Amadori-glycated phosphatidylethanolamine induces angiogenic differentiations in cultured human umbilical vein endothelial cells

Jeong-Ho Oak[a], Kiyotaka Nakagawa[a], Shinichi Oikawa[b], Teruo Miyazawa[a,*]

[a] *Food and Biodynamic Chemistry Laboratory, Graduate School of Life Science and Agriculture, Tohoku University, Sendai 981-8555, Japan*
[b] *Department of Medicine, Nippon Medical School, Tokyo 113-8603, Japan*

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Abstract **Glycation has been implicated in the endothelial dysfunction that contributes to both diabetes- and aging-associated vascular complications. The aim of the present study was to determine whether Amadori-glycated phosphatidylethanolamine (Amadori-PE), a lipid-linked glycation compound that is formed at an increased rate in hyperglycemic states, affected proliferation, migration and tube formation of cultured human umbilical vein endothelial cells (HUVEC). Amadori-PE at a low concentration of less than 5 μM significantly enhanced these three factors involved in angiogenesis. Furthermore, stimulation of HUVEC with Amadori-PE resulted in secretion of matrix metalloproteinase 2 (MMP-2), a pivotal enzyme in the initial step of angiogenesis. Our results demonstrated for the first time that Amadori-PE may be an important compound that promotes vascular disease as a result of its angiogenic activity on endothelial cells. We also demonstrated that MMP-2 is a primary mediator of Amadori-PE-driven angiogenesis.**

Key words: Glycation; Phosphatidylethanolamine; Amadori product; Angiogenesis; Matrix metalloproteinase; Diabetes mellitus

1. Introduction

Proteins react non-enzymatically with glucose, leading to the production of unstable Schiff bases and ultimately Amadori products. These early glycation products are transformed further into a wide spectrum of compounds termed advanced glycation end products (AGEs) [1]. Numerous studies have described the formation and accumulation of Amadori products, such as glycated hemoglobin and serum albumin, and AGEs, such as carboxymethyllysine and carboxyethyllysine, in blood and a range of tissues [2–5]. This glycation of protein progresses during normal aging and at an extremely accelerated rate in patients with diabetes mellitus [6,7]. These changes have been implicated in the development of diabetic vascular complications including altered angiogenesis, and thereby contribute to the disabilities and high mortality rate found in patients with diabetes [8]. Angiogenesis, the growth of new vascular capillary channels from preexisting vessels, is of fundamental importance in pathophysiological events associated with diabetes [9]. Recent studies have demonstrated AGEs initiate angiogenesis and therefore play an active part in the development and progression of diabetic microangiopathy [10–12].

While glycation of proteins has been investigated thoroughly, little attention has been paid to glycation of aminophospholipids such as phosphatidylethanolamine (PE). Recent intensive researches on lipid glycation have shown that PE reacts with glucose to form a Schiff base, followed by rearrangement to the PE-linked Amadori product shown in Fig. 1 [13–17]. This Amadori product accelerates membrane lipid peroxidation [17], with its concentration in human erythrocytes being proportional to the glycated hemoglobin value [18]. There is also evidence in rats that the level of Amadori-PE in plasma increases with aging [19]. A recent study has demonstrated that oxidative stress was directly involved in the angiogenic response [20]. Amadori-PE is therefore considered to be a key compound for generating oxidative stress that disrupts cellular responses and alters cell integrity and survival. Eventually, Amadori-PE may be one of the origin compounds causing enhancing angiogenesis in vivo. Angiogenesis normally involves a series of steps including endothelial cell activation and breakdown of the basement membrane, followed by migration, proliferation and tube formation of the cells. The purpose of this study was to obtain direct evidence of the effects of Amadori-PE on these key steps of angiogenesis using human umbilical vein endothelial cells (HUVEC).

2. Materials and methods

2.1. Materials

1,2-Di(*cis*-9-octadecenoyl)-*sn*-glycero-3-phosphoethanolamine (dioleoyl-PE) was purchased from Funakoshi (Tokyo, Japan). Amadori-PE was prepared from a glycation system of dioleoyl-PE/glucose by a high-performance liquid chromatography technique as described previously [17]. All other reagents were of analytical grade.

