Fetuin-B Interacts With Insulin Receptor-β and Promotes Insulin Resistance in Retina Cells

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Received: July 8, 2024
Accepted: September 19, 2024
Published: October 9, 2024

Citation: Zhang W, Wang X, Tian S, Wang J, Zhou A. Fetuin-B interacts with insulin receptor-β and promotes insulin resistance in retina cells. *Invest Ophthalmol Vis Sci.* 2024;65(12):16. https://doi.org/10.1167/iovs.65.12.16

Purpose. The purpose of this study was to investigate the correlation between insulin and Fetuin-B (FETUB) and the influence of FETUB on insulin signaling pathway in diabetic retinopathy (DR).

Methods. Enzyme-linked immunosorbent assay (ELISA) was used to analyze FETUB and insulin levels in the serum and aqueous fluid of patients with DR and healthy controls. Quantitative PCR (q-PCR), Western blotting, and ELISA were used to examine FETUB expression in ARPE-19, BV2, and Müller cells under insulin stimulation. Co-immunoprecipitation was used to investigate the interaction of FETUB with insulin receptor-β (IRβ). Insulin resistance (IR)-BV2 and IR-Müller cells were treated with FETUB recombinant protein or FETUB short hairpin RNA (shRNA) to explore the influence of FETUB on insulin signaling pathway in DR. LY294002 (a PI3K pathway inhibitor) was used to determine whether FETUB affects glucose metabolism via the PI3K/Akt pathway.

Results. In aqueous fluid, FETUB concentrations were positively correlated with insulin levels. FETUB expression increased in Müller and BV2 cells under insulin regulation, and FETUB interacted with IRβ in retinal cells and mice retina. The interaction between IRβ and FETUB increased in BV2 and Müller cells under high-glucose than in controls. Insulin signaling pathway activation was suppressed in FETUB recombinant protein-treated BV2 and Müller cells but increased in FETUB shRNA-transfected cells. FETUB shRNA could not reverse LY294002-mediated inhibition of glucose transporter-4 expression.

Conclusions. Retinal cells are the source of insulin-regulated FETUB. The FETUB interacts with IRβ and affects insulin signaling pathway in BV2 and Müller cells. FETUB may aggravate IR in BV2 and Müller cells via the PI3K/Akt pathway.

Keywords: diabetic retinopathy (DR), Fetuin-B (FETUB), insulin, insulin resistance (IR), insulin signaling pathway

Diabetic retinopathy (DR) is a common complication of diabetes[1] and the leading cause of vision loss in working-age individuals. Owing to the complex pathogenesis, there is no radical cure for DR. Early diagnosis and new therapeutic target are particularly important.

Fetuin-B (FETUB) is a protease inhibitor, which is mainly secreted by the liver.[2] The correlation between FETUB and glucose and lipid metabolic diseases has been reported. In previous studies, we determined that the FETUB concentrations in plasma, aqueous fluid, and tissue specimens increased in patients with DR.[3] Pasmans et al.[4] found a strong association between FETUB levels in white adipose tissue and peripheral insulin resistance (IR) in mice and humans. FETUB is also involved in the regulation of adipose insulin sensitivity[5] and can cause abnormal glucose tolerance in mice.[6] Compared with control patients, individuals with type 2 diabetes (T2D) exhibit significantly increased serum FETUB levels, which are associated with increased fasting insulin levels and the induction of IR and T2D.[7,8] Elevated serum FETUB levels are also observed in young women with IR or impaired glucose tolerance.[9] In gestational diabetes mellitus (DM), IR is an independent predictor of serum FETUB levels.[10] These studies underscore the association between serum FETUB levels and impaired glucose tolerance and DM.

Previous studies have revealed that IR is an important cause of DR.[11,12] However, research on insulin signaling in the retina is limited. Investigating whether FETUB affects the retinal insulin signaling pathway in the underlying mechanism of DR is, therefore, of great interest.

Materials and Methods

Participants and Exclusive Criteria

Six patients without DM, 3 patients with DM (without DR), and 11 patients with DR participated in the study. All patients needed cataract surgery. DM was diagnosed based on fasting plasma glucose ($\geq$7.0 mmol/L) and oral glucose tolerance tests (2-hour postprandial blood glucose $\geq$11.1 mmol/L). Patients with DR were diagnosed through clinical examination. Patients with acute or chronic inflammatory diseases, cognitive disorders, and pregnant women were excluded. All the patients signed an informed consent form. The Ethics Committee of the Second Affiliated Hospital of Xi'an Jiaotong University approved all the experimental protocols.

Enzyme-Linked Immunosorbent Assay

Before cataract surgery, venous blood and aqueous fluid samples were collected simultaneously without requiring patients to fast. Venous blood samples were allowed to sit for 2 hours before centrifugation for 10 minutes at 1000 × g at 4°C. Aqueous fluid samples were collected using 25 G needles. The concentrations of FETUB and insulin were determined using ELISA kits (Ruixin Biotech, Quanzhou, China). Each sample was analyzed in triplicate.

Cell Culture

Adult retinal pigment epithelial cell line ARPE-19 (Procell, Wuhan, China) was cultured in DMEM/F12 medium (Procell) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA). The BV2 mouse microglia cell line (Procell) was grown in DMEM medium (Procell) containing 10% FBS. The cell lines were identified using short tandem repeats and were found to be free of mycoplasma contamination.

Mouse retinal primary Müller cells (Procell) were grown in DMEM medium containing 15% FBS. Müller cells were identified using immunofluorescence staining for glial fibrillary acidic protein (GFAP) and glutamine synthetase (GS; Supplementary Fig. S1).

All the cells were cultured at 37°C in 5% CO_2. Cells were stimulated with insulin (Beyotime Biotechnology, Shanghai, China) at zero (non-insulin), low (1×10^{-7} mol/L), medium (1×10^{-6} mol/L), or high (1×10^{-5} mol/L) concentrations for 48 hours. Quantitative PCR (q-PCR) and Western blotting were used to detect FETUB levels.

Establishment of Insulin Resistance Model of BV2 Cells and Primary Mouse Retinal Müller Cells

The 2×10^5 cells were spread in 6-well plates. After 12 hours, different volumes of insulin were added to obtain the following final concentrations: 0, 1×10^{-7}, 5×10^{-7}, 1×10^{-6}, 5×10^{-6}, and 1×10^{-5} mol/L. After 48 hours of incubation, the medium was removed, and 2 mL of normal medium was added. After 24 hours, glucose consumption in each group was measured using a glucose assay kit (Beyotime Biotechnology).

As presented in Supplementary Figure S2A, compared with the control group, BV2 cells under 1×10^{-6} mol/L insulin concentration had the most significantly decreased glucose consumption. Additionally, as shown in Supplementary Figure S2B, compared with the control group, Müller cells under 5×10^{-6} mol/L insulin concentration showed the most significantly decreased glucose consumption. Therefore, insulin concentrations of 1×10^{-6} mol/L and 5×10^{-6} mol/L were selected to establish the IR-BV2 and IR-Müller models, respectively.

