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Tunicamycin-induced photoreceptor atrophy precedes degeneration of retinal capillaries with minimal effects on retinal ganglion and pigment epithelium cells

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Abstract

Endoplasmic reticulum (ER) stress is recognized as a contributing factor to various ocular neurovascular pathologies including retinitis pigmentosa, glaucoma, and diabetic retinopathy (DR). ER stress in particular is implicated in the development of DR, which is significantly influenced by inflammation driven retinal vascular degeneration and dysfunction. Ultimately, loss of vision occurs if left untreated. However, the identity of the target cells and their temporal involvement in diabetes-mediated dysfunction need further investigation. Early diabetes-induced stress in photoreceptor cells is proposed as the driver of inflammatory mediated neurovascular changes during diabetes. Although tunicamycin induced ER stress results in photoreceptor loss, its consequences for retinal vascular degeneration and retinal ganglion (RGC) and pigment epithelium (RPE) cell loss remains unclear. Here we show intravitreal delivery of tunicamycin primarily induced ER stress in photoreceptor cells resulting in their loss by apoptosis. This was concomitant with induced expression of the unfolded protein response marker CHOP in these

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cells. We also demonstrated significant degeneration of retinal capillaries following the loss of photoreceptor cells with minimal impact on loss of RGC and RPE cells. However, activation of retinal microglial and Muller cells were noticeable. Thus, our data support the notion that ER stress mediated dysfunction and/or loss of photoreceptor cells in response to inflammation and oxidative stress could precede retinal vascular and neuronal dysfunction and degeneration.

Keywords

Diabetic retinopathy; Retinal vasculature; Inflammation; Retinal degeneration

1. Introduction

Ocular complications of diabetes including diabetic retinopathy (DR) are associated with vision impairment and blindness in middle-aged people (Duh et al., 2017; Gardner et al., 2002). Although DR is a major consequence of chronic hyperglycemia, the pathogenesis of DR remains poorly understood. The majority of investigations have focused on diabetes-mediated vascular dysfunction, including endoplasmic reticulum (ER) stress and inflammation, as the primary insult in the onset and progression of DR. However, recent studies indicate a vital role for changes in the neuroretina, mainly retinal ganglion cells (RGC), that could contribute to and/or precede detected vascular damage during diabetes (Barber et al., 2011). How these stress mediated cellular changes are brought about and in what order these changes occur remains unclear and is the focus of current study.

Specialized retinal neuronal cells, namely photoreceptors, have been recently implicated as the primary source of inflammation and oxidative stress in the onset and advancement of diabetes ocular complications (Kern and Berkowitz, 2015), as well as proper retinal vascularization (Fu et al., 2018). Although diabetes induced degeneration of retinal capillaries in wild type mice, in mice lacking photoreceptors diabetes did not result in capillary degeneration (Du et al., 2013). These data suggest that the diabetes impact on retinal vasculature is indirect, and could occur through metabolic dysfunction and/or loss of photoreceptor cells (Fu et al., 2018; Liu et al., 2016; Tonade et al., 2016). Furthermore, a survey of patients with diabetes, who also had retinitis pigmentosa (lacked photoreceptors), found that these patients had less retinopathy than the diabetic patients without retinitis pigmentosa (Chen et al., 2012). Thus, there is great interest in determining the contribution of these neurovascular alterations to the pathogenesis of DR and susceptibility to retinopathy of prematurity.

A number of studies have demonstrated a role for ER stress in retinal neurovascular changes during diabetes (Elmasry et al., 2018; Kroeger et al., 2018; Li et al., 2011; Ma et al., 2014; Oshitari et al., 2008; Yang et al., 2017; Zhang et al., 2015). However, the major cellular source of this ER stress and its contribution to neurovascular dysfunction and retinal vascular degeneration is not well characterized. ER stress is observed in the retinal blood vessels and its cellular components during diabetes (Adachi et al., 2011; Li et al., 2011; Li et al., 2012; Li et al., 2009), and in human photoreceptor diseases retinitis pigmentosa and achromatopsia (Chan et al., 2016). However, whether ER stress mediated loss of

photoreceptor cells drives retinal neurovascular dysfunction or the reported ER stress in the retinal neurovasculature drives loss of photoreceptor cells and retinal neurons remains unknown.

Here we investigated whether ER stress could contribute to retinal neurovascular changes, and whether toxicity in retinal neuronal cells or ER stress drive retinal capillary degeneration or the other way around. As previously demonstrated, we showed tunicamycin causes ER stress in the retina, more specifically in the photoreceptor cells, which was followed by the degeneration of retinal capillaries. These changes mimic the early non-proliferative changes observed during diabetes including the formation of acellular capillaries, and were concomitant with activation of microglial and Muller cells. Tunicamycin treatment resulted in retinal vascular degeneration without a significant effect on RGC and RPE cell integrity. We observed a significant loss of photoreceptor cells with tunicamycin treatment prior to retinal vascular degeneration. These findings support a model whereby retinal photoreceptor stress drives retinal capillary degeneration, and is in line with the recently suggested role of hyperglycemia in photoreceptor cell oxidative stress, inflammation, and neurovascular dysfunction.

2. Material and methods

2.1. Animals

All animal studies were performed following the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and approved by the Animal Care and Use Committee of the University of Wisconsin School of Medicine and Public Health (Assurance number D16–00239). Six-week-old C57BL/6j mice were used in these studies and housed on a 12-hour light–dark cycle, without food and water restrictions. C57BL/6J and CX3CR1$^{gfp/gfp}$ knock in mice were obtained from Jackson Laboratories and a colony was established in our laboratory. All the mice were on C57BL/6J background, and free of retinal degeneration mutations. Both male and female mice were used. Each group was consisted of at least five mice.

