O-GlcNAcylation under hypoxic conditions and its effects on the blood-retinal barrier in diabetic retinopathy

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Abstract. An increase in O-linked N-acetylglucosamine (O-GlcNAc) protein modifications has been observerd in db/db mouse retinas. O-GlcNAc-modified proteins in the db/db mouse retina have been shown to be localized in the ganglion cell layer, the inner nuclear layer, the retina pigment epithelium (RPE) layer and the inner plexiform layer, in which hypoxia-inducible factor 1α (HIF1α) has also been shown to be localized. In the current study, we examined whether hypoxia increases O-GlcNAcylation in retinal vascular cells under high glucose conditions and whether HIF1α activation is consistent with the response to and activation of O-GlcNAcylation in retinal lesions in diabetic retinopathy. In addition, the effects of O-GlcNAcylation on the blood-retinal barrier were verified *in vitro* by the inhibition of O-GlcNAcylation. A time-dependent increase in the O-GlcNAcylation in bovine retinal vascular endothelial cells (BRVECs) was observed following incubation of the cells with high glucose medium (glucose 4.5 g/l) under hypoxic (1-3% O_2) conditions. Hypoxia-induced BRVEC O-GlcNAcylation was not observed when the BRVECs were transfected with siRNA targeting O-GlcNAc transferase (OGT) or treated with alloxan (an OGT inhibitor) prior to exposure to high glucose. The increase in BRVEC O-GlcNAcylation induced by high glucose, as well as by thiamet G [an O-GlcNAcase (OGA) inhibitor] led to a reduction in occludin expression levels *in vitro,* which was prevented by treatment with OGT siRNA and alloxan. In conclusion, the current study demonstrates the relationship between O-GlcNAc glycosylation and hypoxia during diabetic retinopathy and that hyperglycemia induced O_2 consumption activates HIF1α and O-GlcNAc modification protein in the same retinal layer. The reduced protein BRVEC O-GlcNAcylation levels exert protective effects on the blood-retinal barrier and thus represent a potential therapeutic target for the treatment of diabetic retinopathy.

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Introduction

In 1984, Torres and Hart found that serine and threonine residues in nuclear and cytoplasmic proteins can undergo O-linked N-acetylglucosamine (O-GlcNAc) modification (1). The difference with various other modifications, is that O-GlcNAc modification is regulated by a single pair of enzymes: the glycosyltransferase, O-GlcNAc transferase (OGT) (EC 2.4.1.94; GI: 6006036), and the glycosidase, O-GlcNAcase (OGA) (EC .3.2.1.52; GI: 13646137). There are >1,000 types of proteins that can be O-GlcNAcylated. O-GlcNAcylation affects protein interactions, cellular localization, protein degradation and participates in the regulation of various biological functions (2). The role of O-GlcNAc modification in type 2 diabetes, neurological degenerative diseases, as well as its role in cardiovascular and cerebrovascular diseases has been investigated in depth and is becoming a key factor in regulating cellular processes (3).

Certain studies have revealed that O-GlcNAc modification mediates diabetic vascular dysfunction (4). Increased O-GlcNAcylation has been shown to enhance reactivity to constrictor stimuli (5); O-GlcNAcylation has also been shown to enhance vascular contraction contribute through the activation of the endothelin-1 (ET-1) or RhoA kinase pathway (6).

Although O-GlcNAcylation and diabetes mellitus (DM) have been extensively studied in the cardiovascular and neuroendocrine fields, reports on the alterations that occur in retinal O-GlcNAcylation levels in diabetic retinopathy are limited. More precisely, the location of the retinal cells which are affected by protein O-GlcNAcylation under hypoxic conditions, as well as the effects of O-GlcNAcylation on the blood retinal barrier, have not been reported. In the current study, we examined whether hypoxia increases O-GlcNAcylation in retinal vascular cells under high glucose conditions and whether hypoxia-inducible factor 1α (HIF1α) activation is consistent with the response to and activation of O-GlcNAcylation in retinal lesions in diabetic retinopathy. In addition, the effects of O-GlcNAcylation on the blood-retinal barrier were verified *in vitro* by the inhibition of O-GlcNAcylation.

An adequate supply of oxygen and nutrients is critical to retinal function. Prolonged hypoxia induced by high glucose can induced changes in the retina. Any changes that occur in oxygen or glucose levels can lead to cellular damage at the molecular level. Elucidating these mechanisms will not only enhance our understaning of the pathogenesis of diabetic retinopathy, but may also lead to the development of novel therapeutic strategies (7).

Materials and methods

Reagents. The reagents purchased were as follows: HIF1α antibody (Bethyl Laboratories, Inc., Montgomery, TX, USA); CTD110.6 antibody (Cell Signaling Technology, Inc., Beverly, MA, USA); OGT antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); anti-von Willebrand Factor antibody (Abcam, Cambridge, MA, USA; VWF, ab6994); Alexa Fluor 488-labeled goat anti-Rabbit IgG and occludin (Abcam); 4',6'-diamidino-2-phenylindole hydrochloride (DAPI) and heparin (Sigma, St. Louis, MO, USA); Dulbecco's modified Eagle's medium (DMEM; high glucose medium, 4.5 g/l glucose and low glucose medium, 1 g/l glucose) (Gibco, Carlsbad, CA, USA); MEM, HEPES, collagenase type IV, DNase, Pronase, thiamet G, alloxan, AG490 and bovine serum albumin (BSA) (all obtained from Sigma); fetal calf serum (FBS) Trypsine-EDTA (Invitrogen, Carlsbad, CA, USA); extracellular matrix (ECM), ECGS (100X), penicillin, streptomycin, ascorbic acid, and amphotericin-B (ScienCell, Carlsbad, CA, USA); plastic tissue culture flask (Costar, Cambridge, MA, USA); and Immobilon-NC Transfer Membrane (Millipore, Billerica, MA, USA). Leica confocal laser scanning microscope (Leica Microsystems, Mannheim, Germany) was used for scanning. The db/db mice and db/m mice were purchased from SLRC Laboratory Animal Centre of Shanghai Institutes for Biological Sciences.

