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Endoplasmic reticulum stress induces retinal endothelial permeability of extracellular-superoxide dismutase

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Abstract

The aim of this study was to determine the reasons why the intravitreal level of extracellular-superoxide dismutase (EC-SOD) increases in proliferative diabetic retinopathy patients by the investigation of two possibilities: first, change of EC-SOD expression in the retina; and secondly, leakage of EC-SOD through the endothelial monolayer by the treatment with endoplasmic reticulum (ER) stress inducers because ER stress is known to be involved in the vascular impairment in diabetic retinopathy. Intravitreous injection of tunicamycin in mice increased the permeability of tracer dye across retinal blood vessels while the retinal EC-SOD mRNA level was not changed. The leakage of EC-SOD through the retinal endothelial cell layer was elevated by the treatment with thapsigargin or tunicamycin. The expression of claudin-5 was significantly decreased by the treatment with the ER stress inducers. These phenomena were significantly suppressed by the pretreatment of endothelial cells with a chemical chaperone 4-phenylbutyric acid. Our observations suggest that ER stress leads to the down-regulation of claudin-5 among tight junction proteins and may induce the elevation of endothelial permeability and leakage of EC-SOD into the vitreous body.

Keywords: *Diabetic retinopathy, endoplasmic reticulum stress, extracellular-superoxide dismutase, tight junction protein, endothelial permeability.*

Introduction

Diabetic retinopathy (DR) is a common and severe complication caused by diabetes mellitus and is a leading cause of acquired blindness. The initial clinical stage of DR is characterized by the development of intraretinal microvascular abnormalities. Reactive oxygen species (ROS) are produced continuously in cells to maintain cellular homeostasis. However, excessive endogenous and/or exogenous production of ROS or insufficient removal of ROS could result in oxidative stress and increased oxidative stress is thought to be one of the main contributors to the pathogenesis of DR [1]. The endoplasmic reticulum (ER) is a critical intracellular organelle and functions in processes such as protein synthesis and transport [2]; however, it is also the earliest site of transduction, responding to cellular stresses [3]. Unresolved accumulation of unfolded protein in the ER causes calcium leakage from the ER lumen and uptake into the mitochondrial matrix and this phenomenon increases ROS generation [4]. Agents or conditions that adversely affect ER protein folding result in accumulation of unfolded or misfolded proteins in the ER, a state known as ER stress. ER stress can be induced by agents that interfere with (a) protein glycosylation (e.g. tunicamycin (Tm), glucosamine), (b) disulphide-bond formation (e.g. dithiothreitol, homocysteine), (c) calcium balance (e.g. thapsigargin (Tg), A23187) and others [5]. Moreover, oxidative stress and ER stress have been shown to be key mediators of

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hyperglycaemia because glucose affects unfolded protein response signalling under ER stress [6]. Recently, it has been reported that ER stress plays a pathogenic role in retinal inflammation and vascular impairment in DR [2,3,7]. There is evidence suggesting that ER stress-mediated cell death is the common pathology in the death of not only pancreatic β-cells but also retinal neurons and vascular cells in type 2 diabetes [8,9]. Expressions of pro-inflammatory factors such as vascular endothelial growth factor (VEGF) and tumour necrosis factor-α (TNF-α) are known to be up-regulated by oxidative stress and ER stress [10]. These pathophysiological changes lead to the development of increased vascular permeability and neovascularization and ultimately retinal detachment and blindness [11].

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. SOD works 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 with a molecular weight of 135 kDa, whereas Cu,Zn-SOD and Mn-SOD are intracellular enzymes [12]. EC-SOD is the major SOD isozyme in extracellular fluids but is distributed mainly in blood vessel walls [13]. After secretion, EC-SOD slowly diffuses and binds to heparan sulphate proteoglycan in the glycocalyx on the surface of most cell types in the vascular wall. In a previous report, we described the intravitreal concentrations of EC-SOD and VEGF as being significantly higher in proliferative diabetic retinopathy (PDR) patients than in macular hole (MH) patients as controls [14]. However, the mechanisms causing the increase in intravitreal EC-SOD in PDR are completely unknown.

The aim of this study was to determine the reasons for the higher intravitreal EC-SOD level in PDR patients by the investigation of two possibilities by the treatment with ER stress inducers because ER stress is known to be implicated in DR: first, expression of EC-SOD in retina is increased and, secondly, the plasma EC-SOD leaks by the disruption of retinal vascular permeability.

