

Low-molecular weight fractions of Japanese soy sauce act as a RAGE antagonist via inhibition of RAGE trafficking to lipid rafts

メタデータ	言語: eng 出版者: 公開日: 2017-10-03 キーワード (Ja): キーワード (En): 作成者: メールアドレス: 所属:
URL	https://doi.org/10.24517/00013643

This work is licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 3.0 International License.



1 **Low-molecular weight fractions of Japanese soy sauce act as RAGE**
2 **antagonist via inhibition of RAGE trafficking to lipid rafts**

3

4 Seiichi Munesue,^{*a} Yasuhiko Yamamoto,^a Ryouta Urushihara,^a Kouhei
5 Inomata,^b Hidehito Saito,^a So Motoyoshi,^a Takuo Watanabe,^{ac} Hideto
6 Yonekura^d and Hiroshi Yamamoto^a

7

8 *^aDepartment of Biochemistry and Molecular Vascular Biology, Graduate*
9 *School of Medical Sciences, Kanazawa University, 13-1 Takara-machi,*
10 *Kanazawa 920-8640, Japan*

11 *^bDepartment of Organic and Medicinal Chemistry, Graduate School of*
12 *Pharmaceutical Sciences, Tohoku Pharmaceutical University, 4-4-1*
13 *Komatsushima, Aobaku, Sendai 981-8558, Japan*

14 *^cDepartment of Food and Nutrition, Kanazawa Gakuin Collage, 10*
15 *Sue-machi, Kanazawa 920-1392, Japan*

16 *^dDepartment of Biochemistry, Kanazawa Medical University School of*
17 *Medicine, 1-1 Daigaku, Uchinada 920-0293, Japan*

18

19 *Corresponding author: Seiichi Munesue, Department of Biochemistry and
20 Molecular Vascular Biology, Kanazawa University Graduate School of
21 Medical Sciences, 13-1 Takara-machi, Kanazawa 920-8640, Japan. E-mail:
22 smunesue@med.kanazawa-u.ac.jp; Tel: +81 76 265 2184; Fax: +81 76 234
23 4217

1 **Abstract**

2 Advanced glycation end-products (AGE) have been implicated in
3 aging and the pathogenesis of diabetic complications, inflammation,
4 Alzheimer's disease, and cancer. AGE engage the cell surface receptor for
5 AGE (RAGE), which in turn elicits intracellular signaling, leading to
6 activation of NF- κ B to cause deterioration of tissue homeostasis. AGE are
7 not only formed within our bodies but are also derived from foods, endowing
8 them with flavor. In the present study, we assessed the agonistic/antagonistic
9 effects of food-derived AGE on RAGE signaling in a reporter assay system
10 and found that low-molecular weight AGE can antagonize the action of
11 AGE-BSA. Foods tested were Japanese soy sauce, coffee, cola, and red wine,
12 all of which showed fluorescence characteristics of AGE. Soy sauce and
13 coffee contained *N*^ε-carboxymethyl lysine. Soy sauce, coffee, and red wine
14 inhibited the RAGE ligand-induced activation of NF- κ B, whereas cola had
15 no effect on the ligand induction of NF- κ B. The liquids were then
16 fractionated into high-molecular weight fractions (HMF) and low-molecular
17 weight fractions (LMF). Soy sauce-, coffee-, and red wine-derived LMF
18 consistently inhibited the RAGE ligand induction of NF- κ B, whereas the
19 HMF of these foods activated RAGE signaling. Using the LMF of soy sauce
20 as a model food-derived RAGE antagonist, we performed a plate-binding
21 assay and found that the soy sauce LMF competitively inhibited
22 AGE-RAGE association. Further, this fraction significantly reduced
23 AGE-dependent MCP-1 secretion from murine peritoneal macrophages. The
24 LMF from soy sauce suppressed the AGE-induced RAGE trafficking to lipid
25 rafts. These results indicate that small components in some, if not all, foods
26 antagonize RAGE signaling and could exhibit beneficial effects on
27 RAGE-related disease.

1 **Introduction**

2 Advanced glycation end products (AGE) are stable end products of the
3 Maillard reaction. The Maillard reaction was first described by
4 Louis-Camille Maillard in 1912.¹ Reducing sugars such as glucose react
5 non-enzymatically with amino groups of proteins through a series of
6 reactions including Schiff's base formation, Amadori rearrangement,
7 dehydration, condensation, and crosslinking to yield irreversible AGE.² In
8 diabetes, AGE have been implicated in the development of diabetic vascular
9 complications.³

10 Among a variety of cell surface proteins that have been described to
11 bind AGE, the receptor for AGE (RAGE) has been qualified to transduce
12 signals into the cell upon exposure to AGE, thereby eliciting cellular
13 responses and phenotypic changes.^{4,5} RAGE belongs to pattern recognition
14 receptors, and binds to not only AGE but also S100/calgranulins,⁶ Mac-1,⁷
15 transthyretin,⁸ high mobility group box-1 proteins (HMGB-1)/amphoterin,⁹
16 lipopolysaccharides (LPS),¹⁰ phosphatidylserine,¹¹ and amyloid- β peptides.¹²
17 RAGE engagement by these ligands activates NF- κ B and downstream
18 effector gene expression and contributes to various pathological processes
19 including aging, cancer, inflammation and Alzheimer's disease.¹³⁻¹⁵ We have
20 demonstrated that RAGE overexpression accelerates, but RAGE deficiency
21 ameliorates, the development of diabetic nephropathy,^{16,17} and that RAGE is
22 involved in the brain uptake of amyloid- β_{1-42} .¹⁸

23 AGE are formed within our bodies during aging and under diabetic
24 conditions and in foods through cooking and storage.^{19,20} Human studies
25 revealed that about 10% of diet-derived AGE were absorbed, two-thirds of
26 which remained in the body.^{19,20} It is reported that orally absorbed AGE are
27 an environmental risk factor in diabetic nephropathy, and that AGE-rich

1 meals increase serum levels of AGE.^{19, 21} However, biologic activities of
2 food-derived AGE have been not fully evaluated, because of the lack of
3 suitable *in vitro* assay systems applicable to foods concerned. In this study,
4 we employed a RAGE-dependent reporter assay system and evaluated the
5 agonistic/antagonistic effects of AGE-containing liquids on RAGE signaling.
6 With a model soy sauce low-molecular weight fraction, effects on
7 AGE-RAGE association, MCP-1 secretion from murine peritoneal
8 macrophages, trafficking to lipid rafts were also assessed. We demonstrate
9 for the first time that small AGE components in some, if not all, foods
10 antagonize RAGE signaling and can provide beneficial effects on
11 RAGE-related disease.

1 **Experimental**

2 **Food**

3 Japanese soy sauce, coffee, red wine, and cola were purchased from
4 SHODA SHOYU CO. LTD. (Gunma, Japan), CARAVAN SERAI KC
5 (Ishikawa, Japan), Notowine (Ishikawa, Japan), and Coca-Cola Japan LTD
6 (Tokyo, Japan), respectively.

7

8 **Column chromatography**

9 Liquid foods were filtered through a 0.22- μ m filter (Millipore). Soy
10 sauce was applied to a column (2 x 7cm) of cosmocil 75C18-OPN (Nacalai
11 Tesque, Japan) equilibrated with H₂O for desalting. The column was washed
12 extensively with water. The bound material was eluted with 100%
13 methanol/0.1% TFA. The filtrates of coffee, cola, red wine, and the desalted
14 soy sauce were used as total crude preparations. All preparations were
15 freeze-dried and the resultant lyophilized powder was fractionated. Size
16 fractionation was performed using a column (5 mL) of PD-10 (GE
17 Healthcare) equilibrated with H₂O. Total crude preparations were applied to
18 the column and separated into pass-through fractions and incorporated
19 fractions; these were named HMW fractions and LMW fractions,
20 respectively. The LMW fraction of soy sauce was further applied to a
21 column of cosmocil 75C18-OPN equilibrated with H₂O. The column was
22 washed extensively with H₂O. The bound material was eluted by stepwise
23 elution with H₂O, 20% methanol, 50% methanol, 100% methanol and 100%
24 methanol/0.1% TFA. The eluates were freeze-dried and the lyophilized
25 powder was used in subsequent experiments. Endotoxin was not detected in
26 the preparations and the fractions when tested with Limulus HS-test Wako
27 (Wako Pure Chemical Industries, Osaka, Japan).

1

2 **Preparation and characterization of low-molecular weight AGE**

3 Twenty millimolar *N*^α-carbobenzoxy (CBZ)-L-lysine (Sigma) was
4 incubated at 37 °C for 1 week with 20 mM DL-glyceraldehyde or
5 glycolaldehyde (Nacalai Tesque, Kyoto, Japan) in 0.2 M phosphate buffer
6 (pH 7.4); the products were analyzed by SDS-PAGE (15%) and by surface
7 plasmon resonance assay with a BIAcore CM5 sensor chip, on which human
8 endogenous secretory RAGE (esRAGE), a decoy form generated by
9 alternative RNA splicing,²¹ had been immobilized. The surface plasmon
10 resonance assay was performed as described.¹⁸

11

12 **AGE assays**

13 *N*^ε-carboxymethyl lysine (CML) was determined with the CML ELISA
14 kit (CycLex, Nagano, Japan). Fluorescence was measured with a TriStar
15 LB941 multireader (Berthold Technologies, Bad Wildbad, Germany).
16 Samples were excited at 355 nm and emission was recorded at 460 nm.

