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Relationship between aldose reductase enzyme and the signaling pathway of protein kinase C in an *in vitro* diabetic retinopathy model

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This experimental study was conducted on cell lines in Ankara University, Faculty of Medicine, Internal Medicine, Pathophysiology Department and Faculty of Pharmacy, Biochemistry Department Laboratories, Ankara, Turkey
Abstract

Protein kinase C (PKC) and aldose reductase (AR) enzyme activities are increased in diabetes and its complications are such as retinopathy, nephropathy and neuropathy. However, the relationship between PKC and AR, and the underlying molecular mechanisms are still unclear. We aimed to evaluate the relationship between these two enzymes and clarify the underlying molecular mechanisms by the related signaling molecules.

The effects of hyperglycemia and oxidative stress on AR and PKC enzymes and the signalling molecules such as nuclear factor-kappa B (NF-kB), inhibitor kappa B-alpha (IkB-α), total c-Jun, phospho c-Jun, stress-activated protein kinases (SAPK)/Jun amino-terminal kinases (JNK) were evaluated in human retinal pigment epithelial cells (ARPE-19). AR, PKC protein levels and related signalling molecules were increased with hyperglycemia and oxidative stress. AR inhibitor sorbinil is decreased PKC expression and activity and all signalling molecules’ protein levels.

Increased AR expression during hyperglycemia and oxidative stress is found to be correlated with the increase in PKC expression and activity in both conditions. Decreased expression and activity of PKC and the protein levels of related signaling molecules with the AR inhibitor sorbinil are showed that AR enzyme may play a key role in the expression of PKC enzyme and oxidative stress during diabetes.

Key Words: Aldose Reductase, Protein Kinase C, Hyperglycemia, Oxidative Stress, Retinopathy.
1. Introduction

Diabetes mellitus (DM) (Vincent et al.) is a metabolic disease which is characterized by high blood glucose levels due to a deficiency in the production of insulin by the pancreas and a resistance to insulin’s effects or both. According to the World Health Organization (WHO), DM is an important health issue affecting over 422 million people worldwide (WHO 2016). Furthermore, DM is strongly associated with a range of complications which are the main causes of mortality and morbidity in people with DM. There are two major types of complications; these are acute and chronic complications including nephropathy, neuropathy and retinopathy (Forbes and Cooper 2013).

Diabetic retinopathy (DR) is an important microangiopathy which affects the pre-capillary arterioles, capillaries and venules of retina. DR is a leading cause of visual disability and blindness among diabetic patients aged between 20-74 (Fong et al. 2003; Frank 2004; Hirai et al. 2011). There are several pathological mechanisms which are involved in diabetic complications such as retinopathy and others as well. These include increased polyol pathway flux, activation of the PKC pathway, oxidative stress and accelerated advanced glycation end product (AGEs) (Beckman et al. 2002) formation (Brownlee 2005; Forbes and Cooper 2013).

Aldose reductase (AR; ALR2; EC 1.1.1.21) is the first enzyme in the polyol pathway and it is responsible for the conversion of glucose to sorbitol in the presence of NADPH. Sorbitol is converted to fructose by the second enzyme of polyol pathway which is sorbitol dehydrogenase (SDH) and NAD$^+$ is a cofactor of the reaction (El-Kabbani et al. 2004; Hers 1956). In the hyperglycemic condition, AR enzyme activity increases due to high glucose levels (Srivastava et al. 2005). It has been reported that abnormally activated AR has a critical role in the pathogenesis of the diabetic complications such as retinopathy (Alexiou et al. 2009; Srivastava et al. 2005). Activated AR leads to cell damage through several mechanisms, including sorbitol accumulation which causes osmotic stress (Frank 2004; Sheetz and King 2002), NADPH depletion which causes oxidative stress (Brownlee 2001; Yabe-Nishimura 1998), increase in NADH/NAD$^+$ ratio which causes pseudohypoxia (Obrosova et al. 2003) and increase in fructose levels which causes a formation of AGEs (Yan et al. 2003). Furthermore, increased NADH/NAD$^+$ ratio leads to a formation of diacylglycerol (DAG) and activates the PKC (Ramana et al. 2005).
PKC which is a group of enzyme member of the serine/threonine protein kinase family can phosphorylate various target proteins (Geraldes and King 2010; Koya and King 1998). The PKC family regulates various biological pathways such as cell growth, differentiation, apoptosis, transformation, and tumorigenicity (Das Evcimen and King 2007). The activation of PKC is one of the major pathway involved in the pathogenesis of DR (Koya and King 1998). In the diabetic retina, high glucose levels are increased the total DAG levels which activated the PKC (Shiba et al. 1993). PKC can also be activated by reactive oxygen species (ROS) (Nishikawa et al. 2000). Activated PKC affects many vascular and cellular processes including vascular permeability, angiogenesis, cell growth, apoptosis, endothelial dysfunction, basement membrane (BM) thickening and regulation of vascular endothelial growth factor (VEGF) (Geraldes and King 2010; Koya and King 1998).

