling activation in retinas of *C57BL/6J-Ins2<sup>Akita</sup>* mice

Next, we clarified whether fenofibrate could attenuate oxidative stress and block Wnt/ $\beta$ -catenin signaling in the retinas of *C57BL/6J-Ins2<sup>Akita</sup>* mice. *C57BL/6J-Ins2<sup>Akita</sup>* mice were fed with chow containing fenofibrate for 4 weeks. Retinal oxidation was determined by H<sub>2</sub>DCF-DA assay and immunostaining of 3-NT formation, respectively. We found that retinal fluorescence intensity of DCF and immunoreactivity of 3-NT expression were drastically reduced in fenofibrate-treated *C57BL/6J-Ins2<sup>Akita</sup>* mice compared with vehicle-treated ones (Fig. 5a–a''', b, b'''), indicating a significant anti-oxidation effect of fenofibrate on type 1 DR. Furthermore, fenofibrate intervention also suppressed nuclear  $\beta$ -catenin accumulation in the retinas of *C57BL/6J-Ins2<sup>Akita</sup>* mice (Fig. 5c, c'), which suggested that fenofibrate attenuated activation of Wnt/ $\beta$ -catenin signaling during DR. In addition, our results clearly demonstrated that retinal Nox4 and Nox2 expression were markedly reduced in *C57BL/6J-Ins2<sup>Akita</sup>* mice treated with fenofibrate (Fig. 5d–d''', e, e'''). Furthermore, retinal levels of SOD1, the pivotal antioxidant enzyme, were markedly elevated by fenofibrate in *C57BL/6J-Ins2<sup>Akita</sup>* mice (Fig. 5f–f'''). These results suggest that fenofibrate could attenuate retinal oxidative stress by reducing ROS-generating enzymes as well as by upregulating the antioxidant systems.

### Fenofibrate reduced vascular leakage in retinas of *C57BL/6J-Ins2<sup>Akita</sup>* mice

Finally, we addressed fenofibrate's salutary effect on retinal microvasculopathy of diabetic *C57BL/6J-Ins2<sup>Akita</sup>* mice. Vascular leakage, a hallmark of diabetic retinopathy, was analyzed by FITC-dextran vascular permeability assay in mouse retinas. Compared with littermate controls, *C57BL/6J-Ins2<sup>Akita</sup>* mice showed a dramatic increase of retinal vascular leakage of FITC-dextran (Fig. 6a). However, fenofibrate significantly attenuated retinal hyperpermeability of FITC-dextran in *C57BL/6J-Ins2<sup>Akita</sup>* mice. Meanwhile, the protective role of fenofibrate on diabetes-induced vascular leakage was further verified by immunostaining of extravasated albumin in retinas of *C57BL/6J-Ins2<sup>Akita</sup>* mice. As shown in Fig. 6(b, b'), albumin was weakly detected within blood vessels in non-diabetic retinas. Extravasated albumin was more diffusely distributed in retinas of diabetic *C57BL/6J-Ins2<sup>Akita</sup>* mice (Fig. 6b'', b'''), especially in inner retinal layers, which was markedly ameliorated by fenofibrate treatment (Fig. 6b''''', b'''''). These results suggested that fenofibrate protect against diabetes-caused blood-retinal barrier breakdown.

**Fig. 3** Fenofibrate suppressed WCM-induced Wnt/ $\beta$ -catenin activation and ROS generation in ARPE-19 cells. ARPE-19 cells were treated with WCM for 16 h in the presence or absence of fenofibrate. (a–a') Fenofibrate inhibited WCM-induced LRP6 phosphorylation. (b) WCM increased ROS generation in ARPE-19 cells, which was inhibited by fenofibrate treatment. (c–c') WCM does not alter Nox2 and Nox4 expression in ARPE-19 cells. (d–d') WCM decreased SOD1 and SOD2 expression in ARPE-19 cells, which was reversed by fenofibrate. (e) WCM decreased SOD1 and SOD2 at the mRNA level but had no effect on Nox2 and Nox4 mRNA.  $n = 3$ , \* $p < 0.05$ , \*\* $p < 0.01$  vs LCM and † $p < 0.01$  vs WCM



