Oxidative and Endoplasmic Reticulum Stresses Mediate Apoptosis Induced by Modified LDL in Human Retinal Müller Cells

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Purpose. We previously showed that extravasated, modified LDL is implicated in pericyte loss in diabetic retinopathy (DR). Here, we investigate whether modified LDL induces apoptosis in retinal Müller glial cells.

Methods. Cultured human retinal Müller cells (MIO-M1) were treated with highly oxidized glycated LDL (HOG-LDL, 200 mg protein/L) or native LDL (N-LDL, 200 mg protein/L) for up to 24 hours with or without pretreatment with N-acetyl-cysteine (NAC, a blocker of oxidative stress) and 4-phenylbutyrate (4-PBA, a blocker of endoplasmic reticulum [ER] stress). Effects of HOG-LDL on cell viability, apoptosis, oxidative stress, and ER stress were assessed by cell viability, TUNEL, and Western blot assays. In separate experiments, Müller cells were treated with 7-ketocholesterol (7-KC, 5–20 μM) or 4-hydroxynonenal (4-HNE, 5–40 μM) for up to 24 hours. The same markers were measured.

Results. HOG-LDL induced apoptosis (decreased cell viability, increased TUNEL staining, increased expression of cleaved PARP, cleaved caspase-3, and BAX; decreased Bcl-2), oxidative stress (increased NOX4 and antioxidant enzymes, catalase, and superoxide dismutase 2), and ER stress (increased phospho-eIF2α, KDEL, ATF6, and CHOP). Pretreatment with NAC or 4-PBA partially attenuated apoptosis. In addition. NAC attenuated activation of ER stress. Similar to HOG-LDL, 7KC, and 4HNE also induced apoptosis, oxidative stress, and ER stress.

Conclusions. Our data suggest that extravasated, modified lipoproteins may be implicated in apoptotic Müller cell death, acting at least partially via enhanced levels of oxidative and ER stresses. They support our main hypothesis that, in addition to hyperglycemia, extravasated and oxidized LDL is an important insult to the diabetic retina. (*Invest Ophthalmol Vis Sci.* 2012;53:4595–4604) DOI:10.1167/iovs.12-9910

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Diabetic retinopathy (DR) is a major cause of blindness in working age people in developed countries.[1] Retinal vascular and neuronal changes occur at an early stage and are central to the disease process.[2–5] Müller cells are the principal glia in the retina, spanning its entire thickness.[6] Besides supporting retinal neurons, Müller cells form processes around retinal vessels in the deep, intermediate, and superficial vascular beds, contributing to the maintenance of the blood-retinal barrier.[5] In addition, Müller cells are involved in regulating retinal glucose metabolism, controlling blood flow and extracellular potassium concentration, and modulating neuronal activity.[7,8] Previous reports have shown that diabetes (hyperglycemia) adversely affects function and accelerates apoptotic cell death of Müller cells,[9] but may also promote their proliferation.[10] Further studies revealed that upregulation of receptors for advanced glycation end-products (AGEs) causes proinflammatory responses in Müller cells.[11]

In previous work, we proposed that in addition to hyperglycemia, extravasation of plasma lipoproteins through leaking blood retinal barriers (BRB) and their subsequent modification (glycation, oxidation) are important in the propagation of DR.[12–18] Several lines of evidence support this concept. Clinical studies indicate that dyslipidemia is associated with the severity of DR In particular, DR is positively associated with serum levels of LDL, apolipoprotein B (ApoB), and LDL particle concentration in type 1 diabetic patients.[13,19–21] However, dyslipidemia in the absence of diabetes does not cause retinal injury, and we suggest that breakdown of the BRB is the critical factor. Using immunohistochemistry (for ApoB and oxidized LDL [ox-LDL]), we identified the presence of intraretinal modified LDL in type 2 diabetic patients who had not yet developed clinical DR, with larger amounts proportionate to disease severity in patients with clinical DR This staining initially surrounded the inner retinal capillaries. We also confirmed the absence of ApoB and ox-LDL in normal human retina.[16] In ex vivo studies, ox-LDL was associated with apoptotic figures in human diabetic retinas.[16] In more severe DR cases with proliferative DR, the staining of ox-LDL and ApoB was found throughout all layers of the retina,[16] indicating that extravasated and modified LDL might contact Müller cells and induce Müller cell dysfunction and apoptosis. Indeed, animal studies have shown that accumulation of advanced lipoxidation end-products contributed to Müller glial abnormalities in the early stages of DR.[22] For the present work, we used not only in vitro–modified LDL to assess its effects on Müller cells, but also 7-ketocholesterol (7KC) and 4-hydroxynonenal (4HNE), two of the most important products of lipid peroxidation, which may mediate lipoprotein-induced injury.

Oxidative stress is recognized as a critical and early risk factor in the development of DR.[23,24] Imbalance of oxidants

and antioxidants, mediated by altered activity of the polyol, hexosamine, AGE, and protein kinase C pathways results in inflammation, increased vascular permeability, apoptosis, and eventually, neovascularization.[25,26] Endoplasmic reticulum (ER) stress is another novel and important mediator of retinal cell injury and death in DR.[27–30] Our own recent data indicate that modified LDL can increase oxidative stress and ER stress in aortic endothelial cells, and in animal models related to diabetes.[31]

In the present study, we hypothesized that extravasated and modified LDL, and the lipid oxidation products 7KC and 4HNE, cause Müller cell injury in diabetes. Our findings confirm that modified LDL, 7KC, and 4HNE can all decrease Müller cell viability and increase apoptosis, and suggest that enhanced oxidative stress and ER stress are implicated. Understanding these effects of modified lipoproteins and lipid oxidation products on Müller cells in DR may open up new opportunities to block or inhibit retinal injury in diabetes.