17

18 **Luciferase reporter assay**

19 Rat C6 glioma cells that had been stably transformed with an
20 expression plasmid containing human full-length RAGE cDNA and with a
21 firefly luciferase reporter gene under the control of the NF-κB promoter¹⁷
22 were used. Reporter activation is dependent on ligand-RAGE interactions, as
23 evidenced by (1) induction by AGE, (2) inhibition by siRNA against RAGE,
24 (3) inhibition by cotransfection of intracytoplasmic domain-lacking
25 dominant negative RAGE, and (4) neutralization by soluble RAGE.¹⁷ After a
26 24 h preincubation in Dulbecco's modified Eagle's medium supplemented
27 with 0.1% fetal bovine serum, the cells were stimulated by

1 glycerinaldehyde-derived AGE-BSA²² in the presence or absence of
2 food-derived fractions for 4 h. Luciferase activity was determined with a
3 Luciferase Assay System (Promega) and measured in a luminometer
4 (Fluoroskan Ascent FL; Labotal Scientific Equipment Ltd., Abn Gosh,
5 Israel).

6

7 **Plate binding assay**

8 Competitive inhibition with LMW fractions from soy sauce was
9 performed using a 96-well AGE-BSA-coated plate as described.¹⁷

10

11 **Determination of monocyte chemoattractant protein-1 (MCP-1)**

12 The MCP-1 ELISA kit (R&D Systems Inc.) was used to determine
13 MCP-1 concentrations in the medium of primary culture of mouse peritoneal
14 macrophages.

15

16 **Sucrose density gradient centrifugation and western blotting**

17 Lipid rafts were isolated essentially according to the detergent
18 extraction method described by Mitsuda *et al.*²³ The same cell line used for
19 the luciferase-reporter assay, the C6 glioma cells, was plated at a density of 1
20 x 10⁶/10 cm-dish and cultured to 90% confluence. After washing each well
21 with 0.1% FBS/DMEM, AGE-BSA were added with or without the soy sauce
22 LMF-4 fraction. After 20 h incubation, the cell layer was washed with cold
23 PBS, and the cells were collected, suspended in 1 mL of a buffer containing
24 1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1
25 mM PMSF, 1 µg/mL aprotinin, and disrupted by 5 rounds of 30 sec
26 sonication. Samples were placed on the bottom of Ultra-Clear centrifuge
27 tubes (Beckman Instruments) and mixed with an equal volume of 80% (w/v)

1 sucrose in buffer. This was overlaid with 5 mL 35% sucrose (w/v) and 5%
2 (w/v) sucrose in buffer without Triton X-100. The samples were centrifuged
3 at 55,000 rpm in a Beckman SW28.1 rotor for 18 h at 4 °C. After
4 centrifugation, 1.5 mL of each fraction was collected from the top of the
5 gradient to yield 8 fractions.

6 After determination of protein concentrations with BCA Protein Assay
7 kit (Pierce), equal amounts of proteins were separated by SDS-PAGE
8 (12.5%) and electroblotted onto PVDF membranes (Millipore). The
9 membranes were blocked with 5% (w/v) non-fat dried milk in PBS and 0.1%
10 (v/v) Tween 20, and incubated with goat anti-RAGE antibody (1:1000,
11 Ab5484, Millipore), which recognized human and mouse RAGEs, and with
12 rabbit anti-GM1 antibody (1:1000, orb10299, Biorbyt). Donkey anti-Rabbit
13 IRDye 680 and goat anti-Rabbit IRDye 800 were diluted 10,000-fold and
14 used as the secondary antibodies. The antigen-antibody complex was
15 visualized using the Odyssey Infrared Imaging system (LI-COR
16 Biotechnology, Lincoln, Nebraska, USA).

17

18 **Statistical analysis**

19 Statistical analysis was performed using Student's *t* test. $p < 0.05$ was
20 considered significant.

1 **Results**

2 *RAGE-dependent NF- κ B reporter assay*

3 We and other researchers previously observed that ligand engagement
4 causes oligomerization of RAGE for the initiation of signal transduction.²⁴
5 This led us to speculate that small AGE ligands may exert rather antagonistic
6 effects on RAGE. To test this hypothesis, we prepared low-molecular weight
7 AGE by incubating N ^{α} -CBZ-L-lysine with glyceraldehyde or
8 glycolaldehyde; the former lysyl derivative can react with the latter
9 carbonyls only on the ϵ -amino group but without further Maillard reaction.
10 As shown in Fig. 1A, incubation of CBZ-lysine and glyceraldehyde or
11 glycolaldehyde yielded brown products that migrated as a single band much
12 faster than bromophenol blue on polyacrylamide gel. Both glyceraldehyde-
13 and glycolaldehyde-derived small AGE bound human esRAGE as evidenced
14 by positive sonograms in surface plasmon resonance assay (Fig. 1B). We
15 next tested the effect of the small AGE on post-RAGE signaling. For this, we
16 employed rat C6 glioma cells expressing human RAGE cDNA and carrying
17 the firefly luciferase reporter gene under the control of NF- κ B promoter. As
18 shown in Fig. 1C, HMW AGE-BSA induced the luciferase, but this was
19 completely abolished by glyceraldehydes-derived and CBZ-lysine-derived
20 AGE, indicating that LMW AGE antagonized RAGE signaling.

21 These observations provided a rationale to evaluate food AGE by
22 testing them with the C6 reporter system to judge whether they are agonistic
23 or antagonistic to RAGE.

24

25 *Soy sauce, coffee, red wine, and cola contained AGE*

26 Japanese soy sauce, coffee, red wine and cola were tested in this study.
27 We first determined the fluorescence characteristic of AGE and the content

1 of CML, the representative non-fluorescence AGE structure, to see whether
2 soy sauce, coffee, red wine and cola contained AGE. AGE-BSA and
3 CBZ-lysine-derived AGE were employed as positive controls and
4 non-glycated BSA as negative controls in these determinations. As shown in
5 Table 1, soy sauce, coffee, red wine, and cola exhibited AGE-derived
6 fluorescence and soy sauce and coffee contained CML. CML was not
7 detected in red wine and cola in this assay.

8

9 *The net activities of soy sauce, coffee, and red wine were RAGE*
10 *antagonizing*

11 Japanese soy sauce, coffee, red wine, and cola were used in the
12 RAGE-dependent reporter assay. After desalting or degassing, total crude
13 preparations were added to cultures of human RAGE-expressing, luciferase
14 reporter gene-carrying rat C6 glioma cells. The crude preparations from soy
15 sauce, coffee, and red wine significantly inhibited AGE-induced NF- κ B
16 activation (Fig. 2 A, B and C). The crude preparation from cola yielded no
17 change in reporter activation (Fig. 2D). No significant change in cell
18 viability was observed.

19

20 *The antagonistic effects of soy sauce, coffee and red wine resided in LMW*
21 *fractions*

22 The food-derived preparations were separated by PD-10 column
23 chromatography and fractionated by molecular size. Fractions larger than
24 5000 molecular weight were designated HMW fractions, and fractions
25 smaller than 5000 were categorized as LMW fractions. We determined the
26 content of CML in the HMW and LMW fractions of soy sauce, coffee, red
27 wine and cola. CML was detected in both HMW and LMW fractions from

1 soy sauce and coffee but not in the HMW and LMW fractions from red wine
2 or cola in the conditions employed in this study (Table 2).

3 When the NF- κ B-luciferase-carrying C6 cells were exposed to the soy
4 sauce HMW fraction, AGE-dependent NF- κ B activation was significantly
5 enhanced (Fig. 2A). In contrast, addition of the soy sauce LMW fraction
6 significantly inhibited the AGE induction of NF- κ B (Fig. 2A). HMW
7 fractions of coffee and red wine also enhanced AGE-dependent NF- κ B
8 activation, while their LMW fractions significantly inhibited activation (Fig.
9 2 B and C), similar to the soy sauce-derived LMW fraction. In contrast, the
10 cola-derived HMW fraction had no effect, but the LMW fraction enhanced
11 reporter activity (Fig. 2D). Toxicity to the cells was not observed in the
12 concentration range of 0.5-1.0 mg/mL in any of the HMW and LMW
13 fractions from the four food samples tested, when the cells had been
14 incubated with them for 24 h (supplemental Fig. 1). Soy sauce, coffee and
15 red wine have HMW fractions that engage RAGE and LMW fractions that
16 act as competitive inhibitors. To examine whether the effect of LMW
17 fractions from these three foods on AGE-RAGE signaling is predominant
18 over that of HMW fractions, we performed the RAGE-dependent reporter
19 assay using a mixture of HMW and LMW fractions that had been separated
20 from total crude fractions of those foods. When equal amounts of HMW and
21 LMW fractions from soy sauce, coffee or red wine were combined and
22 assayed, they inhibited the AGE-induced NF- κ B activation as did the
23 respective total crude fractions (Supplemental Fig. 2A). The weight ratios of
24 HMW and LMW fractions from soy sauce, coffee and red wine were 3 : 2,
25 3 : 7 and 3 : 97, respectively, and the average molecular weights of HMW
26 and LMW fractions were 400,000 and 4,000 (soy sauce), 450,000 and 4,500
27 (coffee) and 400,000 and 4,000 (red wine), respectively. This indicates that

1 the number of molecules in the LMW fraction was much larger than that in
2 the HMW fraction. We then conducted the RAGE-dependent reporter assay
3 using mixtures of soy-sauce-derived HMW and LMW fractions at different
4 ratios. Even when the ratio of HMW and LMW was up to 100 : 1, the
5 mixture of the HMW and LMW fractions significantly inhibited the AGE
6 induction of NF- κ B activation (Supplemental Fig. 2B).