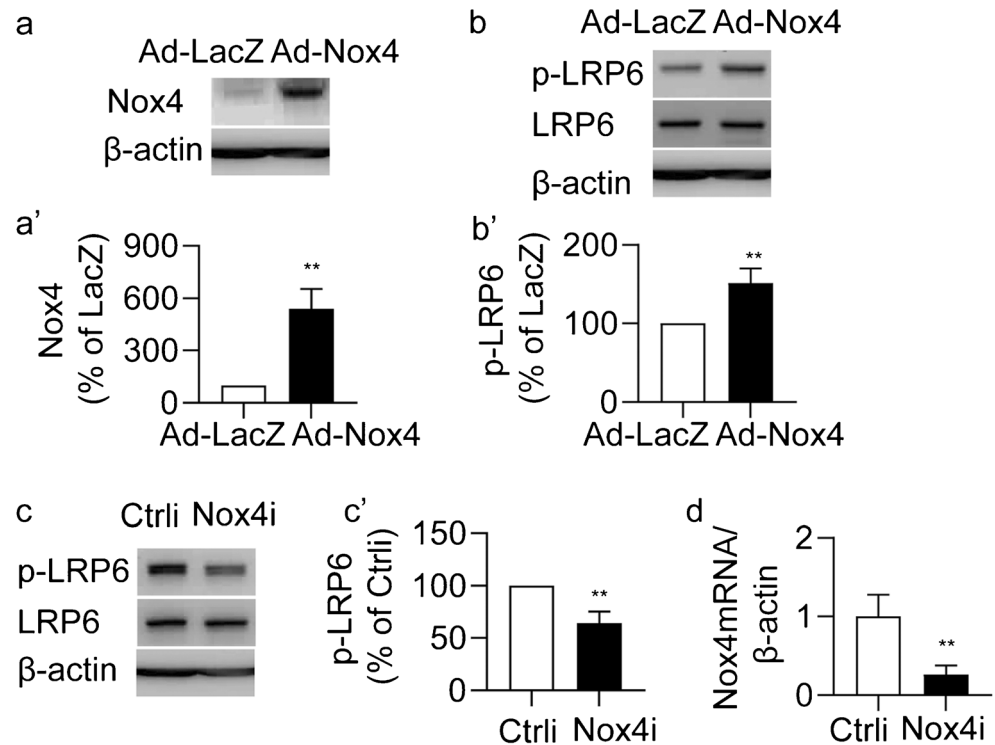
## Discussion

The robust and consistent evidence from the FIELD study and ACCORD-EYE study reported that fenofibrate has therapeutic effects in patients with DR. Here, we utilized *C57BL/6J-*

*Ins2<sup>Akita</sup>* mice as a type 1 diabetic animal model and identified a possible mechanism underlying fenofibrate's protective effect against DR. We found that fenofibrate ameliorated retinal ROS burden by downregulating oxidant enzyme Nox2 and Nox4 expression and upregulating antioxidant enzyme