Effect of FETUB on Glucose Consumption in Insulin-Resistant Cells

IR cells were cultured in the medium containing 0 (control group), 100, 200, and 300 ng/mL FETUB recombinant protein. Additional groups included untreated normal control, FETUB short hairpin RNA (shRNA)-transfected cells, and negative control (NC) shRNA-transfected cells. Glucose assay kits were used to detect glucose consumption in each group. Additionally, LY294002 (Beyotime Biotechnology; 30 µM), a PI3K pathway inhibitor, was used to examine whether FETUB affects cellular glucose metabolism through the PI3K/Akt pathway.

Animal Studies

The Animal Experimentation Ethics Committee of Xi'an Jiaotong University approved all animal procedures. Male C57BL/6 mice (Chengdu Gembio, Chengdu, China) were bred at 26°C (12/12 hours, light/dark cycle), 5-week-old mice were randomly categorized into control and DR groups.

DR mice were established by feeding them a high-fat diet (20% kcal protein, 45% kcal fat, and 35% kcal carbohydrate) for 12 weeks. In the sixth week, DR mice were treated with 120 mg/kg streptozotocin (Solarbio) after 12 hours of fasting via intraperitoneal injection. The control group received a normal diet and an equal volume of citric acid-sodium citrate buffer. Tail vein blood glucose levels were measured to confirm hyperglycemia (≥16.7 mmol/L) in the DR group. The mice were euthanized by cervical dislocation 12 weeks after the establishment of DM, and retinal tissues were collected to perform further experiments (Supplementary Fig. S3).

Co-Immunoprecipitation

Co-immunoprecipitation (CO-IP) was used to detect the interaction between FETUB and insulin receptor-β (IRβ) and to determine the level of their interaction.

RIPA lysis buffer (Epizyme Biotech, Shanghai, China) containing a protease and phosphatase inhibitor cocktail (both 1:1000; Beyotime Biotechnology) was used to extract proteins. Then, 480 µL 1 × tris-buffered saline (TBS) was added to 20 µL protein A+G magnetic beads (Beyotime Biotechnology). The suspension was separated on a magnetic separation rack (Beyotime Biotechnology) for 10 seconds, and the supernatant was discarded. This procedure was repeated three times. FETUB antibody and normal IgG solution were diluted with 1 × TBS to 5 µg/mL. The magnetic beads were then resuspended in 500 µL antibody working solution and incubated for 1 hour at room temperature on a rotary mixer. Next, 20 µL of the magnetic bead suspension was added to 500 µL of freshly prepared protein sample and incubated for 2 hours at room temperature on a rotary mixer.

Subsequently, 100 µL 1 × SDS-PAGE loading buffer was added to the 20 µL magnetic beads and placed in a metal bath at 95°C for 10 minutes. The samples were separated on a magnetic separation rack for 10 seconds, and the supernatant was collected for further analysis.

Western Blot Analysis

Twenty micrograms of protein was resolved by SDS gel electrophoresis, transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA), and incubated for 16 hours at 4 °C with primary antibodies (Supplementary Table S1). The membranes were then incubated with corresponding secondary antibodies (see Supplementary Table S1) for 2 hours at room temperature.

Electrochemiluminescence (Bio-Rad, Hercules, CA, USA) was used to capture the protein bands. ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to measure the blot intensity.

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Table 1. Groups of the Effect of FETUB Recombinant Protein on Insulin Signaling Pathway

	a	b	c	d	e	f	g	h
Insulin resistance	−	−	−	−	+	+	+	+
FETUB recombinant protein	−	−	+	+	−	−	+	+
10^{-7} mol/L insulin	−	+	−	+	−	+	−	+

Table 2. Groups of the Effect of FETUB shRNA on Insulin Signaling Pathway

	a	b	c	d	e	f	g	h	i	j	k	l
Insulin resistance	−	−	−	−	−	−	+	+	+	+	+	+
NC shRNA	−	−	+	+	−	−	−	−	+	+	−	−
FETUB shRNA	−	−	−	−	+	+	−	−	−	−	+	+
10^{-7} mol/L insulin	−	+	−	+	−	+	−	+	−	+	−	+

Immunofluorescence

Müller cell sections were incubated overnight with the primary antibodies (see Supplementary Table S1). Then incubated them with the corresponding secondary antibodies (see Supplementary Table S1), followed by DAPI staining (Beyotime Biotechnology). Imaging was performed using Panoramic MIDI (3DHISTECH, Budapest, Hungary).

The Activation Levels of Insulin Signaling Pathway

Western blotting was used to assess the impact of FETUB recombinant protein on the insulin signaling pathway. BV2 and Müller cells were cultured in a high glucose medium for 48 hours and categorized into 8 groups (Table 1).

Western blot was used to examine the effect of FETUB shRNA transfection on insulin signaling. BV2 and Müller cells were cultured in a high-glucose medium for 48 hours under different conditions. The cells were categorized into 12 groups (Table 2).

Quantitative Real-Time Polymerase Chain Reaction

RNA isolation kit (Beyotime Biotechnology) and cDNA synthesis kit (Servicebio, Wuhan, China) were used to extract RNA and produce the cDNA. The q-PCR was performed using the q-PCR master mix (Servicebio) and CFX connect system (Bio-Rad). The primers used for q-PCR are shown in Supplementary Table S2.

Statistical Analysis

All statistical analyses were performed using SPSS software (version 24.0; IBM Corp., Armonk, NY, USA) and GraphPad Prism version 8.0 software (GraphPad, San Diego, CA, USA). Each group included at least three data points for analysis. Data are presented as mean ± standard error of the mean (SEM). Student's *t*-test (unpaired) or one-way analysis of variance (ANOVA) was used to determine statistical significance. Inter-relationships between variables were analyzed using Spearman's correlation test. Statistical significance was set at $P < 0.05$.

Results

FETUB Concentrations in Aqueous Fluid Were Increased in Patients With DM and Correlated With Insulin in Aqueous Fluid

ELISA was used to quantify FETUB and insulin levels in aqueous fluid and serum. Although serum FETUB levels in the DM group were higher than those in the control group, the difference was not significant (Fig. 1A), which may be due to the small sample size. Serum insulin concentration was significantly higher in patients with DM than in controls ($P < 0.01$; Fig. 1B). Concentrations of FETUB and insulin in the aqueous fluid were significantly higher in patients with DM than those in the control groups (both $P < 0.01$; Figs. 1C, 1D). In addition, aqueous fluid FETUB concentrations were positively correlated with insulin levels ($r = 0.457$, $P < 0.05$; Fig. 1E), and aqueous fluid insulin concentrations were positively correlated with serum insulin levels ($r = 0.802$, $P < 0.001$; Fig. 1F). No correlation was observed between the serum and aqueous fluid FETUB concentrations (Fig. 1G).

