



# Glucagon-like peptide-1 (GLP-1) inhibits advanced glycation end product (AGE)-induced up-regulation of VCAM-1 mRNA levels in endothelial cells by suppressing AGE receptor (RAGE) expression

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## ABSTRACT

Glucagon-like peptide-1 (GLP-1) is one of the incretins, a gut hormone secreted from L cells in the intestine in response to food intake. It has been proposed as a potential therapeutic target for the treatment of patients with type 2 diabetes. However, the direct effects of GLP-1 on vascular injury in diabetes are largely unknown. Since there is a growing body of evidence that advanced glycation end products (AGE) and their receptor RAGE axis plays an important role in vascular complications in diabetes, this study investigated whether and how GLP-1 blocked the deleterious effects of AGE on human umbilical vein endothelial cells (HUVEC). GLP-1 receptor (GLP-1R) was expressed in HUVEC. GLP-1 dose-dependently inhibited RAGE gene expression in HUVEC, which was blocked by small interfering RNAs raised against GLP-1R. An analogue of cyclic AMP also decreased RAGE mRNA level in HUVEC. Further, GLP-1 decreased reactive oxygen species generation and subsequently reduced vascular cell adhesion molecule-1 mRNA levels in AGE-exposed HUVEC. Our present study suggests that GLP-1 directly acts on HUVEC via GLP-1R and it could work as an anti-inflammatory agent against AGE by reducing RAGE expression via activation of cyclic AMP pathways.

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## Introduction

Non-enzymatic modification of proteins by reducing sugars, a process that is also known as Maillard reaction, progress at an extremely accelerated rate under diabetes, thus leading to the formation of advanced glycation end products (AGE) [1–3]. Many types of study, ranging from *in vitro* experiments to pathological analyses to epidemiological studies, have shown that AGE play a central role in the pathogenesis of accelerated atherosclerosis in diabetes [1–3]. Indeed, AGE and their receptor (RAGE) interaction evokes oxidative stress generation and elicits vascular inflammation and thrombosis, thus being involved in vascular complications in diabetes [1–3]. These observations suggest that the inhibition of the AGE–RAGE axis may be a novel therapeutic target for diabetic vascular complications.

Glucagon-like peptide-1 (GLP-1) is one of the incretins, a gut hormone secreted from L cells in the intestine in response to food intake [4]. Since GLP-1 not only augments glucose-induced insulin release from pancreatic  $\beta$ -cells, but also suppresses glucagon secretion and slows gastric emptying [4], it has been proposed as a potential therapeutic target for the treatment of patients with type 2 diabetes. The

biological actions of GLP-1 on pancreatic cells are mainly mediated by high-affinity receptor for GLP-1 [5]. In addition, GLP-1 receptor (GLP-1R) is shown to exist in extra-pancreatic tissues, including brain, lung, kidney, and heart [6]. These observations suggest that GLP-1 could act on extra-pancreatic tissues as well to elicit diverse biological reactions. However, whether GLP-1 could have direct vasoprotective properties are largely unknown. Therefore, this study investigated whether and how GLP-1 blocked the deleterious effects of AGE on human umbilical vein endothelial cells (HUVEC).

## Materials and methods

**Materials.** Bovine serum albumin (BSA) (essentially fatty acid free and essentially globulin free, lyophilized powder), GLP-1 (7–36) amide and 8-bromo-cyclic adenosine monophosphate (8-Br-cAMP), an analogue of cyclic AMP, were purchased from Sigma (St. Louis, MO, USA). D-glyceraldehyde from Nakalai Tesque (Kyoto, Japan). Antibody (Ab) directed against human GLP-1 receptor from Santa Cruz Biotechnology Inc. (Delaware, CA, USA).

**Preparation of antiserum directed against RAGE.** Antiserum directed against human RAGE for western blots was prepared as described previously [7].

**Preparation of AGE–BSA.** AGE–BSA was prepared as described previously [7]. Briefly, BSA (25 mg/ml) was incubated under sterile conditions with 0.1 M glyceraldehyde in 0.2 M NaPO<sub>4</sub> buffer (pH 7.4) for

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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. Preparations were tested for endotoxin using Endospecy ES-20S system (Seikagaku Co., Tokyo, Japan); no endotoxin was detectable. The extent of chemical modification was determined as described with 2,4,6-trinitrobenzenesulfonic acid as a difference in lysine residues of modified and unmodified protein preparations. The extent of lysine modification (%) of modified BSA preparations was 65% for AGE-BSA.

