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Increased Expression of Ecto-NOX Disulfide-thiol Exchanger 1 (ENOX1) in Diabetic Mice Retina and its Involvement in Diabetic Retinopathy Development

YU-CHUEN HUANG[1,2], SHIH-PING LIU[3,4], SHIH-YIN CHEN[1,2], JANE-MING LIN[1,5], HUI-JU LIN[1,5], YU-JIE LEI[2], YEH-HAN WANG[6], WAN-TING HUANG[7], WEN-LING LIAO[8,9] and FUU-JEN TSAI[1,2,10,11]

[1]*School of Chinese Medicine, China Medical University, Taichung, Taiwan, R.O.C.;*
[2]*Department of Medical Research, China Medical University Hospital, Taichung, Taiwan, R.O.C.;*
[3]*Center for Translational Medicine, China Medical University Hospital, Taichung, Taiwan, R.O.C.;*
[4]*Graduate Institute of Biomedical Science, China Medical University, Taichung, Taiwan, R.O.C.;*
[5]*Department of Ophthalmology, China Medical University Hospital, Taichung, Taiwan, R.O.C.;*
[6]*Department of Anatomical Pathology, Taipei Institute of Pathology, Taipei, Taiwan, R.O.C.;*
[7]*Department of Public Health, China Medical University, Taichung, Taiwan, R.O.C.;*
[8]*Center for Personalized Medicine, China Medical University Hospital, Taichung, Taiwan, R.O.C.;*
[9]*Graduate Institute of Integrated Medicine, China Medical University, Taichung, Taiwan, R.O.C.;*
[10]*Children's Hospital of China Medical University, Taichung, Taiwan, R.O.C.;*
[11]*Department of Medical Genetics, China Medical University Hospital, Taichung, Taiwan, R.O.C.*

Abstract. *Background/Aim: Diabetic retinopathy (DR) is a type of retinal damage caused by a complication of diabetes and is a major cause of blindness in working-age adults. Ecto-NOX disulfide-thiol exchanger 1 (ENOX1) is a member of the ecto-NOX family involved in the plasma membrane electron transport pathway. This study aimed to investigate the role of ENOX1 in the development of DR. Materials and Methods: Human retinal endothelial cells (HRECs) and human retinal pigment epithelial cells (HREpiCs) exposed to a high concentration (25 mM) of D-glucose and type 2 diabetes (T2D) mice (+Lepr^{db}/+Lepr^{db}, db/db) with retinopathy were used as models to determine the ENOX1 expression levels there. Results: Our results showed that ENOX1 expression levels did not significantly change in both HRECs and HREpiCs under hyperglycemic conditions for 48 h. Nevertheless, ENOX1 expression increased significantly in T2D mouse retinas, particularly in the photoreceptor layer, compared to the control mouse retinas. Conclusion: Different retinal ENOX1 expression in T2D mice and control mice suggested that ENOX1 may be involved in DR development.*

Correspondence to: Fuu-Jen Tsai and Wen-Ling Liao, Department of Medical Research, China Medical University Hospital, No. 2, Yuh-Der Road, Taichung 404, Taiwan, R.O.C. Tel: + 886 422052121 (Ext. 2041), Fax: +886 422053425, e-mail: d0704@www.cmuh.org.tw (F.J.T) and wl0129@mail.cmu.edu.tw (W.L.L)

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Diabetic retinopathy (DR) is a severe microvascular complication of diabetes and is the leading cause of blindness in working-age adults (1). Risk factors, including poor glycemic control, long duration of diabetes, hypertension, hyperlipidemia and albuminuria, have been found to be associated with DR development (2-6). Nevertheless, the mechanisms underlying DR have not yet been clarified, and the pathogenesis of the condition is believed to be complex and multifactorial (7).

Ecto-NOX disulfide-thiol exchanger 1 (ENOX1) is a member of the ecto-NOX family, which is involved in plasma membrane electron transport pathways that are essential for a variety of functions, including cellular defense, intracellular redox homeostasis, control of cell growth and survival (8). ENOX1 exhibits both NADH oxidase activity and protein disulfide-thiol interchange activity, and normally responds to hormones and growth factors (9-11). It is expressed in several cell types, including the endothelial cells (12). A previous study demonstrated that the interferon RNA-mediated inhibition of ENOX1 expression suppressed endothelial cell migration as well as their ability to form tubule-like structures (13). A subsequent