7
8 *Further fractionation and characterization of the RAGE-antagonizing*
9 *Japanese soy sauce LMW fraction*

10 Next, using the LMW fraction of soy sauce as a model food-derived
11 RAGE antagonist, we further fractionated the soy sauce LMW fraction by
12 reversed-phase chromatography into 5 fractions named LMF-1, LMF-2,
13 LMF-3, LMF-4, and LMF-5 (Fig. 3). When assayed with the
14 RAGE-dependent luciferase reporter system, LMF-1, LMF-3, LMF-4, and
15 LMF-5 significantly inhibited AGE-induced NF- κ B activation in a
16 dose-dependent manner (Fig. 4). LMF-2 did not inhibit NF- κ B activation.

17 Plate assays were used to determine whether the antagonistic LMW
18 fractions from soy sauce inhibit AGE-RAGE association. LMF-1 most
19 strongly inhibited human esRAGE binding to immobilized AGE-BSA (Fig.
20 5). LMF-4 and LMF-5 also inhibited binding in a dose-dependent manner.
21 LMF-2 and LMF-3 did not affect AGE-BSA-esRAGE binding.

22
23 *LMF-4 and LMF-5 inhibited AGE-induced MCP-1 secretion from mouse*
24 *peritoneal macrophages*

25 We then sought to identify the biological activities of fractions that
26 antagonize RAGE signaling and inhibit AGE-RAGE association. For this,
27 we employed mouse peritoneal macrophages, which release MCP-1, an

1 inflammatory cytokine, in response to AGE-RAGE binding.²⁵ As shown in
2 Fig. 6, AGE-BSA increased MCP-1 secretion in comparison to control
3 non-glycated BSA. In the presence of LMF-4 and LMF-5, AGE-induced
4 MCP-1 secretion was significantly inhibited. On the other hand, LMF-1 had
5 no effect on AGE-induced MCP-1 secretion.

6

7 *LMF-4 inhibited RAGE trafficking to lipid rafts*

8 We then sought to determine how the LMF fractions halt AGE-RAGE
9 activity using LMF-4, which showed higher inhibitory activity of MCP-1
10 secretion than LMF-5. Since lipid rafts have recently been reported to be
11 involved in receptor trafficking²⁶ and signal transduction²⁷, we investigated
12 the relationship between RAGE and lipid rafts. As shown in Fig. 7, when the
13 C6 cells were treated with non-glycated BSA, RAGE was recovered in the
14 fractions near the bottom. After exposure to AGE-BSA, RAGE moved to the
15 less dense fractions to which GM-1, the marker of lipid rafts, sedimented,
16 indicating that ligand binding to RAGE induced RAGE trafficking to lipid
17 rafts. However, coexistence of LMF-4 completely inhibited RAGE
18 movement to the lipid raft fractions.

19

1 **Discussion**

2 We have demonstrated that Japanese soy sauce, coffee, red wine, and
3 cola contain AGE (Table 1), and that soy sauce, coffee, and red wine,
4 particularly their LMW fractions, exert RAGE signaling inhibitory effects
5 (Fig. 2 A-C) as do N^α -CBZ-L-lysine-derived small AGE (Fig. 1C). HMW
6 fractions from soy sauce, coffee, and red wine exhibited agonistic effects,
7 but the net activities of the 3 kinds of foods were RAGE-antagonistic. The
8 weight ratios of HMW and LMW fractions in total crude fractions of these
9 three kinds of foods were 3 : 2, 3 : 7 and 3 : 97, respectively, and the average
10 molecular weights of the HMW fractions were 100-fold larger than those of
11 LMW fractions in either kind of the foods. Moreover, the mixture of the
12 HMW and LMW fractions from soy sauce combined at the differing weight
13 ratios significantly inhibited the AGE-induced NF- κ B activation at the ratio
14 up to 100 : 1 (HMW : LMW) (Supplemental Fig. 2B). These results
15 indicated that the absolute number of antagonistic components in LMW
16 fractions from these foods is extremely large compared with that of agonistic
17 components in HMW fractions, and that the effect of LMW fractions on
18 RAGE signaling is predominant over that of HMW fractions. Though HMW
19 fractions from these foods showed a potent RAGE-agonistic activity, the net
20 activity of the total crude fractions was antagonistic, and when the soy
21 sauce-derived HMW and LMW fractions were combined at differing ratios,
22 the agonistic activity was observed only with the ratio of 1,000 : 1 (HMW :
23 LMW) (Supplemental Fig. 2B). The results suggested that the HMW
24 fractions might be too small to exert the RAGE-ligand effect in the total
25 fraction. The results are consistent with our previous observations that
26 heparin acts as RAGE agonist and that LMW heparin acts as RAGE
27 antagonist¹⁷ and with the observation by Penfold *et al.* that HMW serum

1 fractions enhanced post-RAGE signaling.²⁸ It was reported that dimerization
2 of RAGE represents an important component of RAGE-mediated cell
3 signaling.²⁹ And, as the CBZ-lysine-derived LMW AGE completely
4 abolished the HMW AGE-BSA induction of the RAGE-dependent luciferase
5 activation (Fig. 1C), most of the food-derived LMW but not HMW
6 components abolished the AGE induction of the reporter enzyme in the same
7 assay (Figs. 2 and 4). Thus, it may be reasonable to posit that small AGE or
8 food components engage RAGE, but that they interfere the formation of
9 RAGE dimer or oligomer, thereby inhibiting RAGE signaling.

10 In the case of cola, the LMW fraction increased NF- κ B activity, while
11 the total preparation and HMW fraction yielded no changes in RAGE
12 signaling (Fig. 2D). This suggests that the cola HMW fraction contains
13 components capable of suppressing NF- κ B activation, and that this activity
14 supersedes the agonistic effect of the cola LMW fraction. The role of LMW
15 fraction from cola on AGE-RAGE signaling remains to be investigated.

16 In this study, we used food samples at the concentration range of
17 0.5-1.0 mg/mL in the cellular experiments. This was based on the following
18 calculations. First, Koschinsky *et al.*¹⁹ estimated that the total amount of
19 orally absorbed AGE found in blood was equal to about 10% of that
20 estimated to be present in the ingested meal, and that only 30% of the
21 circulating AGE was excreted in the urine of persons over the subsequent 48
22 h. Second, according to data from the Japan Soy Sauce Brewers
23 Association³⁰, the daily consumption of soy sauce in Japan is estimated at
24 about 30 mL per person, and, according to Hamano *et al.*³¹, the average of
25 dry weight of soy sauce is estimated to be 1.19 g/mL. Assuming that a blood
26 volume of the average adult is 5,000 mL, the concentration of Japanese soy
27 sauce *in vivo* would then be at the mg/mL order (approximately 7.1 mg/mL),

1 the concentration near those employed in this study. There is a report that
2 coffee was used for *in vivo* experiments at 15 mg/mL.³²

3 To learn how the food-derived LMW fractions antagonized RAGE, we
4 further fractionated and characterized the LMW fraction from soy sauce.
5 Four of 5 soy sauce subfractions (LMW-1, LMF-3, LMF-4 and LMF-5)
6 possessed RAGE antagonistic activity (Fig. 4). Three of 5 subfractions
7 (LMW-1, LMF-4 and LMF-5) competitively inhibited AGE-RAGE binding
8 (Fig. 5). The results suggest that soy sauce contains plural components with
9 RAGE antagonistic activities, and that some component in LMW-3 could
10 inhibit post-RAGE signaling in a ligand-independent manner.

11 Further, 2 of 3 ligand-association-inhibitory and antagonistic
12 subfractions (LMF-4 and LMF-5) inhibited MCP-1 secretion from mouse
13 peritoneal macrophages (Fig. 6), indicating that those soy sauce-derived
14 LMW subfractions antagonized RAGE *in vivo*.

15 The soy sauce LMW subfraction with the most potent antagonistic
16 activity and the strongest inhibition of macrophage MCP-1 secretion
17 (LMF-4) were assayed for its mechanistic properties. We found for the first
18 time that LMF-4 efficiently halted AGE-induced RAGE trafficking to lipid
19 rafts, the membrane microdomain that compartmentalizes select signaling
20 and functional events.³³ Powers *et al.*³⁴ reported that Toll-like receptor 4,
21 another pattern recognition receptor, was recruited to lipid rafts. The present
22 findings that RAGE can accumulate in lipid rafts and that this can be
23 controlled are previously unreported. We propose that small RAGE ligands,
24 such as soy sauce LMF-4 and CBZ-lysine-derived AGE, may inhibit RAGE
25 dimerization and subsequent trafficking to lipid rafts.

26 The total preparation and the LWF fraction of red wine also exhibited
27 RAGE antagonism. The antagonistic effect of red wine may partly be

1 ascribed to polyphenol. Resveratrol, a natural polyphenol found in red wine,
2 attenuates NF- κ B activation and reduces RAGE expression.³⁵

3 The results thus indicate that small AGE components in some, if not
4 all, foods antagonize RAGE signaling and could provide health benefits.

