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Glycyrrhizin protects IGFBP-3 knockout mice from retinal damage

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Abstract

We previously reported that insulin-like growth factor binding protein 3 (IGFBP-3) knockout (KO) mice have neuronal and vascular damage to the retina. We also reported that glycyrrhizin, a high mobility growth factor binding protein 1 (HMGB1) inhibitor, is protective to the diabetic retina. In this study, we investigated whether glycyrrhizin could reduce neuronal and vascular damage in the IGFBP-3 KO mouse retina. We used measurements of retinal thickness, cell number in the ganglion cell layer, degenerate capillaries, reactive oxygen species (ROS) and protein levels of HMGB1, tumor necrosis factor alpha (TNFα), interleukin-1-beta (IL-1β) and sirtuin 1 (SIRT1) to determine whether glycyrrhizin could protect the retina. Data show that glycyrrhizin in the drinking water was effective in reducing neuronal damage at 2 months and vascular damage at 6 months. Glycyrrhizin reduced ROS levels at 6 months, and reduced levels of HMGB1, TNFα, and IL-1β at both 2 and 6 months. Taken together, the data suggest that glycyrrhizin is protective to the retina of IGFBP-3 KO mice through anti-inflammatory mechanisms.

1.0 Introduction.

We have previously reported that β-adrenergic receptor activation required active insulin like growth factor binding protein 3 (IGFBP-3) to protect the retina against streptozotocin-induced diabetes [1], and that IGFBP-3 actions were insulin like growth factor 1 (IGF-1) receptor independent. We also showed that IGFBP-3 knockout mice have neuronal changes with increased tumor necrosis factor alpha (TNFα) levels [2]. IGFBP-3 reduced TNFα signaling through casein kinase 2 actions in REC grown in high glucose [3]. We also have reported that a mutant form of IGFBP-3, which cannot bind the IGF-1 receptor, inhibited ICAM-1-mediated cellular adhesion. Our data agrees with others, which reported that IGFBP-3 enhanced cell proliferation in REC and decreased the formation of neovascular tufts in a murine model of oxygen-induced retinopathy (OIR) [4–6]. IGFBP-3 is reported to

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Declaration of interests

No competing interests

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be neuroprotective in the retina and reduce injury-induced retinal inflammation [7]. Others have also reported that IGFBP-3 can reduce hepatic inflammation through a reduction in NFkB and JNK actions [8].

The mechanism by which IGFBP-3 is protective to the retina is less clear. Data suggest that IGFBP-3 is protective to the retina and reduces retinal inflammation. Yet, the intermediary factors for this reduced retinal inflammation are unclear. We recently reported that Compound 49b reduced HMGB1 levels in REC and the diabetic mouse retina [9]. In support of the work with Compound 49b, work in a corneal wound healing model showed that IGFBP-3 increased sirtuin 1 (SIRT1) to promote wound healing, despite high glucose conditions [10]. We have recently reported that IGFBP-3 regulates HMGB1 in retinal endothelial cells (REC) grown in high glucose [11]. In addition to our work on IGFBP-3 in the retina, others reported that IGFBP-3 reduced inflammatory mediators and reactive oxygen species (ROS) levels in a colon model [12]. IGFBP-3 also suppressed ROS levels in an esophageal cancer model [13].

Thus, based on our cell culture work and literature on IGFBP-3, we wanted to investigate whether glycyrrhizin could protect the retina of IGFBP-3 KO mice through activation of SIRT1 and reduced inflammatory mediators.

2.0 Methods.

2.1 Mice.

Insulin like growth factor binding protein 3 knockout (IGFBP-3 KO) mice were generously provided by Dr. John Pintar (Rutgers University) [2]. Confirmation of the knockout of the IGFBP-3 gene was completed using Southern blotting. Some IGFBP-3 KO mice were given glycyrrhizin (150mg/kg, Sigma, St. Louis MO) in their drinking water for up to 6 months [14]. IGFBP-3 KO mice are on a C57BL/6J background, and we recently published that glycyrrhizin had no effects on control mice [15].

