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Orginal article

Exenatide modulates expression of metalloproteinases and their tissue inhibitors in TNF-α stimulated human retinal pigment epithelial cells

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ABSTRACT

Background: Diabetic retinopathy (DR) is one of the most common complications of diabetes and the leading cause of acquired blindness in adults. In diabetic patients hyperglycemia induces complex metabolic abnormalities affecting retinal homeostasis, and promotes retinal inflammation and angiogenesis. Incretin mimetic drugs such exenatide, are a relatively new group of drugs used in the treatment of diabetes. We investigated the potential direct effects of exenatide on human retinal pigment epithelium (HRPE).
Methods: cAMP production was measured after stimulation of HRPE cells with GLP-1 and exenatide. Intracellular signaling pathways were also examined. HRPE cells were stimulated with TNF-α and subsequently incubated with exenatide. The concentration of metalloproteinases, MMP-1, MMP-2 and MMP-9, and tissue inhibitors of metalloproteinases, TIMP-1, TIMP-2, and TIMP-3 were evaluated. Viability, cytotoxicity and caspase 3/7 activation were determined. Activity of dipeptidyl peptidase-4 (DPP-4), an enzyme involved in GLP-1 inactivation, was also determined.
Results: Both GLP-1 and exenatide stimulation in HRPE cells caused no effect in cAMP levels suggesting alternative signaling pathways. Signaling pathway analysis showed that exenatide reduced phosphorylation of Akt-Ser473, PRAS40, SAPK/JNK, Bad, and S6 proteins but not Akt-Thr308. Exenatide also decreased MMP-1, MMP-9, and TIMP-2 protein levels whereas MMP-2 level in HRPE cells was increased. Finally, we show that exenatide decreased the activity of DPP-4 in TNF-α stimulated HRPE cells.
Conclusions: These findings indicate that exenatide modulates regulation of extracellular matrix components involved in retinal remodeling.

Introduction

Globally, an estimated 415 million people are suffering from diabetes mellitus [1]. Clinical complications of diabetes include blindness, kidney failure, increased risk of cardiovascular disease, and a need for lower extremity amputations following neuropathy and tissue necrosis, all of which have long term impact on quality of life [2,3]. One of the major microvascular complications of diabetes is diabetic retinopathy (DR), which constitutes the leading cause of acquired vision loss. Among diabetic patients, the number of DR and vision-threatening DR (VTDR) will be estimated to rise to 191 and 56 million, respectively by 2030 [1,4]. The hyperglycemic state in diabetic patients is one of the main risk factors in DR leading to production of reactive oxygen species (ROS), advanced glycation end-products (AGEs), inflammatory factors, and vascular growth factors [5–9]. Such substances promote endothelial dysfunction, and enhanced permeability of vascular structures. Furthermore, tissue ischemia, hemostatic abnormalities, and neovascularization can also develop [10].

The blood-retinal barrier (BRB) plays a fundamental role in preserving and maintaining optimal visual cell function and homeostasis. The BRB consists of an inner and outer components. The inner BRB, is located in the inner retinal microvasculature and comprises the microvascular endothelial cells attached to each other by tight junctions, which mediate highly selective diffusion of molecules from blood to retina. The outer BRB is formed by the retinal pigment epithelium (RPE), in part, to regulate the movement of solutes and nutrients from the choroid to the subretinal space [11,12]. Additionally, RPE plays a role in phagocytosis

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of photoreceptor membranes, cytokine and chemokine production and angiogenic factors [13,14]. Hyperglycemia alters the functioning of the BRB, by activation of several metabolic pathways in endothelial and retinal cells, resulting in retinal neurodegeneration [15–17]. These changes result in oxidative stress, damage of pericytes, increased vascular permeability, activation of pro-angiogenic factors, and finally inflammation. The inflammatory milieu in DR leads to extracellular matrix remodeling by matrix metalloproteinases (MMP) [18–20]. MMPs are a family of zinc-dependent endopeptidases controlled by their endogenous inhibitors: Tissue inhibitors of metalloproteinases (TIMPs), involved in the degradation and re-building of ECM proteins such as collagen, elastin, gelatin, and casein [21,22]. In the retina, MMPs partly synthesized by RPE cells, are involved in remodeling of Bruch's membrane, a structure located between RPE and the choriocapillary basement membrane [23]. In particular, MMP-2 modulates the levels of pigment epithelium derived factor (PEDF), a protein secreted by the RPE, which is involved in retinal cell survival, and also acts as an anti-angiogenic factor. MMP-9 modulates angiogenesis by proteolytic degradation of PEDF, hence controlling the VEGF/PEDF angiogenic balance [24,25]. Another substrate for MMPs is opticin, a small, leucine rich protein which contributes to vitreoretinal adhesion [26]. Several researchers have observed increased MMP-2 and MMP-9 in the diabetic retinas and vitreous [27,28]. In parallel, DR is associated with increased expression of inflammatory markers in the serum, vitreous, and retina in preclinical and clinical studies of diabetes [29–31]. MMP-1 and MMP-9 may play an important role in the progression of angiogenesis associated with proliferative DR (PDR) [27]. Despite great progress in understanding of diabetic complications, the mechanism responsible for the development of diabetic retinopathy remains unknown.

