Biochemical and Biophysical Research Communications 444 (2014) 264–269

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Effect of hyperglycemia on insulin receptor signaling in the cultured retinal Müller glial cells

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ARTICLE INFO

Article history:
Received 7 January 2014
Available online 22 January 2014

Keywords:
Insulin
Hyperglycemia
Neurodegeneration
Retina
Muller cells

ABSTRACT

Hyperglycemia and impaired insulin signaling are considered as major factors in the retinal pathology in diabetic retinopathy (DR). Numerous reports support that these two factors damage retinal glial as well as neuronal cells early in diabetes. However, it is not known whether diabetic induced hyperglycemia causes a depression to the insulin signaling. In this study we utilized a well characterized cultured Muller cells (TR-MUL) where we found a high expression of insulin receptor molecules. TR-MUL Cells were treated with high glucose, glutamate and hydrogen peroxide, and activated with insulin. Following treatments, cell lysates were analyzed by immunoblotting experiments for insulin receptor (IRβ) and insulin receptor substrate (IRS1). In addition, cell lysates were immunoprecipitated using antibodies against insulin receptor proteins to analyze tyrosine phosphorylation and serine phosphorylation of insulin receptor proteins. Results indicate that hyperglycemia did not affect the expression of insulin receptor proteins in cultured TR-MUL cells. Although, hyperglycemia seems to inhibit the interaction between IRS1 and IRβ. Hydrogen peroxide increased the tyrosine phosphorylation of insulin receptor proteins but excess glutamate could not affect the insulin receptor proteins indicating that glutamate may not cause oxidative stress in TR-MUL cells. Hyperglycemia lowered serine phosphorylation of IRS^{ser632} and $IRS^{ser1101}$ however, IRS^{ser307} was not affected. Thus, hyperglycemia may not affect insulin signaling through tyrosine phosphorylation of insulin receptor proteins but may inhibit the interactions between insulin receptor proteins. Hyperglycemia induced phosphorylation of various serine residues of IRS1 and their influence on insulin signaling needs further investigation in TR-MUL cells.

1. Introduction

Hyperglycemia and impaired insulin signaling are considered as major factors in the retinal pathology in diabetic retinopathy (DR). Numerous studies suggest that hyperglycemia activates several metabolic pathways mediating oxidative stress which plays a central role in the pathogenesis of DR as summarized in few recent reviews [1–3]. In addition, a number of studies have shown that diabetes depresses retinal insulin signaling and thereby increases retinal cell death [4–7]. Recently, Fort et al., [8] demonstrated that normalization of glycaemia and restoration of insulin signaling reversed diabetes induced insulin signaling defects in the retina. However, the cross talks between hyperglycemia and insulin signaling are poorly understood in retina and especially in the cells of neuronal component of the retina.

Retina consists of two major cell components, vascular and neuronal cells which are compromised in diabetes. However, within the neuronal component, glia and neuronal cells are compromised in the early stages of diabetes before any signs of vascular damage [4,5,9–11].

Within neural retina, Muller glial cells are metabolically very active cells which exert a pivotal role in supplying metabolites including glucose, lactate and other nutrients from the blood vessels to retinal neurons. These cells also support neuronal activities such as neurotransmission by maintaining glutamate/glutamine cycle. However, diabetes induced metabolic dysregulation and lack of neurotropic support may affect glial cell function in the retina. Since, Muller cells are tightly bound to retinal vasculature, therefore, these cells might become susceptible to high metabolic stress conditions including high glucose in diabetic conditions. Insulin is an important neuroactive hormone essential for regulating growth, development and metabolism in brain and retina [12]. However, the role of insulin and insulin signaling in glial cells is not well known except insulin stimulates glycogen storage in astrocytes [13]. Recently, Jiang et al., [14] reported regulation of insulin signaling by high glucose in a cultured Muller cells (RMC-1). In this study, we focused on another cultured Muller glial cells (TR-MUL) to understand the influence of hyperglycemia on insulin receptor signaling.