2.2 Measurement of Retinal Thickness and Loss of Cells of the Ganglion Cell Layer (GCL).

Formalin-fixed cyrostat sections from IGFBP-3 KO only and IGFBP-3 KO+glycyrrhizin at 2 months of treatment were stained with hematoxylin and eosin for light microscopy and morphometry of retinal thickness as described [16]. Photomicrographs were assessed for retinal thickness and the number of cells in the GCL using methods previously described [17].

2.3 Vascular Analyses.

Retinas from control and IGFBP-3 knockout only or treated with glycyrrhizin for 6 months were used to count degenerate capillaries. Eyes were enucleated, suspended in 10% buffered formalin for 5 days, retina were dissected out, and retinal vascular tree was dried onto a glass slide and stained with hematoxylin-periodic acid-Shiff. Degenerate capillaries were counted and identified as previously described [18, 19].

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2.4 Western blotting.

Whole retinal lysates from mice or cell culture lysates were collected in into lysis buffer with protease and phosphatase inhibitors. Proteins were separated onto a pre-cast tris-glycine gel (Invitrogen, Carlsbad, CA), and blotted onto nitrocellulose membrane. After blocking in TBST (10mM Tris-HCI buffer, pH 8.0, 150 mM NaCl, 0.1% Tween 20) and 5% (w/v) BSA, the membranes were treated with HMGB1, IL-1β (Abcam, Cambridge, MA) or beta actin (Santa Cruz Biotechnology, Santa Cruz, CA) primary antibodies followed by secondary antibodies labeled with horseradish peroxidase. Antigen-antibody complexes were visualized using a chemilluminescence reagent kit (Thermo Scientific, Pittsburgh, PA) and data was acquired with an Azure C500 (Azure Biosystems, Dublin, CA). Western blot data were assessed using Image Studio Lite software.

2.5 Measurement of Reactive Oxygen Species (ROS).

Protein lysates from all groups of mice at both 2 and 6 months of diabetes and treatment were processed for measurement of reactive oxygen species using the 2′-7′-Dichlorodihydrofluorescein diacetate (DCFDA) method as we have done previously [14].

2.6 ELISA.

A TNFα (ThermoScientific, Pittsburgh, PA) and SIRT1 ELISA (Abcam, Cambridge, MA) were done according to manufacturer's instructions with the exception that the primary antibody was allowed to incubate overnight.

2.7 Statistical Analyses.

One-way ANOVA with Student Newman Keul's post-hoc test was used for animal work. $P<0.05$ was considered statistically significant.

3.0 Results.

3.1 Glycyrrhizin improved neuronal measurements of IGFBP-3 KO mice.

We recently reported that glycyrrhizin, a HMGB1 inhibitor, improved retinal thickness and cell numbers in the ganglion cell layer of diabetic mice [14]. Additionally, we have reported that IGFBP-3 regulates HMGB1 levels in retinal endothelial cells (REC) grown in high glucose [11]. Since we have previously showed that IGFBP-3 KO mice have retinal damage similar to diabetes [2], we wanted to determine if glycyrrhizin could protect the retina in these mice. Figure 1 demonstrates that IGFBP-3 KO mice have thinner retinas (B) and fewer cells in the ganglion cell layer (C) than IGFBP-3 KO mice treated with glycyrrhizin in the water for 2 months.

3.2 IGFBP-3 KO mice treated with glycyrrhizin have fewer degenerate capillaries.

We have reported that HMGB1 inhibition protected the retinal vasculature of diabetic mice [15] and diabetic Epac1 floxed and endothelial cell specific Epac1 knockout mice [14]. Figure 2 demonstrates that glycyrrhizin protected the retinal vasculature of IGFBP-3 KO mice by reducing the numbers of degenerate capillaries.

