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Sulforaphane reduces advanced glycation end products (AGEs)-induced inflammation in endothelial cells and rat aorta

T. Matsui, N. Nakamura, A. Ojima, Y. Nishino, S.-i. Yamagishi*

Department of Pathophysiology and Therapeutics of Diabetic Vascular Complications, Kurume University School of Medicine, Kurume, Japan

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Abstract *Background and Aims:* Advanced glycation end products (AGEs)-receptor RAGE interaction evokes oxidative stress and inflammatory reactions, thereby being involved in endothelial cell (EC) damage in diabetes. Sulforaphane is generated from glucoraphanin, a naturally occurring isothiocyanate found in widely consumed cruciferous vegetables, by myrosinase. Sulforaphane has been reported to protect against oxidative stress-mediated cell and tissue injury. However, effects of sulforaphane on AGEs-induced vascular damage remain unclear.
Methods and Results: In this study, we investigated whether and how sulforaphane could inhibit inflammation in AGEs-exposed human umbilical vein ECs (HUVECs) and AGEs-injected rat aorta. Sulforaphane treatment for 4 or 24 h dose-dependently inhibited the AGEs-induced increase in RAGE, monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecular-1 (VCAM-1) gene expression in HUVECs. AGEs significantly stimulated MCP-1 production by, and THP-1 cell adhesion to, HUVECs, both of which were prevented by 1.6 μM sulforaphane. Sulforaphane significantly suppressed oxidative stress generation and NADPH oxidase activation evoked by AGEs in HUVECs. Furthermore, aortic RAGE, ICAM-1 and VCAM-1 expression in AGEs-injected rats were increased, which were suppressed by simultaneous infusion of sulforaphane.
Conclusion: The present study demonstrated for the first time that sulforaphane could inhibit inflammation in AGEs-exposed HUVECs and AGEs-infused rat aorta partly by suppressing RAGE expression through its anti-oxidative properties. Inhibition of the AGEs-RAGE axis by sulforaphane might be a novel therapeutic target for vascular injury in diabetes.
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List of abbreviations: AGEs, advanced glycation end products; RAGE, receptor for AGEs; Nrf2, nuclear factor erythroid-related factor 2; EC, endothelial cell; HUVECs, human umbilical vein ECs; BSA, bovine serum albumin; RT-PCR, reverse-transcription polymerase chain reactions; MCP-1, monocyte chemoattractant protein-1; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; RAGE-Ab, IgG polyclonal antibody directed against human RAGE; ELISA, enzyme-linked immunosorbent assay; HR, heart rate; BP, blood pressure; BG, blood glucose; AST, asparate aminotransferase; ALT, alanine aminotransferase; T-Chol, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein-cholesterol; BUN, blood urea nitrogen; SEM, standard error; ROS, reactive oxygen species; DBP, diastolic BP; 8-OHdG, 8-hydroxy-2′-deoxyguanosine.

* Corresponding author. Department of Pathophysiology and Therapeutics of Diabetic Vascular Complications, Kurume University School of Medicine, 67 Asahi-machi, Kurume, 830-0011, Japan. Tel.: +81 942 31 7873; fax: +81 942 31 7895.
E-mail address: shoichi@med.kurume-u.ac.jp (S.-i. Yamagishi).

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Introduction

Sugars, including glucose, fructose and trioses can react non-enzymatically with the amino groups of proteins, lipids and nucleic acids to form reversible Schiff bases, and then Amadori products [1,2]. These early glycation products undergo further complex reactions such as rearrangement, dehydration and condensation to become irreversibly cross-linked, heterogeneous macroprotein derivatives called "advanced glycation end products (AGEs)" [1,2]. The formation and accumulation of AGEs in various tissues have been known to progress at a physiological normal aging process and at an accelerated rate under hyperglycemic and oxidative stress conditions [1,2]. Recent understandings of this process have revealed that AGEs and their receptor (RAGE) interaction evokes oxidative stress generation and inflammatory, thrombogenic and fibrotic reactions in a variety of cells, thereby playing a central role in vascular complications in diabetes [3–7].

Sulforaphane is generated from glucoraphanin, a naturally occurring isothiocyanate found in widely consumed cruciferous vegetables such as broccoli, kale, cabbage and brussels sprouts, by myrosinase [8]. Sulforaphane is an inducer of phase II anti-oxidant and detoxification enzymes with potential anti-cancer properties [8]. Recently, sulforaphane has been shown to protect against oxidative stress-mediated cell and tissue damage [8–11]. Indeed, sulforaphane has improved metabolic derangement, reduced albuminuria and inhibited glomerulosclerosis in type 1 diabetic rats by suppressing oxidative stress generation via activation of nuclear factor erythroid-related factor 2 (Nrf2) [9]. However, as far as we know, there is no paper to examine the effects of sulforaphane on AGEs-induced endothelial cell (EC) damage and vascular injury in animal models. Therefore, in this study, we investigated whether and how sulforaphane could inhibit inflammation in AGEs-exposed human umbilical vein ECs (HUVECs) and AGEs-injected rat aorta.

Methods

Materials

Sulforaphane and bovine serum albumin (BSA) (essentially fatty acid free and essentially globulin free, lyophilized powder) were purchased from Sigma (St. Louis, MO, USA). D-glyceraldehyde and normal rabbit IgG were purchased from Nakalai Tesque (Kyoto, Japan) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively.

Preparation of AGEs-BSA

AGEs-BSA was prepared as described previously [12]. 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) at 37 °C 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 as described previously [12].

Cells

HUVECs obtained from Lonza Group Ltd. (Basel, Switzerland) 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 manufacturer's recommendation. AGEs or sulforaphane treatment was carried out in a medium lacking epidermal growth factor and hydrocortisone.

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

HUVECs were treated with 100 μg/ml AGEs-BSA or non-glycated BSA in the presence or absence of the indicated concentrations of sulforaphane for 4 or 24 h. Then total RNA was extracted with RNAqueous-4PCR kit (Ambion Inc., Austin, TX, USA) according to the supplier'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 manufacturer's recommendation. IDs of primers for human RAGE, monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and 18S gene were Hs00542592_g1, Hs00234140_m1, Hs00164932_m1, Hs01003372_m1, and Hs99999901_s1, respectively.

