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The influence of elastin degradation products, glucose and atorvastatin on metalloproteinase-1, -2, -9 and tissue inhibitor of metalloproteinases-1, -2, -3 expression in human retinal pigment epithelial cells

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Purpose: Hyperglycemia and increased concentrations of elastin degradation products (EDPs) are common findings in patients with diabetes, atherosclerosis and hypertension. The aim of this study was to assess the influence of high glucose, EDPs and atorvastatin on MMP-1, MMP-2, MMP-9 and TIMP1-3 gene expression in human retinal pigment epithelial cells (HRPE) *in vitro*. Method: HRPE were cultured for 24 hours with the substances being tested (glucose, EDPs), alone or in combination. Additionally, the cells were treated with atorvastatin in two different concentrations (1 or 10 μM). After incubation, total cellular RNA was extracted and used for gene expression evaluation. Gene expression was measured using the real-time RT-PCR technique. Results: Glucose, EDPs and atorvastatin had no impact on TIMP-1 and TIMP-3 expression. HRPE cells treated with glucose or EDPs with the addition of atorvastatin had a statistically significant decrease of TIMP-2 expression; glucose alone decreased MMP-1 expression. Atorvastatin decreased expression of all assessed genes, except TIMP-1 and TIMP-3 in a dose-dependent manner. Conclusions: Our results confirm the importance of MMPs and TIMPs in retinal vascular biology. Atorvastatin-induced MMPs gene expression can deeply affect extracellular matrix turnover, which may play an important role in the progression of ocular diseases.

Key words: metalloproteinase, TIMP, atorvastatin, HRPE

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INTRODUCTION

Elastin is an insoluble fibrous protein and a constituent of the extracellular matrix (ECM). Partial proteolysis of elastin by activated proteinases results in the release of soluble elastin degradation products (s-EDPs) into the circulation (Sivaprasad *et al.,* 2005). s-EDPs are produced in large quantities in several pathological processes, such as: atherosclerosis (Petersen *et al.,* 2002), tumor progression (Lapis & Timar, 2002; Ntayi *et al.,* 2004), aneurysm formation and atherogenesis (Hance *et al.,* 2002). Some authors have shown that EDPs are responsible for the up-regulation of MMPs. s-EDPs enhance angiogenesis by promoting endothelial cell migration and tubulogenesis (Brassart *et al.,* 2001; Robinet *et al.,* 2005). In the eye, elastin is present in Bruch's membrane and the choroidal vessels (Chong *et al.,* 2000). Diabetic vascular complications are possibly connected with the elevated degradation of elastic fibers. As a result, s-EDPs are released into the circulating blood, increasing elastase production and elastase-like activity, free radicals generation, induction of LDL oxidation and chemotactic activity. These peptides also act with various growth factors, cytokines and vasoactive molecules released as a response to injury. They also stimulate endothelial cell cytotoxicity and can up-regulate the synthesis and activation of matrix metalloproteinases (MMPs) (Ntayi *et al.,* 2004).

MMPs regulate important biological functions, including ECM integrity. They are expressed in normal or diseased states and participate in ECM degradation, vascular and cardiac remodeling, wound healing, tumor metastasis and neovascularization (Jones *et al.,* 2003; Visse & Nagase, 2003; Pepper, 2001).

MMP activity is modulated through interaction with MMP inhibitors - tissue inhibitors of metalloproteinases (TIMPs) (Brew *et al.,* 2000; Padgett *et al.,* 1997). Altered MMP expression and activity have been observed in several ocular diseases, such as choroid neovascularization (Lambert *et al.,* 2002) and proliferative retinopathies (Webster *et al.,* 1999; Sheridan *et al.,* 2001), suggesting that these enzymes may play an important role in ocular pathology.

