

## Advanced glycation endproducts inhibit prostacyclin production and induce plasminogen activator inhibitor-1 in human microvascular endothelial cells

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**Summary** Several thrombogenic abnormalities are associated with diabetes. To investigate the underlying molecular mechanisms, we examined the effects of advanced glycation endproducts (AGE), non-enzymatically glycated protein derivatives, on the production of prostacyclin (PGI<sub>2</sub>), an anti-thrombogenic prostanoid, and of plasminogen activator inhibitor-1 (PAI-1), a fast-acting serine protease inhibitor of fibrinolysis, in human microvascular endothelial cells (EC). Firstly, AGE-bovine serum albumin (BSA) but not non-glycated BSA, was found to considerably decrease the production of PGI<sub>2</sub> to about two-thirds of the control value. Secondly, quantitative reverse transcription-polymerase chain reaction showed that AGE-BSA increased the EC levels of mRNA coding for PAI-1, this being associated with a concomitant increase in the immunoreactive PAI-1 contents and the anti-fibrinolytic activity. Thirdly, the effects of AGE on PGI<sub>2</sub> and PAI-1 syntheses in EC were found to be

mediated by a receptor for AGE (RAGE) because antisense DNA against RAGE mRNA could reverse the AGE effects. Further, it was found that AGE decreased the intracellular cyclic AMP concentrations in EC and that cyclic AMP agonists such as dibutyryl cyclic AMP, forskolin and PGI<sub>2</sub> analogue reduced the AGE-stimulated PAI-1 production, suggesting the involvement of cyclic AMP in the AGE-signalling pathway. The results thus suggest that AGE have the ability to cause platelet aggregation and fibrin stabilization, resulting in a predisposition to thrombogenesis and thereby contributing to the development and progression of diabetic vascular complications. [Diabetologia (1998) 41: 1435–1441]

**Keywords** Advanced glycation endproducts, plasminogen activator inhibitor-1, endothelial cells, microangiopathy, thrombogenesis.

Accelerated atherosclerosis and microvascular complications are perhaps the leading cause of coronary heart disease, blindness and renal failure

which are common complications in patients with prolonged diabetes [1–3]. Plasminogen activator inhibitor-1 (PAI-1) is an important fast-acting serine protease inhibitor that attenuates fibrinolysis [4]. Epidemiological studies have demonstrated that reduced fibrinolytic activity because of elevated plasma PAI-1 is an important factor in various thrombotic diseases such as deep vein thrombosis, ischaemic heart disease [5–8] and diabetic vascular complications [9–10]. In addition to the attenuated fibrinolytic activity, hypercoagulability and platelet hyperaggregation are also prevalent in diabetic patients, contributing to microthrombus formation in diabetic micro- and macroangiopathies [11]. The molecular mechanisms underlying these thrombogenic abnormalities, however, are not fully understood.

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**Abbreviations:** PAI-1, Plasminogen activator inhibitor-1; AGE, advanced glycation endproducts; EC, endothelial cells; PGI<sub>2</sub>, prostacyclin; RAGE, receptor for AGE; BSA, bovine serum albumin; u-PA, urokinase-plasminogen activator; 6-keto-PGF<sub>1α</sub>, 6-keto-prostaglandin F<sub>1α</sub>; RT-PCR, reverse transcription-polymerase chain reaction; nt, nucleotide(s); t-PA, tissue-plasminogen activator; HUVEC, human umbilical vein endothelial cells; NF-κB, nuclear factor kappa B.

Glucose can react non-enzymatically with the amino groups of proteins to form reversible Schiff bases and then, Amadori products. These early glycation products undergo further complex reactions and rearrangements to become irreversibly cross-linked, fluorescent protein derivatives termed advanced glycation endproducts (AGE) [12]. The formation and accumulation of AGE in various tissues as well as in plasma have been known to progress at an extremely accelerated rate in diabetes [13].

Our previous co-culture experiments using pericytes and endothelial cells (EC), the cellular constituents of microvessels, have shown that pericytes not only regulate the growth of EC but also preserve their ability to produce prostacyclin ( $\text{PGI}_2$ ) [14], an antithrombogenic prostanoid [15]; this could explain why proliferative diabetic retinopathy develops following pericyte loss, the earliest histopathological hallmark in diabetic retinopathy [16–17]. Furthermore, we have recently shown that AGE exert an inhibitory effect on the growth of pericytes through their interaction with a receptor for AGE (RAGE), and have proposed a novel mechanism for pericyte loss [18].