5

1 **ACKNOWLEDGMENTS**

2 We thank Ms Yuko Niimura for her assistance. This study was
3 supported by Grants-in-aid for Scientific Research for HY from the Japan
4 Society for the Promotion of Science (grant # 19390085 for HY; grant #
5 21590304 for TW) and by the Adaptable and Seamless Technology transfer
6 Program through target derived R&D from the Japan Science and
7 Technology Agency (grant # AS231Z01903B and # AS242Z02314Q for SM).

1 **References**

- 2 1. L. Maillard, *Comptes Rendus de l'Academie des Sciences, Ser II*, 1912,
3 **154**, 66-68.
- 4 2. D. G. Dyer, J. A. Blackledge, S. R. Thorpe and J.W. Baynes, *J. Biol.*
5 *Chem.*, 1991, **266**, 11654-11660.
- 6 3. H. Vlassara, L. T. Striker, S. Teichberg, H. Fuh, Y. M. Li and M. Steffes,
7 *Proc. Natl. Acad. Sci. U.S.A.*, 1994, **91**, 11704-11708.
- 8 4. R. Nagai, K. Mera, K. Nakajou, Y. Fujiwara, Y. Iwao, H. Imai, T. Murata
9 and M. Otagiri, *Biochim. Biophys. Acta*, 2007, **1772**, 1192-1198.
- 10 5. L. Li, P. Sungha and L. G. Edward, *Front. Biosci.*, 2009, **14**, 1403–1413.
- 11 6. M. A. Hofman, S. Drury, C. Fu, W. Qu, A. Taguchi, Y. Lu, C. Avila, N.
12 Kambham, A. Bierhaus, P. Nawroth, M.F. Neurath, T. Slattey, D. Beach,
13 J. McClary, M. Nagashima, J. Morser, D. Stern and A. M. Schmidt, *Cell*,
14 1999, **97**, 889-901.
- 15 7. T. Chavakis, A. Bierhaus, N. Al-Fakhri, D. Schneider, S. Witte, T. Linn, M.
16 Nagashima, J. Morser, B. Arnold, K. T. Preissner and P. P. Nawroth, *J.*
17 *Exp. Med.*, 2003, **198**, 1507-1515.
- 18 8. M. M. Sousa, S. D. Yan, D. Stern and M. J. Saraiva, *Lab. Invest.*, 2000, **80**,
19 1101-1110.
- 20 9. O. Hori, J. Brett, T. Slattey, R. Cao, J. Zhang, J. Chen, M. Nagashima, E.
21 R. Lundh, S. Vijay, D. Nitecki, J. Morser, D. Stern and A. M. Schmidt, *J.*
22 *Biol. Chem.*, 1995, **270**, 25752-25761.
- 23 10. Y. Yamamoto, A. Harashima, H. Saito, K. Tsuneyama, S. Munesue, S.
24 Motoyoshi, D. Han, T. Watanabe, M. Asano, S. Takasawa, H. Okamoto,
25 S. Shimura, T. Karasawa, H. Yonekura and H. Yamamoto, *J. Immunol.*,
26 2011, **186**, 3248-3257.
- 27 11. M. He, H. Kubo, K. Morimoto, N. Fujino, T. Suzuki, T. Takahasi, M.

- 1 Yamada, M. Yamaya, T. Maekawa, Y. Yamamoto and H Yamamoto,
2 *EMBO Rep.*, 2011, **12**, 358-364.
- 3 12. S. D. Yan, X. Chen, J. Fu, M. Chen, H. Zhu, A. Roher, T. Slattery, L.
4 Zhao, M. Nagashima, J. Morser, A. Migheli, P. Nawroth, D. Stern and A.
5 M. Schmidt, *Nature*, 1996, **382**, 685-691.
- 6 13. S. D. Yan, A. M. Schmidt, G. M. Anderson, J. Zhang, J. Brett, Y. S. Zou,
7 D. Pinsky and D. Stern, *J. Biol. Chem.*, 1994, **269**, 9889-9897.
- 8 14. H. M. Lander, J. M. Tauras, J. S. Ogiste, O. Hori, R. A. Moss and A. M.
9 Schmidt, *J. Biol. Chem.*, 1997, **272**, 17810-17814.
- 10 15. C. H. Yeh, L. Sturgis, J. Haidacher, X. N. Zhang, S. J. Sherwood, R. J.
11 Bjercke, O. Juhasz, M. T. Crow, R. G. Tilton and L. Denner, *Diabetes*,
12 2001, **50**, 1495-1504.
- 13 16. Y. Yamamoto, I. Kato, T. Doi, H. Yonekura, S. Ohashi, M. Takeuchi, T.
14 Watanabe, S. Yamagishi, S. Sakurai, S. Takasawa, H. Okamoto and H.
15 Yamamoto, *J. Clin. Invest.*, 2001, **108**, 261-268.
- 16 17. K. M. Myint, Y. Yamamoto, T. Doi, I. Kato, A. Harashima, H. Yonekura,
17 T. Watanabe, H. Shinohara, M. Takeuchi, K. Tsuneyama, N. Hashimoto,
18 M. Asano, S. Takasawa, H. Okamoto and H. Yamamoto, *Diabetes*, 2006,
19 **55**, 2510-2522.
- 20 18. T. Sugihara, S. Munesue, Y. Yamamoto, S. Sakurai, N. Akhter, Y.
21 Kitamura, K. Shiba, T. Watanabe, H. Yonekura, Y. Hayashi, J. Hamada
22 and H. Yamamoto, *J. Alzheimer's Dis.*, 2012, **28**, 709-720.
- 23 19. T. Koschinsky, C. J. He, T. Mitsuhashi, R. Bucala, C. Liu, C. Buenting, K.
24 Heitmann and H. Vlassara, *Proc. Natl. Acad. Sci. U.S.A.*, 1997, **94**,
25 6474-6479.
- 26 20. C. He, J. Sabol, T. Mitsuhashi and H. Vlassara, *Diabetes*, 1999, **48**,
27 1308-1315.

- 1 21. H. Vlassara and G. E. Striker, *Nat. Rev. Endocrinol.*, 2011, **7**, 526-539.
- 2 22. H. Yonekura, Y. Yamamoto, S. Sakurai, R. G. Petrova, M.J. Abedin, H.
3 Li, K. Yasui, M. Takeuchi, Z. Makita, S. Takasawa, H. Okamoto, T.
4 Watanabe and H. Yamamoto, *Biochem. J.*, 2003, **370**, 1097-1109.
- 5 23. T. Mitsuda, K. Furukawa, S. Fukumoto, H. Miyazaki, T. Urano and K.
6 Furukawa, *J. Biol. Chem.*, 2002, **277**, 11239-11246.
- 7 24. T. Ostendorp, E. Leclerc, A. Galichet, M. Koch, N. Demling, B. Weigle,
8 C. W. Heizmann, P. M. Kroneck and G. Fritz, *EMBO J.*, 2007, **26**,
9 3868-3878.
- 10 25. L. Gu, S. Hagiwara, Q. Fan, M. Tanimoto, M. Kobata, M. Yamashita, T.
11 Nishitani, T. Gohda, Z. Ni, J. Qian, S. Horikoshi and Y. Tomino, *Nephrol.*
12 *Dial. Transplant.*, 2006, **21**, 299-313.
- 13 26. J. L. Estall, B. Yusta and D. J. Drucker, *Mol. Biol. Cell*, 2004, **15**,
14 3673-3687.
- 15 27. K. Simons and D. Toomre, *Nat. Rev. Mol. Cell Biol.*, 2000, **1**, 31-39.
- 16 28. S. A. Penfold, M. T. Coughlan, S. K. Patel, P. M. Srivastava, K. C.
17 Sourris, D. Steer, D. E. Webster, M. C. Thomas, R. J. MacIsaac, G.
18 Jerums, L. M. Burrell, M. E. Cooper and J. M. Forbes, *Kidney Int.*, 2010,
19 **78**, 287-295.
- 20 29. H. Zong, A. Madden, M. Ward, M.H. Mooney, C.T. Elliott and A.W. Stitt,
21 *J. Biol. Chem.*, 2010, **285**, 23137-23146.
- 22 30. Statistical data of soy sauce, *SOYSAUCE INFORMATION CENTER*,
23 2013 (<https://www.soy sauce.or.jp/arekore/>)
- 24 31. M. Hamano and Y. Aoyama, *Nouka*, 1974, **40**, 619-625.
- 25 32. M. Nagao, Y. Fujita, K. Wakabayashi, H. Nukaya, T. Kosuge and T.
26 Sugimura, *Environ. Health Perspect.*, 1986, **67**, 89-91.
- 27 33. M. B. Fessler and J. S. Parks, *J. Immunol.*, 2011, **187**, 1529-1535.

- 1 34. K. A. Powers, K. Szászi, R. G. Khadaroo, P. S. Tawadros, J. C. Marshall,
- 2 A. Kapus and O. D. Rotstein, *J. Exp. Med.*, 2006, **203**, 1951-1961.
- 3 35. W. Yu, Y. C. Fu and W. Wang, *J. Cell. Biochem.*, 2011, **7**, 752-759.

1

2 **Table 1** Determinations of AGE in foods.