Materials and methods

In vivo experiments

Induction of ER stress in the retina by injection of Tm into the mouse eye has been employed as an *in vivo* model of DR [3]. Male adult ddY mice (8–11 weeks) were obtained from Japan SLC (Hamamatsu, Japan) and were kept under controlled lighting conditions (12 h/12 h light/dark). Tm-induced retinal damage was produced as previously reported [15]. Briefly, mice were anaesthetized with 3% isoflurane and maintained with 1.5% isoflurane in 70% N_2O and 30% O_2, delivered via an animal general anaesthesia machine (Soft Lander, Sin-ei Industry Co. Ltd., Saitama, Japan). The body temperature was maintained at 37.0–37.5°C with the aid of a heating pad. Retinal damage was induced by injection (2 μl/eye) of 50 μg/mL Tm dissolved in phosphate-buffered saline (PBS) into the vitreous body of the left eye under the above anaesthesia. Twelve hours after the intravitreal injection, mice were euthanized by intraperitoneal injection of sodium pentobarbital at 80 mg/kg and the eyeballs were quickly removed. The retina was carefully separated from the eyeball, quickly frozen in dry ice and used for assay of the mRNA level of various proteins.

Retinal permeability was induced by the injection (2 μL/eye) of 500 μg/mL Tm dissolved in PBS with 5% dimethyl sulphoxide (DMSO). This solution was injected into the vitreous body of the left eye under the above anaesthesia. One drop of 0.01% levofloxacin ophthalmic solution (Santen Pharmaceuticals Co. Ltd., Osaka, Japan) was applied topically to the treated eye immediately after the intravitreal injection. Vehicle (5% DMSO in PBS) was co-administered with Tm in each mouse. The permeability of the mouse retinal vessels was determined as described [16]. Indicated hours after the intravitreal injection of Tm or vehicle, mice were perfused with 1 ml of PBS containing 100 μg/ml Hoechst 33342 (molecular mass, 616 Da, Sigma-Aldrich, St. Louis, MO) and 20 mg/ml fluorescein isothiocyanate (FITC)-dextran (molecular mass, 2000 kDa, Sigma-Aldrich) under deep anaesthesia with pentobarbital. The isolated retinas were flat-mounted and observed using both an epifluorescence microscope (BX50, Olympus, Tokyo, Japan) fitted with a CCD camera (DP30VW, Olympus) and a confocal microscope (FluoView FV10, Olympus).

All experiments were performed in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research and were approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University.

Cell culture

Conditionally immortalized rat retinal capillary endothelial cell lines [17] were kindly provided by Professor Tetsuya Terasaki (Department of Molecular Biopharmacy and Genetics, Graduate School of Pharmaceutical Sciences, Tohoku University, Japan) and Professor Ken-ichi Hosoya (Department of

Pharmaceutics, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Japan). Endothelial cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin and endothelial cell growth factor (Delia Tech GmbH, Braunschweig, Germany). The cells were maintained at 33°C, which is a temperature at which temperature-sensitive SV40 large T-antigen is activated, in an atmosphere of 95% air and 5% CO_2.

Endothelial monolayer permeability

Endothelial cell suspensions (1×10^4 cells/well) were seeded onto transwell inserts (1.0 μm pore size, 0.3 cm^2 membrane surface area, Millicell hanging cell culture inserts, Japan Millipore Co., Tokyo, Japan) and cultured using the above medium. The culture medium was replaced every other day. Transendothelial electrical resistance (TEER) was measured using a Millicell ERS-2 volt-ohm meter. Blank wells were transwell inserts without endothelial cells. TEER of monolayer was calculated as follows according to the manufacturer's directions: TEER of monolayer ($\Delta\Omega cm^2$) = (sample-well resistance − blank-well resistance) × area of cell monolayer. On the indicated day, the culture media in both inserts and bottom chambers were replaced with fresh culture media supplemented with ER stress inducer or VEGF (PeproTech Inc., Rocky Hill, NJ) and incubated for the indicated hours at 33°C in a CO_2 incubator. After the culture media in inserts and bottom chambers were replaced with fresh media, 70 kDa FITC-dextran (final concentration 100 μg/mL, Sigma-Aldrich), FITC-albumin (final concentration 100 μg/mL, Sigma-Aldrich) or EC-SOD (final concentration 50 ng/mL) were added to transwell inserts and incubated for 4 h at 33°C in a CO_2 incubator. Media were collected from transwell inserts and bottom chambers and the fluorescence intensity of FITC-dextran or FITC-albumin was measured with excitation and emission wavelengths at 485 and 538 nm, respectively. The leakage of FITC-dextran was calculated as follows: Permeability (%) = (FITC-dextran content in bottom chamber/(FITC-dextran content in transwell insert + bottom chamber)) × 100. The concentrations of EC-SOD in media obtained from transwell inserts and bottom chambers were assayed by ELISA [18]. The leakage of FITC-albumin or EC-SOD was calculated similarly to that of FITC-dextran.

