Pigment epithelium-derived factor inhibits advanced glycation end product-induced proliferation, VEGF and MMP-9 expression in breast cancer cells via interaction with laminin receptor

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Abstract. Pigment epithelium-derived factor (PEDF) is one of the adipocytokines with multifaceted functions, which may serve a role in the development of various types of cardiometabolic disorders. Advanced glycation end products (AGEs) have been shown to contribute to numerous aging-associated disorders, such as cancer. However, it remains unclear whether and how PEDF exerts antitumor effects in AGE-exposed human breast cancer MCF-7 cells, and therefore this was explored in the present study. NADPH oxidase activity was measured with luciferase assay, while gene and protein expression levels were evaluated with quantitative PCR and western blot analysis, respectively. AGEs significantly increased NADPH oxidase-driven superoxide generation, cytochrome b-245 β chain (gp91phox) and receptor for AGE (RAGE) mRNA expression, proliferation, mRNA and protein expression levels of vascular endothelial growth factor (VEGF), and matrix metalloproteinase (MMP)-9 mRNA expression in MCF-7 cells, all of which were dose-dependently inhibited by PEDF. Neutralizing antibody against laminin receptor (LR-Ab) significantly blocked these beneficial effects of PEDF in AGE-exposed MCF-7 cells. Furthermore, as in AGE-treated cells, PEDF dose-dependently inhibited the NADPH oxidase-driven superoxide generation, gp91phox, RAGE and MMP-9 mRNA expression, proliferation, mRNA and protein expression levels of VEGF in non-treated control MCF-7 cells, and these effects were also reversed by LR-Ab. LR levels were not affected by the treatment with AGEs, PEDF or LR-Ab. The present study suggested that PEDF may exert antitumor effects in AGE-exposed breast cancer cells by suppressing NADPH oxidase-induced ROS generation and VEGF and MMP-9 expression via interaction with LR. Since PEDF expression is decreased in breast cancer tissues, pharmacological upregulation or restoration of PEDF may inhibit the growth and metastasis of breast cancer.

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Introduction

Pigment epithelium-derived factor (PEDF), a serine protease inhibitor, is first identified from conditioned medium of retinal pigment epithelium and now recognized as one of the adipocytokines with multifaceted functions (1-11); it not only promotes neuronal cell differentiation, but also inhibits pathological angiogenesis and suppresses inflammatory and thrombotic reactions through its anti-oxidative properties in numerous cell culture and animal models (1-11). Moreover, we, along with others, have shown that decreased expression levels of PEDF in tumor tissues are associated with growth expansion, aggressiveness, and metastasis in various types of tumors, such as breast cancer and colorectal cancer, thereby being a poor prognostic marker in tumor-bearing patients (12-20). Since overexpression of PEDF or administration of PEDF-derived peptides have been shown to inhibit the growth and metastasis of a variety of tumors, including breast cancer in animal models, PEDF may be a novel therapeutic target for breast cancer (12-20).

Non-enzymatic modification of amino groups of proteins, lipids, and nucleic acids by sugars has progressed under diabetic conditions, which could alter their structural and functional properties via formation and accumulation of aging molecules called advanced glycation end products (AGEs) (21-25). Accumulating evidence has suggested the pathological involvement of AGEs in aging-related diseases, such as cancer, cardiovascular disease, diabetes, osteoporosis, Alzheimer's

disease (21-25). Indeed, we have previously found that AGEs stimulate proliferation and gene expression of vascular endothelial growth factor (VEGF) in MCF-7 human breast cancer cells, which may contribute to the tumor expansion and metastasis (26). Although PEDF exerted anti-angiogenic effects on endothelial cells through the interaction of a non-integrin laminin receptor (LR) (11), it remains unclear whether and how PEDF could inhibit the AGE-induced growth and VEGF expression in MCF-7 breast cancer cells. In this study, we addressed the issue.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM) and MCF-7 human breast cancer cells were obtained from Sigma-Aldrich; Merck KGaA and American Type Culture Collection, respectively. Neutralizing monoclonal antibody (Ab) raised against LR (clone MLuC5; LR-Ab) was purchased from Abcam (cat. no. ab3099). Normal mouse IgG was purchased from WAKO Pure Chemical Co. (cat. no. 140-09511).

Preparation of PEDF proteins. PEDF proteins were purified as described previously (27). SDS-PAGE analysis of purified PEDF proteins revealed a single band with a molecular mass of about 50-kDa, which showed positive reactivity with monoclonal Ab raised against human PEDF (TransGenic Inc.; cat. no. KM037).