2.2. NMDA and tunicamycin induced retinal damage

N-methyl-D-aspartic acid (NMDA) or tunicamycin mediated retinal damage was produced by intravitreal injection of these compounds. Briefly, mice were anesthetized with ketamine hydrochloride (100 mg/kg) and xylazine (10 mg/Kg), Body temperature was maintained between 37.0 and 37.5°C with the aid of a heating pad. For NMDA (Cat# M3262; Sigma, St. Louis, MO) injections, 2 μl of a 40 mM solution of NMDA prepared in balanced salt solution (BSS; Alcon, Fort Worth, TX) was delivered by intravitreal injection into both eyes. Control animals received the vehicle only, BSS. Tunicamycin (T7765; Sigma) was prepared in dimethyl sulfoxide (1 μg/μl; D8418; Sigma). For tunicamycin injections, the stack solution was further diluted into 0.025 μg/μl or 0.05 μg/μl in BSS and 2 μl of solution was injected into both eyes. Vehicle control group receive a BSS-DMSO mixture (2 μl) representing the higher tunicamycin dose (0.05 μg/μl). Intravitreal injections were carried out using a Harvard pump (Harvard Apparatus, Holliston, MA) and pulled-glass capillaries. Each glass capillary was calibrated to deliver 2 μL of vehicle or test compound using a foot

switch. The pupils of anesthetized mice were dilated using a drop of tropicamide (1%). For intravitreal injections, the sharpened tip of the glass capillary was passed through the sclera, just behind the limbus, into the vitreous cavity. The foot switch was then depressed to inject the test compounds. The mice were sacrificed at different time points post injection and the eyes were enucleated for different analysis as described below.

2.3. Trypsin-digest preparations

Eyes were fixed in 4% paraformaldehyde for at least 24 h, and bisected equatorially to remove the entire retina. Retinas were washed in distilled water overnight, and incubated in 3% trypsin (Trypsin 250, Cat# 215240; Difco, Fisher Scientific, Maple Grove, IL) for approximately 1–1.5 h at 37°C. The trypsin solution was prepared by dissolving the desired amount of trypsin in 0.1 M Tris, 0.1 M maleic acid, pH 7.8 containing 0.2 M sodium fluoride (NaF; Cat# 201154; Sigma). Following digestion, retinal vessels were flattened by four radial cuts, mounted on glass slides, and subjected to Periodic Acid-Schiff (PAS) and hematoxylin staining.

2.4. Quantification of degenerated blood vessels

The slides with retinal blood vessels prepared above were used for these quantifications. Degenerated capillaries were quantified in the whole retina (x200 magnification) in a masked fashion. Degenerated capillaries were identified as capillary-sized ghost vessels having no nuclei anywhere along their length. Ghost vessels with a diameter <20% of the diameter of adjacent capillaries were identified as strands and were not counted.

2.5. Histological analysis

Eyes from mice with different treatments were fixed in 10% formalin for 24 h, and paraffin embedded for sectioning. Serial sections (6 μm), separated by at least 40 μm, were obtained from the vicinity of the optic nerve. The hematoxylin and eosin (H&E) stained sections were evaluated in masked fashion for structural organization and alterations with various treatments.

2.6. Retinal wholemount immunostaining

Mice treated with tunicamycin or NMDA, and untreated controls were sacrificed, eyes were removed, and fixed in 4% paraformaldehyde, 4 min on ice. This was followed by fixing in methanol for at least 24 h at −20°C. Following fixation, retinas were removed in phosphate buffered saline (PBS; Cat# D1408; Sigma) and washed with PBS three times, 10 min each. The retinas were then incubated in a blocking buffer (50% fetal calf serum and 20% normal goat serum in PBS) for 1 h. Following blocking, the retinas were incubated with rabbit anti-mouse collagen IV antibody (Cat# AB756P; Chemicon, Temecula, CA; diluted 1:500 in blocking buffer) at 4°C overnight. Retinas were then washed three times with PBS, 10 min each, and incubated with secondary antibody Alexa Fluor 594 goat-anti-rabbit (Cat # A-11037; Invitrogen, Carlsbad, CA; diluted 1:500 in blocking buffer) for 2 h at room temperature. Following incubation, retinas were washed three times with PBS, 10 min each, and mounted on a slide with PBS/glycerol (2 vol/1 vol). Retinas were examined using a

Zeiss fluorescence microscope and images were captured in digital format (Zeiss, Chester, VA).

2.6. RPE ZO-1 wholemount immunostaining

Mice treated with tunicamycin (0.05 μg) or solvent control were sacrificed after 3 days of injection, eyes were removed, and fixed in 4% paraformaldehyde (1.5 h on ice). The anterior segment and retina were dissected under a dissecting microscope. During the removal of retina special attention was paid to avoid damaging the underlying RPE. The remaining RPE-choroid-sclera complex was relaxed by four radial incisions, and was washed with PBS three times, 10 min each. Following incubation in a blocking buffer (see above) for 1 h, the samples were incubated with rabbit anti-mouse ZO-1 antibody (Cat # 61–7300; Invitrogen; diluted 1:500 in blocking buffer) at 4°C overnight. Samples were then washed three times with PBS, 10 min each, and incubated with the secondary antibody Alexa Fluor 594 goat-anti-rabbit (Cat # A-11037; Invitrogen; diluted 1:500 in blocking buffer) for 2 h at room temperature. Following incubation, samples were washed three times with PBS, 10 min each, and mounted on a slide using PBS/glycerol (2 vol/1 vol). RPE cells were viewed using a Zeiss fluorescence microscopy and images were captured in digital format using (Zeiss, Chester, VA).

2.7. TUNEL staining

To detect retinal cell apoptosis induced by tunicamycin, TUNEL staining was conducted using the DeadEnd™ Fluorometric TUNEL System according to the manufacturer's protocol (Cat# G3250; Promega, Madison, WI USA). Mice were sacrificed at desired times after intravitreal injection of tunicamycin or solvent control, eyes were enucleated, fixed overnight in 4% paraformaldehyde, embedded in paraffin, sectioned, and paraffin embedded tissue slides stored at room temperature. For TUNEL staining, the tissue was first treated with decreasing concentrations of ethanol to remove paraffin (100%, 95%, 85%, 70%, and 50%). The slide samples were incubated with 20 μg/ml Proteinase K solution at room temperature (RT), washed with PBS, and incubated with TdT reaction mix for 60 min at 37°C in a humidified chamber. The reaction was stopped by adding 2X SSC, and followed by adding ProLong™ Gold Antifade Mounting medium with DAPI (Cat# P36935; Invitrogen) to slides. The slide were examined using a confocal fluorescence microscop (Nikon Eclipse 50i fluorescence microscope) for detection of green fluorescent apoptotic cells and DAPI-stained nuclei.