Several investigators have reported that increased AR expression levels in hyperglycemia may be related to oxidative stress (Lorenzi 2007; Obrosova et al. 2010; Srivastava et al. 2005). There are two strong sources of evidence; first one is; AR is effective in reducing the levels of various substances including phospholipids, atherogenic aldehydes, lipid aldehydes and their glutathione (GSH)-conjugates with Km in micro molar range (Ramana et al. 2000; Srivastava et al. 1999; Srivastava et al. 2005; Vander Jagt et al. 2001); second one is the presence of binding sites for redox-regulated transcription factors such as activator protein-1 (AP-1) and NF-kB in the promoter region of the AR gene (Iwata et al. 1999). Hyperglycemia and oxidative stress increase the synthesis of DAG and cause an activation of classic isoforms of PKC -α, -β and -δ (Derubertis and Craven 1994; Koya et al. 1997; Koya and King 1998; Xia et al. 1994). PKC is also involved in the activation of redox-sensitive transcription factors. Furthermore, the reduced products of AR which are GS-aldehydes and glutathionyl-HNE (GS-DHN), are activated NF-kB via PKC pathway (Ramana and Srivastava 2010).

Disturbtion of the blood-retina barrier is resulted in DR development. Most of the researchers focused on vascular structures found on the inner blood-retina barrier. In fact; breakdown of both or one of the inner and outer blood-retina could induce pathologies. Retinal pigment epithelial (RPE) cells are neuroectodermal originated and accepted as a part of the retina. RPE cells are most important part of the outer blood-retina barier, and be found between the choriod plexus and photoreceptors. These cells function as a dynamic barrier and prevents leakage of toxic substances to retina (Xia and Rizzolo 2017, Rizzolo 2014, Simo et al 2010). The present study is indicated the effect of hyperglycemia and oxidative stress on the expressions of PKC,
AR, NF-kB, IκB-α, total c-Jun, phospho c-Jun, SAPK/JNK, cytc in ARPE-19 cells. Furthermore, we also determined the effect of AR inhibitor sorbinil on the expressions of PKC, NF-kB, IκB-α, total c-Jun, phospho c-Jun, SAPK/JNK, cyt c in the hyperglycemic condition. In the presence of sorbinil PKC and all signalling molecule levels were decreased. The effect of sorbinil on PKC and related signalling molecules is probably related to its action on AR enzyme in ARPE-19 cells.

2. Materials and Methods

2.1. Materials

Dulbecco’s Modified Eagle’s Medium/Nutrient F-12 Ham (DMEM/F-12) media, fetal bovine serum (FBS), penicillin/streptomycin solution, trypsin/ethylenediaminetetraacetic acid (EDTA), L-glutamine, D-glucose, H$_2$O$_2$, 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS), hydrochloric acid (HCl), sorbinil, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterrazolium bromide (MTT), dimethyl sulfoxide (DMSO), sulfuric acid (H$_2$SO$_4$), trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), NADPH, sodium azid, GSH, glutathione reductase (GR), bovine serum albumin (BSA), monoclonal Anti-PKC antibody (P5704) and reagents were obtained from Sigma (St. Louis, MO, USA). AR antibody (P-20, sc-17735) and β-Actin (sc-47778) antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). NF-kB, IκB-α, total c-Jun, phospho c-Jun, SAPK/JNK ELISA kit and 3,3',5,5'-tetramethylbenzidine (TMB) were obtained from Cell Signaling Technology (Danvers, MA, USA). Cyt c ELISA kit and bicinchoninic acid (BCA) protein assay kit were obtained from Thermo Fisher Scientific (Waltham, MA, USA). PKC activity ELISA kit was purchased from Enzo Life Sciences (Farmingdale, NY, USA).

2.2. Human ARPE-19 cell culture

Human ARPE-19 cells were obtained from ATCC (CRL-2302) (Manassas, VA, USA). Cells were cultured and passaged in high glucose DMEM supplemented with 1 mM sodium pyruvate for 6-10 times before the experiments. Density and shape of ARPE-19 cells were used for determination of experiments, than the medium of ARPE-19 cells were changed to DMEM/F-12 without glucose and with 20% FBS, 100 U/ml penicilin, 100 mg/ml streptomycin and 4 mM
L-glutamine at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. After one week of medium change experiments were done.

### 2.3. Experimental design

ARPE-19 cells were stimulated with the 5.5, 10, 25, 50 and 100 mM D-glucose for 24 h. The stimulation which is done by the 5.5 mM D-glucose is the control group of this study. In order to evaluate the effect of hyperglycemia under oxidative stress, cells were stimulated by 200 µM H₂O₂ and 200 µM H₂O₂ with 50 mM D-glucose as well. The role of AR enzyme in hyperglycemnic condition (50 mM D-glucose) was determined by using the AR inhibitor sorbinil (10 µM) (Reddy et al. 2009).

### 2.4. Cell Viability Assay

The effects of D-glucose and H₂O₂ on the cells were determined by the MTT assay. ARPE-19 cells were cultured in 96-well plates at a number of 10,000 cells/well. The cells were induced by D-glucose (10-25-50-100 mM) and H₂O₂ (5-25-50-100-200-300-400-500 µM) for 24 h. The media was replaced with fresh media and MTT was added in a final volume of 200 µL media. After 2 h of incubation at 37 °C under 5% CO₂, the media was removed and 200 µL DMSO was added to each well to solve formazan crystals. The quantity of formazan was measured by using a plate reading spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 570 nm.