PCR analysis

Rat retinal capillary endothelial cells were cultured and treated in a 60-mm culture dish. After treatment with reagents, the cells were washed with cold PBS and the total RNA was extracted from cells with TRIzol reagent (Invitrogen, Carlsbad, CA). The preparation of cDNA and RT-PCR was performed by the method described in our previous report [19] with primers indicated below. The pairs of sequence-specific primers used to measure the mRNA level in rat culture cells were as follows: EC-SOD, F 5'-TCA CCA GAG GAA AAA CGT TC-3', R 5'-AAG CCC TCC AGA TTG AAG GA-3'; VEGF, F 5'-AGA AAG CCC ATG AAG TGG TG-3', R 5'-ACT CCA GGG CTT CAT CAT TG-3'; TNF-α, F 5'-AAA GCA TGA TCC GAG ATG TG-3', R 5'-ATC TGC TGG TAC CAC CAG TT-3'; claudin-5, F 5'-CTG TCT ATG CTC GTC ATC G-3', R 5' -CAT TCC CGA TCT AAT GAC GC-3'; occludin, F 5'-TGT CTG CAG GCA CAC AAG AC-3', R 5'-CCT GTC GTG TAG TCG GTT TC-3'; 78 kDa glucose regulated protein also known as BiP (Bip/GRP78), F 5'-TTT CTG CCA TGG TTC TCA CT-3', R 5'-CCC AGA TGA GTA TCT CCA TT-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), F 5'-ACC ACA GTC CAT GCC ATC AC-3', R 5'-TCC ACC ACC CTG TTG CTG TA-3'.

For assay of the mRNA level in mouse retina obtained from the *in vivo* experiments described above, the retina was homogenized in TRIzol reagent. The preparation of cDNA and RT-PCR was performed by the method described above. The pairs of sequence-specific primers used to measure the mRNA level in mouse tissue were as follows: EC-SOD, F 5'-AGG TGG ATG CTG CCG AGA T-3', R 5'-TCC AGA CTG AAA TAG GCC TCA AG-3'; Cu, Zn-SOD, F 5'-GTG TCA GGA CAG ATT ACA GG-3', R 5'-TTC TCG TGG ACC ACC ATA GT-3'; Mn-SOD, F 5'-ACA ATC TGA ACG TCA CCG AG-3', R 5'-AGT GGG TCC TGA TTA GAG CA-3'; VEGF, F 5'-TGT CTA CCA GCG AAG CTA CT-3', R 5'-CTC TGA ACA AGG CTC ACA GT-3'; TNF-α, F 5'-TAC AGG CTT GTC ACT CGA ATT-3', R 5'-ATG AGC ACA GAA AGC ATG ATC-3'; and GAPDH, F 5'-ACC ACA GTC CAT GCC ATC AC-3', R 5'-TCC ACC ACC CTG TTG CTG TA-3'. mRNA levels of mouse Bip/GRP78, claudin-5 and occludin could be detected with primers prepared for the assay of corresponding rat proteins.

We ascertained that there was a linear correlation between the amounts of PCR products and template cDNA under our PCR conditions. Aliquots of the PCR mixture were separated on 2% agarose gel and stained with ethidium bromide. Densitometric analysis of the PCR products was performed with Multi Gauge V3.0 (Fuji Film, Tokyo, Japan). Each mRNA level was normalized relative to the GAPDH mRNA level in each sample.

Western blotting

Cells were cultured and treated in a 60-mm culture dish. After treatment, the cells were washed with cold

PBS, scraped and lysed in 300 μL of lysis buffer (20 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 1 mM Na_3VO_4, 20 mM β-glycerophosphate, 1 mM phenylmethylsulphonyl fluoride, 1 mM dithiothreitol (DTT), 2 μg/mL leupeptin and 1% Triton X-100) followed by centrifugation at 17 000 × g for 5 min. After centrifugation, the protein concentration of extracts was assayed using a Bio-Rad protein assay reagent. Extracts containing 20 μg of protein were boiled with sample buffer (62.5 mM Tris-HCl, pH 6.8, containing 2% sodium dodecylsulphate (SDS), 10% glycerol, 50 mM DTT and 0.01% bromophenol blue) for 5 min and separated by SDS-PAGE on 12 or 15% (w/v) polyacrylamide gel. After being transferred electrophoretically onto PVDF membranes, non-specific binding sites were blocked with PBS containing 1% bovine serum albumin (BSA). Subsequently, the membranes were incubated with the respective specific primary antibodies (1:1000). After the membranes had been washed three times with PBST (PBS containing 0.1% Tween 20), the blots were incubated with biotin-conjugated goat anti-rabbit or -mouse antibody (1:1000). After the membranes had been washed three times with PBST, the blots were incubated with ABC reagents (Vector Laboratories, Inc., Burlingame, CA) (1:5000). After the membranes had again been washed with PBST three times, the bands were detected using SuperSignal West Pico (Thermo Scientific, Rockford, IL) and imaged using an LAS-3000 UV mini (Fuji Film, Tokyo, Japan).

Data analysis

Data are presented as mean ± SD from at least three experiments. Statistical evaluation of the data was performed using ANOVA followed by post-hoc Bonferroni tests. A p-value of less than 0.05 was considered significant.

Results

Effect of ER stress on the mouse retina

BiP/GRP78 mRNA expression in the retina of mice was significantly increased at 12 h after the intravitreal injection of Tm (0.1 μg/eye) compared with that in the sham treatment, as shown in Figure 1. We observed an increase of VEGF mRNA level in Tm-treated retina, while the TNF-α mRNA level was not changed. The mRNA expressions of SODs and TJ proteins such as claudin-5 and occludin were not different between the two groups.