In this study, we examined the direct effects of AGE on  $\text{PGI}_2$  and PAI-1 production by human microvascular EC. We show for the first time that AGE not only inhibit  $\text{PGI}_2$  production but also induce functional PAI-1 synthesis in EC, partly through a decrease in intracellular cyclic AMP levels, and discuss a possible mechanism for thrombogenesis in diabetic microangiopathy.

## Materials and methods

**Materials.** Bovine serum albumin (BSA) was purchased from Boehringer Mannheim GmbH (Mannheim, Germany). Heparin-Sepharose CL-4B was from Pharmacia LKB (Uppsala, Sweden); reverse transcriptase and T4 polynucleotide kinase were from Takara (Kyoto, Japan); Hybond- $\text{N}^+$  nylon membrane from Amersham (Buckinghamshire, United Kingdom); [ $\gamma$ - $^{32}\text{P}$ ]ATP from DuPont NEN (Boston, Mass., USA); urokinase-plasminogen activator (u-PA) from JCR Pharmaceuticals Co. Ltd. (Kobe, Japan); plasminogen from Bio Pur AG (Bubendorf, Switzerland); D-Val-Leu-Lys *p*-nitroanilide, forskolin and dibutyl cyclic AMP from Sigma (St. Louis, Mo., USA).  $\text{PGI}_2$  analogue beraprost sodium was a gift from Toray Industries Inc (Tokyo, Japan). Recombinant human RAGE protein and monoclonal antibody against it were kindly donated from Dr. Toshio Doi (Kyoto University Hospital, Japan).

**Cells.** Subcultivation ratio of EC from human skin microvessels was 1 to 2 and the cells were maintained in E-BM medium supplemented with 5 % fetal bovine serum, 0.4 % bovine brain extracts, 10 ng/ml human epidermal growth factor and 1  $\mu\text{g}/\text{ml}$  hydrocortisone according to the supplier's instructions (Clonetics Corp., San Diego, Calif., USA). Cells at 5 to 10 passages received AGE and other treatments in a medium lacking epidermal growth factor and hydrocortisone when they reached approximately 70 % confluency ( $\sim 7000$  cells/ $\text{cm}^2$ ).

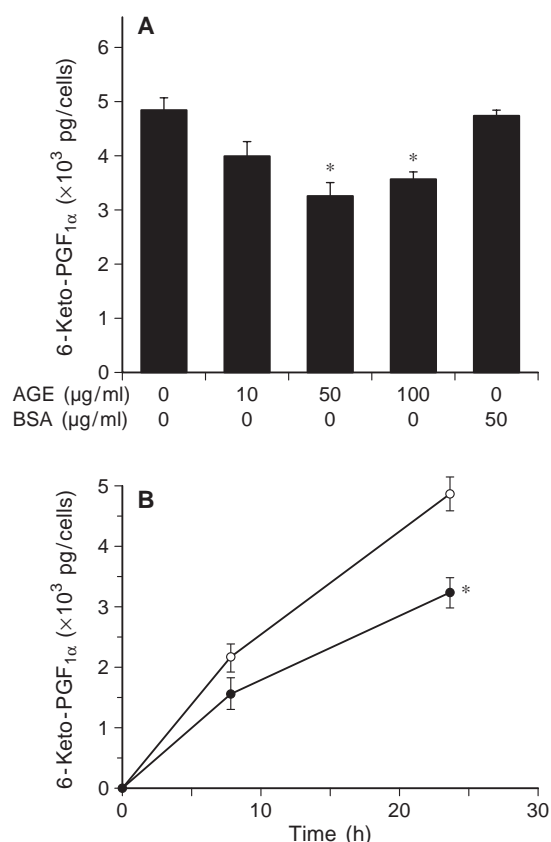
**Preparation of AGE.** AGE were prepared by incubating BSA (fraction V, fatty acid-free, endotoxin-free) with 0.5 mol/l glucose at 37 °C for 6 weeks under sterile conditions as described previously [19]. After unincorporated sugars were removed by dialysis against phosphate-buffered saline, glucose-modified higher-molecular weight materials were purified by heparin-Sepharose CL-4B column chromatography and used as AGE-BSA. Separation of AGE-BSA from non-glycated BSA was confirmed by SDS-PAGE. The concentration of AGE-BSA was determined by the method of Bradford [20]. Control non-glycated BSA was incubated under the same conditions except for the absence of glucose.