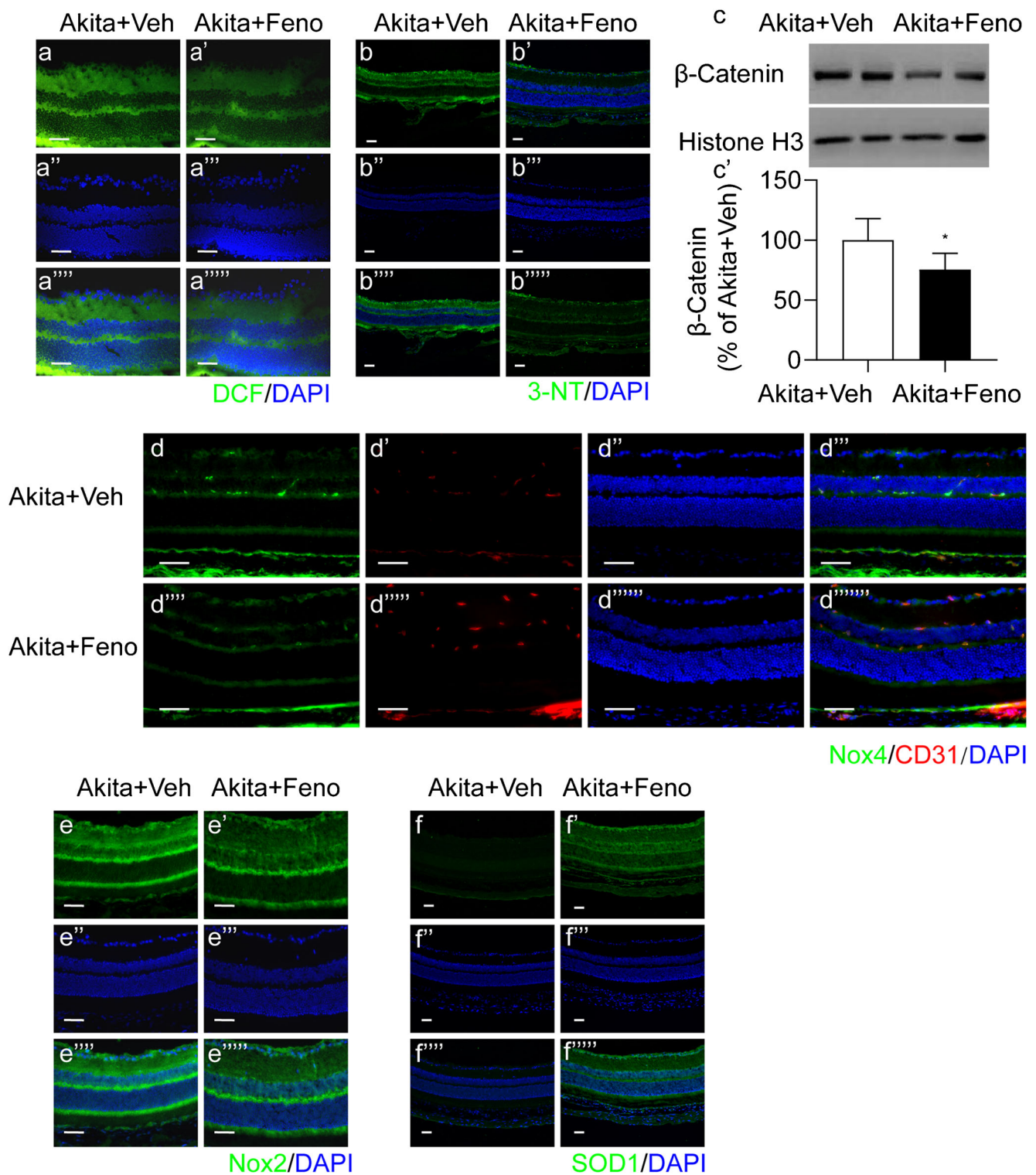
**Fig. 4** Nox4 is required for activation of the Wnt/ $\beta$ -catenin signaling in ARPE-19 cells. ARPE-19 cells were infected with adenovirus expressing Nox4 (Ad-Nox4) or transfected with Nox4-specific siRNA (Nox4i). (a–b') Overexpression of Nox4 promoted phosphorylation of LRP6. Expression of Nox4 (a and a') and p-LRP6 (b and b') was examined by Western blot analysis. (c–d) Knockdown of Nox4 decreased phosphorylation of LRP6. Expression of p-LRP6 was determined by Western blot analysis (c) and semi-quantified by densitometry (c'). Nox4 knockdown efficiency was evaluated by real-time RT-PCR (d).  $n = 3$ ,  $**p < 0.01$  vs Ad-LacZ or Ctrl



