Pigment epithelium-derived factor inhibits advanced glycation end-products-induced cytotoxicity in retinal pericytes

S. Sheikpranbabu, R. Haribalaganesh, S. Gurunathan

Department of biotechnology, division of molecular and cellular biology, Kalasalingam university (Kalasalingam Academy of Research and Education), Krishnankoil-626126, Anand Nagar, Tamilnadu, India

Received 28 October 2010; received in revised form 28 March 2011; accepted 29 March 2011

Available online 1 June 2011

Abstract

Aim. – This study investigated the effects of pigment epithelium-derived factor (PEDF) on advanced glycation end-product (AGE)-induced cytotoxicity in porcine retinal pericytes and the signalling mechanism involved.

Methods. – Retinal pericytes were isolated from porcine eyes and characterized by immunocytochemistry. The effect of AGEs and PEDF on cell proliferation was determined by bromodeoxyuridine (BrdU) assay. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity was analyzed by luminescence assay. Reactive oxygen species (ROS), nitric oxide (NO), superoxide dismutase (SOD) and glutathione peroxidase (GSH) were determined by biochemical assays. Induction of apoptosis was determined by caspase-3 colorimetric assay and DNA fragmentation analysis. Src activity was assessed by transient transfection analysis, and the status of Src phosphorylation at Y419 was analyzed by a competitive ELISA method.

Results. – AGEs significantly increased intracellular ROS generation in pericytes via NADPH oxidase and induced cell death via caspase-3 enzyme activation, whereas PEDF increased cell proliferation in a dose-dependent manner. In addition, PEDF inhibited AGE-induced ROS generation by increasing levels of SOD and GSH, and also blocked the activation of caspase-3. Furthermore, PEDF induced cell survival via the Src pathway by Src phosphorylation at Y419, as evidenced by a pharmacological inhibitor and Src mutants.

Conclusion. – These results suggest that PEDF abrogates AGE-induced oxidative stress and apoptosis in retinal pericytes via the Src pathway, thereby suggesting that PEDF is an effective therapeutic agent for the treatment of loss of pericytes in early diabetic retinopathy.

© 2011 Elsevier Masson SAS. All rights reserved.

Keywords: PEDF; AGE; Pericytes; Src kinase; Oxidative stress; Apoptosis; Diabetic retinopathy

Résumé

Le PEDF (Facteur dérivé de l’ététhélium pigmentaire) inhibe la cytotoxicité des produits de la glycation avancée dans les péricytes rétinien.

Objectif. – Nous avons étudié l’effet du Facteur dérivé de l’ététhélium pigmentaire (PEDF) sur la cytotoxicité induite par les produits de glycation avancée (AGEs) dans des péricytes rétinien porcins et les mécanismes de signalisation impliqués.

Méthodes. – Des péricytes ont été isolés à partir de rétines porcines par méthode enzymatique. Les péricytes rétinien ont été caractérisés par immunocytochimie. Les effets des AGEs et du PEDF sur la prolifération cellulaire ont été déterminés par dosage de la bromodésoxyuridine-uridine. L’activité NADPH a été déterminée par luminescence, les espèces réactives de l’oxygène (ROS), le monoxyde d’azote (NO), la superoxyde dismutase (SOD) et la glutathion peroxydase (GSH) par dosages biochimiques, l’induction de l’apoptose par dosage colorimétrique de la caspase-3 et analyse de la fragmentation de l’ADN, l’activité Src par analyse de transfection transitoire et le statut de phosphorylation Src en Y419 par dosage immuno-enzymatique compétitif (ELISA).

Résultats. – Dans les péricytes, les AGEs augmentent significativement la production intracellulaire de ROS via la NADPH oxydase et la mort cellulaire induite par l’activation de la caspase-3, alors que le PEDF augmente la prolifération cellulaire de manière dose-dépendante. Le PEDF inhibe la production de ROS induite par les AGEs en augmentant les concentrations de SOD et de GSH et en bloquant l’activité caspase-3. En outre, le PEDF induit la survie cellulaire par la voie Src par phosphorylation Src en Y419, mis en évidence par inhibition pharmacologique et les mutants de Src.

1. Introduction

Pericyte loss has been considered a hallmark of the early stages of diabetic retinopathy (DR), as the retinal capillary pericytes undergo premature death, possibly by apoptosis [1,2]. Hyperglycaemia plays a key role in the initiation as well as the progression of DR, which is also associated with several other biochemical abnormalities, including an increase in oxidative stress, non-enzymatic glycation of proteins and activation of the polyl pathway [3,4]. Reducing sugars react non-enzymatically with amino groups in proteins through the Maillard reaction by forming Schiff bases and Amadori products to produce advanced glycation end-products (AGEs) in an irreversible manner [5,6]. AGEs interact with a receptor for AGE (RAGE) that causes apoptotic cell death in retinal pericytes, and are thus involved in the pathogenesis of the early phase of DR [7,8]. However, the underlying molecular mechanism of AGE-induced pericyte loss has yet to be elucidated.