Preparation of IgG polyclonal antibody directed against human RAGE (RAGE-Ab) for cell culture experiments

RAGE-Ab, which recognizes the amino acid residues 167–180 of human RAGE protein, was used for neutralizing assays and prepared as described previously [13].

MCP-1 production

HUVECs were treated with 100 μg/ml AGEs-BSA or non-glycated BSA in the presence or absence of the indicated concentrations of sulforaphane for 24 h. Then MCP-1 levels in the medium were measured with an enzyme-linked immunosorbent assay system (ELISA) (R&D Systems, Inc. Minneapolis, MN, USA).

Assay of THP-1 cell adhesion to HUVECs

Human THP-1 monocytic leukemia cells (American Type Culture Collection, Manassas, VA, USA) were maintained in RPMI 1640 medium supplemented with 1% GultaMAX (Life Technologies Corporation, Carlsbad, CA, USA) and 1% fetal bovine serum (NICHIREI BIOSCIENCES INC, Tokyo, Japan). THP-1 cells were labeled with 3 μM BCECF-AM (Dojindo, Kumamoto, Japan) at 37 °C for 30 min according to the supplier's recommendation as described previously [14].

THP-1 cell adhesion to HUVECs was assayed according to the method described previously [14]. In brief, HUVECs were treated with 100 μg/ml AGEs-BSA or non-glycated BSA in the presence or absence of the indicated concentrations of sulforaphane, 5 μg/ml IgG, or 5 μg/ml RAGE-Ab for 48 h, and then incubated with BCECF-AM-labeled THP-1 cells for 4 h. After the incubation, non-adherent THP-1 cells were removed by washing the HUVECs gently a couple of times. Fluorescent intensities of the adherent THP-1 cells were measured.

Measurement of oxidative stress generation

HUVECs were treated with 100 μg/ml AGEs-BSA or non-glycated BSA in the presence or absence of the indicated concentrations of sulforaphane for 30 or 90 min. Then oxidative stress generation was measured by using the fluorescent probe, 1 μM carboxy-H_2DFFDA (Molecular Probes Inc., Eugene, OR, USA) as described previously [15].

Measurement of NADPH oxidase activity

HUVECs were treated with 100 μg/ml AGEs-BSA or non-glycated BSA in the presence or absence of the indicated concentrations of sulforaphane for 30 min. Then NADPH oxidase activity was measured by lucigenin-enhanced chemiluminescence according to the method of Li et al. [16].

Animal experiments

Seven week-old male Wistar rats (Charles River Laboratories Japan, Inc. Yokohama, Japan) were used in the present experiments. Rats were intraperitoneally injected with 1 mg non-glycated BSA or AGEs in the presence or absence of 0.5 mg/kg body weight sulforaphane (Saint Paul, MN, USA) every day for up to 11 days. At baseline and 11 days after the treatment, body weight and heart rate (HR) were measured, and blood pressure (BP) was monitored by a tail-cuff sphygmomanometer (BP-98A; Softron, Tokyo, Japan) as described previously [17]. Then blood samples were taken from abdominal veins of each rat for measurement of blood glucose (BG), serum insulin, asparate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (T-Chol), triglycerides (TG), high-density lipoprotein-cholesterol (HDL-C) and blood urea nitrogen (BUN). Serum levels of insulin were measured with an enzyme-linked immunosorbent assay kit (ALPCO Diagnostics Salem, NH, USA), and other biochemistry was measured as described previously [17]. The thoracic aortas were excised for immunohistochemical staining. All animal procedures were conducted according to the guidelines provided by the Kurume University Institutional Animal Care and Use Committee under an approved protocol.

Immunohistochemical analysis

Thoracic aorta were fixed for 24 h with 4% paraformaldehyde, dehydrated, embedded in paraffin and sectioned at 4-μm intervals and mounted on glass slides. After blocking endogenous peroxidase activity, the sections were incubated overnight at 4 °C with anti-RAGE (H-300, sc-5563), anti-ICAM-1 and anti-VCAM-1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Then the reactions were visualized with a Histofine Simple Stain Rat MAX-POMULTI kit (Nichirei Co., Tokyo, Japan) as described previously [18]. Intensity of staining in six different fields of each sample was analyzed by microcomputer-assisted ImageJ software version 1.46 (NIH, Bethesda, MD, USA).

Statistical analysis

All values were presented as mean ± standard error (SEM). One-way ANOVA followed by the Tukey's test for Figs. 1–4 or Student's t-test for Table 1 was performed for statistical comparisons; $p < 0.05$ was considered significant.

Results

We first examined the effects of sulforaphane on RAGE, MCP-1, ICAM-1 and VCAM-1 gene expression in AGEs-exposed HUVECs. As shown in Fig. 1A–D, sulforaphane treatment for 4 h dose-dependently inhibited the AGEs-induced increase in RAGE, MCP-1, ICAM-1, and VCAM-1 mRNA levels in HUVECs. Sulforaphane for 24 h also significantly inhibited the AGEs-induced up-regulation of RAGE, MCP-1 and VCAM-1 mRNA levels in a dose-dependent manner, but the ICAM-1 gene induction in AGEs-exposed HUVECs was not affected by sulforaphane (Fig. 1E–H). Sulforaphane treatment at 0.4 μM for 4 h in the presence of non-glycated BSA significantly increased RAGE mRNA levels in HUVECs, whereas 1.6 μM sulforaphane had a tendency to induce RAGE gene expression. Furthermore, sulforaphane at 0.4 or 1.6 μM for 4 or 24 h suppressed MCP-1, ICAM-1 and VCAM-1 gene expression in non-glycated BSA-exposed HUVECs as well (Fig. 1B–D, F and H).