Incretin hormones, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), are a group of metabolic hormones that stimulate postprandial insulin secretion and increase glucose uptake in target tissues. Incretin action is mediated by GLP-1 receptor (GLP-1R), a G-protein coupled receptor present in pancreatic beta cells that stimulates insulin secretion. [32,33]. Rapid hydrolysis of GLP-1 and GIP by dipeptidyl peptidase-4 (DPP-4) has led to the design of two sets of drugs: synthetic incretin analogue drugs with extended half-life, resistant to the actions of DPP-4 and DPP-4 inhibitors. In the clinical setting, incretin-mimetic drugs such as exenatide, also known as Exendin-4, are used for the treatment of type 2-diabetes mellitus (T2DM) [34,35]. Interestingly, incretins exert pleiotropic effects far beyond glycemic control due to the presence of GLP-1R in various tissues. Thus, GLP-1 therapy may be beneficial for the treatment of chronic inflammatory diseases such as atherosclerosis, neurodegenerative disorders, asthma, neoplasms and psoriasis [36–39]. Previous research indicates that exenatide decreases the production of adhesion molecules (ICAM-1 and VCAM-1) in TNF-α stimulated HRPE cells [40]. In the present study, we investigated whether the incretin drug, exenatide, influences human retinal-pigment epithelial cells, and its potential effect in tissue remodeling during DR.

Materials and methods

Cell culture

Human retinal pigment epithelial (HRPE) cell line, ARPE-19 (ATCC, Manassas, VA, USA) was cultured in DMEM/F12 with 10% fetal bovine serum and antibiotics [40]. Before experiments, cells were placed on 6-well cell culture plates and maintained until confluence. Subsequently, cells were incubated for 24 h with TNF-α (10 ng/mL) and exenatide (10 nM). Unless otherwise stated all reagents were purchased from Merck KGaA, Darmstadt, Germany.

Immunoblotting

HRPE cells were lysed in PathScan Cell Lysis Buffer (Cell Signaling, Danvers, MA, USA) supplemented with PMSF and protease inhibitors (Hoffmann-La Roche AG, Basel, Switzerland). Protein concentration was determined with bicinchoninic acid (BCA) method. Equal amounts of protein were loaded and separated by SDS-PAGE. Proteins were transferred into Immobilon-FL PVDF membrane and incubated with antibodies against GLP-1R (ab39072, Abcam, Cambridge, UK) at 1:1000, DPP-4 (#67,138 Cell Signaling, Danvers, MA, USA) at 1:1000, β-actin at 1:2000 and GAPDH at 1:5000. Goat anti-rabbit secondary antibody conjugated with fluorescent dye IRDye800 (LI−COR, Lincoln, NE, USA) was used. The proteins of interest were visualized by using LICOR Odyssey Infrared Imaging System.

Quantification of cAMP

cAMP concentration was measured with cAMP - Gs Dynamic kit (Cisbio, Codolet, France) according to manufacturer's instructions. HRPE cells were cultured on 384-well plates (1000 cells/well) and maintained for 24 h. Subsequently, cells were stimulated for 60 min with GLP-1 (7.8 nM to 1000 nM), exenatide (1.56 nM to 100 nM), and forskolin as a positive control (0.59 μM–75 μM). One step assay protocol including cAMP standard curve was performed. Infinite F200Pro (TECAN, Männedorf, Switzerland) device was used for homogeneous time resolved fluorescence (HTRF) readout.

DPP-4 activity assay

DPP-4 activity was determined by measuring the conversion of a synthetic substrate (Gly-Pro-p-nitroanilide hydrochloride). During hydrolysis a colorful product is released and its absorbance measured at 405 nm. The standard curve was prepared using serial dilutions of the recombinant DPP-4 enzyme. HRPE cells were cultured in a 24-well plate until confluence, cells were then incubated with tested substances for an additional 24 h, and a synthetic substrate in buffer (20 mM Tris; 0.1 M NaCl; 1 mM EDTA pH 8.0) was added to each well for 6 h. To estimate non-specific degradation of a substrate, a DPP-4 inhibitor – KR62436 was used at 5.7 μM. The activity of DPP-4 in HRPE cells was determined as the difference between total activity and activity after addition of the inhibitor.

Intracellular signaling assay

HRPE cells were cultured on 6-well cell culture plates and maintained until confluence. Cells were then co-cultured with tested substances: TNF-α (10 ng/mL) and exenatide (10 nM) for 6 h. After incubation cells were lysed with PathScan Sandwich ELISA Lysis Buffer (Cell Signaling, Danvers, MA, USA) supplemented with phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate and protease inhibitors (Hoffmann-La Roche AG, Basel, Switzerland). Protein concentration in cell lysates was determined by BCA method. PathScan Intracellular Signaling Array Kit (Cell Signaling, Danvers, MA, USA) was used according to the manufacturer's instructions. The array allows for simultaneous detection of 18 signaling molecules, either phosphorylated or cleaved, includes the following proteins, Erk1/2 (Thr202/Tyr204), Stat1 (Tyr701), Stat 3 (Tyr705), Akt (Thr308), Akt (Ser473), AMPKa (Thr172), S6 Ribosomal protein (Ser235/236), mTOR (Ser2448), HSP27 (Ser78), Bad (Ser112), p70 S6 kinase (Thr389), PRAS40 (Thr246), p53 (Ser15), p38 (Thr180/Tyr182), SAPK/JNK (Thr183/Tyr185), PARP (Asp214), Caspase-3 (Asp175), GSK-3b (Ser9). Target-specific capture antibodies have been spotted in duplicates onto nitrocellulose-coated glass slides. Protein lysate was incubated on the slide

followed by a biotinylated detection antibody cocktail. Streptavidin-conjugated fluorochrome was then used to visualize the bound detection antibody. A fluorescent image of the slide was captured with a LICOR Odyssey Infrared Imaging System and spot intensities quantified using Image Studio Lite Ver 5.2 software.