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http://dx.doi.org/10.1016/j.bbrc.2014.01.052

Earlier, we have reported high expression of insulin receptor signaling molecules in the TR-MUL cells [15]. These cells are well characterized to be a unique in vitro model of glial cells, since they express most of the protein markers and metabolic enzymes which are characteristics of glial cells [16,17]. Since Muller cells are part of blood retinal barrier which employ us to investigate the effect of hyperglycemic state on insulin signaling within Muller cells. In this study, we ought to understand whether hyperglycemia affects insulin signaling especially at the level of IRβ and IRS1 proteins in the cultured TR-MUL cells. In addition, we have also investigated the influence of glutamate and oxidant which have been found to be increased diabetic retina, on signaling through insulin receptor proteins in the Muller cells.

2. Materials and methods

2.1. Materials

Cell culture medium, Dulbecco's Modified Eagle Medium (DMEM), heat inactivated fetal bovine serum (FBS), penicillin, streptomycin, and trypsin were purchased from Gibco-Invitrogen Corp. (Grand Island, NY) and insulin (Humulin) was purchased from Sigma Chemicals (St. Louis, MO), and goat anti-mouse IgG was from BD Biosciences, Parmingen (San Jose, CA). ECL Western Blotting Detection Reagents were from Amersham Biosciences (Piscataway, NJ) and MR film from Kodak (Rochester, NY). All other chemicals were from Sigma Aldrich Inc. (Atlanta, GA)

2.2. Cell culture and treatments

The conditionally immortalized rat retinal Muller cell (TR-MUL) was generously supplied to us from the Hosoya Laboratory, Japan [16]. We cultured the cells according to the standard method developed in their laboratory, with slight modifications. Cells were grown in a 75 cm^2 flask at 33 °C in DMEM medium supplemented with normal glucose (5 mM) including 5% (v/v) FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2/95% air. Media was changed every day until cells reached 60–70% confluence. The cells were then grown in low serum concentration (2%) media followed by treatments with high glucose (30 mM), mannitol (25 mM) and glutamate (5 mM). Glutamate treatment to cells was for 24 h. For insulin activation of cells following various treatments, existing low serum containing media were removed, and replenished with serum free media for another 2 h, prior to insulin treatment. Cells were treated either with 10 nM insulin or no insulin for 10 min. For hydrogen peroxide treatments, starved cells were pretreated with 50 μM H_2O_2 for 10 min, followed by insulin treatment for another 10 min. After insulin activation, cells were washed twice with cold phosphate buffer saline, homogenized in the cell lysis buffer, and insulin receptor proteins were analyzed by immunoblotting experiments.

2.3. Immunoblotting analysis

Cultured TR-MUL cells with different treatments as described above were homogenized by brief sonication on ice-cold lysis buffer (10 mM HEPES, 42 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 1 mM phenyl methyl sulfonyl fluoride, 10 mM sodium pyrophosphate, 10 mM benzamidine, 1% Triton X-100, and protease inhibitor tablet; Roche, Mannheim, Germany). Samples were kept on ice for 15 min, and then centrifuged at 15,000*g* for 10 min. The supernatants were decanted and the protein concentrations estimated using the Bio-Rad DC protein assay kit (Bio-Rad). Samples were boiled in Laemmli's sample buffer for 5 min and analyzed by10% of SDS–PAGE. After transferring the proteins onto nitrocellulose membranes, the membranes were blocked for 1.5 h at room temperature with Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% non-fat dry milk. Next, the membranes were incubated overnight with polyclonal anti-insulin receptor-β subunits (Santa Cruz Biotechnology, San Diego, CA) and anti-insulin receptor substrate, (Upstate Biotechnology) primary antibodies. The membranes were washed and probed with secondary antibody conjugated to horseradish peroxidase goat anti-rabbit IgG and goat anti-mouse antibody (Pierce, 1:5000 dilution) for 1.5 h. Membranes were washed with TBST and developed in Super Signal West Pico (Pierce) and exposed to auto radio graphic films. The films were scanned using a GS-800 scanner (Bio-Rad) and the densities were quantified by Quantity One Software (Bio-Rad).