Situated between the neurosensory retina and the choroid, human retinal pigment epithelial (HRPE) cells form the outer blood-retina barrier. Breakdown of this barrier is observed in many ocular diseases, such as proliferative vitreoretinopathy (Troger *et al.,*

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Abbreviations: EDP(s), elastin degradation product(s); MMP-1, matrix metalloproteinase-1; MMP-2, matrix metalloproteinase-2; MMP-9, matrix metalloproteinase-9; TIMP-1, tissue inhibitor of metalloproteinases-1; TIMP-2, tissue inhibitor of metalloproteinases-2; TIMP-3, tissue inhibitor of metalloproteinases-3; HRPE, human retinal pigment epithelium; RNA, ribonucleic acid; RT-PCR, reverse transcriptase polymerase chain reaction; ECM, extracellular matrix; LDL, low density lipoproteins; MMP(s), matrix metalloproteinase(s); TIMP(s), tissue inhibitor(s) of metalloproteinases; RPE, retinal pigment epithelium; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; IL-1, interleukin-1; VEGF, vascular endothelial growth factor; FBS, fetal bovine serum; ATTC, American type culture collection; DMEM/F12, Dulbecco's modified Eagle medium: nutrient mixture F-12; PBS, phosphate buffered saline; DTT, dithiotreitol; PCR, polymerase chain reaction; GADPH, glyceraldehyde 3-phosphate dehydrogenase; AGE(s), advanced glycation end-product(s); VCAM-1, vascular cell adhesion molecule-1

Table 1. Experiment design encompassing all groups tested.

	Control	Glucose + EDP	Glucose + atorvastatin 1 μM	Glucose + EDP + atorvastatin 1 μM	Glucose + atorvastatin 10 μM	Glucose + EDP + atorvastatin 10 μM
Glucose	–	+	+	+	+	+
EDP	–	+	–	+	–	+
atorvastatin 1 μM	–	–	+	+	–	–
atorvastatin 10 μM	–	–	–	–	+	+

2003; Cusick *et al.,* 2003; Spraul *et al.,* 2004; Chan *et al.,* 2010), diabetic retinopathy (Decanini *et al.,* 2008; Villaroel *et al.,* 2009) and age-related macular degeneration (Johnson *et al.,* 2003; Evans, 2001; Dunaief *et al.,* 2002). These pathologies are characterized by increased permeability for serum components, mainly proteins and inflammatory cells. Intraocular influx of serum components is associated with leukocyte infiltration and cellular proliferation, leading to severe complications and visual deterioration (Yoshida *et al.,* 2001).

Retinal pigment epithelial (RPE) cells are an important source of MMPs activity in the retina (Eichler *et al.,* 2004; Ahir *et al.,* 2002; Lee *et al.,* 2004; Hollborn *et al.,* 2007). The expression and activity of MMPs in RPE cells have been shown to be regulated by various cytokines and growth factors, including: transforming growth factor beta (TGF-β), tumor necrosis factor alfa (TNF-α), and interleukin 1 (IL-1) (Eichler *et al.,* 2002; Nagineni *et al.,* 1994; Nagineni *et al.,* 2000). MMP activity can also be controlled by growth factors, mainly the vascular endothelial growth factor (VEGF) (Hollborn *et al.* 2007; Hoffmann *et al.,* 2002). Thus the RPE produces factors that support the survival of photoreceptors and ensure a structural basis for an optimal circulation and supply of nutrients (Strauss, 2005). 3-Hydroxy-3-methylglutaryl-CoA reductase inhibitors (statins) are widely used in the treatment of dyslipidemia (Ludwig & Shen, 2006). They also reduce cardiac death and cerebrovascular events in diabetic patients (Ludwig & Shen, 2006; Collins *et al.,* 2004). Some studies reported beneficial effects of statins on retarding the progression of diabetic retinopathy (Sen *et al.,* 2002; Yamagishi *et al.*, 2006; Al-Shabrawey *et al.,* 2008), hard exudates (Cusick *et al.,* 2003) and macular edema (Gupta *et al.,* 2004) in the retinas of diabetic patients.