### 2.5. Western Blot Analysis

The expression of AR and PKC were analyzed by the Western blot analysis. After stimulation with D-Glucose and H₂O₂, ARPE-19 cells were washed, harvested, and lysed in cold NP-40 lysis buffer which contained protease inhibitor cocktail (phenylmethanesulfonyl fluoride, 1 mmol/L; aprotinin, leupeptin and pepstatin, 2 mg/mL each). Cell lysates were centrifuged at 10,000g at 4 °C for 5 min. The protein concentration in all samples was measured by BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA) using BSA as a standart. Equal amounts of protein from each sample were subjected to 8% and 12% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then were blotted onto polyvinylidenedifluoride membranes (PVDF). The membranes were blocked with 5% nonfat dry milk in 0,1% Tween 20 in phosphate buffered saline (PBS-T) at room temperature for 2 h, and then the membranes were incubated overnight at 4 °C with the relevant primary antibodies (diluted 1:200-1:1000). After incubation, the membranes were washed with PBS-T and were
incubated with horseradish peroxidase (HRP) conjugated goat anti-mouse or rabbit anti-mouse IgG (diluted 1:5000) for 1 h. A chemiluminescence kit (Thermo Fisher Scientific, Waltham, MA, USA) and Gel Logic 2200 Pro imaging system (BioTechniques, Westborough, MA, USA) were used for the visualization of immunoreactive bands. The membranes were stripped and were reprobed with anti-actin antibody. Immunopositive bands were analyzed with an image analyzer and values from three separate experiments were used for statistical analysis.

2.6. Enzyme-linked Immunosorbent Assay (ELISA)

NF-κB, IκB-α, total c-Jun, phospho c-Jun, SAPK/JNK and cyt c protein levels and PKC activity were quantified by ELISA according to kit procedure. Absorbances were measured at 450 nm in a microplate reader. The assay was performed in triplicate.

We were examined the PKC activity by using the PKC activity kit (Enzo Life Science, Farmingdale, NY, U.S.A) according to the manufacturer’s instructions.

2.7. Determination of Malondialdehyde (MDA) Concentration

Oxidative stress levels were measured by the determination of malondialdehyde (MDA) concentration. The MDA concentration was determined according to the modified method of Wills (Wills 1966). Cell lysates were centrifuged at 5,000g at 4 °C for 5 min. 100 μl of cell lysate supernatant and 300 μl of Tris-HCl were incubated at 37 °C for 1 h. After the incubation, 800 μl TCA-TBA-HCl reagent was added and the mixture was kept in boiling water for 30 min. After cooling, samples were cleared by centrifugation and the absorbance was measured at 532 nm. The MDA content was expressed as nmol MDA per mg of protein (Uchiyama and Mihara 1978).

2.8. Determination of Glutathione Peroxidase (GPx) Activity

Oxidative stress levels were measured by determination of glutathione peroxidase (GPx) activity. GPx activity was quantified by using the following method (Wendel 1980). Briefly, 10 μl GSH (0.02 mol/L), 1 μl GR (100 U/mg protein/ml), 10 μl of NADPH solution (8 mmol/L) and 1 μl sodium azide (1 mol/L) were added to a 96-well plate and were filled with phosphate buffer (0.05mol/L, pH: 7.0) up to the volume of 280 μl. Then, 10 μl of cell lysate was added and the reaction was initiated by the addition of 10 μl H₂O₂ (0.020 mol/L). The rate of glutathione disulfide (GSSG) formation was measured by UV spectroscopy at 340 nm.
2.9. Statistical Analysis

The results are expressed as mean ± SE. ANOVA was applied to test significance. Differences were considered significant at $p<0.05$ and $p<0.001$. Each experiment was performed at least three times.

3. Results

3.1. Effects of D-glucose and $\text{H}_2\text{O}_2$ on ARPE-19 cell viability

The effects of D-glucose and $\text{H}_2\text{O}_2$ on ARPE-19 cell viability were determined by the incubation of the cells with various concentrations of D-glucose (10-25-50-100 mM) and $\text{H}_2\text{O}_2$ (5-25-50-100-200-300-400-500 $\mu\text{M}$) for 24 h and the cell viability was measured by MTT assay. The results of the MTT assay showed that 50 mM D-glucose ($\text{HG}$) and 200 $\mu\text{M}$ $\text{H}_2\text{O}_2$ concentrations were caused significant ($p<0.05$) inhibition on cell viability (Fig. 1A and 1B). 25 mM D-glucose did not have any significant effect on ARPE-19 cell viability.

3.2. Effects of D-glucose, $\text{H}_2\text{O}_2$ and sorbinil on AR and PKC protein expression

The roles of AR and PKC in ARPE-19 cells were examined by the incubation of cells with D-glucose, $\text{H}_2\text{O}_2$ and sorbinil. It is well known that D-glucose induces the up-regulation of AR and PKC protein levels in diabetes (González et al. 1984; Koya and King 1998; Yabe-Nishimura 1998). In the present study, we also found that 25, 50 and 100 mM D-glucose concentrations were caused an increase in expression of AR and PKC protein levels compared with control groups ($p<0.05$) (Fig. 2A and 2C). However, 10mM D-glucose had no significant effect on AR and PKC protein expressions.