Quantification of MMPs and TIMPs concentration

Evaluation of protein concentration of MMPs (MMP-1, MMP-2, MMP-9) as well as the inhibitors, TIMPs (TIMP-1, TIMP-2 and TIMP-3) were determined in cell culture medium and measured on 96-well plates by DuoSet ELISA kits (R&D Systems, Minneapolis, MN, USA), according to manufacturer's instructions. Results were normalized to total cellular protein concentration. Cells were lysed in RIPA buffer supplemented with a protease inhibitor cocktail (Hoffmann-La Roche AG, Basel, Switzerland). Protein concentration of lysates were determined by BCA method. To assess the influence of human recombinant TNF-α on MMPs and TIMPs, cells were incubated with TNF-α at 2.5 and 10 ng/mL (data not shown). Each experiment was performed in triplicate and mean values were calculated.

Viability, cytotoxicity and apoptosis assays

HRPE cells were seeded into a 96-well plate at a total density of 10,000 cells per well in DMEM/F12 with 5% fetal bovine serum. Cells were incubated with TNF-α (10 ng/mL) and exenatide (10 nM) for 24 h. Viability, cytotoxicity and caspases activation were determined using ApoTox-Glo Triplex assay (Promega, Madison, WI, USA) according to the manufacturer's protocol using a microplate reader Infinite M200 (TECAN, Männedorf, Switzerland).

Statistical analysis

Measured values were statistically analyzed and presented as mean ± SD or expressed as percent compared to control group. One-way ANOVA followed by the Scheffe or Dunnett *post-hoc* was performed for statistical comparisons; $p < 0.05$ was considered significant. All statistical analyses were performed using Statistica analysis toolpack.

Results

GLP-1R expression and DPP-4 activity in HRPE cells

Expression of GLP-1R and DPP-4 in HRPE cells was confirmed by western blot analysis (Fig. 1A). Upon stimulation with TNF-α (10 ng/mL), DPP-4 activity in HRPE cells was observed to increase by 22%, whereas exenatide alone, or combination of the two decreased DPP-4 levels by 20% and 16% respectively (Fig. 1B). Intracellular signaling pathways involving cAMP were evaluated as well. Stimulation of HRPE cells with forskolin as a positive control decreased the HTRF fluorescence, and this was inversely proportional to cAMP concentration (Fig. 1C). Conversely, GLP-1 (7.8 nM to 1000 nM) and exenatide (1.56 nM to 100 nM) did not result in cAMP level changes in HRPE cells (Fig. 1D).

