

# Fenofibric Acid Reduces Fibronectin and Collagen Type IV Overexpression in Human Retinal Pigment Epithelial Cells Grown in Conditions Mimicking the Diabetic Milieu: Functional Implications in Retinal Permeability

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**PURPOSE.** To determine whether fenofibric acid (FA) reduces high glucose (HG)-induced basement membrane component overexpression and hyperpermeability in human retinal pigment epithelial (RPE) cells.

**METHODS.** Retinal pigment epithelial cells (ARPE-19) were cultured for 18 days in normal glucose (5 mM) or HG (25 mM) medium and studied for the effects of FA on fibronectin (FN) and collagen IV (Coll IV) expression. During last 3 days of the experiment, 100  $\mu$ M FA was added to cells grown in HG medium or in HG medium plus IL-1 $\beta$  (HG + IL-1 $\beta$ ) to mimic, at least in part, the inflammatory aspect of the diabetic milieu. Real-time RT-PCR was performed to determine FN and Coll IV mRNA levels, whereas protein levels were assessed by Western blot analyses. Cell monolayer morphology and barrier function were analyzed by confocal microscopy using specific antibodies against tight junction proteins, ZO-1, and claudin-1 and by measuring apical-basolateral movements of FITC-dextran, respectively.

**RESULTS.** FN and Coll IV expression were significantly increased in RPE cells grown in HG or HG + IL-1 $\beta$  medium compared with cells grown in normal medium. When cells grown in HG or HG + IL-1 $\beta$  medium were treated with FA, significant reductions in FN and Coll IV expression were observed. In addition, exposure to FA decreased excess permeability in a dose-dependent manner in cells grown in HG + IL-1 $\beta$  medium. This effect was unrelated to changes in tight junction protein content.

**CONCLUSIONS.** Findings from this study suggest that the down-regulation of basement membrane components by FA may

have a protective effect against outer blood-retinal barrier leakage associated with diabetic retinopathy. (*Invest Ophthalmol Vis Sci.* 2011;52:6348–6354) DOI:10.1167/iovs.11-7282

The Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) trial has shown beneficial effects of fenofibrate in reducing the risk for cardiovascular disease events and microvascular complications in diabetes.<sup>1,2</sup> In particular, fenofibrate reduced total cardiovascular disease events and macular edema by 31% and proliferative diabetic retinopathy (DR) by 30% in patients with diabetes. In addition, recent data from the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial indicated that ocular complications had 40% odds of progression to DR in the group of patients receiving fenofibrate plus simvastatin compared with the group of patients treated with placebo plus simvastatin.<sup>3</sup> However, it is unknown how fenofibrate, a hypolipemiant drug, improves retinal vascular permeability associated with DR.<sup>4</sup> Fenofibrate reduces cholesterol by lowering low-density lipoprotein, very low-density lipoprotein, and triglyceride levels while increasing high-density lipoprotein levels.<sup>5</sup> In addition, its beneficial effect on insulin resistance has been reported.<sup>6,7</sup> Although the lipid-modifying effects of fenofibrate have been well documented,<sup>8</sup> its mechanistic role in reducing diabetic microvascular complications, specifically diabetic macular edema formation, is unknown.

DR is a leading cause of blindness and vision loss in the working age population.<sup>9</sup> Basement membrane thickening and increased vascular permeability are two major retinal vascular changes associated with the pathogenesis of this disease.<sup>10–12</sup> Studies have reported that HG or hyperglycemia induces the overexpression of basement membrane components, which, in turn, contributes to excess retinal vascular permeability.<sup>11,12</sup> We have shown that normalization of basement membrane component overexpression could lead to beneficial effects in preventing excess retinal vascular permeability and to the development of acellular capillaries and pericyte loss in animal models of DR.<sup>11–14</sup>

Diabetic macular edema (DME) is a prominent clinical manifestation that frequently leads to severe loss of central vision in patients with diabetes.<sup>15</sup> Studies indicate that tight junctions play an important role in maintenance of the inner blood-retinal barrier (BRB) and that compromised tight junctions promote the formation of DME.<sup>16,17</sup> Similarly, the outer BRB, which is formed by RPE cells attached to one another by tight junctions, also plays an essential role in preventing the accumulation of extracellular fluid in the subretinal space of the retina.<sup>18</sup> Compromised tight junctions in the RPE cell monolayer are known to contribute to the disruption of the outer BRB and to the impairment of neural retinal function. Studies

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have shown that fibronectin (FN) and collagen IV (Coll IV) are located in the basement membrane of the RPE<sup>19,20</sup> and that significant thickening develops in the RPE basement membrane with aging and the formation of advanced glycation end products,<sup>21</sup> two phenomena known to contribute to diabetic vascular basement membrane thickening. Because overexpression of basement membrane components and subsequent retinal capillary basement membrane thickening have been implicated in the breakdown of the inner BRB in diabetes, we examined in this study whether the overexpression of FN and Coll IV, two basement membrane components synthesized by RPE cells, may contribute to the outer BRB hyperpermeability seen in DR and whether such hyperpermeability could be prevented by FA.