**Measurement of 6-keto-prostaglandin  $F_{1\alpha}$  (6-keto- $\text{PGF}_{1\alpha}$ ).** 6-Keto- $\text{PGF}_{1\alpha}$ , a stable metabolite of  $\text{PGI}_2$ , released into media was measured with an Amersham enzyme immunoassay system according to the supplier's recommendation.

**Primers and probes.** Oligonucleotide primers and probes for quantitative reverse transcription-polymerase chain reactions (RT-PCR) were chemically synthesized and purified as described previously [21]. Sequences of the upstream and downstream primers and the internal probe were 5'-ATGGGATTCAAGATTGATGA-3', 5'-TCAGTATAGTTGAACCTTGTT-3' and 5'-AGAGAGCCAGATTCATCATCAAT-3' (nucleotides (nt) 379–398, 811–830 and 584–606) for detecting PAI-1 mRNA [22], 5'-ACCACCATCGAGAACCAGCC-3', 5'-AATCAGCTTCACAACAGTCA-3' and 5'-CTGGTTTGCGGCCATCTACA-3' (nt 2494–2513, 4174–4193 and 2514–2533) for u-PA mRNA [23], 5'-CGAAGGATTGCTGGGAAGT-3', 5'-TGCGGTTCTTCAGCACGTGG-3' and 5'-TACGAGGACCAGGGCATCAG-3', (nt 245–264, 746–765 and 294–313) for tissue-plasminogen activator (t-PA) mRNA [24]. The primers and probe used for beta-actin mRNA detection were the same as described previously [21].

**Quantitative RT-PCR.** Poly(A) $^+$  RNAs were isolated [21] from cells treated for various time periods with or without AGE-BSA, and analyzed by RT-PCR as described previously [25]. After the RT-PCR, 6- $\mu\text{l}$  aliquots of each reaction mixture were electrophoresed on 2 % agarose gels, and transferred to Hybond- $\text{N}^+$  nylon membranes, which were then hybridized with the respective  $^{32}\text{P}$ -end labelled probes [19]. The amounts of poly(A) $^+$  RNA templates (30 ng) and the numbers for amplification cycles (20 cycles for beta-actin mRNA, 25 cycles for u-PA and t-PA mRNAs, 35 cycles for PAI-1 mRNA) were chosen in quantitative ranges where reactions proceeded linearly, which had been determined by plotting signal intensities as functions of the template amounts and the cycle numbers [19]. Signal intensities of hybridized bands were measured by a Fujix BAS 1000 Image analyzer (Fuji Photo Film Co. Ltd., Hamamatsu, Japan).

**Measurement of PAI-1.** The amounts of immunoreactive PAI-1 released into media were measured by ELISA (Imulysse PAI-1; Biopool, Umeå, Sweden), according to the supplier's recommendation. Since PAI-1 complexed to u-PA or t-PA is scarcely detectable by this method, the values represent free PAI-1 levels [26]. To determine the biological activity of PAI-1, EC were treated with serum-free medium in the presence or absence of 50  $\mu\text{g}/\text{ml}$  AGE for 24 h. Then, the conditioned medium was assayed for anti-fibrinolytic activity in the presence of u-PA (3.8 units/ml), 20  $\mu\text{g}/\text{ml}$  plasminogen and the chromogenic plasmin substrates (60  $\mu\text{mol}/\text{l}$  D-Val-Leu-Lys *p*-nitroanilide). Substrate hydrolysis was measured from 0 to 240 min and plasmin generation was estimated as the slope of the line defined by plotting relative absorbance vs time, PAI-1 activity thus being judged by the extent of the inhibition of plasmin generation [27].