The effect of oxidative stress on AR and PKC expression was determined by the incubation of cells with 200 $\mu\text{M}$ $\text{H}_2\text{O}_2$ for 24 h. As illustrated in Fig. 2B and 2D, AR and PKC expressions were increased with the oxidative stress. In 50 mM D-glucose with 200 $\mu\text{M}$ $\text{H}_2\text{O}_2$ treated cells, AR and PKC protein levels were also increased significantly ($p<0.05$) compared with control and 200 $\mu\text{M}$ $\text{H}_2\text{O}_2$ groups. These results showed that AR and PKC were affected from oxidative stress and high glucose levels.
In the presence of 10 µM AR inhibitor sorbinil, AR expression levels were not changed but PKC expression was significantly (p<0.05) increased according to the control group. Increased AR and PKC expression were detected in 50 mM D-glucose with 10 µM sorbinil treated cells compared to the control group (p<0.05) (Fig. 2B and 2D).

### 3.3. Evaluation of NF-κB, IkB-α, total c-Jun, phospo c-Jun, SAPK/JNK and cytochrome c protein levels after incubation of the ARPE-19 cells with D-glucose, H$_2$O$_2$, and sorbinil

25, 50 and 100 mM D-glucose were caused significant (p<0.05) increase in the expression of NF-κB (Fig. 3A). Oxidative stress stages with or without HG was also caused significant (p<0.05) increase in the expression of NF-κB. HG stage with the oxidative stress was showed higher NF-κB expression levels than the HG and oxidative stress stages alone (p<0.05). HG with sorbinil incubated cells were showed a decrease in the expression of NF-κB compared with the HG group (p<0.05).

25, 50 and 100 mM D-glucose were caused a significant (p<0.05) increase in the expression of IkB-α compared with the control group (Fig. 3B). The expression of IkB-α levels in HG with H$_2$O$_2$ incubated cells were higher than the H$_2$O$_2$ and HG incubated cells alone(p<0.05). On the other hand, combined treatment of sorbinil with HG resulted in a significant (p<0.05) decrease in IkB-α expression levels compared with the HG group alone. We also determined elevated expression levels of IkB-α with HG plus sorbinil incubated cells compared with the sorbinil group alone (p<0.05) (Fig. 3B).

As it is shown in Fig. 3C, 25, 50 and 100 mM D-glucose were caused a significant (p<0.05) increase in expression of SAPK/JNK compared with the control group. The expression of SAPK/JNK protein levels were significantly (p<0.05) increased in HG with the oxidative stress group compared to the H$_2$O$_2$ and HG groups alone. Sorbinil incubation of cells in the HG stage were also elevated the expression of SAPK/JNK (p<0.05). In addition, combined application of sorbinil with HG was resulted in a significant (p<0.05) decrease in SAPK/JNK expression compared with the HG group.

When ARPE-19 cells were incubated with 25-50-100 mM D-glucose, H$_2$O$_2$ and HG with H$_2$O$_2$, the expression of total c-Jun and phospo c-Jun were increased (p<0.05) compared with the control group (Fig. 3D and 3E). Furthermore, total c-Jun expressions were increased (p<0.05) when the cells were incubated with 10 mM D-glucose. On the other hand, no significant change...
was observed with the sorbinil incubation with or without HG. The combined application of 
H$_2$O$_2$ with HG was resulted in a significant (p<0.05) increase in total c-Jun and phospo c-Jun 
expression levels compared with the HG and H$_2$O$_2$ groups. HG with sorbinil incubated cells, 
were showed decreased expression levels of total c-Jun and phospo c-Jun compared with the 
HG group (p<0.05).

10 and 25 mM D-glucose treatment have no effect on cyt c levels compared with the control 
group (Fig. 3F) but 50 and 100 mM D-glucose application to cells were induced the apoptosis 
(p<0.05). Oxidative stress with or without HG were also caused significant (p<0.05) increase 
in cyt c levels compared to the control group. Incubation of the cells with sorbinil with HG were 
resulted in a significant (p<0.05) decrease in cyt c levels compared with the HG group.

3.4. Determination of Protein Kinase C Activity

25, 50 and 100 mM D-glucose were caused significant (p<0.05) increase in PKC activity 
compared with the control group (Fig. 4). Oxidative stress with HG group was caused a 
significant (p<0.05) increase in PKC activity compared to the control group, HG and H$_2$O$_2$ 
groups were also increased the PKC activity significantly according to the control group In 
contrast, decreased PKC activity was detected in sorbinil incubated cells compared to the 
control group (p<0.05). The contribution of the polyol pathway was examined by treating the 
cells with sorbinil with and without HG. When ARPE-19 cells were incubated with HG with 
sorbinil, PKC activity was decreased compared to the HG group but PKC activity was increased 
compared to sorbinil treated group (p<0.05)(Fig. 4).