2.8. Double antibody and TUNEL staining

Paraffin was removed from embedded eye sections on slides as described above. The slides were stained with TUNEL first as above, without adding the mounting medium. The slides were then blocked with 5% goat serum for 2 h at RT, incubated with 1:100 diluted primary antibody overnight at 4°C, washed with PBS and incubated with florescence labeled goat secondary antibody for 1 h at RT. The specimens were washed in PBS, and mounted using ProLong™ Gold Antifade Mounting medium with DAPI (Invitrogen). The antibodies used were mouse-anti-CHOP (Cat#2895, Cell Signaling, Danvers, MA), and rabbit anti-RBPMS (Cat# ABN1362, Sigma). The secondary Ab was goat anti-rabbit IgG (H+L) highly cross-

adsorbed secondary antibody, Alexa Fluor 594 (Cat# A11037, Invitrogen). Images were obtained in digital format using a Nikon Eclipse 50i fluorescence microscope.

2.9. Immunostaining of frozen sections

Eyes from animals treated with various test compound or solvent control were embedded in Optimal Cutting Temperature (OCT) compound at −80°C. Eyes were sectioned (9 μm) using a cryostat, sections were placed on glass slides, and allowed to dry for 2 h. For immunostaining, sections were fixed in cold acetone (4°C) on ice for 10 min, followed by three washes in PBS, 5 minutes each. Slides were incubated in blocker solution (1% BSA, 0.2% skim milk, and 0.3% Triton X-100 in PBS) for 15 minutes at room temperature, and then incubated with rabbit anti-mouse-GFAP (Cat#: Z0334; DAKO, Denmark; 1:500 dilution) overnight at 4°C in humid environment. After three washes in PBS, 5 min each, samples were incubated with appropriate secondary antibodies for 2 h at room temperature (Jackson Immunoresearch, West Grove, PA; 1:500 dilution). As a control, some sections were incubated only with secondary antibody. Sections were washed three times in PBS, covered with PBS: glycerol (2 vol/1 vol), and mounted with a coverslip. Retina sections were examined using a Zeiss fluorescence microscope and images were captured in digital format.

2.10. Statistical analysis

Data in figures represent means ± SE from animals with $n \geq 5$ (as indicated in figure legends). Student's unpaired *t*-test (2-tailed) or one-way ANOVA with post-Bonferroni test for multiple comparisons were used for statistical evaluations between control and treated samples. $P \leq 0.05$ was considered significant. All data analysis was done in GraphPad Prism or Microsoft Excel.

3. Results

3.1. Tunicamycin mediated ER stress promotes retinal capillary degeneration in a dose dependent manner

An increase in the number of degenerating capillaries are noted in the retinal vasculature with diabetes progression. Available rodent diabetes models, however, show limited levels of acellular capillaries, especially with short duration of diabetes. This makes the detailed study of underlying mechanisms challenging. ER stress, as a risk factor, is known to contribute to retinal vascular degeneration during diabetes (Elmasry et al., 2018; Li et al., 2011; Li et al., 2012). Here to investigate ER stress mediated capillary degeneration, various doses of tunicamycin, a known ER stress inducer, were delivered by intravitreal injection. We assessed the formation of acellular capillaries two weeks after tunicamycin injection by preparing retinal trypsin digests. A significant increase in the number of degenerating capillaries was noted in both tunicamycin treatment groups compared with vehicle controls (Fig. 1). More degenerating capillaries were observed with increasing the dose of tunicamycin from 0.05 mg to 0.1 mg. Thus, the degree of capillary degeneration with tunicamycin was dose-dependent.

3.2. Thinning of the photoreceptor layer, but not RGC layer, following tunicamycin treatment

In diabetic retinopathy studies accumulating data suggest a new role for photoreceptor cell dysfunction and/or loss in the outer retina and the initiation of the degenerative vascular lesions associated with the early stages of DR (Du et al., 2013). We next determined the integrity and organization of the photoreceptors in the tunicamycin induced ER stress mice. Tunicamycin has previously been shown to specifically induce photoreceptor loss (Fliesler et al., 1984). The retinal damage induced by tunicamycin was evaluated by H&E staining of histological sections prepared from mice receiving vehicle and those receiving tunicamycin. A dramatic loss of photoreceptors in eyes injected with tunicamycin was observed compared with vehicle control, which was dose dependent. In the 0.1 μg tunicamycin group, nearly 80% of photoreceptors were uniformly lost throughout the retina. Interestingly, no morphological evidence of disruption was observed in other retinal layers. More specifically, tunicamycin did not cause RGC loss. Thus, photoreceptor loss and capillary degeneration are closely linked in mice receiving tunicamycin (Fig. 2), without significantly affecting RGC. These observations are consistent with the resistance of RGC to adverse effects of photoreceptor loss in retinal degeneration models (Lin and Peng, 2013; Mazzoni et al., 2008).

To further explore the potential contribution of RGC loss to retinal capillary degeneration, mice received NMDA (40 μM) through intravitreal injection, a known inducer of RGC loss. Fig. 2 shows that the RGC layer was intact in tunicamycin treated eyes and did not show a decrease in number of RGC nuclei with the doses of tunicamycin used here. However, as expected in the NMDA treated eyes, the RGC layer was dramatically and uniformly affected and showed loss of RGC throughout the retina. Thus, RGC are more resistant to tunicamycin mediated ER stress compared with photoreceptor cells.

To confirm the status of RGC that was observed by H&E staining, we prepared flatmounts of the eyes from various treatment groups and stained with anti-γ-tubulin to assess the integrity of RGC. Tubulin positive cell density and organization did not differ between vehicle or tunicamycin treated mouse eyes, in striking contrast to the disruption observed in the NMDA treated mice (Fig. 3). These results further confirmed that tunicamycin did not induce RGC loss, while NMDA dramatically decreased the density of tubulin positive RGC.

3.3. Tunicamycin treatment induces apoptosis in the photoreceptors but not RGC

The results described above suggested that tunicamycin spared the RGC layer. To confirm which cells were being lost and whether apoptosis was involved, we examined retinas at earlier time points after tunicamycin treatment by TUNEL staining, along with an antibody to RNA-binding protein with multiple splicing (RBPMS), a RGC marker (Kwong et al., 2010; Rodriguez et al., 2014) (Fig. 4). At 3 and 6 days post injection, no TUNEL positive cells were detected among RGC. The RGC were identified by positive staining for RBPMS. In addition, the staining with RBPMS antibody showed uniform expression in RGC under all conditions with s modest variation in staining intensity among RGC.