Isolation of bovine retinal vascular endothelial cells (BRVECs) and primary cell culture. The isolation of BRVECs was performed according to a modified method originally developed by Banumathi *et al* (8). Briefly, the freshly isolated retinas from bovine eyes were washed with ice-cold CO_2-independent medium, 100 U/ml penicillin and 100 μg/ml streptomycin. In a laminar flow hood, the retinas were washed with the same solution, homogenized on ice 6 times and centrifuged at 400 x g for 10 min at 4˚C. The pelleted retinal tissue was resuspended in 10 ml of serum-free MEM. The retinal sections were transferred to a tube containing 4 ml of an enzyme cocktail which consisted of 500 μg/ml collagenase type IV, 200 μg/ml DNase and 200 μg/ml pronase in 10 mM phosphate-buffered saline (PBS) containing 0.5% BSA. The retinal sections along with the enzyme cocktail were incubated at 37˚C for 30 min. The pellet was passed through a 53-μm steel mesh. The trapped blood vessels were removed with the use of sterile forceps and then washed 3 times with cold MEM by centrifugation at 400 x g for 5 min. The pellet containing microvessel fragments was suspended in DMEM supplemented with 10% FBS, 90 μg/ml heparin, 5 μg/ml ascorbic acid, ECGS 100 U/ml penicillin-G, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin-B. The cells were then incubated at 37˚C in a 5% CO_2 incubator. The culture medium was replaced every 3 days and after reaching confluence, the cells were passaged with a 0.25% solution of trypsin-EDTA. von Willebrand factor (vWF) and CD31 antibodies were used for the identification of BRVECs. The BRVECs were then cultured under high and low glucose conditions for 72 h and were starved in serum-free DMEM for a further 24 h. The cells were then treated with chemicals (thiamet G or OGT siRNA) for 24 h. In the hypoxia group, the cells were cultured in low and high glucose medium, and were then exposed to a culture chamber containing 1-3% O_2 for 2, 6, 12, 24 and 48 h.

Immunofluorescence staining of BRVECs for vWF. The BRVECs were fixed overnight in 4% paraformaldehyde, then washed 3 times with PBS for 5 min. They were pre-treated with 0.5% H_2O_2-methanol and then blocked and permeabilized by incubation in 5% goat serum containing 0.1% Triton X-100 and 3% BSA. After having been washed with PBS, the cells were incubated overnight with the primary vWF antibody, followed by incubation with the Alexa Fluor 488-conjugated secondary antibody in PBS solution for 1 h at room temperature. The cells were then washed several times with PBS and incubated for 10 min with DAPI. After having been washed extensively with PBS, the stained cells were examined under a Leica immunofluorescence microscope.

siRNA tansfection and treatment with thiamet G. Total protein O-GlcNAcylation was inhibited with the use of OGT siRNA or alloxan and was enhanced with the use of thiamet G. siRNA targeting OGT and the negative control siRNA were purchased from GenePharma (Shanghai, China). siRNA (ON-TARGETplus SMARTpool; 100 nM) was incubated with Lipofectamine 2000 (Invitrogen) in 1.0 ml of serum-free medium for 30 min. The siRNA-Lipofectamine 2000 complex was then added to the cells in 4.0 ml of serum-free medium and maintained for days. On the 3rd day, the cells were incubated with high glucose for 48 h. The other cell groups were incubated with thiamet G 48 h followed by high glucose medium.

Treatment with AG490. The BRVECs were treated with 80 μM AG490 for 24 h, then cell lysis was prepared for western blot analysis. AG490, a Janus kinase 2 (JAK2) inhibitor, can reduce the phosphorylation of JAK2, as a protective factor of the retinal barrier. Cells that were treated with AG490 were used as the positive control. AG490 can not only reduce the expression of VEGF, but can also increase the expression of occludin.

Western blot analysis. Cell lysates and the retinal tissues from db/db and db/m mice were treated with lysis buffer [10 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM DTT, 0.1 mM PMSF, 10% glycerol and a protease inhibitor cocktail tablet] for 40 min on ice. The retinal tissues were homogenized in lysis buffer followed by centrifugation at 13,500 rpm for 10 min. The total protein concentration was calculated using the Enhanced BCA protein assay kit (Beyotime Institute of Biotechnology, Haimen, China). Lysate protein (30 μg) was subjected to 4-10% SDS-PAGE and electrophoretically transferred onto a nitrocellulose (NC) membrane. After blotting, the membrane was blocked in 5% fat-free dry milk for 1 h and then incubated with the specific primary anti-

body overnight at 4˚C. Protein bands were detected with the use of an enhanced chemiluminescence (ECL) detection kit (Amersham) following hybridization with the HRP-conjugated secondary antibody or with the use of LI-COR Odyssey infrared laser imaging system in order to detect the scanned image. Densitometric analysis was performed using Image J software (version 1.43, Broken Symmetry Software, Bethesda, MD, USA). For each experiment, the measurements were repeated 3 times.