In the present study we demonstrated that FA, the active metabolite of fenofibrate, prevents the breakdown of the RPE barrier under conditions that mimic the diabetic milieu. This effect is related to the protective role of FA in reducing FN and Coll IV overexpression produced by RPE cells. Results from this study suggest that FA may impart beneficial effects in preventing or arresting the development of DME in diabetic patients by ameliorating abnormal basement membrane component synthesis in the outer BRB.

## MATERIALS AND METHODS

### Cell Culture

ARPE-19 cells representing a spontaneously immortalized human RPE cell line were obtained from American Type Culture Collection (Manassas, VA). Cells from passage 18 were cultured for 18 days at 37°C under 5% (vol/vol) CO<sub>2</sub> in medium (DMEM/F12) supplemented with 10% (vol/vol) fetal bovine serum (HyClone; Thermo Fisher Scientific, Logan, UT) and 1% (vol/vol) penicillin/streptomycin (HyClone; Thermo Fisher Scientific) in N condition (5.5 mM D-glucose) and HG conditions (25 mM D-glucose). To study the potential protective effect of FA on the barrier function of RPE cells, FA (100 μM) was added to the standard culture medium daily for the last 3 days of the experiment (days 15–17). For studies examining the effect of different doses, cells were exposed to 25 or 100 μM FA after the conditions described for 100 μM FA. Cells were also treated with IL-1β (10 ng/mL) for the last 2 days of the experiment (days 16, 17) and were subjected to serum starvation (1% FBS) during the treatments. To rule out a potential bias by an osmotic effect, the experiment was also performed using mannitol (5.5 mM D-glucose + 19.5 mM mannitol vs. 25 mM D-glucose) as an osmotic control agent.

### In Vitro Permeability

For permeability studies, ARPE-19 cells were seeded at 400,000 cells/mL (80,000 RPE cells/well) in 0.33 cm<sup>2</sup> polyester filters (HTS-Transwells; Costar, Corning, NY). For real-time PCR and Western blot analyses, cells were seeded directly on plastic at 20,000 cells/mL. For immunofluorescence and polarization studies, cells were seeded on glass coverslips at 20,000 cells/mL. The permeability of RPE cells was determined at 18 days in culture by measuring the apical-to-basolateral movements of fluorescein isothiocyanate (FITC) dextran (40 kDa) (Sigma, St. Louis, MO). The test molecule was added to the apical compartment of the cells in a concentration of 100 μg/mL. Samples (200 μL) were collected from the basolateral side at baseline and 75

minutes after the addition of the molecules. The medium in the basolateral compartment was replaced by fresh medium after the collection of every sample. A minimum of four wells were used for each time measurement. Absorbance was measured at 485 nm of excitation and 528 nm of emission with a microplate reader (Spectra-Max Gemini; Molecular Devices, Sunnyvale, CA).

### Real-Time RT-PCR

To study the mRNA level of FN and Coll IV, first-strand cDNA was synthesized using a cDNA synthesis kit (Superscript; Invitrogen, Carlsbad, CA). Primer sets for performing real-time quantitative qPCR for Col4a1 (accession no. NM\_001135009) and FN (accession no. X15906) and housekeeping gene hypoxanthine phosphoribosyl transferase 1 (HPRT; accession no. NM\_012583) were designed using a Web-based primer design program (www.roche.com). All real-time qPCR measurements were performed on a PCR system (7500; Applied Biosystems, Foster City, CA) using the standard temperature cycling protocol for the relative quantification assay. Each measurement was run in triplicate for each sample. Selected samples were run after sequential dilution to confirm that the detected signals were within the linear amplification range. Results were first normalized to the expression level of the endogenous housekeeping gene HPRT. Selected samples were tested against two additional housekeeping genes, 18S and glyceraldehyde-3-phosphate dehydrogenase, and the results were no different from the results obtained using HPRT. Further information is presented in Table 1.

### Western Blot Analysis

Western Blot analysis was performed to determine the relative levels of ZO-1, claudin-1, FN, and Coll IV protein in the RPE cells from each group. RPE cells were homogenized, and protein was isolated as previously described.<sup>11</sup> Bicinchoninic acid assay (Pierce Chemical, Rockford, IL) was used to determine total protein concentrations. Western blot analysis were performed with 25 μg protein/lane; after electrophoresis, the gels were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA) using a semidry apparatus according to Towbin's procedure.<sup>22</sup> The membranes were blocked with 5% nonfat dry milk for 2 hours and then exposed to rabbit FN (Millipore, Billerica, MA; 1:1000) and rabbit Coll IV (Fitzgerald Industries, Acton, MA; 1:2500) antibody solution overnight at 4°C. Blots were washed with Tris-buffered saline containing 0.1% Tween-20 and then incubated with goat anti-rabbit IgG secondary antibody (Cell Signaling, Billerica, MA) solution (1:3000) for 1 hour and goat anti-rabbit (1:20,000) or goat anti-mouse (1:10,000) for 1 hour (Pierce; Thermo Scientific). The membranes were again washed as described and then were exposed to a chemiluminescent protein detection system (Immuno-Star; Bio-Rad) to detect the protein signals on x-ray film (Fujifilm, Tokyo, Japan). Protein loading in the gels was confirmed by Ponceau-S staining and tubulin antibody (Cell Signaling; 1:1000), and the densitometric values were used for adjustment of any differences in loading. Densitometric analysis of the Western blot signals was performed at nonsaturating exposures and analyzed using the ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>).