Preparation of AGEs. AGEs were prepared as described previously (28). In brief, BSA (25 mg/ml) was incubated under sterile conditions with 0.1 M glyceraldehyde in 0.2 M $NaPO_4$ buffer (pH 7.4) for 7 days. Then unincorporated sugars were removed by PD-10 column chromatography and dialysis against phosphate-buffered saline. Control non-glycated BSA was incubated in the same conditions except for the absence of reducing sugars.

Cells. MCF-7 cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum. MCF-7 cells were treated with or without 100 μg/ml non-glycated BSA or AGE-modified BSA (AGEs) in the presence or absence of the indicated concentrations of PEDF, 5 μg/ml LR-Ab, or 5 μg/ml mouse IgG in DMEM with 1% fetal bovine serum for 24 h.

NADPH oxidase activity. NADPH oxidase activity of MCF-7 cells was measured by luminescence assay as described previously (6).

Reactive oxygen species (ROS) generation. Intracellular ROS production in MCF-7 cells was measured with a fluorescent probe, 5-(and-6)-carboxy-2',7'-difluorodihydrofluorescein diacetate (carboxy-H_2DFFDA) purchased from Thermo Fisher Scientific (29).

Real-time reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from cells with NucleoSpin RNA Plus (Takara Bio Inc.) according to the manufacturer's instructions. cDNA was obtained using the PrimeScript RT reagent kit (Takara Bio, Inc.). Quantitative real-time RT-PCR was performed using Assay-on-Demand and TaqMan 5 fluorogenic nuclease chemistry (Applied Biosystems) according to the supplier's recommendation. IDs of primers for human cytochrome b-245 α chain (p22phox), cytochrome b-245 β chain (gp91phox), receptor for AGE (RAGE), VEGF, matrix metalloproteinase-9 (MMP-9) and β-actin gene were Hs00609145_m1, Hs00166163_m1, Hs00542592_g1, Hs00900055_m1, Hs00234579_m1 and Hs99999903_m1 respectively. Expression levels of RAGE, VEGF, MMP-9 and β-actin were measured using the $2^{-\Delta\Delta Cq}$ method. Data were normalized by the intensity of internal control β-actin-derived signals. ΔCq and ΔΔCq values were calculated using the following mathematical formulas: ΔCq=Cq(p22phox/gp91phox/RAGE/VEGF/MMP-9)-Cq(β-actin), and ΔΔCq=ΔCq(target sample)-ΔCq(control sample).

Cell proliferation. Cell proliferation was measured with a Cell Proliferation Reagent WST-1 by measuring the absorbance at wavelength 450 nm according to the supplier's recommendations (Darmstadt, Germany).

Measurements of VEGF. VEGF levels in MCF-7 cell culture medium were measured with an enzyme-linked immunosorbent assay kit (Proteintech Group, Inc.).

Western blot analysis. Proteins were extracted from MCF-7 cells using lysis buffer (27). The samples were then separated by SDS-PAGE and transferred to nitrocellulose membranes (Life Technologies Japan, Ltd.). Membranes were probed with LR-Ab (1:100 dilution; Santa Cruz Biotechnology, Inc.; cat. no. sc-20979) for 12 h at room temperature, and then incubated with peroxidase-conjugated polyclonal donkey anti-rabbit IgG Ab (1:20,000 dilution; GE Healthcare UK Ltd.; cat. no. NA934-100UL). Tubulin was visualized with peroxidase-conjugated anti-α-tubulin antibody (1:10,000 dilution; Abcam; cat. no. ab40742). Immune complexes were visualized using an enhanced chemiluminescence detection system (Amersham Bioscience). Protein signals were quantified using ImageJ software (version 1.53; National Institutes of Health, Bethesda, MD, USA). Expression levels of LR were normalized by those of α-tubulin.

Statistical analysis. All values were presented as mean ± standard deviation. Post hoc comparison of means was carried out using Tukey's honestly significant difference test after one-way ANOVA by R software (version 4.0.3. The R Foundation for Statistical Computing Platform). $P<0.05$ was considered significant.

Results

We first examined the effects of PEDF on NADPH oxidase activity, mRNA levels of p22phox and gp91phox, two membrane components of NADPH oxidase, ROS generation, and RAGE gene expression in AGE-exposed MCF-7 cells. As shown in Fig. 1A-C, compared with non-glycated BSA, AGE-modified BSA (AGEs) significantly increased NADPH oxidase activity, gp91phox mRNA levels, and ROS generation in MCF-7 cells, all of which were dose-dependently inhibited by the treatment

with PEDF. Furthermore, the inhibitory effects of 100 nM PEDF on NADPH oxidase activity, gp91phox mRNA levels, and ROS generation in AGE-exposed MCF-7 cells were significantly blocked by LR-Ab (Fig. 1D-F). As is the case in NADPH oxidase activity, gp91phox mRNA levels, and ROS generation, PEDF at 100 nM significantly suppressed the AGE-induced up-regulation of RAGE mRNA level in MCF-7 cells, which was also inhibited by LR-Ab (Fig. 1G and H). LR-Ab alone did not affect NADPH oxidase activity, mRNA levels of p22phox and gp91phox, ROS generation, or RAGE gene expression in non-glycated BSA-exposed MCF-7 cells.