SOD1 and SOD2 expression in diabetic mice; thereby, our study manifested the anti-oxidative property of fenofibrate on DR. Previous studies indicated that activation of Wnt/ $\beta$ -catenin contributes to diabetic complications. In the present study, we showed that Nox4-mediated ROS generation was responsible for activation of the Wnt/ $\beta$ -catenin pathway by inducing phosphorylation of LRP6. Activation of the Wnt/ $\beta$ -catenin pathway resulted in transcriptional downregulation of SOD1 and SOD2, which, in turn, exacerbated retinal oxidative stress and vascular leakage.

Physiological generation of ROS serves as the signaling molecule; however, excessive or sustained ROS generation, especially exceeding the capacity of antioxidant defense systems, increases ROS burden and eventually leads to oxidative damage. The major source of ROS generation in metabolic disorders including diabetes is Nox enzymes, which trigger the transfer of an electron from NADPH substrate to oxygen and catalyze generation of superoxide. Nox family is composed of seven members with distinct cellular distributions and varied activation mechanisms. Notably, Nox1 and Nox2 require the membrane-bound p22<sup>phox</sup> component for membrane stabilization. Nox1, highly expressed in epithelial cells of the gastrointestinal tract, is preferentially activated by assembly with NADPH oxidase activator 1 (Noxa1), NADPH oxidase organizer 1 (Noxo1) and GTPase Rac1. More recently, Nox1 overexpression has been shown to be responsible for retinal neovascularization and ganglion cell (RGC) death in ischemic retinopathy (Dvorianchikova et al. 2012; Wilkinson-Berka et al. 2014). Nox2, also known as gp91<sup>phox</sup>,

first identified in phagocytes, required recruitment of cytosolic regulatory subunits p47<sup>phox</sup>, p40<sup>phox</sup> and GTPase Rac1, forming the active enzyme complex. Nox2 was shown to be expressed in retinal vessels and circulating leukocytes (Al-Shabrawey et al. 2008; Saito et al. 2007; Wei et al. 2016). Nox2-deficient mice displayed reduced aberrant neovascularization and ganglion cell death in ischemic retinopathy (Chan et al. 2013; Yokota et al. 2011). Similarly, diabetes-induced vascular injuries were also attenuated in mice with Nox2 gene depletion or Nox2 activity inhibition (Al-Shabrawey et al. 2008; Rojas et al. 2013). Our findings indicated that fenofibrate might decrease retinal ROS production partially by reducing hyperglycemia-induced Nox2 expression in type 1 diabetes. Nox4 is constitutively active and its activity is mostly regulated at the transcriptional level. A previous study demonstrated that Nox4 is a major source of vascular ROS generation and abundantly expressed in vascular endothelia, smooth muscle cells and fibroblasts (Craigie et al. 2011; Kleinschnitz et al. 2010; Li et al. 2015). Nox4-mediated oxidative stress has been shown to lead to blood-retinal barrier breakdown in DR and retinal neovascularization in ischemic retinopathy (Li et al. 2010, 2015; Wang et al. 2014). Genetic depletion of Nox4 inhibited cerebral ischemia-induced neuronal damage via attenuating breakdown of the blood-brain barrier and neuronal apoptosis (Kleinschnitz et al. 2010). Previous findings indicated that fenofibrate protects retinas from diabetes- or ischemia-induced microvasculopathy by reducing vascular hyperpermeability, pericyte loss, and aberrant neovascularization (Chen et al. 2013; Ding et al. 2014; Moran

