Regulation of extracellular-superoxide dismutase in rat retina pericytes

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Diabetic retinopathy (DR) is regarded as a disease of the retinal microvascular system and metabolic abnormalities that are characteristic of oxidative stress and endoplasmic reticulum (ER) stress have been identified in the retina. Pericytes are known to be susceptible to oxidative stress and selective dropout of pericytes is one of the earliest pathological changes in DR. Extracellular-superoxide dismutase (EC-SOD) is a major antioxidative enzyme and protects vascular cells from the damaging effects of superoxide. Treatment with own conditioned medium significantly decreased EC-SOD expression in pericytes, while the expression of vascular endothelial growth factor and tumor necrosis factor- α were elevated. The addition of chemical chaperone 4-phenyl butyric acid significantly suppressed the effects of conditioned medium on EC-SOD and GRP78, a prominent ER-resident chaperone. Moreover, the cell viability of pericytes changed in a manner similar to that of EC-SOD expression. These results suggest that the expressions of EC-SOD should be regulated, at least partially, through ER stress. Continuous flow of culture media neutralized the ER-stress triggered decrease of EC-SOD expression. The stagnation of factors related to ER-stress around pericytes might reduce EC-SOD expression under pathophysiological conditions such as retinal edema, and this could induce and/or promote the intraretinal microvascular impairment and development of pathogenesis in DR.

Keywords: extracellular-superoxide dismutase, endoplasmic reticulum stress, diabetic retinopathy, pericytes, conditioned medium

Introduction

Diabetic retinopathy (DR) is the most common and severe complication caused by diabetes mellitus and is

a leading cause of acquired blindness. Over 3000 patients with DR lose their vision each year in Japan.¹ The initial clinical stage of DR is characterized by the development of intraretinal microvascular abnormalities. In retinal microvascular systems, endothelial cells are surrounded by pericytes and Müller cell foot processes. Reactive oxygen species (ROS) are produced continuously in cells to maintain cellular homeostasis. However, excessive

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endogenous and/or exogenous production of ROS or insufficient removal of ROS could result in oxidative stress. Increased oxidative stress is thought to be one of the main contributors to the pathogenesis of DR, probably through an increase in expressions of vascular endothelial growth factor (VEGF) and tumor necrosis factor- α (TNF- α),² and dropout of pericytes from microvessels.³ It has been reported that the administration of antioxidants prevented the development of retinopathy in diabetic rats.⁴ The endoplasmic reticulum (ER) is a critical intracellular organelle and functions in processes such as protein synthesis and transport;¹ however, it is also the earliest site of transduction, responding to cellular stresses such as hypoxia and oxidative stress.⁵ ER stress-mediated pericyte loss is known to be one of the characteristic early features of DR.¹ These pathophysiological changes lead to the development of increased vascular permeability and neovascularization, and, ultimately, retinal detachment and blindness.⁶

Since oxidative stress occurs through excess formation and/or impaired removal of ROS, the antioxidant defense system is a crucial component in the maintenance of redox homeostasis. Superoxide dismutase (SOD) is a major antioxidative enzyme that protects cells from the damaging effects of superoxide by accelerating the dismutation reaction of superoxide by approximately 10,000 times. SODs work in conjunction with catalase and glutathione peroxidase to diminish the harmful effects of ROS. There are three SOD isozymes in mammals: copper and zinc-containing SOD (Cu,Zn-SOD or SOD1), manganese-containing SOD (Mn-SOD or SOD2), and extracellular-SOD (EC-SOD or SOD3). EC-SOD is a secretory, tetrameric glycoprotein,⁷ whereas Cu,Zn-SOD and Mn-SOD are intracellular enzymes that are found predominantly in the cytoplasm and mitochondria, respectively. EC-SOD is the major SOD isozyme in extracellular fluids but is distributed mainly in blood vessel walls.⁸ After secretion, EC-SOD slowly diffuses and binds to heparan sulfate proteoglycan in the glycocalyx on the surface of most cell types in the vascular wall. The micro-environment created by the neutrophil-endothelial cell interaction plays an important role in the progression of vascular injury, because the concentration of superoxide released at this site is sufficient to denaturalize the redox-homeostasis. The presence of a high level of EC-SOD throughout the vessel walls might have an important protective role against superoxide in the vascular system.⁹

Pericytes penetrate into basement membranes to make contact with endothelial cells; they can then influence the growth and functions of endothelial

cells.¹⁰ This suggests that intercellular communication between pericytes and endothelial cells or among pericytes themselves may play a role in the maintenance of redox-homeostasis in the vascular system. In the present study, as an initial step toward an understanding of the pathophysiological conditions of DR, we examined the regulation of EC-SOD expression in retinal microvascular cells, especially pericytes.