AGEs significantly stimulated proliferation of MCF-7 cells, which was suppressed by PEDF in a dose-dependent manner (Fig. 1I). LR-Ab blocked the growth inhibitory effects of 100 nM PEDF in AGE-exposed MCF-7 cells (Fig. 1J). Although western blot analysis revealed that LR was actually expressed in MCF-7 cells, expression levels of LR were not changed by the treatment of AGEs, 100 nM PEDF, or LR-Ab (Fig. 1K). We have already confirmed that PEDF directly binds to LR, which is significantly blocked by LR-Ab (27).

We next examined the effects of PEDF on VEGF and MMP-9 expression in AGE-exposed MCF-7 cells. As shown in Fig. 2A and B, VEGF gene and protein expression were significantly stimulated by AGEs, which were inhibited by PEDF in a dose-dependently manner. LR-Ab significantly blocked the effects of PEDF in VEGF expression in MCF-7 cells exposed to AGEs (Fig. 2C and D). Furthermore, PEDF dose-dependently inhibited the AGE-induced increase in MMP-9 gene expression in MCF-7 cells (Fig. 2E). LR-Ab also significantly blocked the PEDF-induced MMP-9 gene suppression in AGE-exposed MCF-7 cells (Fig. 2F).

We further investigated the effects of PEDF in NADPH oxidase activity, mRNA levels of p22phox and gp91phox, ROS generation, RAGE, VEGF, and MMP-9 expression, and proliferation in MCF-7 cells not exposed to BSA or AGEs. As is the case in non-glycated BSA- or AGE-exposed MCF-7 cells, PEDF dose-dependently inhibited the NADPH oxidase activity, gp91phox mRNA levels, ROS generation, RAGE mRNA levels, proliferation, VEGF gene and protein expression, and MMP-9 mRNA levels in MCF-7 cells (Figs. 3, 4A, B and E). LR-Ab significantly blocked all these effects of 100 nM PEDF in MCF-7 cells (Figs. 3 and 4C, D and F). LR expression levels were not affected by PEDF or LR-Ab (Fig. 4G).

Discussion

Breast cancer is the most common cancer in women all over the world, accounting for 10-15% of all cancer deaths in developed countries (30,31). There is a growing body of evidence to show the clinical link between diabetes and breast cancer (20,32,33). Diabetes is associated with the higher incidence and more advanced stage of breast cancer, thereby increasing the mortality rate in these patients compared with non-diabetic individuals (32,33). Moreover, we, along with others, have shown the pathological role of AGEs, which are formed during a physiological aging process and at an accelerated rate under diabetic conditions, in the development and progression of breast cancer (34-37). Indeed, AGEs have been reported to stimulate proliferation, migration, invasion, and VEGF gene expression in cultured human breast cancer cells in association with tamoxifen resistance (26). Moreover, dietary intake of AGEs is associated with the increased risk of breast cancer in two independent cohort studies (38,39). These are reasons why we focused on the effects of PEDF on AGE-exposed human breast cancer cells.

In the present study, we found that AGEs significantly increased NADPH oxidase activity, gp91phox mRNA levels, ROS generation, RAGE gene expression, proliferation, gene and protein expression of VEGF, and MMP-9 mRNA levels in cultured breast cancer cells, all of which were dose-dependently inhibited by the treatment with PEDF. Furthermore, these beneficial effects of PEDF on AGE-exposed MCF-7 breast cancer cells were significantly blocked by neutralizing LR-Ab. In addition, PEDF exerted similar anti-tumor effects in non-glycated BSA- or AGE-exposed MCF-7 cells, whose actions were also inhibited by LR-Ab. VEGF and MMP-9 are crucial factors for tumor growth and invasion, respectively, whose expression levels are associated with breast cancer progression and metastasis (40-42). Given that PEDF expression levels are decreased in breast cancer tissues (15,20), our present study suggests that pharmacological up-regulation or restoration of PEDF may inhibit the growth and metastasis of breast cancer via the interaction with LR in two distinct pathways; one is a direct inhibition of tumor growth, and the other is the suppression of VEGF and MMP-9 expression, which could lead to attenuate tumor angiogenesis, invasion, and metastasis.

