Glucose Regulated Stress Response in ARPE – 19 Cells

By

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ABSTRACT

Among patients with diabetes, many are affected with diabetic retinopathy (DR), a disease affecting their vision and causing blindness. DR is defined as a microvascular complication of diabetes, which leads to secondary damage and degeneration of blood vessels inside the retina. Both Type 1 and Type 2 diabetics are at risk for developing DR. It is well known that neovascularization is the cause of diabetic retinopathy and most investigations have examined endothelial damage. We hypothesized that oxidative stress changes the concentration and localization of stress proteins in retinal pigmented epithelial cells. We used a retinal pigment epithelium cell line known as ARPE-19. The expression of several gene products that are altered under stress conditions were assessed under high (30mM) and low (5mM) glucose conditions. Four different proteins (Grp78, Gp96, ATF4, and Orp150) were assessed by western blot and confocal microscopy. The western blot results were consistent with the preliminary results for Grp78 and Gp96. Specifically, Grp78 showed interesting western blot results as it increased significantly in concentration in low glucose conditions compared to high glucose. The Grp78 protein changed location within the cells under different culture conditions; with high glucose Grp78 was found in the nucleus and with low glucose Grp78 was found in the cytosol. These data show that glucose-induced oxidative stress induces stress protein changes in retinal pigmented epithelium. This is of high significance as it can potentially show that oxidative stress plays a role in the pathogenesis of DR. Targeting treatment of DR to retinal pigmented epithelium may have improved efficacy in diabetic patients.

SPECIFIC AIMS

Diabetes is a common disease of our time affecting millions of people worldwide. It is a particularly impactful disease in the United States as it afflicts 30.3 million people, 7.2 million of which are undiagnosed [1]. After years of having the disease, there are multiple complications that can and are likely to occur. One of the most common complications is Diabetic Retinopathy (DR), which is the leading cause of blindness. It is well known that high levels of glucose cause neovascularization that lead to blindness. This occurs when damage to the endothelium results in retinal capillary occlusions and ischemic damage. However, the neurological effects of high glucose in the retina have yet to be made clear.

The long-term goal of our project is to investigate the mechanisms responsible in neurodegeneration leading to DR. Our hypothesis is that high glucose levels, along with oxidative stress, cause alterations of a specific cell line called human retinal pigment epithelial cells (ARPE-19). Retinal pigment epithelial cells normally produce growth factors responsible in the maintenance and support of neurons in the retina. Research suggests that neurological damage plays a role in the development of DR. Our lab has preliminary data using RT-PCR that specifically shows gene overexpression in these cells that occurs within 24 hours of exposure to high glucose and oxidative stress. This overexpression could lead to lack of growth factors for the neurons of the retina. This has significance as it could lead to the discovery of the mechanism behind neuron damage in DR. This will be accomplished through two specific aims.

Specific Aim 1: We will attempt to confirm our preliminary gene expression data doing western blots for four proteins whose genes showed altered expressions in the RT-PCR test.

Specific Aim 2: Using immunocytochemistry, we will investigate if the proteins of interest have changed location within the cell. Altered location of these proteins could alter the biological activity of this growth factor producing cell.

It is well known that neovascularization is the cause of diabetic retinopathy and most investigations have been targeted at looking at endothelial damage. We will explore a possible different mechanism of damage by looking at the retinal pigment epithelium, as it plays a role in neuron health.

BACKGROUND

Diabetic Retinopathy (DR) is defined as a microvascular complication of diabetes which leads to secondary damage and degeneration. Among patients with diabetes, many are affected with DR [1], a disease affecting their vision and possibly causing blindness. In developed countries, this disease is one of the major causes of visual impairments and blindness among the working age population [2]. Research shows that worldwide in 2010, DR was the cause of blindness in 2.6% of the blind people, thus having a 0.5% rise compared to similar studies in 1990 [3]. According to the World Health Organization (WHO), an increase in the prevalence of DR is expected and by year 2030 the number of people with diabetes is predicted to double [4]. Patients with both Type 1 and Type 2 diabetes are likely to be affected by DR. More specifically, Type 1 diabetic patients present some degree of DR in nearly 77% of cases, whereas more than 60% of type 2 diabetes patients present with retinopathy after 20 years with diabetes [5].

DR pathophysiology is complex and is defined by two stages of progression. The first stage is an early non-proliferative DR, which is followed by an advanced proliferative DR [6]. According to the American Academy of Ophthalmology, non-proliferative DR occurs when tiny blood vessels within the retina leak blood or fluid, thus making the retina swell. The swelling of the macula, macular edema, is a leading cause of vision loss in DR. Another complication of non-proliferative DR may be the occlusion of blood vessels in the retina, a situation known as macular ischemia, which is when blood supply to the macula is compromised. When someone suffers from non-proliferative DR, their vision may be blurry. According to the National Eye Institute (NEI), non-proliferative DR is divided into three categories: mild non-proliferative DR, moderate non-proliferative DR, and severe non- proliferative DR.

The later stage of DR is called proliferative and it is described by the American Academy of Ophthalmology as a more advanced stage of the diabetic eye disease. It occurs when the retina starts growing new blood vessels, known as neovascularization. These fragile new vessels often bleed into the vitreous humor, which fills the cavity of the eye. A small amount of blood can cause a person to see dark floaters and can affect the quality of their vision. However, if a large amount of blood is present, this could lead to visual impairment. These new blood vessels can form scar tissue, thus damaging the macula, and can even lead to a detached retina [7].

Several studies during recent years have contested the notion that retinal microvasculature development is not the only etiology of DR, as they demonstrate that diabetes may also affect the neural retina [8]. Between the neural retina and the choroid, there is a simple epithelium known as the retinal pigment epithelium (RPE). It is a monolayer of pigmented cells that form the outer portion of the blood retinal barrier (BRB). The blood vessels are lined together with endothelium which is the inner portion of the BRB [9-10]. Between the RPE cells of the outer BRB and the endothelial cells of the inner BRB, tight junctions are present. This sealing function maintains the integrity of the retina as it controls fluids and solutes that cross the BRB and prevents toxic molecules and plasma components from entering the retina [10,11]. Functions that are essential for the preservation of structural and physiological integrities of neighboring tissues to the retina performed by the RPE, including light absorption, epithelial transport, spatial ion buffering, phagocytosis, secretion of growth factors for the retinal neuron, and immune modulation [9-12].

One of the most important functions of RPE is the secretion of growth factors. Numerous growth factors are secreted by cultured RPE cells [13]. These growth factors include pigment epithelium derived factor (PEDF) [14], fibroblast growth factor (bFGF) [14], placental growth factor 1 (PLGF-1) [15] and nerve growth factor (NGF) [16]. The growth factors derived from RPE

help the retina maintain its normal structure due to their trophic effects. Neurodegeneration is known to produce neurotoxic factors in the retina. A neuroprotective mechanism to combat those factors is PEDF production. Due to its anti-angiogenic and neuroprotective properties, this peptide is crucial in retinal homeostasis. In the eye the main source of PEDF is the RPE. PEDF is decreased in the diabetic retina verses non-diabetic subjects [14]. There are also studies indicating that DR relates to changes in the levels of NGF. Pro NGF is the immature form of NGF and diabetes causes an increase in pro NGF and a decrease in mature NGF. Furthermore, reduced levels of mature NGF were associated with early retinal neurodegeneration in experimental studies [16]. Lastly, high-levels of PLGF-1 were present in people suffering with DR, which causes RPE blood barrier disruption, making the growth factor crucial regarding DR prevention [15].

Oxidative stress is considered one of the most crucial factors in the development of DR. Hyperglycemia in diabetic patients induces abnormal metabolism, which results in the overproduction of free radicals known as reactive oxygen species (ROS), such as hydroxyl radical and superoxide. DR is the result of damage in the tissue around retinal vessels caused by the accumulation of ROS leading to oxidative stress [17]. The production of ROS is dependent on two factors: Mitochondrial oxidative phosphorylation [17,18], and the nicotinamide adenine dinucleotide phosphate (NADPH-) oxidase (NOX) system [17,19]. Mitochondria metabolize almost 95% of the molecular oxygen inside the body to produce ATP. During this process molecular oxygen metabolizes into ROS. In cases of diabetes, excessive superoxide production due to the mitochondrial functions contributes to the development of DR. On the other hand, the NOX system breaks down molecular oxygen and generates ROS, thus being a major source of oxidative stress in the vascular system [17-19].

In the retinal microvasculature, both functional and structural changes happen due to biochemical changes induced by oxidative stress. The range of structural changes is wide, and it varies from basement membrane thickening and microvascular cell loss to capillary closure and acellular capillary formation. Direct and indirect mechanisms of ROS mediate these changes. An early structural alteration of oxidative stress is basement membrane thickening. This membrane thickening contributes to structural rigidity and limits the transport of various growth factors like the ones mentioned before, thus leading to pericyte and endothelial cell loss [20]. Functional changes, such as altered blood flow, loss of intercellular junctions, and increased vessel permeability may also contribute to structural changes. Some of the earliest functional changes observed in the pathogenesis of DR, like the BRB breakdown and alterations in retinal blood flow, are being attributed to ROS.

Oxidative stress may also activate effector molecules. These effector molecules may be able to play an important role in the retina microvasculature by affecting blood flow and its permeability. Bursts of oxidant production are closely associated with alterations in gene expression in a variety of tissues [21]. The number of genes that studies show to be influenced by the overproduction of ROS is constantly increasing. Many independent studies helped us identify several redox-sensitive genes. For example, several chaperones are known to be transcriptionally activated by increased cellular oxidation.

Some of the redox – sensitive genes include the following:

1. HYOU1/Grp170/HSP12A/ORP150 – Heat shock protein 70 family. These are thought to play an important role in protein folding in the ER. Suppression of the protein is associated with accelerated apoptosis [22].

2. ATF4 - This gene encodes a transcription factor that was originally identified as a widely expressed mammalian DNA binding protein. It is also associated with the Unfolded Protein Response (UPR) [23].

3. Grp78/HSPA5 – Translates for protein Grp78 also known as Binding Immunoglobin Protein (BiP), it can be located in various cell compartments but it is mainly found in the lumen of the ER and binds newly synthesized proteins as they are translocated into the ER [24,25].