Materials and methods

Cell culture

Previously established conditionally immortalized retinal pericytes, Müller cells, and endothelial cells, were used.^{11–13} Pericytes and Müller cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Endothelial cells were grown in the above medium with the exception that endothelial cell growth factor (ECGF; Delia Tech GmbH, Braunschweig, Germany) was added. The cells were maintained at 33°C, which is a permissive temperature at which temperature-sensitive SV40 large T-antigen is activated, in an atmosphere of 95% air and 5% CO₂.

Preparation of conditioned medium

The cells were cultured under the conditions described above. After 48–72 h, conditioned medium (CM) was collected, passed through a filter (pore size 0.2 μ m) to remove cellular debris, and stored at –30°C until use.

PCR analysis

Cells were cultured and treated in a 60-mm culture dish or culture flask. After treatment, the cells were washed with cold phosphate-buffered saline (PBS) and the total RNA was extracted from cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The preparation of cDNA and RT-PCR was performed by the method described in our previous report¹⁴ with primers indicated below. The pairs of sequence-specific primers used were as follows:

EC-SOD, F 5'-TCACCAGAGGAAAAACGTTC-3',
R 5'-AAGCCCTCCAGATTGAAGGA-3';

Cu,Zn-SOD, F 5'-GTGTCAGGACAGATTACAGG-3',
R 5'-TTCTCGTGGACCACCATAGT-3';

Mn-SOD, F 5'-ACAATCTGAACGTCACCGAG-3',
R 5'-AGTGGGTCTCTGATTAGAGCA-3';

VEGF, F 5'-AGAAAGCCCATGAAGTGGTG-3',
R 5'-ACTCCAGGGCTTCATCATTG-3';

TNF- α , F 5'-AAAGCATGATCCGAGATGTG-3',

Figure 1 Expression of SODs and other factors in rat retinal vascular cells. The cells in confluent state were used for RT-PCR analysis. Peri, pericytes; Müll, Müller cells; End, endothelial cells

R 5'-ATCTGCTGGTACCACCAGTT-3'; transforming growth factor- β (TGF- β), F 5'-ATCGACATGGAGCTGGTGAA-3', R 5'-TTGACTTGAATCTCTGCAGG-3'; pigment epithelium-derived factor (PEDF), F 5'-AACAATCCTGACATCCACAG-3', R 5'-TCTCACAGTCCTGCTCTCGT-3'; glucose regulated protein 78 (GRP78), F 5'-TTTCTGCCATGGTTCTCACT-3', R 5'-CCCAGATGAGTATCTCCATT-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), F 5'-ACCACAGTCCATGCCATCAC-3', R 5'-TCCACCACCCTGTTGCTGTA-3'.

Densitometric analysis of the PCR products was performed with Multi Gauge v3.0 (Fuji Film, Tokyo, Japan). Real-time PCR was carried out by intercalater method with SYBR Premix Ex Taq (Takara Bio Inc., Ootsu, Japan) according to the manufacturer's directions. The reverse primers for real-time PCR were as follows:

EC-SOD, R 5'-TATCTGACATGGTCCAGGTG-3'; VEGF, R 5'-ACTCTATCTCATCGGGGTAC-3'; TNF- α , R 5'-AGGAATGAGAAGAGGCTGAG-3'; GRP78, R 5'-GCATCATTGAAGTAAGCTGG-3'; and GAPDH, R 5'-TGCAGGGATGATGTTCTGGG-3'.

These reverse primers were used in exchange for those for RT-PCR.