Materials and Methods

Human Subjects

The study was approved by the Institutional Review Board of the University of Oklahoma Health Sciences Center, and was conducted according to the principles of the Declaration of Helsinki.

Preparation, Modification, and Characterization of LDL

Normal (or Native) LDL (N-LDL) and highly oxidized glycated LDL (HOG-LDL) were prepared by our laboratory as previously described.[12,32] Briefly, human LDL was isolated from pooled plasma obtained from male and female healthy subjects ($n = 4$ for each preparation) aged 20 to 45 years who were not receiving any prescription medications or antioxidant vitamins. N-LDL was prepared by sequential ultracentrifugation. Subsequently, glycated LDL (G-LDL) was prepared by exposing N-LDL to freshly prepared 50 mM glucose for 72 hours at 37°C, and HOG-LDL was made by oxidizing the G-LDL using 10 μM $CuCl_2$ (24 hours, 37°C), followed by extensive dialyses to remove copper ion and glucose.[32] Protein in LDL preparations was quantified with bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). N-LDL and HOG-LDL preparations were characterized by gel electrophoresis (Paragon LIPO Gel; Beckman, Fullerton, CA), and by measuring fluorescence at 360 nm excitation/430 nm emission (Gilford Fluorometer IV; Gilford Instrument Laboratories, Oberlin, OH), and absorbance at 234 nm using a spectrophotometer (model DU650; Beckman). Lipoprotein preparations were used within 1 month of preparation.

Human Retinal Müller Cell Culture

Human Müller (Moorfields/Institute of Ophthalmology-Müller 1 [MIO-M1]) cells[33,34] were provided by G. Astrid Limb (Institute of Ophthalmology, University College London, London, UK).[33] Passage 22-25 was used for current study. Human retinal Müller cells were maintained in Dulbecco's modified Eagle's medium/F12 (DMEM/F12) (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO) and 1% penicillin-streptomycin solution (PS) (Cellgro, Manassas, VA) at 37°C in 5% CO_2, 95% air. After reaching 80% to 85% confluence, cells were treated in serum-free medium (SFM, DMEM/F12 without FBS and PS) for 16 hours to induce quiescence. Cells were then treated with N-LDL, HOG-LDL, 4HNE (Calbiochem, La Jolla, CA), and 7KC (Sigma-Aldrich) at different doses for up to 24 hours, and measures of cell viability, markers for oxidative stress, ER stress, and apoptosis were performed. Each experiment was repeated at least three times.

LDL Concentrations in Cell Culture

In the present study, we used LDL (protein) concentrations of 10 to 200 mg/L in cell culture media. The upper end of this range is approximately one-third to one-half of normal human plasma levels. Although intraretinal concentrations of LDL and modified LDL in the human diabetic retina after BRB breakdown are unknown, it has been estimated that in atherosclerotic plaque, concentrations of ox-LDL may be up to 79 times higher than in plasma,[35] presumably as a result of sequestration and covalent binding. Furthermore, although not quantitative, our previous immunohistochemistry studies suggest copious amounts of oxidized ApoB-containing lipoproteins in the diabetic retina.[16] We therefore consider that the concentrations used in the present study are conservative. Similar or higher concentrations have been used in the past by others and by ourselves for studies on various cell types.[14,15,31,35–39]

Cell Viability Assay

Human MIO-M1 Müller cells were incubated in 96-well-plates (15,000 cells/well). Cells were exposed to SFM (control), N-LDL at 200 mg/L, and HOG-LDL at 10, 50, and 200 mg/L for 1, 6, 12, and 24 hours. In separate experiments, cells were pretreated with N-acetyl-cysteine (NAC) (1 mM) or 4-phenylbutyrate (4-PBA) (100 μM) for 1 hour before spiking SFM (control), N-LDL (to final concentration of 200 mg/L), or HOG-LDL (also 200 mg/L) for 24 hours. In other experiments, cells were exposed to 7KC (5–20 μM) or 4HNE (5–40 μM) for 1 to 24 hours. Cell viability was measured using cell-counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Rockville, MD). Absorbance at 450 nm was used to determine cell viability per manufacturer's instructions. All experiments were performed in triplicate.

Western Blotting

After incubation with medium containing N-LDL, HOG-LDL, 7KC, or 4HNE, cells were harvested, washed three times with ice-cold PBS, and cell lysates were prepared using radio-immunoprecipitation assay lysis buffer (1×; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) containing a protease inhibitor cocktail. Protein concentrations were measured using Pierce BCA protein Assay Kit (Thermo Scientific Pierce Protein Research Products, Rockford, IL). Fifty micrograms of protein were loaded onto each lane of a gel. Proteins were separated by a 12% SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were probed with specific primary antibodies at dilutions of 1:500 to 1:3000. Antibodies against nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4 (NOX4), superoxide dismutase 2 (SOD2), KDEL (Lys-Asp-Glu-Leu), phospho-eIF2α, cleaved activating transcription factor 6 (ATF6), and β-actin were purchased from Abcam (Cambridge, MA). Antibodies against catalase, CCAAT/enhancer-binding protein homologous protein (CHOP), Bcl-2, Bax, cleaved caspase-3, and cleaved Poly (ADP-ribose) polymerase (PARP) were purchased from Cell Signaling Technology (Beverly, MA). After washing three times, the membranes were probed with peroxidase-labeled anti-rabbit IgG (H+L) or anti-mouse IgG (H+L) secondary antibodies (VECTOR, Burlingame, CA). The specific bands were visualized by autoradiography using BioSpectrum Imaging System (UVP, Upland, CA) and SuperSignal West Dura Extended Duration Substrate (Thermo Scientific Pierce Protein Research Products). All experiments were performed in triplicate.