We next examined the involvement of RAGE in AGEs-induced inflammatory reactions in HUVECs. As shown in Fig. 2, 1.5 h-pretreatment with 5 μg/ml RAGE-Ab, but not normal rabbit IgG significantly suppressed the AGEs-induced up-regulation of RAGE, MCP-1, ICAM-1 and VCAM-1 mRNA levels in HUVECs. Moreover, in contrast to the case without RAGE-Ab, 0.4 or 1.6 μM sulforaphane for 4 h did not further reduce RAGE, MCP-1 or ICAM-1 mRNA levels in RAGE-Ab-pretreated, AGEs-exposed HUVECs (Fig. 2A, B and C). However, VCAM-1 mRNA levels were further decreased by sulforaphane treatment in this cell type (Fig. 2D).

Since mRNA levels would not necessarily correspond to protein expression, we next investigated the effects of sulforaphane on MCP-1 production by, and THP-1 cell adhesion to, HUVECs. As shown in Fig. 3A and B, AGEs significantly stimulated MCP-1 production by, and THP-1 cell adhesion to, HUVECs, both of which were prevented by 1.6 μM sulforaphane. Moreover, RAGE-Ab significantly suppressed the increase in THP-1 adhesion to AGEs-exposed HUVECs (Fig. 3C). As the case of mRNA levels (Fig. 1F), sulforaphane dose-dependently suppressed MCP-

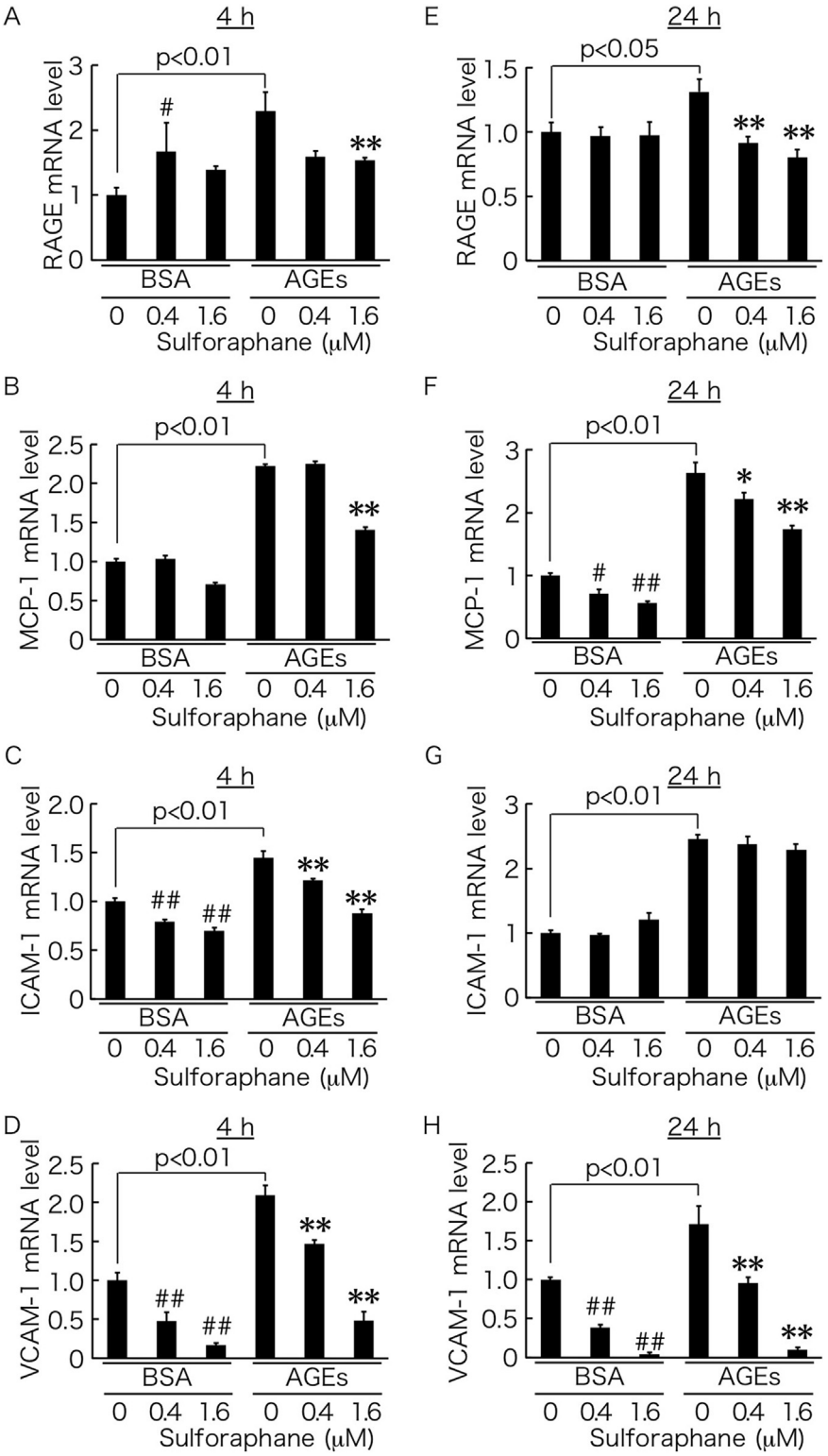


Figure 1 Effects of sulforaphane on RAGE (A and E), MCP-1 (B and F), ICAM-1 (C and G), and VCAM-1 (D and H) mRNA levels in AGEs-exposed HUVECs. HUVECs were treated with 100 μg/ml AGEs-BSA or non-glycated BSA in the presence or absence of the indicated concentrations of sulforaphane for 4 (A–D) or 24 h. (E–H) Then total RNAs were transcribed and amplified by real-time PCR. Data were normalized by the intensity of 18S mRNA-derived signals and then related to the value obtained with non-glycated BSA treatment alone. $N = 4$ per group. # and ##, $p < 0.05$ and $p < 0.01$ compared to the value with non-glycated BSA alone, respectively. * and **, $p < 0.05$ and $p < 0.01$ compared to the value with AGEs-BSA alone, respectively.