Viability assay

Pericytes cells were seeded in 24-well microplates. After the treatment, the medium was removed and 300 μ l of fresh medium containing MTT (0.5 mg/ml) was added to the cells, followed by incubation at 33°C for 4 h. Isopropanol with 0.04 N HCl (300 μ l) was added, mixed thoroughly, and then the optical density was measured at 595 nm. Cellular injury was also estimated by an assay of total cellular protein. Pericyte cells were cultured and treated in a 35-mm culture dish. After the treatment, the medium was removed and the cells were washed twice with cold PBS and then scraped in 400 μ l of PBS. The cell

suspension was homogenized for 10 s using an ultrasonic homogenizer and centrifuged at 18,000 g for 10 min. The total protein concentration in the supernatant was assayed using a Bio-Rad protein assay reagent.

Data analysis

Data were analyzed by the Mann–Whitney U-test. A P -value of less than 0.05 was considered significant.

Results

EC-SOD mRNA expression in retinal vascular cells

The initial study was to investigate EC-SOD expression among three types of cells – pericytes, Müller cells, and endothelial cells. The level of expression of EC-SOD was lower in pericytes compared with the other cell types, while the levels of expression of Mn-SOD, VEGF, and TNF- α were lower in endothelial cells, as shown in Figure 1. The levels of expression of Cu,Zn-SOD, TGF- β 1, and PEDF were at similar levels among the cells. It is known that pericytes are susceptible to oxidative stress.¹⁵ This may be partly due to the lower level of EC-SOD and intermediate levels of expression of VEGF and TNF- α which are known as pro-inflammatory factors.¹⁶ For this reason, we mainly focused on the expression of EC-SOD and other factors in pericytes.

Change in EC-SOD mRNA expression in pericytes by treatment with pericyte conditioned medium

Retinal vascular function is controlled by complex mechanisms involving co-ordinated interactions between pericytes and endothelial cells or among pericytes themselves. In several disorders, decreasing nutrients and oxygen level, and/or increasing deleterious factors secreted by one cell type can induce oxygen stress and ER stress.¹⁷ We hypothesize that the stagnation of factors and/or conditions in micro-surrounding environments of pericytes consequently results in disruption of retinal vascular system.

As the first step toward testing the hypothesis, the changes in the expression of EC-SOD and other proteins in pericytes with the autocrine regulatory system were investigated by treatment with a conditioned medium of this cell line (Peri-CM). As shown in Figure 2a, the EC-SOD mRNA level in pericytes was significantly decreased by treatment with Peri-CM for 8 h. On the other hand, mRNA levels of VEGF and TNF- α were significantly increased, while those of Cu,Zn-SOD and Mn-SOD

Figure 2 Effect of Peri-CM on expression of SODs, VEGF, and TNF- α . (a) Pericytes were treated with Peri-CM (P) or DMEM medium (C) for 8 h, followed by RT-PCR analysis. (b) Time course of Peri-CM treatment (2–24 h) on the expression levels of EC-SOD, VEGF, and TNF- α . Pericytes were cultured for the numbers of hours indicated with DMEM medium (open column) or Peri-CM (closed column). Gray column shows the pre-treatment level. * $P < 0.05$, ** $P < 0.01$ vs DMEM medium. (c) Peri-CM was diluted with DMEM medium by 1/3 or 2/3 and used to treat pericytes for 24 h, followed by RT-PCR analysis of EC-SOD (closed circle), VEGF (open circle), and TNF- α (closed triangle). Gray symbols show the treatment with DMEM medium. * $P < 0.05$, ** $P < 0.01$ vs DMEM medium. (d) After the treatment of pericytes with Peri-CM for 8 h, the media were exchanged for fresh DMEM medium (open circle) or Peri-CM (closed circle) and cultured for another 16 h, followed by RT-PCR analysis. Gray symbol shows the pre-treatment level. ** $P < 0.01$ vs mRNA level at 8 h, # $P < 0.05$, ## $P < 0.01$ between fresh DMEM medium treatment and Peri-CM treatment

were not changed. In the time-course experiment of Peri-CM treatment (2–24 h), we detected a marked decrease in EC-SOD and increase in VEGF and TNF- α at 4–8 h after the medium change to Peri-CM compared to fresh DMEM medium (Fig. 2b). The effects of Peri-CM on the expression of the above proteins were dependent on the concentration of the conditioned medium (Fig. 2c). When the Peri-CM was changed to a fresh medium after treatment for 8 h, the EC-SOD expression level tended to increase and expression levels of VEGF and TNF- α decreased significantly (Fig. 2d). The increase in EC-SOD expression and decrease in expression of VEGF and TNF- α after the medium change may be due to removal of the factors and/or conditions by the replacement with a fresh medium.

