



The effect of insulin and glucose levels on retinal glial cell activation and pigment epithelium-derived fibroblast growth factor-2

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Purpose: The diabetic retina exhibits decreases in endogenous nonangiogenic neurotrophins. This study hypothesized that deficiencies in systemic and retinal pigment epithelium-derived (RPE) neurotrophic factors also influence retinal changes in diabetes.

Methods: Diabetes was established in Lister hooded rats with streptozotocin. Reverse transcriptase coupled polymerase chain reaction (RT-PCR) and immunoblotting were used to determine the expression of fibroblast growth factor-2 (FGF-2) in the retina and RPE, and glial fibrillary acid protein (GFAP) in the retina. In addition, primary human RPE cultures and a transformed Müller cell line were used to determine the effect of insulin, glucose, and insulin-like growth factor (IGF) on the expression of these substances.

Results: FGF-2 and GFAP were increased in retina, but FGF-2 was decreased in the RPE of diabetic animals. Retinal GFAP correlated with RPE FGF-2 expression in these animals. Insulin produced a dose-dependent increase in FGF-2 in RPE cells and decrease in GFAP in Müller cells grown in 15 mM glucose. In 5 mM glucose, insulin had no effect on expression of either protein. Physiological levels of insulin inhibited changes induced by 15 mM glucose. The effect of 9 nM insulin on each culture was mimicked by 1 nM IGF, and blocked with an IGFR-1 inhibitor.

Conclusions: It is suggested that decreased systemic insulin and high glucose levels contribute to decreased FGF-2 production in the RPE and increased glial cell activation in the diabetic retina. Addition of insulin and IGF act to reverse this effect through the IGFR-1. These mechanisms may contribute to the development of diabetic retinopathy.

The traditional view of diabetic retinopathy, based on ophthalmoscopic observations, is of a microangiopathic complication associated with diabetes beginning after a long period of the disease [1]. However, more recent investigations into the effect of diabetes on retinal neural tissue have shown that the disease damages neurons in the inner [2-4] and outer retina [5], induces glial cell activation [4,6-8], and reduces oscillatory potentials on the electroretinogram [9] prior to the onset of microvascular disease. Such studies suggest that virtually all cell types in the retina are affected in the diabetic process before the onset of retinopathy [10]. This phenomenon can be described as diabetic retinal neuropathy.

Animal studies of the diabetic retina in the pre-retinopathic stage show that neuronal cell death is accompanied by a reduction in nonangiogenic neurotrophic factors from endocrine (insulin and erythropoietin [EPO]) [1] and paracrine (brain-derived neurotrophic factor-BDNF [11] and pigment epithelium-derived factor, PEDF) [12,13] sources, with an accompanying increase in locally produced angiogenic neurotrophic factors such as vascular endothelial growth factor (VEGF) [14,15]. Studies involving the supplementation of locally produced nonangiogenic neurotrophic factors to reduce the neuronal and glial signs of diabetes in the retina support this view

[2,11,16]. In humans, supplementation of endocrine neurotrophic factors has also had some success, with EPO administration reducing signs of diabetic retinopathy in a small cohort [17]. Moreover, the Diabetes Control and Complications Trial (DCCT) can be interpreted to show that exogenous administration of the neurotrophic factor insulin prevents diabetic retinopathy [18].

The retinal pigment epithelium (RPE) is a neuroectodermal monolayer of cells situated between the photoreceptors and the choroid. The functions of the RPE are complex, but one of its major roles is to support retinal integrity. It achieves this in part by regulating nutrients [19], phagocytosing rod outer segments [20], and by contributing to the production of cytokines in the eye, including neurotrophic factors such as fibroblast growth factor-2 (FGF-2) [21] and angiogenic factors such as VEGF [22]. While retinal and systemic production of trophic factors have been well studied in diabetes, the growing understanding of the contribution of different retinal cell types to the diabetic process has led to no studies which have investigated whether the RPE contributes to the retinal trophic support in diabetes.

This investigation focuses on the expression of FGF-2 in diabetes. FGF-2 is produced by the RPE [21] and its levels are actively modulated in the tissue in response to a variety of insults [23]. Originally thought to be important in angiogenesis [24], the function of FGF-2 is now thought to be not primarily angiogenic in nature [25] and its main role in the cen-

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tral nervous system is now thought to be neurotrophic and neuroprotective. Constitutive FGF-2 production in the healthy adult is confined to the brain and retina [26,27], where it is expressed widely [28]. Continuous production of the neurotrophin is essential to the health of the retina, and inhibition of FGF-2 signaling in the normal retina induces retinal degeneration in a transgenic model [29]. This is supported by *in vitro* studies, which have shown that the protein enhances survival and neurite outgrowth in a range of neural preparations [30,31]. *In vivo* studies in models of CNS injury reflect the neuroprotective nature of the compound, showing FGF-2 is upregulated, and its distribution altered in models of cortical [32-34] and retinal [35-37] injury. FGF-2 also shows unusual properties in the diabetic retina: Unlike most neurotrophic factors with little angiogenic activity, FGF-2 is elevated in diabetic retinopathy [38,39]. Indeed, laser photocoagulation, a treatment known to be beneficial for diabetic retinopathy [40], causes an increase in FGF-2 production in the retina together with its more accepted role of decreasing oxygen consumption [41]. It therefore seems attractive to postulate that the upregulation of FGF-2 in the diabetic retina is an endogenous mechanism for protecting neural tissue from injury and that if a deficiency in FGF-2 production occurs in the diabetic RPE than this could contribute to diabetic retinal neuropathy.

The investigations performed here used the well characterized model of the streptozotocin-induced diabetic rat. This model is useful because retinal neovascularization does not occur [16] but signs of neural injury and glial cell activation similar to those reported in human retinas (diabetic retinal neuropathy) become evident as the disease progresses [3,7]. Thus, it can be argued that any changes in the levels of neurotrophic factors in this model are predominant associated with a response analogous to diabetic retinal neuropathy, rather than as markers of incipient angiogenesis.

Therefore, an aim of this investigation was to isolate the RPE from a rat model of diabetes and measure the expression of the neurotrophic factor, FGF-2, in the tissue. In addition, experiments were performed to explore the mechanism of any changes in FGF-2 levels in the RPE during diabetes by investigating possible paracrine and endocrine factors likely to be

involved. These included retinal FGF-2 expression, glial cell activation, environmental glucose levels, insulin, and insulin-like growth factor (IGF).