Reactive Oxygen Species

Changes in intracellular reactive oxygen species (ROS) following exposure of cells to N-LDL or HOG-LDL (200 mg/L) for up to 3 hours, with or without pretreatment with NAC for 1 hour, were measured as previously described.[40] Briefly, cells were incubated in 96-well plates with SFM containing 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA,10 μM, 100 μL/well) (Invitrogen, Carlsbad, CA) for 30 minutes. Subsequently, cells were washed once with SFM and medium

was replaced with SFM. Thirty minutes later, the plate was read as baseline (0 minute) by a fluorescence microplate reader with Ex 485 nm; Em 538 nm (VICTOR3 V Multilabel Counter; PerkinElmer Life and Analytical Sciences, Inc., Waltham, MA). Cells were then treated with LDL with or without NAC pretreatment, as described.

TUNEL Assay

Human MIO-M1 Müller cells were incubated in six-well plates with coverslips (3×10^5 cells/well). Cells were exposed for 24 hours to SFM, N-LDL (200 mg/L), HOG-LDL (200 mg/L) with or without 1-hour pretreatment with NAC (1 mM) or 4-PBA (100 μM). Cells in coverslips were fixed by 4% paraformaldehyde in double-distilled water (Electron Microscopy Sciences, Hatfield, PA), permeablized by 0.25% Triton X-100 in PBS, followed by TUNEL immunostaining using Click-iT TUNEL Alexa Fluor Imaging Assay kit (Invitrogen, Eugene, OR). The 4′,6-diamidino-2-phenylindole (DAPI) staining for nuclei used ProLong Gold antifade reagent (Invitrogen). Images were taken under a microscope equipped with fluorescence illumination (Nikon E800 Epifluoresence Microscope; Nikon, Tokyo, Japan).

Statistical Analysis

Results were expressed as means ± SD. Statistical analyses of cell proliferation assay and densitometry data for Western blots (normalized to β-actin) were conducted using Prism 4 software (La Jolla, CA). A value of P less than 0.05 was accepted as significant.

Results

HOG-LDL Decreased Cell Viability and Induced Apoptosis of Human Retinal Müller Cells

Human retinal Müller cells (MIO-M1) were exposed to SFM, N-LDL (200 mg /L), or HOG-LDL (10, 50, 200 mg/L) for 1, 3, 6, 12, and 24 hours. At 50 mg/L, HOG-LDL decreased cell viability at 24 hours, and at 200 mg/L, it decreased cell viability at 12 and 24 hours (Fig. 1A). In comparison, SFM and N-LDL at 200 mg/L had no effect on Müller cell viability at any time point (Fig. 1A).

Cleaved caspase-3, cleaved PARP, Bax, and Bcl-2 were determined by Western blot following exposure of cells to 200 mg/L N- or HOG-LDL for 1, 3, 6, 12, and 24 hours. Densitometric analyses (normalized to β-actin) indicated that protein levels of cleaved caspase-3, cleaved PARP, and Bax increased, and Bcl-2 decreased, following exposure to HOG-LDL, but N-LDL had no effect (Fig. 1B). The data suggest that exposure to modified LDL results in apoptotic Müller cell death.

HOG-LDL Induced Oxidative Stress in Müller Cells

As shown in Figure 2A, exposure of cells to HOG-LDL (200mg/L) caused a significant, 15-fold, increase in intracellular ROS levels over three hours, and this increase was largely prevented by pretreatment with NAC (1 mM). In contrast, N-LDL had no significant effect. HOG-LDL also induced significant increases in NOX4 (consistent with an increase in superoxide production) and in the antioxidant enzymes, catalase and SOD2, as shown by densitometry analyses of Western blot data (Fig. 2B). Again N-LDL had no effect.

HOG-LDL Increased Levels of ER Stress Markers

The results outlined above suggest that HOG-LDL reduces cell viability, increases apoptosis, and increases oxidative stress. We next assessed whether HOG-LDL could induce ER stress. We measured several ER stress markers by Western blot: glucose-regulated protein 78 kDa (GRP78), cleaved ATF6, phosphorylated eukaryotic translation initiation factor 2α (phospho-eIF2α), and CCAAT/enhancer-binding protein homologous protein (CHOP). HOG-LDL significantly increased levels of GRP78, cleaved ATF6, phospho-eIF2α, and CHOP compared with N-LDL or SFM (Fig. 3A). Interestingly, elevation of these markers occurred at different time points, with phospho-eIF2α increasing first at 1 hour, followed by GRP78 at 6 hours, CHOP at 12 hours, and ATF6 at 24 hours (Fig. 3A). When Müller cells were pretreated for 1 hour with 1 mM NAC, an inhibitor of oxidative stress, then exposed to HOG-LDL for 24 hours, all of these HOG-induced responses were significantly reduced (Fig. 3B), indicating that oxidative stress at least partially mediates activation of ER stress.

HOG-LDL Induced Apoptosis via Oxidative Stress and ER Stress

To determine whether HOG-LDL–induced apoptosis resulted from oxidative and/or ER stresses, cells were pretreated with 1 mM NAC or 100 μM 4-PBA (a blocker of ER stress) for 1 hour before incubation with SFM, 200 mg/L N-LDL, or 200 mg/L HOG-LDL for 24 hours. Both NAC and 4-PBA pretreatments partially blocked the reduction in viability induced by HOG-LDL (Fig. 4A, 4B; CCK-8 assay), with NAC being more effective.

Pretreatment of Müller cells with NAC (1 mM) or 4-PBA (100 μM) for 1 hour completely abolished PARP cleavage induced by HOG-LDL 24 hours after HOG treatment, as shown in a representative Western blot and densitometry measures in Figure 4C. Additionally, the number of TUNEL-positive cells induced by HOG-LDL was also significantly reduced by pretreatment with NAC or 4-PBA (Fig. 4D). PARP and TUNEL are markers of DNA fragmentation and apoptosis respectively. These data suggest that oxidative stress and ER stress may both be involved in HOG-LDL–induced Müller cell apoptosis.