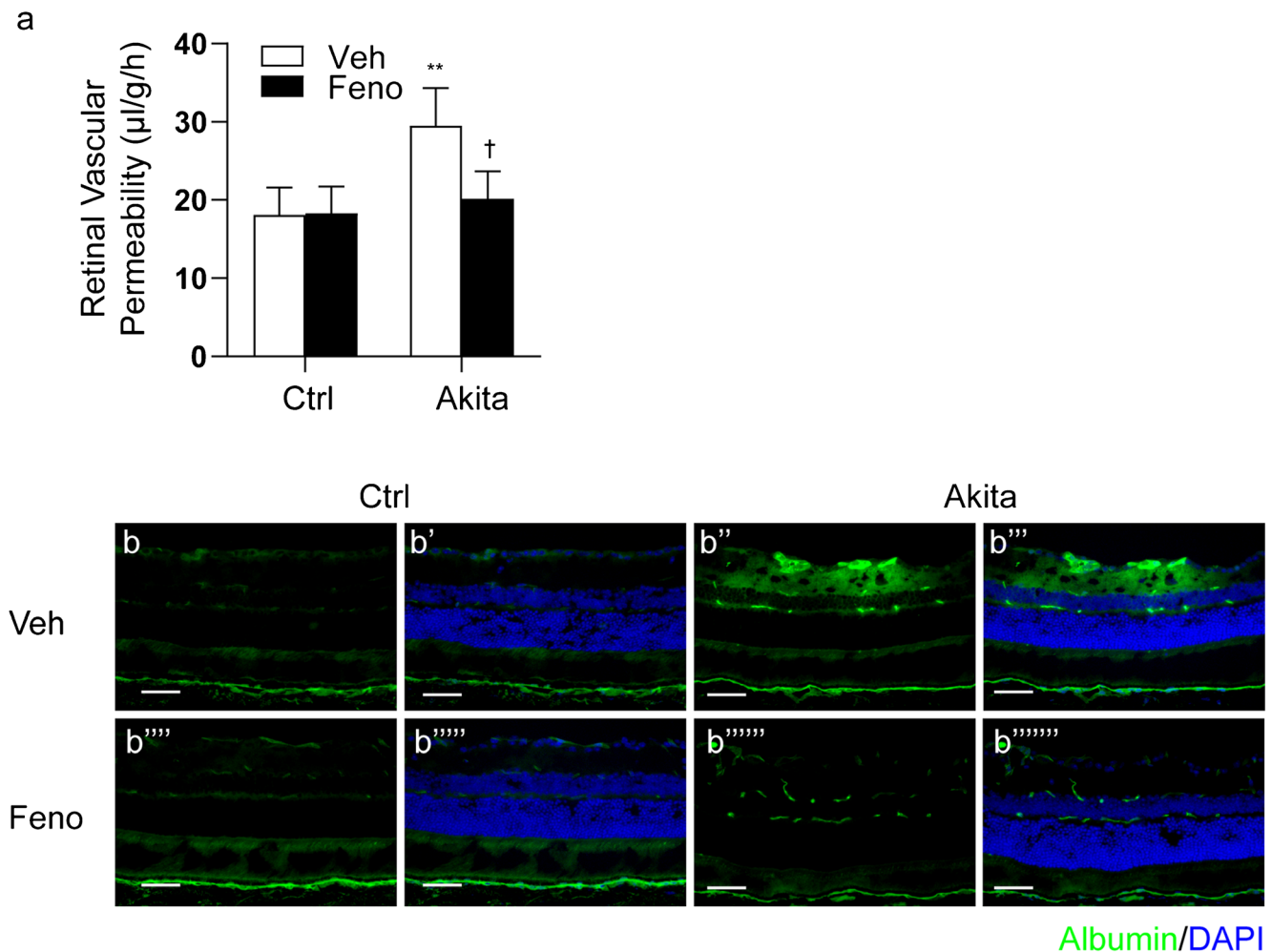


**Fig. 5** Fenofibrate attenuated oxidative stress and inhibited  $\beta$ -catenin expression in the retinas of *C57BL/6J-Ins2<sup>Akita</sup>* mice. *C57BL/6J-Ins2<sup>Akita</sup>* mice were fed with fenofibrate. (a–a''''') Retinal ROS production was detected by H<sub>2</sub>DCF-DA assay. (b–f''''') Expression of 3-NT (b–