4. Grp94/HSP90B1 – Gene responsible for translation of protein Gp96. Gp96 plays a critical role in folding proteins [25].

Grp78/HSPA5

Heat Shock protein, 70 KDa protein 5 (HSPA5), or immunoglobulin heavy chain binding protein (Bip), is also known as Glucose regulated protein 78 (Grp78) [24]. Grp78 is a molecular chaperone and, like in several other members of the HSPA family, has a diverse array of functions (Table 1). The protein maintains other proteins in a folded state and prevents protein intermediates from aggregating in the endoplasmic reticulum (ER). The function of Grp78 is facilitated in multiple cellular areas, including the cytosol, nucleus, mitochondria and the cell surface [24,26]. However, translation of GRP78 is affected by ER stress, which can be triggered by an array of biochemical imbalances, including calcium depletion in the ER lumen, hypoxic conditions, glucose deprivation, expression of mutant proteins, or the overexpression of wild – type proteins.

Grp78 plays a particularly important role in the UPR. The misfolding of proteins in the ER and their subsequent accumulation triggers the initiation of UPR in the ER. Grp78 dissociates from the ER transmembrane proteins, Inositol – requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF6), and protein kinase R – like endoplasmic reticulum Kinase (PERK). This dissociation will activate the main UPR pathways. The pathways of the UPR harmonize to both decrease the ER folding load through selective mRNA degradation and translational repression and to increase ER folding capacity by upregulating the ER folding mechanism, ER – associated degradation (ERAD). [25]

During protein translocation, Grp78 is responsible for maintaining the permeability barrier of the ER. Other functions of Grp78 include guiding protein folding and protein assembly and targeting misfolded proteins for degradation. Grp78 is also an ER calcium binding protein. Through repressing the activation of the pro – apoptotic machinery localized to the outer surface

of the ER, Grp78 also acts as a potent suppressor of apoptosis similar to its suppressing activity of

the UPR. In this way, Grp78 maintains ER integrity. [27-30]

TABLE 1: SUMMARY OF GRP78 LOCATIONS AND FUNCTIONS [31]

GRP78 LOCATION	FUNCTION
Endoplasmic Reticulum (ER)	- Translocating nascent polypeptides - Protein folding and assembly - Targeting misfolded proteins to endoplasmic-reticulum-associated protein degradation (ERAD) machinery - Maintaining calcium homeostasis
Mitochondria and the Mitochondria-Associated ER Membrane	- Involved in calcium mediated signaling between the ER and mitochondria that is important for bioenergetics and cell survival. - GRP78 plays a direct role in controlling efflux of calcium ions from the ER.
Cell surface Grp78	Cytoprotective function
Cytoplasm	- Regulate ER stress signaling - Suppress ER induced apoptosis
Nucleus	Suppress DNA damage induced Apoptosis

GP96:

Glucose regulated Protein 96 (Gp96), also designated as Grp94, CaBP4, TRA-1,

Endoplasmin and Erp99, is a 96 KDa glycoprotein, member of the HSP90 family and a resident

of the ER. It is 50% homologous to cytosolic HSP90 [32]. Early in the evolution of eukaryotic

cells, Gp96 arose through a gene duplication as indicated by sequences of HSP90 family members

[33].

The assistance of ER - resident chaperones will guide nascent synthesized polypeptides

into the ER and will achieve correct folding. "ER quality control" is a term which refers to

membrane proteins and secretory proteins leaving the ER and transporting molecules only if they

are correctly folded. They should also be modified and completely assembled in the ER and Golgi apparatus. Several proteins act in concert to guide protein maturation through each step, including folding, assembly, sorting and translocation to prevent formation of improperly folded proteins. [34]

Many of the chaperones in the ER, including Grp78 and Gp96, have been found to associate with intermediately folded proteins. A multi–chaperone complex is formed by the cooperation of all the above–mentioned chaperones. Different chaperones for folding may be required by each protein substrate. Of these chaperones, Gp96 is of interest because of its abundance and unique restriction to bind a subset of polypeptide chains. [35]

Cell surface ligands, receptors and enzymes localized in the secretory pathways fall into the category of Gp96 - interacting proteins. In innate and adaptive immunity, some of these proteins, such as immunoglobulin chains, MHC class II proteins, integrins, and Toll - like receptors (TLR) are critical players. This implies that for general ER quality control, Gp96 not only serves as a molecular chaperone but also might play an important and unique role in immune recognition. All the above – mentioned information suggests that Gp96 is associated with folding intermediate proteins in the ER [34]. Gp96 can be found in various cell compartments such as the cell surface, the Golgi apparatus and the extracellular milieu. However, the main location of Gp96 is the ER [36]. Lastly, recent studies showed that suppression of Gp96 induction reduced cell viability under stress conditions, thus suggesting that Gp96 and other glucose regulated proteins are believed to protect cells against cell death. [36]

ATF4:

Activating Transcription Factor 4 (ATF4) proteins contain 351 amino acids and they are 38.590 Da. The structure of the protein plays a crucial role in the management of the protein function and its overall stability. The organization of the protein motifs modulate protein stability is response to stress, including hypoxic and anoxic insult. There are two major degradation motifs in ATF4.

- The beta Transducin Repeat Containing Protein (betaTrCP) recognition motif is recognized and targeted for proteosomal degradation by betaTrCP, when phosphorylated.

- The Oxygen-Dependent Degradation Domain (ODDD) motif is recognized by prolyl hydroxylase (PH) domain containing enzymes (PHD), specifically PHD3.

In cells that are exposed to various stress factors such as hypoxia, anoxia, lack of nutrients, and also developing cells, the protein levels are increased. However, ATF4 mRNA is transcribed ubiquitously [37]. ATF4 structure can alter its function as transcriptional repressor or transcriptional activator. Cells exposed to stress factors such as anoxic insult, endoplasmic reticulum stress and oxidative stress can be protected by ATF4. The protein plays an essential role in development of the skeleton and the eyes, and has also been associated with hematopoiesis. ATF4 is also involved in proper function of memory [23].

Metabolism and carbohydrate homeostasis are regulated by ATF4. ATF4 regulates the expression of Asparagine Synthetase (ASNS), a nutrition sensing gene. Diabetes can cause lack of nutrients. Through the regulation of ASNS, a link is shown between ATF4 and the diabetes disease. Furthermore, studies show that ATF4 mutant mice have reduced expression of genes whose

products regulate the intracellular concentration of amino acids. Thus, there is a close relationship between ATF4 function and metabolism. [23]

ORP150:

Oxygen regulated protein 150 (ORP150) is also known as glucose regulated protein 170 (Grp170), CBP-140 and HYOU1. ORP150 has been reported to be localized to the ER in numerous cell types. In those cell types ORP150 acts as a stress – inducible chaperone molecule.

ORP150 is made up of various domains. These domains are an ER retention-like signal at the C – terminus and a signal peptide at the N – terminus. The protein consists of 999 amino acids. ORP150 is a glycoprotein and migrates differentially on SDS – PAGE gels based on its level of secondary modification (150 – 170 KDa). ORP150 shares greater than 91% amino acid sequence identity to its Chinese hamster orthologue Grp170 and is conserved in numerous mammalian species and displays a high degree of similarity to the ATPase domain of the HSP70 family of proteins, suggesting the ability to bind ATP.

Defined by the presence or absence of stress signals such as hypoxia or ER stress, there are three predicted translation products arising from the three separate transcription start sites. After conditions such as hypoxia, serum starvation, ischemia, cancer and treatment with tunicamycin or 2 – deoxy glucose, ORP150 has been observed to be upregulated. The functions of ORP150 have not been fully elucidated, but it appears to play a role in apoptosis, insulin secretion, protein transport, and wound healing. Recently, ORP150 was found to be cytoprotective to the renal tubular epithelium after ischemia/reperfusion injury [22].

Diabetes is affecting millions of people worldwide. The uncontrolled levels of glucose concentration among diabetics is inducing abnormal metabolism and therefore oxidative stress [17]. Oxidative stress is intimately associated with ER stress. Oxidative stress is affecting the function of several proteins listed above. The effect on those special chaperone proteins like Grp78 and Gp96 leads to protein aggregation in the ER and ultimately to what is known as "ER stress".
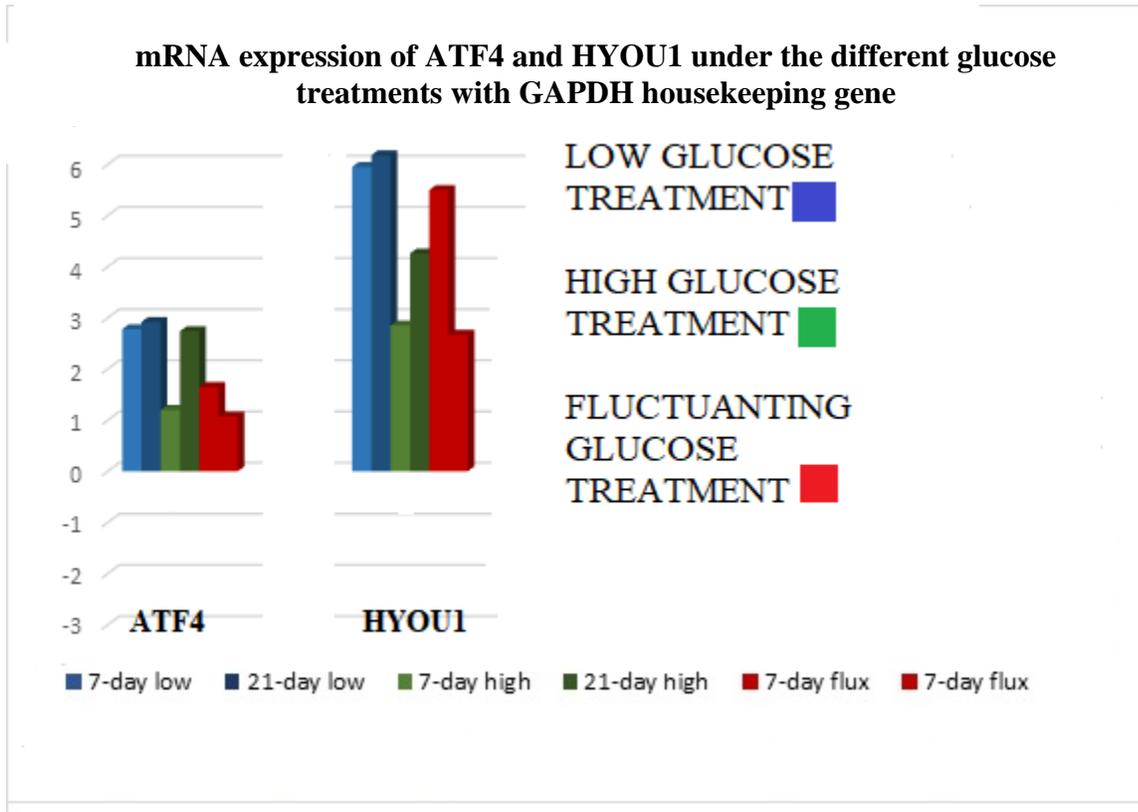
ER stress in the RPE cells causes the misfolding of the growth factors that these cells secrete to maintain healthy BRB and retinal homeostasis. It also contributes to the basement membrane thickening of the RPE which is the outer portion of the BRB. Misfolded growth factor proteins are unable to pass through the increased BRB thickness, thus they cannot enter the retina. This leads to the retina lacking their valuable trophic effects. Nascent and older vessels in the retina start breaking down because of the lack of growth factors. They leak blood and fluid in the retina, which eventually cause macular edema. Macular edema is the major cause of visual impairment in cases of DR individuals.