2.4. Immunoprecipitation and Immunoblotting of insulin receptor proteins

To analyze insulin receptor proteins phosphorylation, an immunoprecipitation buffer was used to homogenize the cell (50 mM HEPES, pH 7.3, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM Na_3VO_4, 10 mM sodium pyrophosphate, 10 mM NaF, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 10 mM benzamidine, 10% glycerol, 1% Nonidet P-40, and 1 protease inhibitor tablet/10 ml). The homogenate was centrifuged at 12,000*g* for 15 min, at 4 °C, to remove cell debris. Supernatant collected and protein concentrations were measured with the Bio-Rad DC protein assay kit (Bio-Rad), and all samples were adjusted for equal protein. Immunoprecipitation was performed overnight at 4 °C with polyclonal anti-insulin receptor-β subunits (Santa Cruz Biotechnology, San Diego, CA) and anti-insulin receptor substrate, (Upstate Biotechnology) antibodies at a concentration of 10 μg/mg protein. Next, 30 μl protein A-Sepharose beads were added and incubated overnight at 4 °C. Supernatants were removed by centrifugation at 10,000*g* for 5 min and precipitated Sepharose bound proteins were mixed with 5X sample buffer (0.32 M Tris, pH 6.8, 62.5% glycerol, 6.25% β-mercaptoethanol, 5% SDS, 5.6 mg/ml bromophenol blue) and boiled for 3 min before SDS–polyacrylamide gel electrophoresis. Phosphotyrosine blotting was performed with mouse monoclonal anti-phosphotyrosine (1:1000, Upstate Biotechnology, Inc.) followed by sheep anti-mouse IgG (Amersham Pharmacia Biotech, Piscataway, NJ). For serine phosphorylation of IRS1, we used three different antibodies (anti-phospho-IRS1(ser1101), anti-phospho-IRS1(ser632) and anti-phospho-IRS1(ser307) (1:1000, Upstate Biotechnology).

2.5. Calculations and statistics

Values are shown in figures as means ± SEM. Statistical significance was judged by independent *t*-tests where $P < 0.05$ is considered significant. Numbers of independent determinations (n) are provided in figure legends.

3. Results

3.1. The influence of hyperglycemia on insulin receptor proteins in TR-MUL cells

Cultured TR-MUL cells have been found to express the glial cell specific enzymes GS, S-100, the Na^+-linked glutamate transporters (EAAT1/GLAST) and high expression of insulin receptor proteins [15–17]. Therefore, in this study we wish to elucidate the influence of high glucose on insulin receptor levels. Cells were treated with media supplemented with normal glucose (5 mM), high glucose (30 mM) and normal glucose plus 25 mM mannitol for 5 days.

We quantitated the expression levels of insulin receptor proteins by Western blot analysis. Results are shown in Fig. 1. Quantifications of band intensities are provided in corresponding bar graphs. The results reveal that protein expression of these insulin receptor molecules did not change either under high glucose or with mannitol which is used as osmolar control compared to normal glucose (Fig. 1).

3.2. The influence of high glucose and insulin on tyrosine phosphorylation of IRS1 and IRβ in the cultured TR-MUL cells

After demonstrating the protein expression of insulin receptor proteins, we evaluated the influences of high or normal glucose, with or without insulin treatments on the phosphorylation activity of the insulin receptor proteins using TR-MUL cells. We employed immunoprecipitation methods followed by immunoblotting techniques to analyze the level of tyrosine phosphorylation of IRS1 and IRβ. Our results indicate that high glucose could not influence the phosphotyrosine level of IRS1 and IRβ when compared to normal glucose (Fig. 2). Insulin activated the phoshotyrosine level several folds after 10 min of treatments both under normal and high glucose conditions, but no significant difference was found between normal and high glucose (Fig. 2). For positive insulin signaling IRS1 protein translocate to the cell membrane where it binds to IRβ proteins. Immunoprecipitation of IRS1 with its specific antibody resulted in co-immunoprecipitation of IRβ proteins, showing the binding of IRS1 to IRβ protein which is analyzed by immunoblotting using phopsotyrosine antibody for IRβ. Interestingly under high glucose conditions the level of phosphotyrosine of co-immunoprecipitated IRβ significantly decreased compared to normal glucose (Fig 2A and B). Also the level of phosphotyrosine of co-immunoprecipitated IRS1 when immunoprecipitated using anti-IRβ decreased under high glucose compared to normal glucose when cells were activated with insulin. (Fig. 2C and D). Thus, high glucose caused a significant decrease in the tyrosine phosphorylation of co-immunoprecipitated insulin receptor proteins when activated with insulin.