METHODS

All experiments conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Eighteen age-matched adult male Lister hooded rats (200-250 g) were housed in a temperature- and humidity-controlled room with a 12 h light/12 h dark cycle and provided with food and water *ad libitum*. Random blood glucose concentrations were measured using a Precision PCx Glucometer (Medisense UK Ltd., Abingdon, Oxfordshire, UK). Nine rats were randomly assigned to the diabetic group and given an intraperitoneal injection of 62.5 mg/kg of streptozotocin in 10 mM citrate buffer. A control group of nine rats received an intraperitoneal injection of the citrate buffer alone. Retinas were taken after 15 weeks of diabetes for analysis. One to three RPE sheets from each eye were isolated by washing the eye cup in phosphate-buffered saline (PBS) and then incubating the whole cup in 0.5% trypsin at 37 °C for 5 min. A flame-blunted Pasteur pipette was then used to scrape the RPE sheets from each eye into 1 ml of unsupplemented Hams F-10 containing soybean trypsin inhibitor (type I-S, 5 mg/7.5 ml). Samples were centrifuged at 2000x g at 4 °C for 5 min. The resultant pellet was washed repeatedly in PBS (100 mM, pH 7.4) and centrifuged again to provide samples for analysis.

Semiquantitative PCR: The levels of cyclophilin, FGF-2, and GFAP mRNA were determined using a semiquantitative reverse transcriptase-polymerase chain reaction technique (RT-PCR) as described elsewhere [42]. Primer sequences are listed in Table 1. Briefly, total RNA was isolated, and first strand cDNA synthesis performed on 2 µg of DNase-treated RNA. Aliquots of the resultant cDNA species were amplified in PCR buffer with 4 mM MgCl₂. Reactions were initiated by incubating at 94 °C for 10 min and PCRs (94 °C, 15 s; 52 °C, 30 s; 72 °C, 30 s) performed for a suitable number of cycles followed by a final extension at 72 °C for 3 min. PCR products were separated on 1.5% agarose gels using ethidium bromide for visualization. The relative abundance of each PCR prod-

TABLE 1. SEQUENCE OF PRIMERS USED FOR PCR

mRNA	Primer sequences (5'-3')	Product size	Annealing temp (°C)	Cycles	Accession number
Cyclophilin	TGGTCAACCCACCGTGTTCCTCG TCCAGCATTGTGCCATGGACAAGA	314 bp	52	26	M19533
FGF-2	GCCTTCCCACCCGGCCACTTCAAGG GCACACACTCCCTTGATGGACACAA	179 bp	55	27	M22427
GFAP	ATTCCGCGCCTCTCCCTGTCTC GCTTCATCCGCCTCCTGTCTGT	437 bp	55	27	U03700

Primers were designed online using Primer3 to span an intron, thereby eliminating genomic contamination. Parameters were set to minimize self complementarity and maximize PCR efficiency by checking oligonucleotides for possible formation of primer-dimers, primer cross-dimers, and hairpins. BLASTn (version 2.0) was used to ensure 100% specificity of primers to the target. PCRs were optimized for annealing temperature and Mg²⁺ concentration. Analysis of gels revealed no primer-dimer formation.

uct was determined by quantitative analysis of digital photographs of the gels viewed under UV light using Labworks software (UVP Products, Upland, CA).

Western blotting: Retinal and RPE proteins were isolated simultaneously with RNA using the standard Tri-Reagent technique (Sigma, Poole, UK). After processing, samples were solubilized in homogenization buffer with protease inhibitors (20 mM Tris HCl, pH 7.4, containing 2 mM EDTA, 0.5 mM EGTA, 1% SDS, 0.1 mM phenylmethylsulphonyl fluoride, 50 µg/ml aprotinin, 50 µg/ml leupeptin, and 50 µg/ml pepstatin A). An equal volume of sample buffer (62.5 mM Tris HCl, pH 7.4, containing 4% SDS, 10% glycerol, 10% mercaptoethanol, and 0.002% bromophenol blue) was then added. Electrophoresis of samples was performed using 10% polyacrylamide gels containing 0.1% SDS and proteins blotted onto nitrocellulose (Sigma). Blots were incubated for 3 h at room temperature with primary antibodies against actin (Chemicon, Chandler's Ford, UK; monoclonal antibody, 1:2000), FGF-2 (Santa Cruz Biotechnology, through Insight Biotechnology, Wemberly, UK; polyclonal rabbit sc-79, 1:200), and GFAP (DAKO, Ely, UK; polyclonal rabbit 1:400). Development was then performed using an avidin-biotin peroxidase complex kit (Vector Labs, Peterborough, UK; 1:100) with appropriate secondary antibodies and subsequently processed according to the manufacturer's directions. The final nitrocellulose blots were developed with a 0.016% w/v solution of 3-amino-9-ethylcarbazole (AEC) in 50 mM sodium acetate (pH 5.0) containing 0.05% (v/v) Tween-20 and 0.03% (v/v) H₂O₂. The color reaction was stopped with 0.05% sodium azide solution and scanned at 800 dpi by an Epson Perfection 1200u scanner. Quantitative analysis of the files was performed using Labworks software (UVP Products, CA).

RPE cell culture: Postmortem donor human eyes (donors aged 26 and 58 years) were obtained without their cornea (for transplantation purposes) from Bristol Eye Bank (Bristol, England) up to 48 h after enucleation and were processed immediately. Culture procedures were undertaken in a sterile laminar flow hood (ICN Flow, Thames, UK). Cultures of RPE cells were prepared and characterized by labeling for cytokeratin (KG 8.13) as described previously [43]. Culture medium consisted of Hams-F10 supplemented with 5 mM glucose, 10% (v/v) fetal bovine serum, 0.4% glucose, 2 mM glutamine, amphotericin B (25 µg/mL), and gentamicin (100 µg/mL). Primary cultures were grown in 25 cm² culture flasks and passaged in a ratio of 1:3, and thereafter, in 75 cm² flasks. While growing, cultures were kept in an incubator at 37 °C, with saturating humidity and an atmosphere of 5% carbon dioxide to 95% air. Experiments were performed between the third and seventh passages.

Culture of transformed Müller cells: The RMC-1-transformed Müller cell line was obtained from Dr. V. Sarthy (Northwestern University, Chicago, IL). These cells were grown in modified Eagle's medium (MEM) supplemented with 5 mM glucose, 10% FBS, 2 mM glutamine, 2.5 mg/ml amphotericin B, and 100 µg/ml gentamicin. Cells were passaged at a ratio of 1:3 every 48-72 h and used for the outlined studies when 80% confluent.

