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Inhibitory effect of black raspberry extract on AGE accumulation and degradation, and ROS production in HUVEC cells

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ABSTRACT

Background: A critical event in age-related diseases involves the glycation of various proteins in the animal body to generate advanced glycation end products (AGEs). We have previously found that black raspberry extract (BRE) has effects on age-related diseases. From this observation, we expected that berry extracts, specifically BRE, would have positive effects on AGE-stimulated cell events that link to age-related diseases.

Objective: To discuss the potency of berry extracts against diseases attributable to the AGE-dependent changes of cellular events, in this study, we examined the effects of berry extracts on the cellular events changed upon AGE stimulation of human umbilical vein endothelial cells (HUVECs) through AGE receptors.

Methods: After HUVECs were incubated with AGE-BSA in the presence of serially diluted berry extracts, mRNA and protein levels of AGE receptors, intracellular AGE accumulation, and ROS production in the cell were determined by qRT-PCR and Western blotting, ELISA, and staining with the fluorescent probe, respectively.

Results: Although concentration-dependent effects of berry extracts tested on mRNA levels of AGE receptors in HUVECs were not clear, mRNA level of the AGE receptor RAGE that is involved in the intracellular ROS production was increased by Blabina, which contains BRE, and the well-known anti-glycation compound aminoguanidine (AGD). In contrast, the protein expression level of RAGE was decreased by BRE and Blabina, but not by AGD. It was also found that BRE and Blabina suppressed AGE-BSA-stimulated ROS production in HUVECs. The extent of inhibition in the RAGE protein expression by BRE and Blabina was correlated well with the ROS generation measured in these samples.

Conclusions: The results obtained in this study demonstrate that BRE has the most potent inhibitory effect on ROS accumulation in the cell, probably due to the suppression in the expression level of the RAGE protein. These observations suggest that black raspberry could be a potential nutraceutical to prevent various age-related diseases.

Keywords: AGEs; RAGE; ROS; black raspberry; HUVECs.

INTRODUCTION

Cellular senescence is closely related to age-related pathologies [1], since removal of senescent cells from mice has previously been shown to increase lifespan [2]. A critical event related to the cellular senescence is glycation, which is a non-enzymatic chemical reaction at amino acid residues of various proteins with sugars to generate advanced glycation end products (AGEs) [3]. AGEs play an important role in aging of the whole organism [4], therefore they are involved in a wide variety of diseases. Humans cannot avoid glycation of proteins within blood sugars because they use dietary carbohydrates as their primary energy source [5]. It has been reported that the level of AGEs in skin collagens increases with aging and is higher in diabetic patients than in healthy individuals of the same age [6]. Microvascular densities and the maturation in the skin of diabetic patients are also closely related to AGE accumulation in the cell [7]. In addition, the formation and accumulation of AGEs in the cell has been shown to be involved in skin aging [8], Alzheimer's disease [9], hypertension [10], arteriosclerosis [11] and osteoporosis [12]. Therefore, the mechanism of AGE effects on cell functions has attracted attention to understanding the pathogenesis of various diseases related to AGEs.

Recently, two types of receptors for AGEs, one of which activates the cellular signal pathways linking to inflammation and another of which is involved in the incorporation, degradation, and elimination of AGEs, have been clarified [13,14,15] (Figure 1). AGE receptors including AGE-R1 (OST-48), FEEL-1 and -2 (Stabillin-1 and -2), and CD-36 are involved in the uptake of extracellular AGEs into cells by endocytosis and their degradation. Other types of AGE receptors, such as AGE-R2 and RAGE (receptor for AGEs), activate ROS production through the stimulation of NADPH oxidase [16] (Figure 1). The best characterized AGE receptor, "RAGE," also activates the cellular signaling to stimulate the production of inflammatory cytokines [17] and acts

as an AGE receptor in the pathogenesis of chronic obstructive pulmonary disease, cardiovascular disease, type 2 diabetes, and osteoporosis [18].

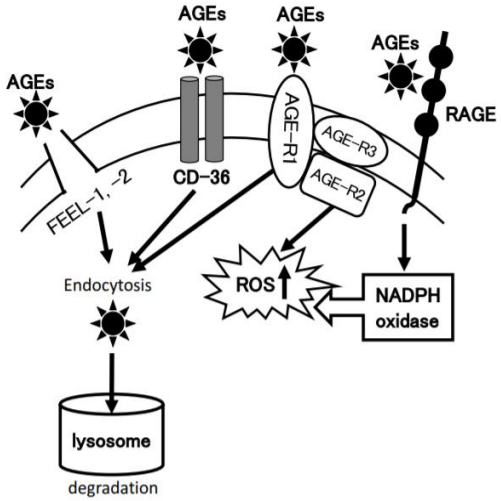


Figure 1. AGE receptors expressed in HUVEC cells and the cell signaling pathways. Receptors including AGE-R1, FEEL-1, -2, and CD-36 endocytose extracellular AGEs into cells then degrade them in the lysosome. Other AGE receptors, such as AGE-R2 and -R3, and RAGE, increase intracellular ROS either directly or indirectly through activation of NADPH oxidase.