We have previously shown that AGEs stimulate growth and VEGF expression in both endothelial cells and malignant melanoma cells by inducing the ROS generation through the interaction with RAGE (29,43). Oxidative stress and redox-sensitive transcriptional factor are involved in VEGF and MMP-9 gene expression in various kinds of cells, including tumor cells (29,43-46). Moreover, p66ShcA has recently been shown to play a role in breast cancer metastasis, while quercetin, an anti-oxidant suppresses the mobility as well as VEGF and MMP-9 expression of breast cancer cells (47,48). Since PEDF has been reported to attenuate the AGE-RAGE-induced proliferative, inflammatory and thrombotic reactions in a variety of cells and tissues through the suppression of NADPH oxidase-driven superoxide generation (6,11), the present findings suggest that PEDF may exert anti-tumor effects on AGE-exposed breast cancer cells by suppressing the NADPH oxidase-induced ROS generation via interaction with LR via down-regulation of gp91phox mRNA levels. In support of our speculation, we have found previously that (1) LR-Ab actually inhibits the binding of PEDF to LR and resultantly restores VEGF mRNA levels in PEDF-exposed myeloma cells and (2) PEDF inhibits tumor necrosis factor-α-induced inflammatory reactions in endothelial cells via inhibition of NADPH oxidase activity through the interaction of *ca*. 60-kDa receptor, which is considered to be LR (16,49). Taken together, although the anti-tumorigenic ability and the anti-invasiveness are different aspects, suppression of oxidative stress by PEDF may connect these two phenomena. We did not know the exact reason why our present results contradicted the effect of PEDF in hepatocellular carcinoma cells; PEDF was found to play a role in metastasis and angiogenesis in this cell type (50). However, there is accumulating evidence that decreased expression levels