Experimental animals and treatment. We used C57BLKS/J db/db mice as an animal model of type 2 diabetes, while C57BLKS/J db/m mice were selected as the control group. Male C57BLKS/J db/db (n=20, 10 weeks old) and db/m mice (n=10, 10 weeks old) were purchased from the SLRC Laboratory Animal Centre of Shanghai Institutes for Biological Sciences. They were housed in cages in a constant environment (room temperature, 20-22˚C; room humidity, 40-60%) with a 12 h/12 h light/dark cycle. Blood glucose levels remained at 19.8-29.8 mM. All procedures were approved by the Animal Ethics Committee of Tongji University, Shanghai, China. The db/db mice were divided into 2 groups: an early-stage diabetic retinopathy group (DM, 12-16 weeks, n=10) and a late-stage diabetic retinopathy group (DM, 24-32 weeks, n=10). The eyes were immediately enucleated, and the retinas were then dissected. Retinal tissues were kept at -80˚C until further analysis.

Immunofluorescence staining of mouse retinas. The eyes from the db/db mice with varying stages of diabetic retinopathy (12, 24 and 32 weeks) and from the control db/m mice were enucleated and embedded with optimal cutting temperature (OCT) compound and then frozen at -80˚C. The 10-mm-thick frozen sections were fixed in 4% paraformaldehyde overnight. Following pre-treatment with 0.5% H_2O_2-methanol for 30 min and PBS containing 5% normal goat serum, 0.5% BSA and 0.1% Triton X-100 for 30 min at room temperature, the sections were incubated overnight at 4˚C with anti-O-GlcNAc [anti-mouse CTD 110.6 (1:100), anti-OGT antibody (1:100)] and anti-HIF1α (1:100) antibodies. The sections were then rinsed with PBS and incubated at room temperature for 1 h with Alexa Fluor 488-conjugated secondary antibody. All antibodies were diluted in PBS containing 0.5% goat serum, 0.5% BSA, and 0.1% Triton X-100. Instead of a primary antibody, goat serum was used as the negative control. The sections were examined with a Leica confocal laser scanning microscope. Images were captured and processed.

In situ hybridization of HIF1α of mouse retinas. The antisense and sense oligonucleotide probes (Boster Biological Technology Co., Wuhan, China) were designed according to the mouse HIF-1a transcript sequences: i) 5'-TTATGAGCTT GCTCATCAGTTGCCACTTCC-3'; ii) 5'-CTCAGTTTGAAC TAACTGGACACAGTGTGT-3'; iii) 5'-GGCCGCTCAATTT ATGAATATTATCATGCT-3'.

The tissue sections were fixed at room temperature for 20-30 min in 4% paraformaldehyde in 0.1 M PBS (PH 7.4), containing 1/1,000 diethylpyrocarbonate DEPC. Endogenous peroxidase and biotin were blocked with 30% H_2O_2-methanol (1:50) for 30 min. The sections were then permeabilized with proteinase K (Catalog no. S3020; Dako Denmark A/S, Glostrup, Denmark) and washed 3 times with Tris-buffered saline. The sections were fixed again with the aforementioned solution. Biotinylated probes designed for HIF1α were mixed in hybridization buffer (50% formamide) at a concentration of 200 ng/ml. Probes were added to the sections, which were then covered with coverslips and heated at 92˚C for 5 min. Hybridization was carried out in a humidified chamber at 37˚C overnight. The sections were washed in 2X saline-sodium citrate (SSC) for 10 min followed by 3 washes in Tris-buffered saline and then blocked at 37˚C for 30 min with blocking solution. The sections were incubated with biotinylated mouse anti-digoxin solution at 37˚C for 60 min, then washed 4 times with PBS. For hybridization signal detection, SABC and DAB kits were used according to the manufacturer's instructions (Boster Biological Technology Co.).

Statistical analysis. Statistical analyses were performed using SPSS software (version 13.0; SPSS, Chicago, IL, USA). The unpaired Student's t-test was used to assess the significance between 2 groups. One-way ANOVA was used to compare 3 or more groups, while the Mann-Whitney U test or Rank Cases method were used to examine the heterogeneity of variance. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Characteristics of BRVECs. The cells initially grew as capillary-like structures and demonstrated the typical cobblestone morphology of endothelial cells at confluence (Fig. 1B and C). These cells were characterized as vascular endothelial cells by VWF antigen expression. Using immunofluorescence, the positive expression of vWF antigen in the BRVECs was demonstrated by green particles in the cytoplasm (Fig. 1A and B). BSA staining was used as a negative control (Fig. 1E) while bovine retinal vascular tissue immunostaining was used as a positive control (Fig. 1D).

Total protein O-GlcNAcylation and HIF1α retinal distribution in db/db mice with varying stages of diabetic retinopathy. Protein O-GlcNAcylation in the retinas of db/db mice was detected by CTD110.6 immunofluorescence and presented a distribution pattern consistent with HIF1α distribution as found by immunofluorescence and *in situ* hybridization (Fig. 2). O-GlcNAcylation appeared in the retinal ganglion cell, inner nuclear and retina pigment epithelium (RPE) layers which are first affected by diabetic retinopathy (Fig. 2B and D), and consequently in the inner plexiform layer (Fig. 2F and H). HIF1α (Fig. 2J and G) and CTD110.6 expression levels (Fig. 2L) were observed at almost undetectable levels in the db/m mouse retinas (negative controls).