MATERIALS AND METHODS

Cell culture. Human retinal pigment epithelial cells (HRPE) were purchased from the American Type Culture Collection (ATCC, CRL-2302). HRPE cells were grown in DMEM:F12 containing 10% FBS and antibiotics as described elsewhere (Zhuge & Xu, 2001; Marin-Castano *et al.,* 2005). HRPE were cultured for 24h at 37ºC in 5% CO_2 atmosphere in a tissue culture incubator with inducers (ϰ-elastin 0.5 mg/L (Elastin Products Company, Inc. Missouri, USA), 5 (control) or 15 mM glucose or ϰ-elastin + 15 mM glucose) and atorvastatin at a concentration of 1 or 10 μM (Table 1). After incubation, the cells were harvested and total cellular mRNA was extracted for further assays.

Isolation of total cellular RNA. Total cellular mRNA was extracted with commercially available RNA isolation kits (RNeasy Mini Kit, Qiagen) according to the manufacturer's protocol, with slight modifications. Cells were rinsed with ice-cold PBS buffer, and later lysed for 5 min on ice in RLN buffer (50 mM TRIS·HCl, pH 8.0; 140 mM NaCl; 1.5 mM $MgCl_2$; 2 mM DTT) with 0.5% (v/v) IGEPAL CA-630 (Sigma-Aldrich). After lysis, the cells were centrifuged for 5 min at 300×*g*, and supernatant was processed according to the kit manufacturer's protocol. The concentration of the obtained RNA and the estimated purity was determined by measuring the absorbance at 260 and 280 nm in a spectrophotometer (GeneQuantII, Pharmacia-Biotech). RNA samples were tested for presence of contaminating DNA and were found negative.

After isolation, RNA extracts were stored at –70ºC until analysis.

Real time RT-PCR. Single step RT-PCR was performed using TaqMan EZ RT-PCR reagents (Applied Biosystems). A 2 μl RNA sample was mixed with specific primer sets and PCR master mix, and was then processed in a thermocycler: 60ºC for 30 min; and 40 cycles: 94ºC for 20 seconds; 65ºC for 60 s. Real time reaction products were detected using FAM-labeled probes (Applied Biosystems) and the LightCycler® 480 instrument (Roche). For the quantitative determination of selected gene expression, ready-to-use Applied Biosystems GeneAssays were used (Table 2). Expression levels of selected genes were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) internal control (GAPDH, TaqMan® pre-developed assay reagents, Applied Biosystems No. 433764T). Genes whose expression did not change significantly were used as additional normalizers, and made possible to assess if GAPDH reference expression remained stable in the different groups tested.

Table 2. Gene expression assays used in the experiment

Gene name	Assay ID	Amplicon length
Matrix metalloproteinase 1 (MMP-1)	Hs00899660_g1	134 bp
Matrix metalloproteinase 2 (MMP-2)	Hs01548733_m1	84 bp
Matrix metalloproteinase 9 (MMP-9)	Hs00957556_g1	93 bp
Metalloproteinase inhibitor 1 (TIMP-1)	Hs01092510_m1	74 bp
Metalloproteinase inhibitor 2 (TIMP-2)	Hs01091319_m1	85 bp
Metalloproteinase inhibitor 3 (TIMP-3)	Hs00165951_g1	76 bp