7KC and 4HNE Induced Apoptosis and Increased Levels of Oxidative Stress and ER Stress

LDL is a large particle and when oxidized, contains numerous products of lipid peroxidation, including 7KC and 4HNE. To determine if these oxidation products might induce responses similar to HOG-LDL, we treated Müller cells with each. The results are shown in Supplementary Figures 1 and 2 (see Supplementary Material and Supplementary Figs. S1, S2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9910/-/DCSupplemental). Cell viability experiments using the CCK-8 assay showed that 20 μM 7KC decreased viability at 12 hours and more severely at 24 hours (see Supplementary Fig. S1A, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9910/-/DCSupplemental). As shown in representative Western blots (see Supplementary Fig. S1B, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9910/-/DCSupplemental) and by densitometry measures (see Supplementary Fig. S1C, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9910/-/DCSupplemental), 7KC increased measures of oxidative stress, ER stress, and apoptosis. Similar results were observed for 40 μM 4HNE (see Supplementary Figs. S2A, S2B, S2C, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9910/-/DCSupplemental); indeed, at the concentrations used, 4HNE exhibited somewhat greater toxicity than 7KC.

Discussion

Previous studies from our own and other laboratories have shown that, in addition to hyperglycemia, qualitative and quantitative lipoprotein abnormalities are implicated in mediating vascular injury in diabetes, and specifically, are associated with the development of DR.[13–21] Our previous studies

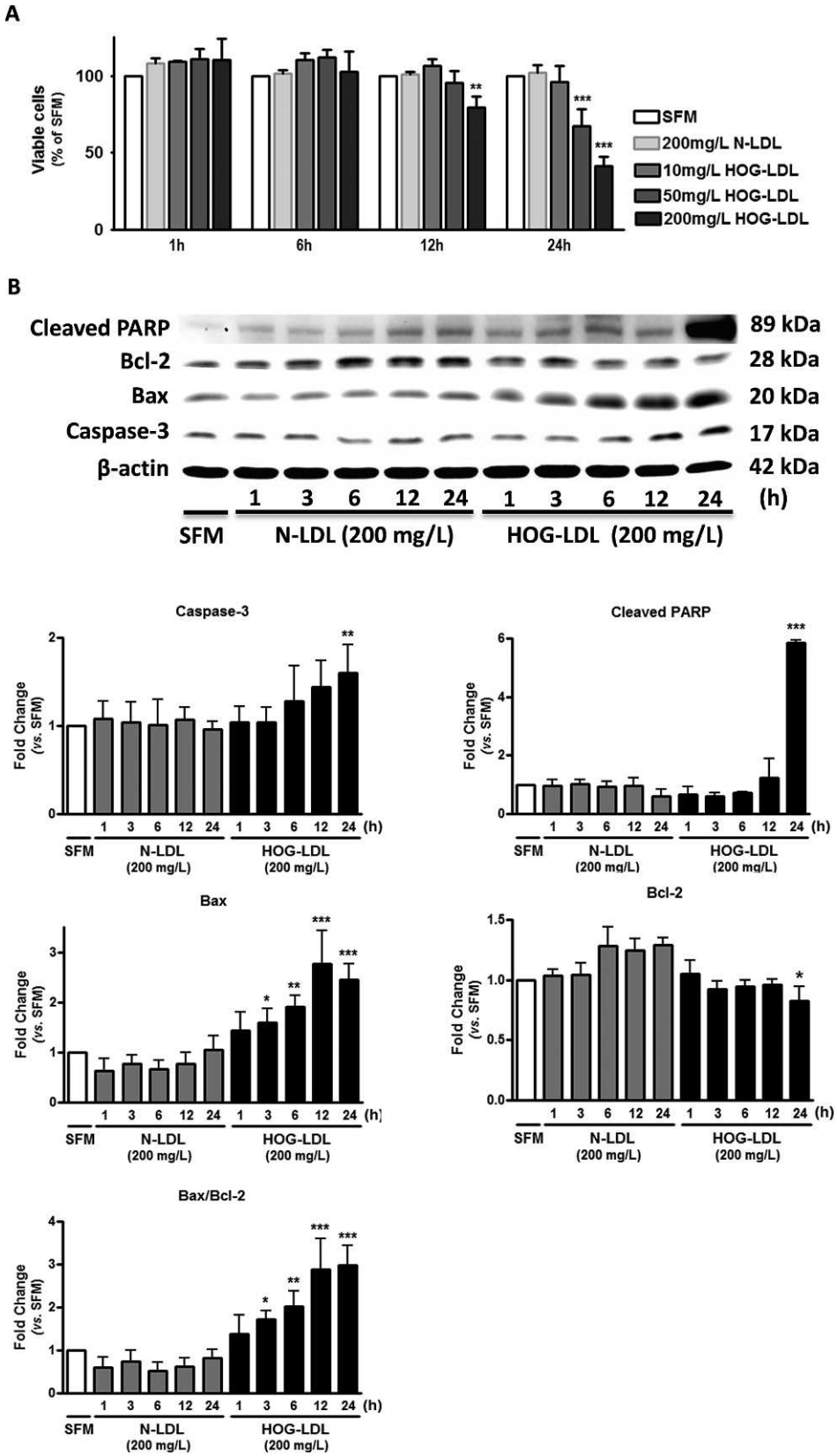

Figure 1. Compared with SFM or N-LDL, HOG-LDL decreased cell viability and caused apoptotic cell death in human retinal Müller cells. (**A**) HOG-LDL reduced viability in a manner that was both dose- (10, 50, and 200 mg/L) and time- (1, 6, 12, and 24 hours) dependent (CCK-8 assay). (**B**) HOG-LDL increased protein levels of caspase-3, cleaved PARP, and Bax; decreased Bcl-2; and increased the ratio of Bax/Bcl-2. Representative Western blots analyzed by densitometry are shown. In the bar graphs, *open columns* represent SFM treatment; *gray columns*, N-LDL treatment; and *black columns*, HOG-LDL treatment. Cells were incubated with HOG- or N-LDL (200 mg/L) for the indicated times ranging from 1 to 24 hours. Data represent the mean ± SD of three experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, versus SFM.