Effect of ER stress on the permeability of rat retinal endothelial cell layer

Rat retinal endothelial cells that had reached confluence on transwell insert (on day 3) were cultured for an additional 2 days and the barrier functions were evaluated. The TEER increased steadily and reached a steady state at day 3. Effect of ER stress on the permeability of retinal endothelial cell layer was assayed at day 5. TEER values of cells treated with Tg or Tm were time-dependently reduced significantly, as shown in Figure 2A. The permeability of exogenously added FITC-dextran or EC-SOD across the monolayer was significantly increased by the treatment with Tg or Tm in a time-dependent manner (Figure 2B). After the treatment with ER stress inducers for 24 h, the permeability of FITC-dextran or EC-SOD was significantly higher compared to vehicle-treatment (Figure 2C). The ratio of leaked/remaining EC-SOD was less than that of FITC-dextran.

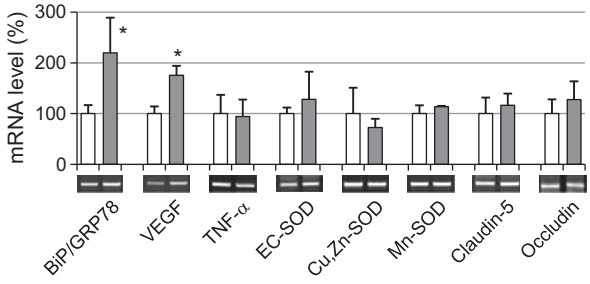

Figure 1. Effect of intravitreous injection of tunicamycin on retinal mRNA levels of various proteins. Mouse retina 12 h after the intravitreous injection of Tm (0.1 μg) was obtained by the method described in Materials and methods, followed by RT-PCR analysis (grey columns). Open columns show values for the sham operation. Each mRNA level was normalized relative to the GAPDH mRNA level in each sample. Data are shown as mean ± SD ($n = 4$). $^*p < 0.05$ vs sham operation.

Effect of ER stress on the expression of tight junction proteins and EC-SOD

The expression of BiP/GRP78 mRNA was significantly increased by the treatment with Tg or Tm for 24 h, as shown in Figure 3A. Induction of C/EBP homologous protein (CHOP) as an ER stress marker was observed by the treatment with Tg or Tm (Figure 3B). The treatment with ER stress inducers resulted in significant elevation of mRNA levels of VEGF and TNF-α. On the other hand, expression of mRNA of claudin-5 was significantly decreased by the treatment with Tg or Tm, while mRNA of occludin was not changed by these reagents. Decrease of claudin-5 protein expression was also confirmed (Figure 3B). The level of EC-SOD mRNA was not changed by the treatment of ER stress inducers. mRNA levels of Cu,Zn-SOD and Mn-SOD were also not changed (data not shown).

Effect of VEGF on the endothelial cell layer

We investigated the effect of exogenously added VEGF on the permeability of rat retinal endothelial

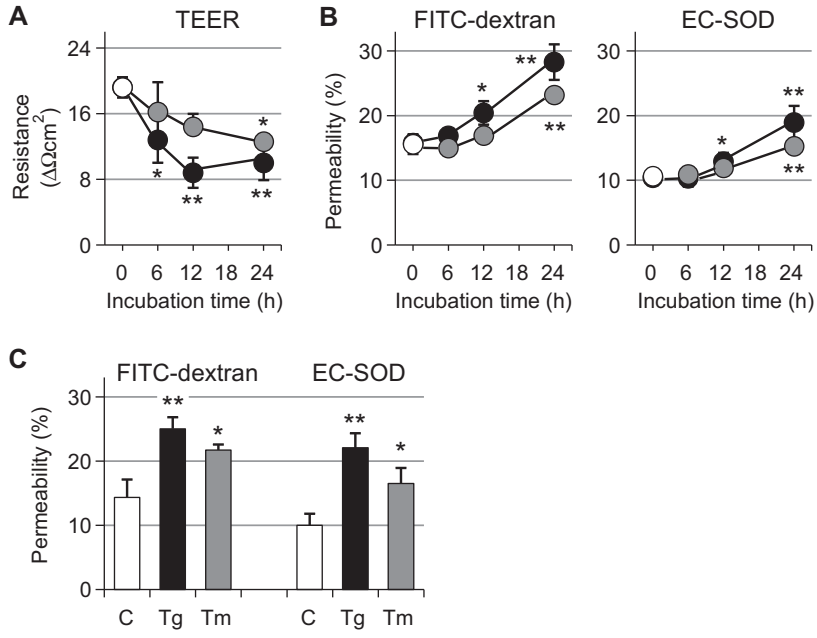

Figure 2. Effect of ER stress on permeability of rat retinal endothelial cells. Rat retinal endothelial cells were treated with 0.1 μM Tg (closed circle) or 2 μg/mL Tm (grey circle) for the indicated hours, followed by the analysis of TEER of endothelial monolayer (A) and permeability of FITC-dextran and EC-SOD (B). Open circle shows the pre-treatment level. Data are shown as mean ± SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$ vs pre-treatment. (C) Rat retinal endothelial cells were treated with 0.1 μM Tg or 2 μg/mL Tm for 24 h, followed by the analysis of permeability of FITC-dextran and EC-SOD. Data are shown as mean ± SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$ vs vehicle-treatment (C).