ER stress on pericytes cells

The expression of VEGF and TNF- α has been found to be induced by ER stress.⁵ We observed significant down-regulation of EC-SOD and up-regulation of VEGF and GRP78, a prominent ER-resident chaperone, in pericytes treated with the common ER stressors thapsigargin and tunicamycin. The expression of TNF- α mRNA changed in a reagent-dependent manner (Fig. 3a). GRP-78 remains bound to three types of ER stress sensors (PERK, ATF6, and IRE1), and keeps them in an inactive state in the absence of ER stress. Under conditions of increased ER stress, up-regulation of GRP78 triggers an unfolded protein response (UPR), such as an increase in protein folding activity and prevention of misfolded protein aggregation.¹ The expression of GRP78

Figure 3 Contribution of ER stress. (a) Pericytes were treated with 0.1 μM thapsigargin (Tg) or 2 μg/ml tunicamycin (Tm) for 24 h followed by real-time PCR analysis. * $P < 0.05$, ** $P < 0.01$ vs vehicle (C). (b) Pericytes were treated with Peri-CM (P) or DMEM medium (C) with or without PBA at 0.1 mM (for EC-SOD analysis) or 1 mM (for GRP78 analysis) for 24 h, followed by real-time PCR analysis. * $P < 0.05$, ** $P < 0.01$ vs DMEM medium without PBA, # $P < 0.05$ vs Peri-CM-treated cells without PBA

mRNA was increased by Peri-CM treatment (Fig. 3b). Inhibition of ER stress by 4-phenyl butyric acid (PBA; LKT Laboratories, St Paul, MN, USA), a chemical chaperone, has been reported to ameliorate inflammatory responses in human retinal endothelial cells *in vitro*⁵ and inhibit the development of streptozotocin-induced diabetic nephropathy *in vivo*.¹⁸ PBA is known to protect cells from the deleterious effects of ER-stress by stabilizing the protein conformation and improving the protein-folding capacity of ER.¹⁹ We confirmed that PBA significantly suppressed the down-regulation of EC-SOD in pericytes treated with thapsigargin (data not shown). The addition of PBA partially, but significantly, suppressed the down-regulation of EC-SOD and up-regulation of GRP78 by treatment with Peri-CM (Fig. 3b).

Change in EC-SOD mRNA expression by treatment with conditioned media from other retinal cells

We observed that the addition of conditioned medium from endothelial cells (End-CM) to pericytes did not affect the levels of expression of EC-SOD, VEGF, and TNF-α, whereas the addition of conditioned medium from Müller cells (Müll-CM) changed the levels of expression of the above mRNAs in a manner similar to that using Peri-CM, as shown in Figure 4. The level of GRP78 mRNA was also significantly increased by the addition of Müll-CM, but not by the addition of End-CM. The addition of each of the three kinds of conditioned media did not affect the expression of Cu,Zn-SOD and Mn-SOD in pericytes (data not shown). The culture medium for endothelial cells contains ECGF, in contrast to those for pericytes and

Figure 4 Effect of conditioned media from pericytes and other cells. Pericytes were treated with Peri-CM (P), Müll-CM (M), End-CM (E), or DMEM-medium (C) for 8 h, followed by real-time PCR analysis. These data were normalized using GAPDH levels. * $P < 0.05$, ** $P < 0.01$ vs DMEM medium

Figure 5 Effect of Peri-CM and other conditioned media on viability of pericytes. (a) Pericytes were treated with Peri-CM (closed column) or DMEM medium (open column) for the indicated time, followed by an assay of cellular protein concentration. Gray column shows pre-treatment level. [§]The media were exchanged for proper media every 24 h. * $P < 0.05$, ** $P < 0.01$ vs DMEM medium. (b) Pericytes were treated with Peri-CM (P), Müller-CM (M), End-CM (E), or DMEM medium (C) for 24 h, followed by an assay of cellular protein concentration. Gray column shows pre-treatment level. ** $P < 0.01$ vs DMEM medium. (c) Pericytes were treated with Peri-CM (P), Müller-CM (M), End-CM (E), or DMEM medium (C) for 48 h, followed by an MTT assay. * $P < 0.05$, ** $P < 0.01$ vs DMEM medium. (d) Pericytes were treated with Peri-CM (P) or DMEM medium (C) with or without 1 mM PBA for 24 h, followed by an assay of cellular protein concentration. ** $P < 0.01$ vs DMEM medium without PBA, ## $P < 0.01$ vs Peri-CM-treated cells without PBA

Müller cells. We confirmed that the existence of ECGF did not influence the effects of conditioned media on the levels of expression of EC-SOD and others in pericytes (data not shown).