In contrast to RGC, a significant number of TUNEL positive cells were uniformly present among photoreceptor cells throughout the retinal ONL. The TUNEL positive cells were not detected in the control solvent treated samples. Thus, these results indicated that tunicamycin did not induce RGC apoptosis, but induced apoptosis in photoreceptor layer neurons.

To determine whether the loss of photoreceptors occurred prior to retinal vasculature degeneration, we prepared trypsin digests from retinas subjected to tunicamycin for three days. At this time, photoreceptor loss is minimal, but apoptosis is well underway (Fig. 4). We observed no significant changes in the number of degenerating capillaries in eyes from mice that received intravitreal tunicamycin after three days compared with solvent control (Fig. 5). Thus, degeneration of retinal vasculature occurs following the initiation of apoptosis in photoreceptor cells and their loss.

3.4. Increased CHOP expression in the apoptotic photoreceptor layer

The unfolded protein response (UPR) is a homeostatic response that allows cells to tolerate ER stress. However, chronic or severe ER stress results in the induction of the pro-apoptotic UPR target gene CHOP (CCAAT-enhancer-binding protein homologous protein). To determine if the apoptosis in the photoreceptor cell layer reflects a proapoptotic UPR induction, the retina samples from 3 days and 6 days post tunicamycin injection were TUNEL and anti-CHOP stained. Fig. 6 shows that 3 days post injection, the majority of TUNEL positive (apoptotic) cells in the photoreceptor layer were also CHOP positive. There were no TUNEL staining or CHOP positive cells in the RGC layer as expected. By day 6, there were fewer TUNEL positive cells and much lower CHOP expression (Fig. 4 and 6), possibly reflecting cell loss and/or CHOP downregulation at this time point. Please also note the co-localization seen in the ONL is not due to bleeding from one channel to the other. The only co-localization seen is in the ONL indicating TUNEL positive cells, which are also positive for CHOP. Thus, photoreceptor cells demonstrate CHOP positive apoptotic loss, which precedes the degeneration of the retinal vasculature.

3.5. Activation of ER stress results in activation of microglial and Muller cells with minimal effect on RPE cell integrity

Since cell death can cause gliosis (Subirada et al., 2018; Winkler et al., 2000), we next investigated whether tunicamycin induced ER stress results in activation of microglial cells, as seen with degenerative retinal diseases with an inflammatory component (Al-Shabrawey et al., 2008; Arroba and Valverde, 2017; Brucklacher et al., 2008; Devi et al., 2012; Ibrahim et al., 2010; Nguyen et al., 2012). Using CX3CR1$^{gfp/gfp}$ knock in mice, a mouse prone to inflammation and retinal degeneration (Eandi et al., 2016; Lavalette et al., 2011a), we found an increase in the number of microglial cells that was concomitant with morphologic changes (Fig. 7A, B). Microglial cells exhibit a ramified morphology with long processes during the resting state. These processes appeared to retract, resulting in an amoeboid morphology indicating their activation in tunicamycin-injected retinas 14 days post treatment.

We next explored the GFAP expression changes in the eyes from tunicamycin treated mice. Retinas from tunicamycin treated mice had activated Muller cells, as evident by enhanced GFAP staining observed across the whole retinal layers. These results are consistent with an ocular inflammatory environment and gliosis (Fig. 7C, D). To determine whether ER stress affects RPE cell integrity and function, we prepared wholemount scleral/RPE flat mounts and stained them with anti-ZO-1. Normally ZO-1 staining exhibits a smooth and uniform localization at the cell-cell junction of RPE cells and can be used to assess changes in size and shape of the cells and function (Georgiadis et al., 2010; Kleinman et al., 2008). The disruption of this staining pattern is an indicator of RPE dysfunction and stress (Ambati et al., 2003). We noted the RPE cells from eyes injected with tunicamycin retained their classic hexagonal packing and were similar in shape and size to those of control eyes (Fig. 7E, F). Thus, tunicamycin had minimal impact on the RPE cells in the studies presented here.

4. Discussion

The tunicamycin induced ER stress model described here mimics the non-proliferative stage of DR and capillary degeneration in a relatively short time, 2 weeks. The induction of ER stress was concomitant with a dose dependent loss of photoreceptors and accompanied by capillary degeneration. Our data support the hypothesis that photoreceptor dysfunction reported during diabetes plays a vital role in the diabetes-mediated neuroinflammation, retinal capillary degeneration, and ischemia. Potential release of cytokines, in response to UPR and ER stress activation, as well as hyperglycemia, by retinal cells including Muller cells, astrocytes, and photoreceptor cells has been previously reported (Dharmarajan et al., 2017; Fu et al., 2018; Lu et al., 2017; Rana et al., 2017; Roche et al., 2016; Yang et al., 2008; Yang et al., 2007). Photoreceptor and glial cells express cytokines and chemokines including CX3CL1, MCP-1, Rantes, Il-1β, and TNF-α in response to light injury, an activator of the UPR, and to LPS treatment (Rana et al., 2017; Rutar et al., 2011; Singh and Kumar, 2015; Yang et al., 2007; Zhang et al., 2012). Incubation of photoreceptor 661W cells with tunicamycin induces the production of Il-1β and Il-6, which is dependent on an intact ER stress/UPR system, including CHOP and ATF-4. In addition, proinflammatory factor Il-1β can induce retinal photoreceptor and vascular degeneration (Eandi et al., 2016; Hu et al., 2015; Lavalette et al., 2011b; Palenski et al., 2013; Rana et al., 2014; Rivera et al., 2013). These observations are consistent with the activation of microglial and Muller cells noted here.

We did not observe a major impact on the RGC with tunicamycin treatment. Thus, loss of RGC was not associated with the retinal vascular degeneration induced by tunicamycin-mediated ER stress. Our results support the findings of previous studies showing RGC number are not affected by retinal degeneration with the loss of photoreceptors (Lin and Peng, 2013; Mazzoni et al., 2008). However, loss of RGC by intravitreal injection of NMDA was accompanied by retinal vascular degeneration (not shown). Thus, the integrity of RGC could also affect the stability and function of retinal vasculature. Unfortunately, the details of these neurovascular interactions, the factors involved, and the cell death pathways activated require further investigation (Awai et al., 2006; Fahrenthold et al., 2018; Syc-Mazurek et al., 2017).