Since AGEs and their receptor RAGE are closely related to a wide variety of diseases, glycation of proteins could be a very important target of drug development for various age-related diseases. We have previously investigated anti-aging effects of several kinds of raspberries and found that black raspberry extract (BRE) has effects on cataract, alopecia, skin whitening, and weight loss [19]. In this study, we examined the effects of berry extracts on expression levels of AGE receptors, AGE accumulation, and ROS production in the cell, and discussed the mechanisms by which berry extracts inhibit these events.

MATERIALS AND METHODS

Materials: D-Glucose, DL-glyceraldehyde, and the anti-glycation compound aminoguanidine (AGD)─ the well-known anti-glycation compound used as a positive control in this study─ was purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). Bovine serum albumin (BSA) (Fraction V) and RAGE polyclonal antibody (Catalog Number PA5-24787) were purchased from SIGMA (St. Louis, MO, USA) and Thermo Fisher (Waltham, MA, USA), respectively. Anti-AGE-R2 (GTX101856), -RAGE (ab54741), and -β-actin antibodies (HRP-60008) were purchased from Gene Tex, abcom, and ProteinTech, respectively. AGEs ELISA Kit (STA-817) was from CELL BIOLABS.

Preparation of berry extracts: Blabina, which is a powdered formulation containing black raspberry extract (BRE) (trademark registration number 5735928), was provided in a frozen state from INNATUS Co. Fruits of black raspberries (Rubus occidentalis; BRs), blueberries (Vaccinium spp.; BBs) and raspberries (Rubus idaeus; RBs). These materials were provided in a frozen state from INNATUS Co., dissolved in 30% ethanol at 9 mg/ml, and evaporated to remove ethanol [19]. Ethanol-depleted berry extracts (ca. 11.5 mg/ml), including BRE, blueberry extract (BBE), and raspberry extract (RBE), were serially diluted with ultrapure water and added to human umbilical vein endothelial cells (HUVECs) (C-12203, PromoCell) in the culture medium (Endothelial Cell Growth Medium 2 Supplement Pack, C-39211: PromoCell) at a volume of 1/100.

Preparation of AGE of BSA (AGE-BSA): AGE-BSA was prepared as previously described [21]. BSA (25 mg/ml) was incubated with 0.1M DL-glyceraldehyde in phosphate-buffed saline, pH 7.4 (PBS), at 37°C for 7 days, and used in the experiments.

Cell culture: HUVECs were cultured in the culture medium at 37°C in a 5% CO_2 incubator. After being harvested with 0.25% trypsin and 0.1% EDTA, cells were

seeded at 2 x 10^5 cells/well in a 35-mm dish and cultured for 24 hrs before use in the experiments.

Expression levels of AGE receptors: mRNA levels of *AGE receptors* including *FEEL-1 (Stabillin-1)* [22], *FEEL-2 (Stabillin-2)* [22], *CD-36* [23], *AGE-R1 (OST-48)* [24], *AGE-R2 (80K-H)* [25], and *RAGE* [26] in HUVECs were measured using one-step quantitative reverse transcription-polymerase chain reaction (qRT-PCR) method. The mRNA level of *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* was also measured as an internal control.

HUVECs were treated with AGE-BSA (100 μg/ml) in the presence or absence of serially diluted berry extracts at 37°C for 4 hrs and total RNA was extracted from cells using TRIzol reagent (Ambion). qRT-PCR was performed in the final volume of 20 μl of solution including 10 μl of 2x Luna Universal One-Step Reaction Mix, 1 μl of Luna WarmStart® RT Enzyme Mix, 2 μλ (500 ng) of total RNA solution, 1.6 μl of primer pair mix (0.4 μM for each primer), and 7.3 μλ of H_2O under the following conditions: at 55°C for 10 min for reverse transcription and then at 95°C for 1 min for initial denaturation, followed by 45 cycles at 95°C for 10 sec and at 60°C for 30 sec. Relative gene expression was calculated using the ΔΔCt method [27] and the stable expression gene, *GAPDH*, was used for normalization. The primers used in qRT-PCR are shown in Table 1