**Cells.** HUVEC were cultured in endothelial basal medium supplemented with 2% fetal bovine serum, 0.4 % bovine brain extracts, 10 ng/ml human epidermal growth factor and 1  $\mu$ g/ml hydrocortisone according to the supplier's instructions (Clonetics Corp., San Diego, CA). AGE or GLP-1 treatment was carried out in a medium lacking epidermal growth factor and hydrocortisone.

**Construction and transfection of small interfering RNAs (siRNAs).** The sense and antisense human GLP-1R siRNAs used in this experiment (5'-UCAUCAAGCUGUUUACAGAtt-3' and 5'-UCUGUAAACAGC UUGAUGAag, respectively) were synthesized by Applied Biosystems (Foster, CA, USA). Control non-silencing siRNAs were also obtained from Applied Biosystems (Silencer Negative Control #1 siRNA). Then the siRNA duplexes were transfected to HUVEC using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. After 2 days of transfection, GLP-1R protein and RAGE gene expression were analyzed.

**Western blotting analysis.** HUVEC were treated with or without 100  $\mu$ g/ml BSA in the presence or absence of 0.03 nM GLP-1 for 4 h. Then proteins were extracted from HUVEC with lysis buffer, and then separated by SDS-PAGE and transferred to nitrocellulose membranes as described previously [7]. Membranes were probed with 1:500 dilution of Ab against GLP-1R, 1:1000 dilution of RAGE antiserum or 1:400 dilution of monoclonal Ab against  $\alpha$ -tubulin (Sigma), and then immune complexes were visualized with an enhanced chemiluminescence detection system (Amersham Bioscience, Buckinghamshire, United Kingdom).

**Real-time reverse transcription-polymerase chain reactions (RT-PCR).** HUVEC were treated with 100  $\mu$ g/ml AGE-BSA or non-glycated BSA in the presence or absence of the indicated concentrations of GLP-1 or 5  $\mu$ M 8-Br-cAMP for 4 h. Then total RNA was extracted with RNAqueous-4PCR kit (Ambion Inc., Austin, TX, USA) according to the manufacturer's instructions. Quantitative real-time RT-PCR was performed using Assay-on-Demand and TaqMan 5 fluorogenic nuclease chemistry (Applied Biosystems, Foster city, CA, USA) according to the supplier's recommendation. IDs of primers for human RAGE, vascular cell adhesion molecule-1 (VCAM-1) and  $\beta$ -actin gene were Hs00153957\_m1, Hs00365486\_m1 and Hs9999 9903\_m1, respectively.

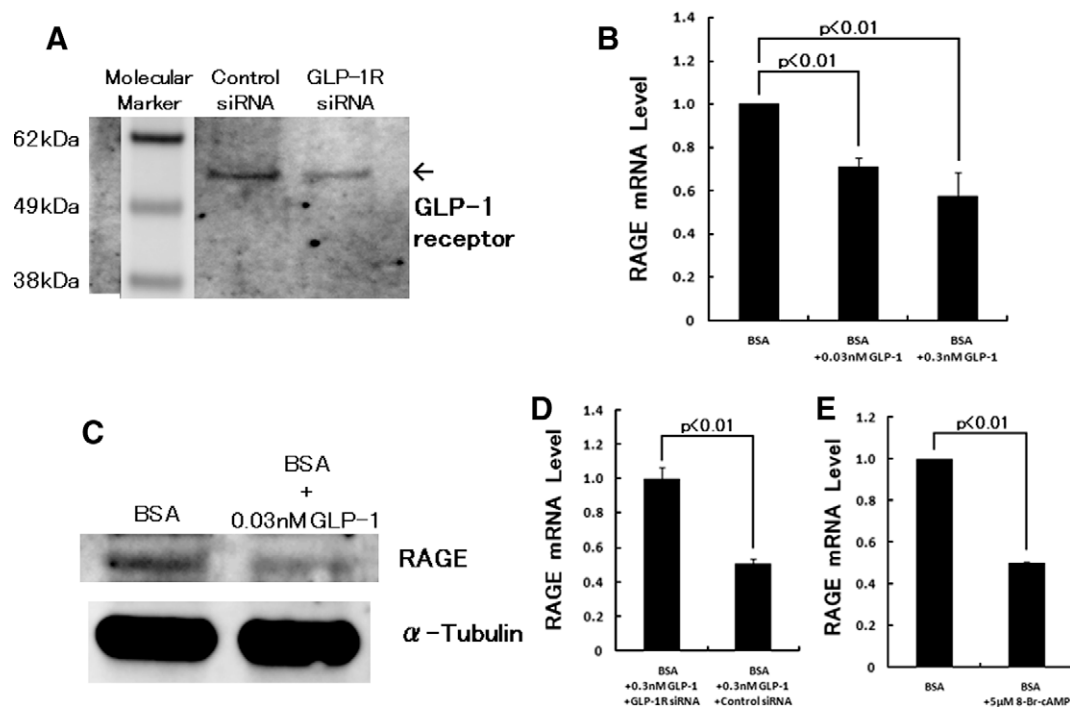
**Dihydroethidium (DHE) staining.** HUVEC were treated with 100  $\mu$ g/ml AGE-BSA or non-glycated BSA in the presence or absence of the indicated concentrations of GLP-1 for 4 h, and then the cells were incubated with phenol red free Dulbecco's Modified Eagle Medium containing 3  $\mu$ M DHE (Molecular Probes Inc., Eugene, OR, USA). After 15 min, the cells were imaged under a laser-scanning confocal microscope. Intensity of DHE staining in 5 different field of each sample was analyzed by microcomputer-assisted NIH image.