	Non-glycated BSA	AGE-BSA	Japanese soy sauce	Coffee	Red wine	Cola	Z-lys
Fluorescence (A.U.)	237 ± 40	3673 ± 115	34358 ± 120	9610 ± 56	1639 ± 35	2901 ± 28	31170 ± 29
CML concentration (µg/mL)	< 0.11	22.5 ± 0.2	2.5 ± 0.0	0.3 ± 0.0	< 0.11	< 0.11	8.0 ± 0.4

3 Hundred µL equivalents to 100 µg/mL BSA that had been added to glycation
4 reaction (non-glycated BSA and AGE-BSA), 100 µL crude preparations (soy
5 sauce, coffee, red wine, and cola), and 100 µL 100 units/mL glyceraldehyde-
6 and *N*^α-CBZ-lysine-derived AGE (Z-lys; 1 unit is defined as the
7 concentration of Z-lys that gives 50 % inhibition of AGE-BSA-RAGE
8 binding) were analyzed by fluorospectrophotometry. Aliquots of each (50µL)
9 were assayed for CML. Values are expressed as means ± S.E. (n = 3). A.U.,
10 arbitrary units.

11

12 **Table 2** Determinations of CML concentrations in LMW and HMW
13 fractions of food-derived samples.

	Japanese soy sauce		Coffee		Red wine		Cola	
	HMW	LMW	HMW	LMW	HMW	LMW	HMW	LMW
CML concentration (µg/mL)	0.45 ± 0.0	1.82 ± 0.1	0.15 ± 0.0	0.14 ± 0.0	< 0.11	< 0.11	< 0.11	< 0.11

14 Fifty µL of each fraction were assayed for CML. Values are expressed as
15 means ± S.E. (n = 3).

1 **Figure legends**

2

3 **Figure 1**

4 Characterization of LMW AGE. A. SDS-PAGE analysis of
5 glyceraldehyde-derived or glycolaldehyde-derived AGE. Closed arrow heads,
6 LMW AGE. Arrows, bromophenol blue. Gels were not stained. B. Surface
7 plasmon resonance sonograms of *N*^α-CBZ-lysine- and glyceraldehyde- or
8 glycolaldehyde-derived AGE. Time 0 indicates addition of AGE analytes to
9 the CM5 sensor chip on which purified human esRAGE proteins were
10 immobilized as ligands. Arrows indicate the start of washing. C. RAGE
11 signaling assay. AGE, glyceraldehyde-derived AGE-BSA; BSA,
12 non-glycated BSA; Glycer-Z-lys, glyceraldehyde-derived *N*^α-CBZ-lysine
13 AGE.

14

15 **Figure 2**

16 Effects of crude preparations and HMW and LMW fractions from Japanese
17 soy sauce (A), coffee (B), red wine, (C) and cola (D) on RAGE signaling.
18 RAGE signaling was assayed in human RAGE-expressing,
19 NF-κB-promoter-luciferase reporter gene-carrying rat C6 glioma cells as
20 described in the Experimental section. AGE-BSA, 50 μg/mL
21 glyceraldehyde-derived AGE-BSA; BSA, 50 μg/mL non-glycated BSA. #, *p* <
22 0.01 (vs. BSA);**, *p* < 0.01 (vs. AGE-BSA); *, *p* < 0.05 (vs. AGE-BSA) (n =
23 3).

24

25 **Figure 3**

26 Fractionation of the Japanese soy sauce LMW fraction by reversed-phase
27 chromatography.

1

2 Figure 4

3 RAGE antagonistic activities of subfractions of the Japanese soy sauce LMW
4 fraction. AGE-BSA, 50 $\mu\text{g}/\text{mL}$ glyceraldehyde-derived AGE-BSA; BSA, 50
5 $\mu\text{g}/\text{mL}$ non-glycated BSA. #, $p < 0.01$ (vs. BSA);**, $p < 0.01$ (vs. AGE-BSA);
6 *, $p < 0.05$ (vs. AGE-BSA) (n = 3).

7

8 Figure 5

9 Effect of soy sauce LMW subfractions on AGE-RAGE binding. A plate
10 competitive inhibition assay was performed as described in the Experimental
11 section. Subfraction (0.063, 0.125, 0.25, 0.5 and 1.0 mg/mL) were incubated
12 with esRAGE on an AGE-BSA-coated plate at room temperature for 1 h.
13 After incubation and washing, europium-labeled anti-RAGE antibody was
14 added and the plate was further incubated for 1 h. After incubation and
15 washing, the europium-labeled antibody, esRAGE and AGE complex was
16 detected by fluorophotometry.

17

18 Figure 6

19 Biological activity of LMW subfractions of Japanese soy sauce. Mouse
20 peritoneal macrophages were incubated for 24 h with non-glycated BSA or
21 AGE-BSA in the presence or absence of LMF-1, LMF-4 and LMF-5, and
22 MCP-1 secreted in the media was measured by ELISA. AGE-BSA, 50
23 $\mu\text{g}/\text{mL}$ glyceraldehyde-derived AGE-BSA; BSA, 50 $\mu\text{g}/\text{mL}$ non-glycated
24 BSA; LMF concentration was 1.0 mg/mL each. #, $p < 0.01$ (vs. BSA); **, p
25 < 0.01 (vs. AGE-BSA) (n = 3).

26

27 Figure 7

1 Localization of RAGE in lipid rafts and its inhibition by soy sauce LMF-4.
2 Human RAGE-expressing and NF- κ B-promoter-luciferase reporter
3 gene-carrying rat C6 glioma cells were treated with AGE-BSA in the
4 presence or absence of LMF-4 for 24 h, followed by sucrose gradient
5 ultracentrifugation and immunoblotting with anti-RAGE and anti-GM1
6 antibodies. Fractions are numbered from the top to the bottom of the
7 gradient.

8

9 Supplemental Experimental

10 Cytotoxicity Assay

11 Cytotoxicity of LMW and HMW fractions of all foods samples was
12 determined by measuring the release of LDH with the CytoTox 96 Assay
13 (Promega) according to the manufacturer's instruction. LDH-release was
14 calculated as percentage of LDH released in the culture media of total LDH
15 inside and outside cells.

16

17 Legend to supplemental Figure

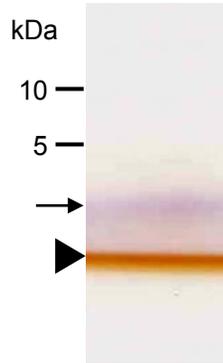
18 Supplemental Fig. 1

19 Cytotoxicity of HMW and LMW fractions from Japanese soy sauce, coffee,
20 and red wine. After a 5 h preincubation in Dulbecco's modified Eagle's
21 medium supplemented with 0.1% fetal bovine serum, rat C6 glioma cells
22 that had been stably transformed with an expression plasmid containing
23 human full-length RAGE cDNA and with a firefly luciferase reporter gene
24 under the control of the NF- κ B promoter were stimulated by AGE-BSA and
25 food-derived fractions (A, 1.0 mg/mL; B, 0.5 mg/mL) for 24 h. After 24 h
26 stimulation, the media and the lysates were assayed for the released and total
27 LDH activity. AGE-BSA, 50 μ g/mL glyceraldehyde-derived AGE-BSA;

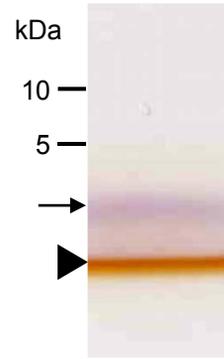
1 BSA, 50 $\mu\text{g}/\text{mL}$ non-glycated BSA.
2
3 Supplemental Fig. 2
4 Effects of mixtures of HMW and LMW fractions from Japanese soy sauce,
5 coffee, and red wine on RAGE signaling. RAGE signaling was assayed with
6 human RAGE-expressing, NF- κ B-promoter-luciferase reporter gene-carrying
7 rat C6 glioma cells as described in the Experimental section. (A) Equal
8 amounts (0.5 mg/mL each) HMW and LMW fractions from soy sauce, coffee
9 and red wine were combined and used for the assay. (B) Soy sauce-derived
10 HMW and LMW fractions were combined at the indicated ratio and used for
11 the assay. AGE-BSA, 50 $\mu\text{g}/\text{mL}$ glyceraldehyde-derived AGE-BSA; BSA, 50
12 $\mu\text{g}/\text{mL}$ non-glycated BSA. #, $p < 0.01$ (vs. BSA);**, $p < 0.01$ (vs. AGE-BSA)
13 (n = 3).

