Clin Exp Med (2011) 11:131–135
DOI 10.1007/s10238-010-0109-2

SHORT COMMUNICATION

Vardenafil, an inhibitor of phosphodiesterase-5, blocks advanced glycation end product (AGE)-induced up-regulation of monocyte chemoattractant protein-1 mRNA levels in endothelial cells by suppressing AGE receptor (RAGE) expression via elevation of cGMP

Yuji Ishibashi · Takanori Matsui · Masayoshi Takeuchi · Sho-ichi Yamagishi

Received: 15 June 2010 / Accepted: 5 August 2010 / Published online: 29 August 2010

Abstract Decreased production and/or impaired action of nitric oxide (NO) play a role in the pathogenesis of atherosclerotic cardiovascular disease and erectile dysfunction (ED) in diabetic patients. Under hyperglycemic conditions, formation and accumulation of advanced glycation end products (AGE) have been known to progress, thus contributing to tissue damage in diabetes. However, effects of inhibitors of phosphodiesterase-5 (PDE-5), an enzyme that catalyzes the degradation of cyclic guanosin-monophosphate (cGMP) and subsequently blocks the actions of NO, on AGE-exposed endothelial cells remain unknown. Therefore, this study investigated whether and how vardenafil, an inhibitor of PDE-5, could block the deleterious effects of AGE on human umbilical vein endothelial cells (HUVEC). Gene and protein expression was analyzed in quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) and western blots, respectively. Intracellular formation of reactive oxygen species (ROS) was evaluated with dihydroethidium staining. AGE increased receptor for AGE (RAGE) mRNA and protein levels in HUVEC, both of which were significantly inhibited by the treatments with 30 nM vardenafil or 5 μM 8-Br-cGMP, an analogue of cGMP. Further, vardenafil reduced the AGE-induced ROS generation and subsequently inhibited up-regulation of monocyte chemoattractant protein-1 (MCP-1) mRNA levels in HUVEC. We demonstrated here for the first time that vardenafil could block the AGE-induced up-regulation of MCP-1 mRNA levels in HUVEC by suppressing RAGE expression and subsequent ROS generation via elevation of cGMP. Our present results suggest that vardenafil directly acts on endothelial cells and it could work as an anti-inflammatory agent against AGE.

Keywords AGE · Atherosclerosis · cGMP · RAGE · Vardenafil

Y. Ishibashi · T. Matsui · S. Yamagishi (✉)
Department of Pathophysiology and Therapeutics of Diabetic Vascular Complications, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011, Japan
e-mail: shoichi@med.kurume-u.ac.jp

M. Takeuchi
Department of Pathophysiological Science, Faculty of Pharmaceutical Science, Hokuriku University, Kanazawa, Japan

Introduction

Erectile dysfunction (ED) is defined as a condition unable to obtain an erection sufficient for sexual intercourse [1, 2]. Since impairment of nitric oxide (NO)/cyclic guanosin-monophosphate (cGMP) pathway in erectile tissues has been reported to play a role in the pathogenesis of ED, phosphodiesterase-5 (PDE-5), an enzyme that catalyzes the degradation of cGMP and subsequently blocks the actions of NO in corpora cavernosa of the penis, now becomes a therapeutic target for the treatment of ED [1, 2]. Indeed, inhibitors of PDE-5 such as vardenafil and sildenafil are most widely used drugs for the treatment of ED [1, 2].

There are several papers to show that ED is associated with cardiovascular disease in both diabetic and non-diabetic subjects [3, 4]. Endothelial dysfunction is an initial step of atherosclerosis, and NO generation and bioavailability are impaired in the settings of endothelial dysfunction, thus being involved in the development and progression of atherosclerosis [5]. These observations suggest that ED and atherosclerotic cardiovascular disease share common pathogenesis, *that is*, endothelial dysfunction. However, effects of PDE-5 inhibitors on endothelial injury are not fully understood.

Non-enzymatic modification of proteins by reducing sugars, a process that is also known as Maillard reaction, progresses at an extremely accelerated rate under diabetes, thus leading to the formation of advanced glycation end products (AGE) [6–8]. There is a growing body of evidence that AGE and their receptor (RAGE) interaction evokes oxidative stress generation and elicits vascular inflammation and thrombosis, thereby participating in vascular complications in diabetes [6–8]. This study investigated whether and how vardenafil, an inhibitor of PDE-5, could block the deleterious effects of AGE on human umbilical vein endothelial cells (HUVEC).

Materials and methods

Materials

Vardenafil was generously provided by Bayer-Pharma (Wupperrtal, Germany). Bovine serum albumin (BSA) (essentially fatty acid-free and essentially globulin-free, lyophilized powder) and 8-bromo-cGMP (8-Br-cGMP), an analogue of cGMP, were purchased from Sigma (St. Louis, MO, USA), D-glyceraldehyde from Nakalai Tesque (Kyoto, Japan).