Animal models have been useful in investigating the RGC death mechanisms in glaucoma (Doh et al., 2010), ischemia (Binet et al., 2013), or optic nerve injury (Lindsey et al., 2015). Although several reports indicated intravitreal injections of higher doses of tunicamycin induced RGC death *in vivo* (Inokuchi et al., 2009; Ito et al., 2006; Shimazawa et al., 2007a; Tsuruma et al., 2012), our results showed mouse RGC were resistant to the doses of tunicamycin used here, without any sign of apoptosis. In contrast, the number of photoreceptors in the outer nuclear layer uniformly decreased and apoptosis appeared almost exclusively throughout this layer. These apoptotic cells showed increased CHOP expression, a marker of ER stress, consistent with terminal UPR signaling. The activation/increased CHOP levels have also been reported in stressed vascular cells including retinal endothelial cells (Shao et al., 2017), pericytes (Ikesugi et al., 2006; Zhong et al., 2012), and RPE cells (Chen et al., 2014; Matsui et al., 2015; Roybal et al., 2004), and its adverse effects are attenuated in CHOP deficient mice (Yang et al., 2017; Zhang et al., 2015; Zhang et al., 2014). Here we observed minimal expression of CHOP in these cells at the time points examined.

In the absence of a stress signal, lack of CHOP expression has a minimal impact on cellular phenotype (Yang et al., 2017). However, under stressed conditions such as oxidative stress/ inflammation as occur in diabetes or ischemia/reperfusion injury, CHOP activation/ expression could show both positive and negative effects (Choi et al., 2016; Loinard et al., 2012; Nashine et al., 2014). This likely contributed to the existence of a threshold level for ER stress (Zhang et al., 2015). CHOP can promote cell survival through modulation of autophagy during early stages/limited ER stress, before ER stress becomes irreversible (Yang et al., 2017). CHOP deficient mice used in studies for retinal degeneration in T17M or P23H Rho transgenic mice or excitotoxicity in RGC cells showed minimal protection for photoreceptor degeneration (Adekeye et al., 2014; Chiang et al., 2016a; Nashine et al., 2013) or excitotoxic RGC death (Fahrenthold et al., 2018). Thus, the cell death pathways activated in these cells under various conditions, and the role CHOP plays in these activities, need further elucidation.

Although changes in CHOP levels do not play a significant role in photoreceptor degeneration associated with retinitis pigmentosa (Adekeye et al., 2014; Chiang et al., 2016b; Nashine et al., 2013), we observed significant CHOP expression in photoreceptors of tunicamycin treated mice. These observations suggest the mechanisms of photoreceptor loss might be different from those seen here, and could likely be through ferroptosis, an iron mediated lipid peroxidation cell death modality (Hong et al., 2017; Shimada et al., 2016; Yang and Stockwell, 2016). Iron chelator deferiprone is shown to attenuate tunicamycin induced photoreceptor degeneration (Shirai et al., 2015). This could be attributed, at least in part, to the ability of CHOP to modulate the production of iron regulatory hormone hepcidin (Mueller et al., 2013), which deserves further exploration.

There are several possible reasons for the marked departure in outcome from previous reports regarding the loss of RGC with ER stress. Commonly, RGC-5, which was first isolated and characterized as being from a “postnatal day 1 rat” in 2001 (Krishnamoorthy et al., 2001) is the only ganglion cell line used in cell culture experiments. Most of the RGC death mechanisms that have been studied have used these cells (Inokuchi et al., 2009;

Shimazawa et al., 2007a; Shimazawa et al., 2007b; Uchibayashi et al., 2011). In vitro experiments using this cell line have shown that tunicamycin was able to induce RGC-5 death or apoptosis in vitro (Shimazawa et al., 2007a; Shimazawa et al., 2007b). In 2009, a group in Australia disputed the source of the RGC-5 cells (Van Bergen et al., 2009). They found these cells were contaminated with mouse mitochondrial DNA, and lacked RGC-specific protein markers. Another group from the University of North Texas Health Science Center (UNTHSC), where the RGC-5 cells were originally isolated, confirmed the species of currently used RGC-5 cells as mouse. Krishnamoorthy concluded that the RGC-5 was 661W (Krishnamoorthy et al., 2013), a mouse photoreceptor cell line developed by Al-Ubaidi et al. in the early 1990's (al-Ubaidi et al., 1992), since RGC-5 demonstrated many similar properties, such as the presence of SV40 large T-antigen, which is expected for 661W but not for RGC-5 cells. Given the confusion over the origin of these cells, it is difficult to draw any relevant conclusions about RGC death in response to ER stress using these cells.

A second possible explanation for the difference in our results reflects the dose of ER stressor utilized. For in vitro experiments, tunicamycin is generally used at 10 μg/ml (Liu et al., 2012). For in vivo experiments, tunicamycin is injected intraperitoneally at the concentration of 1 μg/g bodyweight (Zhang and Kaufman, 2008). In intravitreal injections, one study used 2 doses, 1 μg/eye and 0.1 μg/eye. In our experiments, 1 μg/eye was too strong of a stimulation, and the whole retina was lost within 2 weeks. Here we used 0.05 μg and 0.1 μg per eye, which is consistent with a recently published report examining ER stress in the retina (Alavi et al., 2015). Based on our results, it appears that there is a difference between the sensitivity of RGC and photoreceptors to the ER stressor tunicamycin at these lower doses. The reason why RGC are more tolerant of tunicamycin than photoreceptor cells needs to be further investigated. However, the significant inhibition of membrane morphogenesis in photoreceptor outer segments and its proper development, including incorporation of opsin, by tunicamycin may be involved (Anderson et al., 1988; Fliesler and Basinger, 1985; Fliesler et al., 1984; Fliesler et al., 1985). It will be interesting to determine if RGC and photoreceptors show differences in susceptibility to other types of ER stress, such as that stemming from hyperglycemia. Collectively, our studies suggest degeneration of retinal vasculature, as seen with NMDA, is not sufficient to drive loss of photoreceptor cells, and support an important role for affected photoreceptor cells in retinal vascular dysfunction with alterations in glucose metabolism (Kanow et al., 2017).