Quantitation of intracellular AGEs: HUVECs were stimulated with 100 μg/ml of AGE-BSA or BSA in the presence or absence of serially diluted berry extracts at 37°C for 4 hrs. The intracellular levels of AGEs, which were incorporated from outside or generated inside the cell, were then determined with the AGEs ELISA Kit (STA-817, CELL BIOLABS, INC.), according to the manufacturer's instruction. The intracellular AGE levels in HUVECs incubated with BSA and AGE-BSA were 0.7

Table 1. Primers for PCR

Gene	Primer	References
FEEL-1 (Stabilin-1)	Forward; AGG ACT GCC GCT ACG AAG TA	22
	Reverse; CAC TGC CCT GCT GTG TGT AG	
FEEL-2 (Stabilin-2)	Forward; TCT GAA GGC AGG TCT CAC CTA	22
	Reverse; CTG GGG AGC AGA AAT TTT GTA	
CD-36	Forward; GAG AAC TGT TAT GGG GCT AT	23
	Reverse; TTC AAC TGG AGA GGC AAA GG	
AGE receptor-1	Forward; GTG GGA AAA TGG CAC AAC TT	24
	Reverse; CTG GCC ACG TCC CTA TTT TA	
AGE receptor-2	Forward; AGG GCC GTA AGG AGA GAG AG	25
	Reverse; GTG GCG TCT GTC TGT GTG TC	
RAGE	Forward; GAA ACT GAA CAC AGG CCG GA	26
	Reverse; CAC GGA CTC GGT AGT TGG AC	
GAPDH	Forward; AGG GCT GCT TTT AAC TCT GGT	26
	Reverse; CCC CAC TTG ATT TTG GAG GGA	

μg/ml (basal level) and 5.2 μg/ml (stimulated level) respectively. Based on these intracellular AGE levels, inhibition of AGE accumulation in the cell by berry extracts was calculated and shown as percentages.

Western blot analysis: Protein expression levels of AGE receptors including AGE-R2 and RAGE were also determined by Western blotting after HUVECs were treated for 24 hrs as described above. Protein expression levels of these receptors were normalized by the actin level. HUVECs were treated as described in the section "Expression levels of AGE receptors," washed with cold PBS, scraped off, homogenized by hydrodynamic shearing with a 23-gauge needle, and centrifuged at 12,000 x g at 4°C for 20 min. The precipitant was lysed in the lysis buffer consisting of 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid with a pH of 7.1, 150 mM NaCl, 1% Triton-X-100, 10% glycerol, 2 mM sodium orthovanadate, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 1% phosphatase inhibitor cocktails I and II and 1% protease inhibitor cocktail. After boiling the samples at 95°C for 5 min, 5 μg of proteins in the cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%) and transferred to polyvinylidene difluoride (PVDF; Millipore, Billerica, MA, USA) membranes. After being incubated with a blocking buffer consisting of 5% non-fat milk, 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween-20, the membranes were blotted with specific antibodies to AGE receptors in the blocking buffer at 4°C overnight, followed by incubation with a secondary antibody for 2 hrs. The immunoreactive bands were visualized with an enhanced chemiluminescence detection system (Image Quant LAS 500, GE Healthcare Life Science) [28]. Expression levels of receptor proteins were quantified using Image J software and normalized in regard to the actin control.

Measurement of intracellular ROS production: Intracellular ROS levels were determined using the fluorescent probe CM-H2DCFDA (Molecular Probes Inc., Eugene, OR) [28]. After HUVECs were treated as described in the section "Expression levels of AGE receptors," cells were washed with PBS and incubated with 1 μM fluorescent probe for 60 min at 37°C. Intracellular ROS levels were determined by measuring fluorescence intensity using the micro plate reader (SYNERGY/HT, BioTek, Japan). Basal intensity was 1860 ± 163 and maximum fluorescence was 56500 ± 237 with arbitrary units Images were visualized under a fluorescent microscope (BZ-X710, Keyence, Osaka, Japan) and joined using its software BZ-analyzer (Keyence).

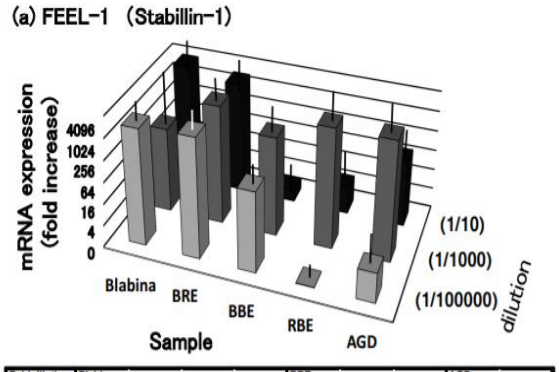
Assessment of inhibitory effects of berry extracts on in vitro AGE formation: To evaluate the anti-glycation effects of berries *in vitro*, a mixture of 0.5% BSA and 300 mg/dl of D-glucose was incubated with or without berry extracts at 60°C for 48 hrs. Subsequently, fluorescence intensity derived from AGE-BSA formed in the reaction (370 nm of excitation wavelength and 440 nm of fluorescence wavelength) [29, 30] was measured using multi-mode plate reader (Synergy HTX, BioTek Japan). The basal fluorescence intensity in the presence of BSA alone and the maximal fluorescence intensity obtained in the reaction of BSA plus D-glucose were 123 and 1768, respectively. Based on these fluorescence intensities, inhibition of *in vitro* AGE formation by berries was calculated as a percentage.