Interaction of FETUB and Insulin Receptor-β in Retinal Cells and Mice Retina

The interaction between FETUB and IRβ in retinal cells was confirmed using CO-IP. The results revealed a moderate interaction of FETUB with IRβ in BV2 cells (Fig. 2A). Conversely, in the Müller cells, FETUB interacted with IRβ more significantly (Fig. 2C). BV2 and Müller cells were cultured in normal and high-glucose (30 mmol/L glucose) media, respectively. The interaction between IRβ and FETUB in BV2 (Fig. 2B) and Müller cells (Fig. 2D) under high-glucose conditions was increased in comparison to that in the control group.

In addition, the interaction between FETUB and IRβ was confirmed in mice retina (Fig. 2E). This interaction was enhanced in the T2D retina compared with that in the control retina (Fig. 2F).

FETUB Expression Level in Retinal Cells Under the Regulation of Insulin

1) FETUB mRNA and protein levels in ARPE-19, BV2, and Müller cells under different concentrations of insulin:

FETUB mRNA and protein levels were assessed in ARPE-19, BV2, and Müller cells treated with different insulin concentrations. In ARPE-19 and BV2 cells, there was no significant differences in FETUB mRNA or protein levels among the groups (Figs. 3A–F). In Müller cells, FETUB mRNA and protein levels were significantly higher in the 1×10^{-5} mol/L group than in the 0, 1×10^{-7}, and 1×10^{-6} mol/L groups ($P < 0.01$; Figs. 3G–I).

The results indicated that FETUB expression increased in Müller cells under high-insulin environment, whereas insulin concentration had no significant effect on FETUB expression in ARPE-19 and BV2 cells.

2) Concentrations of FETUB in the supernatant of ARPE-19, BV2, and Müller cells under different insulin concentrations:

ELISA was used to detect FETUB concentration in the supernatant of the cells. The results showed that insulin concentrations had no significant effect on FETUB levels in the supernatants of ARPE-19 and Müller cells (Figs. 4A, 4C). In BV2 cells, FETUB levels in the supernatants of 1×10^{-6}

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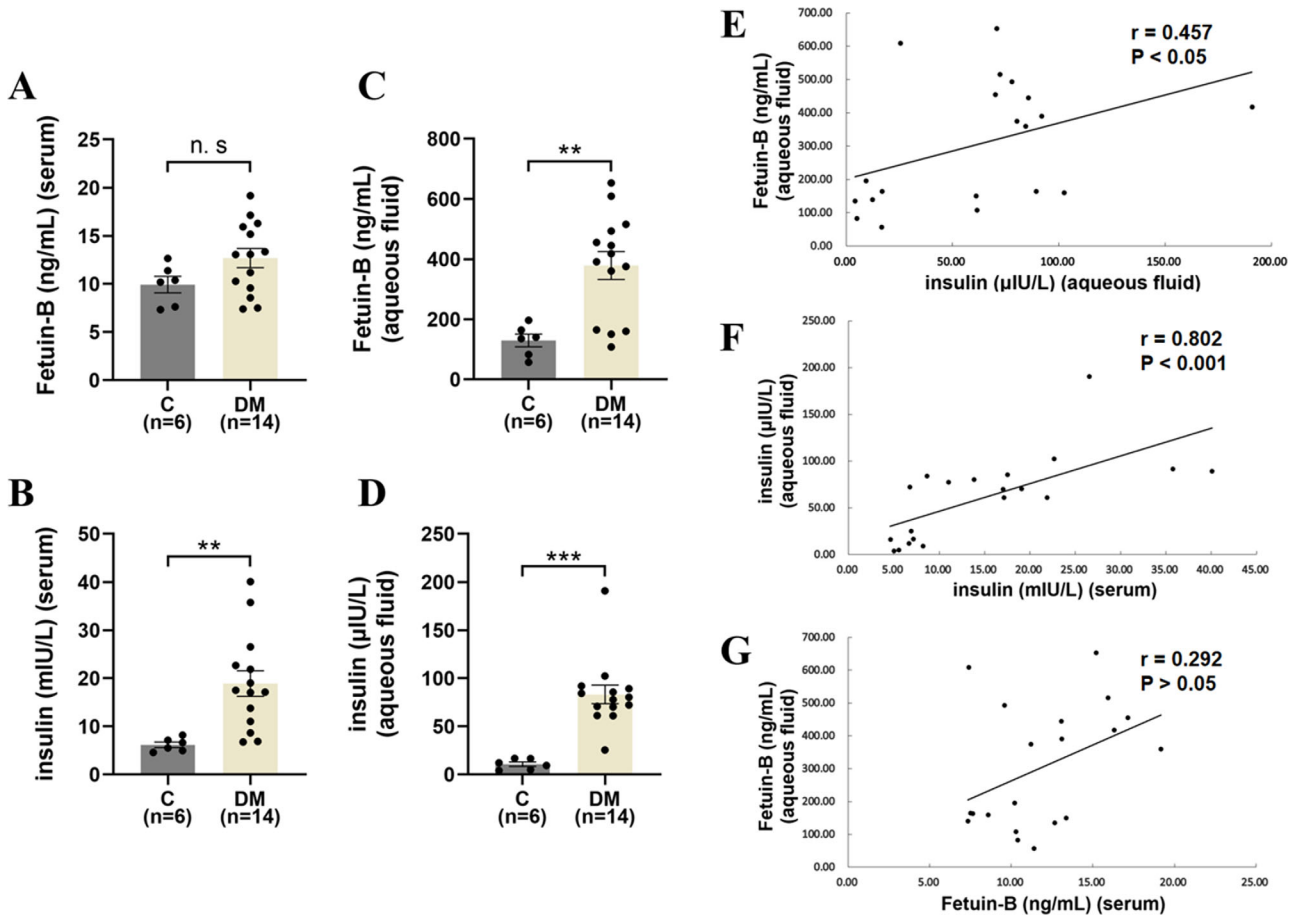

Figure 1. FETUB concentrations in aqueous fluid were increased in patients with DM, and correlated with insulin levels. (**A**) Serum FETUB concentrations in the control and DM groups. (**B**) Serum insulin concentrations in the control and DM groups. (**C**) FETUB concentrations in aqueous fluid in the control and DM groups. (**D**) Insulin concentrations in aqueous fluid in control and DM groups. (**E**) Correlation between insulin and FETUB levels in aqueous fluid. (**F**) Correlation between serum and aqueous fluid insulin concentrations. (**G**) Correlation between serum and aqueous fluid FETUB concentrations. Data are expressed as mean ± SEM. C, control group; DM, diabetes mellitus group. ns, no significance; $^{**}P < 0.01$; $^{***}P < 0.001$.

and 1×10^{-5} mol/L insulin groups were significantly higher than those in 0 and 1×10^{-7} mol/L insulin groups ($P < 0.01$; Fig. 4B).

FETUB Regulated Glucose Metabolism in Retinal Cells

Control (no-insulin resistance) and IR cells were cultured with recombinant FETUB at different concentrations (100, 200, and 300 ng/mL) or were transfected with FETUB shRNA. Glucose consumption was measured after 24 hours.