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Here we show that the selective sensitivity of photoreceptor cells to tunicamycin induced ER stress leads to activation of microglial and Muller cells, and retinal vascular degeneration with minimal effect on retinal ganglion and pigment epithelium cells.

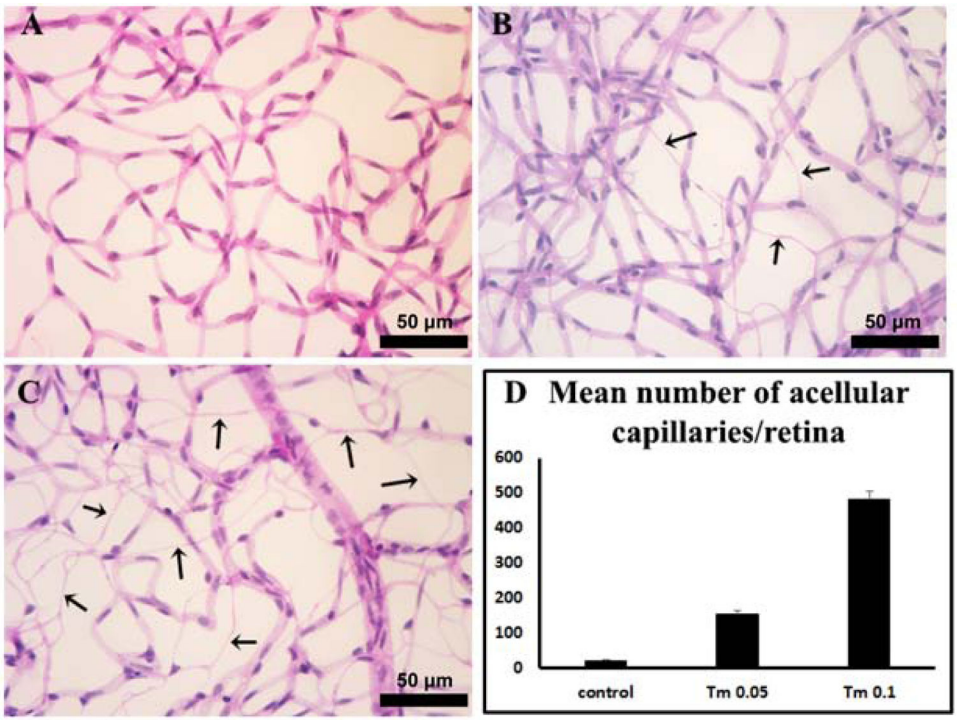

Fig. 1. ER stress induces vascular degeneration in the retina.

Representative images of retinal vasculatures 14 days after intravitreal injection of vehicle (A), 0.05 μg tunicamycin (B) and 0.1 μg tunicamycin (C). Quantitative results of retinas treated with different doses of tunicamycin are shown in (D) ($n = 6$ per group). Arrows indicate acellular capillaries. $^{**}P < 0.01$ compared to vehicle retinas or 0.5 μg tunicamycin-injected retinas. Scale bar, 50 μm.

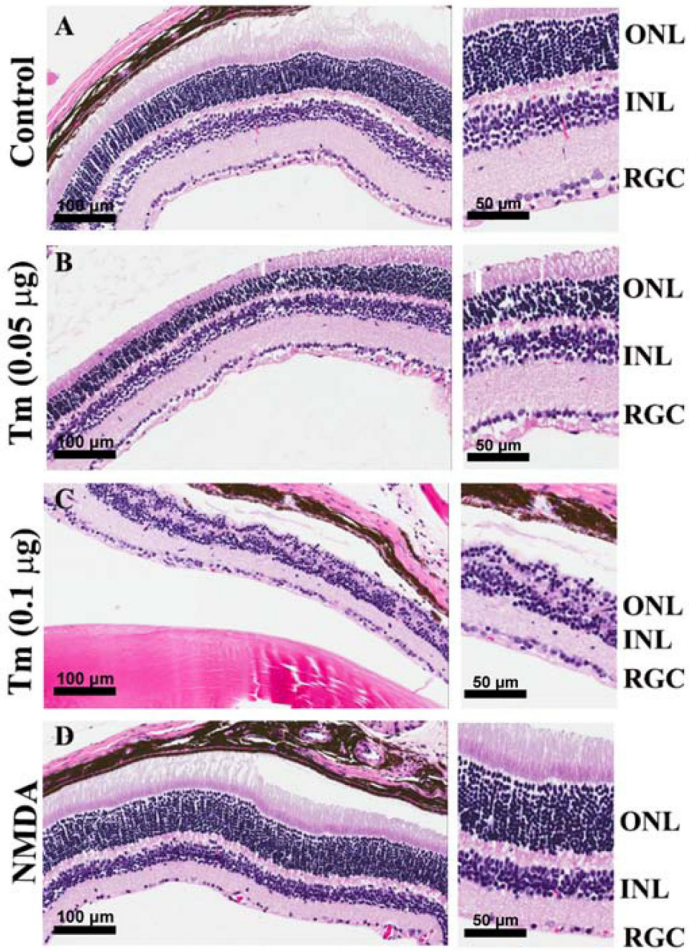

Fig. 2. Photoreceptor loss in tunicamycin injected mouse eyes.

BSS injected control (A), 0.05 µg (B), 0.1 µg of Tunicamycin (C), and NMDA injected mice (D). Eyes were enucleated 2 weeks post injection, and tissue sections examined by H&E staining. The photoreceptor cells loss was dose dependent. In 0.1 µg tunicamycin, nearly 80% of photoreceptors were lost throughout the retina. In contrast, the RGC were minimally affected. The RGC numbers in control and tunicamycin treated groups were similar, while that of NMDA treated eyes was dramatically decreased as shown in histological sections. Scale bars, left panels 100 µm and right panels 50 µm.

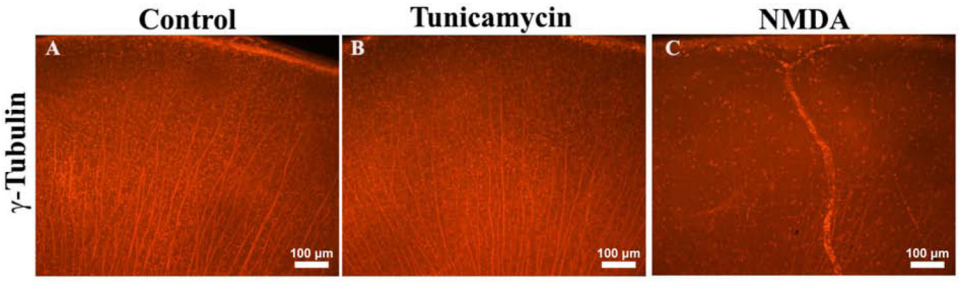

Fig. 3. Minimal RGC loss in the tunicamycin treated wholemount retina.