study showed that the pharmacological targeting of ENOX1 in endothelial cells could influence the expression of proteins involved in cytoskeletal reorganization, and that ENOX1 activity correlated with elevated NADH concentrations to influence cytoskeletal reorganization and angiogenesis (14). A follow-up study, using morpholino technology as well as pharmacological targeting of ENOX1 during embryogenesis in a zebrafish model, revealed that ENOX1 is required for vascular development (12). Moreover, genetic or chemical suppression of ENOX1 significantly increased radiation-mediated caspase 3-activated apoptosis (13). Thus, ENOX1 responds to hormones and growth factors, such as insulin and epidermal growth factor, and is likely involved in angiogenesis and apoptosis pathways, with the ability to regulate the oxidation of NADH to NAD, leading to an increase in reactive oxygen species (ROS). Collectively, these functions indicate that ENOX1 likely plays an important role in the pathogenesis of DR.

Therefore, in the present study, we used human retinal endothelial and pigment epithelial cells exposed to a high concentration of D-glucose to determine the level of ENOX1 expression. In addition, type 2 diabetes (T2D) mice with retinopathy were also used to understand the possible role of ENOX1 in the DR development.

Materials and Methods

Cell culture. Human retinal microvascular endothelial cells (HRECs) purchased from Cell Biologics Inc (Cell Biologics, Inc., Chicago, IL, USA) and human retinal pigment epithelial cells (HRPEpiCs) purchased from ScienCell Research Laboratories (ScienCell Research Laboratories, Carlsbad, CA, USA) were used for *in vitro* experiments. HRECs were maintained in tissue culture flasks pre-coated with a gelatin-based solution and incubated in complete human endothelial medium (Cell Biologics, Inc., Chicago IL, USA) (15). HRPEpiCs were maintained in tissue culture flasks pre-coated with poly-L-lysine overnight and grown in a complete medium consisting of a mixture of epithelial culture medium containing 2% fetal bovine serum, epithelial cell growth supplement, and penicillin/streptomycin solution (ScienCell Research Laboratories, Carlsbad, CA, USA). The cells were incubated with 5 mM (normal condition), 25 mM D-glucose or 25 mM L-glucose for 48 h after inoculation and were maintained at 37˚C in a humidified incubator with 5% CO_2. L-glucose treatment was used as an osmotic control for the experiments. Each set of experiments was performed three times independently.

T2D mouse model. T2D mice (BKS.Cg-*Dock7*^{m}+/+*Lepr*^{db}/JNarl, abbreviation *db*/*db*), and their non-diabetic littermates (control mice, abbreviation +/+) were obtained from the National Laboratory Animal Center (Taipei, Taiwan) (16, 17). Six male mice per group were grown for 32 weeks during the experiment (15). All the mice were housed under a 12 hour light/dark condition with free access to water and food. Blood samples were obtained from the tail veins and the blood glucose levels were monitored by Accu-Chek blood glucose meters every two weeks (Roche, Mannheim, Germany). All animal care and handling were approved by the Institutional Animal Care and Use Committee of China Medical University (CMUIACUC-2017-328-1).

Western blot. Mouse retina tissue protein was extracted busing the radio-immunoprecipitation lysis buffer (Sigma-Aldrich, St. Louis, MO, USA), containing protease inhibitors and phosphatase inhibitors (Roche, Indianapolis, IN, USA). 20 μg of protein extracts were separated using 12% (w/v) sodium dodecyl sulfate-polyacrylamide gel and were then transferred to 0.45 μm pore size nitrocellulose membranes (Millipore, Billerica, MA, USA). The membranes were incubated with anti-ENOX1 primary antibody (dilution 1:500; Novus Biological, Littleton, CO, USA) overnight at 4˚C, followed by incubation with horseradish peroxidase-conjugated secondary antibody (GeneTex, Austin, TX, USA) at room temperature for 1 h. Anti-β-actin (dilution 1:6,000; Novus Biological) was used as an internal control. Protein signal was detected using an enhanced chemiluminescence system (Syngene's ChemiGenius XE Bio Imaging System, Maryland, USA). Protein expression was quantified using the ImageJ program (NIH, Bethesda, MD, USA) and was normalized to the internal control.

Immunohistochemistry. Paraffin-embedded mouse eye tissues were sliced into 5 μm sections. The sections were deparaffinized and soaked in a 3% hydrogen peroxide solution in distilled water for 5 minutes to counteract endogenous peroxidase reactions. Further, the sections were incubated with anti-ENOX1 primary antibody (dilution 1:100; LifeSpan BioSciences, Seattle, WA, USA), followed by incubation with horseradish peroxidase-conjugated secondary antibody. The presence of peroxidase was revealed by the addition of 3, 3'-diaminobenzidine tetrahydrochloride solution and counterstaining with hematoxylin to color the nuclei cell blue.