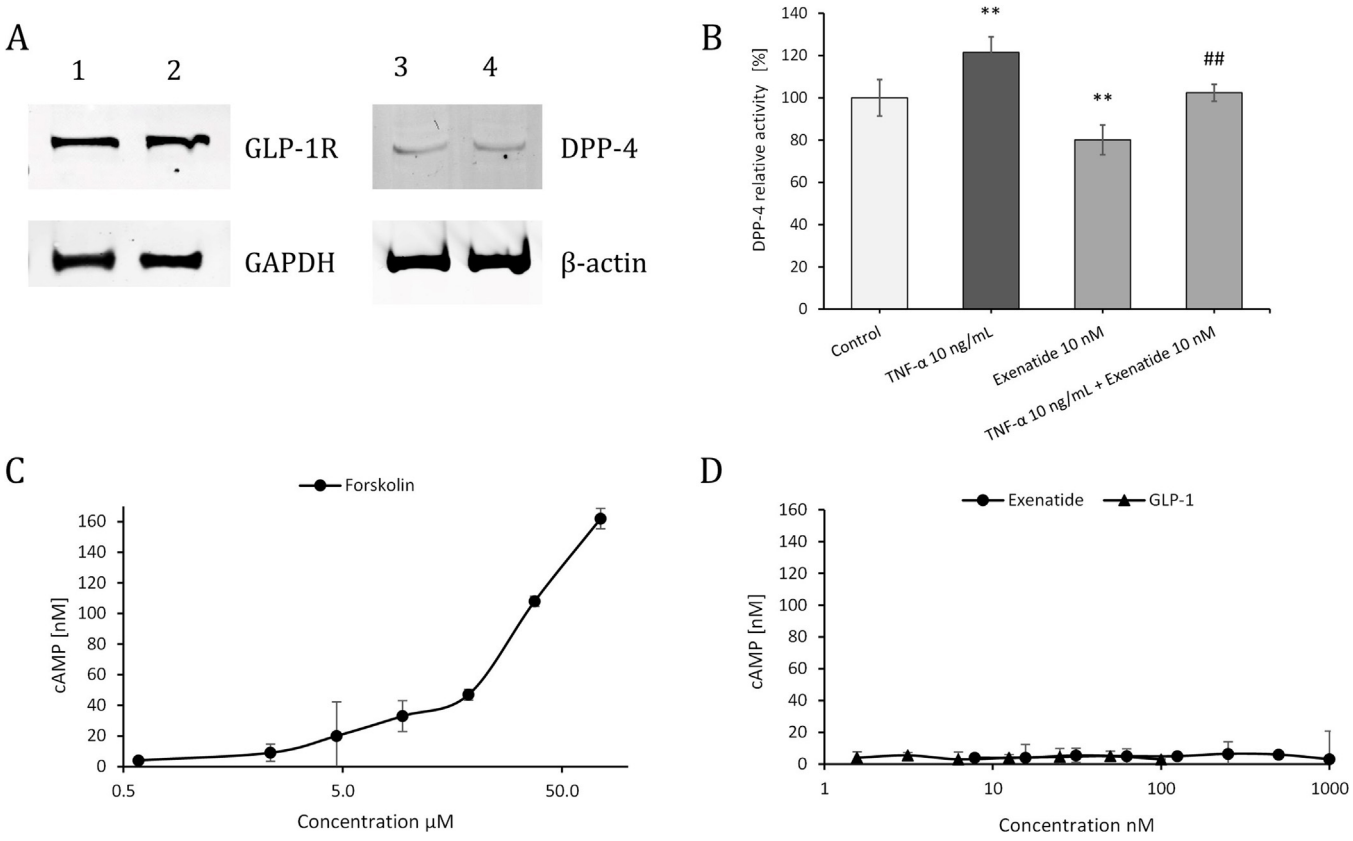

Fig. 1. Western blot of GLP-1R and DPP-4. Activity of DPP-4 and cAMP concentration in HRPE cells. Western blot image of GLP-1 receptor (lanes 1–2) and DPP-4 (lanes 3–4) in protein extracts from HRPE cells. GAPDH or β-actin as loading control (A). Activity of DPP-4 in HRPE cells stimulated with TNF-α (10 ng/mL), or exenatide (10 nM) after 24 h incubation (B). Concentration of cAMP in HRPE cells incubated for 60 min with forskolin as a positive control (0.59 μM to 75 μM) (C) and GLP-1 (7.8 nM to 1000 nM), exenatide (1.56 nM to 100 nM) (D). Mean values ± SD are shown. n = 6 per group. $^{**}p < 0.01$ control *vs.* different conditions. ANOVA followed with Dunnett's *post-hoc* test. $^{\#\#}p < 0.01$ TNF-α *vs.* different conditions. One-way ANOVA followed with Scheffe's *post-hoc* test.

Concentration of intracellular signaling proteins

To gain insights into the molecular mechanism by which exenatide exerts its effects, intracellular signaling pathways were examined. TNF-α stimulation did not change phosphorylation of Akt at Thr308 residue. Exenatide (10 nM) inhibited Akt phosphorylation by 15% compared to control. In TNF-α-stimulated group treated with exenatide, no changes were observed in Akt (Thr308) phosphorylation (Fig. 2A). Conversely, TNF-α stimulation increased activation of Akt(Ser473) residue by 54%. Treatment with exenatide in combination with TNF-α reversed TNF-α-induced Akt activation by 40% to the baseline level. (Fig. 2B). TNF-α stimulation increased activation of PRAS40 by 33%. Conversely, exenatide inhibited PRAS40 phosphorylation by 34%. In the TNF-α-stimulated group treated with exenatide, PRAS40 phosphorylation was reduced by 40% compared to the TNF-α group (Fig. 2C). Interestingly, exenatide decreased S6 Ribosomal Protein phosphorylation by 19% in unstimulated HRPE cells, and by 21% in TNF-α stimulated cells compared to TNF-α alone (Fig. 2D).

Members of the MAPK family, SAPK/JNK, ERK 1/2 and p38 were also analyzed. TNF-α stimulation did not change SAPK/JNK activation when compared to control. Treatment with exenatide alone reduced SAPK/JNK activation by 20%. In TNF-α-stimulated group treated with exenatide, SAPK/JNK was reduced by 15% compared to TNF-α only group (Fig. 2E). Bad, a downstream protein of SAPK/JNK signaling pathway was also analyzed. Exenatide (10 nM) significantly reduced Bad phosphorylation levels by 12% in TNF-α stimulated cells and 15% in controls (Fig. 2F). Other members of the MAPK family such as ERK 1/2 and p38 were analyzed, however, no changes in relevant phosphorylation levels were observed when HRPE cells were stimulated with TNF-α in combination with exenatide. Additionally, exenatide decreased p53 phosphorylation levels when compared to control (Supplementary 1). There were no differences between groups for the rest of assayed proteins.

Distinct extracellular matrix metalloproteinases and its inhibitors concentration after exenatide treatment

HRPE cells were stimulated with TNF-α (10 ng/mL), and treated with exenatide (10 nM) for 24 h. Compared to control, treatment with either TNF-α or exenatide did not change MMP-1 expression. TNF-α-stimulated cells treated with exenatide showed reduced MMP-1 concentration by 8% compared to unstimulated control and by 13% compared to TNF-α stimulated group (Fig. 3A). TNF-α and exenatide individually increased MMP-2 levels by 65% and 150%, respectively. In the TNF-α-stimulated group, exenatide significantly increased MMP-2 concentration compared to control by 150% and by 51% compared to TNF-α group (Fig. 3B). MMP-9 levels were markedly elevated upon TNF-α stimulation by 113% compared to control. Furthermore, exenatide reduced MMP-9 levels by 27% and combination of exenatide and TNF-α reduced MMP-9 by 44% compared to TNF-α only group (Fig. 3C).