O-GlcNAcylation levels in vivo and in vitro. The protein expression of O-GlcNAc was determined by western blot analysis using CTD110.6 and OGT antibodies as described in Materials and methods. O-GlcNAcylation was increased in the retinas of 14- and 24-week-old db/db mice compared to those of the age-matched control mice (Fig. 3A). A similar increase was observed in O-GlcNAcylation in BRVECs cultured under high glucose conditions (Fig. 3B). In addition, culture under

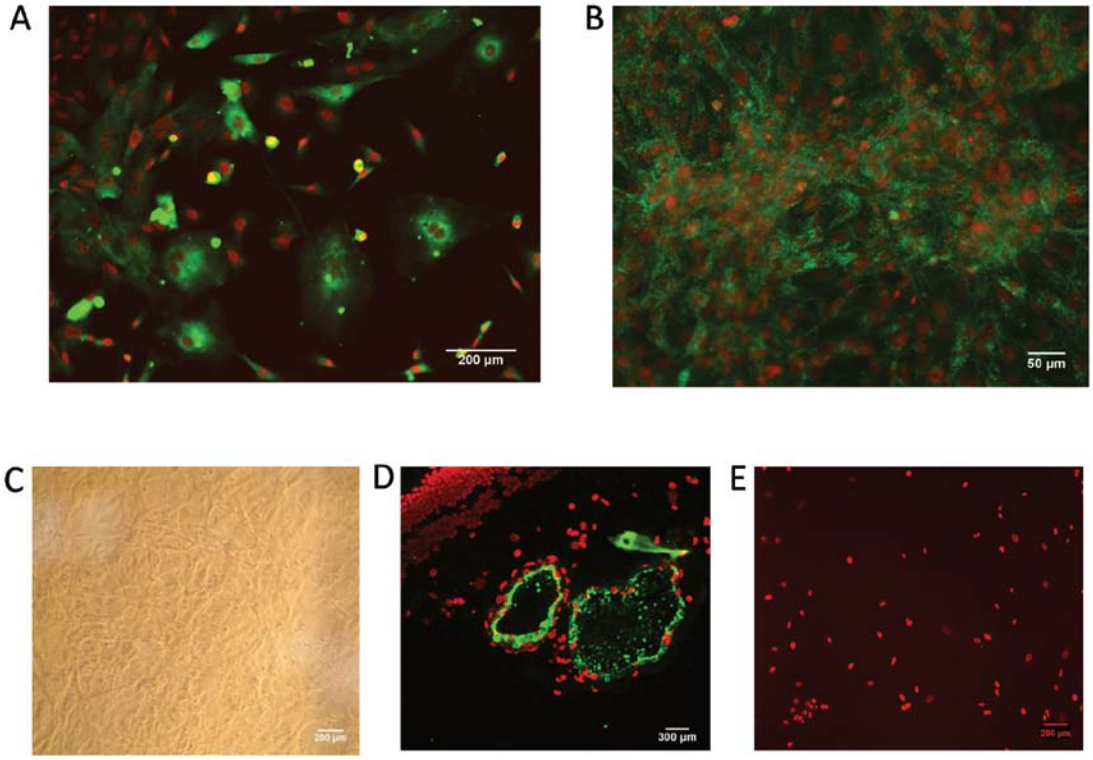

Figure 1. (A and C) Normal cultured bovine retinal vascular endothelial cells (BRVECs). (A and B) Immunostaining: BRVECs were incubated with vWF antibody. (B and C) Cultured BRVECs formed tube-like structures. (D) Bovine retinal vessel tissue was used as a positive control group. (E) Goat serum was used as a negative control group with 5% BSA instead of primary antibody incubation.

hypoxic conditions induced a time-dependent increase in O-GlcNAcylation (Fig. 3C).

Changes in O-GlcNAcylation levels in BRVECs and the blood-retinal barrier. OGT siRNA inhibition of O-GlcNAcylation in BRVECs in under a high glucose state (Fig. 4A) led to an increased expression of occludin and to reduced VEGF expression levels (Fig. 4C), while increased O-GlcNAcylation was observed following treatment with thiamet G (Fig. 4B and C). These results indicate that inhibiting O-GlcNAcylation in BRVECs may protect the blood-retinal barrier and reduce VEGF expression.

Discussion

Diabetic retinopathy is a serious complication of diabetes, leading to blindness in individuals with diabetes (9). O-GlcNAc signaling participates in the pathogenesis of diabetes (10-13). Recent studies have suggested that it also participates in the pathogenesis of diabetic retinopathy (14). The elevated expression of O-GlcNAc has emerged as a regulator of the cellular stress response (15). One of the major causes of cellular stress is hypoxia (16,17) and it is associated with the development of diabetic retinopathy (18,19). In other studies, when multiple cell lines were subjected to diverse types of stress (including hypoxia), there was a rapid and global increase in O-GlcNAc expression levels (20). Hypoxia upregulates the expression of the epitope H containing an O-GlcNAc residue in human ependymal cells (21). Increased OGT levels enhance O-GlcNAcylation and reduce cardiomyocyte death following hypoxia (22). O-GlcNAcylation levels have been shown to increase in cardiac cells folloiwng exposure to hypoxia for 4 h and early reoxygenation; this was confirmed in a follow-up study by the same group (23).

In the current study, it was demonstrated that the O-GlcNAc expression levels increased *in vitro* and *in vivo* not only in a high glucose state but also under hypoxic conditions. BRVEC O-GlcNAcylation in a high glucose state was exacerbated by hypoxia. In diabetic retinopathy, the reduced expression levels of occludin represent a decline in blood-retinal barrier function. OGT siRNA, which inhibits O-GlcNAcylation can protect the blood-retinal barrier by increasing the expression levels of occludin in BRVECs *in vitro* under high glucose conditions. Thus, it is reported herein that O-GlcNAc signaling, which is associated with HIF1α, is one of the regulatory factors of blood-retinal barrier function in the pathogenesis of diabetic retinopathy.