PRELIMINARY RESULTS

Our lab has preliminary data using RT-PCR that specifically shows gene overexpression in ARPE 19 that occurs under exposure to oxidative stress. This overexpression could affect functioning of ARPE-19 cells, which could lead to lack of growth factors for the neurons of the retina. This has significance as it could lead to the discovery of the mechanism behind neuron damage in DR and it was indicative of ER stress responses.

Three control genes were used to determine the ddCT values. The control genes used in our preliminary data were GAPDH, β-actin and HPRT. The reasoning behind the use of three control genes, to suggest the result of the RT – PCR experiment is more convincing. However, all these data are based on mRNA and the actual protein expression data is needed to confirm the RT – PCR results.

FIGURE 1

mRNA expression of ATF4 and HYOU1 under the different glucose treatments with GAPDH housekeeping gene

LOW GLUCOSE TREATMENT

HIGH GLUCOSE TREATMENT

FLUCTUANTING GLUCOSE TREATMENT

ATF4 HYOU1

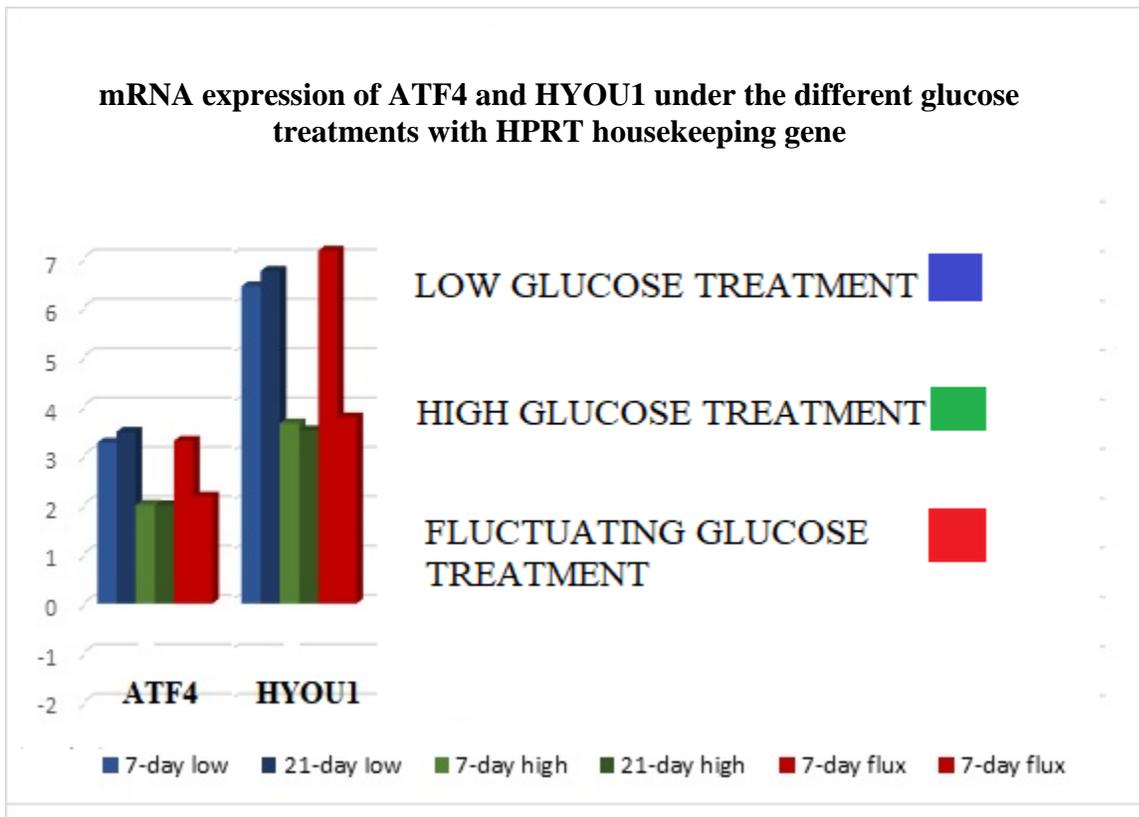■ 7-day low   ■ 21-day low   ■ 7-day high   ■ 21-day high   ■ 7-day flux   ■ 7-day flux
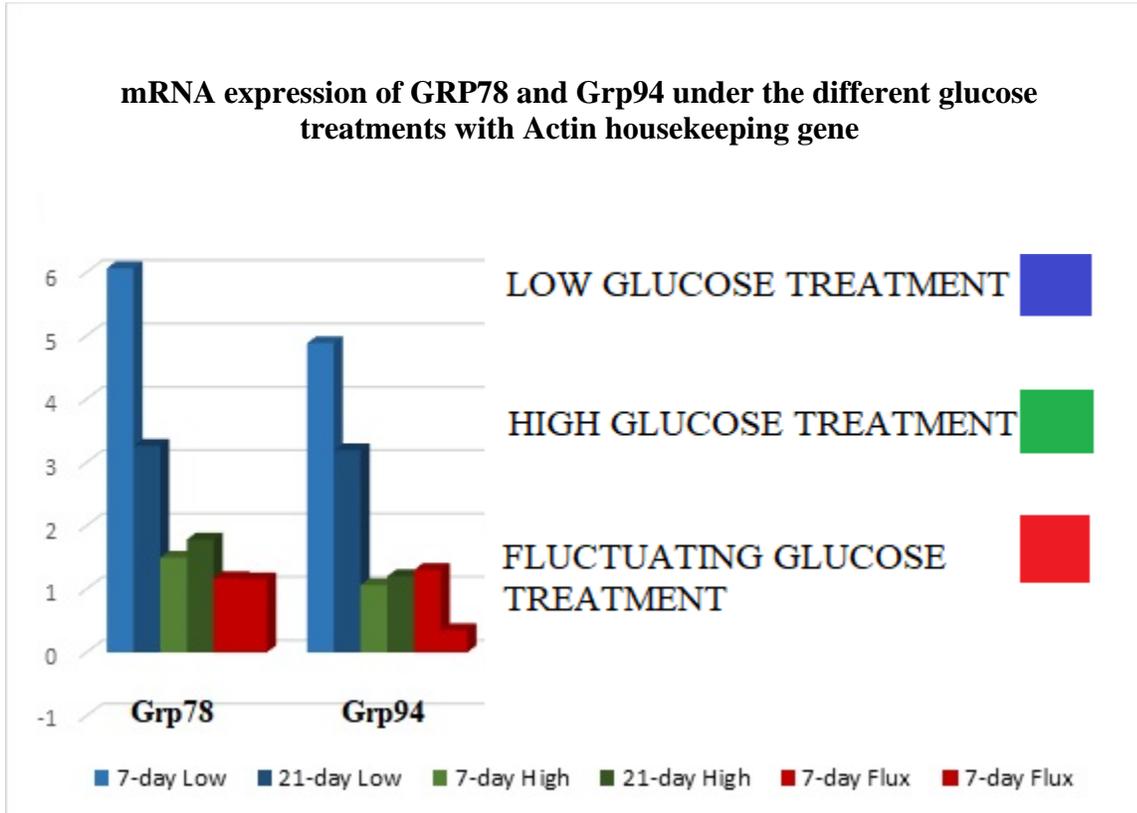
In the x - axis, we show the different treatments and genes. HYOU1, alias ORP150. In the y – axis we show the expression of the genes in ddCT values. The control gene in this graph is GAPDH. The blue bars indicate cells cultured under low glucose concentration. The green bars indicate cells cultured under high glucose concentration. The red bars indicate cells cultured under fluctuating glucose concentration.

In Figure 1, the expression of the ATF4 gene was increased in the low glucose concentration treatments. Within the high glucose concentration treatments, the ATF4 gene was increased at 21 days compared to high glucose at 7 days. The fluctuating treatments were not statistically different with each other. The data were based on mRNA and the actual protein expression data is needed to confirm the RT – PCR data. The expression of the HYOU1 gene was

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increased in the low glucose concentration treatments. In the high glucose concentration treatments, the HYOU1 gene expression was low with a tendency to rise from day 7 to day 21. Lastly, in the fluctuating treatments the expression of the gene was increasing at first only to drop as the glucose concentration remained low.

FIGURE 2

In the x - axis, we show the different treatments and genes. HYOU1, alias ORP150. In the y – axis we show the expression of the genes in ddCT values. The control gene in this graph is HPRT. The blue bars indicate cells cultured under low glucose concentration. The green bars indicate cells cultured under high glucose concentration. The red bars indicate cells cultured under fluctuating glucose concentration.

In the Figure 2, the expression of the ATF4 gene was increased in the low glucose concentration treatments. In the high glucose concentration treatments, the ATF4 gene was not significantly different in expression. In fluctuating treatment ATF4 expression was increased at

first and decreased later. The expression of the HYOU1 gene was increased in the low glucose concentration treatments. In the high glucose concentration treatments, the expression was reduced at day 21 compared to day 7. Lastly, in the fluctuating treatments the expression of the gene was increased at first only to drop as the glucose concentration remained low.