**Fig. 1.** Effects of AGE-BSA on 6-keto-PGF<sub>1α</sub> production by human microvascular EC. **(A)** Dose effects of AGE on 6-keto-PGF<sub>1α</sub> production by EC. EC were treated with 10, 50 or 100 μg/ml AGE-BSA, or 50 μg/ml non-glycated BSA, or without them for 24 h. Each column represents the mean ± SEM of four replicate experiments. \*,  $p < 0.05$  compared with both the control value without AGE-BSA and that with non-glycated BSA (Mann-Whitney test). **(B)** Time course of 6-keto-PGF<sub>1α</sub> production by EC. EC were treated with (●) or without 50 μg/ml AGE-BSA (○) for the indicated periods of time. Amounts of 6-keto-PGF<sub>1α</sub> released by EC are indicated on the ordinate. Each point represents the mean ± SEM of nine replicate experiments. \*,  $p < 0.05$  compared with the control value without AGE-BSA (Mann-Whitney test)

**Assay with antisense oligodeoxyribonucleotides.** A phosphorothioate antisense complement of human RAGE mRNA, and the corresponding sense oligodeoxyribonucleotide, were synthesized and purified as described previously [28]. Sequences of antisense and sense oligonucleotides were 5'-CAACTGCTGTTCCGGCT-3' and 5'-AGCCGGAACAGCAGTTG-3', respectively, which corresponded to nucleotides 6–22 of human cDNA [28]. The oligonucleotides were added to the medium, with or without 50 μg/ml AGE-BSA, in which EC were grown. After 24 h, 6-keto-PGF<sub>1α</sub> and PAI-1 syntheses were assayed.

**Western blotting.** Subconfluent cultures of EC treated with or without 10 μmol/l oligodeoxyribonucleotides for 24 h were lysed in phosphate-buffered saline (pH 7.4) containing 1% (V/V) NP-40, 0.5% (W/V) deoxycholate, 0.1% (W/V) SDS, 2 mmol/l phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 2 mmol/l sodium orthovanadate, 10 mmol/l sodium pyrophosphate, 50 mmol/l sodium fluoride, 10 mmol/l EDTA and 1.5 mmol/l MgCl<sub>2</sub>. Fifteen μg of proteins were loaded per lane

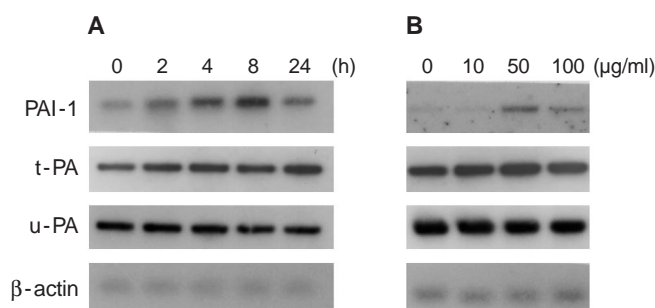
and separated by SDS-PAGE (12.5%), and then transferred onto a nitrocellulose membrane. The filter was then treated with a monoclonal antibody against human RAGE, and the resultant immune complexes were visualized with an Amersham enhanced chemiluminescence system as described previously [29].

**Measurement of cyclic AMP.** EC were treated with or without 50 μg/ml AGE for 4 h, after which trichloroacetic acid was added to the medium to a final concentration of 5%. Cells were collected, sonicated and centrifuged at 300 g for 15 min at 4°C, and the supernatant saved was then extracted with water-saturated ether to remove the trichloroacetic acid. The resultant aqueous layer was dried by a centrifugal vaporizer, and assayed for cyclic AMP levels with an Amersham enzyme immunoassay system according to the supplier's recommendation.

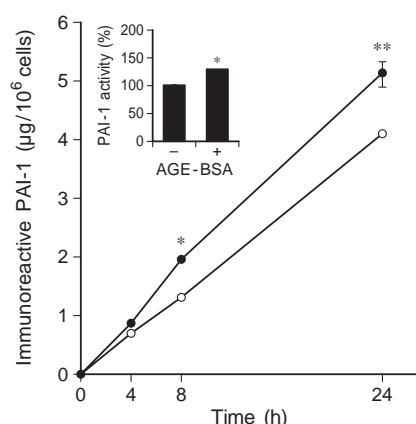
## Results

**AGE inhibition of PGI<sub>2</sub> production by microvascular EC.** PGI<sub>2</sub> is a vasodilating, antithrombogenic prostanoid mainly produced by EC [15]. We tested the effects of AGE on PGI<sub>2</sub> production in microvascular EC by measuring its stable metabolite, 6-keto-PGF<sub>1α</sub> released into media. As shown in Figure 1A, the amounts of 6-keto-PGF<sub>1α</sub> released from EC were decreased with increasing concentrations of AGE-BSA; the lowest level was noted at 50 μg/ml AGE-BSA, about 30% lower than the control value without AGE or that with non-glycated BSA. The inhibition of PGI<sub>2</sub> production began to be observed at 8 h after the addition of AGE-BSA, and at 24 h a statistically relevant decrease was achieved (Fig. 1B).