### Immunohistochemistry

For immunohistochemistry and polarization studies, cells were grown for 18 days at confluence in 24-well plates containing one circle

**TABLE 1.** PCR Primer Sequences Used for Performing Real-Time Quantitative qPCR to Assess FN and Coll IV mRNA Levels

Name	Forward	Reverse	Amplicon Size
FN	cagccctgattggagtc	tgggtgacacctgagtgaac	72
Coll IV	gcccattggtcaggacttg	aaggcatggtgctgaact	61

coverslip of glass (12-mm diameter) (Thermo Scientific, Menzel-Gläser; Braunschweig, GE) inside each well. Cells were washed with PBS and fixed with methanol (ZO-1 and claudin-1) or paraformaldehyde (FN and Coll IV) for 10 minutes, washed again with PBS twice, and blocked with 2% BSA and 0.05% Tween in PBS overnight at 4°C. Mouse anti-ZO-1, rabbit anti-claudin-1 (Zymed Laboratory Gibco, Invitrogen, San Diego, CA), rabbit anti-FN, rabbit anti-Coll IV (Abcam, Cambridge, MA), and mouse anti-N+/K+ ATPase (Millipore), all diluted to 1:200, were incubated for 1 hour at room temperature (RT). After washing with PBS, cells were further incubated with Alexa 488 goat anti-rabbit and Alexa 594 donkey anti-mouse secondary antibodies (Invitrogen) for 1 hour at RT. After washing with PBS, the slides were mounted with mounting medium containing DAPI for fluorescence (Vectashield; Vector Laboratories, Burlingame, CA). Images were acquired with a confocal laser scanning microscope (FV1000; Olympus, Hamburg, Germany).

### Statistical Analysis

Data are presented as mean  $\pm$  SD. The values of the control groups were normalized to 100%, and values from all other groups were expressed as percentages of control; statistical analysis was performed using the normalized values. Comparisons between groups were performed using ANOVA followed by the Student's *t*-test, and  $P < 0.05$  was considered statistically significant.

## RESULTS

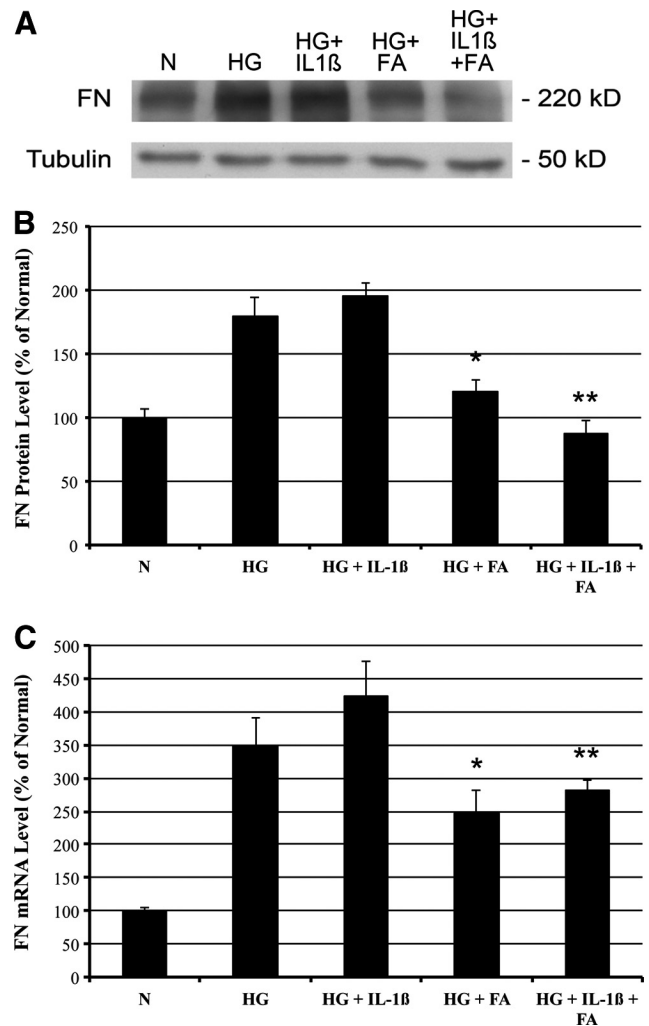
### Effect of FA on High Glucose- and IL-1 $\beta$ -Induced Fibronectin Overexpression in RPE Cells