Statistical analysis: All results are presented as mean ± SD. Statistical significance was determined using one-way analysis of variance (ANOVA) and the difference was considered statistically significant when $P < 0.05$.

RESULTS

Effects of berry extracts on mRNA levels of *AGE receptors:* To evaluate the effects of berry extracts on

the expression of AGE receptors, their mRNA levels in HUVECs were analyzed after treatment of the cell with AGE-BSA in the presence of various berry extracts (Figure 2). In mRNA, levels of *AGE receptors* such as FEEL-1, FEEL-2, CD-36, and AGE-R1, which are involved in AGE incorporation and degradation, incurred neither concentration-dependent changes by berry extracts nor clear different effects among berry extracts (Figure 2a-d).



Fold dilution	Blabina				BRE			AGD	
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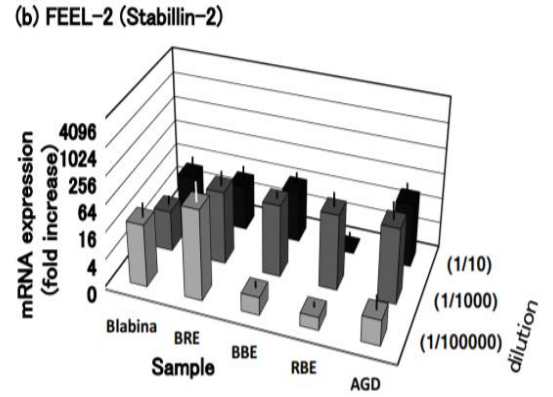
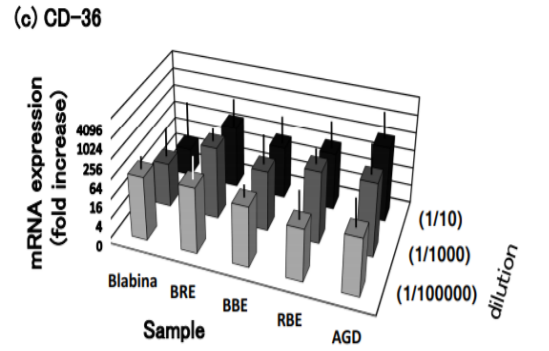
(1/10)	ns	***	***	*	***	***	ns	***	*
(1/1000)	ns	ns	ns	ns	ns	ns	ns	ns	ns
(1/100000)	ns	**	***	***	**	***	***	**	*

ns: not significant, * ; P<0.05, ** ; P<0.01, *** ; P<0.001



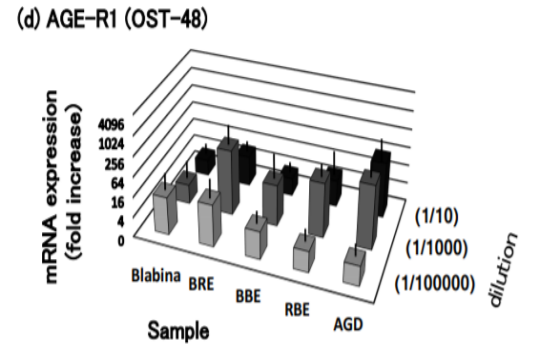
Fold dilution	Blabina				BRE			AGD	
	BRE	BBE	RBE	AGD	BBE	RBE	AGD	BBE	RBE
(1/10)	ns	ns	***	ns	ns	***	ns	ns	***
(1/1000)	*	*	*	*	ns	ns	ns	ns	ns
(1/100000)	*	***	***	**	***	***	***	ns	ns

ns: not significant, * ; P<0.05, ** ; P<0.01, *** ; P<0.001



Fold dilution	Blabina				BRE			AGD	
	BRE	BBE	RBE	AGD	BBE	RBE	AGD	BBE	RBE
(1/10)	ns	ns	ns	ns	ns	ns	ns	ns	ns
(1/1000)	ns	ns	ns	ns	ns	ns	ns	ns	ns
(1/100000)	ns	ns	ns	ns	ns	ns	ns	ns	ns

ns: not significant



Fold dilution	Blabina				BRE			AGD	
	BRE	BBE	RBE	AGD	BBE	RBE	AGD	BBE	RBE
(1/10)	ns	ns	ns	*	ns	ns	*	**	*
(1/1000)	**	*	**	**	*	ns	ns	ns	ns
(1/100000)	ns	ns	*	*	ns	ns	ns	ns	ns