Preparation of antiserum directed against RAGE

Antiserum directed against human RAGE for western blots was prepared as described previously [9].

Preparation of AGE-BSA

AGE-BSA was prepared as described previously [9]. Briefly, 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. 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 μg/ml hydrocortisone according to the supplier's instructions (Clonetics Corp., San Diego, CA, USA). AGE treatment was carried out in a medium lacking epidermal growth factor and hydrocortisone.

Real-time reverse transcription-polymerase chain reactions (RT-PCR)

HUVEC were treated with 100 μg/ml AGE-BSA or non-glycated BSA in the presence or absence of 30 nM vardenafil 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, monocyte chemoattractant protein-1 (MCP-1) and β-actin gene were Hs00153957_m1, Hs00234140_m1 and Hs99999903_m1, respectively.

Western blotting analysis

HUVEC were treated with or without 100 μg/ml BSA in the presence or absence of 30 nM vardenafil 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 [18]. Membranes were probed with 1:1,000 dilution of RAGE antiserum or 1:400 dilution of monoclonal Ab against α-tubulin (Sigma), and then immune complexes were visualized with an enhanced chemiluminescence detection system (Amersham Bioscience, Buckinghamshire, United Kingdom).

Dihydroethidium (DHE) staining

HUVEC were treated with 100 μg/ml AGE-BSA or non-glycated BSA in the presence or absence of 30 nM vardenafil for 4 h, and then the cells were incubated with phenol red–free Dulbecco's Modified Eagle Medium containing 3 μM 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 five different field of each sample was analyzed by microcomputer-assisted NIH image.

Statistical analysis

All values were presented as mean ± SEM. One-way ANOVA followed by the Scheffe F test was performed for statistical comparisons; $P < 0.05$ was considered significant.

Results

RAGE is a signal-transducing receptor for AGE [6–8]. Indeed, engagement of RAGE with AGE activates its down-stream signaling and subsequently evokes inflammatory and thrombogenic responses through reactive oxygen species (ROS) generation in endothelial cells [6–8]. Therefore, we first examined the effect of vardenafil on RAGE expression levels in HUVEC. As shown in Fig. 1a, b, AGE increased RAGE mRNA and protein levels in HUVEC, both of which were significantly inhibited by the treatment with 30 nM vardenafil; vardenafil treatment reduced RAGE mRNA and protein levels in AGE-exposed HUVEC by about 30 and 20%, respectively.

We next examined the involvement of cGMP in the RAGE down-regulation. For this, we investigated effects of an analogue of cGMP and 8-Br-cGMP on RAGE expression in HUVEC. As shown in Fig. 1c, d, 8-Br-cGMP modestly, but significantly, inhibited the AGE-induced increase in RAGE mRNA and protein levels in HUVEC; 5 μM 8-Br-cGMP treatment reduced RAGE mRNA and protein levels in AGE-exposed HUVEC by about 20 and 10%, respectively.

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

Discussion

In this study, we have demonstrated for the first time that vardenafil could block the AGE-induced up-regulation of MCP-1 mRNA levels in HUVEC by suppressing RAGE expression and subsequent ROS generation. We also found here that an analogue of cGMP and 8-Br-cGMP mimicked the effects of vardenafil on RAGE expression in HUVEC. Since cGMP mainly mediates the biological actions of

