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

Mohammad Shamsul Ola *

Department of Biochemistry, College of Science, King Saud University, Riyadh, Saudi Arabia

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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.

* Fax: +966 14775724.
E-mail address: mola@ksu.edu.sa

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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.