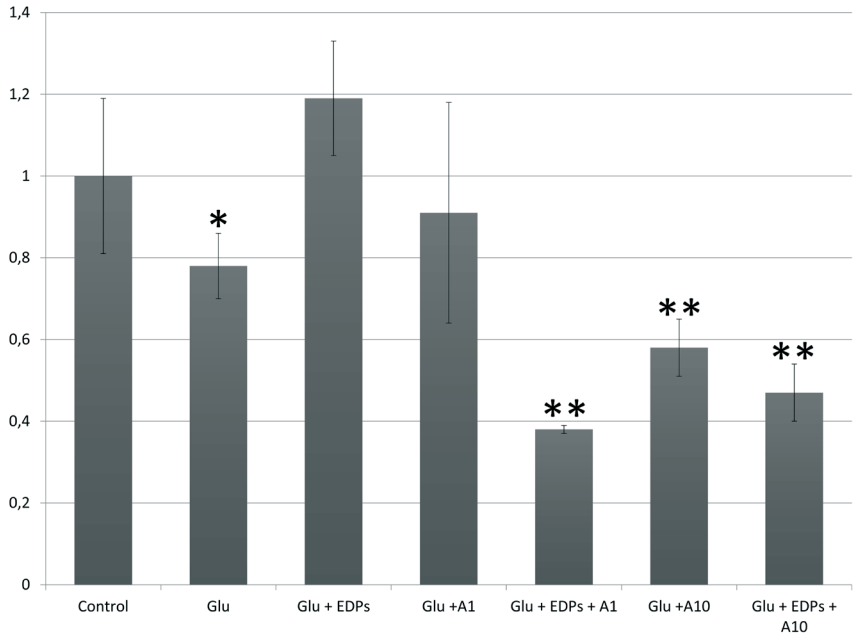

Figure 1. Expression of MMP-1gene in HRPE cells.
All results are normalized to a control group, and expressed as percentage of control. Glu, glucose 15 mM; EDPs, elastin derived peptides (0.5 μg/ml); A1, atorvastatin 1 μM; A10, atorvastatin 10 μM). *p-value<0.05. Data is presented as mean value ±S.D., **p-value<0.01.

RESULTS

Gene expression profiles of MMPs and TIMPs are shown on Figs. 1–4. All expression levels were normalized to a control group, so that control group expression was always set at 100%.

None of the substances tested (glucose, s-EDPs and atorvastatin) had any effect on TIMP-1 and TIMP-3 expression. Neither high glucose concentration nor EDPs influenced TIMP-2 expression. However, in the HRPE cells treated with glucose or EDPs with the addition of atorvastatin, we observed a small but statistically significant decrease of TIMP-2 expression.

The influence of the tested substances on MMPs gene expression was divergent. Atorvastatin at the concentration of 10 μM decreased MMP-9 expression, whereas glucose or EDPs had no effect on this gene. The relatively high standard deviation in this group rendered statistical analysis difficult due to a very low MMP-9 gene expression in HRPE. MMP-9 mRNA was observed at the method detection limit.