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3.3 Glycyrrhizin reduced ROS in IGFBP-3 KO mice at 6 months.

In contrast to the C57BL/6 mice, glycyrrhizin significantly inhibited ROS levels only at 6 months (Figure 3). There was a trend for decreased ROS at 2 months, but this did not reach statistical significance.

3.4 Glycyrrhizin increased SIRT1 and IGFBP-3 levels in IGFBP-3 KO mice, while decreasing HMGB1, TNFα and IL-1β at both 2 and 6 months of STZ.

We previously reported that glycyrrhizin increased SIRT1 levels in the retina of mice [14]. In this study, we demonstrate that glycyrrhizin also increased SIRT1 levels in IGFBP-3 KO mice at both 2 and 6 months (Figure 4B). Figure 4A confirms knockout of IGFBP-3, and it shows that glycyrrhizin increased IGFBP-3 levels in the IGFBP-3 KO mice. Figures 4C–E demonstrate that IGFBP-3 KO mice have increased inflammatory mediators, which are all decreased by glycyrrhizin treatment in the water. Taken together, these data show that glycyrrhizin has anti-inflammatory actions on the IGFBP-3 KO mouse retina.

4.0 Discussion.

We have previously demonstrated that IGFBP-3 KO mice have neuronal and vascular damage [2]. We also showed that IGFBP-3 inhibited TNFα production in retinal endothelial cells (REC) in culture [3], as well as regulated insulin signal transduction in the retina [20]. Since we recently reported that IGFBP-3 regulated HMGB1 levels in REC [11], we wanted to extend these studies to an *in vivo* setting.

Since these mice have neuronal and vascular damage, we did not make the mice diabetic for these studies. We did confirm neuronal and vascular damage in the IGFBP-3 KO mice in Figures 1 and 2, respectively. We recently reported that glycyrrhizin, a natural component of black licorice that inhibits HMGB1, protected the diabetic mouse retina [15]. In this study, we examined whether glycyrrhizin could also protect the retina of IGFBP-3 KO mice. Data demonstrate that glycyrrhizin reduced neuronal and vascular damage noted in the IGFBP-3 KO mice at 2 and 6 months, respectively. Loss of IGFBP-3 significantly increased ROS levels at both 2 and 6 months, with glycyrrhizin reducing ROS levels at 6 months. There was a trend for reduction at 2 months, but this did not reach statistical significance. IGFBP-3 KO mice had increased HMGB1, TNFα, and IL-1β levels at both 2 and 6 months of age, which were significantly reduced by glycyrrhizin. Glycyrrhizin also significantly increased both IGFBP-3 levels in the IGFBP-3 KO mice, as well as SIRT1 levels.

Glycyrrhizin likely works in the IGFBP-3 KO mice through anti-inflammatory actions. The protein data demonstrate that both TNFα and IL-1β levels increase between 2 and 6 months. Glycyrrhizin reduced both proteins. We have previously reported that TNFα can negatively regulate IGFBP-3 in REC grown in high glucose through casein kinase pathways [21]. Thus, glycyrrhizin's inhibition of TNFα could increase IGFBP-3 levels. Furthermore, we recently reported that glycyrrhizin increased SIRT1 levels in Epac1 endothelial cell specific knockout mice [14], which also occurred in the IGFBP-3 KO mice. Through reducing inflammatory mediators, glycyrrhizin likely reduced neuronal and vascular damage in the IGFBP-3 KO mice.

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Since these were *in vivo* experiments, we did not directly test whether glycyrrhizin's actions on TNFα lead to the increase in IGFBP-3. We will be expanding these findings in the future. We did test permeability in these mice, but did not find increased permeability in the IGFBP-3 KO mice, so we did not investigate whether glycyrrhizin could reduce permeability. It may be that IGFBP-3 KO mice without diabetes do not have the increased permeability, and we would have to treat them with STZ to get permeability changes. However, since we previously showed that these mice had neuronal and vascular damage [2], we chose to avoid the STZ treatment and investigate glycyrrhizin actions on only the IGFBP-3 KO mice. Finally, we did not use only C57BL/6 mice for this work since we recently published the actions of glycyrrhizin on control mice [15].