**Statistical analysis.** All values were presented as mean  $\pm$  SEM. One-way ANOVA followed by the Scheffe *F* test was performed for statistical comparisons; *p* < 0.05 was considered significant.

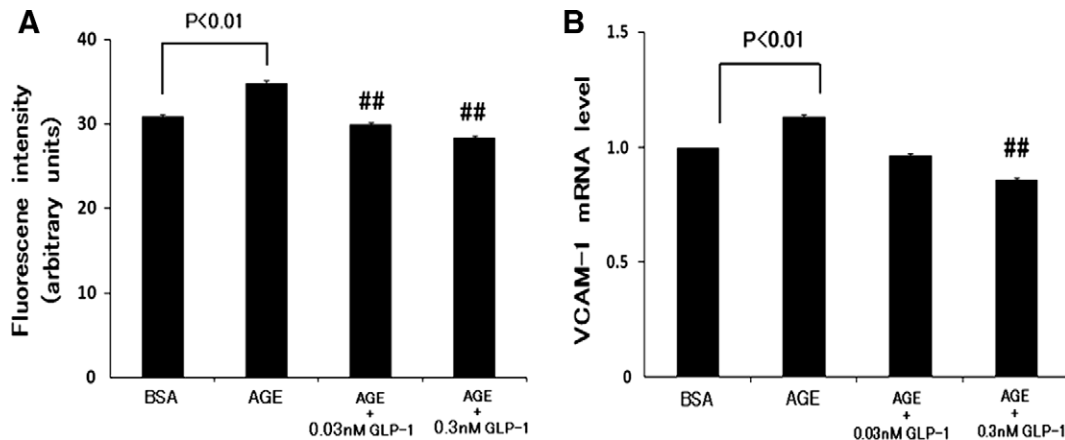
## Results

Because of the ambiguity as to the location of GLP-1R in human blood vessels, we first examined whether HUVEC expressed GLP-1R. As shown in Fig. 1A, western blotting analysis revealed a single band with a molecular mass of 56 kDa, corresponding to the GLP-1R seen in HUVEC. We also confirmed that siRNA molecules specific for human GLP-1R actually reduced GLP-1R level in HUVEC to about 1/3 of those of control siRNA-treated cells (Fig. 1A).

RAGE is a signal transducing receptor for AGE [1–3]. Indeed, engagement of RAGE with AGE activates its down-stream signaling



**Fig. 1.** GLP-1R and RAGE gene and protein expressions in HUVEC. HUVEC were treated with or without 100  $\mu$ g/ml non-glycated BSA in the presence or absence of the indicated concentrations of GLP-1 or 5  $\mu$ M 8-Br-cAMP for 4 h. Then GLP-1R (A), RAGE gene (B, D, and E) and protein (C) expressions were measured. \*\**p* < 0.01 compared to the value with BSA alone. *N* = 3.



**Fig. 2.** Anti-inflammatory effects of GLP-1 on AGE-exposed HUVEC. HUVEC were treated with 100  $\mu$ g/ml AGE-BSA or non-glycated BSA in the presence or absence of the indicated concentrations of GLP-1 for 4 h. Then, ROS generation (A) and VCAM-1 gene expression (B) were measured. ## $p$  < 0.01 compared to the value with AGE alone.  $N$  = 3.

and subsequently evokes inflammatory and thrombogenic responses through reactive oxygen species (ROS) generation in endothelial cells [1–3]. Therefore, we examined the effect of GLP-1 on RAGE gene and protein expression in HUVEC. As shown in Fig. 1B, GLP-1 dose-dependently decreased RAGE mRNA levels in HUVEC; 0.03 nM GLP-1 reduced both RAGE mRNA and protein levels to about 70% of control cells (Fig. 1B and C).