2.2. Cells cultures

HUVEC were purchased from Iwaki (Tokyo, Japan). Cells were cultured in the growth medium HuMedia-EG2™ (Kurabo, Osaka, Japan), and grown at 37°C in a humidified atmosphere of 5% CO_2 in air. The HuMedia-EG2™ medium consisted of the base medium (HuMedia-EB2™) supplemented with 2% fetal bovine serum (FBS), 0.5 μg/ml human epidermal growth factor (hEGF), 2 μg/ml hFGF-B, 5 mg/ml insulin, 50 mg/ml gentamicin and 50 μg/ml amphotericin B. Tightly confluent monolayers of HUVEC, of passage 2 to 6, were used in the experiments.

2.3. Preparation of Amadori-PE for cell cultures

A portion of 1 mM Amadori-PE in methanol was placed in a sterile tube and the solvent removed using a N_2 gas flux. The dried Amadori-PE was dispersed in the culture medium (HuMedia-EG2™) using sonication, followed by dilution with medium to achieve the desired

*Corresponding author. Fax: (81)-22-717 8905.
E-mail address: miyazawa@biochem.tohoku.ac.jp (T. Miyazawa).

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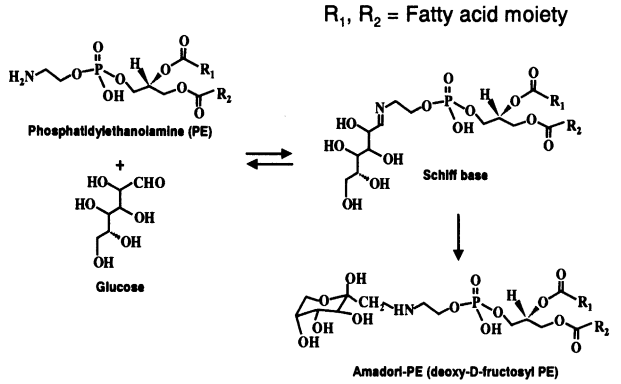

Fig. 1. Scheme for the glycation of PE. Glucose reacts with an amino group of PE to form an unstable Schiff base, which then undergoes rearrangement to yield the stable deoxy-D-fructosyl PE, namely Amadori-PE.

final concentration. Medium containing dioleoyl-PE (non-glycated PE) and sample-free medium were prepared as controls for the study.

2.4. Cell proliferation

HUVEC with 90% confluent growth were trypsinized, transferred into 96-well plates (3000 cells/well) and preincubated in HuMedia-EG2™ medium for 24 h. The culture medium was then changed to a medium containing test samples at various concentrations and incubated for a further 72 h. At the end of these treatments, viable cell numbers were estimated by the WST-1 assay. Briefly, 10 μl of WST-1 solution (Dojindo, Kumamoto, Japan) was added to each well and incubated at 37°C for 2 h, and the absorbance at 450 nm of the cultured medium measured using a microplate reader (Model 550, Bio-Rad, Tokyo, Japan).

2.5. Migration of HUVEC in a wound closure model

HUVEC were cultured on 12-well plates (2×10^5 cells/well) in FBS-free HuMedia-EG2™ medium. After 24 h, the cells were scratched with a yellow pipette tip to obtain a 'wounded' monolayer culture [21]. The media and dislodged cells were aspirated, and fresh medium added to the plates along with the test samples. After incubation at 37°C for 12 h, cell migration was observed using a phase contrast inverted microscope. The width of wounded cells in four randomly chosen fields was then determined.

2.6. Tube formation

An angiogenesis assay kit (Kurabo) was used according to the manufacturer's instructions. Briefly, HUVEC co-cultured with fibroblasts were cultivated in the presence or absence of various concentrations of Amadori-PE. After 11 days, cells were fixed in 70% ethanol, and then visualized with van Willebrand factor antibody. Vascular endothelial growth factor (VEGF, 10 ng/ml) was also tested as a positive control. Tube length was quantified using angiogenesis imaging software (Kurabo).

2.7. Enzyme-linked immunosorbent assays and zymography

HUVEC were cultured on 12-well culture plates (1×10^5 cells/well) in HuMedia-EG2™ medium containing the test sample at various concentrations. After 24 h, aliquots of the conditioned medium were used to measure the levels of matrix metalloproteinases (MMPs) and $VEGF_{165}$, using commercial enzyme-linked immunosorbent assay (ELISA) kits obtained from Biotrak (Amersham-Pharmacia, Freiburg, Germany) and R&D Systems (Minneapolis, MN, USA), respectively. The residual conditioned medium was then subjected to gelatin zymography in order to detect MMP-2.