Immunocytochemistry: Some cells from each culture were transferred to 13 mm glass coverslips in 24 well plates. After appropriate treatments, cells were fixed in 4% paraformaldehyde in sodium phosphate buffer (100 mM, pH 7.4) for 30 min. Cells were washed in PBS and PBS plus Triton X-100 (0.1%, v/v (PBS-T)), and nonspecific antibody binding was blocked with bovine serum albumin in PBS (0.5%, w/v; PBS-B). Cultures were then incubated in PBS-B with primary antibodies against FGF-2 (Santa Cruz Biotechnology; sc-79 polyclonal rabbit, 1:200), vimentin (Sigma, monoclonal 1:1000) and GFAP (Dako, polyclonal rabbit; 1:200). After cells were washed three times (5 min each) in PBS-T, they were immunolabeled with fluorescein isothiocyanate (FITC)-linked antirabbit or antimouse antisera (Sigma, Poole, UK; 1:100). Visualization was by using a Zeiss epifluorescence microscope (Göttingen, Germany).

Experimental protocol in cell culture experiments: Experiments designed to investigate the mechanism of in vivo findings in the RPE were performed on cells grown to 80% confluence in both RPE and Müller cell cultures. To remove insulin from the FBS used in the growth media, appropriate cultures were washed three times in PBS (100 mM, pH 7.4) and maintained for two days in a hormonally defined media (HDM) of Ham's F10 (for RPE cells) or MEM (for Müller cells) supplemented with 5 mM glucose, 2 mM glutamine, 2.5 mg/ml amphotericin B, 100 µg/ml gentamicin plus 1 g/l transferrin, 96.6 µg/ml putrescine, 300 nM sodium selenate, 200 nM progesterone, and 10 pM estrogen, together with treatments of glucose, mannitol, insulin, IGF-1, and the IGF-1 inhibitor, AG1024.

Cultures used for semiquantitative analysis of protein levels were grown in 6 well plates and samples collected for analysis by dislodging cells with a cell scraper, centrifuging the cell suspension at 1000x g for 5 min and then resuspending the pellet in 100 µl of homogenization buffer and processed for immunoblotting as already described.

Statistical analyses: All analyses were performed with the SPSS statistical package (v. 12; SPSS Inc., Chicago, IL). Rat weights and blood glucose levels were analyzed using Student's unpaired t-tests. Other analyses of difference were performed with Student's unpaired t-tests. Intra- and interclass correlations between the two eyes of each animal were accounted for using repeated measures analysis of variance. Bonferroni's correction to the p value was included where multiple endpoints were measured from the same samples. Correlation coefficients were obtained from linear regressions by the least squares method and 95% prediction intervals calculated. All results are presented as mean±standard error of the mean (SEM). Assay readings and analysis of protein levels in cultures were corrected for osmotic control levels, and the results of varying replications in four to six independent cultures were compared using Student's t-test or repeated measures analysis of variance where appropriate. An α level of 0.05 was chosen.

RESULTS

Streptozotocin induces diabetes in the rat: Diabetic rats displayed polyuria, polydipsia, and had unformed feces throughout the experiment. Cataracts became evident in some rats in the diabetic group after nine weeks of diabetes, and all rats displayed cataracts on gross examination after 12 weeks of the experiment. No cataracts were evident in the control group at any time. Rats in the diabetic group showed reduced weight gain, displaying an average gain of 4.5 ± 1.6 g/week compared with 11.2 ± 0.4 g/week in the control group averaged across the experiment ($p < 0.001$). Final random blood glucose concentrations were 6.7 ± 0.5 mM in the control group and 22.3 ± 3.1

mM in the diabetic group ($p < 0.001$). No rats from either group died during the experiment.

Figure 1 shows analysis of retinal and RPE samples taken from diabetic and control rats. Figure 1A shows that the RPE samples contained no detectable neurofilament light (an inner retinal marker found in ganglion cells) or von Willebrand factor (vWF), a marker of endothelial cells as found in the choroid, revealing the relative purity of the sample. Figure 1B,C show that FGF-2 protein and mRNA levels in retinal samples were significantly increased by $25 \pm 6\%$ and $34 \pm 4\%$, respectively, in the diabetic group after 15 weeks of diabetes. GFAP levels were also significantly increased in the diabetic retina,

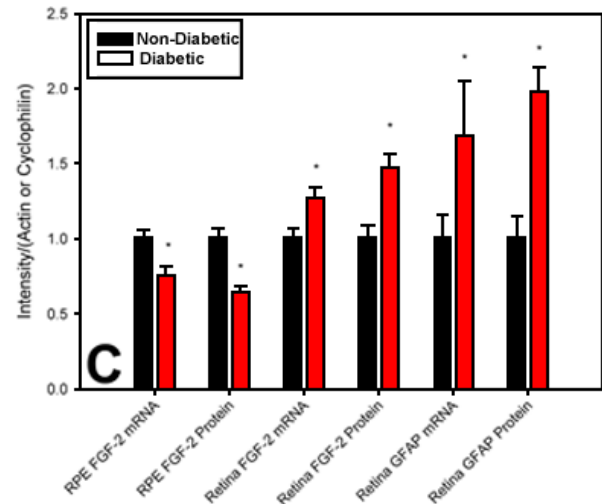
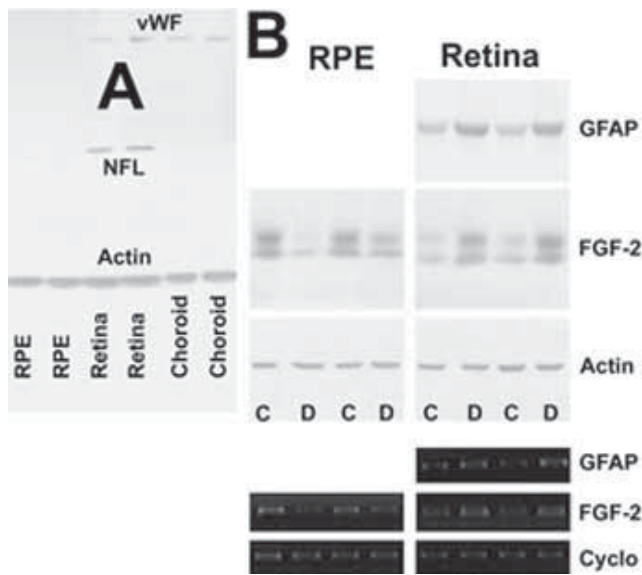
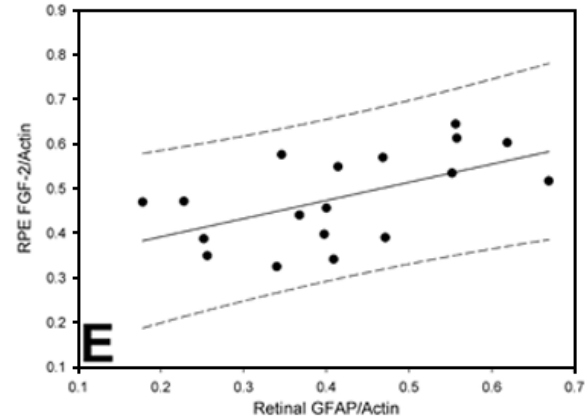
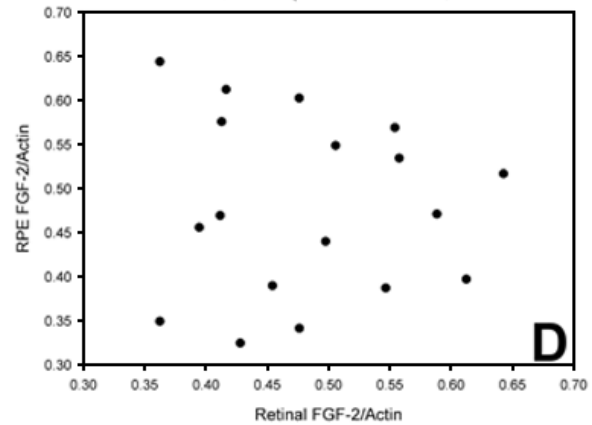