Statistical analyses. Statistical analysis was performed using IBM SPSS Statistics 22 (IBM Co., USA). The relative ENOX1 expression levels are presented as mean±SD, and the differences between the expression levels in T2D and control mice were compared using the Student's *t*-test. The relative ENOX1 expression levels in retinal cells with different treatments were compared by one-way analysis of variance (ANOVA), as specified in the figure legends. $p<0.05$ was considered statistically significant.

Results

We used a western blot assay to determine the expression level of ENOX1 in HRECs and HRPEpiCs under different glucose concentrations (Figure 1A). The retinal cells were treated with normal (5 mM of D-glucose) or high concentration of D-glucose (25 mM) or osmotic control L-glucose (25 mM) for 48 h. The results showed that the expression levels of ENOX1 slightly increased in cells treated with high concentration of D-glucose compared to cells treated with normal glucose condition, but no significant difference was observed in both cell types (Figure 1B).

db/*db* T2D mice at 32 weeks of age exhibited features of the early clinical stages of DR, as reported previously (15). We then compared the protein expression levels of ENOX1 in the retina of T2D and of non-diabetic control mice at 32 weeks of age (Figure 2A). The western blot assay showed

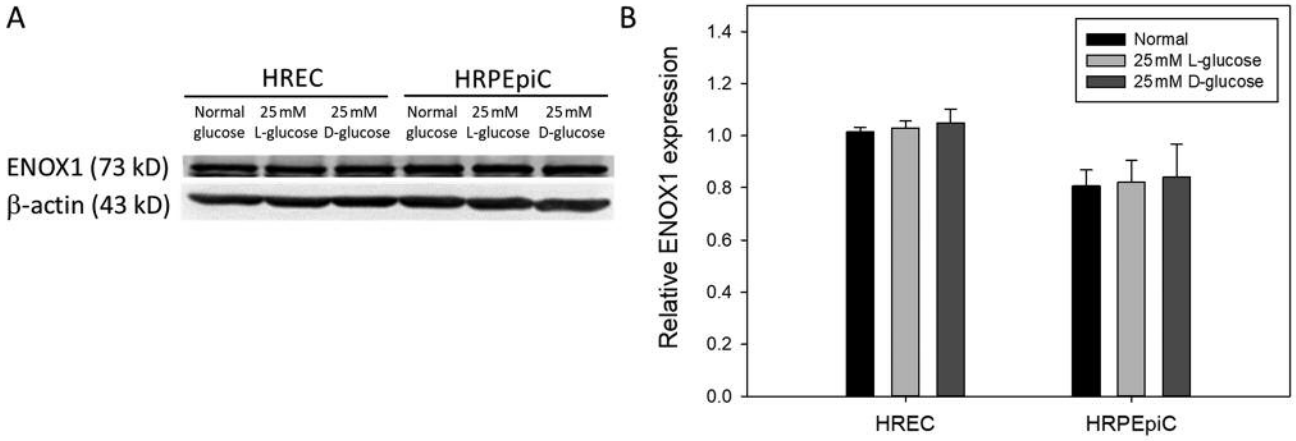

Figure 1. *(A) Representative western blot image of ENOX1 expression in human retinal microvascular endothelial cells (HRECs) and human retinal pigment epithelial cells (HRPEpiCs) under high glucose condition for 48 h. (B) ENOX1 expression relative to that of β-actin in retinal cells under high glucose condition for 48 h. Data are presented as mean±SD and the differences between means were compared by ANOVA.*