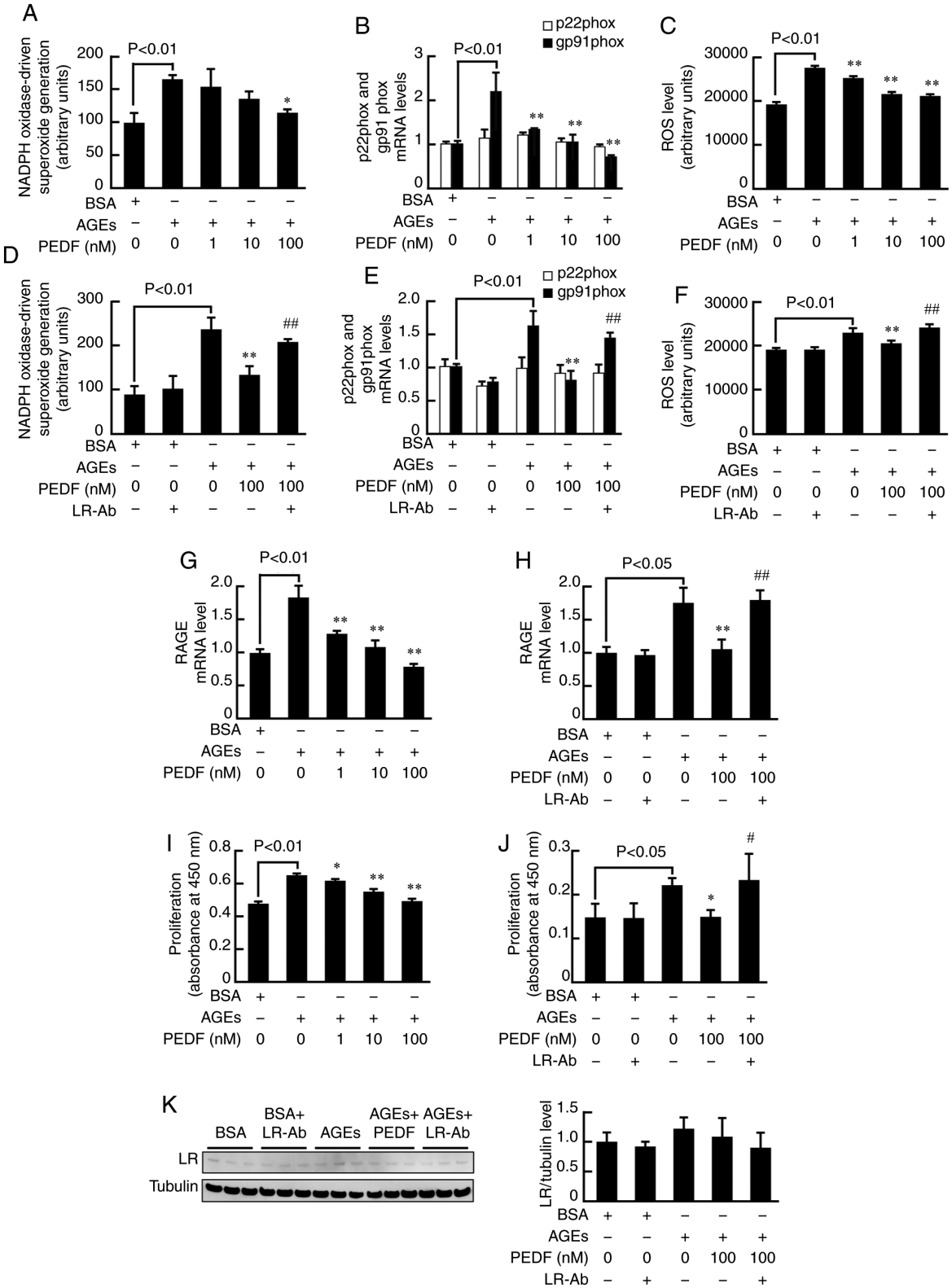

Figure 1. Effects of PEDF on NADPH oxidase-driven superoxide generation, mRNA expression levels of p22phox and gp91phox, ROS production, RAGE mRNA expression, proliferation and LR expression in MCF-7 breast cancer cells exposed to AGEs or BSA. (A) NADPH oxidase-driven superoxide generation (n=4), (B) mRNA expression levels of p22phox and gp91phox (n=3), and (C) ROS production (n=4) in MCF-7 cells treated with 100 μg/ml non-glycated BSA or AGEs in the presence or absence of the indicated concentrations of PEDF. (D) NADPH oxidase-driven superoxide generation (n=4), (E) mRNA expression levels of p22phox and gp91phox (n=3), and (F) ROS production (n=4) in MCF-7 cells treated with 100 μg/ml non-glycated BSA or AGEs in the presence or absence of the indicated concentrations of PEDF or 5 μg/ml LR-Ab. (G) RAGE mRNA expression in MCF-7 cells treated with 100 μg/ml non-glycated BSA or AGEs in the presence or absence of the indicated concentrations of PEDF or (H) 5 μg/ml LR-Ab (n=3). (I) Proliferation of MCF-7 cells treated with 100 μg/ml non-glycated BSA or AGEs in the presence or absence of the indicated concentrations of PEDF or (J) 5 μg/ml LR-Ab (n=4). (K) LR protein expression in MCF-7 cells treated with 100 μg/ml non-glycated BSA or AGEs in the presence or absence of the indicated concentrations of PEDF or 5 μg/ml LR-Ab analyzed by western blotting (n=3). *P<0.05 and **P<0.01 vs. AGEs; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. AGEs and 100 nM PEDF. AGEs, advanced glycation end products; LR, laminin receptor; Ab, antibody; PEDF, pigment epithelium-derived factor; ROS, reactive oxygen species; BSA, bovine serum albumin; p22phox, cytochrome b-245 α chain; gp91phox, cytochrome b-245 β chain; RAGE, receptor for AGE.