3.3. Influence of glutamate and hydrogen peroxide on insulin activated tyrosine phosphorylation of insulin receptor proteins in the TR-MUL cells

In this study, we analyzed if excess glutamate and H_2O_2 (oxidant) treatments to TR-MUL cells influence the tyrosine phosphorylation of insulin receptor proteins. Results indicate that treatments of cells with glutamate (5 mM) for 24 h could not influence the tyrosine phosphorylation of IRS1or IRβ proteins in the insulin activated cells (Fig. 3A and B). However, treatments with 50 μM H_2O_2 significantly increased the tyrosine phosphorylation of IRS1and also robustly increased the tyrosine phosphorylation of IRβ protein compared to cells treated with insulin alone or with glutamate. Thus, the data suggest a positive insulin signaling effect under oxidizing conditions within TR-MUL cells. However, glutamate did not show any influence on signaling through insulin receptor proteins in these cells. The data further indicate that excess extracellular level of glutamate might not cause oxidative stress within TR-MUL cells.

3.4. Influence of high glucose on serine phosphorylation of IRS1 in TR-MUL cells

Following treatments of cells with 5 and 30 mM glucose for 5 days, followed by treatment with or without insulin (10 nM) for 10 min, protein extract was immunoprecipitated using anti-IRS1 antibody, and probed with three specific antibodies having different serine phosphorylation sites in IRS1 (anti $IRS1^{Ser632}$, $IRS1^{Ser1101}$ and $IRS1^{Ser307}$) (Fig. 4). Results revealed that neither 5 nor 30 mM glucose treatments could influence a change in the phosphorylation of $IRS1^{Ser632}$, however insulin significantly decreased the phosphorylation of $IRS1^{Ser632}$ compared to control. To our surprise, high glucose augmented insulin effect in decreasing the phosphorylation of $IRS1^{Ser632}$ compared to treatments either with normal glucose in presence of insulin or high glucose without insulin. Furthermore, phosphorylation of $IRS1^{Ser1101}$ significantly decreased when cells were treated either with high glucose and/or insulin. Again, high glucose seems to augment the effect of

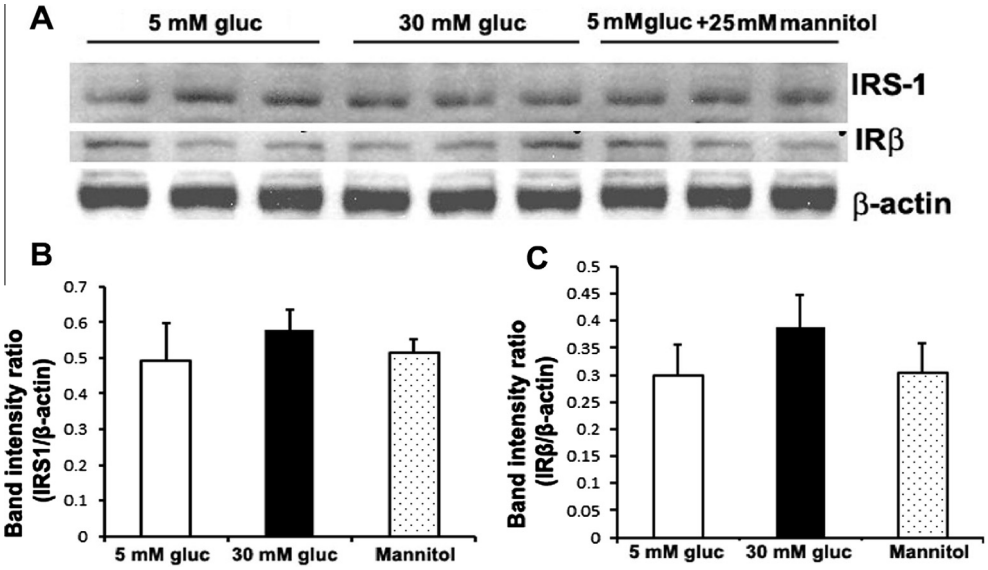

Fig. 1. Effect of hyperglycemia on insulin receptor protein in TR-MUL cells. Cells were grown in normal glucose (5 mM) and treated with high glucose (30 mM) and mannitol (25 mM) for 5 days. Proteins were extracted from both untreated (control) and treated cells. Equal amount of proteins were processed for Western blot analysis. Membranes were probed for IRS1, IRβ and β-actin using specific antibodies against IRS1 and IRβ and β-actin (Panel A). β-Actin was used as a loading control. Resulting bands were quantified by densitometric analyses and represented as band intensity ratio ± SEM (Panel B and C).