2.8. Analysis of the expression of MMP-2 by reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the sample-treated HUVEC (1×10^6 cells) using a commercial kit (RNeasy mini kit, Qiagen, Tokyo, Japan). The RNA was reverse transcribed to cDNA by addition of FPLC*pure* M-MuLV reverse transcriptase (Amersham Biosciences, Piscataway, NJ, USA) and *Not*I-d$(T)_{18}$ primer (Amersham Biosciences). The MPCR kit (Maxim Biotech, San Francisco, CA, USA) was used for the amplification of the cDNA product. PCR thermocycling was carried out with denaturation at 94°C for 30 s, and annealing and extension at 60°C for 30 s using a Zymoreactor II (ATTO, Tokyo, Japan). The amplified cDNA product was then fractionated on a 3% agarose gel electrophoresis, and stained with SYBR-green I (BMA, Rockland, ME, USA).

2.9. Statistical analysis

The data are expressed as the mean and S.D. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's test. $P<0.05$ was accepted as significant.

3. Results

HUVEC were cultured in the presence or absence of 1 μM Amadori-PE, and the number of viable cells was evaluated over 72 h (Fig. 2A). Amadori-PE-treated cells showed an increased proliferation over this time period compared with control cells cultured without Amadori-PE. This effect was apparent 12 h after cultivation, with the number of cells present at 36 h being approximately two-fold greater in the Amadori-PE culture compared with the control. Non-glycated dioleoyl-PE induced no change in cell number compared with the control (Fig. 2A). The enhancing effect of Amadori-PE on cell proliferation peaked at a concentration of 5 μM (Fig. 2B,C).

To assess the impact of Amadori-PE on endothelial cell migration, a denudation injury model was used. Wounded HUVEC were incubated with Amadori-PE, and the rate of

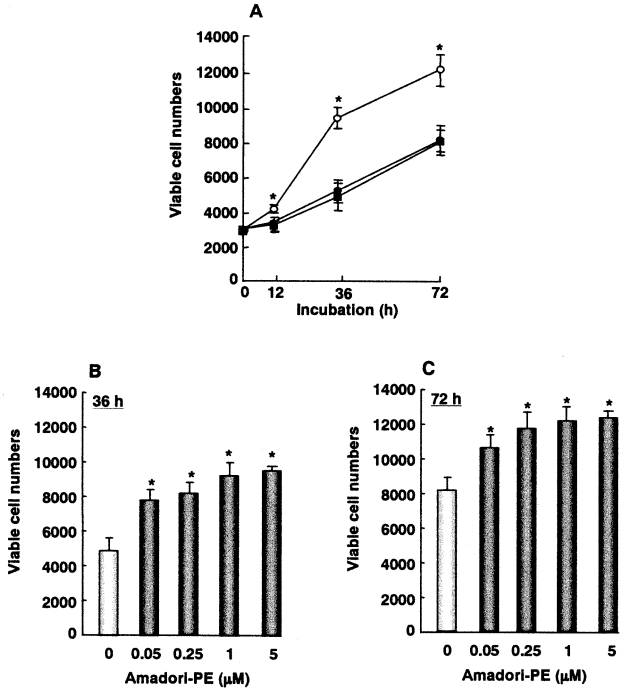

Fig. 2. Effects of Amadori-PE on HUVEC proliferation. A: HUVEC were cultured for 72 h in the absence (■) or presence of either 1 μM Amadori-PE (○) or 1 μM non-glycated dioleoyl-PE (●). HUVEC were also incubated with various doses of Amadori-PE for 36 h (B) or 72 h (C). At each time point, the number of viable cells was estimated by the WST-1 assay. *Significant difference compared to control cells ($P<0.05$). Values are mean ± S.D. of six wells. Replicate experiments (at least three times) demonstrated a similar trend.

closure observed over the following 12 h. In cultures supplemented with 5 μM Amadori-PE, endothelial cells migrated into the denuded area, recovered the exposed surface, and reduced the uncovered area (Fig. 3A). A slight increase in the rate of wound closure was observed with the addition of 1 μM Amadori-PE (Fig. 3A,B). Such migratory activity was not observed in the non-glycated dioleoyl-PE-treated cells (Fig. 3A).

We then examined the effect of Amadori-PE on tubular morphogenesis of endothelial cells. When HUVEC were co-cultured with human fibroblasts and incubated for 11 days with 10 ng/ml of VEGF, an increase in the number of tube-like structures was observed (Fig. 4A). Amadori-PE, like VEGF, was found to enhance the width and length of the endothelial tubes (Fig. 4A), with this effect being maximal at 5 μM Amadori-PE and approximately 1.7-fold higher than untreated control cells (Fig. 4B). Addition of non-glycated PE did not affect the basal level of tube formation (Fig. 4A).