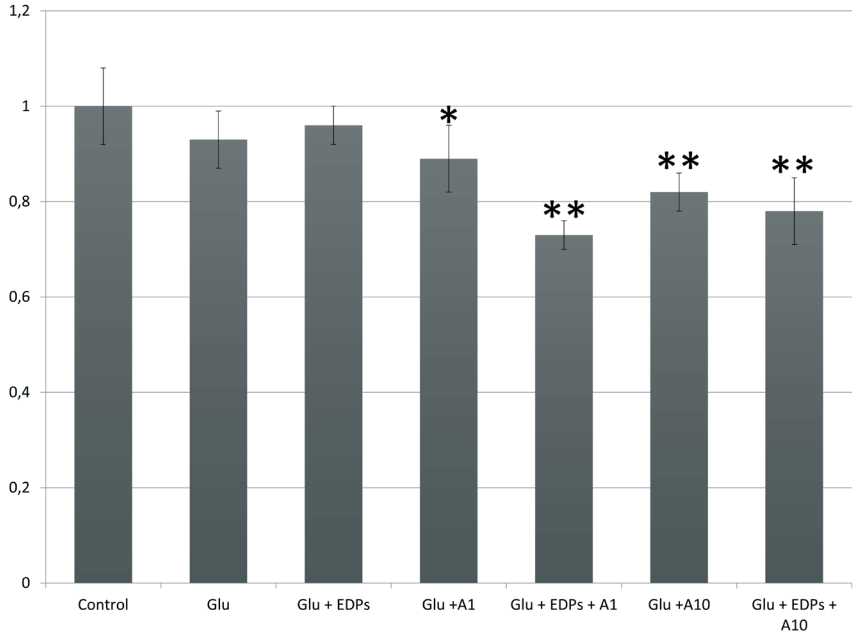

Figure 2. Expression of MMP-2 gene in HRPE cells.
All results are normalized to a control group, and expressed as percentage of control. Glu, glucose 15 mM; EDPs, elastin derived peptides (0.5 μg/ml); A1, atorvastatin 1 μM; A10, atorvastatin 10 μM). *p-value<0.05. Data is presented as mean value ±S.D., **p-value<0.01.

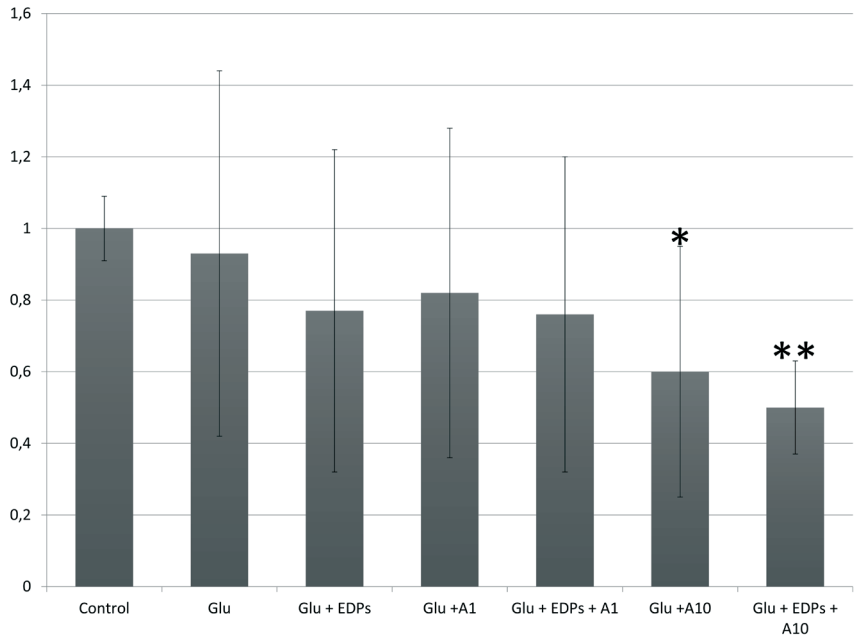

Figure 3. Expression of MMP-9 gene in HRPE cells.
All results are normalized to a control group, and expressed as percentage of control. Glu, glucose 15 mM; EDPs, elastin derived peptides (0.5 μg/ml); A1, atorvastatin 1 μM; A10, atorvastatin 10 μM). *p-value<0.05. Data is presented as mean value ± S.D., **p-value<0.01.

MMP-2 expression was slightly affected by the tested substances. In a dose-dependent manner, atorvastatin decreased metalloproteinase expression, which was amplified by EDPs. Glucose, alone or in combination with s-EDPs, had no influence on MMP-2 gene expression.

The most profound effects were observed on the MMP-1 gene. Glucose alone significantly decreased MMP-1 expression; EDPs alone or in combination with glucose had no effect. Similar to the effects on MMP-2, atorvastatin also decreased the expression of MMP-1 in a dose-dependent manner. What is interesting, EDPs augmented the observed effect of atorvastatin on MMP-2 gene expression.

In conclusion, atorvastatin had an inhibitory effect on MMP-1, MMP-2, MMP-9 and TIMP-2 expression. The observed effect was related to atorvastatin concentration. High glucose concentration had only a minimal effect on the expression of the genes tested, mainly diminishing MMP-1 expression. EDPs alone had no effect on the expression of the genes tested but amplified the inhibitory effect of atorvastatin on MMP-1 gene expression.