As illustrated in Figures 5A and 5B, compared with that in control group, the glucose consumption of control-BV2 and control-Müller cells under FETUB recombinant protein stimulation was significantly reduced ($P < 0.05$). The glucose consumption in control-BV2 and control-Müller cells transfected with FETUB shRNA was significantly higher than that in control group ($P < 0.05$). As for IR-BV2 and IR-Müller cells, compared with that in the IR group, the glucose consumption of IR-BV2 and IR-Müller cells was significantly reduced under 200 ng/mL and 300 ng/mL FETUB recombinant protein stimulation ($P < 0.05$). Additionally, glucose consumption in IR-BV2 and IR-Müller cells transfected with FETUB shRNA was significantly higher than that in the IR group ($P < 0.05$).

These results suggest that FETUB plays a role in glucose metabolism in BV2 and Müller cells during DR.

FETUB Aggravated Insulin Resistance in Retinal Cells

1) FETUB aggravated IR in BV2 cells:

The IR-BV2 model was established by treating BV2 cells with 1×10^{-6} mol/L insulin, as shown in Supplementary Figure S2A.

First, based on previous results, 200 ng/mL of FETUB recombinant protein was chosen to detect the phosphorylation levels of various proteins in the insulin signaling pathway following stimulation with FETUB recombinant protein in BV2 cells. BV2 cells were categorized into eight groups based on IR status, use of FETUB recombinant protein, or addition of a physiological concentration (1×10^{-7} mol/L) of insulin.

Subsequently, to detect the phosphorylation levels of various proteins in the insulin signaling pathway after FETUB shRNA transfection in BV2 cells, the cells

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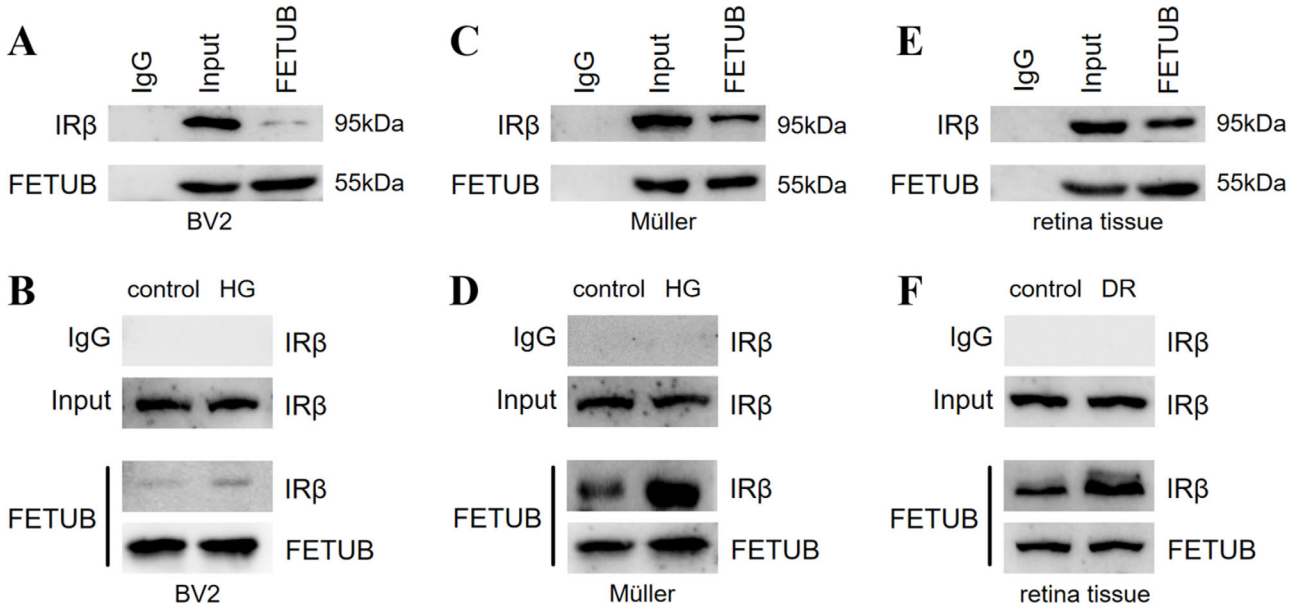

Figure 2. Interaction between FETUB and IRβ in retinal cells and mice retina. (**A, C, E**) CO-IP was used to detect the interaction between FETUB and IRβ in BV2 cells, Müller cells, and mice retina. (**B, D**) Interaction between FETUB and IRβ in BV2 and Müller cells of the control and high-glucose groups. (**F**) Interaction between FETUB and IRβ in mice retina of the control and DR groups. Input, whole cell lysate (positive control group); IgG, IgG control (negative control); HG, high-glucose group; DR, diabetic retinopathy group.