No changes were observed in both TIMP-1 and TIMP-3 concentration when cells were stimulated with TNF-α or treated with exenatide for 24 h (Fig. 3D and F). Conversely, TNF-α caused a reduction of TIMP-2 by 47%. TIMP-2 levels remained unchanged upon exenatide treatment compared to control. Interestingly,

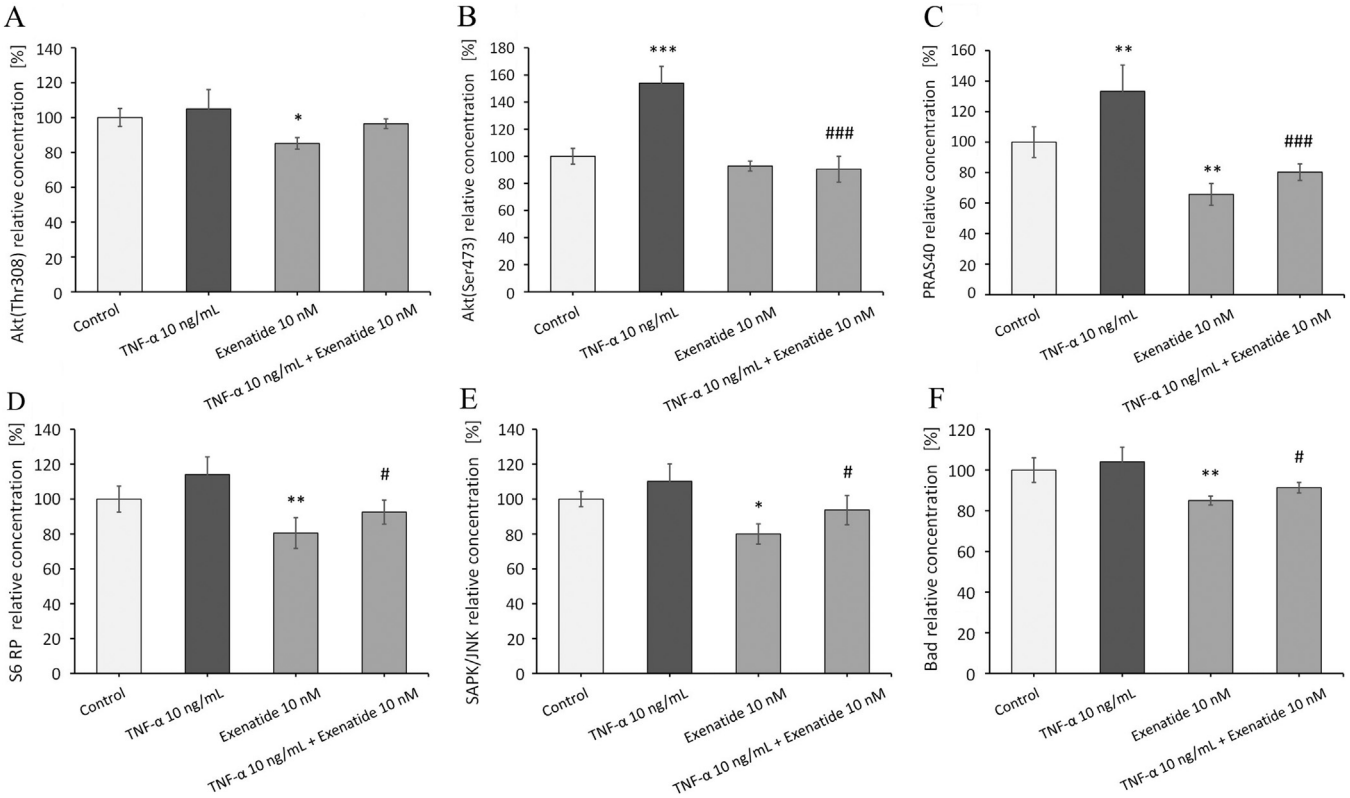

Fig. 2. Phosphorylation of intracellular signaling proteins in HRPE cells stimulated with TNF-α and exenatide. Phosphorylation of Akt(Thr308) (A), Akt(Ser473) (B), PRAS40 (C), S6 RP (D), SAPK/JNK (E) and Bad (F) in HRPE cells stimulated with TNF-α (10 ng/mL) and exenatide (10 nM) after 6 h incubation. Mean values ± SD are shown. n = 4 per group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ control *vs.* different conditions. ANOVA followed with Dunnett's *post-hoc* test. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ TNF-α *vs.* different conditions. One-way ANOVA followed with Scheffe's *post-hoc* test.