Pigment epithelium-derived factor (PEDF), a 50-kDa molecule secreted by the retinal pigment epithelial cells detected in both the aqueous and vitreous humors [9], has been reported to be an inherent antiangiogenic molecule [10], the ocular level of which is reduced in angiogenic-related diseases. Recently, PEDF was reported to inhibit AGE-induced vascular cell injury by suppressing the generation of reactive oxygen species (ROS) during retinal angiogenesis and hyperpermeability [11] in endothelial cells (EC). In addition, PEDF was also able to protect retinal pericytes against the deleterious effects of AGE-induced ROS generation [12]. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is a membrane-bound enzyme complex identified in both phagocytes and non-phagocytes. A relatively low level of NADPH oxidase is produced during conditions of ROS under basal conditions in response to high glucose conditions, thereby generating higher levels of oxidants. However, the protective mechanism of PEDF on AGE-modified bovine serum albumin (AGE–BSA)-induced oxidative stress in retinal pericytes has still not been elucidated.

The present study aimed to explore the antioxidative properties of PEDF during conditions of AGE–BSA-induced oxidative stress and apoptosis in porcine retinal pericytes (PRPCs). The molecular mechanism through which PEDF exerts these protective effects in retinal pericytes was also investigated. The results obtained from this study may offer greater insights into understanding the protective role of PEDF against early DR by attenuating the deleterious effects of AGEs, and may lead to the recognition of PEDF as a potentially important pharmacological agent in the treatment of retinal diseases, such as DR, characterized by pericyte loss.

2. Materials and methods

2.1. Isolation and characterization of PRPCs

PRPCs were isolated and characterized as described previously [13,14], but with slight modifications. They were grown in Iscove’s Modified Dulbecco’s Medium (IMDM) supplemented with 5% fetal bovine serum (FBS) and incubated at 37°C in 5% CO2. The PRPCs were characterized using mouse anti-α-smooth muscle actin (α-SMA; Sigma-Aldrich, St. Louis, MO, USA), and other cell-type contaminations were examined using mouse anti-CD31 (Chemicon, Temecula, CA, USA) for ECs. Donkey anti-mouse IgG (Cy3-conjugated) were used as secondary antibodies. Co-staining with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) was performed to stain the nuclei and to confirm cell purity. For the experiments, the growth medium was replaced by 0.5% FBS containing various concentrations of AGE–BSA and/or PEDF, and incubated at 37°C in 5% CO2 for 24 h. The morphological changes from each set of experiments were observed using a phase-contrast microscope. Total cell counts were determined by DAPI staining, using fluorescence image analysis (Carl Zeiss AG, Germany).

2.2. Bromodeoxyuridine (BrdU) ELISA cell proliferation assay

PRPC proliferation was assessed using a bromodeoxyuridine (BrdU) enzyme-linked immunosorbent assay (ELISA) colorimetric kit (Roche Applied Science, Germany). The pericytes were seeded at a density of 2 × 103 cells per well into 96-well plates. After 12 h, the cells were serum-starved (0.5% FBS) for 5 h, and then treated with various concentrations of AGE–BSA (BioVison, Mountain View, CA, USA), PEDF (Abcam, Cambridge Science Park, UK), PP2 (Src kinase inhibitor) and BrdU labeling reagent (10 μM). Following 24 h of incubation, cell proliferation was assessed according to the manufacturer’s instructions. Absorbance of the samples was measured, using a microplate reader, at 450 nm.

2.3. Transient transfection and phospho-Src assay

Transient transfection assay of the PRPCs was performed, using a Nucleofector II Device (Program P-13; Amaxa Biosystems, Cologne, Germany), as reported previously [15]. Briefly, cells were resuspended in the nucleofector solution, made to a final concentration of 4–5 × 105 cells/100 μL, after which 1–3 μg of plasmid DNA, encoding constitutively active Src (CA...
Src) or dominant negative Src (DN Src) mutants, was added and subjected to electroporation. Later, the transfected cells were resuspended in IMDM, and incubated in 5% CO₂ at 37°C. Phospho-Src was quantified, using a human phospho-Src (Y419) ELISA kit, as described previously [15]. In brief, the samples were loaded at a volume of 100 \( \mu \)L into each well of a 96-well microplate coated with phospho-Src capture antibody, and incubated for 2 h at room temperature. The procedure was followed as per the manufacturer’s instructions, with absorbance measured at 450 nm.