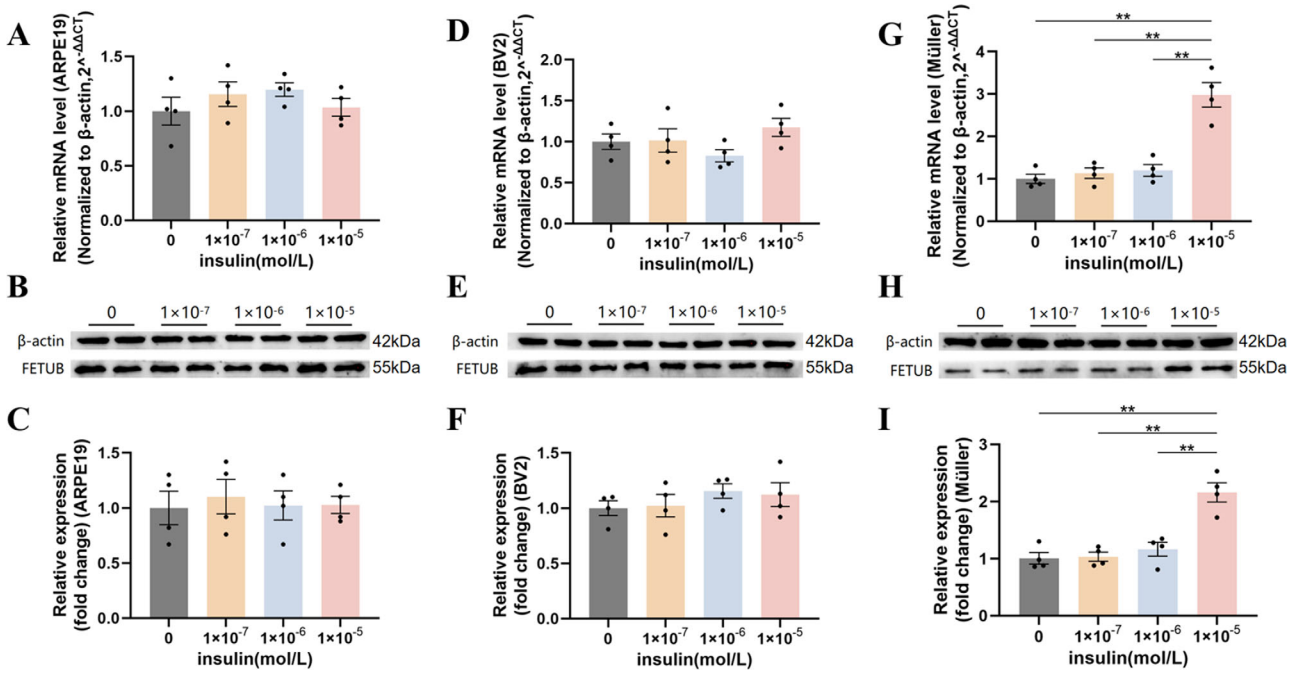

Figure 3. FETUB expression in ARPE-19, BV2, and Müller cells under insulin stimulation. (**A, B, C**) FETUB mRNA and protein levels in ARPE-19 cell groups under different concentrations of insulin. (**D, E, F**) FETUB mRNA and protein levels in BV2 cell groups under different concentrations of insulin. (**G, H, I**) FETUB mRNA and protein levels in Müller cell groups under different concentrations of insulin. β-actin was used as the internal reference control. Data are expressed as mean ± SEM of four independent experiments. $^{**}P < 0.01$.

were categorized into 12 groups based on IR status, use of FETUB shRNA or NC shRNA, and the addition of a physiological concentration (1×10^{-7} mol/L) of insulin.

As shown in Figure 6, 1×10^{-7} mol/L insulin rapidly activated insulin signaling in BV2 cells, leading to increased p-IRβ (Tyr1361), p-PI3K, p-Akt, and glucose transporter-4 (GLUT4) levels, whereas phosphorylation of pho-insulin

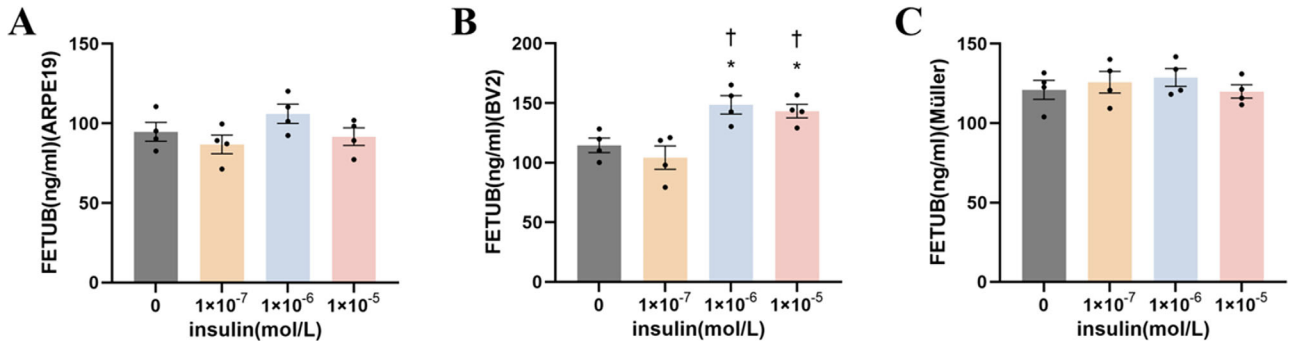

Figure 4. FETUB concentrations in the supernatant under different insulin concentrations. (**A**) FETUB concentrations in the supernatant of ARPE-19 cells under different insulin concentrations. (**B**) FETUB concentrations in the supernatant of BV2 cells under different insulin concentrations. (**C**) FETUB concentrations in the supernatant of Müller cells under different insulin concentrations. Data are expressed as mean ± SEM. *Compared with no insulin group, $P < 0.05$; † Compared with 1×10^{-7} mol/L insulin group, $P < 0.05$.

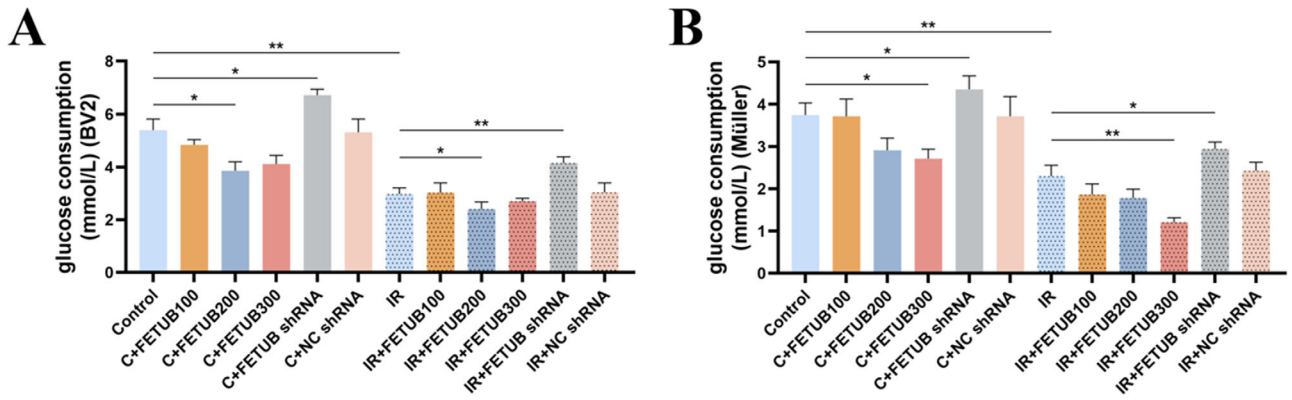

Figure 5. Effect of FETUB on glucose consumption in BV2 and Müller cells. (**A**) Effect of FETUB on glucose consumption in BV2 cells. (**B**) Effect of FETUB on glucose consumption in Müller cells. C, control (no-insulin resistance) cells; IR, insulin resistance group; FETUB 100, cells treated with 100 ng/mL FETUB; FETUB 200, cells treated with 200 ng/mL FETUB; FETUB 300, cells treated with 300 ng/mL FETUB; FETUB shRNA, cells transfected with FETUB shRNA; NC shRNA, cells transfected with NC shRNA. Data are expressed as mean ± SEM. *$P < 0.05$; **$P < 0.01$.

receptor substrates (p-IRS)-1 (Ser307) was reduced. Treatment with FETUB recombinant protein or FETUB shRNA could affect the activation of insulin signaling pathway in control BV2 cells. After establishing the IR model, BV2 cells did not respond to 1×10^{-7}mol/L insulin, and the phosphorylation levels of insulin signaling proteins remained almost unchanged. Treatment with FETUB recombinant protein aggravated IR in BV2 cells, resulting in reduced responsiveness to 1×10^{-7}mol/L insulin (see Figs. 6A–F). IR was effectively relieved in FETUB shRNA transfected BV2 cells, with an enhanced response to 1×10^{-7} mol/L insulin (see Figs. 6G–L).

2) FETUB aggravated IR in Müller cells:

The IR-Müller model was established by treating Müller cells with 5×10^{-6} mol/L insulin, as shown in Supplementary Figure S2B.