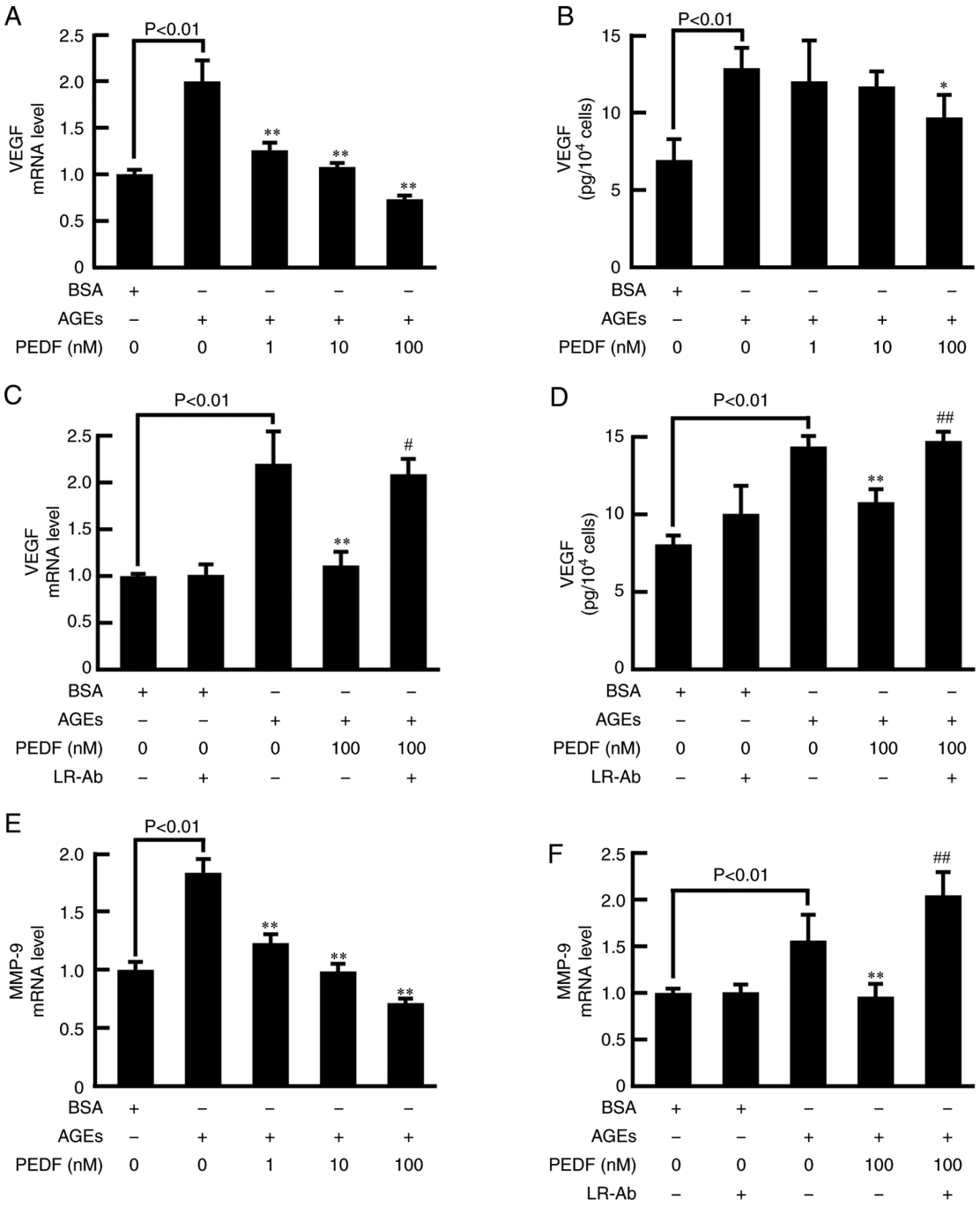

Figure 2. Effects of PEDF on VEGF mRNA and protein expression, and MMP-9 mRNA expression in breast cancer MCF-7 cells exposed to AGEs or BSA. VEGF (A) mRNA (n=3) and (B) protein expression (n=4) in MCF-7 cells treated with 100 μg/ml non-glycated BSA or AGEs in the presence or absence of the indicated concentrations of PEDF. VEGF (C) mRNA (n=3) and (D) protein expression (n=4) in MCF-7 cells treated with 100 μg/ml non-glycated BSA or AGEs in the presence or absence of the indicated concentrations of PEDF or 5 μg/ml LR-Ab. (E) MMP-9 mRNA expression in MCF-7 cells treated with 100 μg/ml non-glycated BSA or AGEs in the presence or absence of the indicated concentrations of PEDF or (F) 5 μg/ml LR-Ab (n=3). *P<0.05 and **P<0.01 vs. AGEs; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. AGEs and 100 nM PEDF. AGEs, advanced glycation end products; LR, laminin receptor; Ab, antibody; PEDF, pigment epithelium-derived factor; VEGF, vascular endothelial growth factor; MMP-9, matrix metalloproteinase 9; BSA, bovine serum albumin.

of PEDF are associated with angiogenesis and metastasis in various types of tumors, whereas PEDF inhibits the growth and metastasis of tumors in animal models (12-20). Differences in expression levels or pattern of PEDF and LR between breast cancer cells and hepatocellular carcinoma cells may partly explain the discrepant results.

Results of all the parameters we examined here were almost the same regardless of whether the cells were treated with AGEs or not. Therefore, AGEs may not affect the anti-tumor effects of PEDF. However, AGEs have been shown to decrease PEDF expression in endothelial cells, mesangial cells, and podocytes via oxidative stress generation, further potentiating the deleterious effects by attenuating the protective action of PEDF against AGEs (51-53). Therefore, there is a bi-directional interaction between AGEs and PEDF. In many aging-related diseases, such as cancer and diabetic vascular complications, the balance may shift towards AGEs. Amelioration of the balance of two molecules may be a novel therapeutic strategy