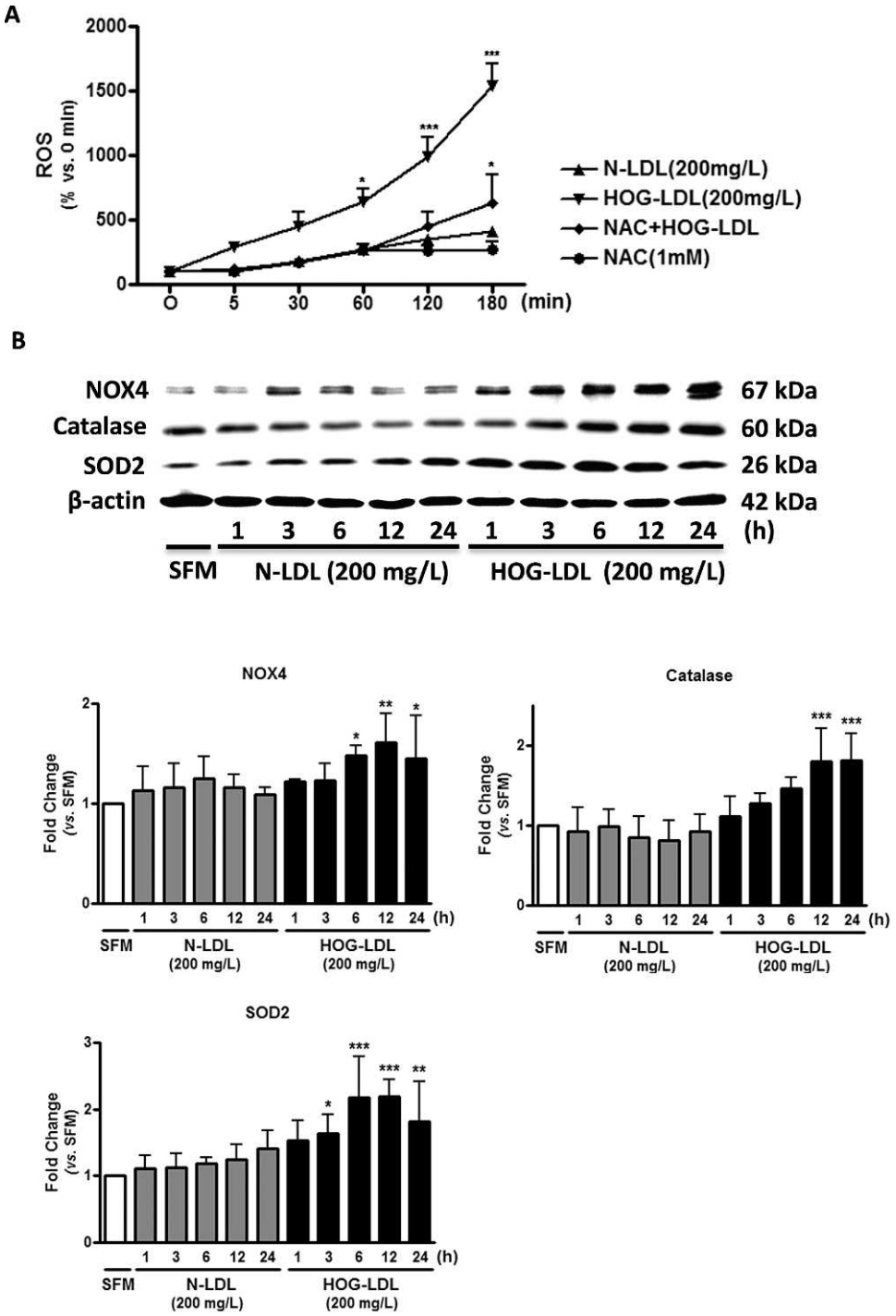

Figure 2. HOG-LDL increased oxidative stress. (**A**) HOG-LDL increased intracellular ROS production 15-fold over 3 hours; the effect was mitigated by pretreatment with NAC. N-LDL had no significant effect. $^{*}P < 0.05$; $^{***}P < 0.001$, versus Time 0. (**B**) Western blot analysis showing that from approximately 6 hours, HOG-LDL increased protein levels of Nox4, catalase, and SOD2, whereas N-LDL had no effect. The bar graphs show densitometry data: *open columns* represent SFM; *gray columns* represent N-LDL treatment; *black columns* represent HOG-LDL treatment. Cells were incubated with HOG- or N-LDL (200 mg/L) for the indicated times ranging from 1 to 24 hours. Data represent the mean ± SD of three experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, versus SFM.

indicated that modified (oxidized and glycated) LDL induced apoptosis in human retinal capillary pericytes in vitro.[12,14,17] In addition, large quantities of extravasated apoB100 and oxidized LDL were identified in retinal tissues from diabetic human retinas.[16] The presence of modified LDL preceded the onset of DR, and the amount was associated with the severity of retinopathy and with apoptotic figures.[16] Müller cells are intimately associated with all of the vascular beds of the retina, and functionally support BRB homeostasis[41]; these cells are "early responders" to any retinal insult.[42,43] The present study demonstrates for the first time that HOG-LDL induces apoptotic cell death in vitro of human retinal Müller cells. The degree of LDL modification and the concentrations used were chosen as conservative estimates of those likely to be