cell layer and expression of TJ proteins and EC-SOD because VEGF mRNA in Tm-injected mouse retina was elevated, as shown in Figure 1. However, significant changes of TEER value and permeability of FITC-dextran and EC-SOD by the treatment of rat retinal endothelial cells with 10 ng/mL VEGF were not detected, as shown in Figures 4A and B. The expressions of mRNAs of claudin-5, occludin and EC-SOD tended to decrease, but not significantly (Figure 4C). The expressions of mRNAs of BiP/GRP78, VEGF and TNF-α were also not changed (data not shown).

Effect of 4-phenylbutyric acid on the endothelial cell layer

Chemical chaperone such as 4-phenylbutyric acid (PBA) is known to be an ER stress inhibitor because it can stabilize the protein conformation and improve the fording capacity of ER [3,20,21]. Thus, we subsequently investigated whether PBA could attenuate the ER stress-induced retinal endothelial permeability. As shown in Figure 5A, the down-regulation of claudin-5 by the treatment with Tg or Tm was significantly suppressed by the pre-treatment with 5 mM PBA for 1 h. Moreover, the pre-treatment with PBA significantly attenuated the decrease of TEER (Figure 5B) and permeability of exogenously added FITC-albumin or EC-SOD across the endothelial monolayer (Figure 5C).

Effect of ER stress on the permeability of mouse retinal blood vessels in vivo

The permeability of the retinal blood vessels in mice treated with Tm was evaluated by *in vivo* tracer experiments using FITC-dextran (2000 kDa) and Hoechst 33342 (616 Da). As shown in Figure 6A, the injected FITC-dextran was detected in the vascular lumen with minimal leakage from blood vessels. The extravasation of the Hoechst 33342 dye was detected at 72 h after the intravitreal injection of Tm. On the other hand, there were no significant changes between Tm and vehicle-treatment at 24 h after the injection. Extensive nuclear staining of surrounding retinal cells by the extravasated Hoechst 33342 was noted at 72 h after the Tm-intravitreal injection, whereas the stained nuclei were localized only in the vicinity of vascular lumen by the vehicle-treatment (Figure 6B).

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. EC-SOD is the major antioxidative enzyme in extracellular space and is widely distributed in blood vessel