Effect of conditioned media of retinal cells on viability of pericytes

The total cellular protein in pericytes was reduced in a time-dependent manner by the addition of Peri-CM (Fig. 5A). A significant decrease in the amount of cellular protein in pericytes was also observed upon the treatment with Müller-CM but not with End-CM (Fig. 5B), which was similar to the changes in EC-SOD mRNA level. The viability of pericytes assayed

by the MTT method also decreased more by the treatment with Peri-CM or Müller-CM compared with that with End-CM (Fig. 5c). As shown in Figure 5d, the decrease of cellular protein by the treatment with Peri-CM was partially but significantly suppressed by the addition of PBA similar to the observation in EC-SOD mRNA level again.

Effect of continuous flow culture

Decrease of EC-SOD mRNA expression by the treatment with Peri-CM described above should indicate that the culture of pericytes induces ER stress. When pericytes was cultured for 48 h in culture flask with continuous flow (5 ml/h) of fresh medium (Fig. 6a), we observed significant enhancement of EC-SOD mRNA level accompanied with decreased GRP78 mRNA level compared to the static control culture, as shown in Figure 6B. On the other hand, the circulation of conditioned media (5 ml/h) did not affect the expressions of EC-SOD and GRP78. The treatment of pericytes cells with Peri-CM resulted in the significant decrease of EC-SOD mRNA and increase of GRP78 mRNA again. We observed a significant high in the amount of cellular protein in culture with continuous flow of fresh medium (Fig. 6c), which was similar to the changes in EC-SOD mRNA level.

Discussion

The body is equipped with an efficient antioxidant system consisting of endogenous antioxidative enzymes and low-molecular-weight antioxidants. However, under pathological conditions, an imbalance between the production and detoxification of ROS can occur, which results in oxidative stress. DR is a major complication in patients with diabetes and can lead to vision impairment in a large proportion of diabetic patients. Several interrelated pathways, such as oxidative stress and ER stress, have been shown to contribute to DR.^{1,6} In addition, a number of factors that are characteristic of inflammation, such as TNF- α and VEGF, are known to be up-regulated in the retina of diabetes patients.²⁰ The up-regulation of TNF- α and VEGF under ER stress was also observed in *in vitro* experiments with cultured cells.^{21,22} EC-SOD is widely distributed in blood vessel walls, including large amounts in the intima.^{23,24} The presence of a high level of EC-SOD throughout the vessel walls might have an important protective role as an anti-inflammatory and anti-arteriosclerosis factor against superoxide in the vascular system.^{9,25} EC-SOD expression in fibroblasts and smooth muscle cells is

Figure 6 Effect of continuous flow culture. (a) Schematic representation of the experiments. Static: Pericytes were cultured for 48 h in static condition after a change of culture medium to a fresh one (5 ml). Circulation: Pericytes were cultured for 48 h with continuous circulation of conditioned medium (5 ml/h) after a medium change. Continuous flow: Pericytes were cultured for 48 h with continuous supply (5 ml/h) of fresh medium and exhaust of conditioned medium (5 ml/h) after a medium change. Peri-CM: Pericytes were treated with Peri-CM (5 ml) for 48 h. (b) Pericytes were cultured in static condition (St), with continuous circulation of conditioned media (Cir), with continuous flow of medium (FI), under the treatment with Peri-CM (P) for 48 h, followed by real-time PCR analysis. These data were normalized using GAPDH levels. * $P < 0.05$, ** $P < 0.01$ vs St. (c) Pericytes were cultured under the conditions described above, followed by an assay of cellular protein concentration. * $P < 0.05$ vs St

known to be regulated by numerous substances such as cytokines, vasoactive factors, and growth factors.^{26–29} Moreover, we observed a decrease in EC-SOD expression in COS7 cells under hypoxia³⁰ and ER stress (unpublished data). We hypothesized from the above results that a decrease in EC-SOD expression contributes to the progress of DR.