Wholemount preparations of the eyes treated with various compounds were stained with a neuron specific marker, γ-tubulin. The tubulin positive cells in control (BSS) (A) and 0.1 μg tunicamycin injected (B) showed no significant differences in terms of organization and number of RGC 2 weeks post injection. In contrast, the NMDA treated eyes showed dramatic disruption in tubulin staining patterns and numbers of RGC (C). Scale bar, 100 μm.

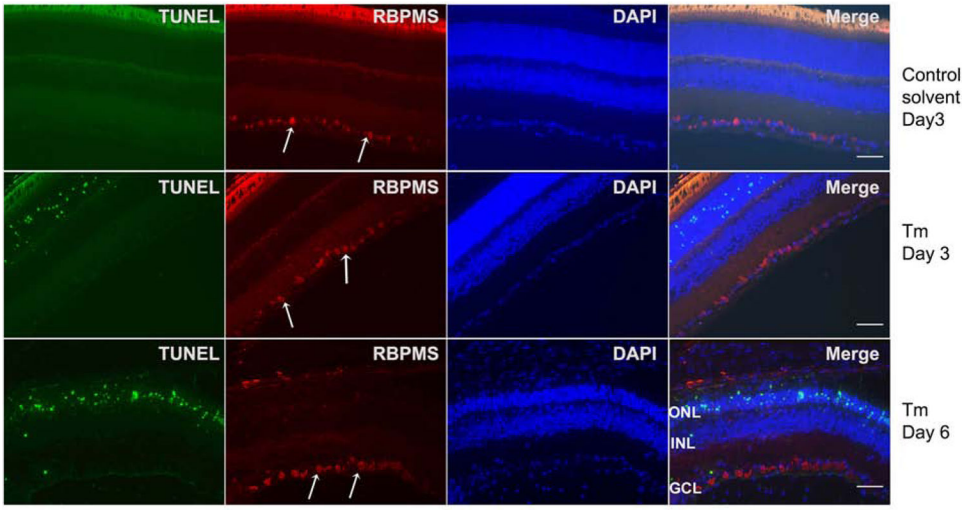

Fig. 4. Tunicamycin induces apoptosis in photoreceptor cells but not RGC.

Sections from eyes subjected to solvent control, tunicamycin (0.1 μg) treatments on day 3 and day 6 post-injection were double stained with TUNEL and RBPMS. TUNEL positive cells were located in the photoreceptor layer, not in the GCL. GCL neurons did not show apoptosis. The white arrows are pointing to RBPMS positive RGC in the GCL. Please note all RGC uniformly stained with RBPMS antibody. No TUNEL staining was noted in sections from control eyes. (GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer). Scale bar, 100 μm.

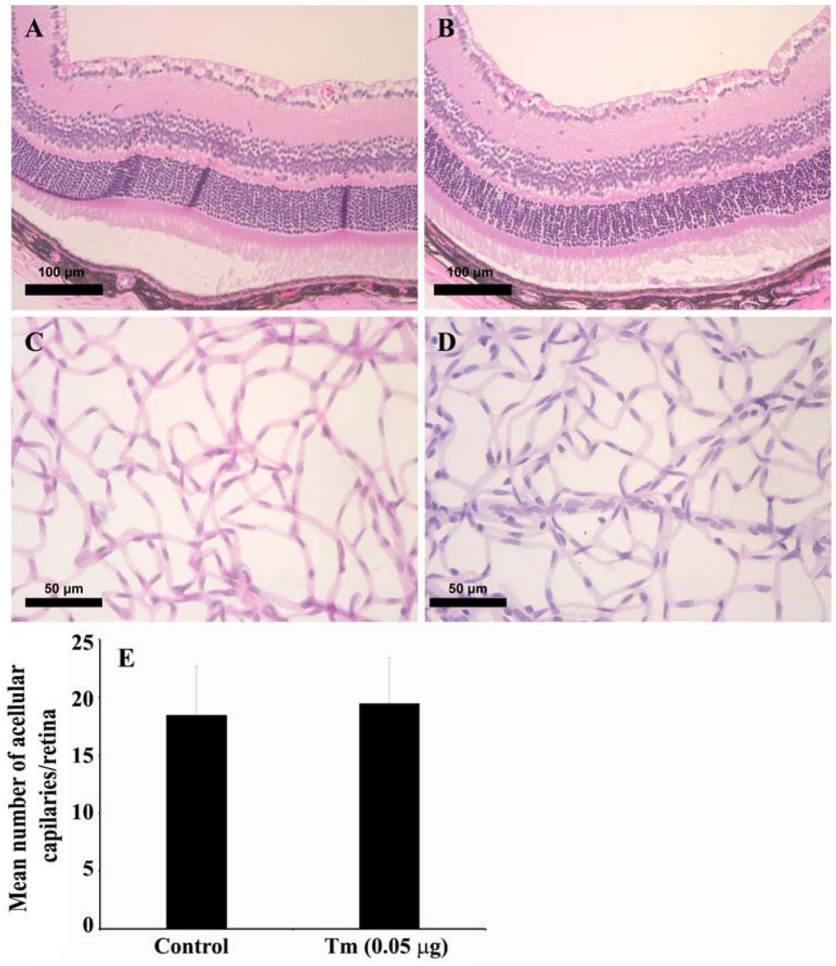

Fig. 5. Tunicamycin treatment minimally affects the organization of retinal layers and acellular capillary formation.

In histological examination of retinas, the organization of retinal layers grossly appeared similar in control and treated (0.1 μg tunicamycin, 3 days) mice, despite the presence of apoptotic cell at this stage (Fig. 4). However, no significant changes in number of acellular capillaries were observed ($P > 0.05$; n =6). Scale bars, top panels 100 μm and lower panes 50 μm.