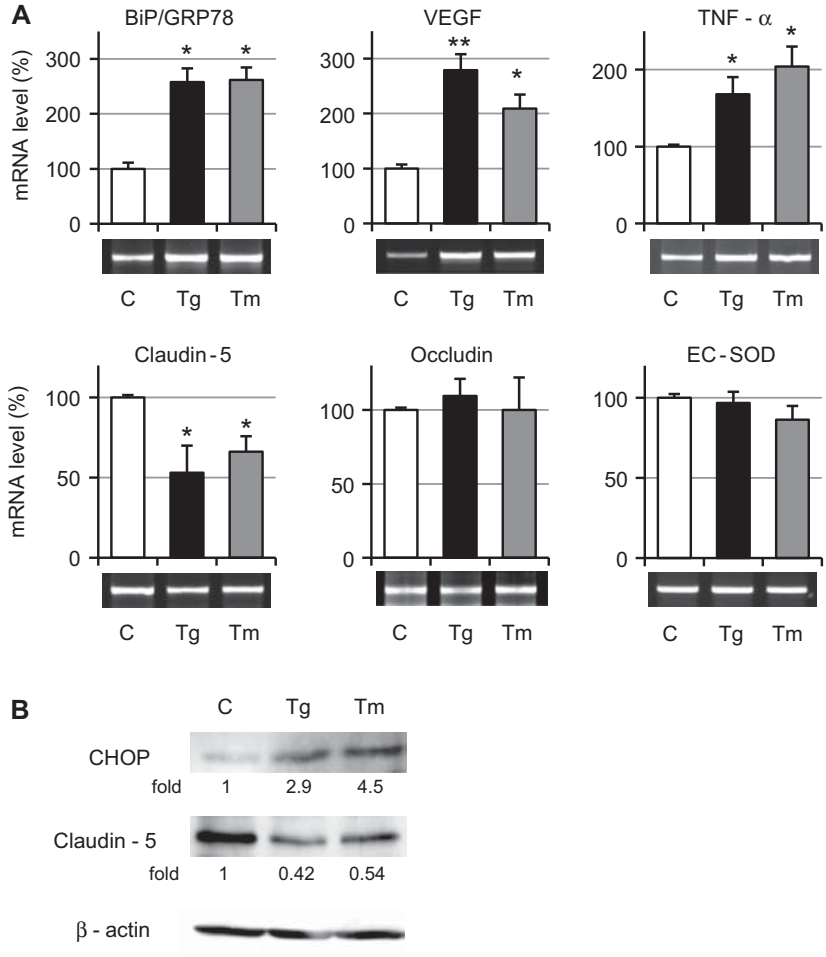

Figure 3. Effect of ER stress on expression of pro-inflammatory proteins, TJ proteins and EC-SOD. (A) Rat retinal endothelial cells were treated with 0.1 μM Tg or 2 μg/mL Tm for 24 h, followed by RT-PCR analysis. The mRNA levels were normalized with the GAPDH mRNA level in each sample. Data are shown as mean ± SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$ vs vehicle (C). (B) Rat retinal endothelial cells were treated with 0.1 μM Tg or 2 μg/mL Tm for 24 h, followed by Western blotting. The protein levels were normalized with the β-actin level in each sample. Values are fold change compared with that of vehicle (C).

walls, including large amounts in the intima [22]. 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 [23]. EC-SOD expression in fibroblasts and smooth muscle cells is known to be regulated by numerous substances such as cytokines, vasoactive factors and growth factors [24–26]. Moreover, we observed a decrease in EC-SOD expression in some cell lines under hypoxia [27,28]. DR is a major complication in patients with diabetes and can lead to vision impairment in a large proportion of diabetic patients. Several inter-related pathways, such as oxidative stress and ER stress, have been shown to contribute to DR [2,11]. From these reports, it is speculated that the impairment of EC-SOD function in vascular system under ER stress contributes to the progress of DR.

In a previous report, we described that the intravitreal concentration of EC-SOD was significantly higher in PDR patients (58.0 ± 23.8 ng/mL, mean ± SD, $n = 14$) than in MH patients (29.3 ± 6.6 ng/mL, $n = 14$) as controls [14], whereas the mechanisms causing the increase in intravitreal EC-SOD in the PDR are completely unknown. On the other hand, the serum levels of EC-SOD were not significantly different between the PDR group (85.3 ± 18.4 ng/mL, $n = 9$) and the MH group (85.0 ± 12.3 ng/mL, $n = 9$). We noted that the EC-SOD level in vitreous body was still lower than the serum EC-SOD level, even in PDR patients. On the other hand, the VEGF level in vitreous body of PDR patients (798.2 ± 882.7 pg/mL, $n = 14$) was much higher than that in MH controls (17.7 ± 15.5 pg/mL, $n = 14$). This observation of intravitreal VEGF level is consistent with studies that originally found that the level of VEGF is increased in vitreous body of PDR patients [29,30]. In this study, we investigated the reasons why the intravitreal EC-SOD level increases in PDR patients by the investigation of two possibilities: first, change of EC-SOD expression in

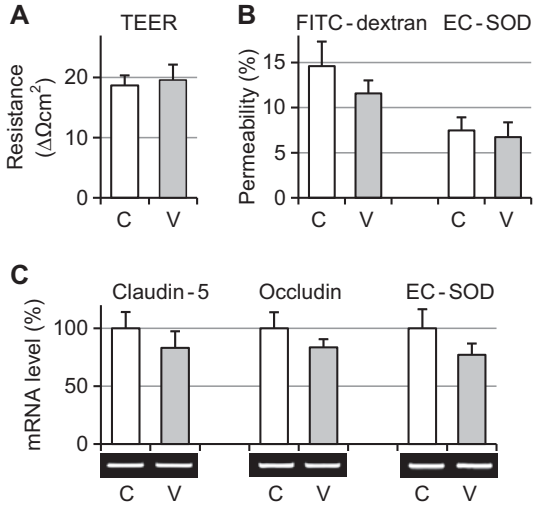

Figure 4. Effect of VEGF on function of rat retinal endothelial cells. Rat retinal endothelial cells were treated with 10 ng/mL rat VEGF (V) or vehicle (C) for 24 h, followed by the analysis of TEER of endothelial monolayer (A), permeability of FITC-dextran and EC-SOD (B) and RT-PCR analyses of claudin-5, occludin and EC-SOD (C). The mRNA levels were normalized with the GAPDH mRNA level in each sample. Data are shown as mean ± SD ($n = 3$).