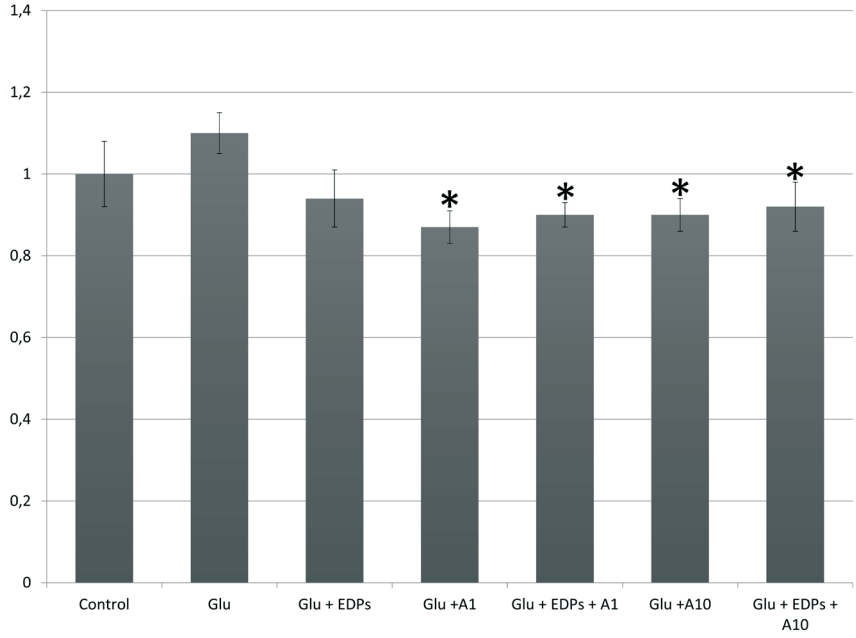

Figure 4. Expression of TIMP-2 gene in HRPE cells.
All results are normalized to a control group, and expressed as percentage of control. Glu, glucose 15 mM; EDPs, elastin derived peptides (0.5 μg/ml); A1, atorvastatin 1 μM; A10, atorvastatin 10 μM). *p-value<0.05. Data is presented as mean value ± S.D., **p-value<0.01.

DISCUSSION

Changes in MMPs and TIMPs expression induced by hyperglycemia were observed in cultured RPE cells (Lee *et al.,* 2004) and in vascular endothelial cells (Uemura *et al.,* 2001).

Up-regulation of MMP-9 expression was indicated as a factor contributing to the formation of choroidal neovascularization in mice (Lambert *et al.,* 2002).

In our study, the expression of MMP-2 in control HRPE was several thousand times higher than MMP-9, thus making MMP-2 the major metalloproteinase expressed under normal conditions. Our results are in agreement with the studies of other authors (Zhuge & Xu, 2001; Marin-Castano *et al.,* 2005). RPE synthesizes collagens type I-IV and fibronectin. MMP-2 degrades collagen I, IV and laminin — the most important components of Bruch's membrane (Zhuge & Xu, 2001). This type of metalloproteinases seems to play an important role in the pathogenesis of retinal disorders.