2.4. Caspase-3 and DNA fragmentation assay

CPP32 activity and DNA fragmentation were performed as described elsewhere [14]. Briefly, cells were lysed, using the lysis buffer, and kept on ice for 15–20 min. The cell lysates were incubated at 37°C with the caspase substrate and assay buffer for 2 h. Absorbance was measured at 405 nm. For DNA fragmentation, DNA from all samples underwent electrophoresis on a 1% agarose gel and then photographed.

2.5. NADPH oxidase and ROS assay

NADPH oxidase activity was analyzed based on superoxide-induced lucigenin photoemission, as described previously [11]. Briefly, PRPCs were treated with AGE–BSA and/or PEDF for 24 h, following which the cells were then suspended in homogenization buffer. In some experiments, the NADPH oxidase inhibitor diphenylene iodinium (DPI, 100 \( \mu \)mol/L) was added, and the activity determined by luminescence assay. ROS generation was analyzed using the nitrotetrazolium blue (NBT) reduction assay, as described elsewhere [16]. Briefly, cells were incubated with 0.1 mM of NBT along with 50% (v/v) acetic acid for 1 h in IMDM, and the absorbance read at 595 nm.

2.6. NO, GSH and SOD assays

Nitric oxide (NO) generation was measured, using Griess reagent, as described previously [4]. Briefly, 300 \( \mu \)L of cell extract was added, with an equal volume of 1% sulphanilamide and 1% N-(1-naphthyl)-ethylenediamine solution, and incubated at 37°C for 10 min; absorbance was read at 540 nm. Concentration of NO generation was extrapolated using an NaNO₂ standard graph. Glutathione peroxidase (GSH) was measured according to the method of Sedlack and Lindsay [17]. Briefly, cell lysate was mixed with 0.02 M of EDTA and 50% trichloroacetic acid (TCA), then centrifuged at 500 \( \times \) g for 15 min at 4°C. The supernatant was mixed with 0.01 M of 5,5’-dithiobis-(2-nitrobenzoic acid), and absorbance measured at 412 nm. Superoxide dismutase (SOD) was measured as described previously [16]. Briefly, cell extract was added to a reaction mixture containing 1 mM of NBT and 0.2 mM of CuCl₂, then incubated for 20 min at 25°C, and the absorbance measured at 560 nm. Enzyme activity was expressed as a percentage, using 0.5% FBS as a control.

2.7. Statistical analysis

All results were expressed as means ± standard error of mean (SEM) values. Statistically significant differences were evaluated using paired two-tailed Student’s t test for comparison with the control. A level of \( P < 0.05 \) was considered significant.

3. Results

3.1. Isolation and characterization of PRPCs

Following isolation, the migration of cells from microvessel fragments began 2–3 days after plating. The PRPCs appeared to be amorphous with extensive cytoplasmic processes. Subconfluent pericytes grew individually and did not readily make contact with other cells. On day seven, a dense, young colony of cells was observed. Elimination of other perivascular cells in subculture was done using the trypsinization method, as described elsewhere [18]. To confirm cell identity, co-immunostaining was performed, using α-SMC along with the nuclear stain DAPI. In addition, purity of the pericytes was analyzed using the astrocyte marker GFAP (glial fibrillary acidic protein), and endothelial cell marker VE-cadherin and CD31 (Fig. S1; see supplementary material associated with this article online). No contamination of astrocytes nor dedifferentiation of ECs was observed.

3.2. PEDF protects AGE–BSA-induced cell death in PRPCs

On microscopy, the proportion of dead cells was increased in the AGE–BSA-treated plates, whereas pericytes appeared to be healthy in all PEDF-treated plates No morphological changes were observed in any of the AGE–BSA and PEDF plates combined. DAPI fluorescence images of PRPCs treated with AGE–BSA showed apoptotic nuclei in the periphery, whereas the PEDF-treated plates with and without AGE–BSA showed no apoptotic nuclei (Fig. S2; see supplementary material associated with this article online). As PEDF (20 and 50 nM) induced effective cell survival and DNA synthesis, the same concentrations were used to analyse the combined effects with optimized concentrations of AGE–BSA (50 and 100 \( \mu \)g; (Fig. S3; see supplementary material associated with this article online). Fig. 1A shows the cell survival of PRPCs incubated with a combination of AGE–BSA and PEDF. The addition of either 20 nM of PEDF with 50 \( \mu \)g/mL of AGE–BSA or 50 nM of PEDF with 100 \( \mu \)g/mL of AGE–BSA significantly inhibited cell death compared with AGE–BSA treatment alone (0.395 ± 0.021 vs. 0.161 ± 0.0430 and 0.474 ± 0.014 vs. 0.1152 ± 0.049, respectively; \( n = 3; P < 0.05 \)). Cell survival was enhanced by the presence of PEDF in a medium containing high concentrations of AGE–BSA. These data suggest that PEDF partially inhibited AGE–BSA-induced cytotoxicity in PRPCs.