Western blot analysis showed significantly increased FN protein expression in RPE cells grown in HG or HG + IL-1 $\beta$  medium compared with those grown in normal medium (179%  $\pm$  14% of normal,  $P < 0.05$ ; 195%  $\pm$  10% of normal,  $P < 0.05$ , respectively). When RPE cells grown in HG medium were treated with FA, a significant reduction in FN protein level was observed compared with RPE cells grown in HG medium (121%  $\pm$  9% of normal vs. 179%  $\pm$  14% of normal,  $P < 0.05$ ). Similarly, when RPE cells grown in HG medium supplemented with IL-1 $\beta$  were treated with FA, FN expression was significantly reduced compared with RPE cells grown in HG medium supplemented with IL-1 $\beta$  (87%  $\pm$  10% of normal vs. 194%  $\pm$  14% of normal,  $P < 0.05$ ) (Figs. 1A, 1B).

Real-time RT-PCR results showed significantly increased FN mRNA levels in RPE cells grown in HG or HG + IL-1 $\beta$  medium compared with RPE cells grown in normal medium (349%  $\pm$  41% of normal,  $P < 0.05$ ; 423  $\pm$  53% of normal,  $P < 0.05$ , respectively). FA significantly reduced FN mRNA overexpression in RPE cells grown in HG or HG + IL-1 $\beta$  medium compared with untreated RPE cells grown in HG or HG + IL-1 $\beta$  medium, respectively (247%  $\pm$  34% of normal vs. 349%  $\pm$  41% of normal,  $P < 0.05$ ; 282%  $\pm$  15% of normal vs. 423%  $\pm$  53% of normal,  $P < 0.05$ , respectively; Fig. 1C).

### Effect of FA on High Glucose- and IL-1 $\beta$ -Induced Collagen Type IV Overexpression in RPE Cells

Western blot analysis showed significantly increased Coll IV protein expression in RPE cells grown in HG or HG + IL-1 $\beta$  medium compared with those grown in normal medium (232%  $\pm$  25% of normal,  $P < 0.05$ ; 276%  $\pm$  21% of normal,  $P < 0.05$ , respectively; Fig. 2). When RPE cells grown in HG medium or HG medium supplemented with IL-1 $\beta$  were treated with FA, a significant reduction in Coll IV expression compared with RPE cells grown in HG medium or HG medium supplemented with IL-1 $\beta$ , respectively, was observed (113%  $\pm$  17% of normal vs. 232%  $\pm$  25% of normal,  $P < 0.05$ ; 168%  $\pm$  22% of normal vs. 276%  $\pm$  21% of normal,  $P < 0.05$ , respectively; Figs. 2A, 2B).



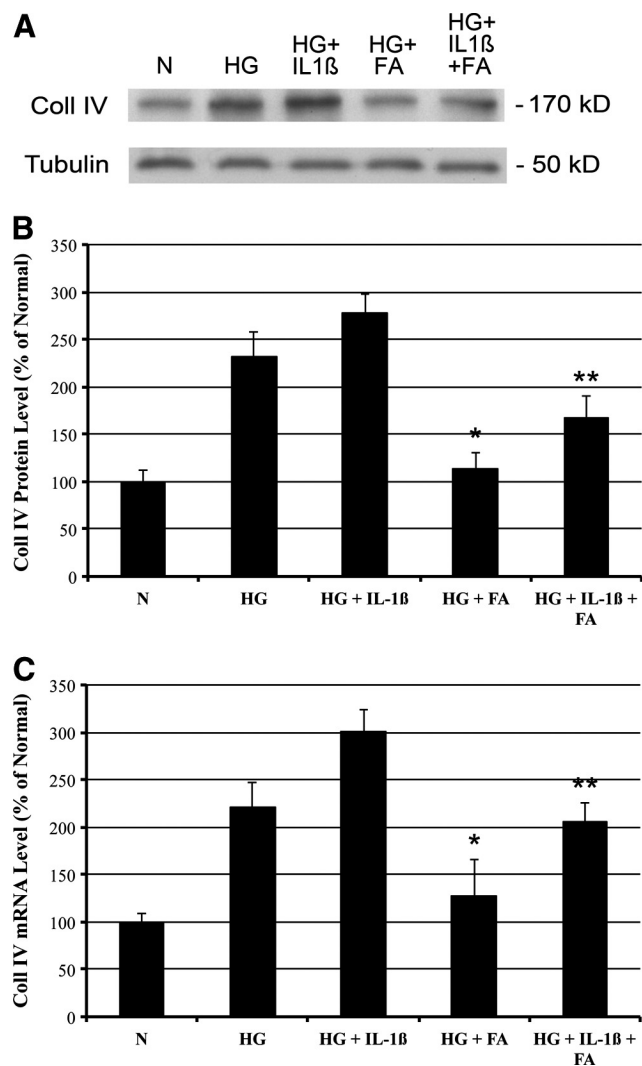
**FIGURE 1.** Effect of FA on FN protein and mRNA levels in RPE cells. (A) Representative Western blot image shows FA reduces HG- and HG + IL-1 $\beta$ -induced FN overexpression. (B) Graphical representation of Western blot data. FN protein level is significantly increased in RPE cells grown in HG or HG + IL-1 $\beta$  medium. When treated with fenofibrate, RPE cells grown in HG medium showed a significant reduction in FN expression compared with untreated HG cells ( $*P < 0.05$ ). Similarly, FA treatment reduced FN overexpression in cells grown in HG + IL-1 $\beta$  medium compared with untreated cells grown in HG + IL-1 $\beta$  medium ( $**P < 0.05$ ). (C) Real-time RT-PCR result indicates increased FN mRNA expression in cells grown in HG or HG + IL-1 $\beta$  medium. FA significantly reduces FN overexpression in both groups ( $*HG$  vs. HG + FA,  $P < 0.05$ ;  $**HG + IL-1\beta$  vs. HG + IL-1 $\beta$  + FA,  $P < 0.05$ ).