First, based on previous results, 300 ng/mL FETUB recombinant protein was chosen to detect the phosphorylation levels of various proteins in the insulin signaling pathway following stimulation with FETUB recombinant protein in Müller cells. The Müller cells were categorized into eight groups based on IR status, use of FETUB recombinant protein, and exposure to physiological concentration (1×10^{-7} mol/L) of insulin.

Subsequently, to detect phosphorylation levels of various proteins of the insulin signaling pathway after FETUB shRNA transfection in Müller cells, the cells were categorized into 12 groups based on IR status, use of FETUB shRNA or NC shRNA, and the exposure to a physiological concentration (1×10^{-7} mol/L) of insulin.

As shown in Figure 7, 1×10^{-7} mol/L insulin rapidly activated insulin signaling in Müller cells, resulting in increased levels of p-IRβ (Tyr1361), p-PI3K and p-Akt, whereas the p-IRS-1 (Ser307) level reduced. Treatment with FETUB recombinant protein or FETUB shRNA could affect the activation of insulin signaling pathway in control Müller cells. After establishing the IR model, Müller cells did not respond to 1×10^{-7} mol/L insulin, and the phosphorylation levels of insulin signaling proteins remained almost unchanged. Treatment with FETUB recombinant protein aggravated IR in Müller cells, resulting in reduced responsiveness to 1×10^{-7} mol/L insulin (see Figs. 7A–F). IR was effectively relieved in FETUB shRNA transfected Müller cells, with an enhanced

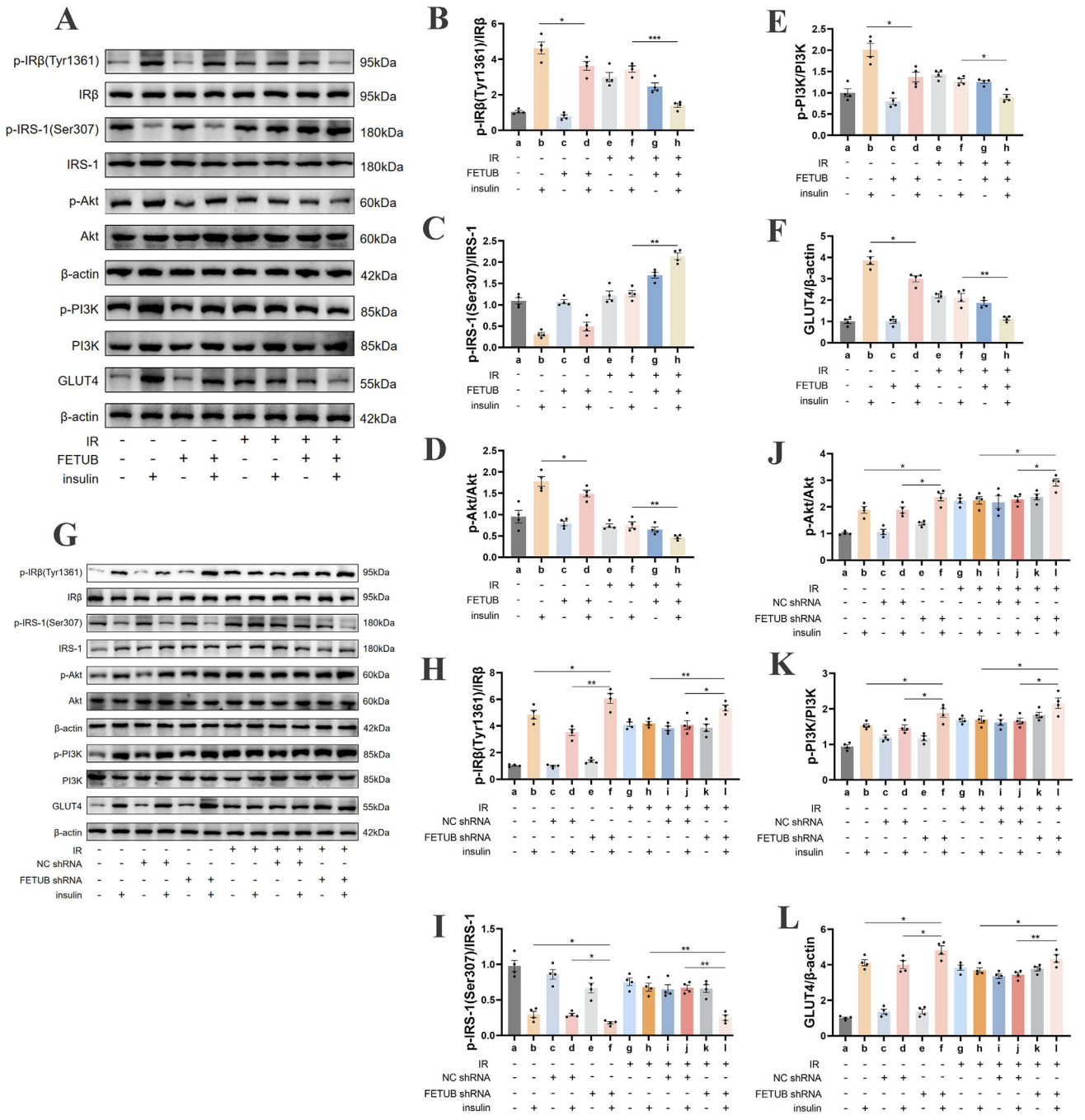

Figure 6. Effect of FETUB on insulin resistance in BV2 cells. (**A–F**) Changes of protein phosphorylation levels of the insulin signaling pathway and GLUT4 level in BV2 cells treated with FETUB recombinant protein. (**G–L**) Changes of protein phosphorylation levels of the insulin signaling pathway and GLUT4 level after FETUB shRNA transfection in BV2 cells. FETUB, 200 ng/mL FETUB recombinant protein; IR, insulin resistance; insulin, physiological concentration (10^{-7} mol/L) insulin. Data are expressed as mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

response to 1×10^{-7} mol/L insulin (see Figs. 7G–L), besides.

LY294002 Inhibited the Effect of FETUB on Insulin Resistance in Retinal Cells

LY294002, an inhibitor of the PI3K pathway, was used to investigate whether FETUB affects cellular glucose metabolism via this pathway.

IR-BV2 and IR-Müller cells were categorized into 8 groups based on the addition of LY294002, use of FETUB recombinant protein, and use of FETUB shRNA or NC shRNA.