In conclusion, data suggest that glycyrrhizin is protective to the retina of IGFBP-3 KO mice through reduction in inflammatory mediators. Data in the IGFBP-3 KO mice matches recent findings in diabetic C57BL/6 mice, as well as Epac1 endothelial cell specific KO mice. Ongoing studies will be focused on the exact mechanisms by which glycyrrhizin protects the retina.

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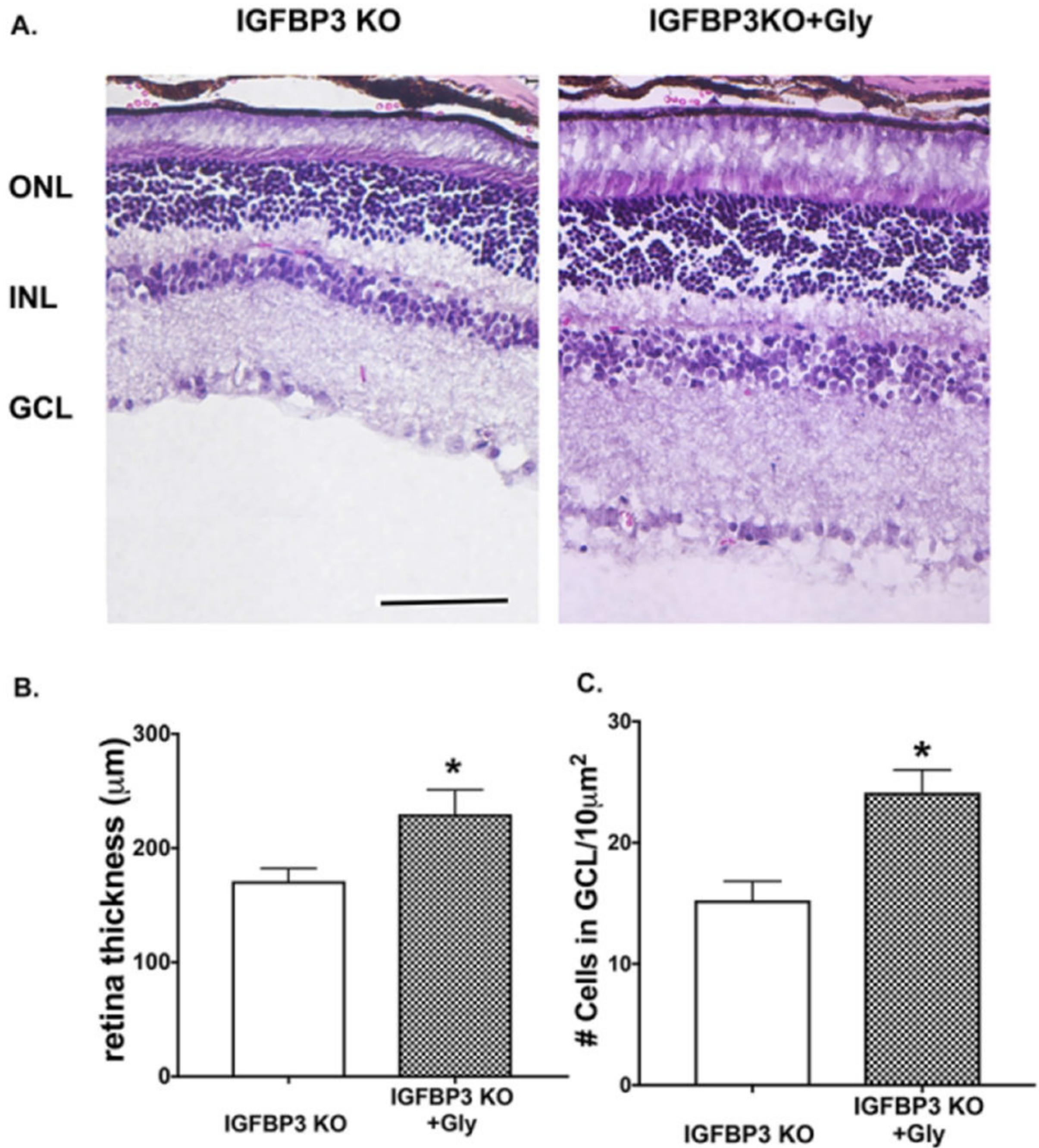