Real-time RT-PCR results showed significantly increased Coll IV mRNA levels in RPE cells grown in HG or HG + IL-1 $\beta$  medium compared with RPE cells grown in normal medium (221%  $\pm$  28% of normal,  $P < 0.05$ ; 301%  $\pm$  23% of normal,  $P < 0.05$ , respectively). FA significantly reduced Coll IV mRNA overexpression in RPE cells grown in HG or HG + IL-1 $\beta$  medium compared with untreated RPE cells grown in HG or HG + IL-1 $\beta$  medium, respectively (127%  $\pm$  39% of normal vs. 221%  $\pm$  28% of normal,  $P < 0.05$ ; 206%  $\pm$  19% of normal vs. 301%  $\pm$  23% of normal,  $P < 0.05$ , respectively; Fig. 2C).

### Effect of FA on High Glucose- and IL-1 $\beta$ -Induced Increased Barrier Permeability in RPE Cells

The effect of different conditions tested on the permeability of ARPE-19 monolayers is displayed in Figure 3. HG alone





**FIGURE 2.** Effect of FA on Coll IV protein and mRNA levels in RPE cells. (A) Representative Western blot image shows FA reduces HG- and HG + IL-1β-induced Coll IV overexpression. (B) Graphical representation of Western blot data. Coll IV protein level is significantly increased in RPE cells grown in HG or HG + IL-1β. When treated with FA, RPE cells grown in HG medium showed a significant reduction in Coll IV expression compared with untreated HG cells (\**P* < 0.05). Similarly, FA treatment reduced Coll IV overexpression in cells grown in HG + IL-1β medium compared with untreated cells grown in HG + IL-1β medium (\*\**P* < 0.05). (C) Real-time RT-PCR result indicates increased Coll IV mRNA expression in cells grown in HG or HG + IL-1β medium. FA significantly reduces Coll IV overexpression in both groups (\*HG vs. HG + FA, *P* < 0.05; \*\*HG + IL-1β vs. HG + IL-1β + FA, *P* < 0.05).

mildly increased excess permeability, whereas IL-1β alone significantly increased permeability. Interestingly, both (HG + IL-1β) dramatically increased permeability in what appeared to be a synergistic effect. Data related to osmotic control experiments using mannitol indicated that the excess permeability and the effects of HG + IL-1β are independent of hyperosmotic effects. When cells grown in HG medium supplemented with IL-1β were treated with 25 μM FA, a significant reduction in permeability was observed (164.6 ± 38.3 vs. 224.9 ± 26.4; *P* = 0.03). This protective effect on monolayer permeability was more evident in cultures treated with 100 μM FA (149.9 ± 15.5 vs. 224.9 ± 26.4; *P* = 0.005).

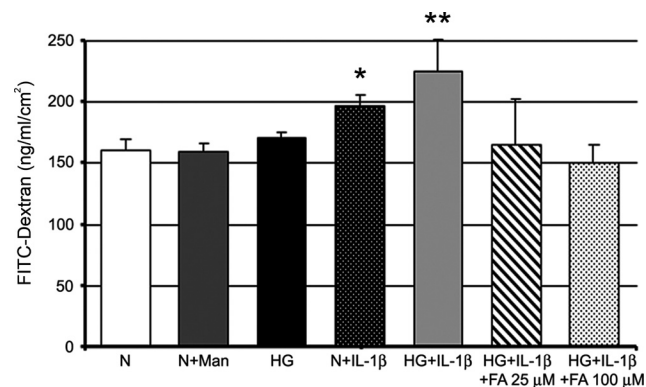
### Effect of FA on Localization and Distribution of High Glucose- and IL-1β-Induced Fibronectin, Collagen Type IV, Claudin-1, and ZO-1 in RPE Cells

To demonstrate that the cells formed a monolayer and exhibited polarity, ARPE-19 cells were stained with the tight junction protein occludin and with the apical marker enzyme Na<sup>+</sup>/K<sup>+</sup> ATPase. As expected, the confocal vertical (X-Z) sections showed a predominant apical Na<sup>+</sup>/K<sup>+</sup> ATPase localization and apical staining pattern for occludin (Fig. 4).