In summary, regardless of the housekeeping gene used ATF4 and HYOU1 (ORP150) mRNA was increased in low glucose treatments compared to high glucose treatments. Even though differences in ATF4 and HYOU1 mRNA were observed in the high glucose from day 7 to day 21 using the GAPDH housekeeping gene, our subsequent studies focus on the day 7 timepoint.
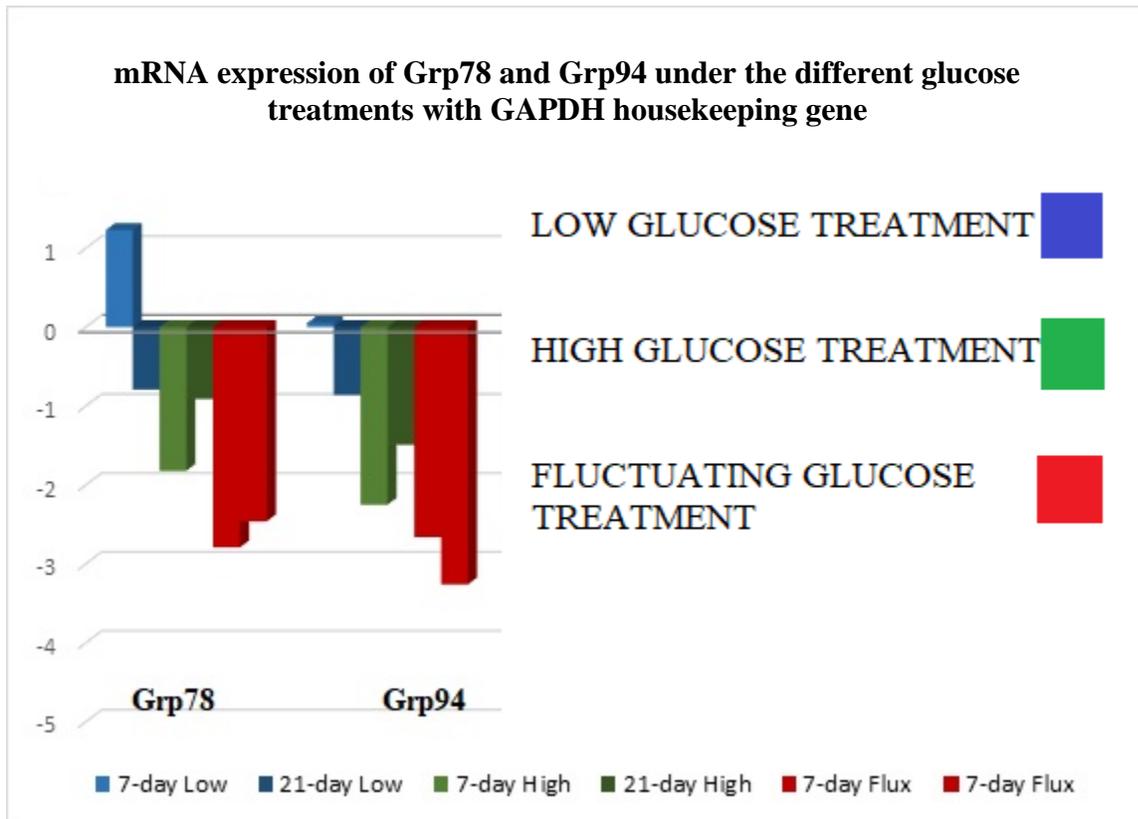
FIGURE 3

In the x - axis, we show the different treatments and genes. In the y – axis we show the expression of the genes in ddCT values. The control gene in this graph is Actin. The blue bars indicate cells cultured under low glucose concentration. The green bars indicate cells cultured under high glucose concentration. The red bars indicate cells cultured under fluctuating glucose concentration.

In Figure 3, the expression of the Grp78 gene was increased in the low glucose treatments. Within the high glucose treatments, expression of Grp78 gene was not significantly different from day 7 to day 21. In fluctuating treatment Grp78 gene expression did not show any statistical difference. We can also see that the expression of the Grp94 gene was increased in the low glucose treatments. In the high glucose treatments, we can see that the expression of the Grp94 gene was lower. Lastly, in the fluctuating treatments the expression of the gene was increasing at first only to drop as the glucose concentration remained low.

FIGURE 4

mRNA expression of Grp78 and Grp94 under the different glucose treatments with GAPDH housekeeping gene

In the x - axis, we show the different treatments and genes. In the y – axis we show the expression of the genes in ddCT values. The control gene in this graph is GAPDH. The blue bars indicate cells cultured under low glucose concentration. The green bars indicate cells cultured under high glucose concentration. The red bars indicate cells cultured under fluctuating glucose concentration.

In Figure 4, the expression of the Grp78 gene was increased in the 7 – day low glucose concentration treatment and then decreased in the 21 – day low glucose. In the high glucose treatments, Grp78 gene was decreased in expression in the two high treatments but the expression of the gene in the 7 – day treatment is lower than that in the 21 - day treatment. In fluctuating treatment Grp78 gene expression does not show any statistical difference but they are decreased in comparison to the other treatments. The expression of the Grp94 gene was increased in the 7-day low glucose concentration treatment and then decreased in the 21 – day low treatment. In the high glucose concentration treatments, Grp94 expression was similar to the Grp78 expression.

Lastly, in the fluctuating treatments the expression of the gene was decreased but was not significantly different between the two treatments.

In summary, Grp78 and Grp94 mRNA was increased in 7 day low glucose treatments compared to 7 day high glucose treatments regardless of which housekeeping gene was used.

MATERIALS AND METHODS

Cell Culture

The human retinal pigment epithelial cell line (ARPE-19) was obtained from the American Type Culture Collection (ATCC, USA) and cultured following the instructions from the ATCC. The cells were maintained in Dulbecco's Modified Eagles Medium (DMEM)/F-12 containing 10% fetal bovine serum and the medium was renewed every other day. Cultures were maintained at 37 °C in humidified atmosphere of 5% CO_2. The cells were passaged by trypsinization every 5-7 days.

Hyperglycemia / Hypoglycemia studies

The ARPE-19 cells were cultured in a CO_2 incubator and manipulated in a certified laboratory cabinet. They were cultured either with a low glucose concertation of 5mM (Sigma Life Science, St. Louis, MO) or with hyperglycemic glucose concentration of 30mM (Life Technologies Corporation, Grand Island, NY) for 8 days to mimic the long course and uncontrolled blood glucose in diabetic patients. Cells were also cultured in fluctuating glucose concentrations; 7 days in high glucose medium, followed by a one-day culture in low glucose medium and vice versa (Table 2).

Table 2: Different Cell Treatments

	TREATMENT (7 DAYS)	TREATMENT (1 DAY)
ARPE 19 CELLS (H/H)	High Glucose treatment	High Glucose treatment
ARPE 19 CELLS (H/L)	High Glucose treatment	Low Glucose treatment
ARPE 19 CELLS (L/H)	Low Glucose treatment	High Glucose treatment
ARPE 19 CELLS (L/L)	Low Glucose treatment	Low Glucose treatment

Low glucose was 5mM and high glucose was 30mM

Cell lysis to extract protein

We washed the ARPE-19 cells in the tissue culture dish by adding cold phosphate buffered saline (PBS) and rocking gently. This PBS was then discarded. More PBS was added and a cell scraper was used to dislodge the cells. The mixture was transferred to microcentrifuge tubes containing 180μL of ice-cold cell RIPA buffer (Cold Spring Harbor protocol) with 20μL fresh Fisher Protease Inhibitor cocktail P187786 (Thermo Fisher Scientific, Waltham, MA).

Protein Assay

The lysis of the cells was followed by a protein assay experiment using Pierce BCA protein assay kit (Thermo Fisher Scientific). A Bovine Serum Albumin Standard (BSA) ampule was used to prepare a set of protein standards diluted from 2000ug/ul to 40ug/ul. Each 96 well plate had a volume of diluted BSA of 25μl. The diluent we used was distilled water.

For the preparation of the BCA Working Reagent (WR), we used the following formula to determine the total volume of WR required:

(# standards + # unknowns) × (# replicates) × (volume of WR per sample) = total volume WR.

We had four unknown samples in our experiment and 8 standards. We used 200μl for each sample and run samples in triplicates. So, the amount of WR we used was:

(8 standards + 3 unknowns) x (3 replicates) x 200μl = 6.6 ml of WR

To prepare the WR, we mixed 50 parts of BCA reagent A with 1 part of BCA reagent B to make a total volume of 6.6 ml. We pipetted 25μl of each standard or unknown sample replicate into the microplate well. Then, we added 200μl of the WR to each well. A 30-minute incubation at 37C followed. Lastly, we used a plate reader to measure the absorbance at 562nm.

Primary Antibodies

The following antibodies were used for both Western Blot and Immunocytochemistry experiments: HYOU1/ORP150 Polyclonal Goat Antibody from R&D systems (Minneapolis,MN), ATF4 Polyclonal Mouse Antibody from R&D systems, Gp96/HSP90B1 Monoclonal Mouse from R&D systems, Grp78/HSPA5 Monoclonal mouse Antibody from R&D systems, B – tubulin Monoclonal Mouse from R&D systems, and GAPDH Polyclonal Rabbit from Jackson ImmunoResearch Laboratories Inc. (Eagan,MN). Antibody concentrations were determined empirically.

Secondary Antibodies

The following secondary antibodies used in these experiments:

<u>Western Blot</u>: Goat anti-Mouse IgG (H+L) Peroxidase – conjugated Affinipure from Jackson ImmunoResearch Laboratories Inc., Donkey anti – Goat IgG (H+L) Peroxidase – conjugated Affinipure from Jackson ImmunoResearch Laboratories Inc.

Immunocytochemistry: Cy 3 – conjugated AffiniPure Donkey anti – Mouse IgG (H+L) from Jackson ImmunoResearch Laboratories Inc., Cy 3 – conjugated AffiniPure Donkey anti – Goat IgG (H+L) Jackson ImmunoResearch Laboratories Inc., and Cy 2 – conjugated AffiniPure Donkey anti – Rabbit IgG (H+L) Jackson ImmunoResearch Laboratories Inc.