Retinal capillary pericytes mediate vascular stability and control endothelial proliferation in the retina, and selective dropout of pericytes triggers early pathological changes that lead to DR.^{31,32} Li *et al.*³² showed that oxidative stress weakened antioxidant defense in pericytes but not in endothelial cells. The mechanism by which oxidative stress can increase pericyte loss appears to be complex, but could involve increases in membrane lipid peroxidation, oxidative injury of proteins essential for cellular functions,³³ and changes in cellular redox potentials, which result in depletion of reduced forms of glutathione and ATP.³⁴ Previously reported results that the altered gene profile of scavenging enzymes correlates with overexpression of the cell death protease gene in retinal pericytes obtained from diabetic patients would suggest an important role of oxidative stress in pericyte loss observed in DR.³⁵ It is possible that the lower EC-SOD level and relatively high levels of pro-inflammatory substances such as VEGF and TNF- α contribute to susceptibility of pericytes to oxidative stress.

The present study demonstrated that factors secreted from pericytes or conditions surrounding pericytes suppressed the expression of EC-SOD and induced VEGF and TNF- α with autocrine and/or

paracrine phases. The experimental results of the culture of pericytes with continuous supply of fresh medium and simultaneous excretion of conditioned medium (Fig. 6) consist with above observations and suggest that the factors would stir up ER-stress.

It was found that the addition of PBA significantly suppressed the effects of Peri-CM such as not only an increase in the level of GRP78 but also a decrease in the level of EC-SOD in pericytes (Fig. 3b). The results that thapsigargin or tunicamycin decreased EC-SOD expression while they increased VEGF and GRP78 expression levels (Fig. 3a) are consistent with the above results. These findings suggest ER-stress should be a candidate for a regulator of EC-SOD, at least in part, in Peri-CM treatment. Down-regulation of EC-SOD and up-regulation of VEGF and TNF- α described above were observed when the pericytes was treated with Müll-CM but not with End-CM. Peri-CM and Müll-CM but not End-CM impaired the pericytes and decreased the cellular viability, as shown in Figure 5b,c. This result resembles the decrease in EC-SOD expression in CM-treated pericytes shown in Figure 4. The cell viability of pericytes changed in a manner similar to that of EC-SOD mRNA by the treatment with PBA (Fig. 5d). Chemical chaperones such as PBA are reported to protect cells from the deleterious effects of ER stress and act as potent anti-diabetic modulators in *in vitro* and *in vivo* models of type 2 diabetes.³⁶ The present data support the hypothesis that the decrease in EC-SOD contributes, at least in part, to the impairment of retinal pericytes. Inadequate EC-SOD in the vascular system may cause a shift to an oxidized inflammatory state. The

importance of EC-SOD has been explored in animal models: mice lacking EC-SOD presented more severe inflammatory changes of endothelial cells in lipopolysaccharide-induced inflammation models in mouse cornea,^{37,38} whereas EC-SOD gene transfer in mice suppressed demyelination of the optic nerve.³⁹

The results presented in this study indicate that the factors secreted by retinal vascular cells tend to impair themselves in an autocrine manner accompanied by a decrease in EC-SOD expression and stimulation of pro-inflammatory factors such as TNF- α and VEGF, and, at least partially, through ER stress. Pericytes are smooth muscle-like cells and envelop capillary endothelial cells through the basement membrane. Müller cells are retinal glia-type cells and make contact with both blood vessels and neurons. These types of cells surround endothelial cells and enable cell-cell interaction to maintain retinal function. Under pathophysiological conditions, deleterious factors secreted by one cell type may affect cell of the same type and other cell types. Continuous flow of culture media neutralized the effects of factors which result in ER-stress (Fig. 6b,c). These results suggest that the stagnation of factors around a cell (for example, under retinal edema) may disturb the retinal vascular system leading to pericyte loss and progressive retinopathy. Unfortunately, we could not identify the factor(s). EC-SOD is produced and secreted by smooth muscle cells, diffuses into the vascular wall, and also binds to glycocalyx on the endothelial cell surface in the retinal vascular system. The decrease in EC-SOD expression and stimulation of pro-inflammatory factors in pericytes might induce and/or promote the intraretinal microvascular abnormalities and development of pathogenesis in DR. These findings enable the development of a hypothesis explaining the molecular mechanisms of oxidative stress and ER-stress-inducible retinopathy.