Figure 1. The effects of diabetes on FGF-2 and GFAP. The effect of 15 weeks of diabetes on retinal and retinal pigment epithelium fibroblast growth factor-2 and retinal GFAP expression was measured by immunoblotting. **A:** Immunoblotting for neurofilament light (NFL, an inner retinal marker), von Willebrand Factor (vWF; monomer, an endothelial marker), and actin (a housekeeping structural protein), showing the method used to produce RPE samples for analysis yielded relatively little contamination from the retina or choroid. **B:** Representative immunoblots showing upregulation of FGF-2 and GFAP protein and mRNA in the diabetic retina but downregulation of FGF-2 protein and mRNA in the diabetic RPE. The control (C) and diabetic (D) lanes are identified. Cyclophilin is abbreviated “Cyclo”. **C:** FGF-2 protein and mRNA was reduced by $25 \pm 6\%$ ($p < 0.01$) and $35 \pm 4\%$ ($p < 0.01$) in the diabetic RPE. In contrast, retinal FGF-2 protein and mRNA were increased by $26 \pm 7\%$ ($p < 0.01$) and $47 \pm 8\%$ ($p < 0.01$). Similarly, GFAP expression was also increased in the diabetic retinas. Asterisk (*) indicates $p < 0.01$ after accounting for intra- and interclass variability between eyes and within animals by repeated measures analysis of variance; 18 eyes were studied in each group. **D:** Retinal FGF-2 protein levels were not related to the FGF-2 expression in the corresponding eye’s RPE ($p < 0.87$). **E:** Retinal GFAP levels were correlated to the FGF-2 expression in the corresponding eye’s RPE ($r^2 = 0.31$, $p < 0.01$). Dotted lines indicate 95% prediction intervals of the regression. Results are expressed proportionally in relation to 5 mM glucose in the absence of insulin.



with a $68 \pm 37\%$ increase in mRNA and $98 \pm 16\%$ increase in protein expression. Figure 1B,C also show that RPE FGF-2 protein and mRNA levels were distinctly reduced in diabetes by $25 \pm 6\%$ and $36 \pm 7\%$, respectively.

In order to determine if retinal production of either protein could relate to a decrease in FGF-2 expression in the RPE, the amount of FGF-2, and GFAP protein in individual eyes were correlated. There was no significant correlation between