3.5. Evaluation of oxidative stress markers in ARPE-19 cells

To determine whether D-glucose, H$_2$O$_2$ and sorbinil causes an oxidative stress, MDA levels and 
GPx activity were examined. ARPE-19 cells which were incubated with 10 mM D-glucose and 
sorbinil had no effect on MDA levels (Fig. 5A) but when ARPE-19 cells were incubated with 
25-50-100 mM D-glucose, H$_2$O$_2$, HG with H$_2$O$_2$, and HG with sorbinil, the MDA levels were 
significantly (p<0.05)increased compared to the control group. MDA levels were determined 
in HG with H$_2$O$_2$ and HG with sorbinil incubated cells which were significantly (p<0.05) higher 
than the H$_2$O$_2$ and sorbinil incubated cells, respectively. The MDA levels were also higher in 
HG with H$_2$O$_2$ and HG with sorbinil treated cells compared to the HG group (p<0.05).
We observed that 10 mM D-glucose and sorbinil have no effect on GPx activity (Fig. 5B). As shown in Fig. 5B, GPx activity was significantly increased (p<0.05) when the cells were treated with 25-50-100 mM D-glucose, H2O2, HG with H2O2 and HG with sorbinil. In HG with H2O2 treated cells, GPx activity was significantly (p<0.05) higher than the H2O2 and HG groups. In addition, the GPx activity in HG with sorbinil treated cells was significantly (p<0.05) higher than the H2O2 treated group, but lower (p<0.05) than the HG treated group (Fig. 5B).

4. Discussion

Increased polyol pathway flux, activation of the PKC pathway, oxidative stress and AGE formation has been implicated to enhance the development of diabetic complications (Brownlee 2005; Forbes and Cooper 2013). More than 20 years, researchers have been looking for a unified mechanism to identify the causes of the complications. Brownlee (Brownlee 2005) suggested that overproduction of superoxides by the mitochondria is the major cause of the complications. Nevertheless, Tang et al. and Obrosova et al. (Obrosova et al. 2003; Tang et al. 2012) concluded that the polyol pathway is the major mechanism for the development of the diabetic complications. In contrast, Qi, et al. (Qi et al. 2017) showed that enzymes in the glycolytic, sorbitol, methylglyoxal and mitochondrial pathways were elevated in individuals without diabetic nephropaty. Elevated level of the enzymes in these pathways including aldose reductase protects podocytes and glomeruli from hyperglycemic toxicity. Therefore, elevated level of aldose reductase activity may not be pathogenic for diabetic complications in some cases. In the present study, our results will help to solve these arising contradictions by unifying the relationship between AR, PKC and oxidative stress in diabetic ARPE cells.

In the present study, cells were incubated with 5.5 mM (100 mg/dl) glucose which is the control group and cells were also incubated with 10-25-50-100 mM (180-450-900-1800 mg/dl) glucose levels for to determine the effect of glucose on ARPE-19 cells.. According to American Diabetes Association quideline (2019); 70-110 mg/dl glucose is accepted as normal fasting plasma glucose (FPG) concentrations and normal FPG level must be below 7 mM. Blood glucose starts to increase after meal and reaches up to 140 mg/dl in healthy individuals. FPG between 100 to 125 mg/dl or after oral glucose tolerance test (OGTT) between 140 to 199 mg/dl are accepted as prediabetes. Postprandial glucose (PG) level or PG ≥200 mg/dL (11.1mmol/L) during OGTT are used for diagnosis of diabetes. The hyperosmolar hyperglycemic state (HHS) is a syndrome characterized with severe hyperglycemia when plasma glucose level is >600 mg/dL and plasma osmolality is >320 mOsm/kg (Pasque and Umpierrez 2014).
Due to its different physiological functions, RPE cells are used for modelling of visual pathologies. RPE cells have differentiation capacity. In normal conditions, nonproliferating RPE cells form a dense barrier; but in the case of disturbance of the barrier, new RPE cells are started to differentiate from stem cells which are near to barrier. RPE cells have roles in retina and barrier renewal process, they prevent drug delivery and toxicity of unwanted substances as well. Nowadays RPE cells are accepting as novel promising therapeutic target in retinal pathologies (Kuznetsova et al. 2014, Chiba 2014, Pavan and Dalpiaz 2018). Cell culture conditions are important for phenotypes and differentiation of ARPE-19 cells. Previous studies showed that naïve RPE cells are similar to ARPE-19 cells. These cells have capacity of differentiation during culture and this depends on the way how they cultured and passaged in the media. DMEM with 4.5 g glucose is a typical and optimum growth condition of the ARPE cells (Ahmado et al. 2011, Samuel et al. 2017). These cells prefer “high glucose conditions” in their normal life. ARPE-19 cells are cultured and passaged 6-10 times with DMEM plus high glucose before the experiments. Our study represents fasting glucose, postprandial glucose and hyperosmolar levels of glucose. The differentiation and culture condition of cells prior to the experiments is determined the dosages of the glucose for our experiments.

Our results showed that AR expression levels in ARPE-19 cells were increased by HG (Fig. 2A). This is in agreement with the previous studies which were demonstrated that 30% of glucose is metabolized by the polyol pathway under hyperglycemia (González et al. 1984; Yabe-Nishimura 1998). However, under normoglycemia, less than 3% of glucose is metabolized with the same pathway (González et al. 1984).