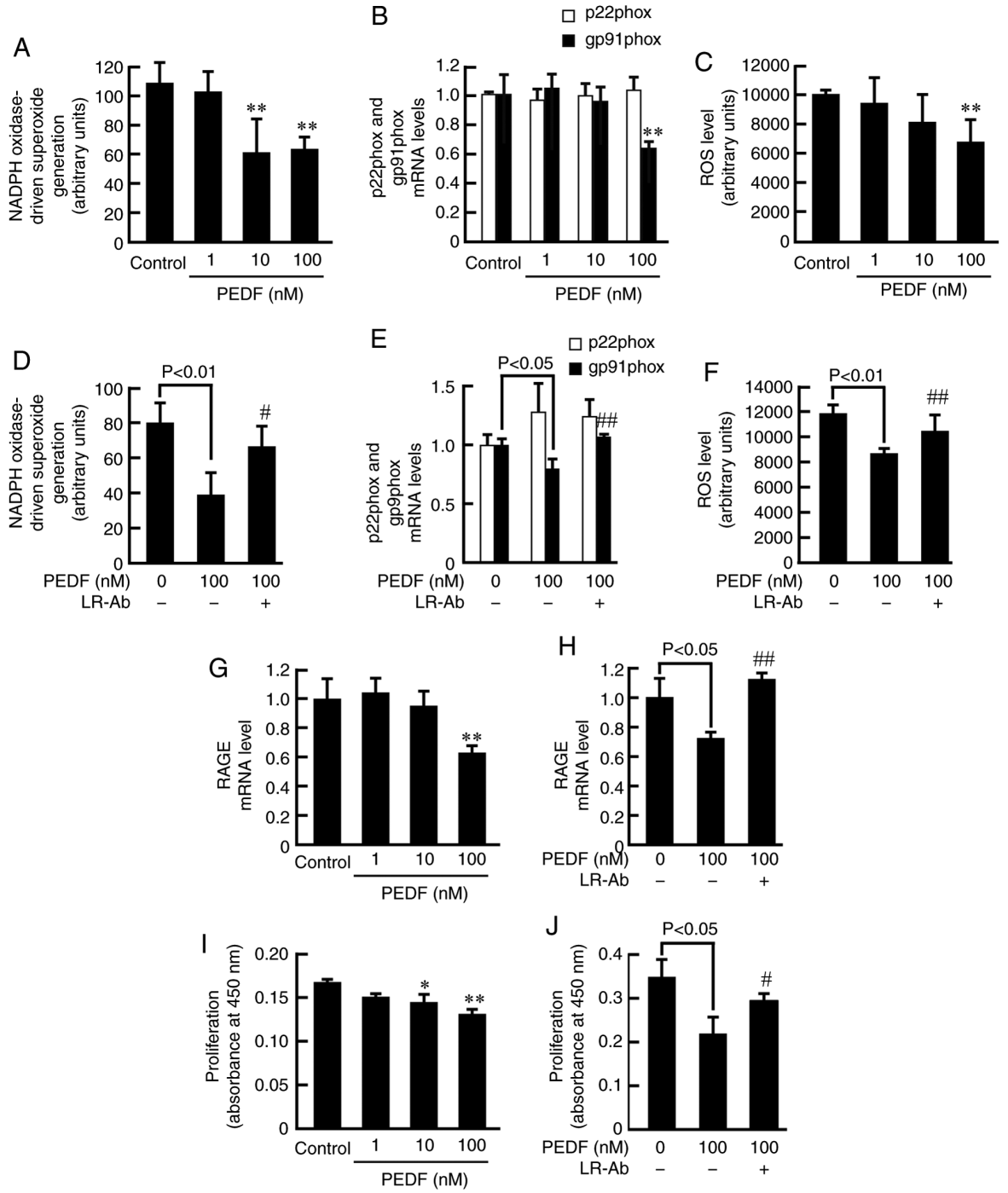

Figure 3. Effects of PEDF on NADPH oxidase-driven superoxide generation, mRNA expression levels of p22phox and gp91phox, ROS production, RAGE mRNA expression and proliferation in MCF-7 breast cancer cells. (A) NADPH oxidase-driven superoxide generation (n=4), (B) mRNA expression levels of p22phox and gp91phox (n=3), and (C) ROS production (n=4) in MCF-7 cells treated with the indicated concentrations of PEDF. (D) NADPH oxidase-driven superoxide generation (n=4), (E) mRNA expression levels of p22phox and gp91phox (n=3), and (F) ROS production (n=4) in MCF-7 cells treated with the indicated concentrations of PEDF in the presence or absence of 5 μg/ml LR-Ab. (G and H) RAGE mRNA expression in MCF-7 cells treated with the indicated concentrations of PEDF in the presence or absence of 5 μg/ml LR-Ab (n=3). (I and J) Proliferation of MCF-7 cells treated with the indicated concentrations of PEDF in the presence or absence of 5 μg/ml LR-Ab (n=4). *P<0.05 and **P<0.01 vs. control; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. 100 nM PEDF. AGEs, advanced glycation end products; LR, laminin receptor; Ab, antibody; PEDF, pigment epithelium-derived factor; ROS, reactive oxygen species; p22phox, cytochrome b-245 α chain; gp91phox, cytochrome b-245 β chain; RAGE, receptor for AGE.

for aging-related diseases, including breast cancer in patients with diabetes.