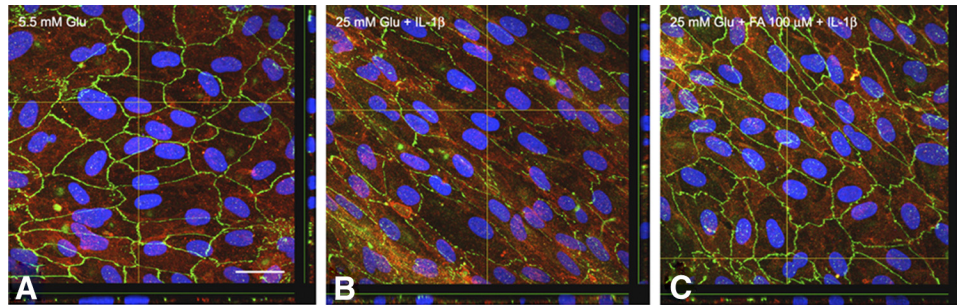
Immunostaining of tight junction proteins, ZO-1 and claudin-1 showed disruption of the cell monolayer induced by HG + IL-1β and the beneficial effect of 100 μM FA in preventing the disorganization of tight junction proteins and maintaining the integrity of the monolayer. Merged images show colocalization of claudin-1 and ZO-1 (Fig. 5A); treatment with 100 μM FA shows reduced disruption of the tight junctions. Increased FN and Coll IV localization was observed in cells grown in HG + IL-1β; treatment with 100 μM FA showed downregulation effects for both FN and Coll IV expression (Figs. 5B, 5C). Western blot analysis showed no significant difference in ZO-1 protein levels under the different experimental conditions compared with cells grown in normal medium. By contrast, HG + IL-1β-treated cultures showed higher levels of claudin-1 than did untreated cells. This increase in claudin-1 after IL-1β supplementation was associated with an increase rather than a decrease in permeability, which was reduced in a dose-dependent manner when the cells were treated with 25 μM or 100 μM FA (data not shown). The apparent contradictory effect of HG + IL-1β upregulating claudin-1 expression but decreasing the sealing function of RPE has been previously observed with respect to the IL-1β effect; the study indicated that IL-1β promotes an aberrant and dysfunctional distribution of claudin-1.<sup>2,3</sup>

### DISCUSSION

Findings from the present study indicate that FA treatment prevents increased RPE permeability induced by HG + IL-1β and that this beneficial effect of FA is associated with decreases in HG- and HG + IL-1β-induced FN and Coll IV overexpression.