3.3. PEDF modulates PRPC proliferation via an Src-dependent pathway

To confirm the role of Src kinase in the regulation of the cell proliferation pathway, PRPCs were preincubated with the
S. Sheikpranbabu et al. / Diabetes & Metabolism 37 (2011) 505–511

Fig. 1. Results of the BrdU ELISA cell proliferation and Src phosphorylation assays. Porcine retinal pericytes (PRPCs) treated with pigment epithelium-derived factor (PEDF) with and without advanced glycation end-product-modified bovine serum albumin (AGE–BSA) and 10 μM PP2 (Src inhibitor) reveal that both AGE–BSA (A) and PP2 (B) significantly blocked PEDF-induced DNA synthesis; (C) DN Src significantly blocked PEDF-induced proliferation whereas CA Src had an additive effect following PEDF treatment compared with wild-type PRPCs; (D) PEDF significantly increased Src phosphorylation compared with the control. Both AGE–BSA and PP2 significantly decreased PEDF-induced Src phosphorylation. Data are presented as means ± SEM, and represent the results obtained from three identical independent experiments (n = 3; *P < 0.05 vs. control, **P < 0.01 vs. control).

Src kinase inhibitor PP2 before PEDF treatment. In addition, cells were transfected with CA Src and DN Src, and the effects of PEDF and AGE–BSA on cell proliferation measured. It is noteworthy that preincubation of pericytes with PP2 totally abrogated the effect of PEDF on cell survival, decreasing cell survival to 2.5-fold compared with the 20-nM and 50-nM PEDF treatments (Fig. 1B; 0.192 ± 0.084 and 0.245 ± 0.077; n = 3; P < 0.05). DN Src significantly reduced PRPC proliferation even during treatment with PEDF compared with the control.

In contrast, transfection of CA Src led to a substantial increase in the number of viable cells, and stimulation of these PRPCs with PEDF treatment had an additive effect on cell proliferation (Fig. 1C). Furthermore, on measuring the status of Src phosphorylation at Y419, AGE–BSA had decreased Src phosphorylation whereas PEDF treatment had significantly increased phosphorylated Src (Y419) in comparison to control (0.87 ± 0.089 vs. 0.41 ± 0.032; n = 3; P < 0.05). The increased phospho-Src (Y419) after PEDF treatment was significantly decreased by preincubation with AGE–BSA and PP2 (Fig. 1D). Thus, it can be concluded that PEDF directly induces Src phosphorylation.

3.4. PEDF inhibits AGE–BSA-induced apoptosis in PRPCs

To confirm whether or not AGE–BSA induces apoptosis in PEDF-protected cells, PEDF treatment was carried out with and without AGE–BSA. Fig. 2A shows that AGE–BSA induced an increase in caspase-3 activity, whereas PEDF inhibited the AGE–BSA-induced caspase increase. The apoptosis induced by AGE–BSA was also inhibited by pretreatment for 30 min with the caspase-3-specific inhibitor Ac-DEVD-CHO (100 nM/mL). Fig. 2B shows the DNA isolated from PRPCs treated with AGE–BSA and PEDF for 24 h. DNA from the AGE–BSA treated plates (Lane 2) had the typical smudged ladder pattern of internucleosomal fragmentation, while such a pattern was not observed in PRPC plates treated with PEDF (Lanes 3 and 4) in both the presence and absence of AGE–BSA (Lanes 5 and 6). These results suggest that PEDF inhibits AGE–BSA-induced apoptosis in PRPCs.
3.5. PEDF inhibits AGE–BSA–induced oxidative stress in PRPCs

AGE–BSA treatment significantly raised NADPH oxidase activity and ROS generation compared with BSA alone at 12 h, whereas increased NADPH oxidase and ROS in pericytes following AGE–BSA (100 μg) treatment was significantly decreased by preincubation with DPI inhibitor (100 μM) and PEDF (50 nM) treatments (Fig. 2C and D). Similarly, PEDF treatment significantly reduced NO generation in both the presence and absence of AGE–BSA (Fig. S4A; see supplementary material associated with this article online). The results confirm that PEDF significantly inhibited AGE–BSA–induced NADPH oxidase activity, ROS and NO generation. Furthermore, PEDF significantly protected against AGE–BSA–induced cell death by increasing levels of GSH and SOD activity (Fig. S4B; see supplementary material associated with this article online). This finding shows that PEDF suppresses AGE–BSA–induced oxidative stress.