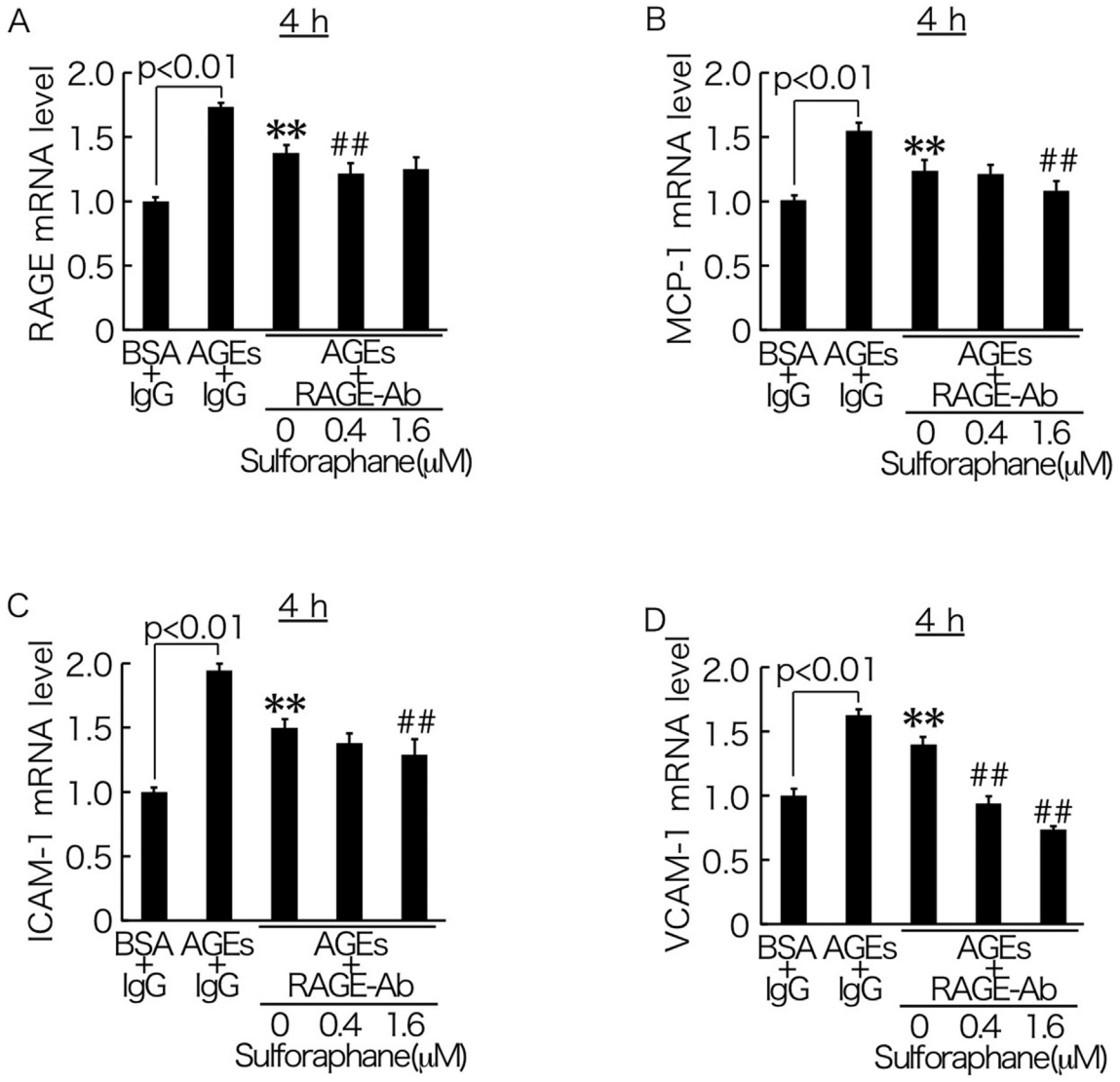


Figure 2 Effects of sulforaphane on RAGE (A), MCP-1 (B), ICAM-1 (C), and VCAM-1 (D) mRNA levels in RAGE-Ab-pretreated, AGEs-exposed HUVECs. HUVECs were preincubated with 5 μg/ml rabbit RAGE-Ab or normal rabbit IgG (IgG) for 1.5 h, and treated with 100 μg/ml AGEs-BSA or non-glycated BSA in the presence or absence of the indicated concentrations of sulforaphane for 4 h. Then total RNAs were transcribed and amplified by real-time PCR. Data were normalized by the intensity of 18S mRNA-derived signals and then related to the value obtained with non-glycated BSA + IgG. $N = 4$ per group. **, $p < 0.01$ compared to the value with AGEs + IgG. ##, $p < 0.01$ compared to the value with AGEs + RAGE-Ab.

1 production by non-glycated BSA-exposed HUVECs (Fig. 3A).

AGEs-RAGE interaction could exert pleiotropic actions on various types of cells through oxidative stress generation [3–7]. So we further studied the effects of sulforaphane on reactive oxygen species (ROS) generation in HUVECs. As shown in Fig. 4A–C, incubation of AGEs for 30–90 min significantly induced oxidative stress generation and NADPH oxidase activity in HUVECs, which were also blocked by the treatment with 1.6 μM sulforaphane.

We next investigated the effects of sulforaphane on vascular damage in AGEs-infused rats. As shown in Table 1, 0.5 mg/kg body weight sulforaphane administration to normal rats significantly decreased diastolic BP (DBP) and increased HDL-cholesterol. Infusion of AGEs increased TG in normal rats, which was prevented by the treatment with sulforaphane. HDL-C tended to decrease in AGEs-treated rats, which was also restored by sulforaphane. Moreover, RAGE, ICAM-1 and VCAM-1 expression in thoracic aorta of AGEs-injected rats were significantly increased, all of which were suppressed by simultaneous infusion of sulforaphane (Fig. 5). Although expression of MCP-1 and 8-hydroxy-2′-deoxyguanosine (8-OHdG), a marker of oxidative stress, and lymphocyte infiltration was not significantly increased by the treatment with AGEs injection, sulforaphane administration significantly reduced these parameters in AGEs-infused rats (data not shown).