The possible mechanisms involved in triggering the increase in O-GlcNAcylation under hypoxic conditions are the following: Firstly, O-GlcNAc-mediated cytoprotection, as well as hypoxia which causes endoplasmic reticulum (ER) stress, have been shown to be involved in the pathogenesis of diabetes (24-26). The enhancement of O-GlcNAcylation has been shown to exert cytoprotective effects during hypoxia, ischemia and oxidative stress (27-29). The mitochondria are critical targets of O-GlcNAc-mediated cytoprotection (22,27,29,30). Moreover, O-GlcNAc signaling can directly attenuate oxidative stress-induced cellular dysfunction (27) and calcium overload (29,31). Further studies are required in order to gain a better understanding of diabetic retinopathy.

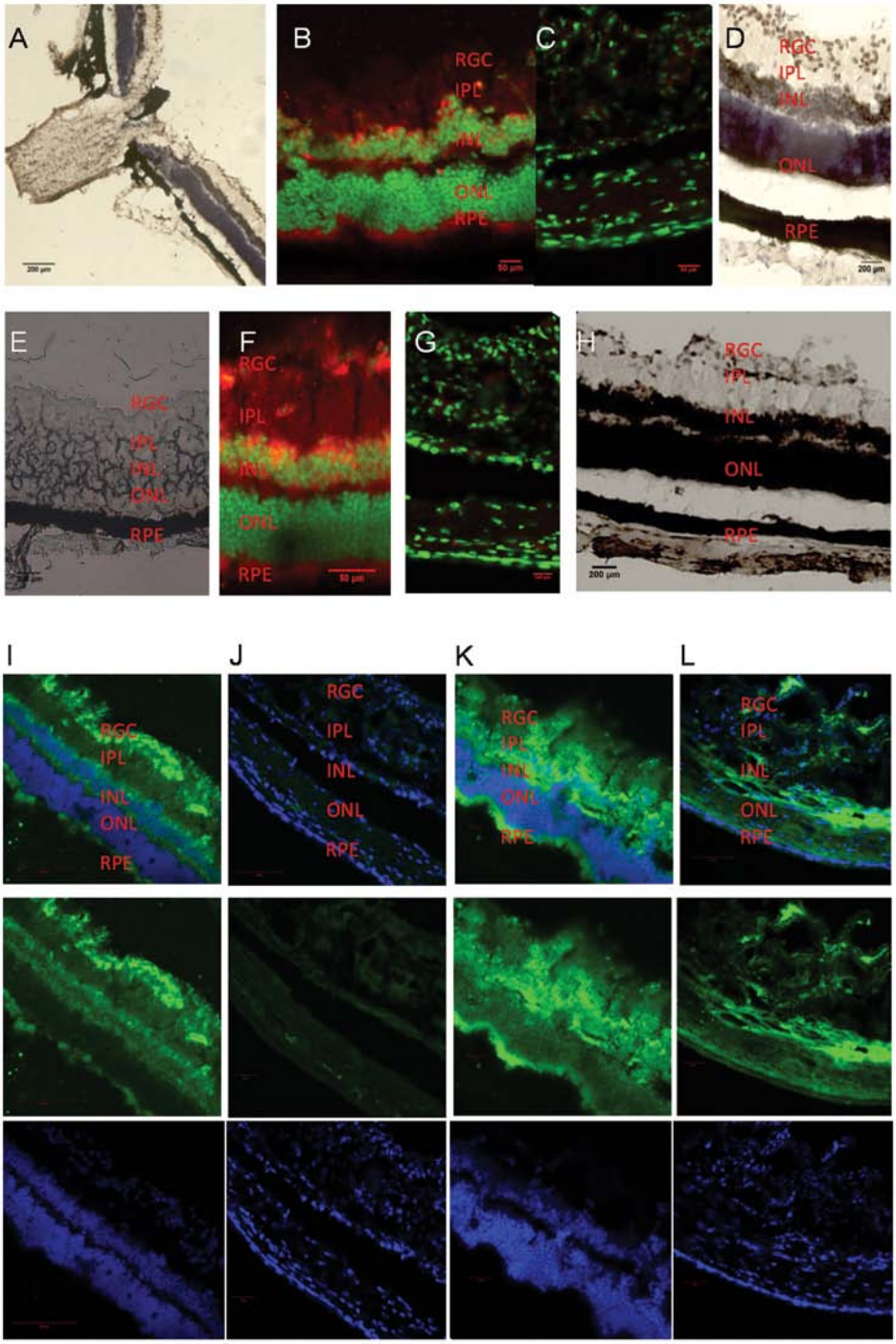

Figure 2. (A) HIF1α *in situ* hybridization of db/db mouse retinas (magnification, x100). (B) HIF1α immunofluorescence of retinas of 12-week-old db/db mice (magnification, x200). HIF1α is stained red and is expressed mainly in the retinal ganglion cell (RGC) layer, the inner nuclear layer (INL) and the retinal pigment epithelium (RPE) layer. The outer nuclear layer (ONL) shows negative staining. (C) HIF1α immunofluorescence of 12-week-old db/m mouse retinas of the control group, (magnification, x400). (D) HIF1α *in situ* hybridization of the retinas of 12-week-old db/db mice (magnification, x400) present the same distribution of immunostaining. (E) H&E staining of db/db mouse retinas. (F) HIF1α immunofluorescence of 32-week-old db/db mouse retinas (magnification, x400). HIF1α is stained red and appears mainly in the RGC layer, the INL, the inner plexiform layer (IPL) and the RPE layer. The ONL shows negative staining (G) HIF1α immunofluorescence of 32-week-old db/m mouse retinas of the control group (magnification, x400). (H) HIF1α *in