4. Discussion

In the present study, it was demonstrated that PEDF significantly inhibited AGE–BSA–induced ROS generation and the subsequent decrease in DNA synthesis, as well as apoptosis in retinal pericytes. Pericytes are known to regulate growth together with preserving prostacyclin-producing ability, thereby protecting against lipid-peroxide-induced injury of co-cultured ECs. These features play a vital role in the maintenance of microvascular homoeostasis. In addition, the effective role of glyceraldehyde (glycer–AGE) and glycolaldehyde (glycol–AGE) to induce apoptotic cell death of pericytes through interaction with RAGE and induction of VEGF has already been elucidated [12]. The results of our present study reveal the ability of AGE–BSA to affect cell survival as well as the generation of ROS and NO, leading to apoptosis. Such generation of ROS as a result of hyperglycaemia may be increased via activation of AGE-sensitive cell surface receptors such as RAGE [19] or non-receptor-dependent pathways, leading to activation...
of MAP kinase/Ras and nuclear factor (NF)-κB, as well as the Akt and p38 pathways, which can promote both inflammatory responses and cellular apoptosis [20]. Our present results show that ROS derived from NADPH oxidase is involved in caspase-3-mediated apoptosis of pericytes induced by AGE–BSA.

PEDF has been shown to be a potent putative inhibitor of angiogenesis that is seven times as potent as endostatin and more than two times as potent as angiostatin [10]. Recently, it was reported that PEDF generates the antioxidative signal through inactivation of Bax expression in retinal pericytes. Thus, to validate PEDF as a potent antioxidant molecule, we studied the effect of PEDF on AGE–BSA-induced cytotoxicity action in PRPCs. Consistent with previous observations [12], our present data have demonstrated the antioxidative effects of PEDF on deleterious AGE–BSA-induced effects in PRPCs. In addition, we observed that PEDF alone inhibits ROS and NO generation, thereby returning the antioxidant system to normal by bringing the levels of antioxidant enzymes, such as GSH and SOD, back to normal under conditions of oxidative stress.

In the present study, the effective role of NADPH in the generation of increased AGE–BSA-induced ROS was also demonstrated. The results obtained corroborate the relative dependence of Src phosphorylation on the activation of NADPH oxidase for the generation of ROS and activation of caspase enzyme, leading to an apoptosis that is cell-specific. Previous studies have reported on the antiangiogenic properties of PEDF in retinal ECs by inhibiting cell survival [14] via the PI3 K/Akt pathway. However, in the present study, the other property of PEDF that exhibits protective effects through antioxidative functions has been demonstrated. These effects may be due to the fact that the PEDF molecule is specific to the cell types and conditions induced. As previous reports describing the antiangiogenic properties of PEDF were carried out under growth-factor-mediated conditions, the present study analyzed the effect of PEDF under AGE–BSA-induced conditions during hyperglycaemia.

The pro-survival function of growth-factor signalling occurs through various signalling pathways, including the Src pathway that is interrelated with oxidative stress during conditions of hyperglycaemia. In the present study, the generation of ROS through activation of NADPH and NO in pericytes induced with AGE–BSA were protected against cell damage and apoptosis by PEDF through Src phosphorylation. Hence, our present study demonstrates the ability of PEDF to activate Src that, in turn, leads to the prevention of AGE-induced apoptosis, thereby protecting pericytes by enhancing antioxidants. Taken altogether, these findings support the argument that PEDF possesses therapeutic antioxidative properties in addition to its antiangiogenic and antiproliferative properties. Such a combination of effects indicates that PEDF can be potentially harnessed as a therapeutic agent for complications related to pericyte loss.

**Disclosure of interest**

The authors declare that they have no conflicts of interest concerning this article.

**Acknowledgements**

This study was supported by DBT (No. BT/PR/7704/MED/14/1065/2006), DST (No. SR/FT/L-118/2006) and UGC (No. F.10-4/2005/SA-I) in New Delhi, India. We thank Umamaheshwaran and Sriram for the critical reading of this manuscript.

**Appendix A. Supplementary data**

Supplementary material (Figs. S1–S4) associated with this article can be found at http://www.sciencedirect.com, at doi:10.1016/j.diabet.2011.03.006.

**References**