Figure 1.

Neuronal measurements in IGFBP-3 KO and IGFBP-3 KO+glycyrrhizin treatment. Two month of glycyrrhizin treatment restored retinal thickness (B) and cell numbers in the ganglion cell layer (C) in the IGFBP-3 KO mice. *P<0.05 vs. IGFBP-3 KO. Scale bar is 50um.

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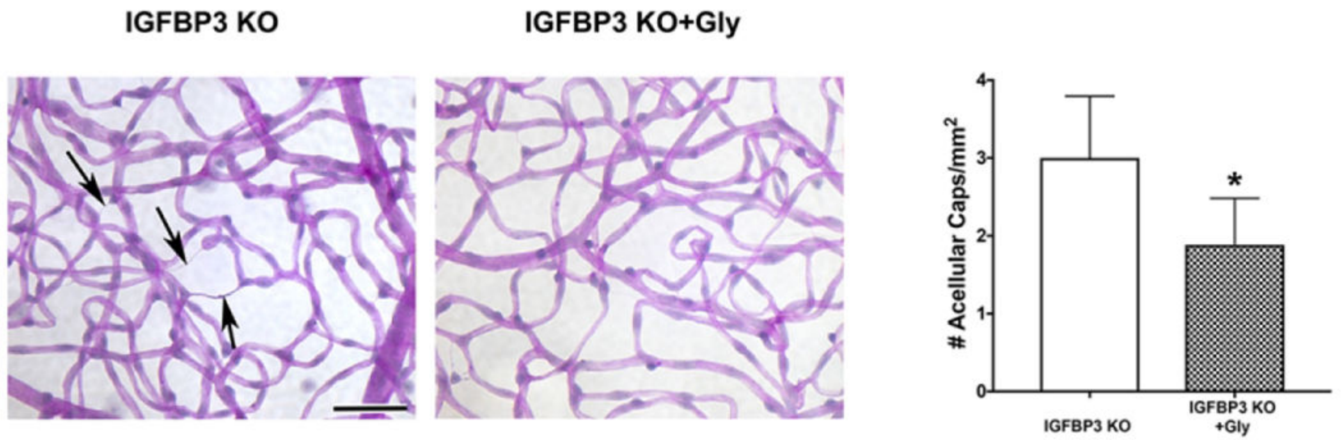

Figure 2.
Degenerate capillaries in IGFBP-3 KO mice. IGFBP-3 KO and IGFBP-3 KO mice treated with glycyrrhizin for 6 months were processed for measurement of degenerate capillaries. *P<0.05 vs. IGFBP-3 KO. Scale bar is 50um.