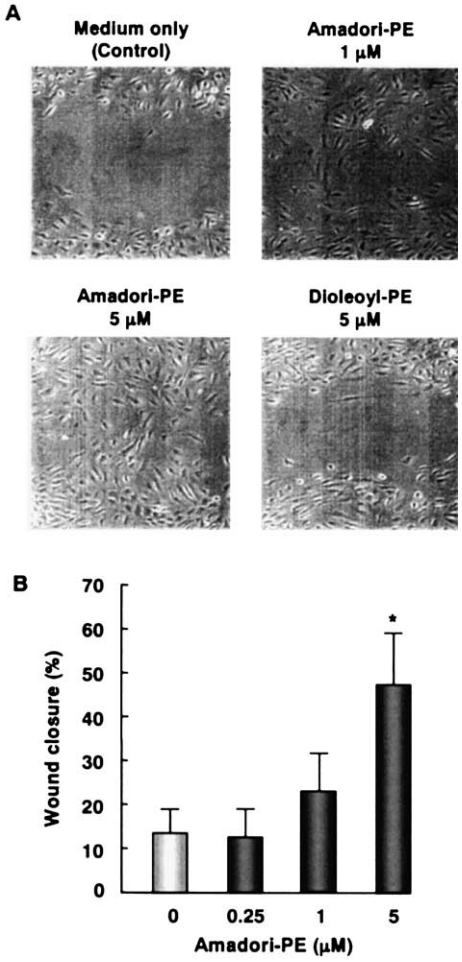

Fig. 3. Effects of Amadori-PE on HUVEC migration. A: A confluent HUVEC monolayer was sheared with a yellow pipette tip, followed by exposure to a fresh medium containing Amadori-PE (0–5 μM), or 5 μM dioleoyl-PE. After 12 h, closure of the wound was observed using a phase contrast inverted microscope. B: Each well was observed in four randomly chosen fields, and the width of wound measured. *Significant difference compared to control ($P < 0.05$). Values are mean ± S.D. of six wells. Replicate experiments (at least three times) demonstrated a similar trend.

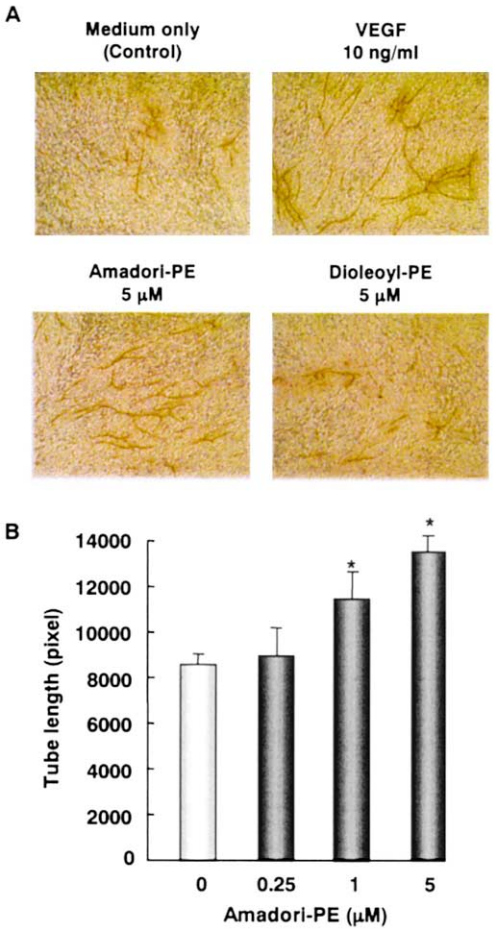

Fig. 4. Effects of Amadori-PE on tube formation in HUVEC. A: HUVEC co-cultured with fibroblasts were cultured in the absence (control) or presence of test samples (5 μM Amadori-PE and 5 μM dioleoyl-PE) for 11 days. VEGF (10 ng/ml) was tested under identical conditions as a positive control. B: Tube formation was observed in four randomly chosen fields, and the tube lengths measured. *Significant difference compared to control ($P < 0.05$). Values are mean ± S.D. of six wells. Replicate experiments (at least three times) demonstrated a similar trend.