**PAI-1, u-PA and t-PA mRNA levels in AGE-exposed EC.** Both u-PA and t-PA can stimulate the conversion of plasminogen to plasmin, promoting fibrinolysis, while PAI-1 inhibits plasmin generation by forming inactive complexes with u-PA and t-PA or both [4]. To determine whether AGE could affect fibrinolysis, poly(A)<sup>+</sup> RNAs were isolated from EC, which had been treated with various concentrations of AGE-BSA at various times, and assayed for their contents of PAI-1, u-PA and t-PA mRNAs by a quantitative RT-PCR technique. As shown in Figure 2, AGE-BSA were found to increase considerably the concentration of PAI-1 mRNA. The PAI-1 mRNA concentration began to increase at 2 h in the presence of AGE-BSA, and reached a maximum at 8 h (Fig. 2A); the peak value was about threefold higher than the basal concentration when standardized with the signal intensities of beta-actin mRNA as an internal control. Among the concentrations tested, the maximal effect was observed with 50 μg/ml AGE-BSA (Fig. 2B), the same concentration giving the maximal inhibition of PGI<sub>2</sub> production (Fig. 1A). Non-glycated BSA induced no change in PAI-1 mRNA concentrations (data not shown). On the other hand, the concentrations of u-PA and t-PA



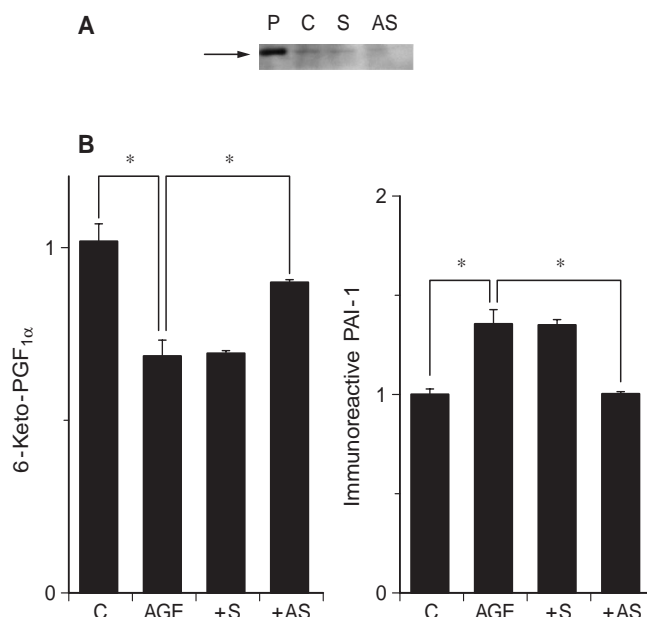
**Fig. 2.** PAI-1, t-PA and u-PA gene expressions in AGE-exposed EC. **(A)** Time course of changes in PAI-1, t-PA and u-PA mRNA levels. Poly(A)<sup>+</sup> RNAs were isolated from EC treated for the indicated time periods with 50 µg/ml AGE-BSA, 30 ng of which were then transcribed and PCR-amplified. **(B)** Dose response of PAI-1, t-PA and u-PA mRNA concentrations to AGE. After incubation for 4 h with the indicated concentrations of AGE-BSA, poly(A)<sup>+</sup> RNAs were isolated from EC and 30 ng of them were amplified by RT-PCR. The PCR products were electrophoresed on 2% agarose gels, transferred onto nylon membranes and hybridized with <sup>32</sup>P-end labelled probes as described in Materials and methods. PCR amplification for beta-actin mRNA was done for 20 cycles