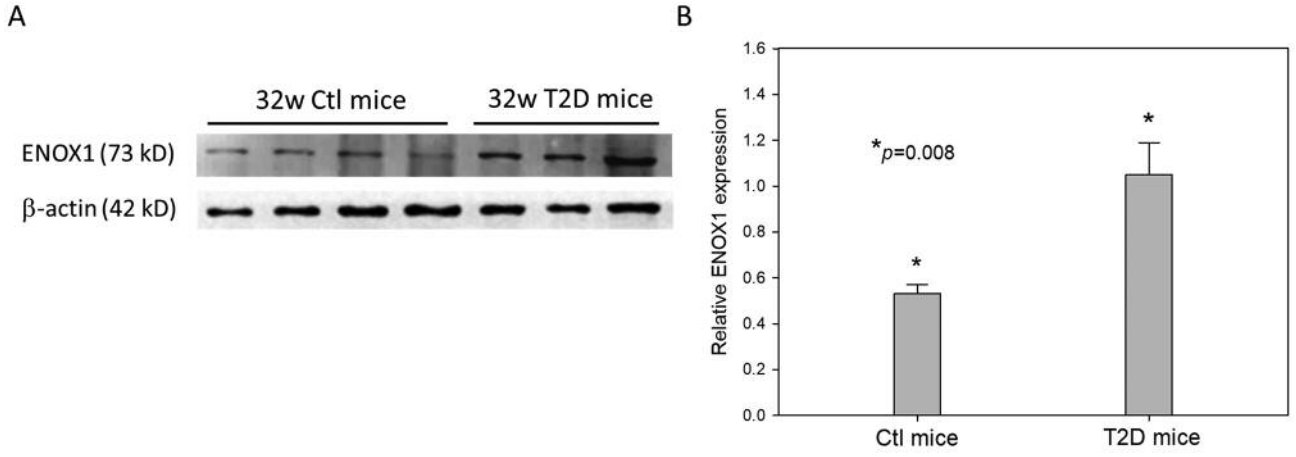

Figure 2. *(A) Representative western blot image of ENOX1 expression in T2D and control mouse retina. (B) ENOX1 expression relative to that of β-actin in mouse retina at 32 weeks of age. Data are presented as mean±SD. *p=0.008 (Student's t-test).*

that ENOX1 was much more highly expressed in the retinas of the T2D mice compared to the control mice (relative ENOX1 expression: T2D mice:1.05±0.14 *versus* control mice:0.53±0.04; p=0.008, Figure 2B). Further results obtained from immunohistochemical staining also showed higher expression levels of ENOX1 in the T2D mice retina compared to those of the control mouse retinas, with particularly abundant expression detected in the photoreceptor layer (arrows in Figure 3).

Discussion

To the best of our knowledge, the present study is the first report of increased ENOX1 expression in T2D mouse retina, particularly in the photoreceptor layer, suggesting a potential role in retinopathy development. We have previously conducted a genome-wide association study and have identified several susceptibility loci associated with DR in the Taiwanese population (18-22). Based on this genome-wide association study, we have also identified that the T allele of single nucleotide polymorphism rs7985254 located in the *ENOX1* gene is associated with increased DR risk (odds ratio=2.04, 95% confidence interval=1.37–3.02, p=0.00041). This suggested that ENOX1 also plays an important role in T2D patients with DR.

Several studies indicate that the inhibition of ENOX1 expression in endothelial cells can influence the cytoskeletal reorganization and angiogenesis (12-14). Angiogenesis plays