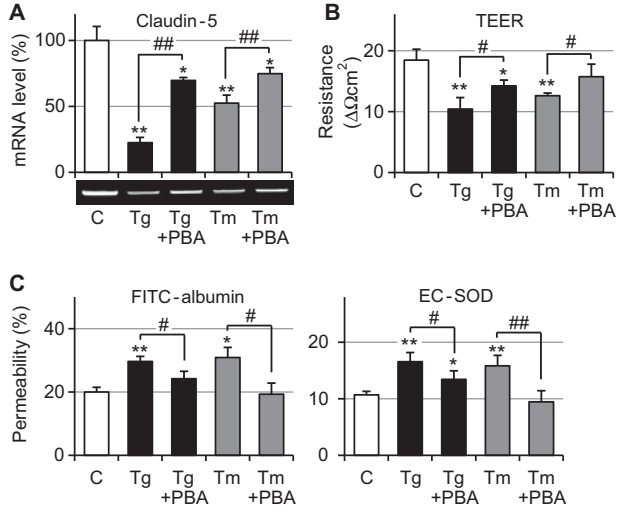

Figure 5. Effect of pre-treatment with PBA on ER stress-induced endothelial permeability. (A) Rat retinal endothelial cells were pre-treated with or without 5 mM PBA for 1 h and then treated with 0.1 μM Tg or 2 μg/mL Tm for 24 h. Claudin-5 mRNA was determined by RT-PCR. The mRNA levels were normalized with the GAPDH mRNA level in each sample. Data are shown as means ± SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$ vs vehicle (C), $^{\#\#}p < 0.01$ vs Tg or Tm only-treated cell. The cells in transwell inserts were pre-treated with or without 5 mM PBA for 1 h and then treated with 0.1 μM Tg or 2 μg/mL Tm for 24 h, followed by analyses of TEER (B) and permeability of FITC-albumin and EC-SOD (C). Data are shown as means ± SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$ vs vehicle (C), $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs Tg or Tm only-treated cell.

retina; secondly, leakage of EC-SOD in plasma through retinal endothelial monolayer by increase of vascular permeability, with *in vivo* or *in vitro* experiments under ER stress as a pathological condition of DR.

We observed a significant increase of retinal BiP/GRP78 mRNA level accompanied with VEGF elevation in mice injected with Tm into the vitreous body as an *in vivo* model of ER stress-induced retinal injury [3,15]. However, the level of EC-SOD mRNA in retina from Tm-injected mice was not different from that of the sham operation group. No significant change of EC-SOD mRNA was also observed in retinal endothelial cells treated with Tg or Tm as an *in vitro* experiment, whereas the BiP/GRP78 mRNA and CHOP protein levels were significantly elevated again. It is well known that ER stress is involved in the injury of vascular cells in DR via the induction of VEGF [2,3,8]. In this experiment, we also observed that the treatment with ER stress inducers significantly increased the mRNA levels of VEGF in both *in vivo* and *in vitro* experiments. However, exogenous addition of VEGF to retinal endothelial cells could not induce EC-SOD, similarly to the addition of ER stress inducers. From these results, the high level of EC-SOD in the vitreous body of DR patients is not associated with the up-regulation of EC-SOD expression in the retina.

In diabetic eyes, retinal vascular permeability is significantly increased, which leads to macular oedema and finally retinopathy [31,32]. Elevation of retinal vascular leakage of albumin and decreased TJ protein level in retina were observed in db/db rodent type 2 diabetes mice [33]. The blood–retinal barrier (BRB) is formed by the endothelial cells of retina, providing a dynamic interface between the peripheral circulation and the retinal nervous system. TJ between vascular endothelial cells creates the BRB and is an intricate complex of transmembrane proteins such as occludin, claudins and junction adhesion molecules (JAMs). Breakdown of the retinal TJ is a major causative factor in DR and correlates with macular oedema leading to vision loss [34]. Of these molecules, occludin and claudin are the most extensively studied and several studies have shown that claudin contributes to create a barrier with a major function compared with that of occludin [16,35,36]. In this study, we observed that expression of claudin-5 but not occludin was decreased by the treatment with ER stress inducers. The leakage of FITC-dextran as a marker of permeability through the endothelial monolayer was significantly elevated by the treatment with Tg or Tm accompanied with a decrease of TEER. The permeability of exogenously added EC-SOD was almost proportional to that of FITC-dextran. Moreover, we confirmed that the permeability of FITC-labelled albumin was also significantly elevated by the treatment with ER stress inducers. The result that the ratio of leaked/remaining FITC-albumin was more than that of EC-SOD may be due to the difference of molecular weight between