We next examined the involvement of GLP-1R in GLP-1 actions on HUVEC. For this, we investigated the effects of siRNAs raised against GLP-1R on RAGE gene expression in HUVEC. GLP-1R siRNAs were found to inhibit the GLP-1-induced decrease in RAGE mRNA levels in HUVEC (Fig. 1D). Since the actions of the GLP-1R are mediated by cAMP production and subsequent protein kinase A activation [8], we further studied the effects of an analogue of cyclic AMP, 8-Br-cAMP on RAGE gene expression in HUVEC. As shown in Fig. 1E, 8-Br-cAMP significantly reduced RAGE mRNA levels in HUVEC.

We next investigated whether GLP-1 could actually exert anti-inflammatory effects on HUVEC by suppressing the down-stream signaling evoked by AGE-RAGE. For this, we studied the effect of GLP-1 on ROS generation and VCAM-1 gene expression in AGE-exposed HUVEC. As shown in Fig. 2A and B, GLP-1 dose-dependently inhibited the AGE-induced ROS generation and subsequent up-regulation of VCAM-1 mRNA levels in HUVEC.

## Discussion

In this study, we have demonstrated for the first time that GLP-1 could block the AGE-induced up-regulation of VCAM-1 mRNA levels in HUVEC by suppressing RAGE expression and subsequent ROS generation. Since GLP-1R was expressed in HUVEC and that GLP-1R siRNAs inhibited the GLP-1-induced down-regulation of RAGE mRNA levels, our present study suggests that GLP-1 directly acts on endothelial cells via GLP-1R and it could work as an anti-inflammatory agent against AGE by reducing RAGE expression.

We found here that an analogue of cyclic AMP, 8-Br-cAMP mimicked the effects of GLP-1 on RAGE gene expression in HUVEC. Since GLP-1R mainly mediates the biological actions of GLP-1 on pancreatic cells via cyclic AMP pathways [5,8], our present results suggest that the anti-inflammatory actions of GLP-1 on HUVEC are mediated in part through the GLP-1R and cyclic AMP signaling pathways. We have previously shown that AGE decrease the intracellular cyclic AMP levels in human cultured endothelial cells and that cyclic AMP agonists such as dibutyryl cyclic AMP reduce the AGE-RAGE-stimulated endothelial plasminogen activator inhibitor-1 production [9]. Taken together, these findings suggest the

active involvement of cyclic AMP in the AGE-signaling pathways and that RAGE is a molecular target for anti-inflammatory effects of GLP-1-GLP-1R-cyclic AMP axis in AGE-exposed HUVEC.

One early phase of atherosclerosis involves the recruitment and firm adhesion of inflammatory cells to endothelial cells, whose process is mainly mediated by VCAM-1 [10]. Given the active participation of the AGE-RAGE axis in diabetic vascular complications [1–3], pharmacological up-regulation or substitution of GLP-1 may play a protective role against accelerated atherosclerosis in diabetes by suppressing VCAM-1 expression via blockade of the deleterious effects of AGE. With this regard, strategies to enhance the biological actions of GLP-1, including GLP-1 receptor agonists or analogues or dipeptidyl peptidase-4 inhibitors (exemplified by exenatide, liraglutide, sitagliptin, vildagliptin and linagliptin) may be promising for not only ameliorating hyperglycemia, but also protecting against vascular injury in type 2 diabetic patients because the effects of GLP-1 are attenuated in these patients [11].

The plasma concentration of GLP-1 in type 2 diabetic patients is reported to be about 0.01–0.025 nM [12]. However, a dipeptidyl peptidase-4 inhibitor, sitagliptin treatment has been shown to increase its plasma level by approximately 2-fold [12]. So, the concentration of GLP-1 having beneficial effects on HUVEC (0.03 nM) may be comparable to the therapeutic levels, which are achieved in the treatments of patients with type 2 diabetes. Further, in this study, *in vitro*-modified AGE were prepared by incubating BSA with glyceraldehyde for 1 week; this process produced relatively highly-modified proteins in comparison to those *in vivo*. However, it is unlikely that extensively-modified, unphysiologic AGE that were formed under the *in vitro*-conditions may exert non-specific and toxic effects on HUVEC for the following reasons: (1) we have previously found that immunological epitope of glyceraldehyde-modified AGE was actually present in serum of diabetic patients and that the concentration (100  $\mu$ g/ml) of *in vitro*-prepared AGE used here were comparable with those of the *in vivo* diabetic situation, (2) we have also shown previously that the AGE-rich serum fractions obtained from diabetic patients on hemodialysis have the same biological effects as did the *in vitro*-prepared AGE, and (3) pre-incubation of AGE-containing media with 1  $\mu$ g/ml polymyxin B, an inhibitor of endotoxin for 30 min did not affect the AGE-induced ROS generation [7,13–15].

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