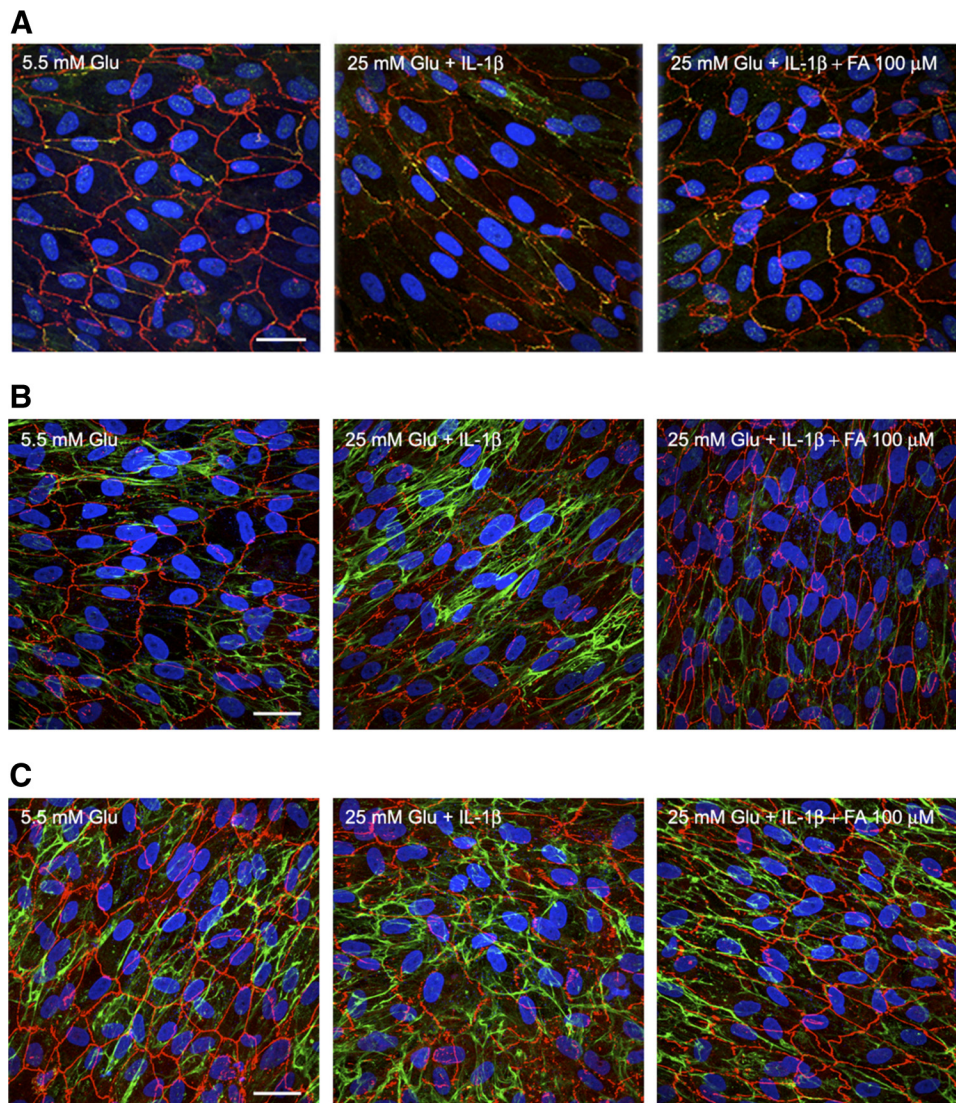
**FIGURE 3.** Effect of FA on ARPE-19 cell monolayer permeability. Data from permeability assays indicate that FA has a protective effect on HG + IL-1β-induced increased barrier permeability in a dose-dependent manner. Monolayer permeability of cells grown in 5.5 mM D-glucose medium (white bar), 5.5 mM D-glucose + 19.5 mM mannitol (dark gray bar), 25 mM D-glucose (HG; black bar), N + IL-1β (dotted black bar), HG + IL-1β (light gray bar), HG + IL-1β + FA (25 mM; striped bar), and HG + IL-1β + FA (100 mM; dotted white bar). Results are expressed as the mean ± SD (*n* = 4). \**P* < 0.05 compared with N. \*\**P* < 0.01 in comparison with N.



**FIGURE 4.** Evidence for tight junction and polarity in ARPE-19 monolayer. Confocal image showing the expression of occludin (green) and the apical marker enzyme Na<sup>+</sup>/K<sup>+</sup> ATPase (red). Nuclei were stained with DAPI (blue). (A) Confocal vertical (X-Z) sections showing predominant apical Na<sup>+</sup>/K<sup>+</sup> ATPase localization and apical staining pattern for the tight junction protein occludin in cells grown in NG medium. (B) ARPE-19 cells cultured under HG supplemented with IL-1 $\beta$  showing disruption of the cell monolayer and partial loss of polarization, which is prevented after treatment with FA 100  $\mu$ M (C).

This suggests that FA can prevent the breakdown of BRB permeability at least in part by normalizing ECM protein overproduction. In addition, we confirmed previous reports showing that the altered amount of tight junction proteins was not necessarily the only factor regulating tight junction functionality and that the distribution of the tight junction proteins plays

an important role in barrier permeability.<sup>23,24</sup> In fact, the protective effect of FA on RPE disruption induced by HG + IL-1 $\beta$  is in part mediated by its ability to prevent the aberrant distribution of tight junction proteins. The capacity of FA in maintaining the tight junction distribution and its suppressive effect on ECM overproduction could be involved in the beneficial



**FIGURE 5.** Effect of FA on localization and distribution of tight junction and ECM proteins in ARPE-19 cells. (A) Immunohistochemistry of ARPE-19 cells showing disruption of the monolayer induced by HG + IL-1 $\beta$  and the beneficial effects of FA in preventing the disorganization of tight junction proteins in the cell monolayer. Merged images show colocalization of claudin-1 and ZO-1 (yellow). Note that claudin-1 immunostaining appears green and ZO-1 immunostaining appears red. (B) Immunohistochemistry of ARPE-19 showing downregulation effect of 100  $\mu$ M FA on FN (green). (C) Immunohistochemistry of ARPE-19 showing the downregulation effect of 100  $\mu$ M FA on Coll IV expression (green). Nuclei were stained with DAPI (blue). Scale bar, 20  $\mu$ m.



effects of fenofibrate on DME. However, further investigation to determine the mechanisms by which FA affects ECM protein expression and tight junction protein distribution are needed.