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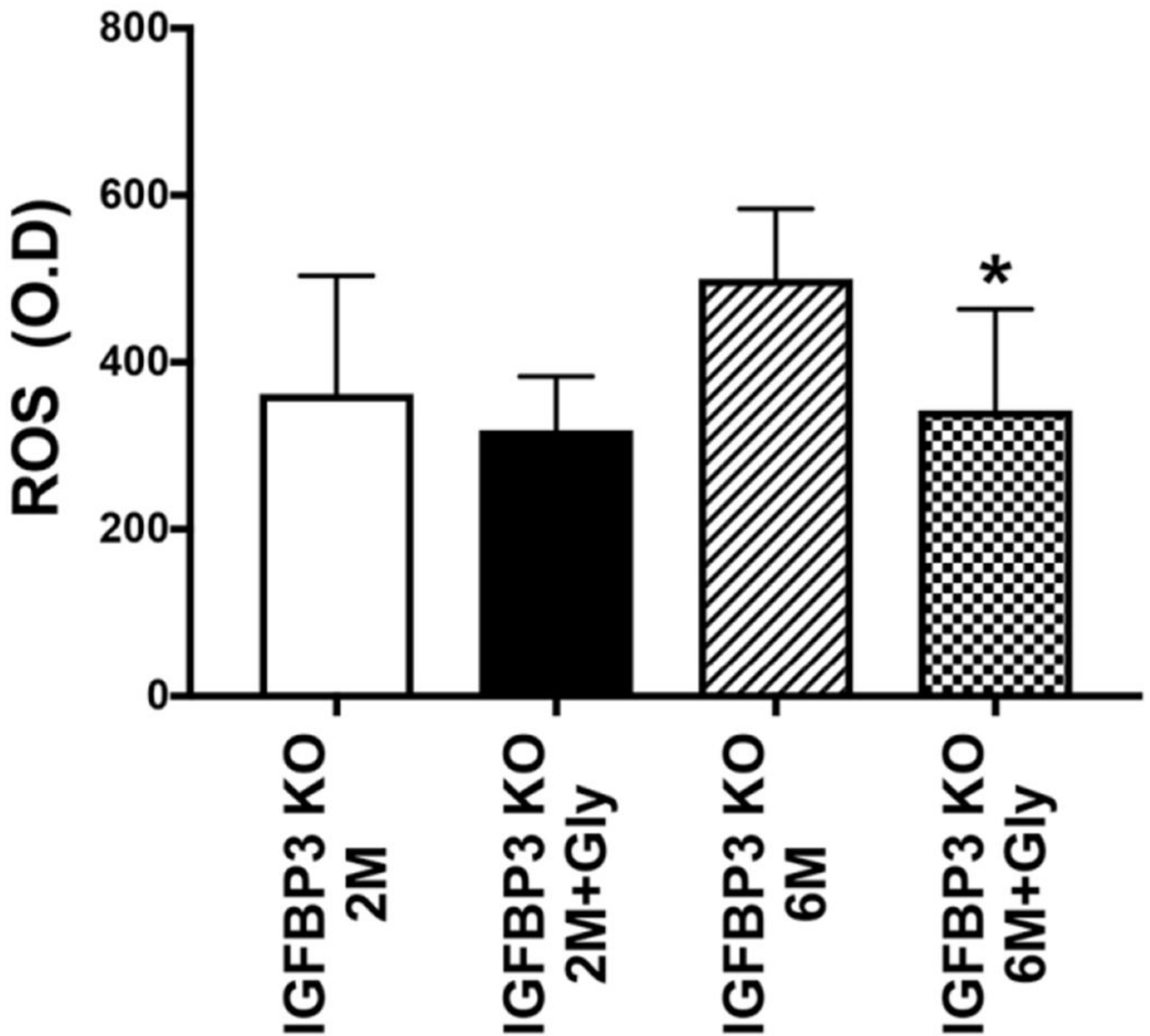

Figure 3.

Reactive oxygen species (ROS) measurement in IGFBP-3 KO mice and IGFBP-3 KO +glycyrrhizin treatment mice at 2 and 6 months of glycyrrhizin treatment. *P<0.05 vs. IGFBP-3 KO at 6 months. Data are mean±SEM. N=5.