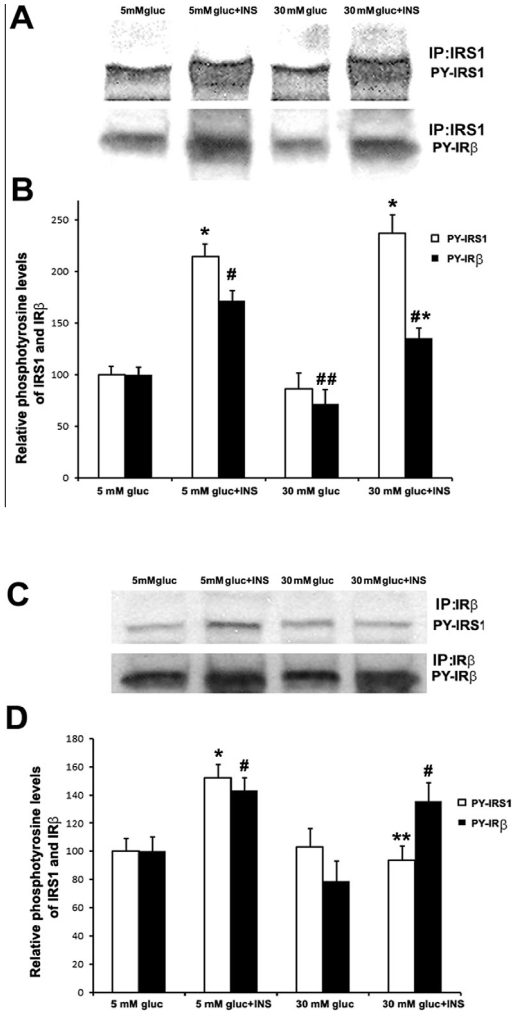

Fig. 2. Effect of hyperglycemia and insulin on tyrosine phosphorylation of insulin receptor proteins in TR-MUL cells. Following incubations with normal (5 mM) and high (30 mM) glucose in presence or absence of insulin (10 nM) for 10 min, equal amount of cell lysates were immunoprecipitated using anti-IRS1 and anti-IRβ antibodies, and subjected to immunoblotting for immunoprecipitated phosphotyrosine (PY-IRS1) of IRS1 and (PY-IRβ) of IRβ. Resulting bands were quantified as relative densitometric units in terms of PY-IRS1 and PY-IRβ with means in percent ± SEM compared to 5 mM glucose set at 100. Values are obtained from three separate experiments. Asterisks on bars indicate significant differences from cells cultured with 5 mM glucose ($P < 0.05$).