Fig.1

Glyceraldehyde-AGE

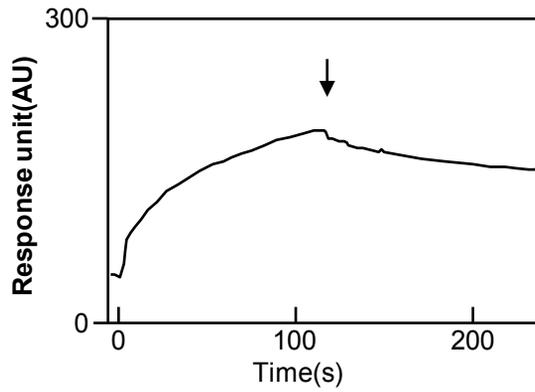


Glycolaldehyde-AGE

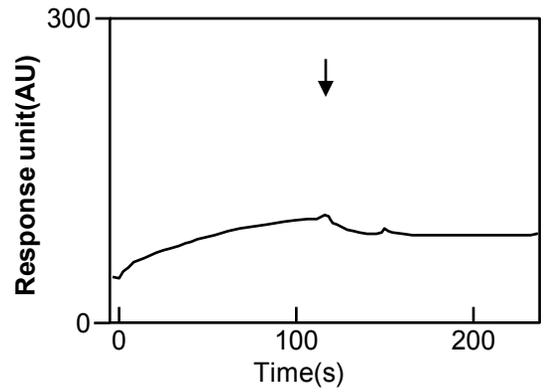


A

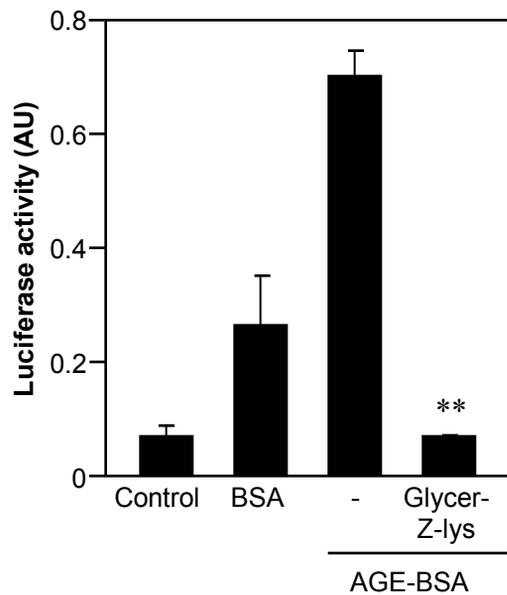
Glyceraldehyde-AGE



Glycolaldehyde-AGE



B



C

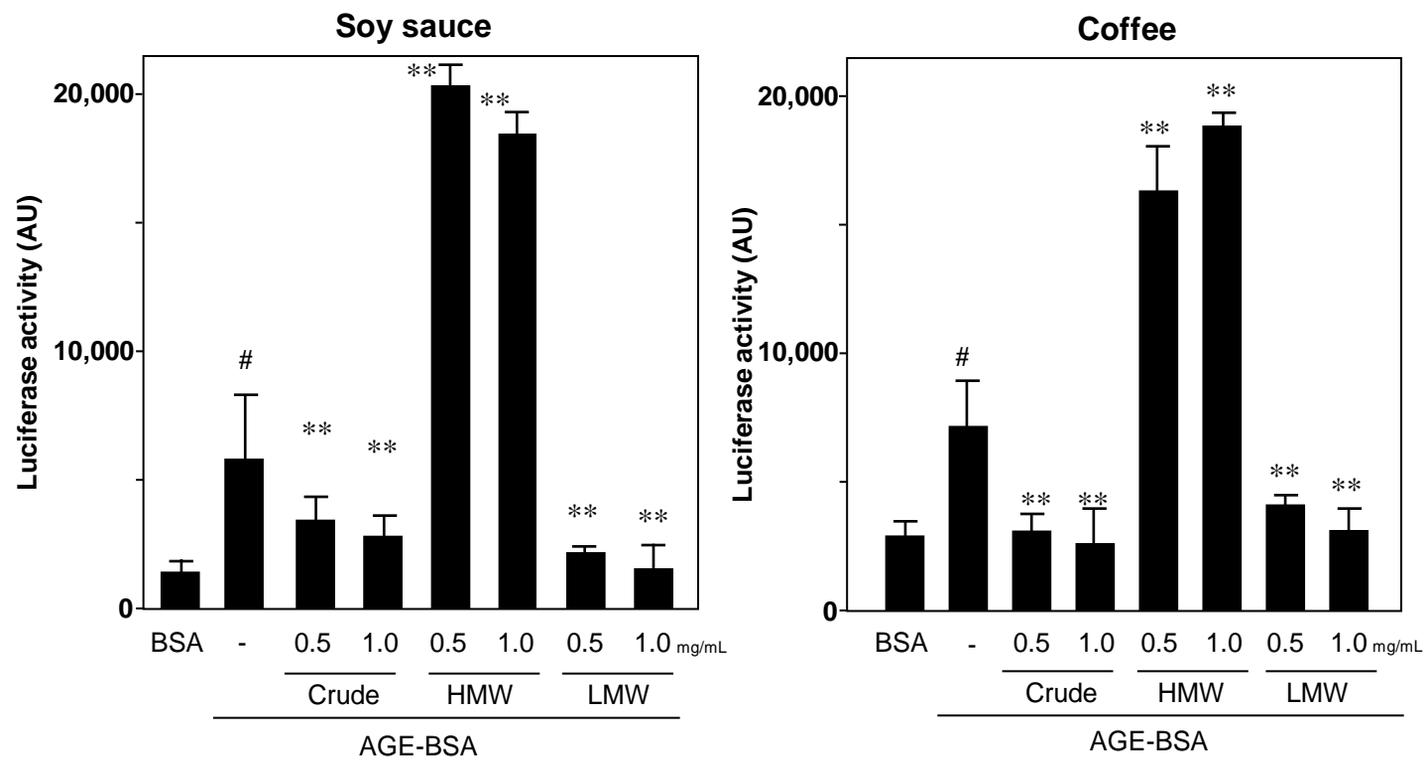
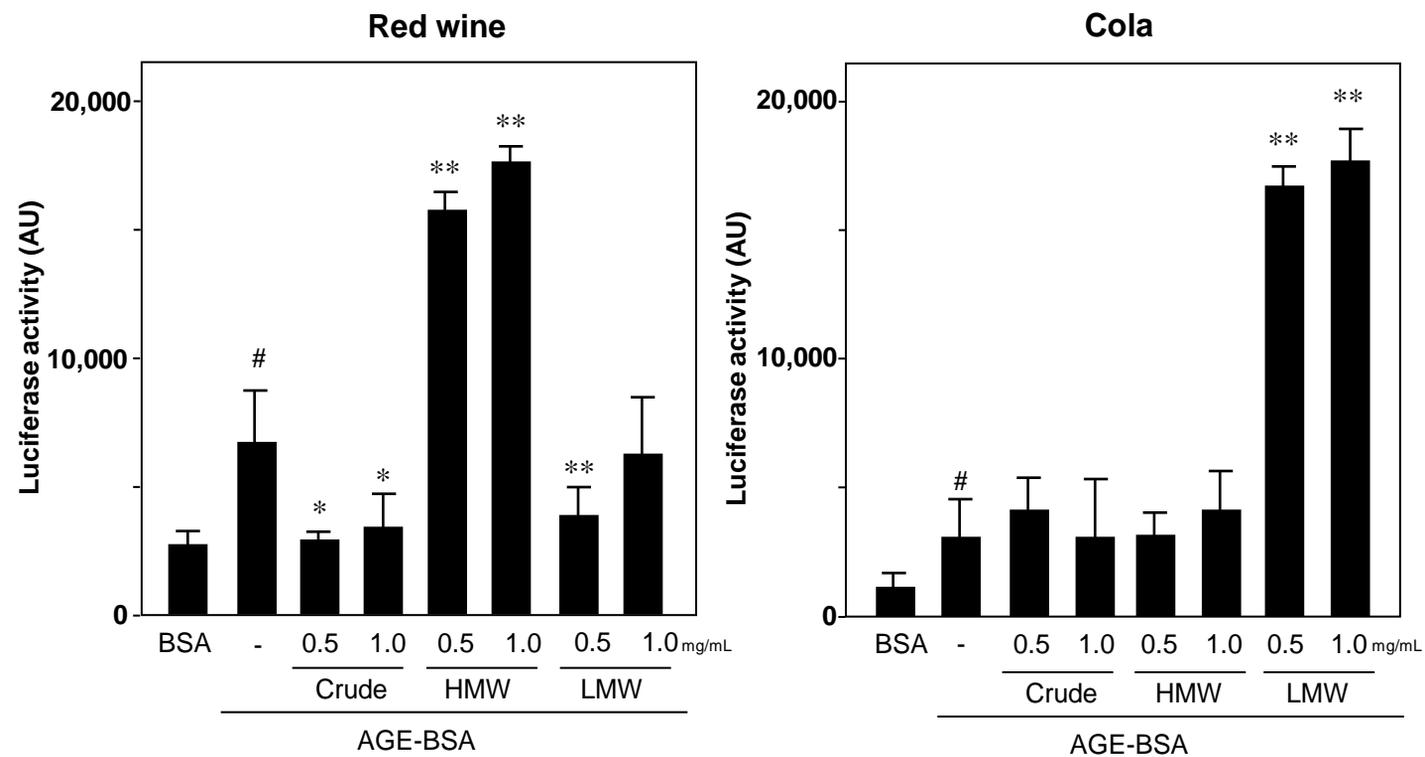
Fig.2**A****B****C****D**