Discussion

In this study, we found for the first time that (1) sulforaphane significantly suppressed the AGEs-induced RAGE,

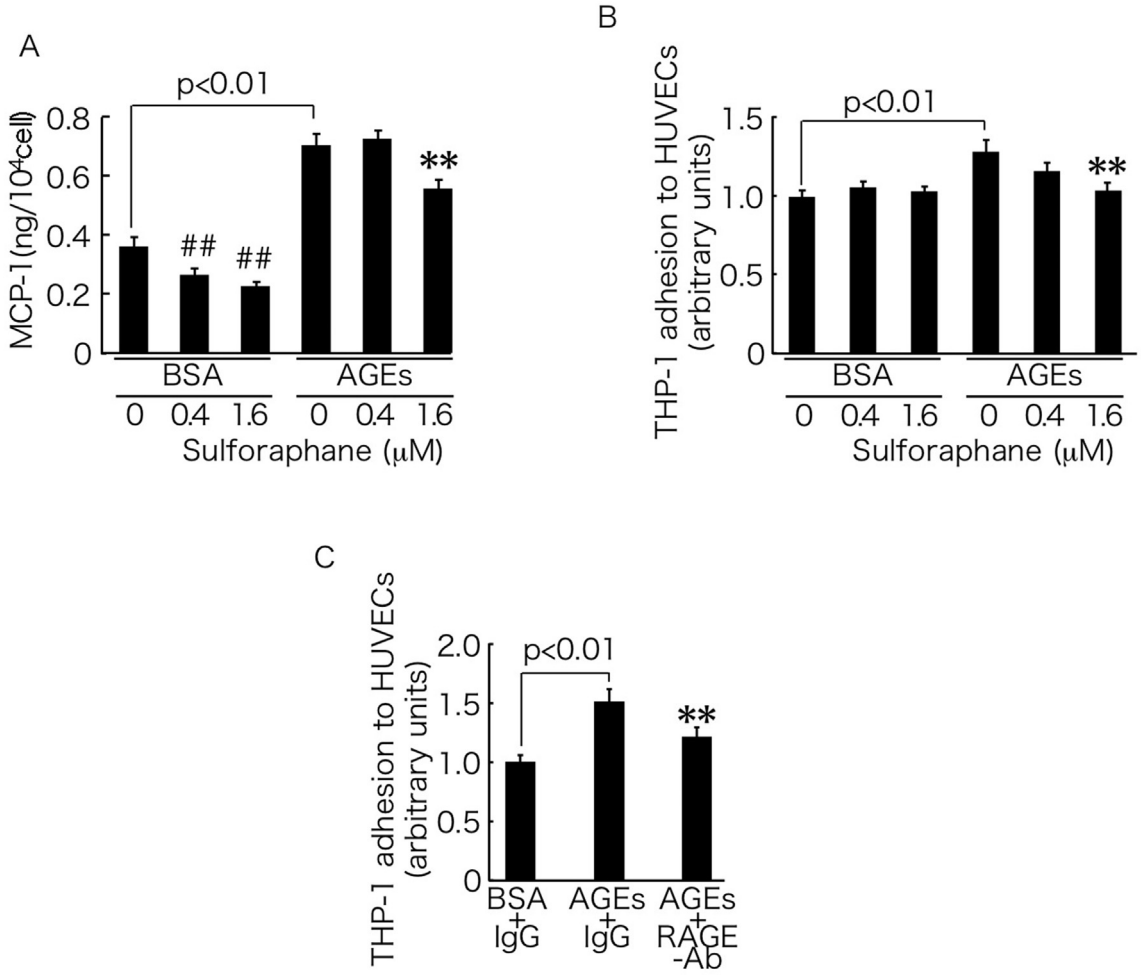


Figure 3 Effects of sulforaphane on MCP-1 production by (A), and THP-1 cell adhesion to, AGEs-exposed HUVECs (B and C). HUVECs were treated with 100 μg/ml AGEs-BSA or non-glycated BSA in the presence or absence of the indicated concentrations of sulforaphane, 5 μg/ml IgG or 5 μg/ml RAGE-Ab. (A) After 24 h, MCP-1 levels in the medium were measured with ELISA. $N = 4$ per group. (B) and (C) After 48 h, HUVECs were incubated with BCECF-AM-labeled THP-1 cells for 4 h. Then nonadherent THP-1 cells were removed. Fluorescent intensities of the adherent THP-1 cells were measured. $N = 8$ per group for (B) and $N = 6$ per group for (C). ##, $p < 0.01$ compared to the value with non-glycated BSA. **, $p < 0.01$ compared to the value with AGEs-BSA alone.

MCP-1, ICAM-1 and VCAM-1 gene expression in HUVECs, (2) pretreatment with 5 μg/ml RAGE-Ab significantly suppressed the AGEs-induced up-regulation of RAGE, MCP-1, ICAM-1 and VCAM-1 mRNA levels in HUVECs, (3) in contrast to the case without RAGE-Ab, additional treatment with 0.4 or 1.6 μM sulforaphane for 4 h did not further reduce these mRNA levels except VCAM-1 in RAGE-Ab-pretreated, AGEs-exposed HUVECs, (4) sulforaphane inhibited the MCP-1 production by, and the THP-1 monocytic cell adhesion to, AGEs-exposed HUVECs, (5) RAGE-Ab significantly suppressed the AGEs-induced increase in THP-1 cell adhesion to HUVECs, (6) AGEs stimulated the ROS generation and increased the NADPH oxidase activity in HUVECs, both of which were significantly suppressed by sulforaphane, and (7) sulforaphane administration significantly inhibited the increase in RAGE, ICAM-1 and VCAM-1 expression in thoracic aorta of AGEs-injected rats.