These results indicated Amadori-PE enhanced several in vitro endothelial cell activities that are relevant to angiogenesis, including proliferation, migration and tube formation. We next evaluated the mechanism of Amadori-PE-driven angiogenesis in vitro by measuring the secretion of well-characterized angiogenic factors such as VEGF and MMPs. ELISA analysis of VEGF and MMP-1, MMP-2 and MMP-9 revealed that Amadori-PE over the concentration range 1–5 μM resulted in upregulation of MMP-2 secretion from HUVEC in a dose-dependent manner (Fig. 5A). Amadori-PE has no significant effect on MMP-1, MMP-9 and VEGF secretion from HUVEC although there was a slight increase in MMP-9 secretion (Fig. 5A). To determine the effect of Amadori-PE on MMP-2 secretion in greater detail, we carried out a zymogram analysis that enabled us to visualize gelatinolytic activity of the secreted proteinase. As shown in Fig. 5B, the results of the ELISA assay were confirmed by zymography. In addition, treatment of HUVEC with 1–5 μM Amadori-PE significantly enhanced MMP-2 mRNA expression (Fig. 5C), with this effect being maximal at 5 μM.

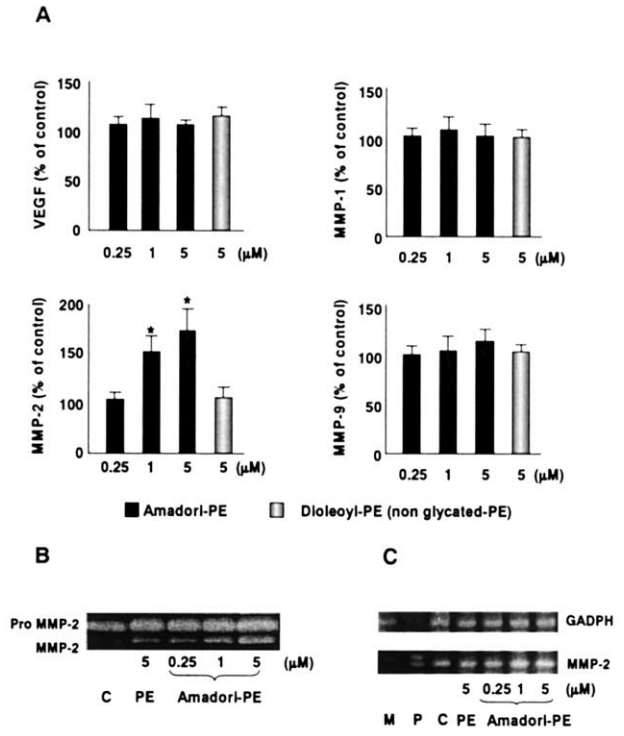

Fig. 5. Effect of Amadori-PE on the secretion of MMPs and VEGF from HUVEC. A: HUVEC were cultured in the absence (control) or presence of test samples (0.25–5 μM Amadori-PE and 5 μM dioleoyl-PE) for 24 h. The culture medium was then harvested and the yields of MMPs and VEGF in the medium measured by ELISA as described in Section 2. *Significant difference compared to control ($P < 0.05$). Values are mean ± S.D. of six wells. Replicate experiments (at least three times) demonstrated a similar trend. B: HUVEC were exposed to Amadori-PE (0.25–5 μM) or 5 μM di1oleoyl-PE for 24 h. The MMP-2 released from the HUVEC into the medium was analyzed by zymography. C, control (without test sample); PE, dioleoyl-PE. C: HUVEC were incubated with Amadori-PE (0.25–5 μM) or 5 μM dioleoyl-PE for 24 h. The level of MMP-2 mRNA in the HUVEC was then measured using an RT-PCR method as described in Section 2. M, DNA marker; P, positive control; C, control (without test sample); PE, dioleoyl-PE.

4. Discussion

In this study we investigated the in vitro angiogenic effects of Amadori-PE, a non-enzymatically glycated lipid formed under hyperglycemic conditions, on endothelial cells. This series of experiments included measuring the growth, migration and tube formation of HUVEC under a variety of culture conditions. To date there has been no study on the physiological activity of Amadori-PE with the exception of one report that showed it induced lipid peroxidation in vitro [17]. Several recent in vivo and in vitro studies have shown that the formation and accumulation of AGEs is proportional to aging and the severity of diabetic complications and that these changes promote angiogenesis [10–12]. These results led us to hypothesize that lipid glycation products may also induce angiogenic differentiations.