Fig.3

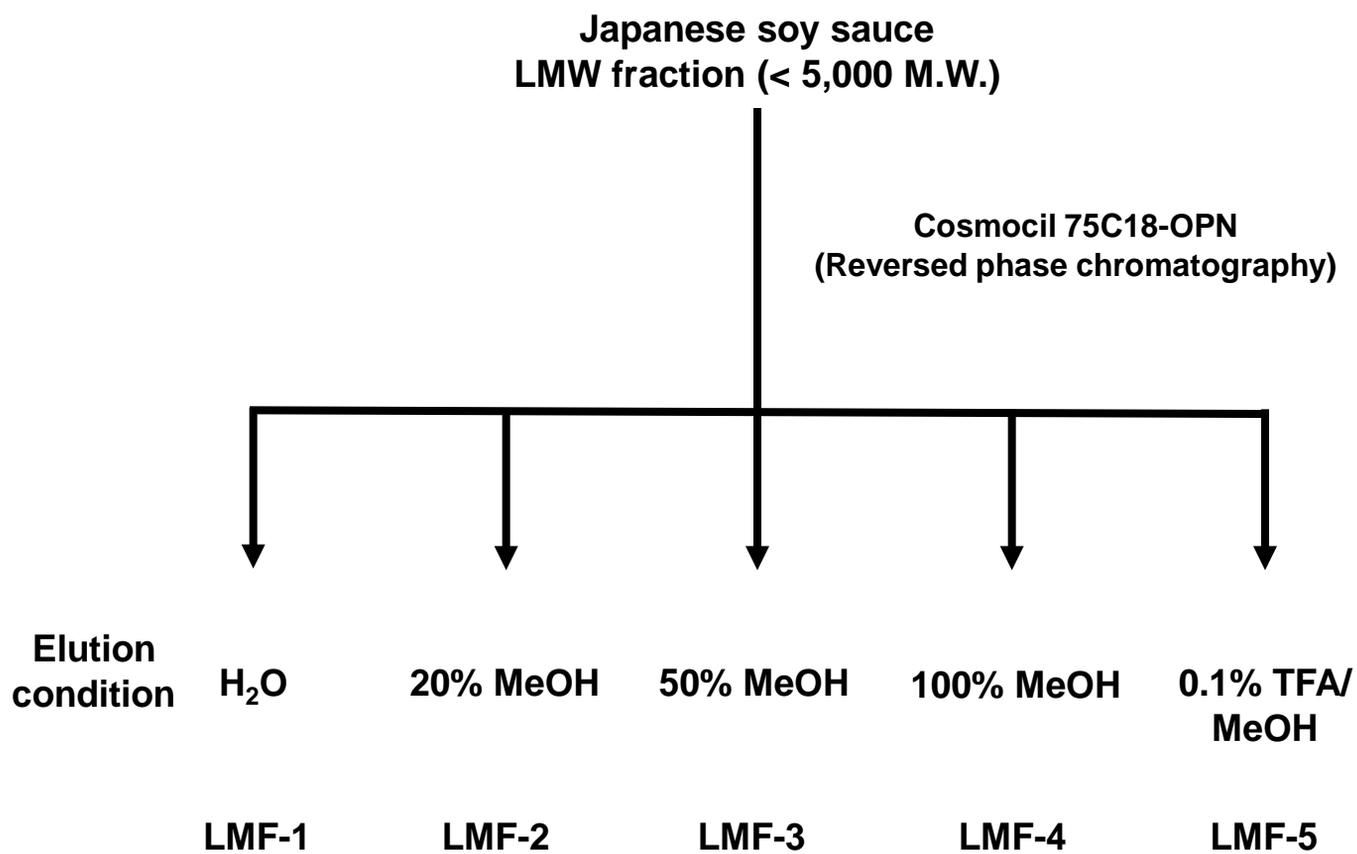


Fig.4

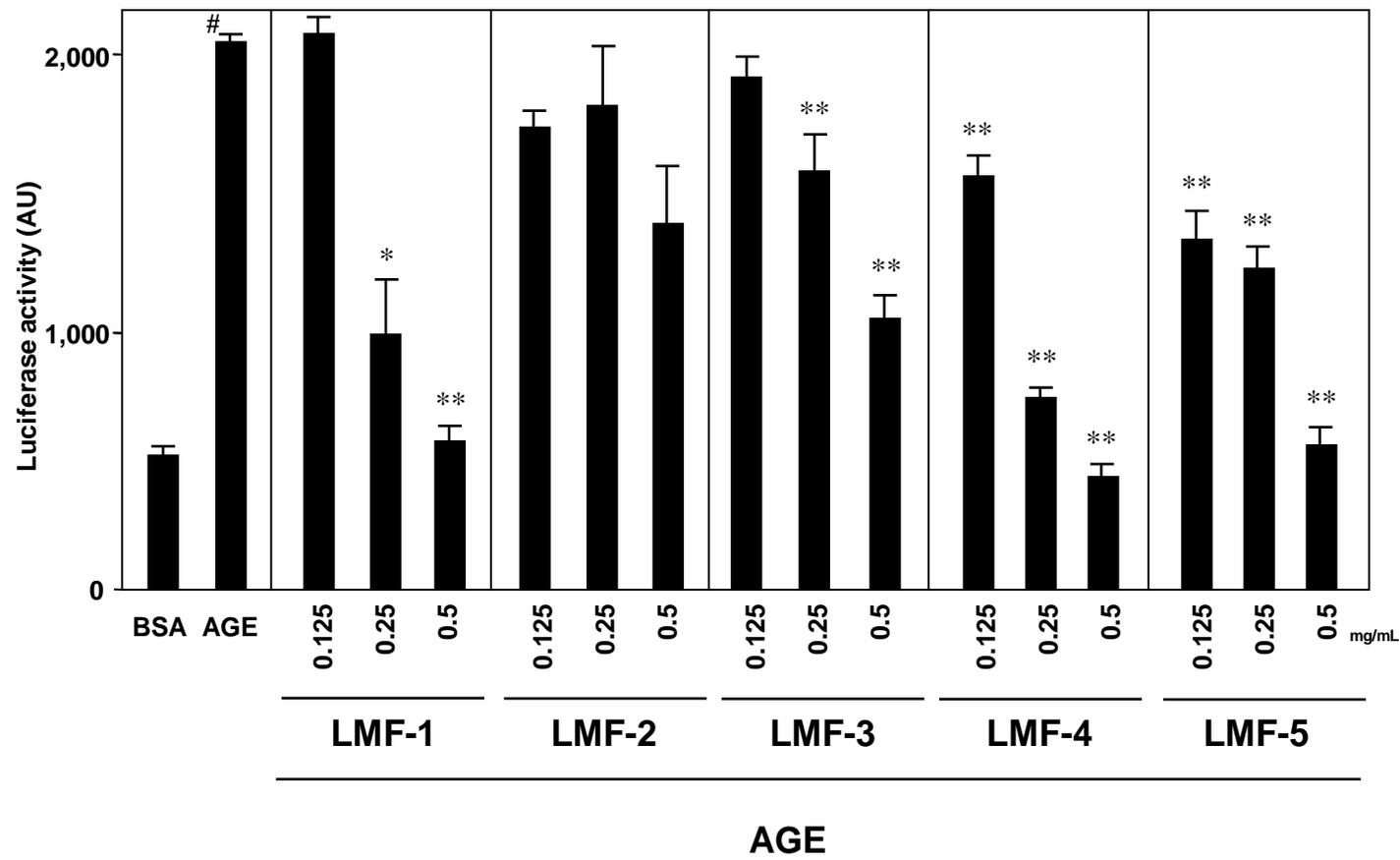


Fig.5

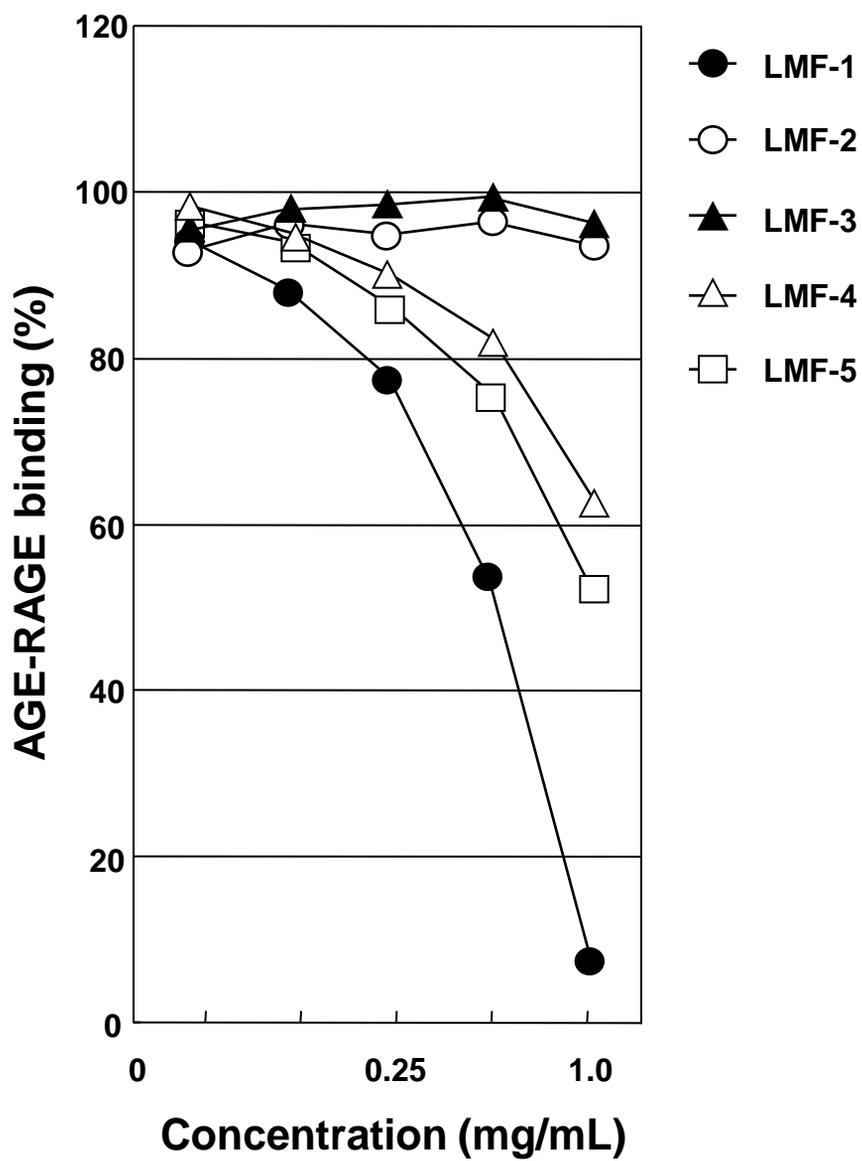


Fig.6

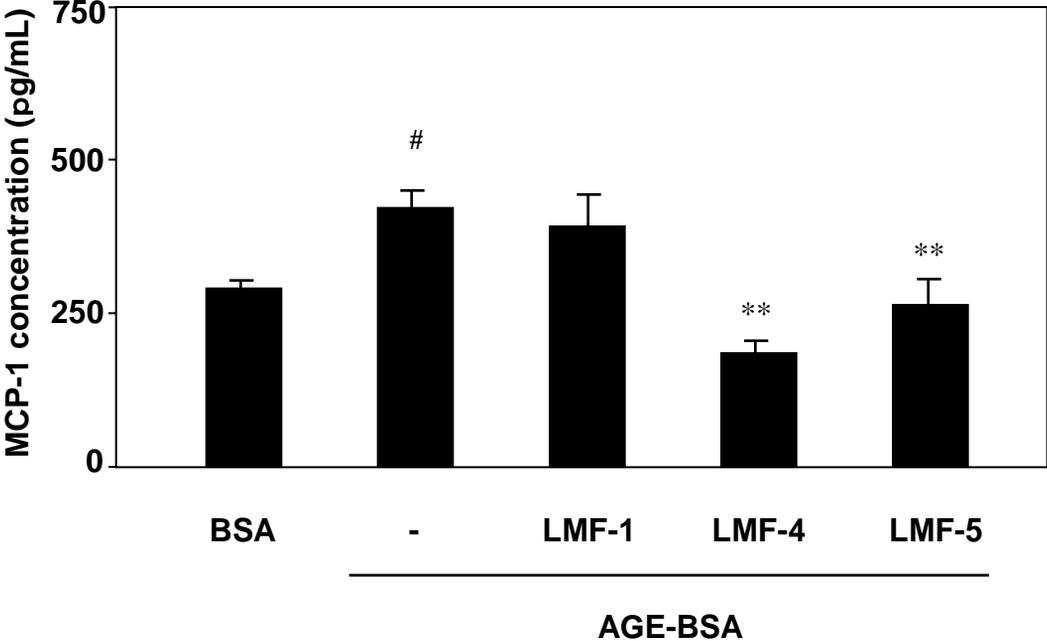


Fig.7

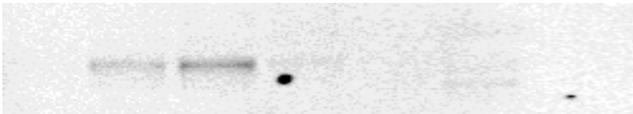