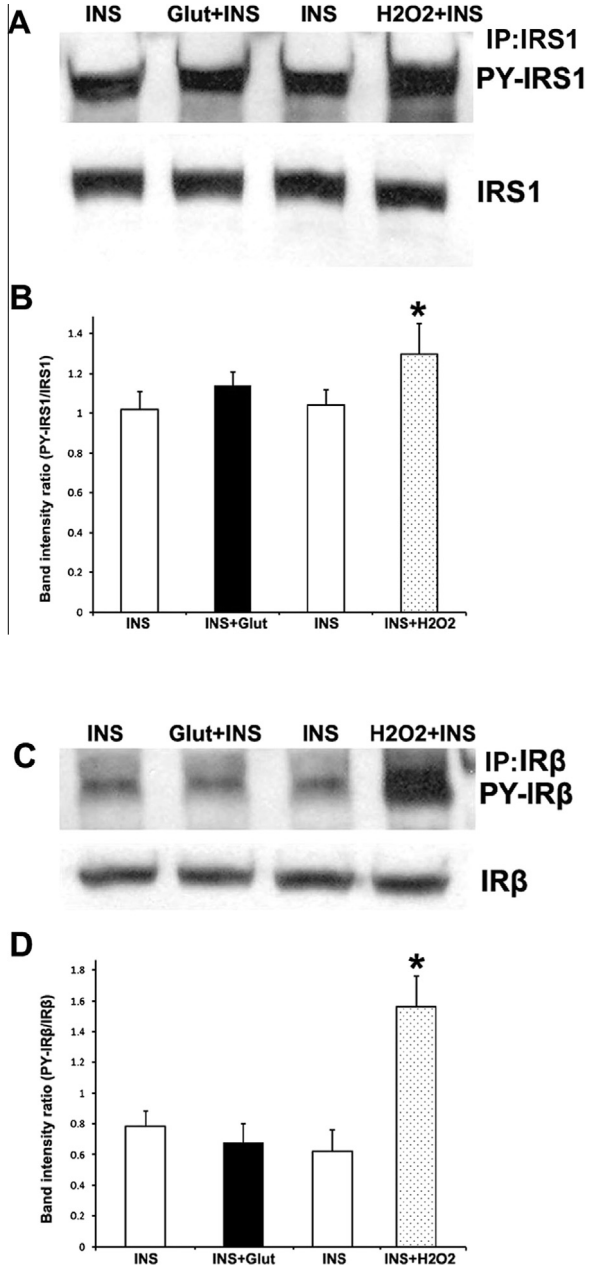

Fig. 3. Hydrogen peroxide induced insulin signaling by activation of tyrosine phospohorylation of IRS1 and IRβ. Cells were treated with hydrogen peroxide (50 μM, 20 min) and insulin (10 nM, 10 min). The cell lysates (500 μg) were immunoprecipitated and analyzed by immunoblotting. Densitometric analysis was used to determine the band intensity ratio of phosphotyrosine level of IRS1 and IRβ. Values are the means ± SEM ($n = 3$), $^{*}P < 0.05$ (H_2O_2 + insulin vs. insulin).

insulin in decreasing the phosphorylation of IRS1^{Ser1101} compared to high glucose alone or normal glucose with insulin. In addition, the phosphorylation of IRS1^{Ser307} was not affected either with glucose levels or insulin treatment conditions.

4. Discussion

In this study, we utilized the cultured rat Muller cells (TR-MUL) which have been found to be well differentiated, expressing glial specific markers including GLAST and S-100 [16]. Recently, we have demonstrated the expression and activities of glial specific enzymes such as mitochondrial branched chain aminotransferase, pyruvate carboxylase and glutamine synthetase required for glutamate metabolism in Muller glial cells, thus making these cells a unique model of cultured Muller cells [17] compared to other Muller cells available [18,19]. In addition, we observed an abundance of insulin receptor proteins in these Muller cells [15] similar to reported in the intact Muller cells of the retina and in cultured Muller cells (RMC-1) [20]. In diabetic retina, a disrupted insulin receptor signaling observed which suggest that hyperglycemia might modulate insulin receptor signaling. Therefore, in this study, first, we demonstrated the effect of hyperglycemia on the expression levels of insulin receptor proteins in TR-MUL cells. Second, we analyzed the influence of hyperglycemia on tyrosine phosphorylation of insulin receptor proteins. Third, we investigated the effect of excess glutamate and oxidant (H_2O_2) on the tyrosine phosphorylation of IRS1, and finally investigated the serine phosphorylation of IRS1 under hyperglycemic conditions.

Insulin signaling proceeds with phosphorylation of tyrosine residues in the insulin receptor proteins which activate the downstream signaling molecules, and in contrast phosphorylation of