We found that proliferation and migration of HUVEC were enhanced to a moderate extent by treatment of Amadori-PE in a dose- and time-dependent manner (Figs. 2 and 3). Furthermore, Amadori-PE increased the formation of capillary-like networks by the HUVEC (Fig. 4). In contrast, exposure to dioleoyl-PE, a non-glycated PE, caused no change in angiogenic differentiation. This indicated that the angiogenic action was specific to the glycated lipid, but not to intact lipids. Amadori-PE over the concentration range 1–5 μM resulted in the maximal effect in all cases (Figs. 2–4). Ravandi et al. [14] reported that the mean Amadori-PE level in plasma from patients with diabetes was 4.8% per mg of phospholipids, equivalent to approximately 50 μg/ml of plasma. The concentration range of Amadori-PE of 1–5 μM that we used in our in vitro experiments for the evaluation of endothelial differentiations was therefore comparable to the concentration of this lipid in the plasma of patients with diabetes.

It is well known that VEGF is one of most potent factors for accelerating angiogenesis. Recently, Yamagishi et al. [11] suggested that angiogenic activity of AGEs on endothelial cells was mediated mainly by induction of autocrine vascular VEGF. In order to confirm whether Amadori-PE also stimulated the release of VEGF from HUVEC, we determined whether VEGF proteins were present in the conditioned medium using ELISA. However, we were unable to confirm clear secretion of VEGF from the HUVEC regardless of the addition of Amadori-PE or non-glycated PE (Fig. 5A). This suggested that the enhancing effect of Amadori-PE on angiogenesis in endothelial cells was not attributable to autocrine vascular VEGF.

An initial step in the angiogenic process is the degradation of subendothelial basement membrane and surrounding extracellular matrix (ECM) [22]. Following matrix breakdown, endothelial cells migrate and proliferate to form new vessels. Proteolytic enzyme activity degrading ECM is therefore essential for the initial angiogenesis process. The MMPs, a family of Zn^{2+}-dependent endopeptidases, are responsible for the digestion of a variety of ECM components [23]. Endothelial cells express various MMPs, including MMP-1 (collagenase), MMP-2 (gelatinase A) and MMP-9 (gelatinase B) [24]. Although the contribution of MMPs to angiogenesis has been attributed primarily to their ability to break down the ECM, they also have the ability to promote migration and proliferation of endothelial cells [25,26]. Given this important role of MMPs in angiogenesis we measured the concentration of MMP-1, MMP-2 and MMP-9 in conditioned medium of HUVEC using ELISA. We found the secretion of MMP-2 from the HUVEC was significantly enhanced when the cells were exposed to Amadori-PE (Fig. 5A). This result was reflected by increases in both enzymatic activity (Fig. 5B) and mRNA levels (Fig. 5C).

It has been suggested that Amadori-PE may be present in atherosclerotic lesions of individuals with diabetes [27]. We have shown previously that Amadori-PE may be an origin compound for generating reactive oxygen species [17]. A recent study demonstrated MMPs were activated by reactive oxygen species, and were implicated in atherosclerotic plaque stability associated with diabetic complications [28]. Therefore, the elevation of MMP-2 secretion which we observed following addition of Amadori-PE (Fig. 5) also provides a possible mechanism to account for the adverse changes in MMP activity that occur in diabetic atherosclerosis.

Under long-term hyperglycemia, early stimuli elicit adaptive reactions of tissues showing acute inflammatory processes of vessel walls and then late irreversible changes of microangiopathy [29]. The mechanisms of how these changes occur remain speculative; increased polyol pathway, excessive for-

mation of AGEs, increased protein kinase C activity, and oxidative stress are all interrelated for the cause and development of the microangiopathy [30]. Focusing on AGEs, it is reported that AGEs promote vascular cell changes typical of diabetes, including angiogenic and thrombogenic responses of endothelial cells, and pericyte loss [31]. Thus, our future aim is to explore whether Amadori-PE also has these abnormal properties.

To date, although a number of studies [10–12] have suggested a relationship between glycation and angiogenesis, there is no information on the potential interactions between lipid glycation and angiogenesis. In this study, Amadori-PE, a lipid glycation product, stimulated the growth, migration and tube formation of HUVEC in a dose-dependent manner. Hence, Amadori-PE may be one of the important inducers of angiogenesis in vivo. Now, this possibility is being investigated with in vivo angiogenesis models (i.e. chick embryo chorioallantoic membrane assay).

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