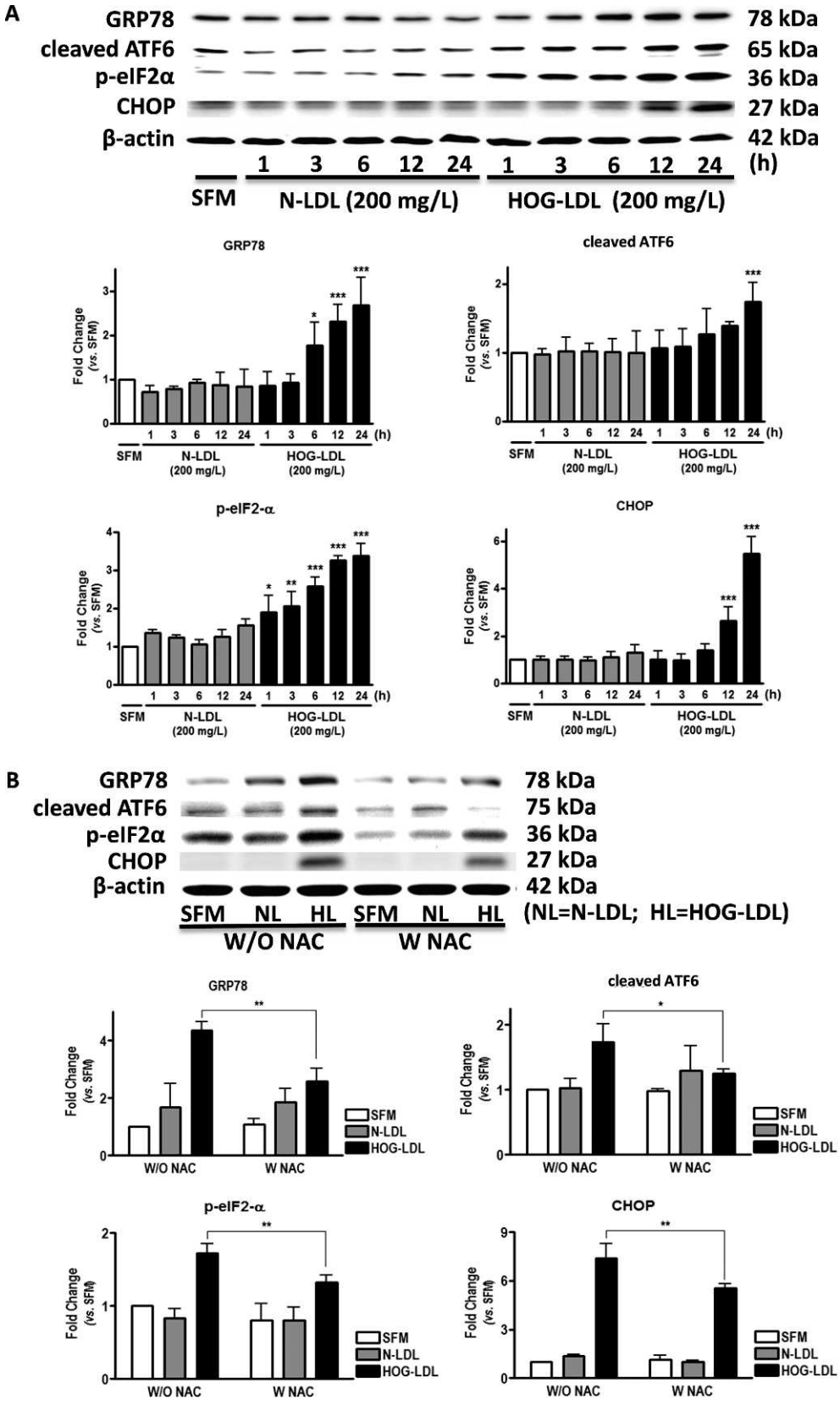

Figure 3. HOG-LDL increased ER stress in human retinal Müller cells via oxidative stress pathways. (**A**) Protein levels of ER stress markers GRP78, split-ATF-6, phospho-eIF2-α, and CHOP increased by HOG-LDL (200 mg/L) in a time-dependent manner (1, 3, 6, 12, and 24 hours), but were unaffected by the same concentration of N-LDL. Representative Western blots are shown. In the bar graphs showing densitometry, *open columns* represent SFM; *gray columns* represent N-LDL treatment; *black columns* represent HOG-LDL treatment. Cells were incubated with HOG- or N-LDL (200 mg/L) for the indicated times ranging from 1 to 24 hours (means ± SD; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, versus SFM). (**B**) After 24-hour exposure, the increase in ER stress induced by HOG-LDL (200 mg/L) was suppressed by pretreatment with NAC (1 mM, 1 hour; W/O NAC, W NAC:

without, with NAC). Data shown are representative Western blots and, in bar graphs, the results of three experiments using densitometry (mean ± SD; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, W/O versus W NAC).

found in vivo in the retinas of people with diabetes who have leakage of the BRB. Furthermore, our study suggests that in Müller cells, modified LDL promotes oxidative stress, then ER stress, and that both stresses contribute to apoptosis. Finally, we show that two well-known lipid peroxidation products, 7KC and 4HNE, have effects similar to HOG-LDL on Müller cells. The preparation of LDL and its in vitro modification is laborious and time-consuming, and therefore substitution of these commercially available products of lipid oxidation may facilitate future studies to explore the effects of extravasated plasma lipoproteins.