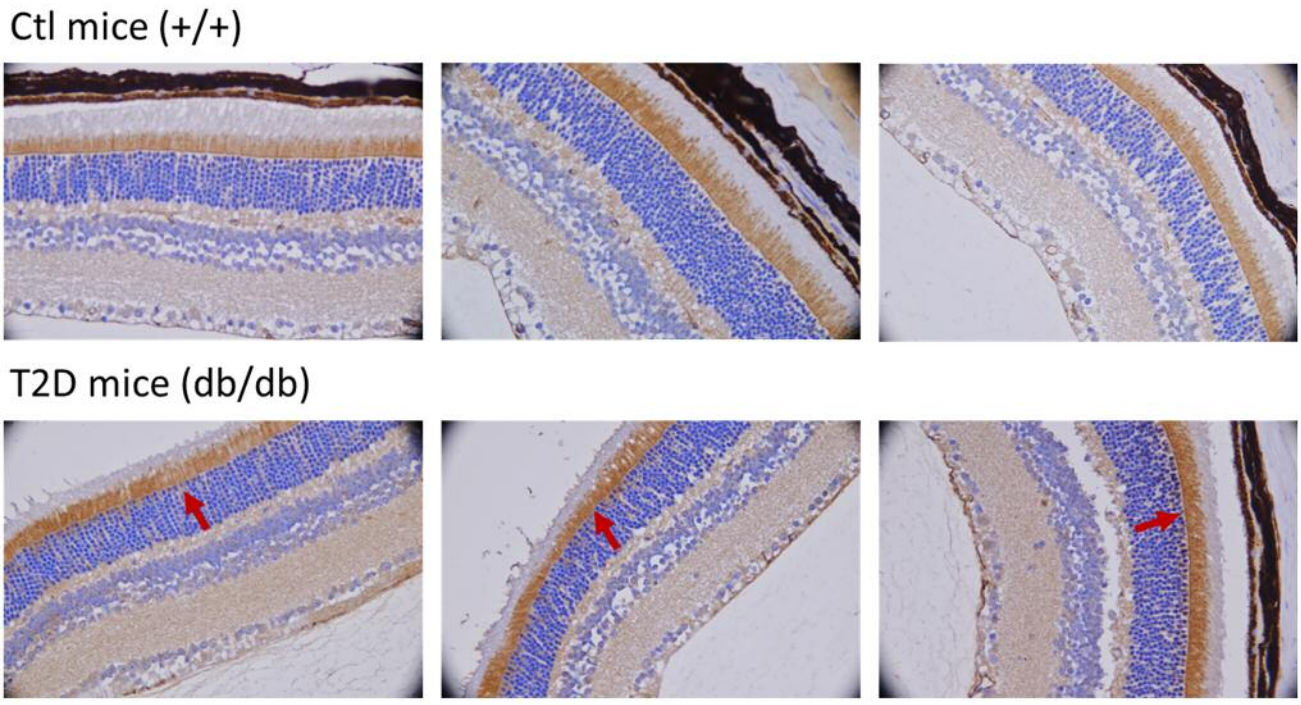

Figure 3. *Representative images of immunohistochemical staining of ENOX1 expression in mouse retina at 32 weeks of age (magnification=400X). Hematoxylin was applied to color the nuclei cell blue. ENOX1 expression is increased in T2D mice retina and prominently expressed in the photoreceptor inner segments (arrow).*

a crucial role in the development of DR, particularly in the proliferative DR stage, which is characterized by the formation of new leaky vessels spreading in the retina (23). In this study, we observed that the ENOX1 expression level increased in the T2D mice with DR compared to control mice. Further studies should investigate the mechanism of action of ENOX1 in the pathogenesis and progression of DR. In addition, ENOX1 was shown to exhibit NADH oxidase activity, which catalyzes the one-electron reduction of oxygen to superoxide anion *via* oxidizing cytosolic NADH to NAD (24). NADH oxidase and the mitochondrial transport chain can produce ROS, a major source of which in vascular cells is the activity of NADH oxidase (25). When ROS overwhelm the cellular antioxidant defense system, either through an increase in ROS levels or a decrease in the cellular antioxidant capacity, oxidative stress occurs. Several clinical and experimental evidence has clearly demonstrated that oxidative stress is increased in the retina and its capillary cells in diabetes, which is thus considered to be a key event in the pathogenesis of DR (26). Previous reports have also indicated that ROS derived from NADH oxidase are involved in the apoptosis of retinal pericytes, which is caused by their chronic exposure to high glucose (27).

In the present study, we observed an increased ENOX1 expression in the photoreceptor layer in T2D mice retina. Previous studies have suggested that diabetes-induced structural and functional alterations in photoreceptors may play a role in DR pathogenesis (15, 28). Such retinal abnormalities have also been reported in other studies in *db/db* mice over 8-24 weeks of diabetes (29, 30) and our previous study at 32 weeks of age (15). In addition, mitochondria are abundantly present in the photoreceptor inner segments. Mitochondria not only cross-talk with NADH oxidases (31), but also play a key role in activating intrinsic apoptosis in mammalian cells (32). Studies of ENOX1 co-localization with mitochondria-specific proteins may reveal valuable information in the future. Since photoreceptors may play an important role in diabetic-induced degeneration of the retinal capillaries (28), increased ENOX1 expression in T2D mouse retina photoreceptor should be further investigated to elucidate the mechanism of DR pathogenesis.

In conclusion, different ENOX1 expression levels in T2D and control mouse retinas suggest that ENOX1 may be involved in DR development. Experiments that can prove that ENOX1 can reverse some phenotypic characteristics of DR, could make it an ideal drug target for future DR therapeutic strategies.

Conflicts of Interest

None of the Authors have any financial interests to disclose.

Authors' Contributions

FJT and WLL conceived and supervised all work, YCH and WLL designed, analyzed and drafted the article, SYC, SPL, JML, and HJL participated in the interpretation of the data, YJL finalized the experimental work, YHW performed the histopathology of the mouse retinas. All authors read and approved the final manuscript.

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