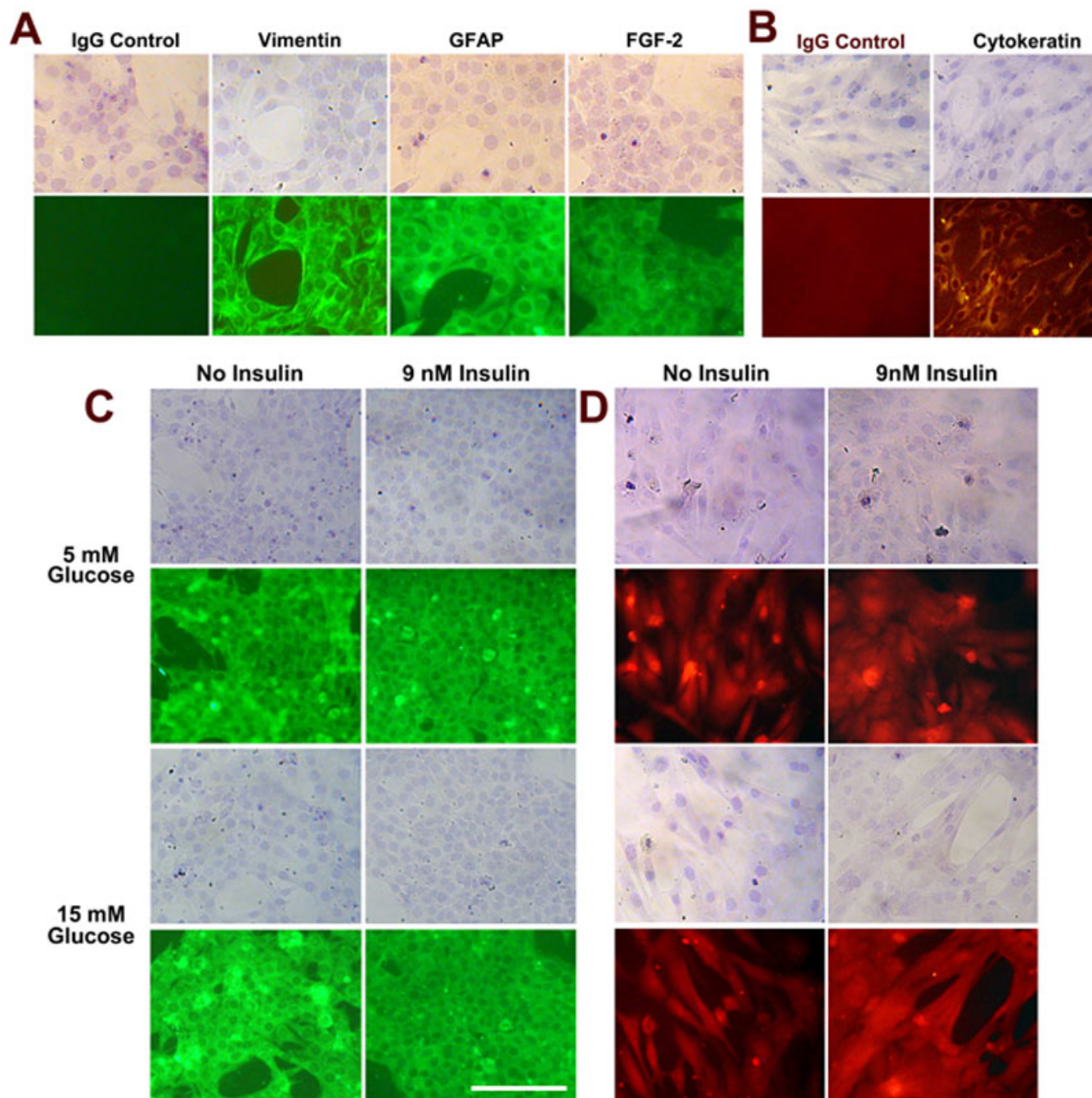


Figure 2. Immunohistochemical characteristics of cultures. Each pair of panels shows the cellular architecture of the culture: The top panel presents a hematoxylin stain and the corresponding immunolabeling is beneath it. **A:** Cultured Müller cells grown in hormonally defined medium (HDM) and 5 mM glucose for 24 h with no added insulin. Cells demonstrate expression of vimentin, a specific Müller cell marker, along with GFAP and FGF-2. **B:** Cultured retinal pigment epithelium (RPE) cells grown in HDM and 5 mM glucose for 24 h with no added insulin. Cells are immunoreactive to cytokeratin (K 8.13). **C:** Representative Müller cell GFAP labeling after 24 h of treatment. It can be seen that cultures in 15 mM glucose display increased GFAP production relative to cultures treated in the 5 mM glucose environment. This increase appears to be evident in all cells, however some cells appear to be more intensely labeled. **D:** Representative RPE cell labeling after 24 h of treatment. It can be seen that cultures in 15 mM glucose display quantitatively increased fibroblast growth factor-2 production when treated with 9 nM insulin. Subtly decreased FGF-2 labeling was seen after 24 h in cultures in 15 mM glucose without insulin. The scale bar represents 100 μ m.

retinal and RPE FGF-2 levels ($r^2=0.01$, $p<0.87$; Figure 1D). In contrast, there was a significant correlation between RPE FGF-2 expression and retinal glial cell activation as indicated

by GFAP protein expression ($r^2=0.31$, $p<0.01$; Figure 1E).

Culture experiments: Studies on RPE and Müller cell cultures in low (5 mM) or high (15 mM) glucose environment