There is accumulating evidence that engagement of RAGE with AGEs elicits oxidative stress generation and resultantly causes inflammatory reactions in ECs, thus being involved in vascular complications in diabetes [3–7]. Indeed, we have previously shown that binding of AGEs to RAGE induces the MCP-1, ICAM-1 and VCAM-1 gene expression in ECs by stimulating oxidative stress generation via activation of NADPH oxidase [14,19–25]. We found here that pretreatment of RAGE-Ab significantly inhibited the AGEs-induced RAGE, MCP-1, ICAM-1 and VCAM-1 gene expression in HUVECs. Moreover, an anti-oxidant *N*-acetylcysteine has been shown to block up-regulation of RAGE mRNA levels in AGEs-exposed ECs, whereas antibody or antisense DNA raised against RAGE completely inhibits the AGEs-evoked EC ROS generation [12,22,26–29]. These findings indicate that there could exist a positive feedback loop between the AGEs-RAGE axis and ROS production; the AGEs-RAGE interaction-mediated oxidative stress generation could further potentiate the deleterious effects of AGEs on HUVECs via overexpression of RAGE.

In the present study, ROS was increased by 30–90-min exposure of AGEs, while inflammatory gene expression as

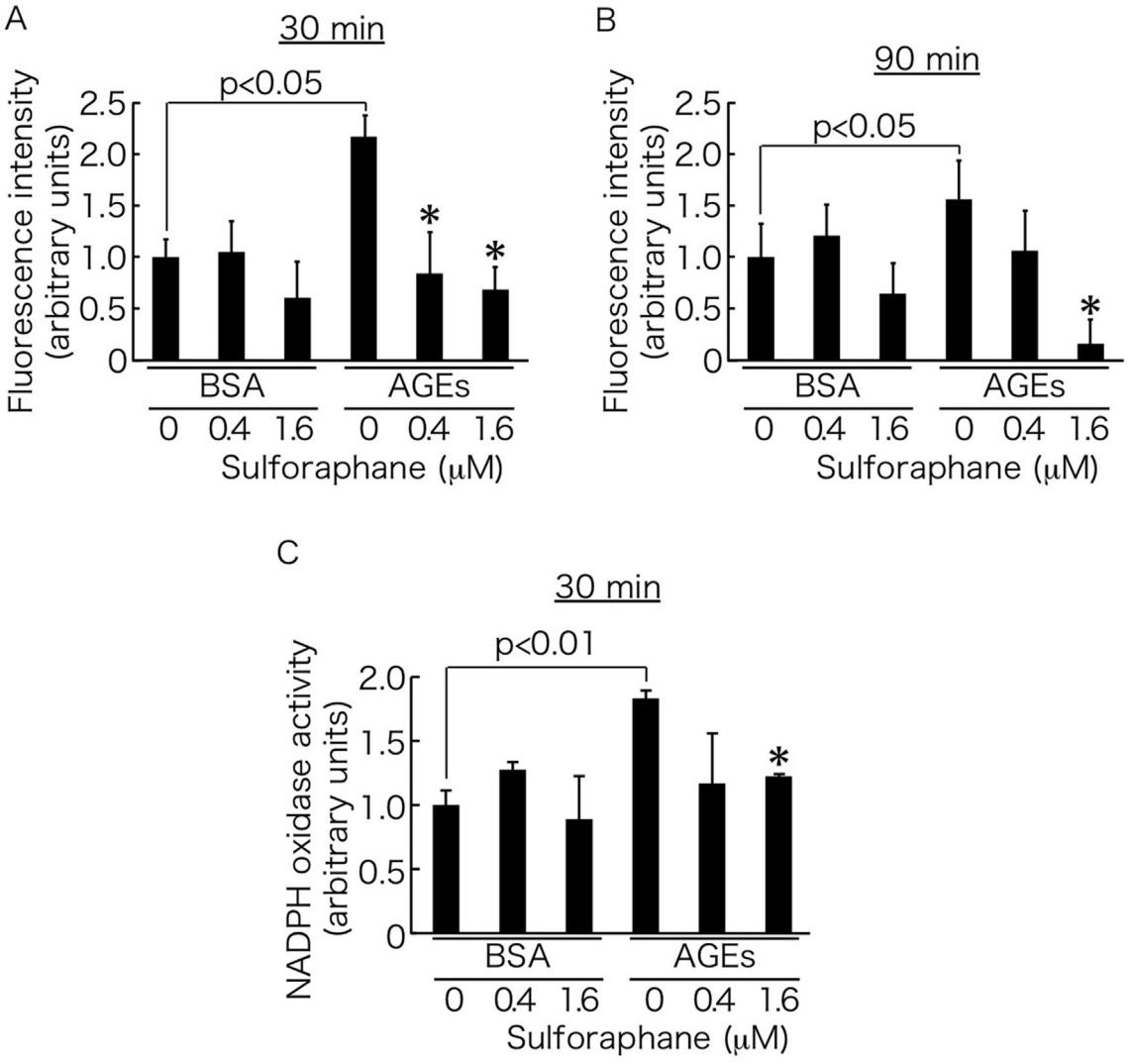


Figure 4 Effects of sulforaphane on oxidative stress generation (A and B) and NADPH oxidase activity (C) in AGEs-exposed HUVECs. HUVECs were treated with 100 μg/ml AGEs-BSA or non-glycated BSA in the presence or absence of the indicated concentrations of sulforaphane for 30 min (A and C) or 90 min (B). (A) and (B) Then oxidative stress generation was measured by using the fluorescent probe carboxy-H_2DFFDA. (C) NADPH oxidase activity was measured by lucigenin-enhanced chemiluminescence. $N = 5$ per group. *, $p < 0.05$ compared to the value with AGEs-BSA alone.