b'''''),  $\beta$ -catenin (c and c'), Nox4 (d–d'''''), Nox2 (e–e'''''), and SOD1 (f–f''''') were determined by immunofluorescence staining or Western blot analysis.  $n = 6$ ,  $*p < 0.05$  vs Akita+Veh. Veh, vehicle; Feno, fenofibrate. Scale bar 50  $\mu$ m

et al. 2014). In the present study, we provided evidence that fenofibrate may protect the blood-retinal barrier by inhibition of diabetes-induced Nox4 expression and ROS production.

Hyperglycemia or high glucose-triggered ROS generation by Nox has been shown to activate diverse aberrant signaling pathways resulting in vascular injuries in diabetic retinas.



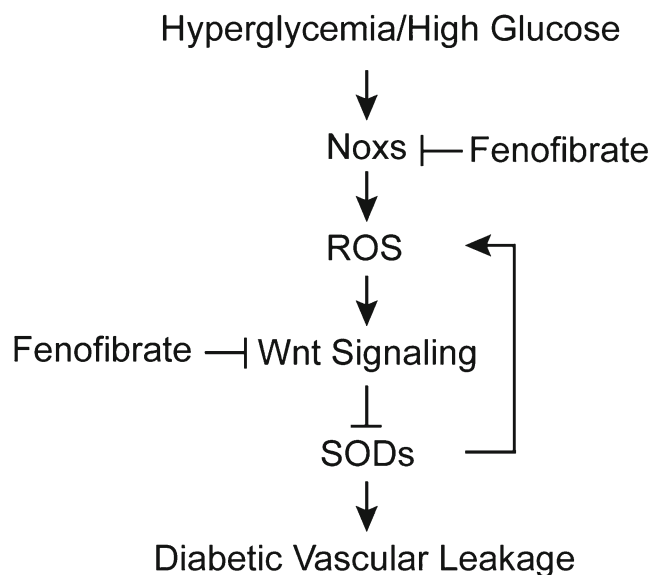
**Fig. 6** Fenofibrate prevented retinal vascular leakage in *C57BL/6J-Ins2<sup>Akita</sup>* mice. (a–b) Retinal vascular leakage was evaluated by FITC-dextran permeability assay (a) and immunostaining of extravasated

albumin (b–b'''''''), respectively.  $n = 6$ ,  $**p < 0.01$  vs Ctrl+Veh,  $†p < 0.05$  vs Akita+Veh. Veh, vehicle; Feno, fenofibrate. Scale bar 50  $\mu\text{m}$

Accumulating evidence suggests that Wnt/ $\beta$ -catenin cascade could be activated by redox signaling. A previous study reported that nucleoredoxin (NRX) interacted with Dvl and negatively regulated the Wnt/ $\beta$ -catenin signaling pathway (Funato et al. 2006). A further study demonstrated that Nox1-derived ROS caused oxidative-inactivation of the NRX and dissociation of NRX and Dvl, which accelerated activation of Wnt/ $\beta$ -catenin signaling (Kajla et al. 2012). Upon binding of Wnt ligands to the transmembrane receptor Fz and co-receptor LRP5/6, Dvl was recruited to Fz, which results in the relocation of Axin and associated GSK-3 to the plasma membrane to initiate LRP6 phosphorylation. Phosphorylation of LRP5/6 at ProlineProlineProlineSerine/ThreonineProline (PPPS/TP) motifs by GSK-3 in the intracellular domain provides the docking site for Axin and then attacks more GSK-3 to form a LRP6 signaling complex (LRP6 signalosome) and further enhances LRP6 phosphorylation, which accelerates dissociation of “destruction complex” and propagates stabilization of  $\beta$ -catenin and activation of  $\beta$ -catenin-dependent transcription. In