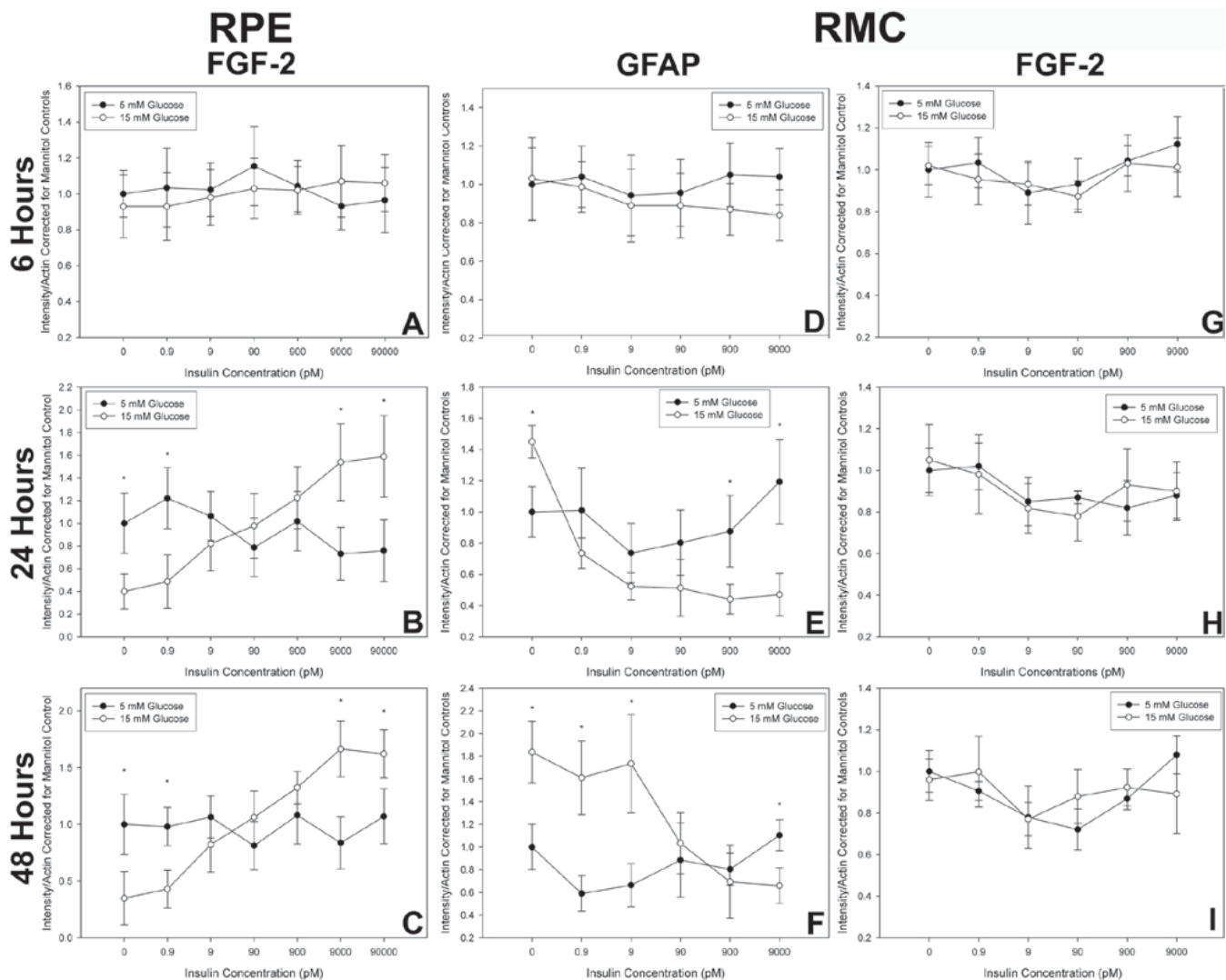


Figure 3. The effect of insulin on retinal pigment epithelium (RPE) and Müller cell protein expression. The effect of insulin on RPE and Müller cell protein expression was measured by immunoblotting, in conditions of high (15 mM) and low (5 mM) glucose. Results are expressed as a proportion of the expression of the substance in 5 mM glucose in the absence of insulin. **A,B,C:** The effect of insulin on fibroblast growth factor-2 (FGF-2) production in RPE culture. After 6 h, insulin and glucose levels have no effect on RPE FGF-2 expression. However, after 24 h, FGF-2 production is significantly decreased ($p<0.01$) in conditions of low insulin but only in the 15 mM glucose environment. Conversely, high insulin levels increased FGF-2 production in the RPE significantly in the 15 mM glucose environment relative to the 5 mM environment ($p<0.01$). This effect is maintained in other cultures analyzed 48 h after treatment. In a 5 mM environment, insulin levels in this range do not alter FGF-2 expression in cultured RPE cells at any of the times tested. **D,E,F:** Similar experiments in cultured Müller cells are shown. GFAP expression in Müller cells is unaffected by insulin or glucose treatments after 6 h, however, after 24 h of treatment, very low levels of insulin are noted to increase GFAP expression significantly in an environment of 15 mM glucose ($p<0.01$). GFAP expression is decreased with increasing insulin concentrations in the 15 mM environment and is significantly reduced relative to the 5 mM environment after treatment with 900 pM and 9 nM of insulin ($p<0.05$). After 48 h of treatment in other cultures, low insulin levels increased GFAP levels to a significantly greater degree ($p<0.05$ at the lowest insulin levels), and the increase was significant relative to the 5 mM environment across a greater range of concentrations. After 48 h in the 15 mM glucose environment, 9 nM insulin was still effective in reducing GFAP expression both relative to the insulin free conditions ($p<0.01$) and to all treatments in the 5 mM environment. In the environment of 5 mM glucose, GFAP expression was not significantly altered with insulin treatment across the range of concentrations and treatment durations tested here. **G,H,I:** FGF-2 production in cultured Müller cells was not significantly altered by insulin in either glucose environment across the range of concentrations and treatment durations investigated here. Asterisk indicates $p<0.05$ by Student's unpaired t-tests comparing protein expression in the 5 and 15 mM glucose environments with five independent samples for each treatment condition and at each time.

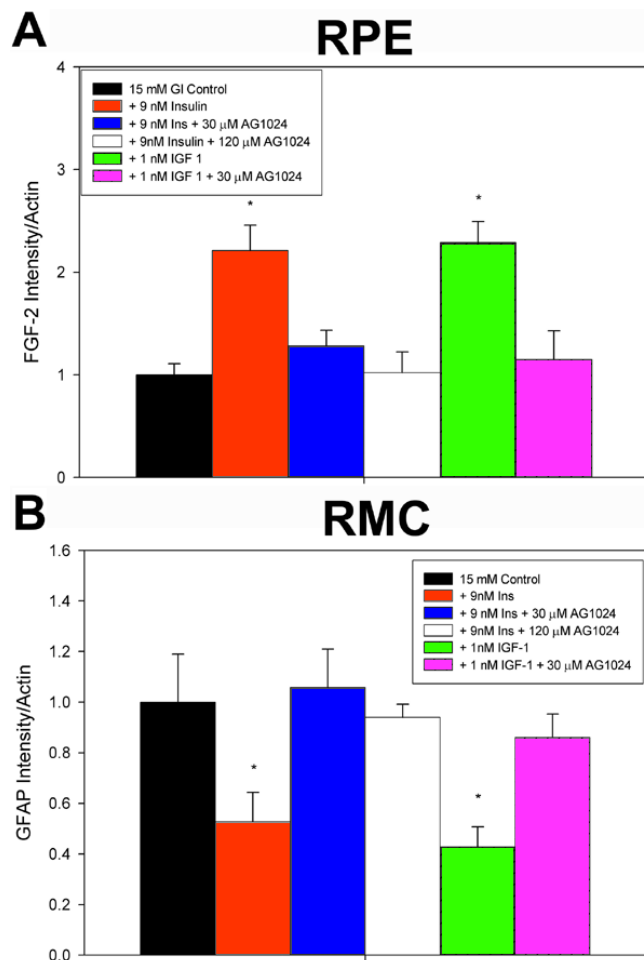


Figure 4. Mechanisms of changes to FGF-2 and GFAP expression. The effect of insulin-like growth factor and the insulin like growth factor receptor 1 antagonist AG1024 was measured on retinal pigment epithelium (RPE) fibroblast growth factor-2 (FGF-2) expression and Müller cell GFAP expression in culture. Treatment with 9 nM insulin increases FGF-2 production in this system, and this effect is mirrored by 1 nM IGF. **A:** Expression of FGF-2 by cultured retinal pigment epithelium (RPE) cells after 24 h of treatment. The two effects are not statistically different. Treatment with 30 μ M AG1024, a dose effective in blocking IGFR-1 receptors but not insulin receptors [46], reduces both insulin, and IGF-induced FGF-2 expression to levels which are not significantly different to baseline in the 15 mM glucose environment. Addition of 120 μ M AG1024, a dose which affects both IGFR-1, and insulin receptors [46] has no additional effect in blocking insulin induced FGF-2 expression. **B:** Expression of GFAP in cultured Müller cells (RMC) after 24 h of treatment. 9 nM insulin decreases GFAP production in this system and this effect is also evident in cultures treated with 1 nM IGF. The effects of 9 nM insulin and 1 nM IGF are not statistically different. AG1024 (30 μ M) blocks the effect of insulin and IGF on GFAP expression to levels that are not significantly different to insulin-free baseline in the 15 mM glucose environment. Addition of 120 μ M AG1024 has no additional effect in blocking the action of insulin on GFAP expression in an environment of 15 mM glucose. Asterisk indicates $p < 0.05$ comparison to 15 mM glucose baseline by Student's unpaired t-test with five independent repetitions for each culture treatment.

were undertaken in order to determine if the relationship between GFAP and RPE FGF-2 production in the diabetic retina in vivo was causal or if a separate dose-dependent mechanism was responsible for these phenomena.

The characteristics of Müller and RPE cell cultures used in these studies are shown in Figure 2. RPE cultures at the passages used in these studies showed immunolabeling for cytokeratin (K_G 8.13) an RPE specific protein [44], and FGF-2 (Figure 2B,D). Müller cell cultures were positively labeled for vimentin, GFAP, and FGF-2 (Figure 2A).

In "type 1 diabetic" conditions (15 mM glucose and no insulin for 1 day), immunolabeling for FGF-2 in RPE cells (Figure 2D) showed less intense staining than in conditions of 5 mM glucose. Addition of 9 nM insulin resulted in more intense FGF-2 labeling in 15 mM of glucose than in 5 mM glucose (Figure 2D). In the Müller cell cultures, GFAP staining in 15 mM glucose and no insulin was more intense than when insulin was present in either 5 mM glucose or 15 mM glucose. (Figure 2C) In contrast, the intensity of FGF-2 staining in the Müller cell culture appeared unaffected by insulin in either a low or high glucose environment (results not shown).