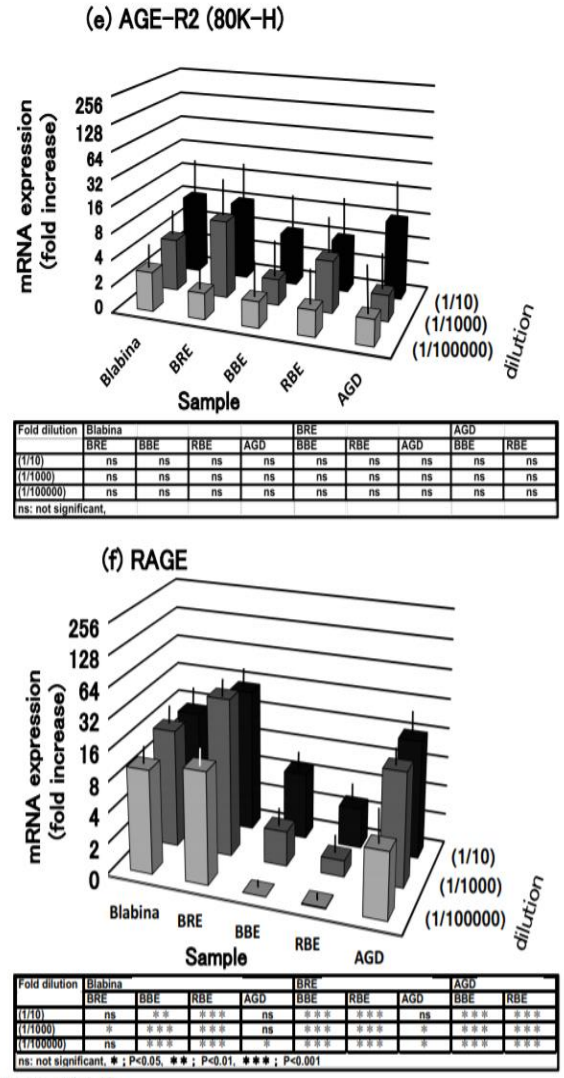
ns: not significant, * ; P<0.05, ** ; P<0.01

Figure 2 a, b: The mRNA expression levels of AGE receptors in HUVECs including FEEL-1 (a) and FEEL-2 (b), which are involved in glycation stress-induced AGE endocytosis and degeneration.

Figure 2 c, d: The mRNA expression levels of AGE receptors in HUVECs including CD-36 (c) and AGE-R1 (d), which are involved in glycation stress-induced AGE endocytosis and degeneration.

In contrast, the mRNA level of the receptor, RAGE, which is involved in the intracellular ROS generation, was highly increased by Blabina and BRE compared with other berry extracts (Figure 2f). Although there was no clear difference in the mRNA level of another receptor involving in ROS production, *AGE-R2,* was observed among berry extracts (Figure 2e). The positive control

AGD, Blabina, and BRE increased the mRNA level of *RAGE* (Figure 2f).



Fold dilution	Blabina				BRE			AGD		
	BRE	BBE	RBE	AGD	BBE	RBE	AGD	BBE	RBE
(1/10)	ns	ns	ns	ns	ns	ns	ns	ns	ns
(1/1000)	ns	ns	ns	ns	ns	ns	ns	ns	ns
(1/100000)	ns	ns	ns	ns	ns	ns	ns	ns	ns

ns: not significant,

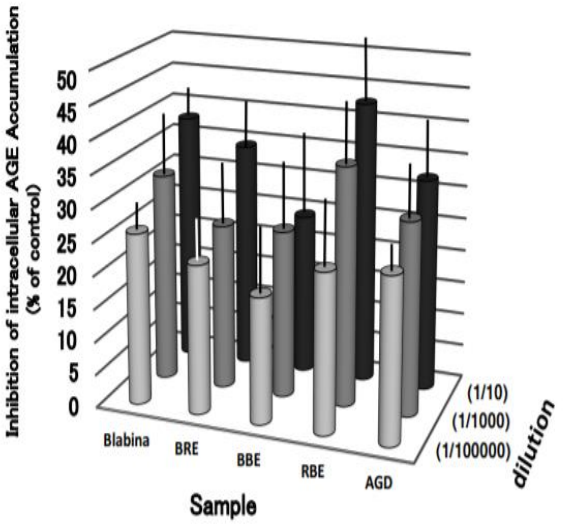
Fold dilution	Blabina				BRE			AGD		
	BRE	BBE	RBE	AGD	BBE	RBE	AGD	BBE	RBE
(1/10)	ns	**	***	ns	***	***	ns	***	***
(1/1000)	*	***	***	ns	***	***	*	***	***
(1/100000)	ns	***	***	*	***	***	*	***	***

ns: not significant, * ; P<0.05, ** ; P<0.01, *** ; P<0.001

Figure 2 (e) (f).