the present study, we found that overexpression of Nox4 can directly increase phosphorylation of LRP6; in contrast, knock-down of Nox4 attenuated HG-induced LRP6 phosphorylation, suggesting a causal role of Nox4-derived ROS in activation of the Wnt/ $\beta$ -catenin signaling pathway. One of the intriguing observations in our study was that fenofibrate suppressed HG- and WCM-induced LRP-6 phosphorylation in retinal pigment epithelial cells. Fenofibrate is a PPAR- $\alpha$  agonist. A previous study reported that loss of PPAR- $\alpha$  augmented Wnt/ $\beta$ -catenin signaling by increasing LRP6 stability in cultured murine tubular cells; however, overexpression of PPAR- $\alpha$  accelerated LRP6 degradation and exerted an inhibitory effect on Wnt/ $\beta$ -catenin signaling activation (Cheng et al. 2016a). All of these findings suggested that fenofibrate could be involved in post-transcriptional regulation of LRP6 in a ROS-related and PPAR- $\alpha$ -dependent manner.

SOD is a ubiquitous family of antioxidant enzymes. Among three distinct isoforms, SOD1 (Cu-Zn-SOD) is mainly distributed in the cytoplasm; SOD2 (Mn-SOD) is



**Fig. 7** A schematic diagram summarizing the effect and mechanism for fenofibrate to prevent retinal vascular leakage in diabetic animal models. Hyperglycemia upregulated Nox and contributed to excessive ROS generation, which activated the Wnt/ $\beta$ -catenin signaling pathway. Activation of the Wnt/ $\beta$ -catenin pathway downregulated expression of anti-oxidative enzymes such as SOD1 and SOD2, which further exacerbated cellular oxidative stress. Fenofibrate suppressed hyperglycemia-induced Nox expression, thus attenuated Wnt/ $\beta$ -catenin activation. Inhibition of the Wnt/ $\beta$ -catenin pathway activation may contribute to fenofibrate's effect on upregulating SOD1 and SOD2

compartmentalized in the mitochondrial matrix; SOD3 (EC-SOD) is an extracellular enzyme. SOD catalyzes the dismutation of superoxide into hydrogen peroxide, which could be further converted to water by glutathione peroxidase or catalase, thus serving a pivotal antioxidant role. Retinal SOD expression or activity was reduced by diabetes, which compromised the antioxidant defense capacity and aggravated retinal oxidative injuries. In this study, we demonstrated that both SOD1 and SOD2 were downregulated at the transcriptional level via Wnt/ $\beta$ -catenin signaling cascade, which was consistent with a previous finding that SOD activity was dramatically decreased by Wnt3a (Zhang et al. 2013). Fenofibrate treatment abrogated hyperglycemia- and Wnt/ $\beta$ -catenin-induced SOD downregulation. However, the detailed mechanisms by which Wnt/ $\beta$ -catenin activation negatively regulated SOD transcription remain to be elucidated in the future.

Taken together, hyperglycemia or HG induced Nox2 and Nox4 upregulation and thus increased ROS generation. The overproduction of ROS led to Wnt/ $\beta$ -catenin activation. Activation of Wnt/ $\beta$ -catenin signaling resulted in downregulation of SOD1 and SOD2, which, in turn, further accentuated retinal ROS burden (Fig. 7). Fenofibrate reduced Nox-mediated ROS production and subsequently suppressed Wnt/ $\beta$ -catenin signaling activation. Inhibition of Wnt/ $\beta$ -catenin activation by fenofibrate attenuated SOD downregulation, thereby promoting ROS

decomposition in diabetic retinas. Thus, our study indicated that Nox-derived ROS contributed to LRP-6 phosphorylation and subsequent Wnt/ $\beta$ -catenin signaling activation. Activated Wnt/ $\beta$ -catenin led to transcriptional downregulation of SOD to accelerate retinal ROS burden. Fenofibrate ameliorated diabetic vascular leakage likely through coordinate attenuation of oxidative stress and blockade of Wnt/ $\beta$ -catenin signaling.

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### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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