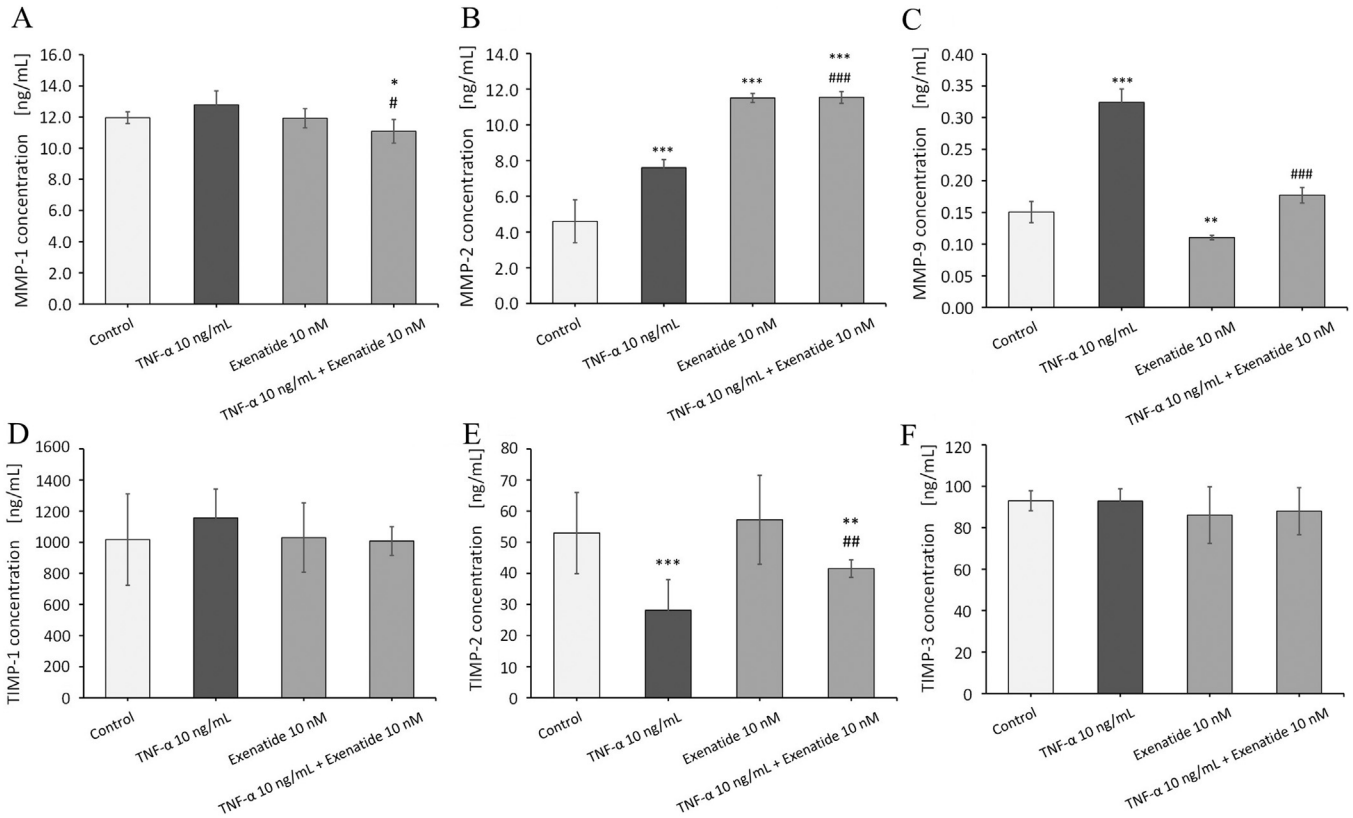

Fig. 3. Production of metalloproteinases and tissue inhibitors of metalloproteinases by HRPE cells stimulated with TNF-α and exenatide. Concentration of MMP-1 (A), MMP-2 (B), MMP-9 (C), TIMP-1 (D), TIMP-2 (E) and TIMP-3 (F) in medium of HRPE cells stimulated with TNF-α (10 ng/mL) and exenatide (10 nM) after 24 h incubation. Mean values ± SD are shown. n = 4 per group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ control *vs.* different conditions. ANOVA followed with Dunnett's *post-hoc* test. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ TNF-α *vs.* different conditions. One-way ANOVA followed with Scheffe's *post-hoc* test.

exenatide partly reversed TNF-α-induced TIMP-2 downregulation by increasing TIMP-2 levels by 50% (Fig. 3E).

Viability, cytotoxicity and apoptosis assays

ApoTox-Glo Triplex assay was performed in HRPE cells stimulated with TNF-α (10 ng/mL), and treated with exenatide (10 nM) for 24 h. TNF-α caused a reduction of HRPE viability by 29%. Compared to control, exenatide partly reversed the negative effects of TNF-α on viability leading to an increasing in viability of 17% (Fig. 4A). Compared to control, incubation with TNF-α increased cytotoxicity by 147%. TNF-α-stimulated cells treated with exenatide showed reduced cytotoxicity actions by 37% compared to TNF-α alone (Fig. 4B). Treatment with exenatide increased cytotoxicity by 67% compared to control. No changes were observed in caspase 3/7 activity when cells were stimulated with TNF-α or treated with exenatide for 24 h (Fig. 4C).