Oxidative stress has an important role in the pathogenesis of diabetic complications (Kowluru 2003). It is believed that mitochondria is the major source of ROS in diabetes (Pitocco et al. 2013). Previous studies showed that increased AR expression levels in hyperglycemia may be related to oxidative stress (Lorenzi 2007; Obrosova et al. 2010; Srivastava et al. 2005). In our study we detected that AR expression levels in ARPE-19 cells were increased by H$_2$O$_2$ (Fig. 2B). Our results supports the idea of relation between the hyperglycemia and oxidative stress.

We also measured the levels of MDA which is the major lipid peroxidation product (Slatter et al. 2000; Uchida 2000). On the other hand, we also determined the GPx activity which is one of the main endogenous enzymatic defense systems in oxidative stress (Fisher-Wellman et al. 2009; Milic et al. 2009). MDA levels and GPx activity were increased in oxidative stress and hyperglycemic conditions. (Fig. 5A and 5B). These results are related with the increased level...
of AR expression during both condition. In the presence of AR inhibitor sorbinil in HG, intracellular MDA levels were increased as well (Fig. 5A). These results support the view that AR is a key enzyme in the antioxidant defence system. Therefore, AR inhibition must be evaluated attentively in order to protect metabolism from oxidative stress’ damage. It has been reported that oxidative stress causes enhanced AR expression levels in T cells, vascular smooth muscle cells (VSMC), macrophages, liver, lens and retina and support the antioxidant defence system (Brown et al. 2005; Jedziniak et al. 1981; Ramana et al. 2004a; Ramana et al. 2004b; Rittner et al. 1999). In contrast, several investigators have shown that the polyol pathway can induce oxidative stress through different mechanisms such as depletion of NADPH and accumulation of sorbitol which causes osmotic stress (Lee & Chung, 1999; Obrosova et al., 2003; Obrosova et al., 2005; Drel et al., 2006; Drel et al., 2008). AR takes part in reduction of glutathionyl-4-hydroxynonenal (GS-HNE) to glutathionyl-1,4-dihydroxynonene (GS-DHN) which is one of the products of lipid peroxidation. Inhibition of AR might cause accumulation of lipid peroxidation products in the cells (Drel et al. 2008; Drel et al. 2006; Lee and Chung 1999; Obrosova et al. 2003; Obrosova et al. 2005). This effect could be more prominent in hyperglycemic conditions. Because of the relative contribution of polyol pathway to oxidative stress, physiological function of AR is still under investigation.

In the second part of our study, we measured the PKC expression and also the activity. It is well known that hyperglycemia activates PKC isoforms in a tissue specific manner (Beckman et al. 2002; Brownlee 2001; Sheetz and King 2002). Previous studies have shown that hyperglycemia activates PKC-α, -β1, -β2, -δ, -ε and -ζ in ARPE-19 cells (Moriarty et al. 2000; Wood and Osborne 1997, 1998). Young et al. showed that activation of PKCδ in ARPE-19 was associated with retinopathy (Young et al. 2005). Our results showed that PKC expression and also activity were increased by HG and oxidative stress (Fig. 2C, 2D and Fig. 4). Our results confirmed the studies which were done by different researchers (Koya et al. 1997; Koya and King 1998; Sheetz and King 2002; Young et al. 2005). In our study we detected the expression of redox-sensitive transcription factor NF-kB and NF-kB inhibitor IκB-α level for to show the relationship between AR ve PKC. PKC causes activation of NF-kB in DM. NF-kB has a pro-apoptotic role in the retina during DM (Kowluru 2003). Additionally, AR gene has a promoter region for NF-kB(Iwata et al. 1999). In the present study, we showed that NF-kB and IκB-α levels were increased in HG and oxidative stress groups (Fig. 3A and 3B). Therefore, we may conclude that the activation of NF-kB via PKC is due to an increased AR expression level in ARPE-19 cells during hyperglycemia.
We cultured the ARPE-19 cells with AR inhibitor sorbinil whether it affects the PKC levels and activity. Our results showed that the inhibition of AR with sorbinil prevents hyperglycemia induced activation of PKC and expression of NF-kB and IkB-α as well compared to 50 mM glucose levels (Fig. 4, 3A and 3B). This is in agreement with previous observations showing that AR activates PKC and NF-kB that lead to increased vascular inflammation and adhesion (Ramana et al. 2004b). Ramana and Srivastava, (Ramana and Srivastava 2010) showed that the reduced products of GS-aldehydes and glutathionyl-HNE (GS-DHN), activates PKC which leads to increased expression of NF-kB. Choudhary et al. (Choudhary et al. 2005) also have reported that GS-DHN levels were decreased by sorbinil in ARPE-19 cells. These results showed that AR is likely to play a direct role in PKC expression. Our results are parallel with other investigations. Therefore, we can say that enhanced AR expression which is induced by oxidative stress during hyperglycemia may be the reason of increased PKC activity and NF-kB (Fig. 6).