Western Blot

After the different treatments in triplicate (n=3), the cells were lysed. The lysed cells were heated to 60 degrees Celsius to allow the denaturation of the proteins. After that, 15ul of 2 – mercaptoethanol was added in 45ul of lysed cells. The 2 – mercraptoethanol gave a blue color to our cell aliquots that helped during the gel loading procedure. We decided to load the gels with equal amounts of protein (25μg). Proteins were separated by 10% SDS-PAGE (Thermo Fisher Scientific) at 110-120 V for about 2 hours and then transferred from the gel to nitrocellulose membranes (GE Healthcare Life Sciences, Pittsburgh, PA). The transfer was occurring at 220 – 240 mA for 5-6 hours. Membranes were blocked for an hour in 3% BSA dissolved in Tris Buffer Saline with Tween (TBST). Primary antibody (1:1000 dilution) was added in 3% BSA and incubated for 1 hour at room temperature on a shaker. The purpose of the shaker was to incubate the whole surface of the membrane with antibody. Next, the membrane was washed 3 times with TBST for 5 minutes each. Then we added secondary antibody in 1:10000 dilution (Jackson immunoResearch Laboratories Inc.) in 3% BSA in TBST and incubated for 1 hour. The membrane was washed 3 times with TBST for 5 minutes each. For protein detection we used SuperSignal West Pico Chemiluminescent SubstrateTM (Pierce, Rockford, IL). We prepared the ECL mix. Membrane was incubated for 1-2 minutes. Then the UVP chemidoc – it imaging system (Analytic jena, Upland, CA) was used for the acquisition of images.

Western Blot Data and Graphs

Western Blot analysis was performed using a program available for free from the National Institute of Health (NIH) called image J. Using the rectangle tool, each band (experimental and control) was selected analyzed for number of pixels. The control protein that we used for our western blot experiment was B – tubulin. For each lane in the western blots, there was a corresponding B – tubulin band. The data that we collected were normalized against those B – tubulin bands. The concentration of B – tubulin should not change due to the different treatments. This means that any change in the concentration of B – tubulin between the different treatments was due to loading errors. We measured the differences between the B – tubulin bands for our different treatments. By doing that we were able to find the "loading error" concentration differences for our proteins of interest. We then normalized our protein bands by dividing their intensity to that of the B – tubulin.

Statistical Analysis

For the statistical analysis of our results we used the SPSS statistics software. A one-way Analysis of Variance (ANOVA) statistical test was performed to our results. In order to check if there are statistically important different results for the different treatments, we proceeded with post-hoc tests. The post – hoc tests that were chosen were Tukey and Tukey b. Statistical differences were considered significant at a *P* value less than 0.05.

Immunocytochemistry

ARPE 19 cells were plated on polylysine-coated coverslips. Cells were then fixed with 4% paraformaldehyde in Phosphate Buffer Saline (PBS) for 10 minutes at room temperature and

washed with PBS. For the permeabilization of the cells 0.2% Triton was used for 10 minutes. Blocking was performed using 1% BSA in PBS for 1 hour. Cells were then washed in PBS 0.2% Tween, 4 times for 5 minutes each time. The incubation was performed for 1 hour at Room Temperature (RT) with primary antibody in 1:100 dilution (Grp78/HSPA5 Monoclonal mouse R&D systems, GAPDH polyclonal Rabbit) and 1:100 dilution of Hoechst solution (ThermoFisher Scientific) specific for nuclei staining. We washed the cells in PBS 0.2% Tween 4 times for 5 minutes each again. Then, we incubated the cells with the secondary antibody in 1:100 dilution (Cy3 labeled donkey anti - Mouse, Cy2 labeled Donkey anti-Rabbit) for 1 hour. We mounted the coverslips onto the slides using a Vecta – Shield glycerol based medium (Vector Laboratories, Inc. Burlingame, CA) and nail polish sealant. Images were captured using a DMi 8 Leica Microsystems confocal digital microscope. The microscopy magnification was 63X. The lens used for the microscopy was a Leica 1.3 NA oil objective (ACS APO). During microscopy we used three different lasers. We used the laser on the 405nm channel; specific for the excitation of the Hoechst dye. The dye stains the nucleus of the cells blue. The laser was used in 80.4% power and the emission filter bandwidth was 445 – 465nm. The second laser we used was on the 488nm channel and it was specific for the excitation of the Cy2 secondary fluorescent antibodies specific for GAPDH. GAPDH was visualized with green color. The laser was used in 85% power and the emission filter bandwidth was 500 – 530nm. The last laser emitted at 532nm channel and it excited the Cy3 secondary fluorescent antibody specific for our proteins of interest. The laser was also used in 90% power and the emission filter bandwidth was 550 - 580nm. All the images acquired were averaged using three image frames.

RESULTS

Protein Assay

Figure 5 shows the standard curve for the standard assay method as described in the Material and Methods section. The figure shows that the protein concentration for our samples was in the 1100μg/μl to 1300μg/μl range. This allowed us to verify that our cells were rich in protein, but it could not give us any information about the identity and concentration of our proteins of interest; thus, we proceeded with the Western Blot experiments.

Western Blots

From the four proteins, Grp78 showed the most interesting results (Fig 6). Grp78 showed a significant change in its quantity under all the different treatments. The figure shows that Grp78 levels increased in low glucose conditions and decreased under high glucose conditions.

Figure 7 shows the reaction of the Gp96 protein under the four different treatments. The figure shows that there is a significant difference between the high glucose and the low glucose treatments. Expression of Gp96 was higher in the low glucose treatment compared to high glucose treatment. However, there was no significant difference between the fluctuating H/L and L/H treatments. No significant difference can be observed in the fluctuating treatments between them or in the comparison between them and the H/H or L/L.

The expression of the ATF4 protein can be seen in Figure 8. Even though Figure 8 shows that there was not a significant difference between the different treatments for ATF4, the Western blots show something interesting for the protein itself. The protein showed multiple bands in the blot; this can be due to proteolysis of that specific protein or cross-reactivity of the primary antibody that we used for our experiment. We addressed this problem by choosing to measure the

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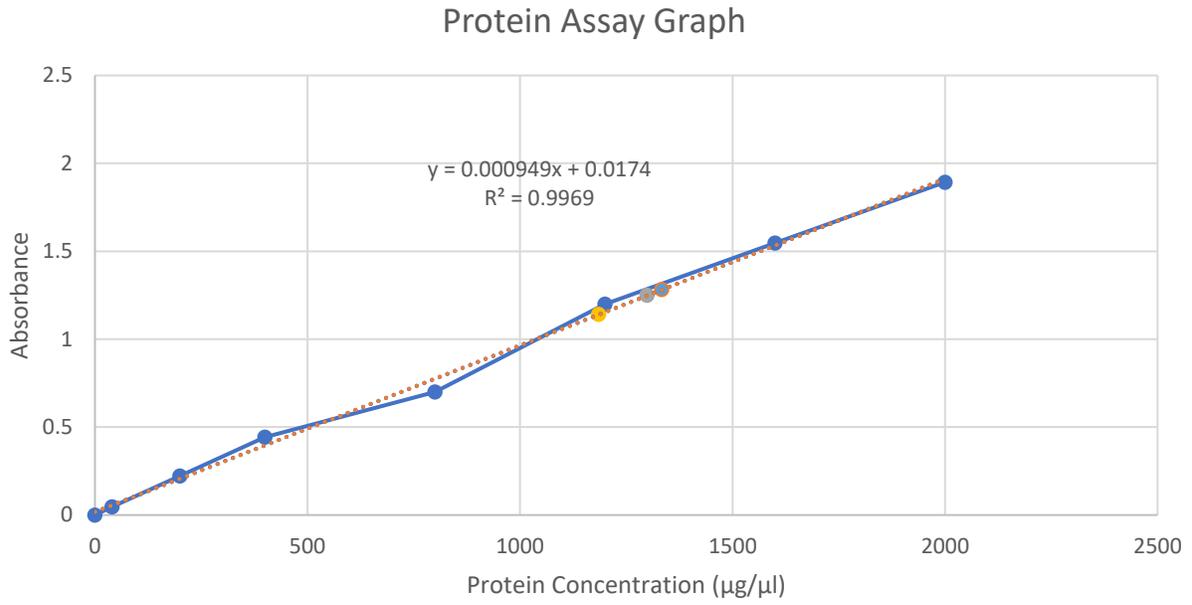band that corresponded the molecular weight of the protein as it was found in the literature [23].

Figure 9 shows that ORP150 levels increased during the fluctuating treatments. This can be supported by the fact that the H/L treatment is significantly higher than the H/H treatment and also the L/H treatment is significantly higher than the L/L treatment. Unlike Gp96 and Grp78, compared to low glucose treatment expression of ORP150 was increased with high glucose treatment.

Immunocytochemistry

For immunocytochemistry, we tested Grp78, Gp96 and ORP150. The Western Blot results of protein ATF4 (Fig. 8) demonstrated concerns with the primary antibody used which did not encourage follow through with ATF4 localization within the cell under different glucose conditions. Figures 10 and 11 show the immunocytochemistry results for proteins Gp96 and ORP150 respectively. Unfortunately, there is no specific protein binding in the figures all binding was considered background.

On the other hand, Grp78 showed interesting results on the immunocytochemistry experiment. Figure 12 shows that under the high glucose conditions for a week the Grp78 was observed in the nuclei of the ARPE19 cells. However, Grp78 relocates from the nucleus to the cytoplasm when exposed to low glucose for 5 days.