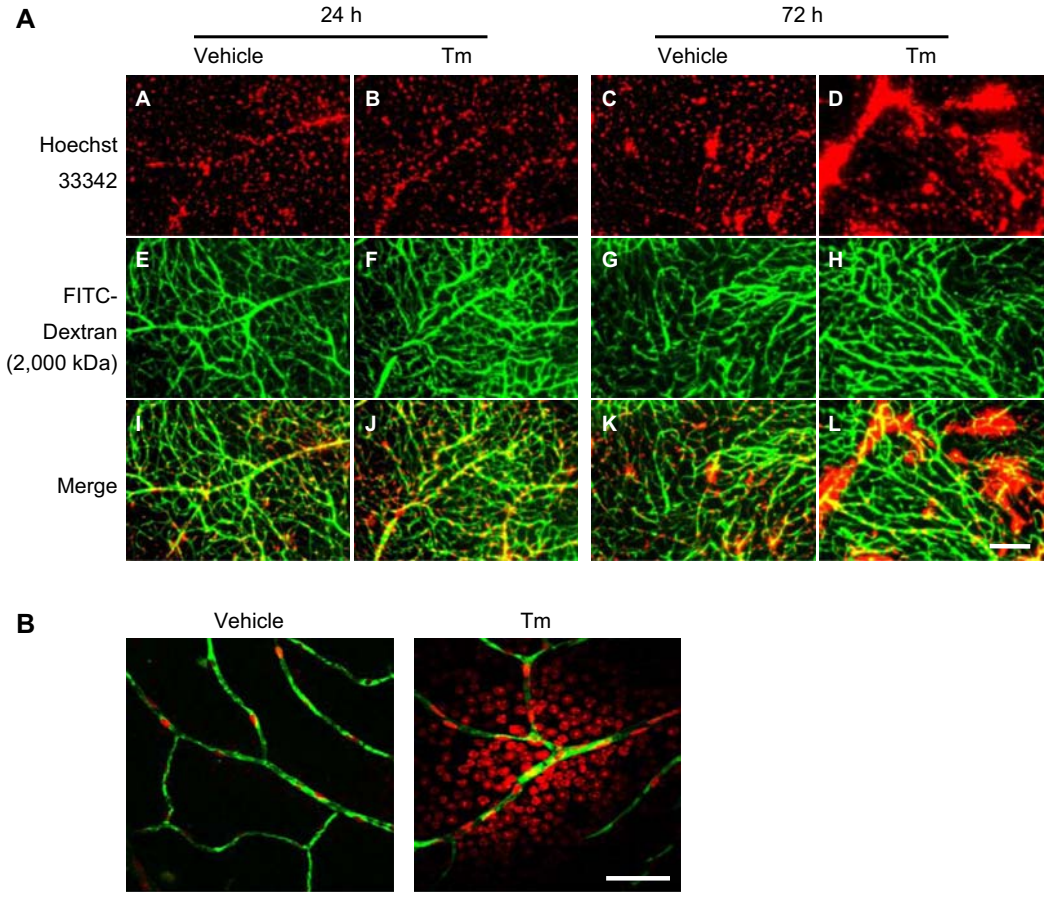

Figure 6. Effect of intravitreous injection of tunicamycin on the permeability of mouse retinal blood vessels *in vivo*. (A) Hoechst 33342 stain (A–D) and FITC-dextran (2000 kDa) (E–H) were detected at 24 h or 72 h after the intravitreous injection of Tm (1 μg/eye) or vehicle by the method described in Materials and methods. Merged views (I–L) of the signals of Hoechst 33342 and FITC-dextran are presented. The scale bar represents 200 μm. (B) Confocal fluorescence micrographs show higher-magnification versions of part of the vehicle or the Tm-treated mouse retinal blood vessels at 72 h after the intravitreous injection. The scale bar represents 50 μm.

them. It was reported that the leakage of albumin from blood vessels into the retina was higher in db/db diabetic mice [33]. These results suggest that plasma EC-SOD and other proteins leak out by the elevation of vascular permeability. PBA is a chemical chaperone that can inhibit ER stress. The pre-treatment with PBA significantly attenuated the increase of permeability of FITC-albumin or EC-SOD and decrease of TEER, which were induced by the treatment with Tg or Tm. The result that the down-regulation of claudin-5 by Tg or Tm was suppressed by PBA is consistent with the above observation.

Finally, the change of retinal endothelial permeability by Tm-treatment was assessed *in vivo* by using Hoechst 33342 and FITC-dextran as tracers. The extravasation of the injected Hoechst stain was detected in the retina of Tm-treated mice. In contrast, FITC-dextran (2000 kDa), a large molecular weight tracer, remained inside the vessels. Thus, the barrier function of blood vessels was selectively disrupted by ER stress.

DR has recently been recognized as a chronic inflammatory disease [37]. The inflammation process is initiated by endothelial cell activation comprising secretions of pro-inflammatory cytokines (TNF-α, interleukin-6, monocyte chemoattractant protein-1 and others) and up-regulation of cell adhesion molecules (intercellular and vascular cell adhesion molecules, P-selectin and others). It has been reported that over-expression of EC-SOD significantly reduced the expression of the above inflammatory molecules [38]. It is known that the vascular endothelial glycocalyx contributes to endothelial barrier formation [39]. EC-SOD is produced and secreted from vascular cells and binds to the endothelial cells by the interaction of C-terminal basic amino acids with anionic charged residues in glycocalyx [40]. It is possible that the disruption of glycocalyx weakens the ability to retain EC-SOD around the endothelial cell surface and facilitates leakage through the BRB.

Taken together, the present results suggest that ER stress leads to the down-regulation of mainly claudin-5 among TJ proteins, the elevation of endothelial permeability and leakage of EC-SOD. Our previous observation that the intravitreal concentrations of EC-SOD in PDR patients were significantly

higher than those in controls may depend on the enhancement of EC-SOD leakage through the retinal endothelial monolayer by an increase of vascular permeability. The decrease of EC-SOD on the endothelial cell surface and elevation of pro-inflammatory factors in the retinal vascular system might induce and/or promote intraretinal microvascular impairment and finally lead to 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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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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