**Fig. 3.** Effects of AGE-BSA on PAI-1 production by human microvascular EC. EC were treated with (●) or without 50 µg/ml AGE-BSA (○) for the indicated time periods. Amounts of PAI-1 antigen released from EC are indicated on the ordinate. Each point represents the mean for six replicate experiments; vertical bars show SEM when larger than the symbol; \* and \*\*,  $p < 0.05$  and  $p < 0.01$ , respectively, compared with the value without AGE-BSA (Mann-Whitney test). Inset, biological assay for PAI-1 activity. Microvascular EC were treated with (+) or without (-) 50 µg/ml AGE for 24 h. Conditioned medium was removed and assayed for PAI-1 activity in the presence of u-PA, plasminogen and the chromogenic plasmin substrates as described in Materials and methods. Data represent the mean  $\pm$  SEM of three replicate experiments. \*,  $p < 0.05$  compared with the value without AGE-BSA (Mann-Whitney test)

mRNAs (Fig. 2, A and B) were essentially unchanged by the exposure to AGE-BSA.

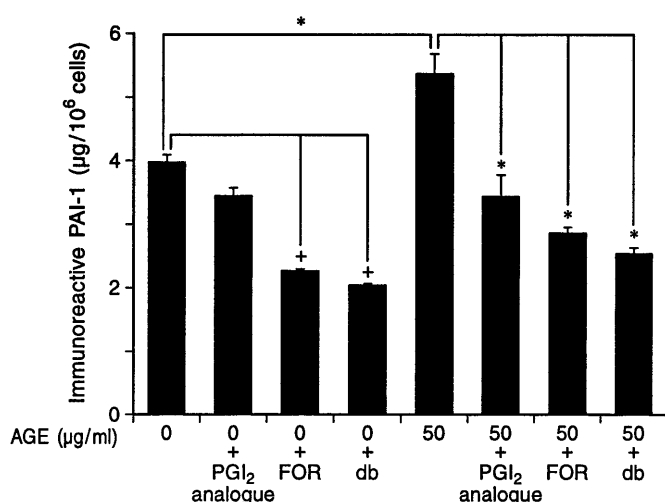
**AGE induction of functional PAI-1.** To confirm whether AGE-BSA increased the synthesis of PAI-1



**Fig. 4.** Effects of antisense oligonucleotides on EC. **(A)** EC were treated with or without 10 µmol/l oligomers for 24 h, and then proteins were extracted and analyzed by Western blotting as described in Materials and methods using a monoclonal antibody against human RAGE. P, human recombinant RAGE protein; C, untreated EC; S, sense oligomer-treated EC; AS, antisense oligomer-treated EC. Arrow indicates the position of RAGE protein. **(B)** EC were cultured with 50 µg/ml AGE in the presence or absence of 10 µmol/l oligomers or without AGE-BSA or oligomers. The relative amounts of 6-keto-PGF<sub>1α</sub> and PAI-1 released from EC are indicated on the ordinate, the value without additives being 1. Each column represents the mean value of four replicate experiments. Bars show SEM. \*,  $p < 0.05$  compared with the values obtained in the presence of AGE alone (Mann-Whitney test). C, control without additives; AGE, 50 µg/ml AGE-BSA; + S, 50 µg/ml AGE-BSA plus 10 µmol/l sense oligomers; + AS, 50 µg/ml AGE-BSA plus 10 µmol/l antisense oligomers

proteins, we used ELISA to measure the amounts of PAI-1 antigen released from EC treated with or without AGE-BSA. As shown in Figure 3, immunoreactive PAI-1 was measurably increased by 50 µg/ml AGE; after the 24-h incubation, the value was about 30% above the control. This correlated strikingly with the anti-fibrinolytic activity of PAI-1 induced by AGE-BSA (Fig. 3 inset).