Figure 3 quantifies the relationships described in Figure 2 for 6 h, 24 h, and 48 h treatment periods using immunoblotting. A dose-dependent relationship exists between insulin and FGF-2 production in RPE cells and GFAP suppression in Müller cells, respectively. These relationships are evident only after a 24 h period and only for cultures in the 15 mM when results are normalized with what occurs in 5 mM glucose in the absence of insulin.

After two days of exposure to high glucose in the absence of insulin, RPE cells contained $65 \pm 23\%$ less FGF-2 than cells in 5 mM glucose ($p < 0.05$) and $71 \pm 26\%$ less FGF-2 than cells in 15 mM glucose and treated with physiological (36-179 pM) [45] levels of insulin ($p < 0.05$; Figure 3C). When cultures exposed to 15 mM glucose are treated with 9 nM insulin, FGF-2 levels are increased $64 \pm 28\%$ ($p < 0.05$) relative to the same treatment in cultures exposed to 5 mM glucose (Figure 3C). Insulin had no significant effect on FGF-2 expression in cultures exposed to an environment of 5 mM glucose (Figure 3A-C). FGF-2 levels in cells exposed to 15 mM of glucose and treated with physiological levels of insulin (90 pM) were also not significantly different to the levels in cultures maintained in 5 mM glucose (Figure 3A-C).

Insulin treatment had the opposite effect on expression of GFAP in Müller cell cultures (Figure 3D-F). After two days of exposure to high glucose in the absence of insulin, Müller cells produced $83 \pm 27\%$ more GFAP than cells in 5 mM glucose ($p < 0.05$) and $81 \pm 26\%$ less GFAP than cells in 15 mM glucose that were treated with physiological levels of insulin ($p < 0.05$; Figure 3F). When treated with 9 nM insulin, GFAP levels decreased $44 \pm 15\%$ relative to the same treatment in cultures exposed to 5 mM glucose. Insulin had no significant effect on GFAP expression in cultures in an environment of 5 mM glucose. Cells exposed to 15 mM glucose and treated with physiological levels of insulin did not display significantly different FGF-2 or GFAP levels than the cells maintained in 5 mM glucose. Müller cell FGF-2 levels were not significantly af-

ected by either glucose or insulin treatment in the ranges tested (Figure 3G-I).

Figure 4 shows the results of investigations designed to explore the possible mechanism by which insulin influences RPE FGF-2 levels and Müller GFAP expression in an environment of 15 mM glucose. Figure 4A shows that in RPE cultures a significant increase ($121 \pm 24\%$, $p < 0.05$) in FGF-2 occurs after 24 h of treatment with 9 nM insulin in a high (15 mM) glucose environment. This effect is reduced to levels not significantly different to the insulin-free baseline by addition of 30 μ M AG1024 which is known to specifically block IGFR-1 receptor signaling [46]. Higher concentrations of AG1024 (120 μ M) also blunts the effect of insulin. This concentration of AG1024 is known to block both IGFR-1 and insulin receptors [46], and from the data shown in Figure 4A had no clear effect on FGF-2 expression. In addition, treatment with 1 nM IGF led to an increase in FGF-2 expression of $128 \pm 28\%$ ($p < 0.05$) which was not significantly different to that induced by treatment with 9 nM insulin (Figure 4A). This IGF-induced increase in FGF-2 production in the RPE was reduced to basal levels by 30 μ M AG1024.

Figure 4B demonstrates a similar effect in Müller cell cultures. In the presence of 15 mM glucose, 9 nM insulin reduced GFAP expression by $48 \pm 11\%$, ($p < 0.05$) and this was significantly inhibited by 30 μ M AG1024, increasing GFAP expression to baseline levels. Addition of 120 μ M AG1024 had no additional effect on GFAP expression. Treatment with 1 nM IGF caused a decrease in GFAP expression of $58 \pm 9\%$ ($p < 0.05$), similar to that induced by 9 nM insulin. The IGF-induced reduction of GFAP was inhibited by 30 μ M AG1024 to levels observed in the absence of insulin.

DISCUSSION

The experiments detailed here show that FGF-2 expression is decreased in the RPE after 15 weeks of diabetes in an experimental model. Surprisingly, this decrease is not correlated with retinal FGF-2 production but showed instead a strong correlation with retinal GFAP levels, a marker of glial cell activation. While a causal effect of one phenomenon upon the other is a possibility, it is more likely that the statistical correlation results from a dose-dependent effect of a separate factor responsible for both observations.

To explore these possibilities, primary cultures of RPE cells and Müller cells (the primary source of GFAP in the diabetic retina) [7,8] were carried out. Addition of a hormonally defined medium to achieve insulin-free culture conditions and high glucose (15 mM) led to significantly reduced levels of FGF-2 in RPE cells and increased GFAP levels in Müller cells, reflecting the findings *in vivo*. Insulin reversed both effects in cultures in an environment of 15 mM glucose in a dose-dependent manner. The effect of insulin was mimicked by IGF, and both influences were blocked by a specific inhibitor of IGFR-1.

Streptozotocin-induced diabetes in the rat: The animals used in these experiments were pigmented Lister hooded rats, which display pigmented RPE. Although streptozotocin-induced diabetes and diabetic retinopathy is better character-

ized in other strains, the use of Lister hooded rats was found to be necessary to assist visualization and handling of the RPE during the delicate manipulations necessary to separate the RPE sheets while avoiding choroidal contamination. Streptozotocin appeared to induce experimental diabetes in the rats, with increased blood glucose concentrations, polyuria, polydipsia, cataract formation, and a failure to gain weight noted in the diabetic group. In addition, FGF-2 and GFAP, two proteins known to be upregulated in the diabetic retina [4,6-8,38,39], were increased in these rats, giving an indication that diabetic retinal neuropathy was present in the samples after 15 weeks of diabetes.

The isolation of the RPE from rats with minimal contamination from the retina or choroid are shown by a lack of retinal and choroidal markers in the sample. FGF-2 mRNA and protein were detected in the RPE samples, and were downregulated in the diabetic rats. Initially it could be considered that this was the logical result of increased FGF-2 expression in the retina causing a paracrine negative feedback loop with the RPE, but no correlation was found between FGF-2 protein levels in each retina and FGF-2 expression in the adjacent RPE. However, GFAP, a marker known to be induced in Müller cells in diabetes, was found to correlate with RPE FGF-2 protein expression. Cell cultures were chosen to investigate this relationship since possible confounding factors central to the disease, such as ischemia, can be controlled. In these studies, the culture medium was manipulated to reflect the core metabolic insults known to occur in type 1 diabetes: low insulin (<36 pM) and high glucose (>5 mM).