situ* hybridization of the retinas of 32-week-old db/db mice (magnification, x200); brown shows the positive staining. (I) HIF1α immunofluorescence of 24-week-old db/db mouse retinas (magnification, x200). HIF1α is expressed mainly in the RGC, the INL, the IPL and the RPE layer. (K) Retinal CTD110.6 immunofluorescence of 24-week-old db/db mice (magnification, x200), presented a positive staining pattern similar to that of HIF1α. Mainly the RGC, the INL, the RPE layer and the IPL were stained. (J and L) Retinal HIF1α (magnification, x200, J) and CTD110.6 (magnification, x200, L) immunofluorescence of the control group db/m mice. Low HIF1α and CTD110.6 expression levels were observed.

Secondly, it has been demonstrated that the hexosamine signaling pathway (HSP) is linked to O-GlcNAc cycling and plays an important role in nutrient sensing (32). HSP expression represents an ubiquitous molecular mechanism which prevents the deleterious effects caused by stress (33), while O-GlcNAcylation plays an important role in regulating DNA

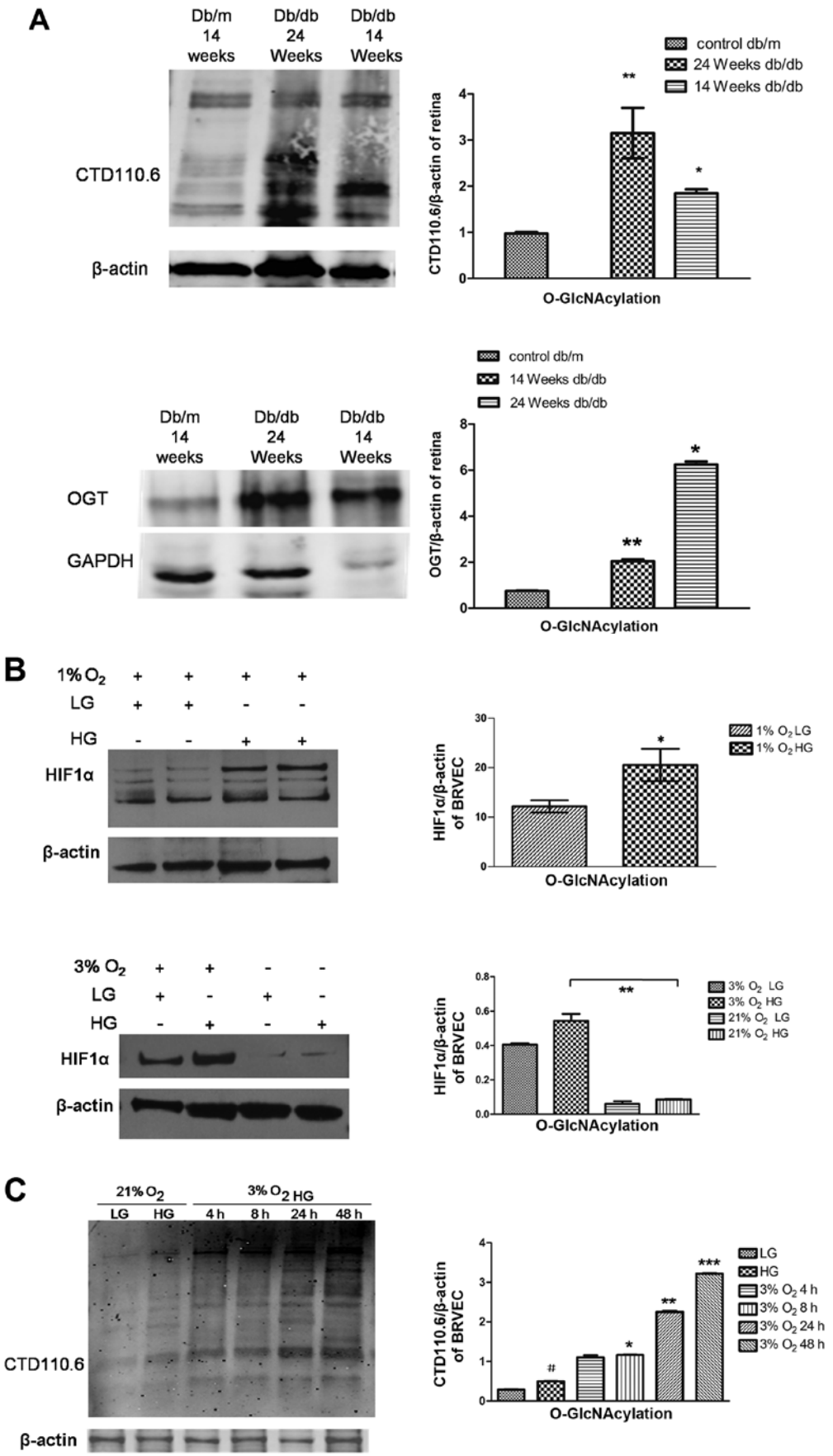

Figure 3. (A) The protein expression levels of CTD110.6 and OGT (O-GlcNAcylation) in the retinas of db/db mice were markedly increased compared with those of the control group (*P<0.05, **P<0.01). (B) HIF1α expression levels in the high glucose group (HG) were higher under hypoxic conditions than those in the low glucose group (LG) (*P<0.05). The western blots and graph depict the elevated expression levels of HIF1α when *in vitro* oxygen levels are low (**P<0.01). (C) High glucose increases CTD110.6 expression levels, and O-GlcNAcylation, in bovine retina vascular endothelial cells (BRVECs) ($^{\#}$P<0.05). CTD110.6 expression levels in BRVECs cultured under high glucose conditions. present a time-dependent increase which is due to hypoxia (*,**,***P<0.05).