As shown in Figure 8, LY294002 reduced the protein levels of GLUT4 and glucose consumption. Notably, neither FETUB recombinant protein nor FETUB shRNA significantly altered the LY294002-induced reduction in GLUT4 expression and glucose consumption. This suggests that FETUB shRNA was unable to reverse LY294002-mediated inhibition. These findings collectively indicate that FETUB reduced

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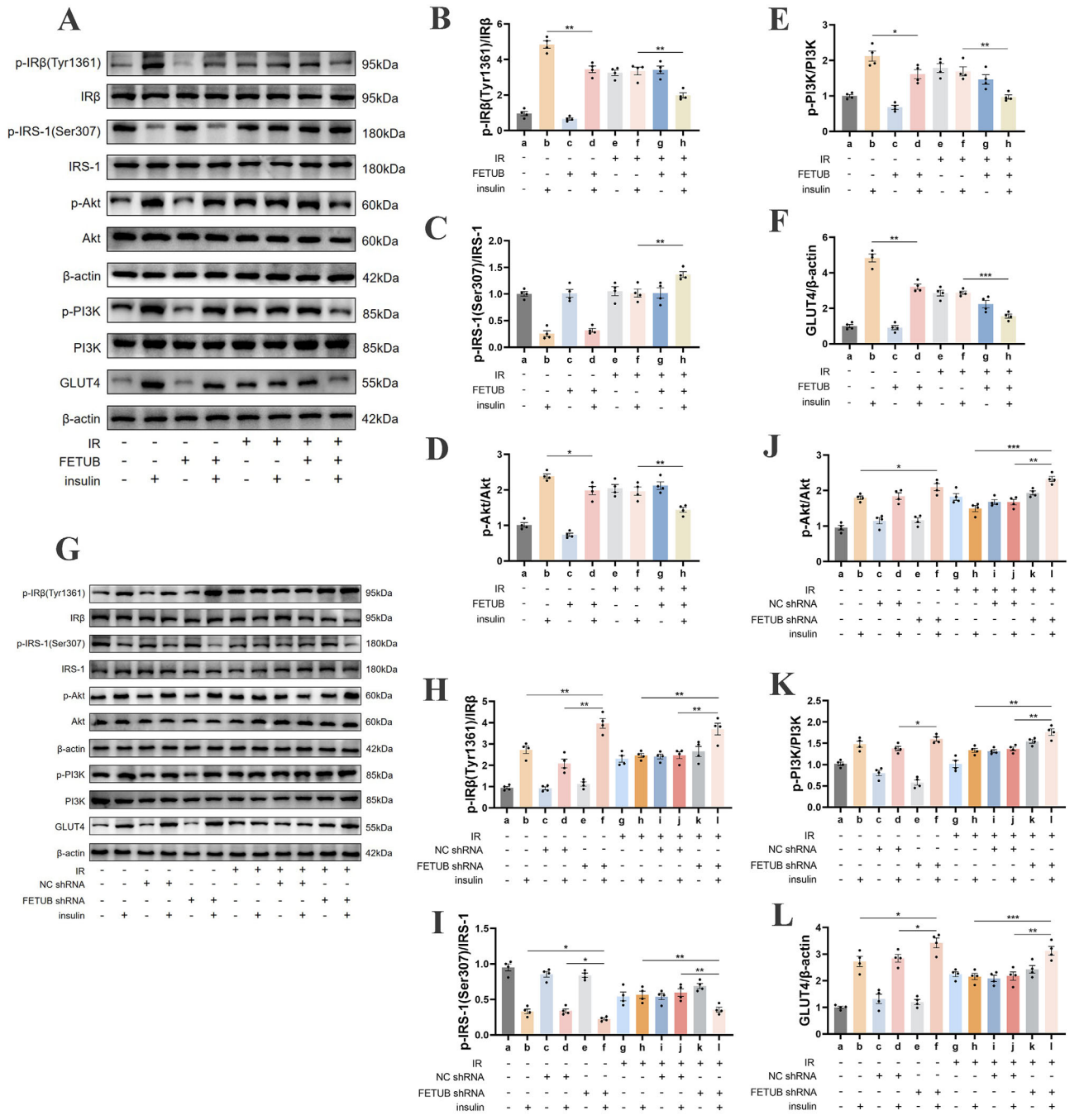

Figure 7. Effect of FETUB on insulin resistance in Müller cells. (**A–F**) Changes of protein phosphorylation levels of the insulin signaling pathway and GLUT4 level in Müller cells treated with FETUB recombinant protein. (**G–L**) Changes of protein phosphorylation levels of the insulin signaling pathway and GLUT4 level after FETUB shRNA transfection in Müller cells. FETUB, 300 ng/mL FETUB recombinant protein; IR, insulin resistance; insulin, physiological concentration (10^{-7} mol/L) insulin. Data are expressed as mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

GLUT4 expression and glucose consumption in BV2 and Müller cells via the PI3K/Akt signaling pathway.

Discussion

Insulin signaling is initiated by the binding of insulin-to-insulin receptors, specifically the insulin receptor-α (IRα) subunit, triggering downstream signaling pathways. This activation induces structural changes in the receptor, leading to autophosphorylation of the tyrosine kinase domain within the IRβ subunit. Phosphorylated insulin receptors subsequently phosphorylate tyrosine residues of insulin receptor substrate proteins, some of which are recognized by the p85 regulatory subunit of PI3K. This activation in turn stimulates the PI3K/Akt signaling pathway, regulating GLUT4 expression, influencing tissue glucose metabolism.[13,14] Insulin

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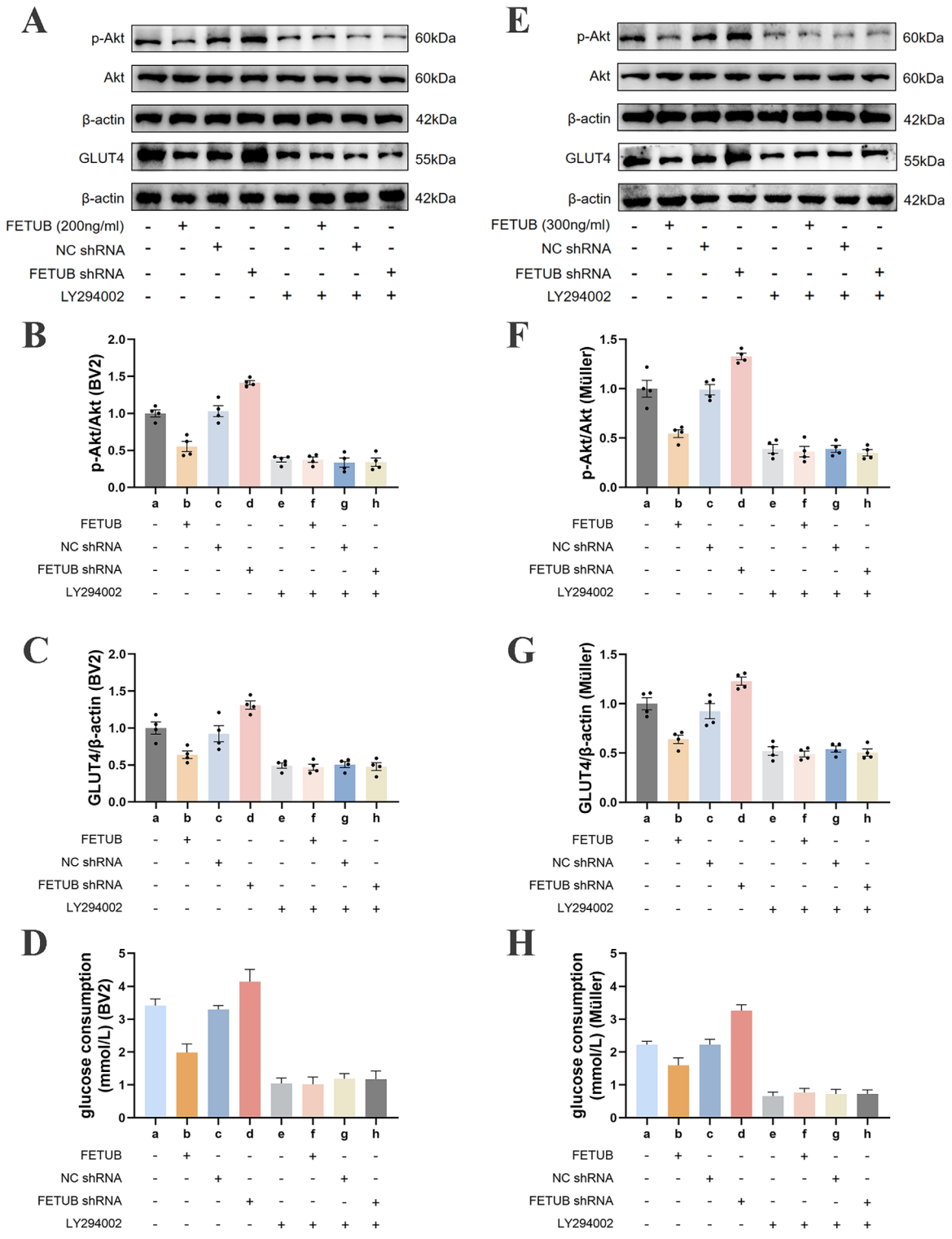