Figure 2 e, f: The mRNA expression levels of AGE receptors in HUVECs including AGE-R2 (e) and RAGE (f), which are involved in the stimulation of ROS production by glycation stress.

Inhibition of intracellular AGE accumulation by berry extracts: Figure 3 shows the effects of berry extracts on intracellular AGE accumulation augmented by AGE-BSA stimulation of HUVECs. All berry extracts inhibited the intracellular AGE accumulation augmented by AGE-BSA in a concentration-dependent manner: about 20% inhibition by 100,000-fold diluted berry extracts was observed. The extent of the inhibition by these berry extracts was not significantly different from that by the anti-glycation compound AGD.



Fold dilution	Blabina				BRE			AGD		
	BRE	BBE	RBE	AGD	BBE	RBE	AGD	BBE	RBE
(1/10)	ns	ns	ns	ns	ns	ns	ns	ns	ns
(1/1000)	ns	ns	ns	ns	ns	ns	ns	ns	ns
(1/100000)	ns	ns	ns	ns	ns	ns	ns	ns	ns

ns: not significant

Figure 3. Inhibition of AGE accumulation in HUVECs by berry extracts. HUVECs were treated as described in Figure 2 and intracellular AGE levels were determined as described in the Materials and Methods.

Inhibition of the protein expression level of RAGE by Blabina and BRE in HUVECs: Since Blabina and BRE increased the mRNA level of *RAGE* (the AGE receptor involved in the intracellular ROS production), the effects of the berry extracts on the expression level of RAGE protein in HUVECs were subsequently examined by Western blotting in comparison with that of AGE-R2 protein (Figure 4). The expression level of the AGE-R2 protein was not significantly affected by berry extracts,

but that of the RAGE protein was evidently suppressed by Blabina and BRE.

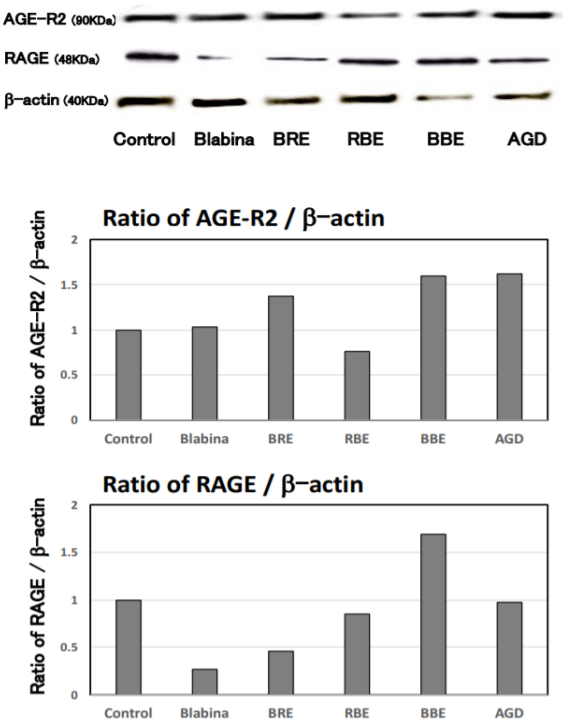
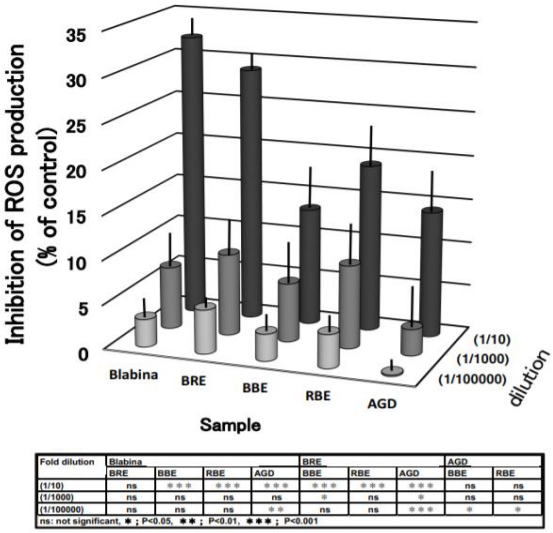


Figure 4. Protein expression levels of AGE receptors, AGE-R2, and RAGE. HUVECs were treated with AGE-BSA in the presence or absence of berry extracts as described in the section of Materials and Methods. Receptor proteins expressed were determined by Western blotting and the quantitative data for expression levels of receptor proteins was shown as the ratio of the target protein / actin.