Vascular changes are the hallmark of DR.[44–46]; however, increasing evidence has suggested that, in addition to microangiopathy, degeneration of other retinal cells is also involved.[47] In fact, many glial and neuronal changes have been observed in animal models and in diabetic patients before the onset of clinical vascular injury.[47] Müller cells span the entire thickness of the retina, are positioned between retinal vasculature and neurons,[41] and are important in establishing and maintaining the BRB.[48] Müller cells have been shown to become dysfunctional in the early stages of DR.[49–51] Dysfunctional Müller cells may contribute to vessel leakage, and later, to proliferative retinal vascular disease through the secretion of VEGF.[52,53] We hypothesize that plasma lipoproteins (and specifically LDL), are extravasated through a leaking BRB in diabetes, become further modified by additional glycation and oxidation, and then contribute to Müller cell injury and death. In diabetes, glycation of lipoproteins that has occurred in plasma[54] will increase further after extravasation in the retina. Lipoproteins that have been further modified by glycation and oxidation may be implicated in reactive gliosis[55] of Müller cells, which may eventually lead to cell death. Our present in vitro data are consistent with the contention that oxidized and glycated lipoproteins induce Müller cell death, demonstrating dose-dependent reduction in cell viability (Fig. 1A) and enhancement of apoptosis (Fig. 1B).

Oxidative stress has been implicated in DR,[26,56] and in animal studies, has been shown to contribute to Müller cell gliosis.[47] Our results show that HOG-LDL is a potent inducer of intracellular free radicals (Fig. 2A), and that HOG-LDL–induced cell death is inhibited by NAC (Fig. 4), indicating an important primary role for oxidative stress. Furthermore, the pro-oxidant enzyme, NOX4, a novel isoform of NADPH oxidase,[47] was significantly elevated in Müller cells after treatment with HOG-LDL (Fig. 2B). Whether increased NOX4 expression is responsible for the increased oxidative stress remains to be determined; however, the increased expression of antioxidant enzymes, catalase and SOD2, suggests that these presumably protective responses are unable to suppress HOG-LDL–induced oxidative stress.

The ER is a sensor for cellular stresses such as hyperglycemia, dyslipoproteinemia, hypoxia, and oxidative stress, and ER stress may play an important role in DR.[28–30] In the current study, we found that HOG-LDL induced phosphorylation of eIF2α and increased expression levels of GRP78, cleaved ATF6, and CHOP, whereas N-LDL was without effect (Fig. 3). It is interesting to note that in response to HOG-LDL, these molecules were induced at different time points, with phospho-eIF2α being the earliest, followed by GRP78, CHOP, and ATF-6. Consistent with a previous report that CHOP induces the transcription of pro-apoptotic genes and suppresses the transcription of Bcl-2,[47] our data showed increases in the active forms of caspase-3 and PARP, and a decrease of Bcl-2 at 24 hours (i.e., after CHOP activation [at 12 hours]) (Figs. 1, 3). Bax expression, however, increased at an earlier time point, perhaps via other synergistic pathways. The timing of responses suggests that HOG-LDL first triggers the unfolded protein response, which in turn leads to activation of apoptosis. Although ATF6 was also activated, the late response to increasing protein degradation by the ATF6 pathway could not prevent the deleterious process. Indeed, blockage of ER stress with the inhibitor, 4-PBA, could inhibit HOG-LDL–induced activation of PARP (Fig. 4C), thereafter reducing apoptosis (Fig. 4D) and improving cell viability (Fig. 4B). Of interest, blockade of oxidative stress with NAC attenuated both ER stress and apoptosis induced by HOG-LDL (Figs. 3B, 4D), suggesting that oxidative stress is an initial step of HOG-LDL–induced ER stress and apoptosis. These results are consistent with the idea that HOG-LDL–induced oxidative stress results in an accumulation of damaged proteins, thereby eliciting the unfolded protein response and subsequent apoptosis. We have observed the same timing in HOG-LDL–treated human capillary pericytes and retinal pigment epithelial cells (Yang S, Wu M, Fu D, Du M, Wilson K, Lyons T, unpublished data, 2012).

Lipid peroxidation products have been implicated as a major source of oxidative stress in retina,[39,57] and may induce ER stress and apoptosis.[58,59] New in vivo evidence showed FDP-lysine (N"-[3-formyl-3,4-dehydropiperidino]-lysine) accumulates in retinal Müller glia, and FDP-lysine–modified proteins caused Müller cell dysfunction in vitro, including a decrease in protein levels of the potassium channel subunit Kir4.1, and upregulation of transcripts for VEGF, IL-6, and TNF-α.[22] In the current report, we investigated 7KC and 4HNE, two well-known lipid oxidation products,[60,61] to determine whether their effects on Müller cells resembled those of HOG-LDL. We found that, as with HOG-LDL, both 7KC and 4HNE decreased cell viability; increased expression of Nox4, catalase, and SOD2; increased ER stress proteins (phosphor-eIF2α, GRP78, CHOP, and ATF6); and triggered activation of pro-apoptotic Bax, caspase-3, and PARP (see Supplementary Figs. S1, S2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9910/-/DCSupplemental). However, 7KC showed a closer resemblance to the effects of HOG-LDL than 4HNE. The data show that oxidative stress and ER stress activated by HOG-LDL can be partially reproduced by 7KC and 4HNE.

Previously, using immunostaining, we demonstrated the presence of large quantities of extravasated and oxidized LDL in retinal tissues from diabetic human retinas.[16] The extent of staining correlated with the severity of retinopathy: it was absent in normal, nondiabetic retinas, but present in diabetic retinas even in the absence of clinical DR This suggests that retinal injury mediated by extravasated, modified lipoproteins may commence in the preclinical phase of DR. The current study extends our knowledge of the effects of modified LDL to Müller glial cells, and expands on our previous results showing toxicity of modified LDL on retinal endothelial cells and pericytes.[12,14–18] Due to their critical functions in maintaining the healthy retina, any damage to Müller cells is likely to promote both retinal neuronal and vascular injury. The resulting enhancement of vascular leakage means that this effect may contribute to the initiation and progression of DR. Therefore, Müller cell dysfunction and death resulting from exposure to modified lipids may be a new target for novel therapeutic strategies to prevent or slow the development and progression of DR.