well as THP-1 adhesion to HUVECs were induced by the treatment with AGEs for 4–48 h, all of which were suppressed by sulforaphane. Therefore, the AGEs-RAGE axis could induce ROS generation and subsequently up-regulate RAGE expression in HUVECs, which might further augment the RAGE downstream pathway, thereby enhancing the inflammatory reactions in this cell type. Furthermore, since RAGE, MCP-1, ICAM-1, VCAM-1 and 8-OHdG levels in thoracic aorta of AGEs-infused, but not BSA-infused rats were significantly suppressed by the treatment with sulforaphane injection, sulforaphane might block the inflammation in AGEs-infused rat aorta partly by suppressing RAGE expression through its anti-oxidative properties. Therefore, although exposure to sulforaphane has been reported to reduce expression levels of MCP-1, ICAM-1 and VCAM-1 in ECs even in the absence of exposure to AGEs [30–32], our present study suggests that anti-inflammatory effects of sulforaphane observed in AGEs-treated HUVECs and rat aortas could be partly ascribed to its inhibitory actions on RAGE expression. Sulforaphane is not a direct anti-oxidant [33]. Therefore, we did not know why 30–90-min treatment with sulforaphane strongly reduced the ROS generation in AGEs-exposed HUVECs. However, since 1 h-treatment with sulforaphane has been shown to stimulate the Nrf2-mediated phase II anti-oxidative enzymes and resultantly inhibit the redox-sensitive transcriptional factor, nuclear factor-κB (NF-κB) activation [34,35], sulforaphane may reduce the AGEs-induced ROS generation partly through the Nrf2 pathway.

In this study, even under the condition of RAGE-Ab pretreatment, sulforaphane at 0.4 and 1.6 μM further decreased VCAM-1 mRNA levels in AGEs-exposed HUVECs (Fig. 2D). The findings suggest that other mechanism than RAGE suppression may also be responsible for VCAM-1 gene suppression by sulforaphane. Sulforaphane has

Table 1 Clinical data of animals.

N	BSA + Vehicle	BSA + Sulforaphane	AGEs + Vehicle	AGEs + Sulforaphane
	6	5	6	6
Body weight (g)	444 ± 13	441 ± 14	439 ± 10	439 ± 12
HR (beats/min)	361 ± 7	324 ± 12	333 ± 13	344 ± 16
SBP (mmHg)	140 ± 7	128 ± 3	134 ± 4	130 ± 4
DBP (mmHg)	100 ± 2	84 ± 4*	96 ± 3	94 ± 3
BG (mg/dL)	162 ± 8	158 ± 4	146 ± 4	146 ± 4
Insulin (μU/mL)	62 ± 14	58 ± 17	58 ± 13	44 ± 10
AST (U/L)	57 ± 4	88 ± 21	60 ± 4	59 ± 2
ALT (U/L)	21 ± 2	47 ± 19	23 ± 1	19 ± 2
T-Chol (mg/dL)	54 ± 5	63 ± 4	49 ± 6	56 ± 5
TG (mg/dL)	72 ± 6	69 ± 5	88 ± 7*	57 ± 7##
HDL-C (mg/dL)	34 ± 2	40 ± 3*	29 ± 3	36 ± 2#
BUN (mg/dL)	14 ± 1	15 ± 1	13 ± 1	13 ± 1

Values are mean ± SEM. *P < 0.05 compared with BSA + Vehicle, #P < 0.05, ##P < 0.01 compared with AGEs + Vehicle.
HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; BG, blood glucose; AST, aspartate aminotransferase; ALT, alanine aminotransferase; T-Chol, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; BUN, blood urea nitrogen.