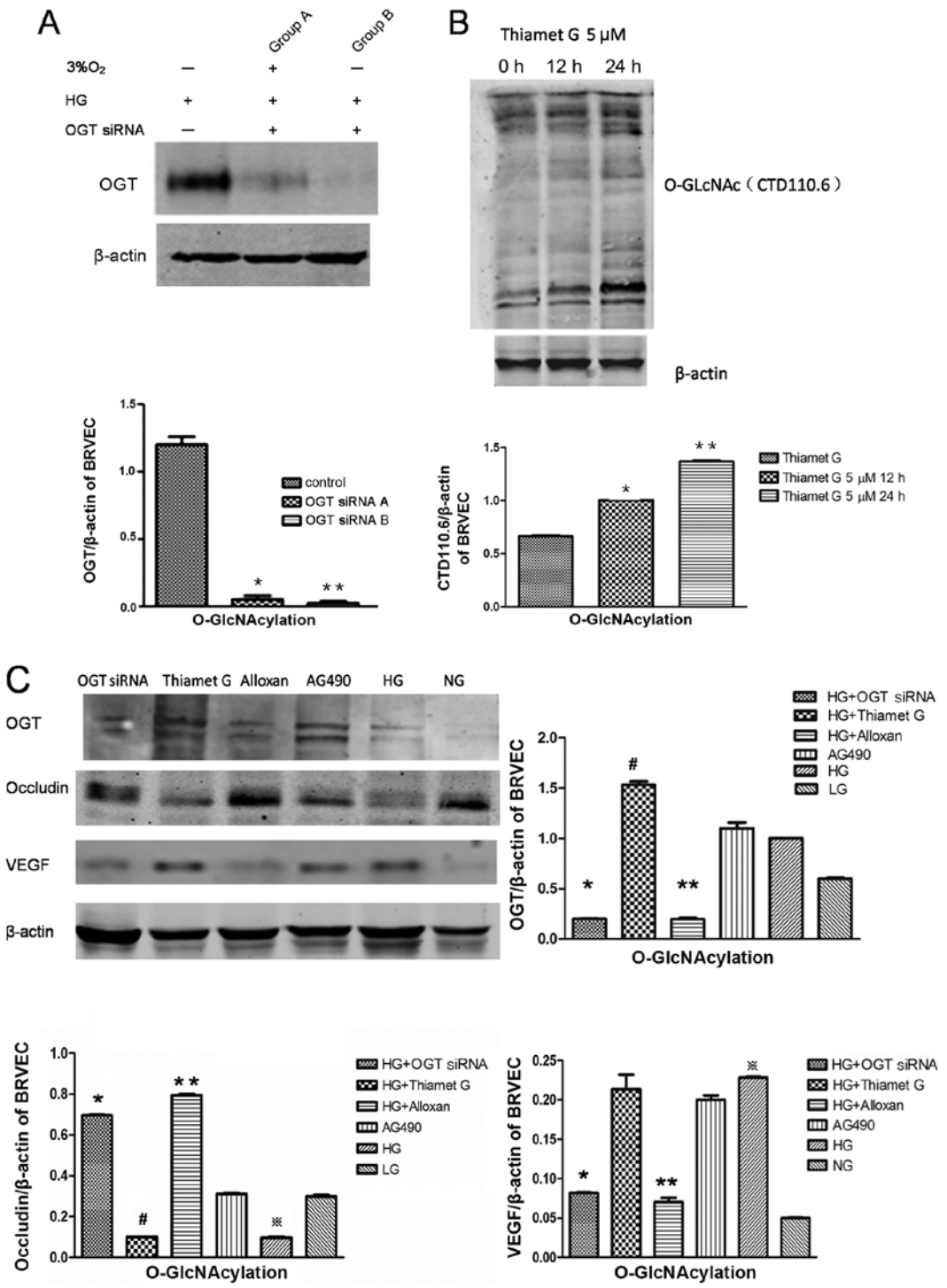

Figure 4. (A) OGT siRNA inhibition of O-GlcNAcylation in bovine retina vascular endothelial cells (BRVECs) in 3% O_2 (OGT siRNA group A) and high glucose (HG, OGT siRNA group B). Very low expression levels of OGT (O-GlcNAcylation) were observed in the OGT siRNA groups ($P<0.05$). (B) Increase in O-GlcNAcylation in BRVECs following treatment with thiamet G 5 μM at 12 and 24 h under high glucose conditions. According to the graph and the western blot, a gradual increase in O-GlcNAcylation (CTD110.6 antibody) was observed. (C) In a high glucose state occludin protein expression was decreased in BRVECs ($^{※}P<0.05$), but increased following the administration of alloxan (inhibitor of O-GlcNAcylation) or OGT siRNA ($^{**,*}P<0.05$). Thiamet G enhancement of O-GlcNAcylation leads to reduced expression levels of occludin ($^{\#}P<0.05$) in BRVECs, while AG490 (a STAT3 inhibitor) on the other hand leads to the opposite result, in other words to increased occludin expression levels. High glucose levels lead to increased expression of VEGF in BRVECs, which can be reduced by inhibiting O-GlcNAcylation with OGT siRNA or alloxan ($^{**,*,※}P<0.05$).

damage or repair through signaling pathways following stress (15).