FIGURE 5

Protein Assay Graph

$y = 0.000949x + 0.0174$
$R^2 = 0.9969$

Standard Curve. Linearization of the calibration graph (dotted line). Quantification of total protein in our lysed cells (orange dots). The blue dots are the protein standards.
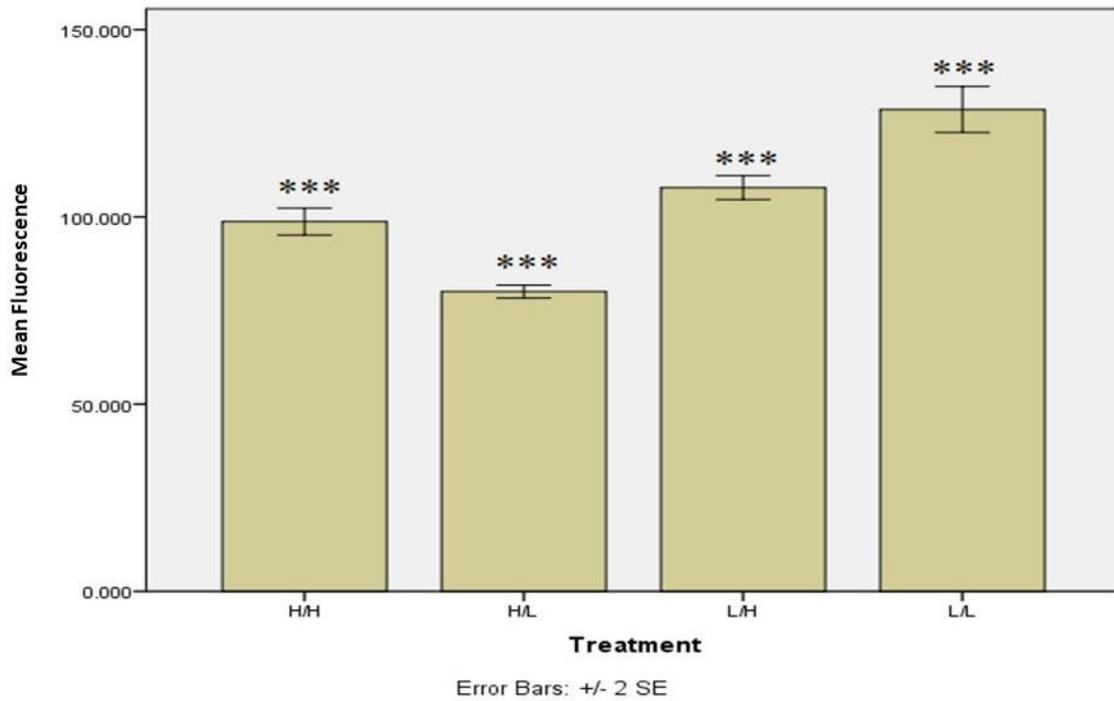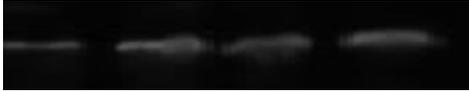
FIGURE 6

A)

B)

C)

A) Western Blot result for the Grp78 protein. B) Western Blot result for the B-tubulin protein. C) Normalized Grp78 expression in different glucose treatments. The number of three asterisks indicates that all the results are significantly different from each other. *** $P<0.05$ as predicted by one-way ANOVA statistical test.
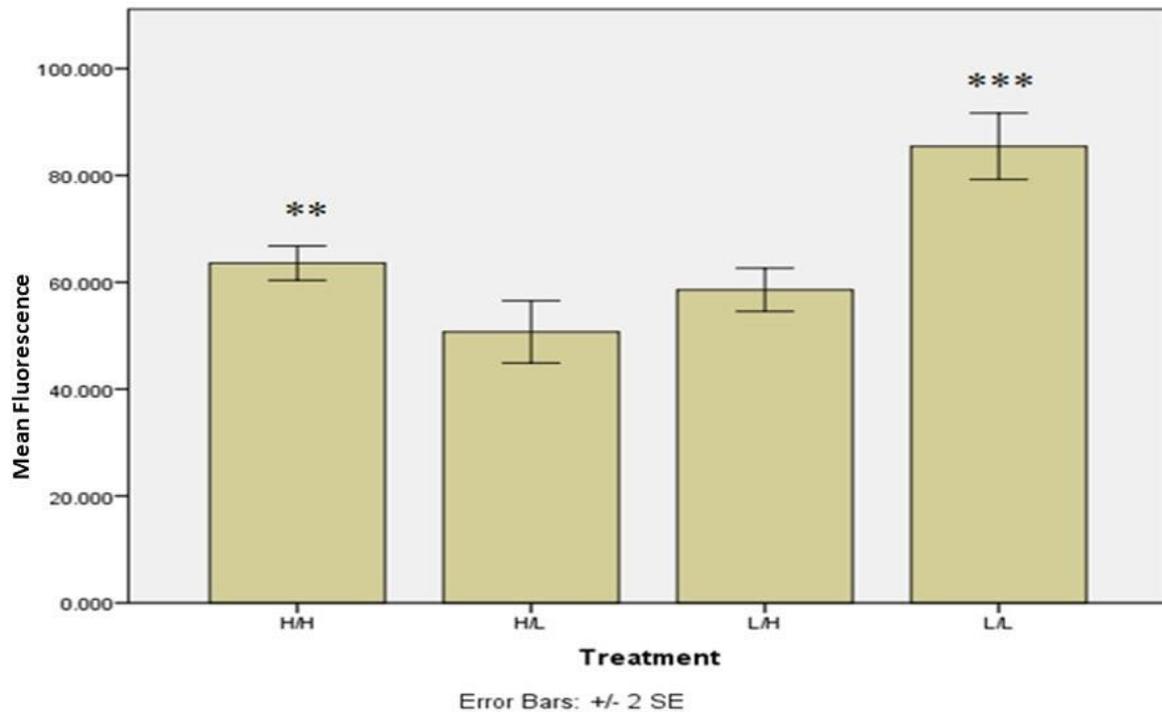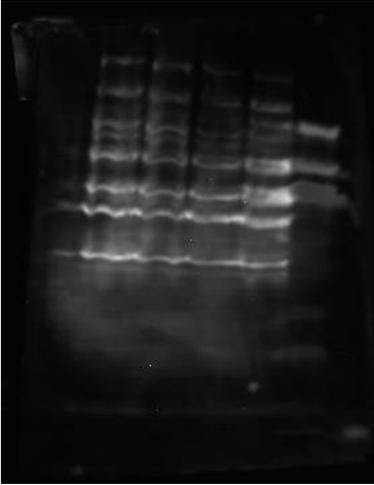
FIGURE 7

A)

B)

C)

A) Western Blot result for the Gp96 protein. B) Western Blot result for the B-tubulin protein. C) Normalized Gp96 expression in different glucose treatments. The number of three asterisks in L/L treatment indicates that the result is significantly different from all three others. The two asterisks on H/H treatment indicates that the result is significantly different from the H/L and L/L treatments. **/*** $P<0.05$ as predicted by one-way ANOVA statistical test.
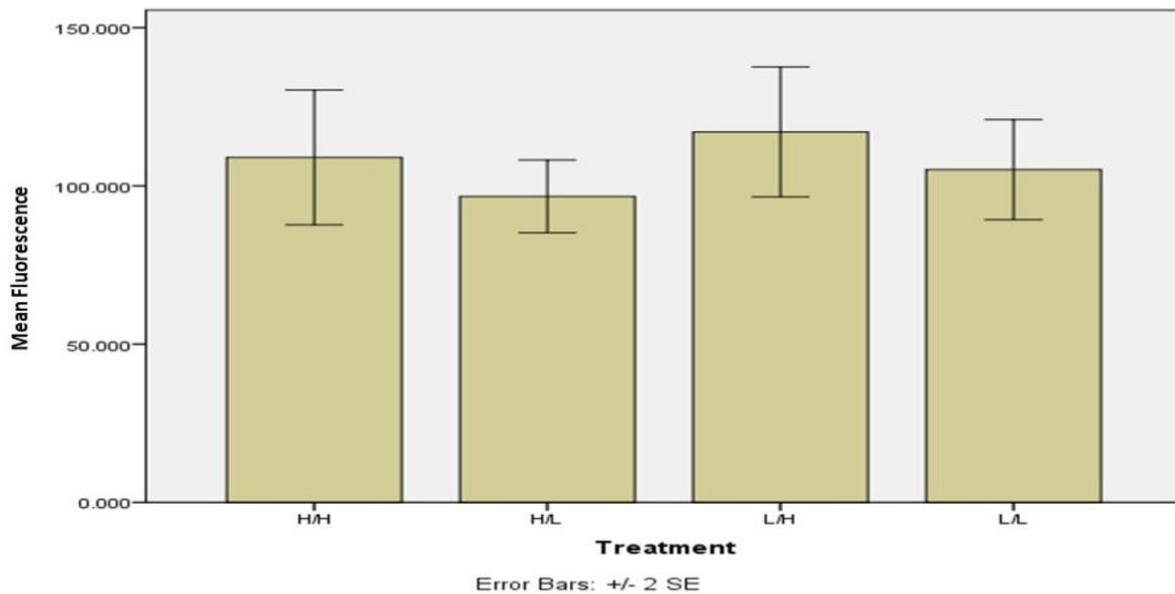
FIGURE 8

A)

B)

C)