In conclusion, our data suggest that modified lipoproteins may be implicated in the death of Müller glial cells, and their

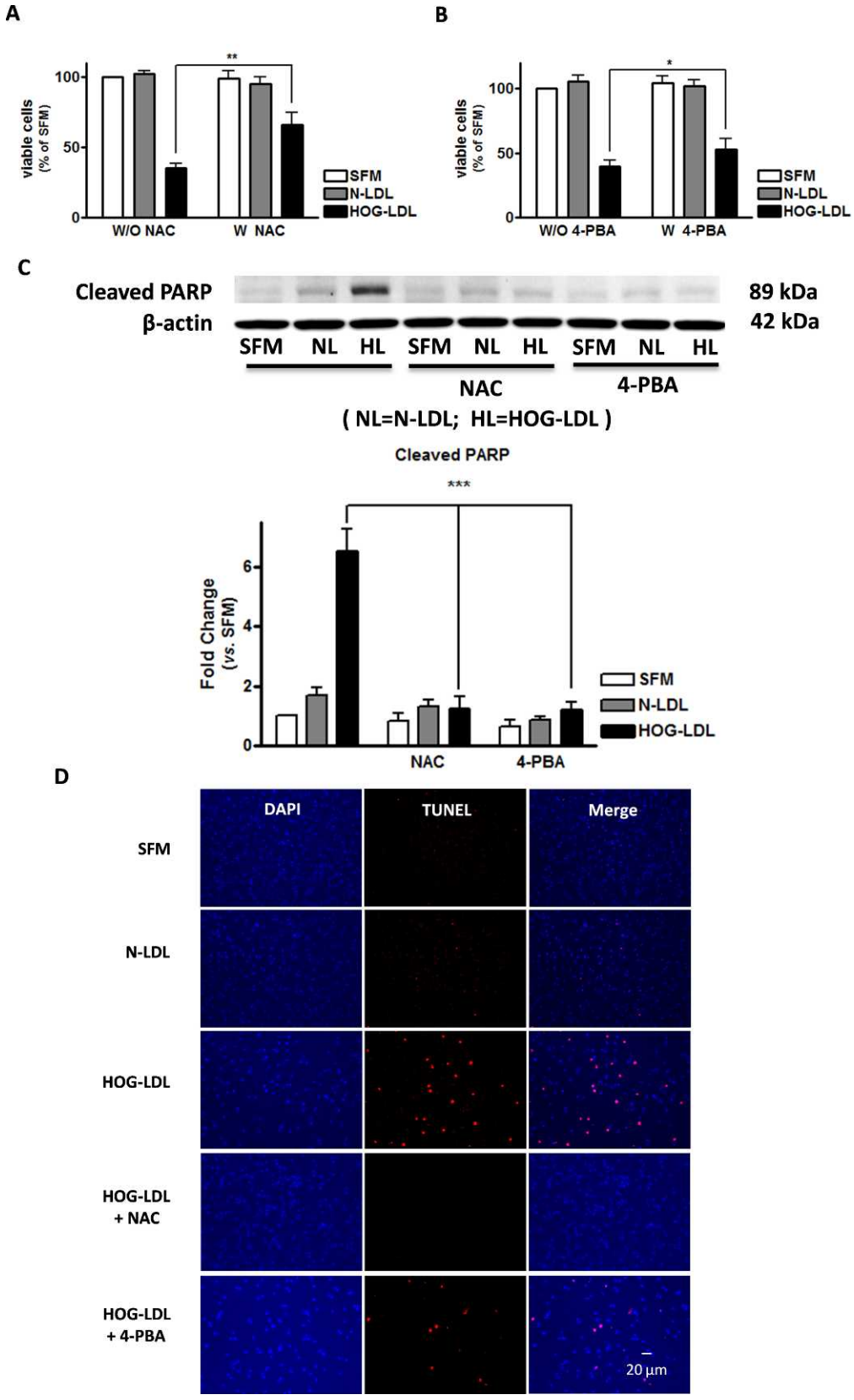

Figure 4. In human retinal Müller cells, HOG-LDL–induced apoptosis is mediated by oxidative and ER stress. (**A**) Pretreatment with NAC (1 mM, 1 hour) mitigated the decrease in cell viability induced by HOG-LDL (200 mg/L, 24 hours) (W/O NAC, W NAC: without, with NAC; $n = 3$; mean ± SD; $^{**}P < 0.01$). (**B**) Pretreatment with 4-PBA (100 μM, 1 hour) also mitigated the decrease in cell viability induced by HOG-LDL (200 mg/L, 24 hours) (W/O 4-PBA, W 4-PBA: without, with 4-PBA; $n = 3$; mean ± SD; $^{*}P < 0.05$). (**C**) Pretreatment (for 1 hour) with NAC (1 mM, 1 hour) or 4-PBA (100 μM, 1 hour) attenuated the increased expression of cleaved PARP induced by HOG-LDL (200 mg/L, 24 hours) ($n = 3$; mean ± SD; $^{***}P < 0.001$ versus HOG-LDL alone). (**D**) Representative images showing that both NAC (1 mM, 1 hour) and 4-PBA (100 μM, 1 hour) pretreatment reduced the

number of TUNEL-positive cells induced by HOG-LDL (200 mg/L, 24 hours). Blue color represents DAPI staining of nuclei and red indicates TUNEL-positive cells.

effect is mediated, at least partially, by both oxidative and ER stresses. Future studies are needed to explore these effects in vivo, but such effects are likely, given strong evidence that plasma lipoproteins flood the retina once BRB breakdown occurs in diabetes. The realization that extravasated, modified lipoproteins in the retina may be a central stimulus to the development of DR has therapeutic implications. New treatments to preserve the integrity of the BRB, thus preventing the extravasation of lipoproteins, or to block the effects of extravasated, modified lipoproteins on various retinal cell types, including Müller cells, may hold great promise. If such treatments can be realized, and combined with more effective means to achieve euglycemia, it may be possible to arrest DR at the earliest stages of its development.

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