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References

- Oshitari T, Hata N, Yamamoto S. Endoplasmic reticulum stress and diabetic retinopathy. *Vasc Health Risk Manage* 2008; **4**: 115–122.
- Al-Shabraway M, Bartoli M, El-Remessy AB *et al.* Role of NADPH oxidase and Stat3 in statin-mediated protection against diabetic retinopathy. *Invest Ophthalmol Vis Sci* 2008; **49**: 3231–3238.
- Ejaz S, Chekarova I, Ejaz A, Sohail A, Lim CW. Importance of

- pericytes and mechanisms of pericyte loss during diabetes retinopathy. *Diabetes Obes Metab* 2008; **10**: 53–63.
- Kowluru RA, Odenbach S. Effect of long-term administration of alpha-lipoic acid on retinal capillary cell death and the development of retinopathy in diabetic rats. *Diabetes* 2004; **53**: 3233–3238.
- Li J, Wang JJ, Yu Q, Wang M, Zhang SX. Endoplasmic reticulum stress is implicated in retinal inflammation and diabetic retinopathy. *FEBS Lett* 2009; **583**: 1521–1527.
- Kowluru RA, Chan P-S. Oxidative stress and diabetic retinopathy. *Exp Diabetes Res* 2007; 2007: ID43 603.
- Marklund SL. Human copper-containing superoxide dismutase of high molecular weight. *Proc Natl Acad Sci USA* 1982; **79**: 7634–7638.
- Ookawara T, Imazeki N, Matsubara O *et al.* Tissue distribution of immunoreactive mouse extracellular superoxide dismutase. *Am J Physiol* 1998; **275**: C840–C847.
- Takatsu H, Tasaki H, Kim H-N *et al.* Overexpression of EC-SOD suppresses endothelial-cell-mediated LDL oxidation. *Biochem Biophys Res Commun* 2001; **285**: 84–91.
- Orlidge A, D'Amore PA. Inhibition of capillary endothelial cell growth by pericytes and smooth muscle cells. *J Cell Biol* 1987; **105**: 1455–1462.
- Kondo T, Hosoya K, Hori S *et al.* Establishment of conditionally immortalized rat retinal pericyte cell lines (TR-rPCT) and their application in a co-culture system using retinal capillary endothelial cell line (TR-iBRB2). *Cell Struct Funct* 2003; **28**: 145–153.
- Hosoya K, Tomi M, Ohtsuki S *et al.* Conditionally immortalized retinal capillary endothelial cell lines (TR-iBRB) expressing differentiated endothelial cell functions derived from a transgenic rat. *Exp Eye Res* 2001; **72**: 163–172.
- Tomi M, Funaki T, Abukawa H *et al.* Expression and regulation of L-cystine transporter, system x_c⁻, in the newly developed rat retinal Müller cell line (TR-MUL). *Glia* 2003; **43**: 208–217.
- Adachi T, Inoue M, Hara H, Suzuki S. Effects of PPAR γ ligands and C/EBP β enhancer on expression of extracellular-superoxide dismutase. *Redox Rep* 2004; **9**: 207–212.
- Liu B, Bhat M, Padival AK, Smith DG, Nagaraj RH. Effect of dicarbonyl modification of fibronectin on retinal capillary pericytes. *Invest Ophthalmol Vis Sci* 2004; **45**: 1983–1995.
- Yang LP, Sun HL, Wu LM *et al.* Baicalein reduces inflammatory process in a rodent model of diabetic retinopathy. *Invest Ophthalmol Vis Sci* 2009; **50**: 2319–2327.
- Choi YK, Kim K-W. Blood-neural barrier: its diversity and coordinated cell-to-cell communication. *BMB Rep* 2008; **41**: 345–352.
- Luo Z-F, Feng B, Mu J *et al.* Effects of 4-phenylbutyric acid on the process and development of diabetic nephropathy induced in rats by streptozotocin: regulation of endoplasmic reticulum stress-oxidative activation. *Toxicol Appl Pharmacol* 2010; **246**: 49–57.
- Yam GH, Gaplovska-Kysela K, Zuber C, Roth J. Sodium 4-phenylbutyrate acts as a chemical chaperone on misfolded myocilin to rescue cells from endoplasmic reticulum stress and apoptosis. *Invest Ophthalmol Vis Sci* 2007; **48**: 1683–1690.
- Kern TS. Contributions of inflammatory processes to the development of the early stages of diabetic retinopathy. *Exp Diabetes Res* 2007; 2007: ID95 103.
- Hu P, Han Z, Couvillon AD, Kaufman RJ, Exton JH. Autocrine tumor necrosis factor alpha links endoplasmic reticulum stress to the membrane death receptor pathway through IRE1 α -mediated NF- κ B activation and down-regulation of TRAF2 expression. *Mol Cell Biol* 2006; **26**: 3071–3084.
- Roybal CN, Yang S, Sun C-W *et al.* Homocysteine increases the expression of vascular endothelial growth factor by a mechanism involving endoplasmic reticulum stress and transcription factor ATF4. *J Biol Chem* 2004; **279**: 14844–14852.
- Strålin P, Karlsson K, Johansson BO, Marklund SL. The interstitium of the human arterial wall contains very large amounts of extracellular superoxide dismutase. *Arterioscler Thromb Vasc Biol* 1995; **15**: 2032–2036.
- Marklund SL. Expression of extracellular superoxide dismutase by human cell lines. *Biochem J* 1990; **266**: 213–219.
- Heistad DD. Oxidative stress and vascular disease: 2005 Duff lecture. *Arterioscler Thromb Vasc Biol* 2006; **26**: 689–695.
- Marklund SL. Regulation by cytokines of extracellular superoxide dismutase and other superoxide dismutase isoenzymes in fibroblasts. *J Biol Chem* 1992; **267**: 6696–6701.