A) Western Blot result for the ATF4 protein. B) Western Blot result for the B-tubulin protein. C) Normalized ATF4 expression in different glucose treatments. The absence of asterisks indicates that the results are not significantly different from each other. * $P<0.05$ as predicted by one-way ANOVA statistical test.
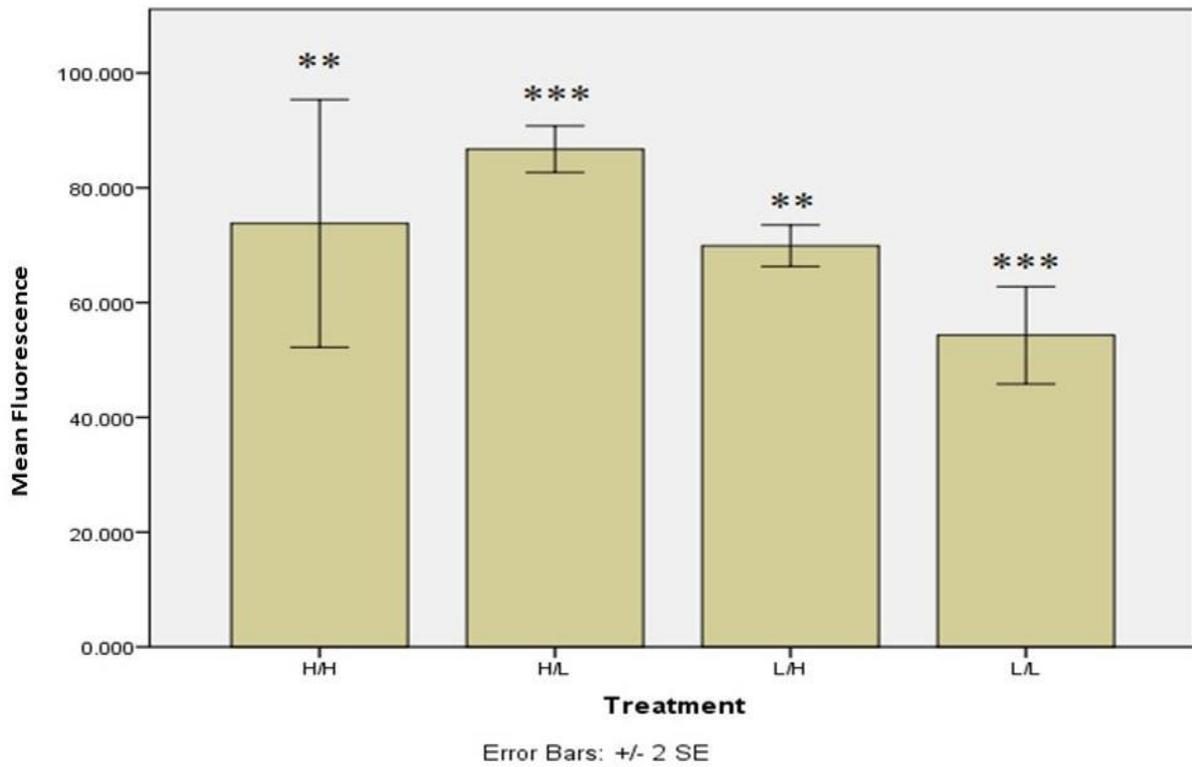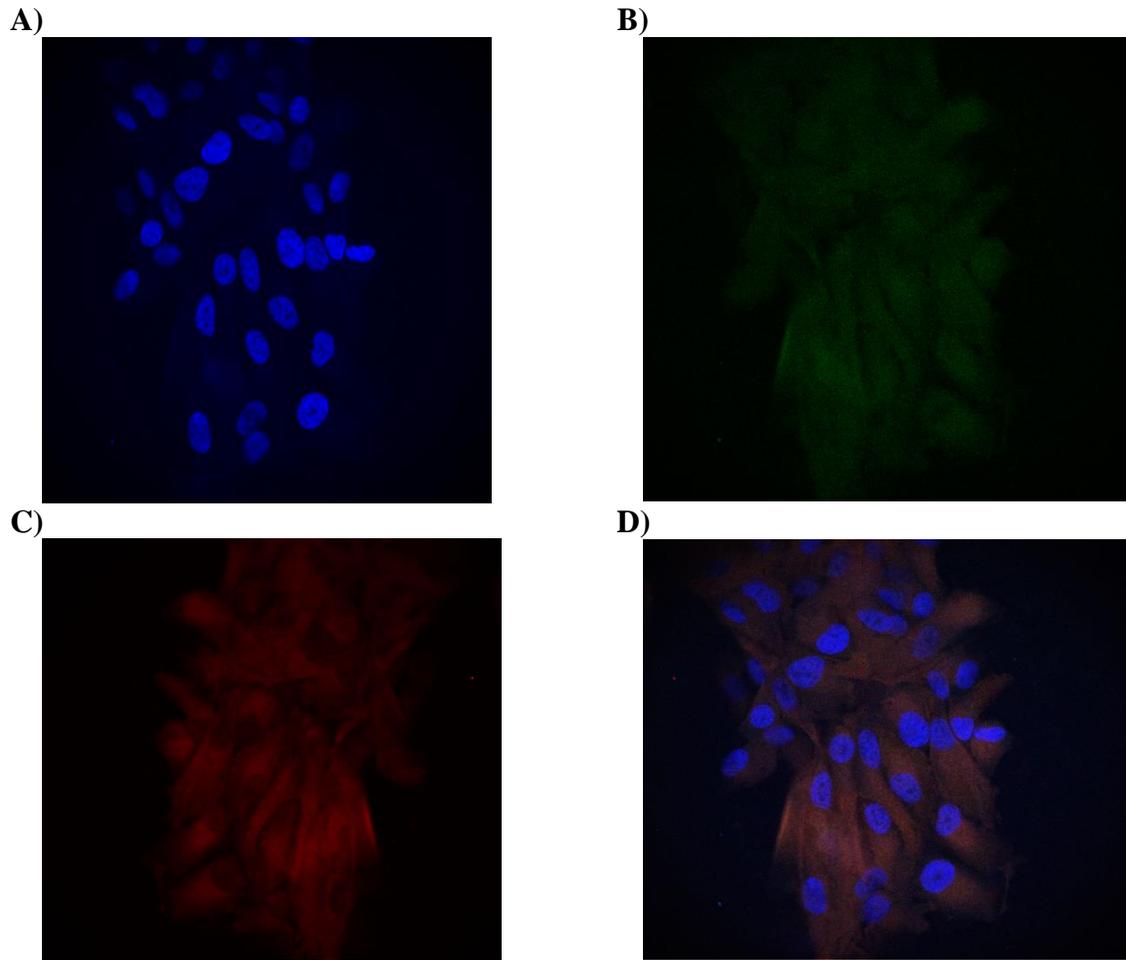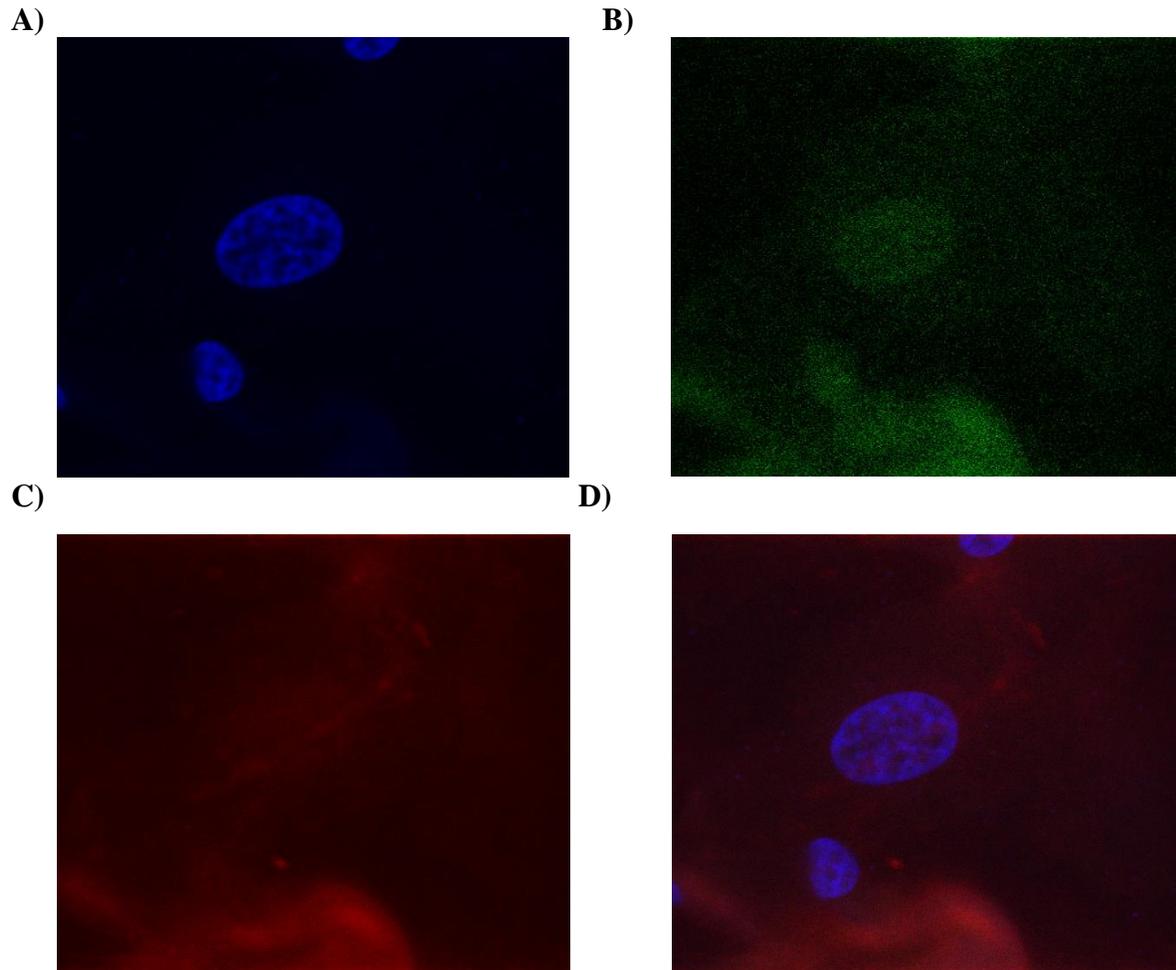
FIGURE 9

A)

B)

C)