Effects of berry extracts on ROS production stimulated by AGE-BSA in HUVEC cells: The effects of berry extracts on ROS production stimulated by AGE-BSA in HUVECs were examined using a fluorescent probe (Figure 5). All berry extracts as well as the positive control AGD inhibited ROS production stimulated by AGE-BSA in a concentration-dependent manner. It is noteworthy that the inhibition by Blabina and BRE at 1:10 dilution was markedly higher than those by other berry extracts and AGD. Furthermore, the fluorescent images shown in Figure 6 show that ROS production was inhibited by BRE. Although the inhibition of ROS production analyzed by the fluorescent image was not statistically significant, AGD tended to inhibit ROS production.



Fold dilution	Blabina				BRE			AGD	
	BRE	BBE	RBE	AGD	BBE	RBE	AGD	BBE	RBE
(1/10)	ns	✱✱✱	✱✱✱	✱✱✱	✱✱✱	✱✱✱	✱✱✱	ns	ns
(1/1000)	ns	ns	ns	ns	✱	ns	✱	ns	ns
(1/100000)	ns	ns	ns	✱✱	ns	ns	✱✱✱	✱	✱

ns: not significant, ✱ ; P<0.05, ✱✱ ; P<0.01, ✱✱✱ ; P<0.001

Figure 5. Inhibition of intracellular ROS production by berry extracts. HUVECs were treated as described in the section of Materials and Methods and ROS levels produced in the cell were determined using a fluorescence probe.

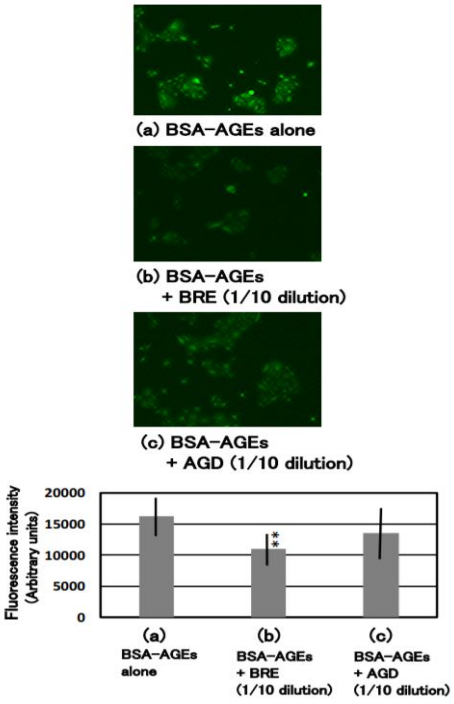
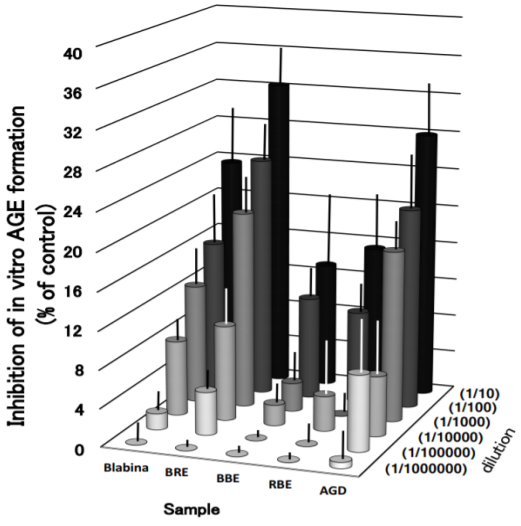


Figure 6. Images for inhibition of intracellular ROS generation by BRE or AGD. After HUVECs were treated

with AGE-BSA in the presence of BRE or AGD, ROS generated in the cell was detected using the fluorescent probe as described in the Materials and Methods. Images of ROS production were shown in the upper panels of Figure 6. The lower panel of Figure 6 shows the quantitative data for intracellular ROS production determined as fluorescence intensity. **P<0.01 vs AGEs alone.

Inhibition of in vitro AGE formation by berry extracts:

The effects of berry extracts on the *in vitro* AGE formation were examined as described in the Materials and Methods section (Figure 7). All berry extracts including Blabina inhibited the *in vitro* AGE formation in a concentration-dependent manner. Extents of inhibition by Blabina, BRE, and the positive control AGD were significantly higher than those by BBE and RBE.