Importantly, our findings from this study implicate a down-regulation effect of FA on extracellular matrix protein levels, which could play a role in preventing vascular permeability and in underscoring the importance of FN and Coll IV in forming a selective permeable outer BRB. In this regard we have previously shown that reducing basement membrane thickening by downregulating extracellular matrix components including FN and Coll IV is effective in preventing the apoptosis and increased permeability associated with DR.<sup>11,25</sup> Additionally, studies on RPE monolayers cultured on laminin-coated filters indicated that extracellular matrix components promote RPE morphology and the formation of a selective permeability barrier to various tracers.<sup>26</sup>

Increased levels of proinflammatory cytokines play a key role in the pathogenesis of DME.<sup>17,27,28</sup> Treatment of RPE cells with either serum, interferon- $\gamma$ , tumor necrosis factor- $\alpha$ , hepatocyte growth factor (HGF), interleukin (IL)-1 $\beta$  or placental growth factor-1 increases permeability and alters the expression or content of tight junction molecules.<sup>23,29-31</sup> Because IL-1 $\beta$  plays an important role in the development of DR,<sup>32-34</sup> we decided to use the cytokine together with HG conditions to mimic the diabetic milieu. A significant overexpression of FN and Coll IV was observed after treating ARPE-19 cells with IL-1 $\beta$  in the presence of HG, and this overexpression was associated with an increase in permeability. Overall, these findings indicate that a higher content of basement membrane components may contribute to the impairment of barrier function, leading to excess permeability. In addition, the overexpression of basement membrane components known to be induced by inflammatory cytokines such as IL-1 $\beta$ <sup>35,36</sup> may be involved in hyperpermeability, which occurs in DR.

Microvascular basement membrane is an important component of the blood barrier system, which participates in the regulation of vascular permeability. Thus, any changes to the basement membrane structure or its composition may adversely affect its function. Previous studies demonstrated the ability of fenofibrate to decrease extracellular matrix accumulation in renal cortex of streptozotocin-induced diabetic rats<sup>37</sup> and in kidneys of spontaneously hypertensive rats.<sup>38</sup> In addition, fenofibrate treatment was shown to affect extracellular matrix changes associated with systolic failure seen in ascending aortic constriction in chronic pressure overload mice.<sup>39</sup> Our results from this study parallel these findings and demonstrate fenofibrate treatment's beneficial effects on pathologic changes associated with the overexpression of extracellular matrix proteins.

The exact cellular mechanisms by which FA influences extracellular matrix component levels is unclear. Recent studies have focused on the ability of FA to activate peroxisome proliferator-activated receptor alpha (PPAR $\alpha$ ), a transcription factor that regulates the genes involved in cellular lipid catabolism. The activation of PPAR $\alpha$  increases lipolysis and the elimination of triglyceride-rich particles from plasma and also increases the synthesis of apoproteins, which leads to a reduction in very low-density and low-density fractions and an increase in the high-density lipoprotein fraction containing apoprotein. PPAR $\alpha$  may regulate extracellular matrix turnover through consequently inhibiting matrix metalloproteinases<sup>38,39</sup> or decreasing plasminogen activator inhibitor-1.<sup>37</sup> However, the exact pathway involving PPAR $\alpha$  and its downstream effectors has not been completely defined.

Other studies have investigated how fenofibrate may suppress oxidative stress and MAPK activation, thus decreasing TGF- $\beta$  levels and ultimately affecting extracellular matrix accumulation.<sup>38</sup> Finally, one cannot rule out other mechanisms

whereby fenofibrate may affect vascular permeability. One report demonstrated that fenofibrate is able to reduce apoptosis in human retinal endothelial cells, which is associated with DR.<sup>40</sup> The mechanism by which fenofibrate exerted its anti-apoptotic effect was found to be AMP-activated protein kinase (AMPK)-dependent and PPAR $\alpha$ -independent. Preventing unwanted apoptosis in the retinal vasculature may help maintain vessel integrity and prevent leakage associated with DR. In addition, we have recently shown that RPE disruption induced by IL-1 $\beta$  is prevented by FA because of its ability to suppress AMPK activation.<sup>24</sup> This finding indicates that suppression rather than activation of AMPK is the mechanism by which FA prevents the hyperpermeability induced by HG + IL-1 $\beta$ . In the same paper, we reported that AMPK activation in human RPE from diabetic donors was significantly higher than from non-diabetic donors and very similar to that obtained in ARPE-19 cells cultured under high (25 mM) glucose + IL-1 $\beta$ . Taken together, our results suggest that the suppression of AMPK activation is a mechanism by which fenofibrate may prevent or arrest diabetic macular edema.

A limitation of the present study is that it focuses on the effects of FA only on the outer BRB. As such, further studies are needed to investigate the effect of FA on the inner BRB and the contribution of FA on overall BRB breakdown. However, findings from this study documented an important proof of concept that HG-induced excess accumulation of basement membrane components of the outer BRB is involved in increased retinal permeability and that the protective effect of FA against leakage of the outer BRB is at least in part linked to the inhibitory effect of FA on specific basement membrane component expression in the RPE cells. The ability of FA to prevent basement membrane component overexpression may have significance for other diabetic microangiopathies beyond DME.

### Acknowledgments

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