27. Strålin P, Marklund SL. Multiple cytokines regulate the expression of extracellular superoxide dismutase in human vascular smooth muscle cells. *Atherosclerosis* 2000; **151**: 433–441.
28. Strålin P, Marklund SL. Vasoactive factors and growth factors alter vascular smooth muscle cell EC-SOD expression. *Am J Physiol* 2001; **281**: H1621–H1629.
29. Adachi T, Hara H, Yamada H *et al*. Heparin-stimulated expression of extracellular-superoxide dismutase in human fibroblasts. *Atherosclerosis* 2001; **159**: 307–312.
30. Kamiya T, Hara H, Yamada H, Imai H, Inagaki N, Adachi T. Cobalt chloride decreases EC-SOD expression through intracellular ROS generation and p38-MAPK pathways in COS7 cells. *Free Radic Res* 2008; **42**: 949–956.
31. Hammes HP, Lin J, Renner O *et al*. Pericytes and the pathogenesis of diabetic retinopathy. *Diabetes* 2002; **51**: 3107–3112.
32. Li W, Liu X, He Z, Yanoff M, Jian B, Ye X. Expression of apoptosis regulatory genes by retinal pericytes after rapid glucose reduction. *Invest Ophthalmol Vis Sci* 1998; **39**: 1535–1543.
33. Kaneto H, Kajimoto Y, Miyagawa J *et al*. Beneficial effects of antioxidants in diabetes: possible protection of pancreatic β -cells against glucose toxicity. *Diabetes* 1999; **48**: 2398–2406.
34. Hancock JT, Desikan R, Neill SJ. Does the redox status of cytochrome C act as a fail-safe mechanism in the regulation of programmed cell death? *Free Radic Biol Med* 2001; **31**: 697–703.
35. Li W, Yanoff M, Jian B, He Z. Altered mRNA levels of antioxidant enzymes in pre-apoptotic pericytes from human diabetic retinas. *Cell Mol Biol* 1999; **45**: 59–66.
36. Özcan U, Yilmaz E, Özcan L *et al*. Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. *Science* 2006; **313**: 1137–1140.
37. Behndig A, Karlsson K, Brännström T, Sentman ML, Marklund SL. Corneal endothelial integrity in mice lacking extracellular superoxide dismutase. *Invest Ophthalmol Vis Sci* 2001; **42**: 2784–2788.
38. Behndig A. Corneal endothelial integrity in aging mice lacking superoxide dismutase-1 and/or superoxide dismutase-3. *Mol Vis* 2008; **14**: 2025–2030.
39. Qi X, Hauswirth WW, Guy J. Dual gene therapy with extracellular superoxide dismutase and catalase attenuates experimental optic neuritis. *Mol Vis* 2007; **13**: 1–11.