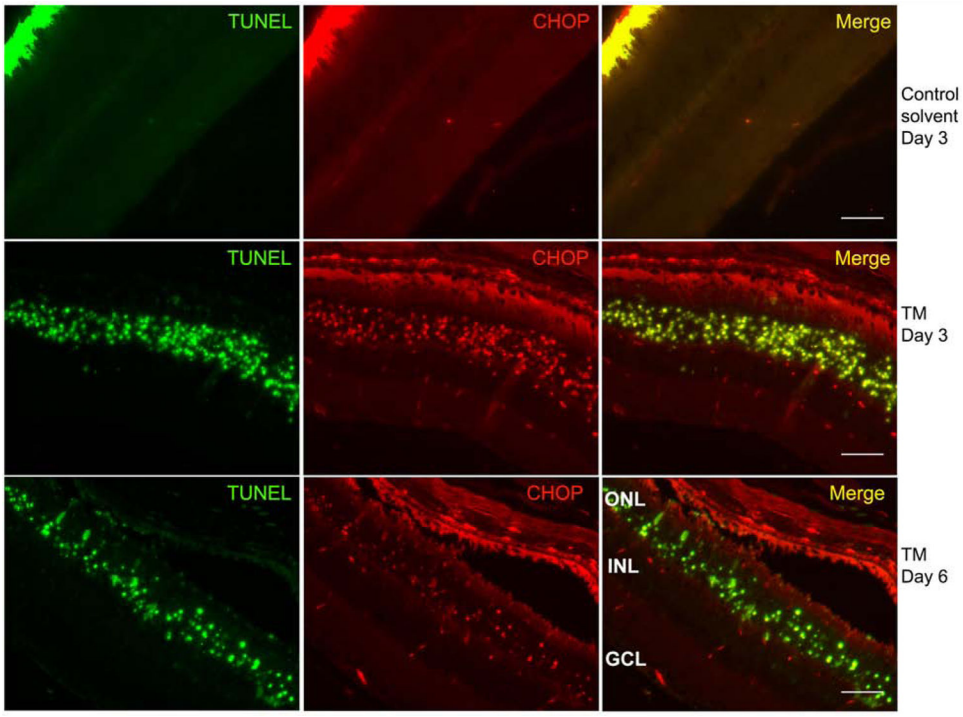

Fig. 6. CHOP expression in photoreceptor cells undergoing apoptosis.

Eye section from mice subjected to solvent control, tunicamycin treatments (0.1 μg) for 3 or 6 days were double stained with TUNEL (green) and CHOP (red). Tunicamycin treatment at day 3 (middle row) and day 6 (lower row) showed the apoptotic (TUNEL positive) and ER stress positive (CHOP positive) cells, which are located in the ONL. No staining was observed in sections from solvent control eyes (top row). Significant overlap staining is only seen in the ONL. (GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer). Scale bar, 100 μm.

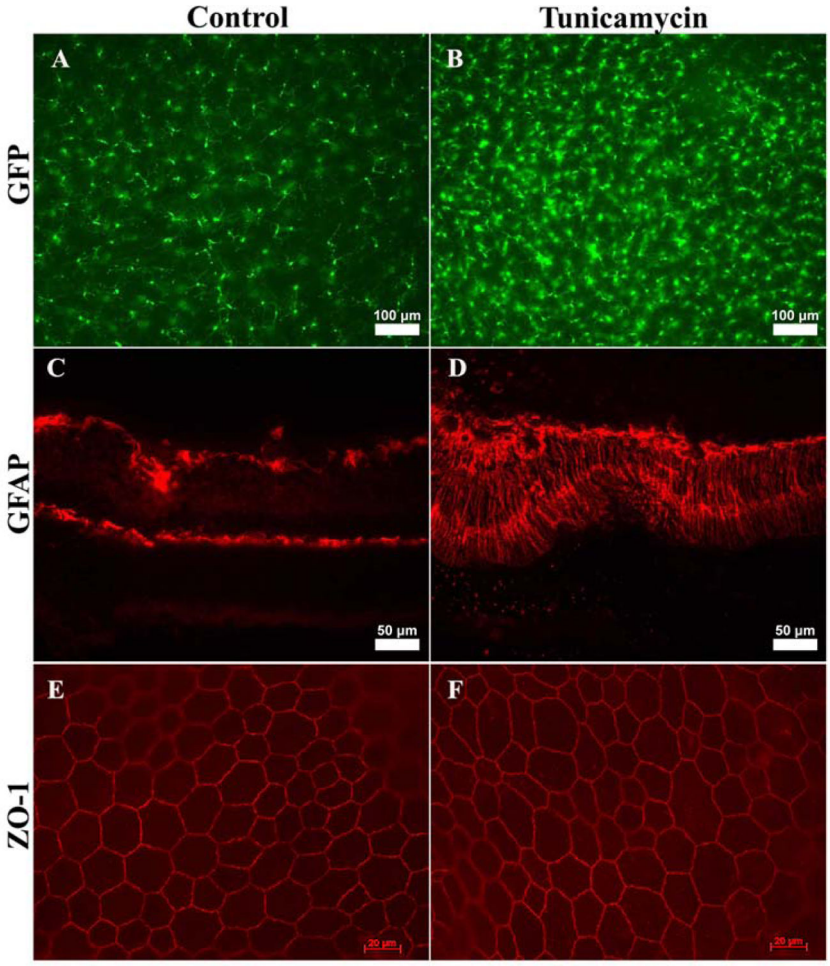

Fig. 7. Increased microglial and Muller cell activation in retinas from tunicamycin injected mice.

Wholemount retinas prepared from CX3CR1$^{gfp/gfp}$ mice treated with vehicle or tunicamycin (0.05 µg, 2 weeks post injection) were evaluated for microglial cells (A, B). GFAP staining of the retinal sections from control and tunicamycin injected mice (C, D). RPE/scleral flatmounts from mice treated with control or tunicamycin stained for ZO-1 (E, F). Please note the increase in microglial cell number and morphological changes indicting activation. Enhanced GFAP expression in treated mice compared to control mice demonstrate Muller glial cell activation. The smooth junctional localization and similar shape of cells demonstrated by ZO-1 staining indicates minimal changes in RPE cells of eyes injected with tunicamycin. These experiments were repeated with eyes from 5 mice with similar results. Scale bars, top panels 100 µm, middle panels 50 µm, and lower panels 20 µm.