Insulin-free conditions were required for treatment of Müller cell cultures with "diabetic" levels of glucose (15 mM) to mirror the changes in GFAP noted in the streptozotocin-induced diabetic rat retina. The effect was reversed in a dose-dependent manner by the addition of insulin, with GFAP expression returning to levels equivalent to those of cultures grown in 5 mM glucose when treated with physiological concentrations of insulin (36-179 pM) [45]. Cultures displayed less GFAP expression than cultures grown in 5 mM glucose even when treated with supraphysiological levels of insulin. Care should be taken in correlating these results with the conditions of hyperinsulinaemia or type 2 diabetes, where insulin resistance and receptor malfunction is the primary defect [1].

"Diabetic" glucose levels in insulin-free conditions also decreased FGF-2 production in cultured human RPE cells, mirroring the results presented in the animal model and perhaps indicating that the phenomenon may extend to human disease. Once again, this effect was reduced with the addition of physiological levels of insulin, which restored FGF-2 expression to levels equivalent to those of cultures grown in 5 mM glucose. The results also indicate that the trend continues with supraphysiological insulin treatments, but care should again be taken in extending this analysis to the type 2 diabetic situation for the reasons already given.

The results also demonstrate that no concentration of glucose or insulin tested produced a significant change in Müller cell FGF-2 production. While acknowledging the inherent errors in using monolayers of isolated, transformed, actively di-

viding or passaged cells in investigations, these results together support the hypothesis that high glucose levels in the absence of insulin contribute to both the increased GFAP expression in the retina (which is mostly of Müller cell origin) [6], and decreased FGF-2 production in the RPE. Therefore, the combined effects of low insulin and high glucose could account for the correlation between the retinal GFAP and RPE FGF-2 noted in the streptozotocin-induced diabetic rat. Therefore, this may be an important contributor to similar observations in the human condition.

The insulin receptor (IR) is known to be present in the CNS [47] and in the retina [48], where it appears to be localized in photoreceptors, other neuronal elements [49], and in the RPE [50]. However, the retina is unusual in that IGF binding is 10-20 times greater than insulin binding [51]. Like insulin and IGF-1, the IR and IGFR-1 display extensive structural homologies [52,53], with both insulin and IGF acting as ligands for each, although the extent of the modest cross-affinity of each substance to the other receptor is controversial. Binding studies suggest each ligand has a 100 fold increased affinity for its own receptor [54,55]; however, studies of binding and pharmacological effects of the ligands in the retina estimate up to a 30 fold greater cross reactivity than these results predict [51,56,57]. This may be due to nonclassical insulin binding patterns that have been reported in the retina, where a proportion of binding sites have a similar affinity for both IGF and insulin [51]. Given the presence of both the IR and IGFR1 in the RPE and retina, the unusually high cross-reactivity between the two and the fact that the literature supports the strong possibility of a differential regulation of FGF-2 and IGF-1 [58-60], it was hypothesized that the effect of insulin on FGF-2 and GFAP could be modulated through the IGFR-1 receptor.

This hypothesis was supported by the investigations presented here, in which treatment with AG1024 at a concentration known to specifically inhibit IGFR-1 [46] blocked the effect of insulin on FGF-2 expression in cultured RPE cells and its effect on GFAP expression in cultured Müller cells. In addition, treatment with IGF at a concentration ten times lower than that tested for insulin resulted in an effect of the same magnitude on both cell types. This effect was also blocked by the IGFR-1 receptor blocker AG1024. Addition of AG1024 at the much higher concentration necessary to also block the IR [46] had no further influence on either RPE FGF-2 expression or Müller cell GFAP expression. This evidence may be interpreted to support the tentative conclusion that the dose-dependent effect of insulin on RPE FGF-2 production and Müller cell GFAP production is mediated through the IGFR-1 rather than, as originally expected, the IR.

These results could explain the findings that treatment of cultures with diabetic levels of glucose is a prerequisite for the withdrawal of insulin to induce phenotypical changes in RPE and Müller cells. Previous studies have reported that IGF-induced pancreatic b cell proliferation is glucose dependent, displaying maximal synergy at 15 mM of glucose [61]. Similarly, IGF and glucose have been observed to act synergistically in stimulating fibrosis in renal fibroblasts [62], contrib-

uting to diabetic nephropathy. It appears consistent to argue that, in the culture experiments, insulin acts in synergy with "diabetic" glucose levels through the IGFR-1 receptor to alter RPE FGF-2 expression and Müller cell GFAP expression. This mechanism could induce similar changes consistent with the observations presented here in the streptozotocin-induced rodent model of diabetic retinal neuropathy and with the known manifestations of diabetes in retinal glial cells in human diabetes [8].

It should be noted that the IGF system is complex in the intact retina and consists of at least insulin, IGF-1, IGF-2, and the primarily inhibitory IGF binding proteins 2 and 3 [63]. In addition, it is known that IGF itself has an important role in diabetes. IGF is downregulated in the retina of diabetic rats [64]. In contrast, IGF is upregulated in the diabetic vitreous after the onset of microvascular disease [65] and appears to be disproportionately active at this stage of the disease [66]. Additionally, IGF is important in upregulating VEGF expression in the diabetic retina [67], and IGFR-1 receptors are required for retinal neovascularization in a neonatal hypoxia model [68]. Recent data from a normoglycemic transgenic model overexpressing IGF reported that the model displays most of the signs of human diabetic retinopathy [69]. Together these reported results imply that IGF is decreased in the streptozotocin-induced diabetic retinal neuropathy in the rat and possibly the human, but an increase may mark the onset of neovascularization in both the rat and human [69]. Therefore, changes in IGF appear to be complex and pathogenically important in the diabetic retina. Indeed these experiments do not rule out the possibility that autocrine production of IGF in the culture systems may have contributed to some of the effects noted here; however, it was not the purpose of these experiments to fully investigate the IGF system in the diabetic retina and RPE. It is worth noting that the previously described decrease in retinal IGF levels reported in diabetes would be expected to exacerbate the effects of low insulin on Müller cell GFAP expression and RPE FGF-2 levels in a diabetic environment through this mechanism.

It is concluded that low insulin, high glucose environments in culture mirror phenotypical changes in an animal model of experimental diabetes. It is likely that decreased activation of the IGFR-1 in diabetes contributes, perhaps significantly, to the retinal effects of the disease.

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