Figure 8. LY294002 inhibited the effect of FETUB on insulin resistance in retinal cells. (**A–C**) Western blot showing that FETUB recombinant protein and FETUB shRNA did not significantly change LY294002-induced reduction of p-Akt/Akt and GLUT4 expression in IR-BV2 cells. (**D**) FETUB recombinant protein and FETUB shRNA did not significantly change LY294002-induced reduction of glucose consumption in IR-BV2 cells. (**E–G**) Western blot showing that FETUB recombinant protein and FETUB shRNA did not significantly change LY294002-induced reduction of p-Akt/Akt and GLUT4 expression in IR-Müller cells. (**H**) FETUB recombinant protein and FETUB shRNA did not significantly change LY294002-induced reduction of glucose consumption in IR-Müller cells. Data are expressed as mean ± SEM.

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receptors (IRα and IRβ subunits) are widely expressed in the human retina.[15] Insulin is not only produced in pancreatic islets by the paracrine pathway but also locally within the retina. Using insulin antisera, insulin immunoreactivity has been detected in the human retinal tissue and the optic nerves of mice.[16] Insulin is located in the ganglion cell, inner nuclear, and inner and outer plexiform layers. Glial cells from the optic nerve also exhibit positive insulin staining.[17] Based on these findings, insulin signaling may play an important role in retinal glucose metabolism and physiological functions, and the retina should be considered as a major target of insulin action.[17,18]

Impaired insulin receptor activity leads to IR, a concept originating from Himsworth's experimental results.[19] IR refers to the failure of target tissues to carry out normal coordinated glucose-lowering responses, including the inhibition of endogenous glucose production, stimulation of cellular glucose uptake, and glycogen synthesis at normal serum insulin levels. To alleviate this phenomenon, pancreatic β cells need to increase insulin secretion, resulting in elevated fasting serum insulin levels in patients with IR. The IR is essential in the pathogenesis of fasting hyperglycemia and T2D,[20] potentially leading to retinal damage in patients with T2D. Dysfunction of insulin signaling has been observed in the retinas of T2D mice.[11,12] Although extensive research exists on insulin-sensitive organs, such as adipocytes and the liver, fewer studies have been conducted on the retina.[11,21] Further studies are required to determine the effects of diabetes on retinal insulin signaling.

In this study, we found a positive correlation between insulin and FETUB levels in the aqueous humor and a positive correlation between insulin levels in the serum and aqueous humor, suggesting that insulin may promote the secretion of FETUB in the eye. A previous study[3] has shown that high-glucose environments increase FETUB expression. In this study, FETUB expression levels in BV2 and Müller cells increased in a high-insulin environment. However, compared with glucose, insulin had a lesser impact on retinal FETUB expression levels, indicating that high glucose levels might be the primary factor affecting FETUB secretion in the retina.

Through the CO-IP experiment, we demonstrated that FETUB interacted with IRβ in retinal cells. Moreover, the interaction between FETUB and IRβ was enhanced in the high-glucose or DR groups compared with in the control group. This result suggests that FETUB may exert its effect on the insulin pathway by interacting with IRβ. Subsequently, we confirmed that FETUB affected IRβ phosphorylation and aggravated IR in retinal cells. Simultaneously, FETUB inhibited PI3K/Akt pathway activation and GLUT4 expression. Finally, LY294002 was used to inhibit the PI3K/Akt pathway. Using LY294002, we determined that FETUB inhibited GLUT4 expression and glucose consumption in BV2 and Müller cells through the PI3K/Akt signaling pathway, disrupting glucose metabolism in retinal cells.

In a previous study, Xing et al.[22] reported that FETUB could bind to IRβ in cardiomyocytes, enhancing their interaction compared with that in the control group. This binding inhibits the tyrosine site phosphorylation and downstream signaling pathways, aggravating IR and myocardial ischemia/reperfusion injury in the myocardium of patients with T2D. These findings align with our results. In addition, fetuin-A (FETUA) interacts with activated insulin receptors and inhibits their phosphorylation in the liver and skeletal muscles.[23,24] Phosphorylation of FETUA is critical for the inhibition of insulin function and is associated with obesity and IR.[25] FETUA downregulates GLUT4 translocation to the cell membrane by inhibiting Akt phosphorylation, thereby impairing insulin-mediated glucose uptake.[26] According to these studies, FETUB appears to function similarly to FETUA, promoting IR.

In conclusion, our experimental results indicate that insulin may promote FETUB secretion and regulate its expression in retina cells. FETUB interacts with IRβ in retinal cells during DR and exacerbates IR in BV2 and Müller cells through the PI3K/Akt pathway. However, several questions remain unanswered, such as whether FETUB binds directly to IRβ and the specific mechanism of this interaction. Additionally, the impact of FETUB on retinal cell energy metabolism and its involvement in other physiological and pathological activities through insulin signaling pathways necessitate further investigation.

Acknowledgments

Supported by the Key Program of Research and Development of Shaanxi, China (Grant No. 2023-YBSF-585, 2022SF-154).

Disclosure: **W. Zhang**, None; **X. Wang**, None; **S. Tian**, None; **J. Wang**, None; **A. Zhou**, None

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