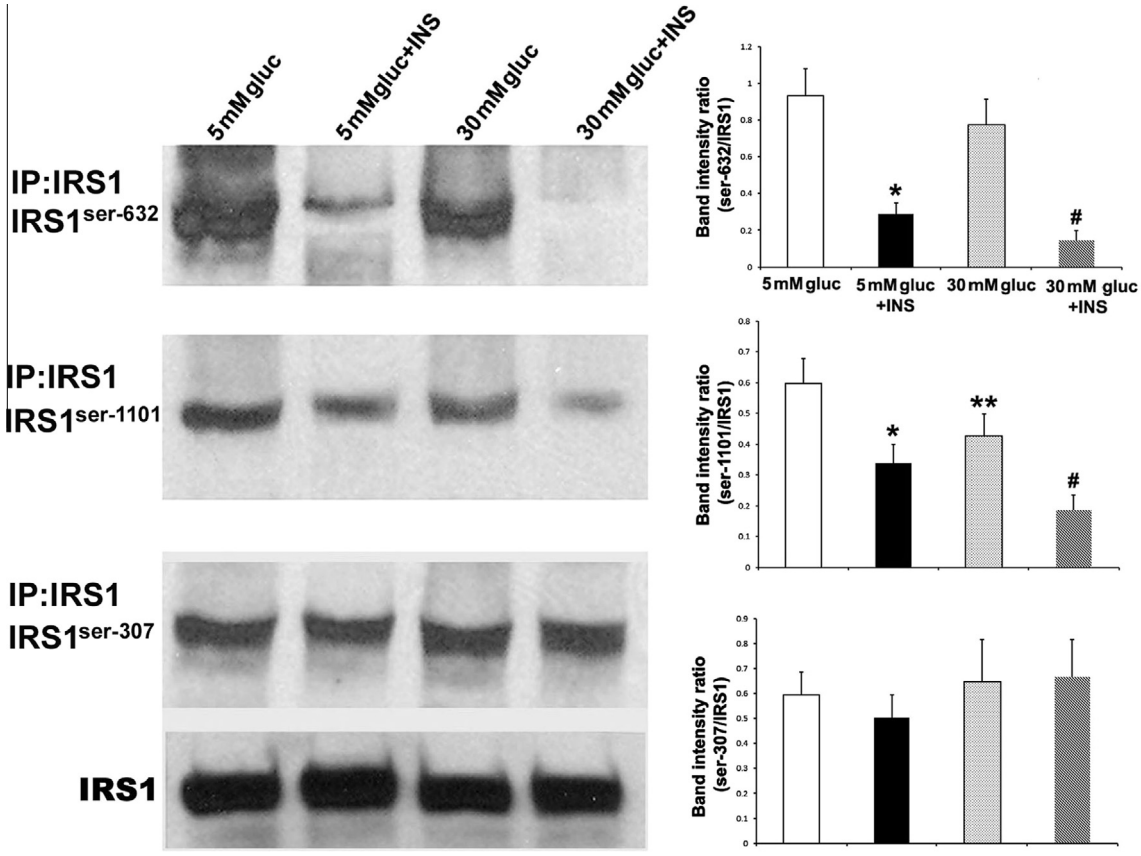

Fig. 4. Immunoblotting for phosphorylated IRS1(ser307, ser632, ser1101) to total IRS1 in TR-MUL cells cultured in normal glucose or high glucose and treated with Insulin (10 nM) Representative blots are shown in the left panel and corresponding bar graphs in the right panel showing the normalized values of phospho-IRS1 with total IRS1. Ratio of normalized value of $IRS1^{ser632}$ to IRS1: $^{*}P < 0.05$ (5 mM glucose + Insulin vs. 5 mM glucose), $^{\#}P < 0.01$ (high glucose + Insulin vs. normal glucose, normal glucose + Insulin and high glucose). Ratio of $IRS1^{ser1101}$ to IRS1: $^{*}P < 0.05$ (5 mM glucose + Insulin vs. 5 mM glucose), $^{**}P < 0.05$ (30 mM glucose vs. 5 mM glucose), $^{\#}P < 0.01$(30 mM glucose + Insulin vs. 5 mM glucose, 5 mM glucose + Insulin, 30 mM glucose). Ratio of $IRS1^{ser307}$ to IRS1: no significant differences were found between different treatments. Data are means ± SEM. Values are obtained from three independent experiments.

various serine/threonine residues of IRS1 inhibit the signaling, thus these phosphorylations of insulin receptor proteins modulate the signaling. Insulin receptor phosphorylation is considered the key upstream regulator in insulin signaling cascades. Our immunoblotting experiments indicate that insulin increased the phosphotyrosine levels of both IRβ and IRS1 to several fold but high glucose alone could not influence their levels compared to normal glucose. However, when IRS1 and IRβ were immunoprecipitated with their respective antibodies, IRS1 also co-immunoprecipitated IRβ and vice versa, suggesting a complex formation between IRS1 and IRβ which is required for positive insulin signaling. However, high glucose caused a decrease in the phosphotyrosine level of co-immunoprecipitated IRβ compared to normal glucose. Similarly, high glucose also decreased the phosphotyrosine level of co-immunoprecipitated IRS1, when cells were activated with insulin. Based on these results, we suggest that high glucose may inhibit the interaction between IRS1 and IRβ, which in turn may negatively regulate insulin signaling in these Muller cells. Earlier, Nakamura et al., [21] reported that exposure of retinal neurons to elevated glucose and glucosamine impaired trophic support provided by insulin [21]. Furthermore, recently Jiang et al., [22] suggested that hyperglycemia/diabetes induced cytokine TNFα suppresses insulin signaling by inducing the suppressor of cytokine signaling 3 which may inhibit complex formation between IRS1 and IRβ.