Fold dilution	Blabina	BRE	BBE	RBE	AGD	BRE	RBE	AGD	AGD	
	BRE	BBE	RBE	AGD	BBE	RBE	AGD	BBE	RBE
(1/10)	ns	ns	ns	ns	***	***	ns	***	*
(1/100)	ns	ns	ns	ns	***	ns	ns	***	***
(1/1000)	*	***	***	ns	***	***	ns	***	***
(1/10000)	ns	***	ns	ns	***	ns	ns	ns	ns
(1/100000)	ns	ns	ns	ns	***	*	ns	*	ns
(1/1000000)	ns	ns	ns	ns	ns	ns	ns	ns	ns

ns: not significant, * ; P<0.05, *** ; P<0.001

Figure 7. Inhibitory effects of berry extracts on *in vitro* AGE formation. The levels of AGE formed under these conditions were determined as described in the Materials and Methods section and the inhibition by berry extracts were calculated.

DISCUSSION

In this study, we demonstrate that Blabina and BRE of the already established anti-glycation compound AGD and berry extracts tested in this study have the most potent inhibitory effect on AGE-induced ROS production in HUVECs. This observation suggests that black raspberry could be a potential nutraceutical preventing various age-related diseases.

The inhibition of ROS production by BRE seems to be attributable to the suppression of RAGE protein expression in the cell as demonstrated by Western blotting probed with anti-RAGE antibody (Figure 4). This notion is supported by the fact that the extent of inhibition by BRE or AGD in ROS production was closely correlated with the RAGE protein expression (Figures 4-6), although AGD did not show the inhibition of ROS production when this was assessed by the fluorescence image (Figure 7). If the result showing that AGD inhibits ROS production but did not inhibit the RAGE protein expression is in fact correct, AGD may inhibit ROS production through mechanisms other than the inhibition of BRE. This issue remains to be clarified. It is also conceivable that the inhibition of ROS production by BRE resulted from the prevention of AGE production/accumulation in the cell. However, this is not likely since the mode of inhibition of AGE production and formation in the cell and *in vitro* by berry extracts was not correlated with that of ROS production; all berry extracts examined in this study showed the inhibitory effect on AGE accumulation in the cell to an almost comparable extent (Figure 3), whereas inhibition of ROS production by BRE was much greater when compared with other berry extracts (Figure 5).

Blabina and BRE suppressed the expression level of RAGE protein in the AGE-BSA-stimulated HUVECs (Figure 4). Contrasting this observation, the mRNA level of *RAGE* was increased by these berry samples under the same conditions (Figure 2f). This discrepancy can be accounted for by the assumption that these berry samples stimulate the production of a splicing variant of RAGE. It is known that, in addition to the full-length form of RAGE (full length RAGE: F-RAGE), two types of splicing variants of the RAGE protein are produced in the cell; one is an intracellular domain-deficient form of RAGE (C-terminally truncated RAGE: C-RAGE) which is a soluble RAGE protein to be released from the cell membrane, and another an extracellular V domain-deficient form (N-terminally truncated RAGE: N-RAGE) [31, 32]. If it is true that Blabina and BRE stimulate the mRNA expression of *RAGE* to increase the protein expression level of C-RAGE rather than that of F-RAGE and N-RAGE, the discrepancy described above can be explained by the expression of different types of RAGE proteins in the presence of these berry samples: C-RAGE cannot be detected by Western blotting of the washed cells since it is released from the cell and removed by washing the cell. It is also plausible that full length RAGE is post-translationally modified to produce these deficit forms of RAGE, e.g. proteolysis by the action of proteases. This point requires further clarification.

The components of various berry extracts have been investigated in detail [20]. It is of interest to analyze the components of BRE and investigate which component(s) of BRE is effective on the inhibition of ROS production.

CONCLUSION

In this study, we examined the effects of berry extracts on the expression levels of AGE receptors, intracellular AGE accumulation, and ROS production in HUVECs. The results demonstrate that BRE from the berry extracts examined in this study has the most potent inhibitory effect on ROS accumulation in the cell, probably due to the suppression in the expression level of the RAGE protein. These observations suggest that black raspberry could be a potential nutraceutical to prevent various age-related diseases.

Abbreviations: advanced glycation end products (AGEs), receptor for AGEs (RAGE), nicotinamide adenine dinucleotide phosphate (NADPH), reactive oxygen species (ROS), bovine serum albumin (BSA), phosphate-buffered saline (PBS), aminoguanidine (AGD), black raspberry extract (BRE), blueberry extract (BBE), raspberry extract (RBE), human umbilical vein endothelial cells (HUVECs).

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Authors' contributions: All authors contributed to this work. H. Banno, Y. Kusumi, K. Dan, and Y. Kanaho designed the research. K. Dan and A. Takada carried out the experiments and analyzed the results. All authors interpreted the results and designed the research strategy. K. Dan, A. Takada and Y. Kanaho prepared the manuscript.

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