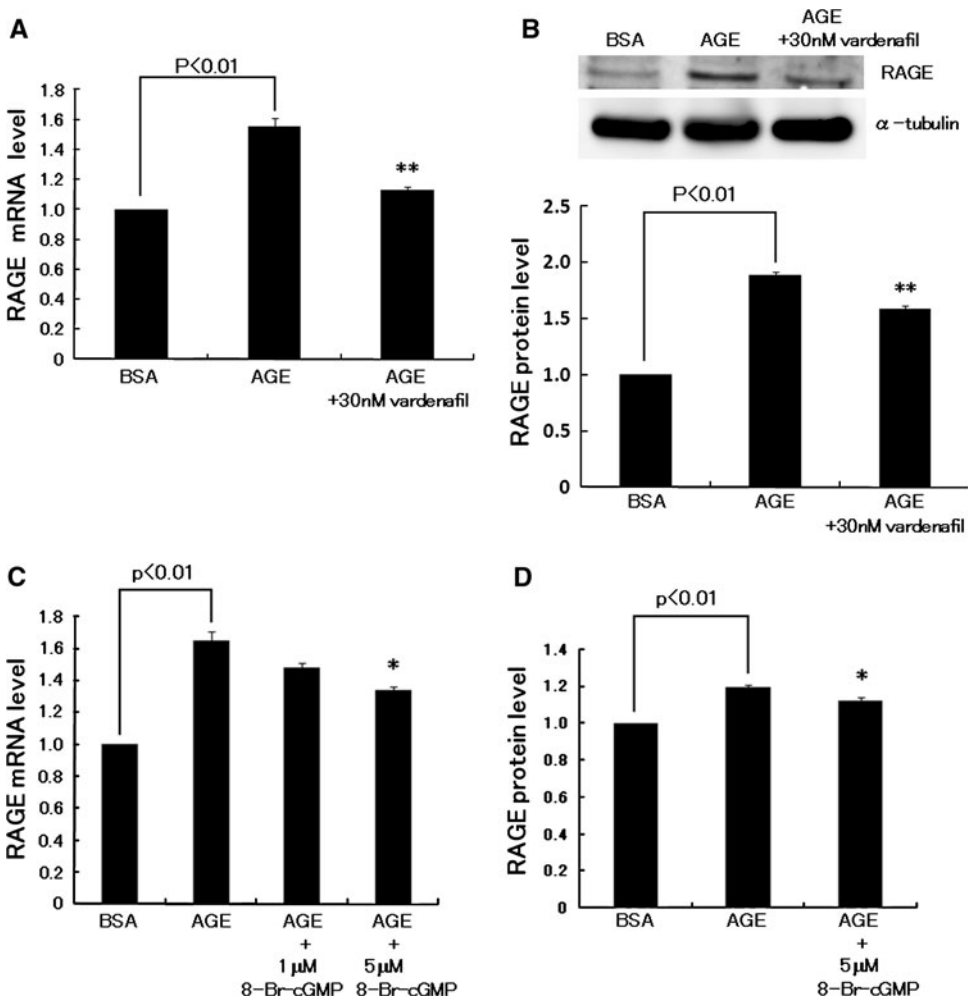

Fig. 1 Effects of vardenafil (**a** and **b**) and 8-Br-cGMP (**c** and **d**) on RAGE expression in HUVEC. HUVEC were treated with 100 μg/ml AGE-BSA or non-glycated BSA in the presence or absence of 30 nM vardenafil or the indicated concentrations of 8-Br-cGMP for 4 h. Then, RAGE gene (**a** and **c**) and protein (**b** and **d**) expressions were measured. * and **, $P < 0.05$ and $P < 0.01$, compared to the value with AGE alone. $N = 3$–5

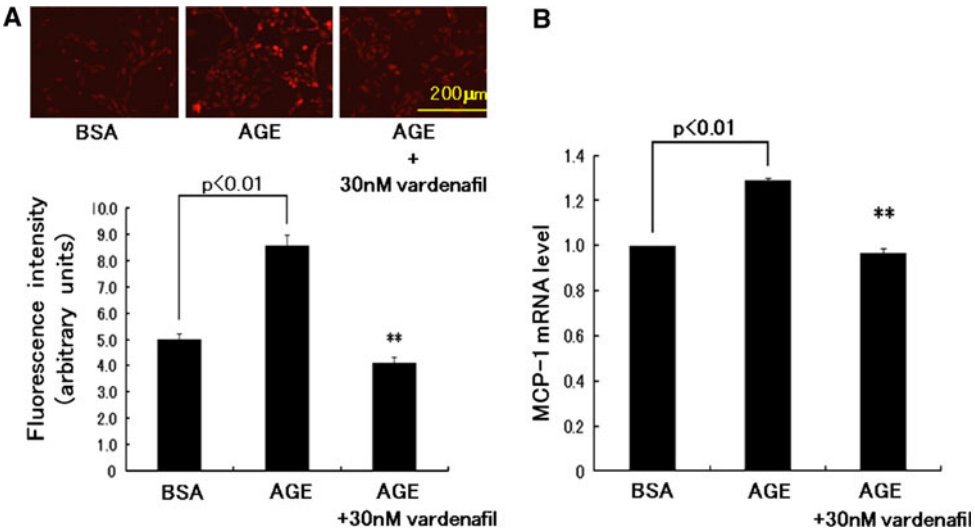

Fig. 2 Anti-inflammatory effects of vardenafil on AGE-exposed HUVEC. HUVEC were treated with 100 μg/ml AGE-BSA or non-glycated BSA in the presence or absence of 30 nM vardenafil for 4 h. Then, ROS generation (**a**) and MCP-1 gene expression (**b**) were measured. **, $P < 0.01$ compared to the value with AGE alone. $N = 3$–5

vardenafil on endothelial cells [1, 2], our present results suggest that vardenafil directly acts on endothelial cells and it could work as an anti-inflammatory agent against AGE by reducing RAGE expression probably via elevation of cGMP. In the present study, although the inhibitory effect of 30 nM vardenafil on RAGE protein expression was partial (Fig. 1a, b), the vardenafil treatment completely inhibited the AGE-induced ROS generation in HUVEC (Fig. 2a). The observation suggests that anti-oxidative properties of vardenafil in AGE-exposed HUVEC may be ascribed partly to mechanisms other than RAGE protein reduction.