Discussion

Diabetic retinopathy is a major microvascular complication of diabetes that can lead to visual impairment and vascular abnormalities in the retina. Hyperglycemia leads to neurodegeneration (neural apoptosis and reactive gliosis) and neuroinflammation of the blood-retina barrier (BRB) [10]. Oxidative stress, due to excessive production of reactive oxygen species (ROS),

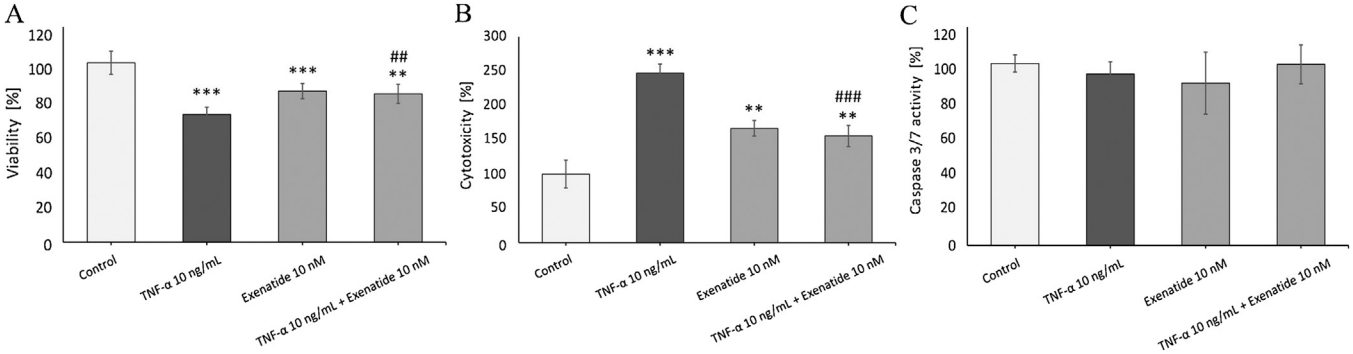

Fig. 4. Cell viability, cytotoxicity and activation of caspase 3/7 in HRPE cells stimulated with TNF-α and exenatide. Cell viability (A), cytotoxicity (B), activation of caspase 3/7 (C), in HRPE cells stimulated with TNF-α (10 ng/mL) and exenatide (10 nM) after 24 h incubation. Mean values ± SD are shown. n = 6 per group. $^{**}p < 0.01$, $^{***}p < 0.001$ control *vs.* different conditions. ANOVA followed with Dunnett's *post-hoc* test. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ TNF-α *vs.* different conditions. One-way ANOVA followed with Scheffe's *post-hoc* test.

contributes to the development of DR. Synthesis of extracellular matrix metalloproteinases contributes to the thickening of the basement membrane and modulation and proliferation of HRPE in DR [41]. Breakdown of the BRB can expose RPE cells to a variety of growth factors, cytokines and neurotransmitters in the subretinal space and in the vitreous, which can trigger the activation, migration, and proliferation of RPE cells leading to continuous ECM remodeling [42]. In the present study, we evaluated the effect of exenatide in HRPE function in the presence of the inflammatory mediators involved in the pathogenesis of DR. Consistent with Puddu and collaborators [10], we detected GLP-1R in HRPE. We also detected DPP-4 protein by western blot. DPP-4 is involved in the degradation of endogenous incretins GLP-1 and GIP. Our study showed that exenatide decreases the activity of DPP-4 in HRPE, after its activation by TNF-α. This effect may be involved in the beneficial action of incretin agonists reported in experimental studies [43]. In a recently published study, topical administration of DPP-4 inhibitors prevented neurodegeneration and vascular leakage in *ex vivo* diabetic retina of both human and mice [44].

Incretins exert glucose-lowering effects by increasing insulin release from pancreatic beta cells in response to a meal. Upon GLP-1 receptor activation, adenylyl cyclase is activated and cAMP generated, leading, in turn, to cAMP-dependent signaling pathways [45]. cAMP-dependent pathway is a signal transduction pathway after activation of GLP-1 receptor in pancreatic beta cells. Forskolin, a natural diterpene, was used as a positive control due to its ability to activate adenylyl cyclase leading to an increase in the intracellular cAMP (EC50 = 5–10 μM). In our study, we observed that both GLP-1 and exenatide stimulation in HRPE cells caused no effects in cAMP levels, suggesting an alternative signaling pathway independent of cAMP. Signaling pathway analysis revealed that exenatide reversed TNF-α-induced Akt phosphorylation at Serine 473. Phosphorylation of PRAS40 by Akt results in its dissociation from mTORC1, leading to release of inhibitory constraints on mTORC1 activity [46]. Exenatide inhibited activation of PRAS40 by Akt, potentially promoting inhibitory actions to mTORC1 and leading to a reduction in S6 protein levels, and therefore protein synthesis and cell proliferation.

Development of DR involves synthesis of growth factors, cytokines, proteases and chemokines such as intercellular adhesion molecule-1 (ICAM-1), monocyte chemo- attractant protein-1 (MCP-1), vascular endothelial growth factor (VEGF), and MMPs. Such factors promote connective tissue growth, neovascularization and fibrosis of the retina [47]. The balance between MMPs and its inhibitors seems crucial for ECM remodeling and DR complications. Hoffmann and collaborators showed that stimulation of HRPE cells with TNF-α leads to increased secretion of MMP-2 and MMP-9 [48]. These metalloproteinases preferentially degrade basement membrane components such as type IV collagen promoting *in vitro* HRPE cell migration. In fact, in HRPE, MMP-9 is a potent angiogenic factor [24,25]. Furthermore, activation of MMP-2 and MMP-9 in retina capillaries induces apoptosis of retinal capillary cells [49]. Exenatide demonstrated potentially beneficial effects on MMP-1 and MMP-9 by reducing their levels after TNF-α stimulation. Additionally, TNF-α induces MMP-9 activation through Akt phosphorylation at Ser 473 [50], which is an indicator of mTORC1 activity. In this study, we showed that exenatide reversed TNF-α-induced MMP-9 activation. TIMP-2 has been shown to have anti-proliferative and anti-angiogenic effects [51]. Stimulation with TNF-α decreases TIMP-2 levels and exenatide partially reversed this effect. Moreover, TIMP-2 concentrations have been shown to