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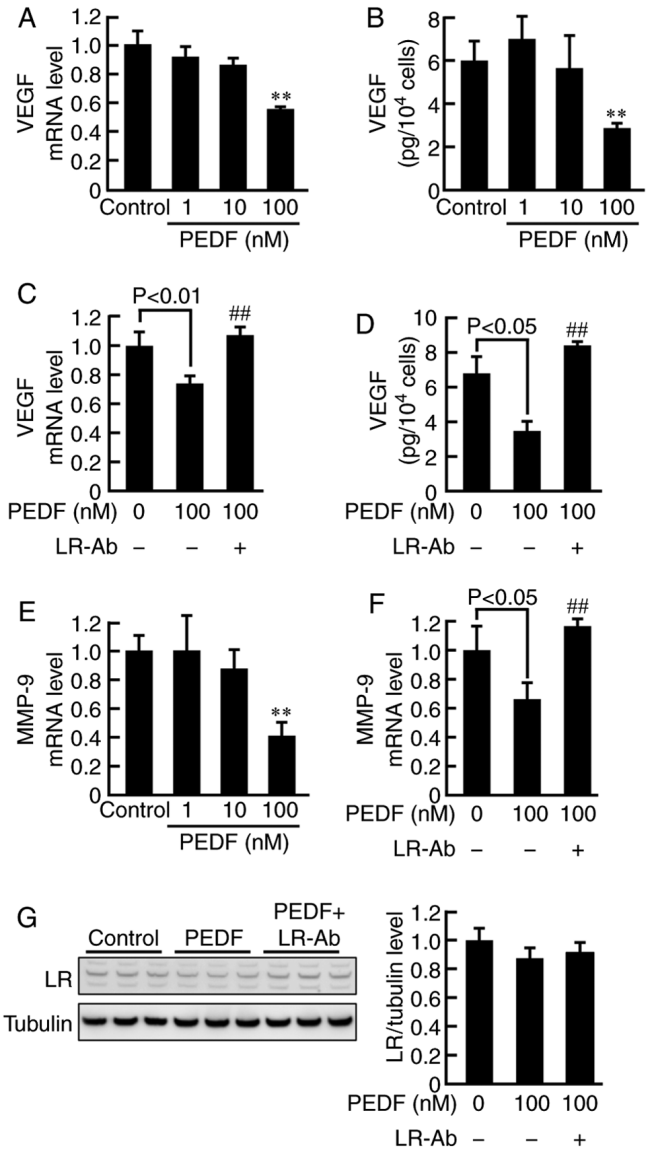

Figure 4. Effects of PEDF on VEGF mRNA and protein expression, MMP-9 mRNA expression and LR protein expression in MCF-7 breast cancer cells. VEGF (A) mRNA (n=3) and (B) protein expression (n=4) in MCF-7 cells treated with the indicated concentrations of PEDF. VEGF (C) mRNA (n=3) and (D) protein expression (n=4) in MCF-7 cells treated with the indicated concentrations of PEDF in the presence or absence of 5 μg/ml LR-Ab. (E and F) MMP-9 mRNA expression in MCF-7 cells treated with the indicated concentrations of PEDF in the presence or absence of 5 μg/ml LR-Ab (n=3). (G) LR protein expression in MCF-7 cells treated with the indicated concentrations of PEDF in the presence or absence of 5 μg/ml LR-Ab analyzed by western blotting (n=3). **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. 100 nM PEDF. AGEs, advanced glycation end products; LR, laminin receptor; Ab, antibody; PEDF, pigment epithelium-derived factor; VEGF, vascular endothelial growth factor; MMP-9, matrix metalloproteinase 9.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SIY conceptualized and designed the study, acquired, analyzed and interpreted the data, drafted the manuscript and takes responsibility for all the integrity of the data and accuracy of the data analysis. ST, TM, YK, AS and MY conceptualized and designed the study, as well as acquired, analyzed and interpreted the data. MY revised the manuscript critically for intellectual content. ST and TM confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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