MCP-1 plays an important role in the early phase of atherosclerosis by initiating monocyte recruitment to the vessel wall, and its expression is elevated in human atherosclerotic plaques [5, 6]. Further, the selective targeting of CCR2, the receptor for MCP-1, was shown to markedly decrease atheromatous lesion formation in apoE knockout mice [5, 6]. Therefore, our present study suggests that vardenafil may play a protective role against the development and progression of atherosclerosis by suppressing MCP-1 expression in diabetes. In support of this, Gazzaruso et al. recently reported that use of PDE-5 inhibitors was associated with a lower rate of major adverse cardiac events in diabetic ED patients with angiographically proven coronary artery disease [3]. A positive correlation between increased levels of AGE in penile tissues and diabetes-related ED was reported [10]. Further, administration of an inhibitor of AGE formation or an AGE cross-link breaker has been shown to reverse erectile dysfunction in diabetic rats [10]. Taken together, these observations suggest that AGE–RAGE axis is a molecular target of PDE-5 inhibitors and that vardenafil may provide a new therapeutic strategy for preventing cardiovascular disease in diabetic patients with ED.

The plasma concentration of vardenafil following oral administration of 20 mg vardenafil after an overnight fast is about 20–100 nM [11]. So, the concentration of vardenafil having beneficial effects on HUVEC (30 nM) is comparable to the therapeutic levels, which are achieved in the treatments of patients with ED. Further, in this study, in vitro-modified AGE was prepared by incubating BSA with glyceraldehyde for 1 week; this process produced relatively highly modified proteins in comparison with 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 μg/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 μg/ml polymyxin B an inhibitor of endotoxin for 30 min did not affect the AGE-induced ROS generation [6, 9].

Acknowledgments This work was supported in part by Grants of Collaboration with Venture Companies Project from the Ministry of Education, Culture, Sports, Science and Technology, Japan (S.Y.).

Conflict of interest None.

References

1. Lin CS (2009) Phosphodiesterase type 5 regulation in the penile corpora cavernosa. J Sex Med Suppl 3:203–209

2. Gur S, Sikka SC, Hellstrom WJ (2008) Novel phosphodiesterase-5 (PDE5) inhibitors in the alleviation of erectile dysfunction due to diabetes and ageing-induced oxidative stress. Expert Opin Investig Drugs 17:855–864
3. Gazzaruso C, Solerte SB, Pujia A, Coppola A, Vezzoli M, Salvucci F, Valenti C, Giustina A, Garzaniti A (2008) Erectile dysfunction as a predictor of cardiovascular events and death in diabetic patients with angiographically proven asymptomatic coronary artery disease: a potential protective role for statins and 5-phosphodiesterase inhibitors. J Am Coll Cardiol 51:2040–2044
4. Speel TG, van Langen H, Meuleman EJ (2003) The risk of coronary heart disease in men with erectile dysfunction. Eur Urol 44:366–370
5. Yamagishi S, Nakamura K, Matsui T (2008) Role of oxidative stress in the development of vascular injury and its therapeutic intervention by nifedipine. Curr Med Chem 15:172–177
6. Yamagishi S, Imaizumi T (2005) Diabetic vascular complications: pathophysiology, biochemical basis and potential therapeutic strategy. Curr Pharm Des 11:2279–2299
7. Bierhaus A, Stern DM, Nawroth PP (2006) RAGE in inflammation: a new therapeutic target? Curr Opin Investig Drugs 7:985–991
8. Yamagishi S, Matsui T, Nakamura K (2007) Kinetics, role and therapeutic implications of endogenous soluble form of receptor for advanced glycation end products (sRAGE) in diabetes. Curr Drug Targets 8:1138–1143
9. Yoshida T, Yamagishi S, Nakamura K, Matsui T, Imaizumi T, Takeuchi M, Koga H, Ueno T, Sata M (2006) Telmisartan inhibits AGE-induced C-reactive protein production through downregulation of the receptor for AGE via peroxisome proliferator-activated receptor-gamma activation. Diabetologia 49:3094–3099
10. Usta MF, Kendirci M, Gur S, Foxwell NA, Bivalacqua TJ, Cellek S, Hellstrom WJG (2006) The breakdown of preformed advanced glycation end products reverses erectile dysfunction in streptozotocin-induced diabetic rats: preventive versus curative treatment. J Sex Med 3:242–252
11. Rajagopalan P, Mazzu A, Xia C, Dawkins R, Sundaresan P (2003) Effect of high-fat breakfast and moderate-fat evening meal on the pharmacokinetics of vardenafil, an oral phosphodiesterase-5 inhibitor for the treatment of erectile dysfunction. J Clin Pharmacol 43:260–267