**Reversal of the AGE actions on EC by antisense oligonucleotides against RAGE mRNA.** We next examined the role of RAGE in the AGE actions. For this, septadecamer antisense oligodeoxyribonucleotides complementary to human RAGE mRNA, and the corresponding sense control, were added to culture media in which EC were grown. As shown in Figure 4A, Western blot analysis showed that the antisense oligomer but not sense control could inhibit the expression of the RAGE protein in EC. Further, as shown in Figure 4B, the antisense oligomer was



**Fig. 5.** Effects of cyclic AMP agonists on the AGE-induced PAI-1 production by human microvascular EC. EC were treated with or without 50 µg/ml AGE-BSA in the presence or absence of various types of cyclic AMP agonists for 24 h. Amounts of PAI-1 antigen released by EC are indicated on the ordinate. Each column represents the mean  $\pm$  SEM of four replicate experiments. PGI<sub>2</sub> analogue, 1 µmol/l PGI<sub>2</sub> analogue; FOR, 10 µmol/l forskolin; db, 1 mmol/l dibutyryl cyclic AMP. +,  $p < 0.05$  compared with the control value without additives, \*,  $p < 0.05$  compared with the value with AGE-BSA alone (Mann-Whitney test)

**Table 1.** Effects of AGE on cyclic AMP concentrations in microvascular EC

Treatment	cyclic AMP concentrations (pmol/10 <sup>5</sup> cells)
None (0 h)	1.30 $\pm$ 0.09
None (4 h)	1.37 $\pm$ 0.07
AGE-BSA (4 h)	0.74 $\pm$ 0.03 <sup>a</sup>
AGE-BSA + PGI <sub>2</sub> analogue (4 h)	1.20 $\pm$ 0.08
PGI <sub>2</sub> analogue (4 h)	1.38 $\pm$ 0.08

Microvascular EC were treated with or without 50 µg/ml AGE for the indicated time period. After fixation with 5 % trichloroacetic acid solution, cells were collected, sonicated and centrifuged and the resultant supernatant was assayed for cyclic AMP contents as described in Materials and methods. Data represent the mean  $\pm$  SEM of nine replicate experiments. <sup>a</sup>  $p < 0.05$  compared with the value without additives (Mann-Whitney test). PGI<sub>2</sub> analogue, prostacyclin analogue beraprost sodium added together with AGE-BSA at a concentration of 1 µmol/l

found to diminish considerably both the AGE-induced inhibition of PGI<sub>2</sub> production and the increase in PAI-1 production by EC.

*Reduction by AGE in EC cyclic AMP concentrations and protection against the AGE-induced PAI-1 synthesis by cyclic AMP agonists.* Constitutive secretion of PAI-1 is reported to be inhibited by cyclic AMP agonists in human umbilical vein EC (HUVEC) [30]. Accordingly, we tested the effects of AGE on the intracellular cyclic AMP concentrations in micro-

vascular EC. As shown in Table 1, 50 µg/ml AGE were found to decrease noticeably the cyclic AMP concentration to about half of the control value. To determine the functional link between the cyclic AMP and PAI-1 levels in EC, we examined the effects of cyclic AMP agonists on the AGE-induced PAI-1 stimulation. As shown in Figure 5, cyclic AMP elevating compounds such as PGI<sub>2</sub> analogue beraprost sodium, forskolin and dibutyryl cyclic AMP not only decreased the basal concentration of immunoreactive PAI-1 but also inhibited the AGE induction of this protein in microvascular EC.

## Discussion

In this study we showed for the first time that AGE, non-enzymatically glycated protein derivatives formed at an accelerated rate during hyperglycaemia, could inhibit the production of PGI<sub>2</sub> by microvascular EC through interaction with RAGE. This finding extends our previous observation with HUVEC [28], and supports our hypothesis that AGE have thrombogenic activities. Since oxidant stress is reported to inhibit the production of PGI<sub>2</sub> in EC [31], and since vitamin E treatment can restore the reduction of PGI<sub>2</sub> synthesis in diabetic rat aortae [32], the AGE-RAGE-induced intracellular oxidant stress [33] may account for the impaired PGI<sub>2</sub> production by EC.

This study has also showed that AGE could cause the stabilization of preformed fibrin clots. That is, AGE can induce EC expression of the gene for PAI-1, a potent inhibitor of fibrinolysis. As the AGE-induced PAI-1 mRNA upregulation (Fig. 2) coincided with the increase in both the immunoreactive protein and the plasminogen-activator inhibiting activity (Fig. 3), it could be postulated that the de novo synthesis of functional PAI-1 is elicited by AGE. The AGE-induced PAI-1 synthesis was also found to be RAGE-mediated (Fig. 4B). Since free radical scavengers such as glutathione are reported to reduce considerably the PAI-1 level in diabetic subjects [34], oxidant stress elicited by AGE-RAGE interaction could also be involved in the PAI-1 overproduction.