The data concerning MMP expression in retinal pigment epithelial cells is inconsistent, probably due to the differences in study models, the cells used and the duration of experiments. In animal studies, glucose increases retinal MMP-2 and MMP-9 gene expression in streptozotocin-induced diabetes in rats (Giebel *et al.,* 2005). We were unable to confirm these findings as well as the findings of Lee *et al.* (2004) who observed that hyperglycemia after stimulation of HRPE with glucose, increased the expression of MMP-2 and MMP-9. In our experiment, 15 mM glucose did not change the expression of MMP-2 or -9 during incubation. In the animal model and the *in vitro* HRPE model, other factors, such as the formation of advanced glycation end-products (AGE) are likely to play an important role. In our study, we only incubated cells for 24 hours, which is too short to create a significant concentration of AGE in vitro. Thus we could evaluate the influence of sole hyperglycemia and exclude a component of AGE. Giebel and coworkers (2005) did not present MMPs mRNA expression in animals before the 12th week of study, probably because the results were not significantly different or they were simply not evaluated. In the study by Lee *et al.* (2004), an increase in the expression of MMPs was observed after 14 days of incubation. Therefore we cannot exclude that in order to observe any significant changes in MMPs gene expression, the cells must be incubated with glucose for longer periods of time. The oxidative stress associated with hyperglycemia may also be due to increased concentrations of AGEs (Sulochana *et al.,* 2001). AGEs interact with specific receptors and induce oxidative stress, enhance the expression of vascular cell adhesion molecule 1 (VCAM-1) and increase endothelial adhesiveness to monocytes (Schmidt *et al.*, 1995). Hoffman *et al.* (2002) reported that AGEs stimulate MMP-2 expression in choroidal endothelial cells (CECs). Hoffmann and coworkers (2006) found that TNF-alfa and VEGF stimulate the secretion of MMP-2 and MMP-9 in RPE cells. Combining both studies, it seems that over-expression of MMP-2 and -9 can take place in pathological conditions, such as diabetes, and in processes related to neovascularization. Thus, factors diminishing MMP-2 and -9 expression may possibly have a beneficial role. In the presented study, atorvastatin decreased the expression of the two metalloproteinases in a dose-dependent manner. It also decreased the expression of TIMP-2 and MMP-1.

In the study performed by Alge-Priglinger *et al.* (2009), oxidative stress induced MMP-1 expression. Thus, atorvastatin action which leads to the inhibition of MMP-1 mRNA expression should have a beneficial effect. Surprisingly, glucose at a concentration of 15 mM significantly decreased MMP-1 mRNA expression. We were unable to find any studies related to the influence of glucose on MMP-1 expression in RPE cells. However, the effect of glucose on MMP-1 expression in other cells is divergent. Kim and coworkers (2008) found that whereas in human peritoneal mesothelial cells glucose decreases MMP-1 expression, in endothelial cells, it has the opposite effect. Endothelial cells incubated with high glucose concentration over-express the MMP-1 gene (Death *et al.,* 2003).

In our experiment, we did not observe any direct influence of the tested components on the expression of the TIMPs gene in any of the examined groups, with the exception of atorvastatin with glucose and with glucose + EDPs, which statistically significantly decreased TIMP-2 gene expression. These results enable us to conclude that TIMPs expression seems not to be regulated by hyperglycemia or EDPs. Therefore hyperglycemia and EDPs are not involved in the process of regulation of MMPs activation.

The addition in our study of atorvastatin significantly decreased all the examined MMPs expression, but atorvastatin alone influenced TIMP-2 expression. The observed effect was concentration-dependent. Although some authors confirmed the positive effect of statins on MMPs activity, we found no data on the effects of statins on RPE cells. Luan and coworkers (2003) noticed that statins inhibit the secretion of several MMP-1, -2, -3 and -9 from the vascular smooth muscle and macrophages in humans and rabbits. In the rat model of heart failure, Ichihara and coworkers (2006) reported that pravastatin suppressed an increase in myocardial MMP-2 and -9 activity. The influence of atorvastatin on MMPs expression in our study reflects *in vivo* results obtained in different vascular localizations. Kajimoto *et al.* (2009) observed that atorvastatin therapy reduces MMP-2 and MMP-9 expression in human abdominal aortic aneurysmal wall. Collin and coworkers (2006) observed the same effect of atorvastatin on MMP-2 expression reduction in coronary arteries of rabbits treated with atorvastatin. Atorvastatin-reduced MMPs gene expression can deeply affect extracellular matrix turnover, which may play an important role in the progression of ocular diseases, including diabetic retinopathy.

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