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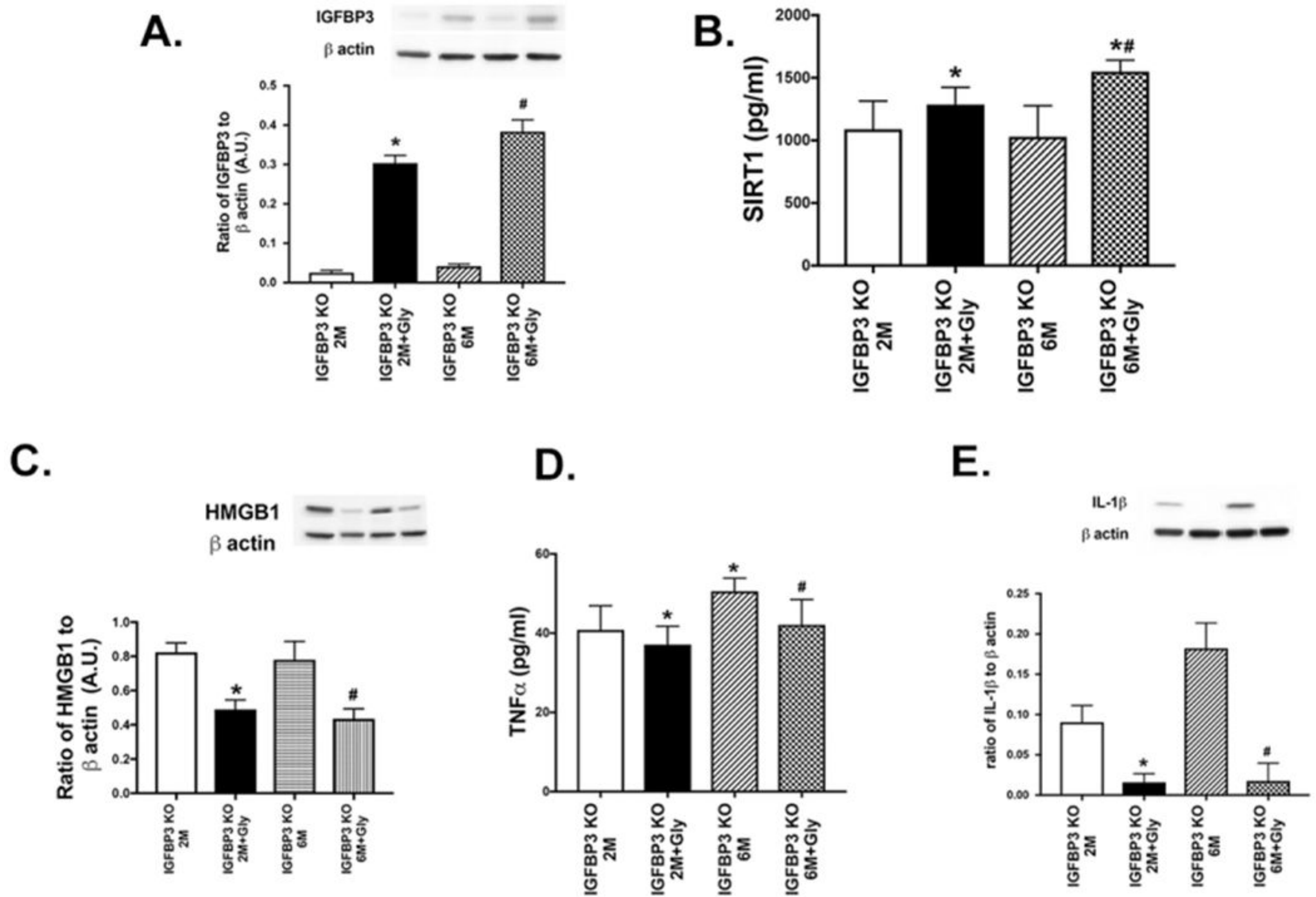

Figure 4.

Protein measurements in IGFBP-3 KO and IGFBP-3 KO+glycyrrhizin mice at 2 and 6 months for IGFBP-3 (A), SIRT1 (B), HMGB1 (C), TNFα (D), and IL-1β (E). *P<0.05 vs. IGFBP-3 KO at 2 months, #P<0.05 vs. IGFBP-3 KO at 6 months. Data are mean±SEM. N=5.