Although epidemiological studies have established the plasma PAI-1 elevation in diabetes [9, 10], there is no clinical evidence suggesting plasma AGE concentrations as a major contributor to the PAI-1 concentration. Further studies would be required to determine a cause-to-effect relationship between AGE and PAI-1 in diabetic patients, including intervention studies with AGE inhibitors. Geiger et al. [35, 36] described that non-enzymatic glycation of lysine residues of plasminogen itself can result in the impaired formation of plasmin. AGE may exert their anti-fibrinolytic activities by plural mechanisms.

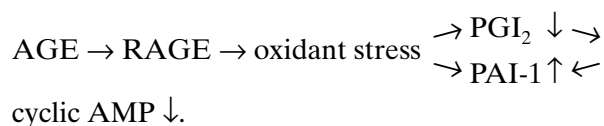
The time-response relationship in the two indices was a striking mirror-image (Figs. 1 B and 3), suggest-

ing that both the AGE-induced PGI<sub>2</sub> inhibition and the PAI-1 stimulation could take place simultaneously in EC. Further, the concentration of AGE that gave the maximal effect (50 µg/ml) is the same between the two and is comparable to the concentration of plasma AGE in human diabetic subjects [37]. This would suggest that such AGE-driven thrombogenic and anti-fibrinolytic changes could occur in vivo. Although the AGE effects appeared to be modest, we speculate that chronic exposure to AGE during prolonged hyperglycaemia causes blood vessels to become more susceptible to fibrin formation and stabilization. Actually, a decrease of approximately 30% in plasma PGI<sub>2</sub> was a risk factor for diabetic vascular complications [38], and about a one and one half-fold increase in plasma PAI-1 activity resulted in an impaired fibrinolytic capacity in various thrombogenic disorders [6, 8, 10]. In addition, a synergistic involvement of glucose and AGE on reduced fibrinolysis was suggested by Maiello et al. [39] who showed that high glucose increased the PAI-1 activity in HUVEC by approximately 120%.

This study has also shown for the first time that AGE decrease the intracellular cyclic AMP concentration in EC (Table 1), and that cyclic AMP agonists can protect against the AGE-induced increase in the EC production of PAI-1 (Fig. 5), indicating that AGE might exert their anti-fibrinolytic activity at least in part through the cyclic AMP reduction. This finding could be important in several respects.

Firstly, it suggests a reciprocal link between PGI<sub>2</sub> and PAI-1 productions under the influence of AGE. As PGI<sub>2</sub> exerts its biological action through the increase of intracellular cyclic AMP [40], AGE inhibition of PGI<sub>2</sub> synthesis would result in further exacerbation of the PAI-1 stimulation. Constitutive and phorbol ester-stimulated expression of PAI-1 in HUVEC is also known to be inhibited by cyclic AMP [30, 41].

Secondly, it suggests an involvement of cyclic AMP in the AGE-signalling pathway. The AGE-RAGE interaction is reported to induce oxidant stress and then to activate nuclear factor kappa B (NF-κB) [42]. Since NF-κB binding sites are identified in the PAI-1 promotor region [43], AGE probably enhanced the transcription of PAI-1 gene through the NF-κB-mediated pathway. Agonists that increase intracellular cyclic AMP concentrations inhibit the NF-κB-mediated gene transcription in HUVEC [44]. Accordingly, AGE-induced PAI-1 mRNA upregulation would be enhanced by the decrease in cyclic AMP. An overall scheme for the AGE-induced thrombogenesis would thus seem to be



Thirdly, as shown previously, hypoxia [21] and AGE themselves [19] cause angiogenesis, another hallmark of diabetic microangiopathy, through the induction of autocrine vascular endothelial growth factor in EC. The AGE-driven microthrombus formation would thus give rise to local hypoxia, superdriving angiogenesis and thereby leading to further progression of diabetic microangiopathy.

Fourthly, cyclic AMP agonists such as PGI<sub>2</sub> analogue, which ameliorate effectively the thrombogenic effects of AGE (Fig. 5), may have therapeutic potential in the treatment of diabetic vascular complications, the main cause of diminished quality and expectancy of life in patients with diabetes.

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