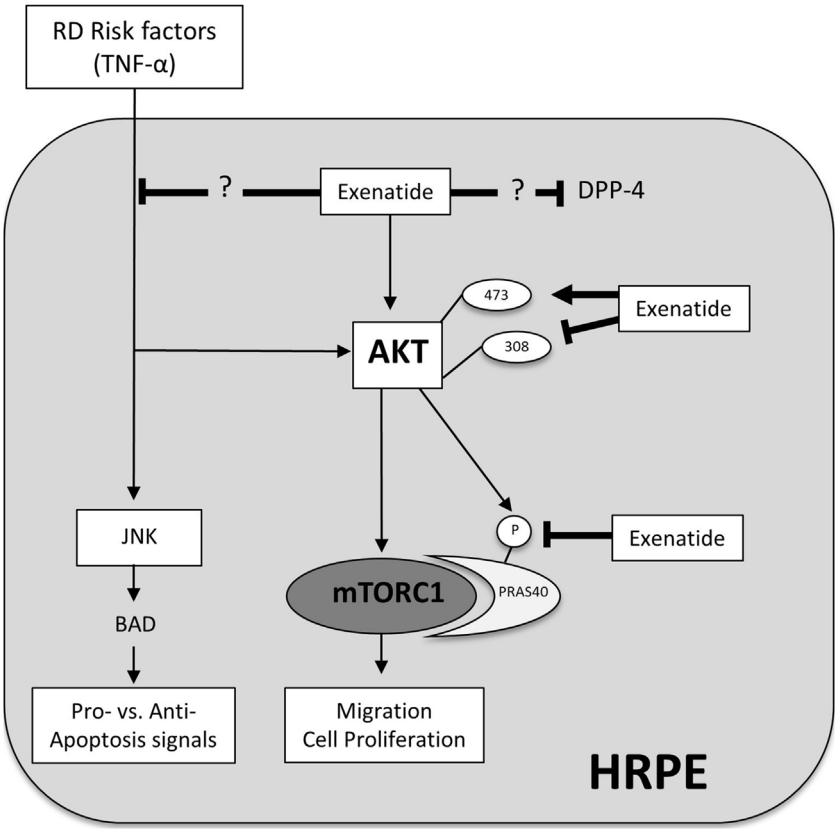

Fig. 5. Model of exenatide and DPP-4 actions in HRPE cells. In cultured RPE cells, exenatide modulates MMPs through the AKT/AMPK signaling pathway. Exenatide inhibits pro-survival mTORC1 activation and PRAS40-AKT-mediated phosphorylation activation. Exenatide inhibits JNK activation modulating Bad-mediated HRPE cell apoptosis.

be markedly different in the vitreous humor of diabetic patients with retinal detachments and diabetic retinopathy [52]. The four main TIMPs share a largely redundant property of broadly blocking MMP-mediated proteolysis by non-covalent binding to the MMP active site in a 1:1 stoichiometric ratio [53]. The pleiotropic effects of incretin-based therapies have been shown to improve the antioxidative potential in cultured human monocytes/macrophages [42,54]. MMP-2 has been shown to be a potent sensitizer for oxidative stress hence and a potential target to prevent the development of DR [55,56]. Noda et al. also demonstrated colocalization of MMP-2 and MMP-9 in endothelial and glial cells of fibrovascular tissues from patients with PDR [56]. Additionally, MT1-MMP and TIMP-2 an activator and an activation-enhancing factor for proMMP-2, respectively also colocalized with MMP-2. The results demonstrated that proMMP-2 is activated in fibrovascular tissues of PDR patients, probably *via* MT1-MMP and TIMP-2 crosstalk. In our study, we observed that TNF-α and exenatide influence of both MMP-2 and TIMP-2 levels in HRPE cells. After treatment with TNF-α - proinflammatory cytokine, exenatide revealed cytoprotective properties to HRPE cells. However, compared to control, exenatide moderately reduced viability and increased cytotoxicity. We have also observed induction of MMP-2 concentration by exenatide. This undesirable effect of incretin agonists may alter ECM remodeling. Although experimental data on animal and human models had suggested a possible protective effect of incretin agonists, the SUSTAIN-6 trial suggested that treatment with semaglutide could be associated with a progression of DR [49]. Modulation of ECM remodeling by the incretin drug exenatide may ameliorate DR-mediated activation of RPE cells and potentially be beneficial in patients with diabetic retinopathy. Further investigations are warranted to clarify the mechanisms underlying the partial inhibitory effect of exenatide by AMPK activation, the role of MMPs during ECM remodeling and concomitant effects of incretin agonist with other anti-diabetic drugs, especially gliptins (DPP-4 inhibitors) on HRPE cells. The main limitation of *in vitro* models requires further corroboration by *in vivo* models and, eventually, clinical trials. Taken together, this data supports the idea that a net overall reduction in matrix breakdown may ameliorate initiation of proliferation of HRPE, hence reducing basement membrane thickening and fibrosis (Fig. 5).

Conflict of interest statement

The authors affirm that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.pharep.2018.10.003.

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