We also measured the expression of total c-Jun, phospo c-Jun and SAPK/JNK protein levels in HG and oxidative stress stages. c-Jun, c-Fos or activating transcription factor (ATF) are the subunits of AP-1 transcription factor (Angel and Karin 1991). Phosphorylation of c-Jun and c-Fos or ATF subunits causes AP-1 activation and leads to the transcription of target genes including AR gene (Angel and Karin 1991; Iwata et al. 1999). AP-1 activity is regulated with specific protein kinases (Karin et al. 1997). SAPK/JNK are members of the MAPK family and has 3 isoform including JNK1, JNK2 and JNK3. SAPK/JNK can regulate the activity of transcription factors such as c-Jun and ATF-2 (Nishina et al. 2004). Therefore, SAPK/JNK family closely related with AP-1 which leads the transcription of AR gene (Lewis et al. 1998). Furthermore, activated PKC isoforms leads to MAPKs activation (Vincent et al. 2004). We showed that in hyperglycemia and oxidative stress, activated PKC leads to SAPK/JNK activation and causes increased levels of total c-Jun and phospo c-Jun. Phosphorylation of c-Jun subunit induces AP-1 activation and effects the transcription of AR gene. These signalling molecules activate AR enzyme through the feed back activation. There might be a loop between these products and AR. Increased glucose levels and oxidation activate AR, PKC, NF-kB and JNK. Following the activation of signalling molecules AR transcription starts (Fig. 6).

We also detected that the inhibition of AR was caused decreased expression levels of SAPK/JNK, total c-Jun, phospo c-Jun similarly NF-kB and IkB-α levels in hyperglycemic condition (Fig. 3A-E). The decrease in signaling molecule levels alike PKC expression level
and activity by the inhibition of AR support our idea about the relationship between AR and PKC.

We measured the cyt c levels in ARPE-19 cells for to show the connection between AR, PKC and apoptosis. Mitochondria has a pivotal role in the apoptosis and regulates the release of cytochrome c (Kroemer and Reed 2000; Rego and Oliveira 2003). Our results showed that hyperglycemia and oxidative stress causes apoptosis in ARPE-19 cells (Fig. 3F). Similar results have also been reported by Barak et al. in ARPE-19 cells (Barak et al. 2001). On the other hand, the inhibition of AR with sorbinil prevents hyperglycemia induced apoptosis via PKC compared with the 50 mM glucose group (Fig. 3F). Our results are consistent with the study (Ramana et al. 2005) which is showing that AR inhibitors prevent activation of PKC which leads apoptosis.

In conclusion, the relationship between PKC and AR is crucial for the development of diabetic complications such as retinopathy. In diabetes, PKC and AR were linked together with redox-sensitive transcription factors NF-kB and signaling molecules total c-Jun, phospho c-Jun and SAPK/JNK family and were related to oxidative stress as well. Our results support the view that AR is a vital component of the antioxidant defence system. On the other hand, it triggers the apoptosis via PKC in ARPE-19 cells. In this context, the AR enzyme inhibition in the treatment of diabetic complications must be attentively evaluated.

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Figure legends

Fig. 1. The effects of D-glucose and H₂O₂ on ARPE-19 cell viability. Concentration-
dependent effect of D-glucose (A) and H₂O₂ (B) on ARPE-19 proliferation. Cell viability was
determined by MTT and results were expressed as percentage of viable cells compared with the
control groups. *p<0.05 vs control group.
Fig. 2. Effects of D-glucose, H₂O₂, and sorbinil on AR and PKC protein expression. Cells were treated with various concentrations of D-glucose, H₂O₂ and sorbinil; AR and PKC protein expression levels were measured by Western blot. (A) and (C) show AR and PKC protein expression with 10-25-50-100 mM D-glucose treated cells respectively. (B) and (D) show AR and PKC protein expression with 200 µM H₂O₂, 200 µM H₂O₂ + 50 mM D-glucose, 10 µM sorbinil, 10 µM sorbinil + 50 mM D-glucose treated cells respectively. Western blot analysis was carried out at least three times and the figures are showing representative of three independent analyses. β-Actin is used as a control protein. *p<0.05 compared with control group.

Fig. 3. Evaluation of NF-κB, IκB-α, total c-Jun, phospho c-Jun, SAPK/JNK and cytochrome c protein levels after incubation of the ARPE-19 cells with D-glucose, H₂O₂ and sorbinil. In the presence of 10-25-50-100 mM D-glucose, 200 µM H₂O₂, 200 µM H₂O₂ + 50 mM D-glucose, 10 µM sorbinil and 10 µM sorbinil + 50 mM D-glucose, NF-κB(A), IκB-α (B), SAPK/JNK (C), total c-Jun(D), phospho c-Jun (E) cyt c (F) protein levels were assessed by ELISA. ELISA was carried out at least three times and the figures are showing representative of three independent analyses. *p<0.05 vs control group. #p<0.05 vs 50 mM D-glucose (HG) group. &p<0.05 vs H₂O₂ group. +p<0.05 vs Sorbinil group.