In diabetes, an increased level of glutamate and oxidative stress were observed in diabetic retina, which may cause neurodegeneration early in diabetic retina. Muller cells is the major cell in the retina involve in clearance of the excitotoxic level of glutamate by transporting and metabolizing using Muller specific transporter proteins and enzymes [17]. Therefore, we tested if excess extracellular glutamate has any influence on signaling through insulin receptor in the Muller cells. We did not find any effect of glutamate at the level of insulin receptor protein or phosphotyrosine levels. However, when cells were treated with H_2O_2 (oxidant) for short duration, tyrosine phosphorylation of insulin receptor proteins significantly increased. Our results are in agreement with a previous study in cultured podocytes showing that H_2O_2 enhanced the insulin signal transduction [23]. Thus, the data indicate a positive insulin signaling under oxidant stress conditions implying a potential role in the prevention of insulin resistance under conditions associated with oxidative stress [24]. Our data also indirectly suggest that excess extracellular level of glutamate or high glucose might not cause oxidative stress within TR-MUL cells since insulin receptor signaling was not affected. This is in accordance with our unpublished data in which we found no increase in free radicals under hyperglycemic conditions in TR-MUL cells.

Hyperglycemia and hyperinsulinemia have been found to stimulate serine phosphorylation of IRS1 and decrease tyrosine phosphorylation that leads to IRS1 degradation causing insulin resistance [25]. A number of studies reported several phosphorylation sites at multiple serine residues of IRS1 in a variety of cultured cells under diabetic conditions [22,26,27]. In addition, dysfunction of insulin receptor signaling has been reported in diabetic retina, but the mechanism of dysfunction at cellular level is not much known [6–8,14]. However, a recent report suggested that hyperglycemia phosphorylates serine residue at $IRS1^{ser307}$ in cultured

Muller cells causing inhibition of insulin signaling [14]. In this study, we analyzed serine phosphorylation at IRS1^{ser307}, in addition, we also investigated the phosphorylation of two other sites (IRS1^{ser632}, IRS1^{ser1101}) known to inhibit insulin receptor signaling in cultured cells and tissues under diabetic conditions [26]. Contrary to the study by Jiang et al., [14], we found that the phosphorylation level of IRS1^{Ser307} was not affected either with high glucose levels and/or with insulin treatments in TR-MUL cells. In addition, high glucose treatments could not influence a change in the level of phosphorylation of IRS1^{Ser632}. To our surprise, high glucose augmented the effect of insulin in decreasing the phosphorylation of IRS1^{Ser632}. Furthermore, the phosphorylation of IRS1^{Ser1101} was significantly high in normal glucose and insulin treatment decreased its level. Again, high glucose seems to augment the effect of insulin in decreasing the phosphorylation of IRS1^{Ser1101}. Therefore, insulin resistance under high glucose conditions in Muller cells does not seem to be due to serine phosphorylation of IRS1 as suggested by Jiang et al. [14]. Reasons for the discrepancy in these results may be due to the genetic difference in the two model of cultured Muller cells or the treatment conditions.

Taken together, these data suggest that hyperglycemia does not affect the expression of insulin receptor proteins in cultured TR-MUL cells. Although, hyperglycemia seems to inhibit the interaction between IRS1 and IRβ which may influence the insulin signaling cascade. Hydrogen peroxide increased the tyrosine phosphorylation of insulin receptor proteins but excess glutamate could not affect the signaling indicating that excess extracellular glutamate may not cause oxidative stress within Muller cells. Hyperglycemia lowered serine phosphorylation of IRS1^{ser632} and IRS1^{ser1101}, however, IRS1^{ser307} was not affected. Further investigation is required to understand the effects of longer treatments (more than 5 days) of hyperglycemia on both upstream and downstream insulin receptor signaling in the TR-MUL cells.

Acknowledgments

The author thanks Professor Kathryn F. LaNoue, Department of Cellular and Molecular Physiology, College of Medicine, Penn-State University, Hershey, PA for providing facilities in carrying out a major part of this study. The author also thanks Dr. Ken-Ichi Hosoya, Japan for providing cultured TR-MUL cells. Funding from Deanship of Scientific Research (RGP-VPP-052) at King Saud University Riyadh, KSA is gratefully acknowledged.

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