It is still unclear as to the exact changes that occur in O-GlcNAcylation levels in the diabetic retina. In the present study, we aimed to determine which retinal layer is affected first and to a greater extent by O-GlcNAcylation during diabetic retinopathy in db/db mice.

The results of immunofluorescence and *in situ* hybridization (Fig. 2) revealed that the distribution of the O-GlcNAcylated factors is consistent with HIF1α distribution in the db/db

mouse retina. During the early stages of diabetic retinopathy (12 weeks), protein O-GlcNAcylation is distributed in the retinal ganglion cell layer, inner nuclear layer and RPE layer. During the later stages of the disease (24 and 32 weeks), it is not only distributed in the aforementioned layers but also in the inner plexiform layer. During the early stages of diabetic retinopathy, high glucose levels lead to retina hypoxia (34). According to a recent study, HIF1α expression levels have been shown to increase significantly in the vitreous fluid of surgically-treated eyes with proliferative diabetic retinopathy (PDR) (35), which also verifies the existence of hypoxia throughout the entire duration of diabetes. The data reported herein demostrate that hypoxia is accompanied by protein O-GlcNAcylation, a finding that reveals that oxygen is necessary for O-GlcNAcylation of the retinal protein.

The photoreceptors of the inner layer, the outer plexiform layer and the deeper regions of the inner plexiform layer are considered dominant oxygen consumers of the rat retina (36). The higher oxygen demand of these layers including the 'OFF' pathway ganglion cells which are normally located in the deeper inner plexiform layer, may render them more sensitive to hypoxia (36).

In the hyperoxia state, such as respired 100% oxygen, oxygen consumption of the inner retinal layer exceeds that of the photoreceptor layer (37-39). Studies have also shown that VEGF mRNA expression levels in the retina of diabetic rats are significantly increased, mainly in the retinal ganglion cell layer and the inner nuclear layer (40). These data are iin accordance with our observation that HIF1α is distributed first in the inner nuclear layer, retinal ganglion cell layer and RPE layer, and then in the inner plexiform layer in db/db mouse retinas. HIF1α is a molecular level oxygen sensor and represents the degree of hypoxia (41). Hyperglycemia-induced O_2 consumption activates HIF1α and other hypoxia-associated genes (42). Hypoxia induces a decrease in adenosine triphosphate (ATP) levels, ion imbalance and free radicals which cause ganglion cell, inner nuclear layer cell and RPE cell apoptosis (43). The distribution of O-GlcNAc is consistent with the distribution of HIF1α in the db/db mouse retina and reveals that the change in retinal O-GlcNAcylation levels is mainly influenced by the location where hypoxia occurs. This may be a compensatory response which leads to tissue damage.

In this study, we also investigated the decreased expression levels of occludin in cultured BRVECs under high glucose conditions; these levels increased following transfection with OGT siRNA. Further studies are required to confirm whether this change occurs due to the direct regulation of occludin phosphorylation, or to other kinase-mediated signal transduction pathways.

Occludin is known as a transmembrane component of tight junctions (TJs) and together with ZO-1, constitute the TJ between cells (44). It has also been confirmed that occludin plays a role in the blood-retinal barrier breakdown during diabetic retinopathy (45, 46). Under hypoxic conditions, the transient increase in intracellular Ca^{2+} activates extracellular signal-related kinases, such as calmodulin-dependent kinase, triggering the cascade reaction, causing changes in the mRNA levels and protein expression of occludin and other TJ-associated proteins, eventually leading to cell barrier function damage (47).

It is more evident when occludin is phosphorylated. Occludin serine/threonine phosphorylation aggregates in the TJ location, while fewer occludin phosphorylated residues are distributed in the cytoplasm of the basement membrane side. The increased serine/threonine phosphorylation of occludin can enhance the barrier function (48). Factors, such as lack of calcium, phorbol (phorbol ester) stimulation (44) and pathological conditions (45), that destroy TJs, can change the state of occludin phosphorylation, which influences TJ barrier functions (46). It is well known that O-GlcNAc modification due to glycosylation and phosphorylation, is a complementary adjustment, which functions in a regulatory mode, as suppressing glycosylated occludin expression may enhance its phosphorylation (1), leading to the increase of TJ barrier functions. There exists a novel interplay between occludin and O-β-glycosylation (49). Due to the complexity of the measurement methods, further studies arerequired to confirm whether occludin itself exerts O-GlcNAcylation.

The present study demonstrates that during the course of diabetic retinopathy, the degree of retinal hypoxia becomes more severe and O-GlcNAcylation is closely associated with hypoxia in the retinas of db/db mice with type 2 diabetes. Protein O-GlcNAcylation appears mainly first in the ganglion cell layer, the inner nuclear layer and the RPE layer and at a later stage, in the inner plexiform layer. In addition, we found that the inhibition of BRVEC O-GlcNAcylation under high glucose conditions can prevent the increase in occludin and the decrease in VEGF expression levels, and may thus contribute to the protection of blood-retinal barrier in diabetic retinopathy. Further studies are required to gain a better understanding of the molecular basis of O-GlcNAcylation which plays a role in the pathogenesis of diabetic retinopathy.

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