Fig. 4. Determination of Protein Kinase C Activity. In the presence of 10-25-50-100 mM D-glucose, 200 µM H₂O₂, 200 µM H₂O₂ + 50 mM D-glucose, 10 µM sorbinil, 10 µM sorbinil + 50 mM D-glucose, PKC activity were assessed by ELISA. ELISA was carried out at least three times and the figures are showing representative of three independent analyses. *p<0.05 vs control group. #p<0.05 vs 50 mM D-glucose (HG) group. &p<0.05 vs H₂O₂ group. +p<0.05 vs Sorbinil group.

Fig. 5. Evaluation of oxidative stress markers in ARPE-19 cells. Effect of 10-25-50-100 mM D-glucose, 200 µM H₂O₂, 200 µM H₂O₂ + 50 mM D-glucose, 10 µM sorbinil and 10 µM sorbinil + 50 mM D-glucose on oxidative stress markers were assessed by MDA concentration (A) and GPx activity (B). Experiments were carried out at least three times and the figures are showing representative of three independent analyses. *p<0.05 vs control group. #p<0.05 vs 50 mM D-glucose (HG) group. &p<0.05 vs H₂O₂ group. +p<0.05 vs Sorbinil group.
Fig. 6. Molecular pathway of aldose reductase. Relationship between hyperglycemia, oxidative stress, PKC and apoptosis. AR: Aldose reductase, DHAP: Dihydroxyacetone phosphate, NADPH: Nicotinamide adenine dinucleotide phosphate, NADH: Reduced form of nicotinamide adenine dinucleotide, SDH: Sorbitol dehydrogenase, AGE: Advanced glycation end-product, ROS: Reactive oxygen species, DAG: Diacylglycerol, GS-HNE: Glutathione-4-hydroxynonenal, GS-DHE: glutathionyl-1,4-dihydroxynonene, PKC: Protein kinase C, AP-1: Activator protein-1, JNK: Jun N-terminal kinase, Nf-kb: Nuclear factor kappa B
229x140mm (96 x 96 DPI)
Fig. 1. The effects of D-glucose and H2O2 on ARPE-19 cell viability. Concentration-dependent effect of D-glucose (A) and H2O2 (B) on ARPE-19 proliferation. Cell viability was determined by MTT and results expressed as percentage of viable cells compared with control groups. *p<0.05 vs control group.
Fig. 2. Densitometric quantification of AR and PKC protein expression. Cells were treated with various concentrations of D-glucose, H2O2 and sorbinil; AR and PKC protein expression levels were measured by Western blot. (A) and (C) show AR and PKC protein expression with 10-25-50-100 mM D-glucose treated cells respectively. (B) and (D) show AR and PKC protein expression with 200 µM H2O2, 200 µM H2O2 + 50 mM D-glucose, 10 µM sorbinil, 10 µM sorbinil + 50 mM D-glucose treated cells respectively. Western blot analysis was carried out at least three times and the figures shown are representative of three independent analyses. β-Actin is used as a control protein. *p<0.05 as compared with control group. #p<0.05 vs H2O2. &p<0.05 vs Sorbinil group. +p<0.05 vs 50 mM D-glucose.
Fig. 3. Evaluation of NF-κB, IκB-α, total c-Jun, phospho c-Jun, SAPK/JNK and cytochrome c protein levels after incubation of the ARPE-19 cells with D-glucose, H₂O₂ and sorbinil. In the presence of 10-25-50-100 mM D-glucose, 200 µM H₂O₂, 200 µM H₂O₂ + 50 mM D-glucose, 10 µM sorbinil and 10 µM sorbinil + 50 mM D-glucose, NF-κB (A), IκB-α (B), SAPK/JNK (C), total c-Jun (D), phospho c-Jun (E) and cyt c (F) protein levels were assessed by ELISA. ELISA was carried out at least three times and the figures shown are representative of three independent analyses. *p<0.05 vs control group. #p<0.05 vs 50 mM D-glucose (HG) group. &p<0.05 vs H₂O₂ group. +p<0.05 vs Sorbinil group.

338x190mm (96 x 96 DPI)
Fig. 4. Determination of Protein Kinase C Activity. In the presence of 10-25-50-100 mM D-glucose, 200 µM H2O2, 200 µM H2O2 + 50 mM D-glucose, 10 µM sorbinil, 10 µM sorbinil + 50 mM D-glucose, PKC activity were assessed by ELISA. ELISA was carried out at least three times and the figures shown are representative of three independent analyses. *$p<0.05$ vs control group. #$p<0.05$ vs 50 mM D-glucose (HG) group. &$p<0.05$ vs H2O2 group. +$p<0.05$ vs Sorbinil group.
Fig. 5. Evaluation of oxidative stress markers in ARPE-19 cells. Effect of 10-25-50-100 mM D-glucose, 200 µM H2O2, 200 µM H2O2 + 50 mM D-glucose, 10 µM sorbinil and 10 µM sorbinil + 50 mM D-glucose on oxidative stress markers were assessed by MDA concentration (A) and GPx activity (B). Experiments were carried out at least three times and the figures shown are representative of three independent analyses. *p<0.05 vs control group. #p<0.05 vs 50 mM D-glucose (HG) group. &p<0.05 vs H2O2 group. +p<0.05 vs Sorbinil group.
Fig. 6/Molecular pathway of aldose reductase

229x140mm (96 x 96 DPI)