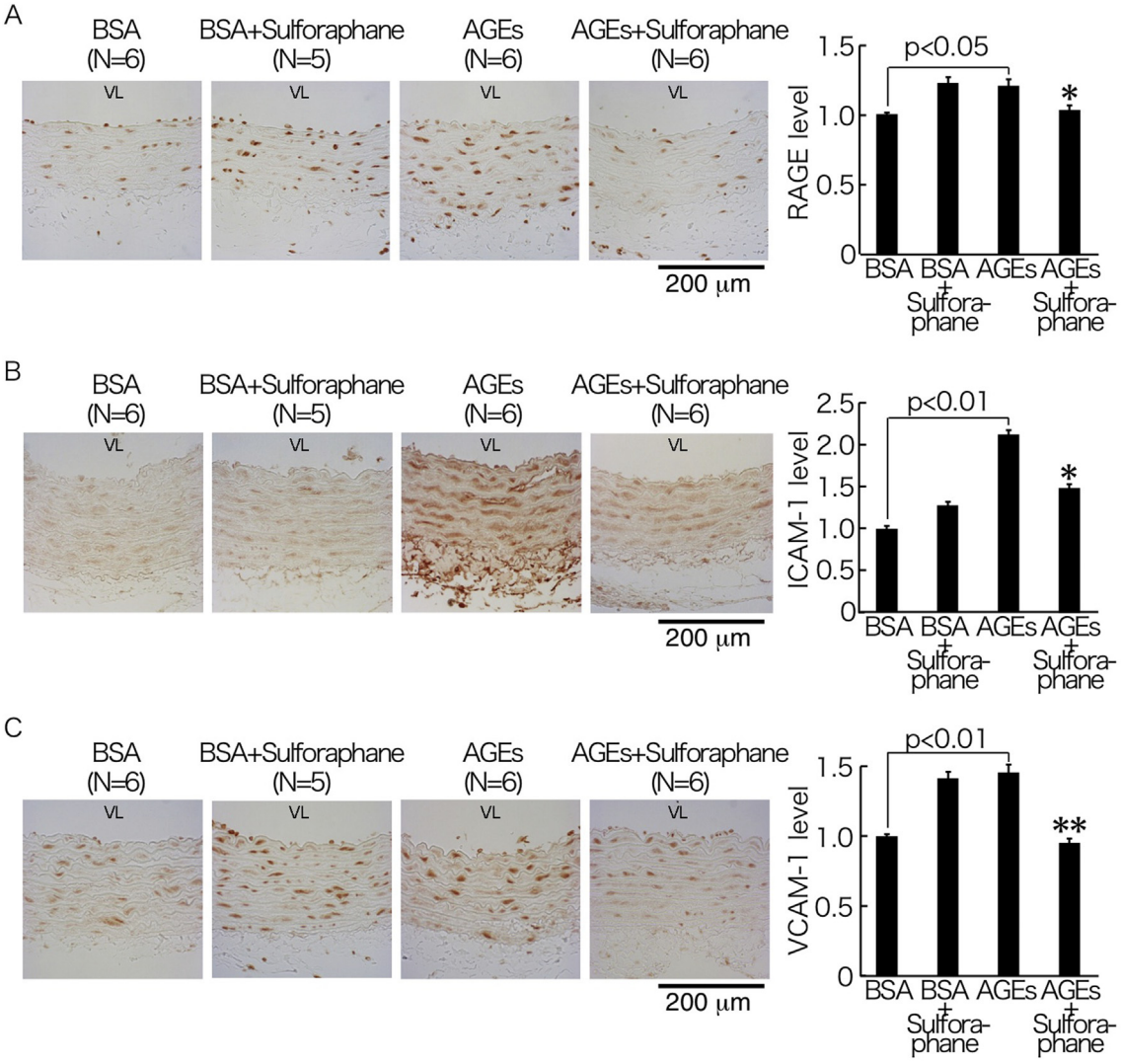


Figure 5 Effects of sulforaphane administration on MCP-1 (A), ICAM-1 (B), and VCAM-1 expression (C) in thoracic aorta of BSA or AGEs-injected rats. (A)–(C) Each left panel shows the representative results of immunostainings in thoracic aorta. Each right panel shows the quantitative results. Immunoreactivity in 6 different fields of each sample was measured. * and **, $p < 0.05$ and $p < 0.01$ compared to the value with AGEs + Vehicle rats, respectively. VL means vascular lumen.

been reported to inhibit tumor necrosis factor-α-induced p38 mitogen-activated protein kinase and VCAM-1 gene expression in ECs without affecting Nrf2 or NF-κB pathway [30]. Sulforaphane may inhibit the AGEs-induced VCAM-1 gene expression in HUVECs partly through the inhibition of p38 mitogen-activated protein kinase.

Hanlon et al. have reported that plasma concentration of sulforaphane is about 70 nM after intake of liquidized broccoli containing 3.9 mg sulforaphane [36]. Glucoraphanin content in cruciferous vegetables, such as broccoli, kale and cabbage is estimated to be about 4–250 mg/100 g [36–39], So the concentration of sulforaphane which could exert beneficial effects on HUVECs (0.4–1.6 μM) may be comparable to the physiological levels, which are achieved after daily consumption of 100 g cruciferous vegetables.

One early phase of diabetic retinopathy, nephropathy and atherosclerosis involves the recruitment and firm adhesion of inflammatory cells to ECs, whose process is mainly mediated by adhesion molecules, such as ICAM-1 and VCAM-1 [40–42]. Further, MCP-1 also plays an important role in EC and kidney damage by initiating monocyte recruitment to the vessel wall and renal area, and its expression levels are elevated in human atherosclerotic plaques, aqueous humor and vitreous of patients with proliferative diabetic retinopathy, and tubulointerstitial lesions of human diabetic nephropathy [43–46]. Selective targeting of MCP-1 has been shown to not only decrease atheromatous lesion formation in atherosclerosis-prone, low-density lipoprotein receptor deficient mice, but also suppress albuminuria, renal injury and fibrosis in streptozotocin-induced diabetic rats [47,48]. Taken together, these observations suggest that sulforaphane could protect against vascular complications in diabetes partly by suppressing MCP-1, ICAM-1 and VCAM-1 expression through the blockade of the harmful effects of AGEs-RAGE axis. Sulforaphane has been recently found to inhibit a precursor of AGEs, methylglyoxal-induced neuronal cell damage and apoptosis by reducing ROS production and increasing intracellular glutathione levels as well [49].

We, along with others, have found that AGEs induce ROS generation in ECs through the interaction with RAGE via activation of NADPH oxidase activity [12,22–25,50]. In this study, we found that sulforaphane inhibited ROS generation and NADPH oxidase activity in AGEs-exposed HUVECs (Fig. 4). NADPH oxidase-mediated ROS generation is enhanced under intracellular glutathione depleted-conditions [51]. Sulforaphane is an inducer of phase II antioxidant and detoxification enzymes [8], and has been shown to restore cellular glutathione levels in an Nrf2-dependent manner [51]. Therefore, the present findings further support the concept that sulforaphane may inhibit the AGEs-RAGE-induced NADPH oxidase-mediated ROS generation in HUVECs partly via activation of Nrf2. However, it remains unclear whether sulforaphane may reduce the expression of RAGE in AGEs-exposed HUVECs through Nrf2-mediated transcriptional repression. Since we have previously shown that hyperglycemia induces ROS generation and exacerbates renal dysfunctional in Nrf2-deficient mice [52], it would be interesting to further examine whether sulforaphane could reduce RAGE gene expression and inflammatory reactions in AGEs-infused Nrf2-knockout mice. In this study, we did not know why 0.4 and 1.6 μM sulforaphane alone for 4 h induced RAGE gene expression in HUVECs (Fig. 1A). Given the facts that sulforaphane did not increase *rather than* decrease MCP-1, ICAM-1 or VCAM-1 mRNA levels in non-glycated BSA-exposed HUVECs, the mechanism other than RAGE suppression may account for its anti-inflammatory actions of sulforaphane on non-AGEs-exposed HUVECs.

Conclusions

The present study suggested that sulforaphane may inhibit the inflammation in AGEs-exposed HUVECs and AGEs-infused rat aorta partly by suppressing RAGE expression through its anti-oxidative properties. Suppression of the AGEs-RAGE axis by sulforaphane might be a novel therapeutic target for vascular injury in diabetes.

Authors' contributions

S.Y. conceptualized and designed the study; acquired, analyzed, and interpreted data; and drafted the manuscript; and took responsibility for the integrity of the data and the accuracy of the data analysis. T.M., N.N., A.O., and Y.N. acquired, analyzed, and interpreted data.

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Conflict of interest

There is no conflict of interest in this paper.

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