A) Western Blot result for the ORP150 protein. B) Western Blot result for the B-tubulin protein. C) Normalized ORP150 expression in different glucose treatments. **/*** $P<0.05$ as predicted by one-way ANOVA statistical test.
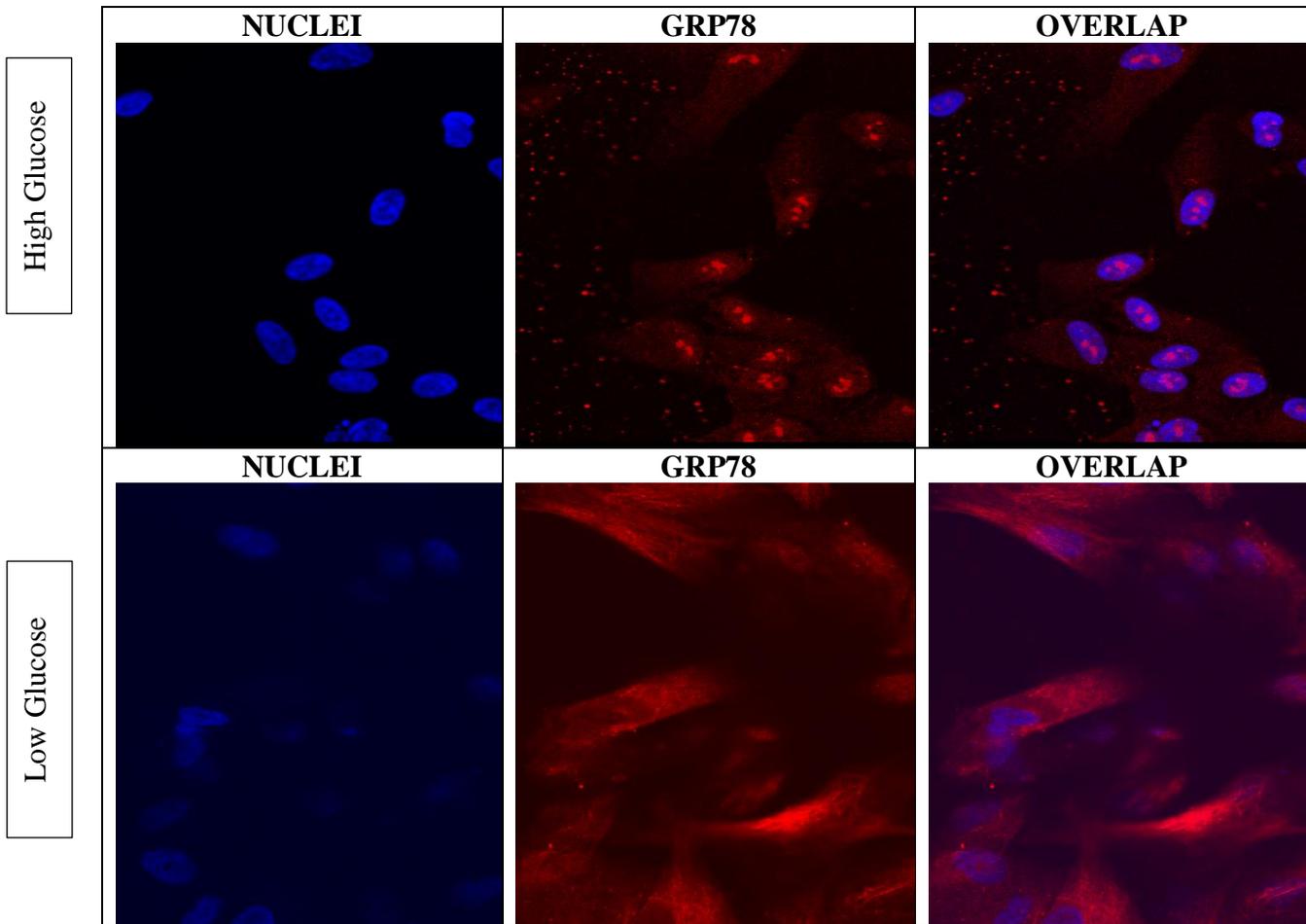
FIGURE 10

Immunocytochemistry images for protein Gp96. A) ARPE 19 Nuclei
B) Cy2 dye specific for GAPDH C) Cy3 dye specific for Gp96. D) Merge figure of A, B and C

FIGURE 11

A)

B)

C)

D)

Immunocytochemistry images for protein Orp150. A) ARPE 19 Nuclei
B) Cy2 dye specific for GAPDH C) Cy3 dye specific for Orp150. D) Merge figure of A, B, C

FIGURE 12

Immunocytochemistry images for protein Grp78. The top pictures show the nuclei and the Grp78 staining under high glucose stress conditions. The bottom pictures show the nuclei and the Grp78 staining under low glucose stress conditions.

DISCUSSION

The preliminary data (Figures 1-4), along with the western blots (Figure 6-9) and the immunocytochemistry results (Figure 12) support the hypothesis of this project. Overall, most of the data collected during this two-year research project suggests that oxidative stress plays a crucial factor in the development of DR. Oxidative stress significantly affects a number of redox sensitive genes and their products. Genetic and environmental risk factors in DR pathogenesis, along with oxidative stress increase ER stress response and activate the UPR in RPE cells [38-42]. Our findings concerning the effect of oxidative stress on Grp78, Gp96, and Orp150 extend the work of previous studies exploring the effect of oxidative stress on other proteins like Nrf2 and XBP-1 [42]. In this earlier experiment, the results demonstrate that XBP1 is required for Nrf2 expression. The data demonstrated in our project shows something similar with the ER/oxidative stress related proteins. The proteins in this pathway could result in altered growth factor production by the RPE.

Oxidative stress was found to affect the expression of Grp78 and Gp96 (Figure 6, Figure 7) which are chaperone proteins involved in folding and trafficking of proteins including RPE secreted growth factors. Previous studies have shown that induced ER stress can upregulate the expression of Endoplasmic reticulum protein 29 [41]. Overexpression of this protein is also increasing the expression of Grp78 and enhances the function of both chaperone proteins, Grp78 and Gp96 [41]. These data, in addition to our data (Figure 6, Figure 7, Figure 12), suggest that all the above-mentioned chaperone proteins attenuate protein aggregation, reduce apoptosis, and mitigate the damage of tight junctions in the RPE cells. These tight junctions are extremely important in the development of DR as they are the ones that are formed in the BRB and basically allow or prevent secreted growth factors to enter the retina.

ATF4 and ORP150 western blot data (Figure 8, Figure 9) were not consistent with the preliminary results (Figure 1, Figure 2). This suggests that there is an underlying suppressing mechanism stopping the translation of the proteins. This mechanism might be the model for PERK activation and the role of Grp78 in that. Under normal stress conditions Grp78 binds to and inactivates PERK. This allows the translation and translocation of other proteins to continue without problems. However, when the cells are induced to ER stress Grp78 unbinds from PERK to perform chaperone functions. This activates PERK, and when activated it phosphorylates eukaryotic translation initiation factor 2 (elF2a). elF2a phosphorylation inhibits the initiation step in protein biosynthesis. Thus, when PERK is activated protein synthesis and translocation into the ER lumen are attenuated. This can explain why even though the mRNA expression of ORP150 under low glucose treatment is elevated, the protein synthesis of ORP150 under the same conditions is decreased. Another surprising observation is that the translation of ATF4 is directly linked to PERK and elF2a. The ATF4 mRNA has several upstream open reading frames (uORFs). uORFs are located at the 5' end of the ATF4 mRNA, and they are out of frame with the main protein – coding region. This means that uORFs mediate basal repression of ATF4 translation. Expression of ATF4 in ER – stressed cells is dependent on PERK mediated phosphorylation of elF2a, because phosphorylated elFa2 allows the ATF4 mRNA to translate more efficiently [43].

Another very important part of our research is the various growth factors that can be secreted from the RPE. During our experiments, testing the effect of oxidative stress on chaperone and transport proteins expression was the focus. This is important as it can give us information on the molecular mechanisms that affect the secretion of the growth factors; however, it is worth exploring the effect that oxidative stress might have on the growth factors themselves. There are several studies that have tried to determine the implications of oxidative stress on growth factors.

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Specifically, there are studies regarding Vascular endothelial growth factor [44,45] and Fibroblast growth factor 1 [46]. These studies were completely independent from each other and even used different types of eukaryotic cells than our study, but showed similar results. Overproduction of growth factors has been shown to mitigate ER stress. In addition, ER stress shows suppression of the production of growth factors. This suggests that ER stress might have a similar effect on the growth factors secreted by the RPE.

The immunocytochemistry results for Grp78 protein also showed some interesting results. Under high glucose conditions the protein localizes within the nuclei of the cells. A change in the treatment from high to low glucose changes the localization from nuclear to cytoplasmic. This suggests that the fluctuation of the concentration of glucose in the cell cultures affect the localization of the Grp78 protein. The localization of the protein is consistent with the existing literature, and research shows that Grp78 can be found in the nuclei of the cells along with the ER localization. When localized in the nucleus, Grp78 has a DNA damage suppressing function [31]. This, along with the findings of Figure 12, suggest that the oxidative stress induced during the high glucose treatments is damaging the ARPE19 cells. The results on our cell model suggest that the accumulation of ROS related to oxidative stress may also cause damage in the retinal vessels and RPE which triggers the relocation and anti – apoptotic function of Grp78. Studies show that ER stress causes a redistribution of GRP78 in the nuclei and the ER of the cells [31,47,48,49]. This can also be supported by Figures 6 and 12 shown earlier.

The mechanism that causes the redistribution of Grp78 in the nucleus of the cells has not yet been fully elucidated. Previous studies transfected COS cells with Grp78 cDNA. They used cDNA with or without the 5' sequence responsible for encoding the hydrophobic leader sequence. The transfection was followed by an analysis of the Grp78 subcellular distribution of the cells.

Cells transfected with cDNA containing the hydrophobic leader sequence exhibited a typical granular expression pattern in the cytosol, thus suggesting specific expression in the ER. Conversely, cells transfected with Grp78 cDNA lacking the hydrophobic leader sequence exhibited a nuclear distribution. The above – mentioned experiment [50], along with our data (Figure 12), suggest that the absence of this hydrophobic sequence in Grp78 is the key to its nuclear redistribution. Perhaps, oxidative stress causes a deletion of that 5' specific sequence thus changing the location of the protein.

Overall, our experiments showed that oxidative stress is a crucial factor in changes to Grp78, Gp96, ATF4, and Orp150 in the gene and the protein level. These changes might affect the functions of the proteins thus reduce the secreted growth factors to pass the BRB and enter the retina. The lack of their growth effects in the retina leads to the breaking of the nascent and older blood vessels. Blood and fluids accumulate in the retina causing macular edema which is the main reason of visual impairment during DR. This is of high significance because by understanding the pathway to the progression of the disease, we can plan future treatments.

Future experimentation:

Firstly, the immunocytochemistry technique experiments should continue. Purchase of new primary antibodies should be achieved in order to test the Gp96 and ORP150 proteins under the different glucose conditions. Further investigation of the Grp78 protein is also recommended. Lastly, a possible future experiment should involve luciferase assay.

To study gene expression, gene reporters are used as indicators. Typically, a reporter gene is cloned with a DNA sequence of interest into an expression vector that uses an unusual form of luciferase that is secreted through the ER, called *Gaussia* Luciferase [51]. The expression vector

is then transferred into cells. Following the transfer, the cells are assayed for the presence of the reporter by directly measuring the activity of the reporter protein. Luciferase is an enzyme that interacts with a luminescent substrate and produces light. By measuring the amount of light produced in the cells under normal conditions and then also under stressed conditions, we can measure the changes in the function of the proteins under those conditions [52]. Glucose stress may reduce protein secretion, so it may reduce luciferase secretion.

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