Fraction number

2 3 4 5 6 7 8

RAGE



BSA

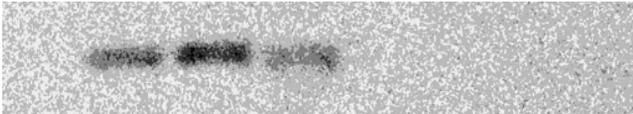


AGE

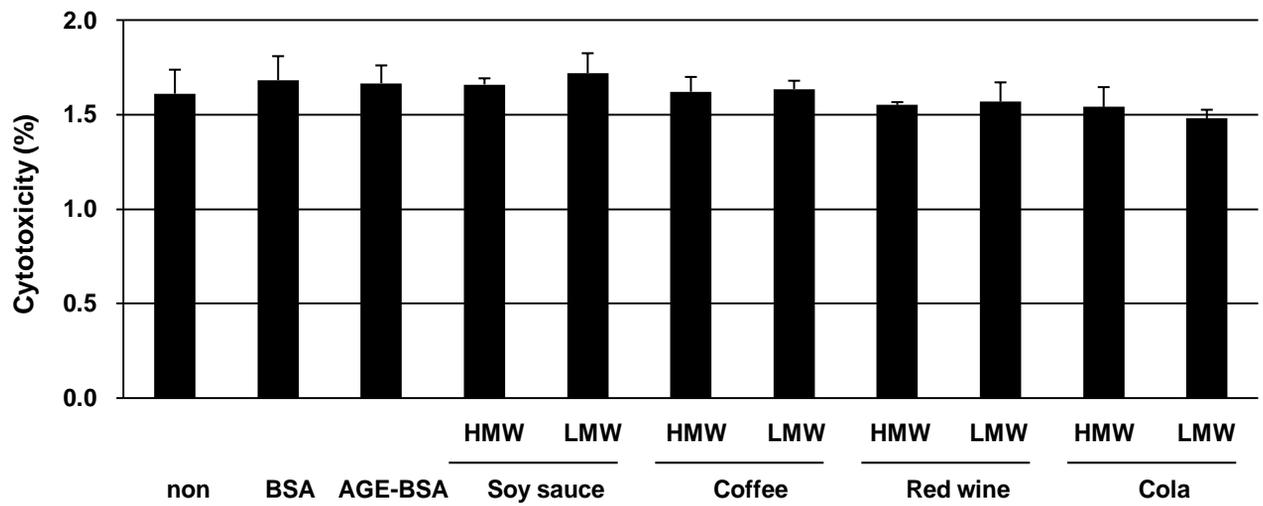


AGE+LMF-4

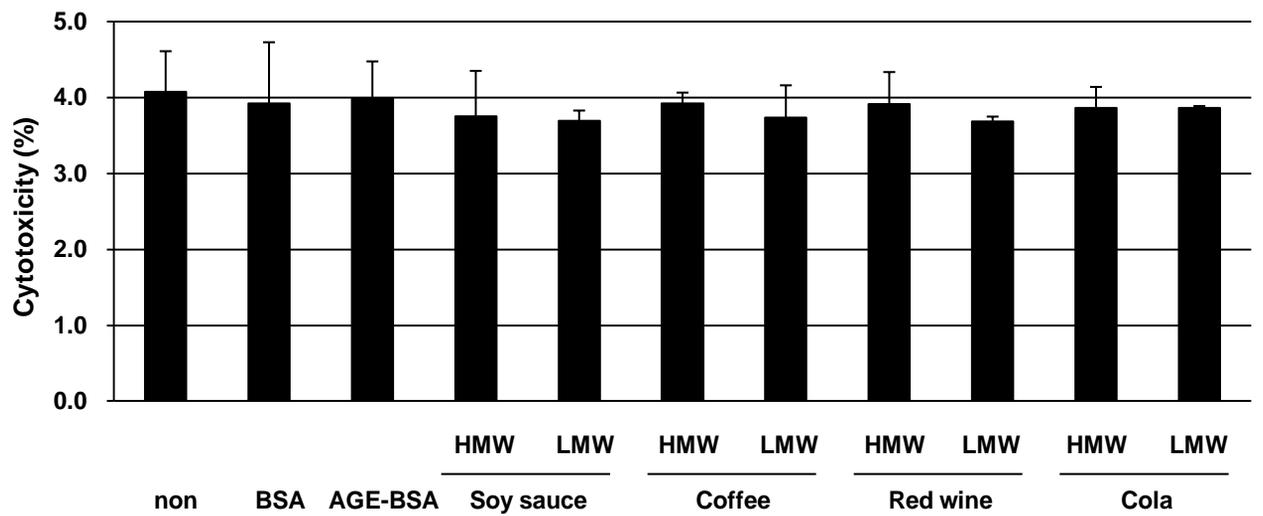
GM1



Supplemental Fig. 1

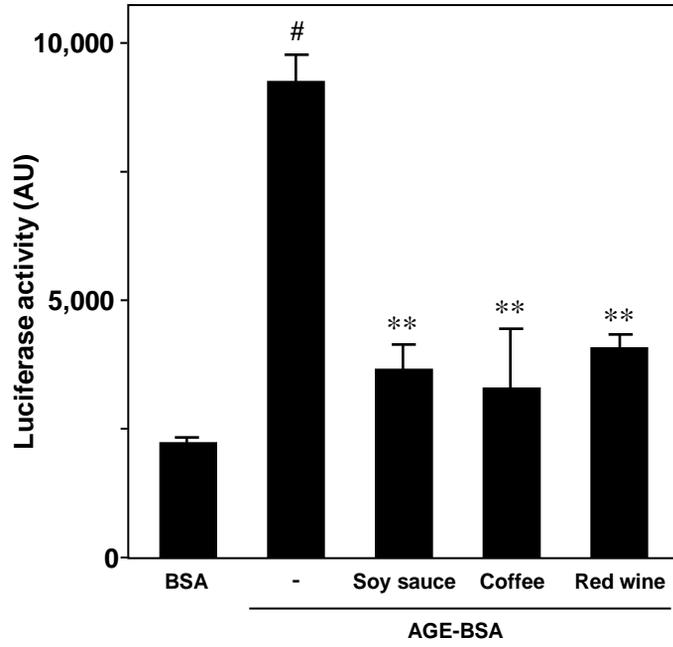


A

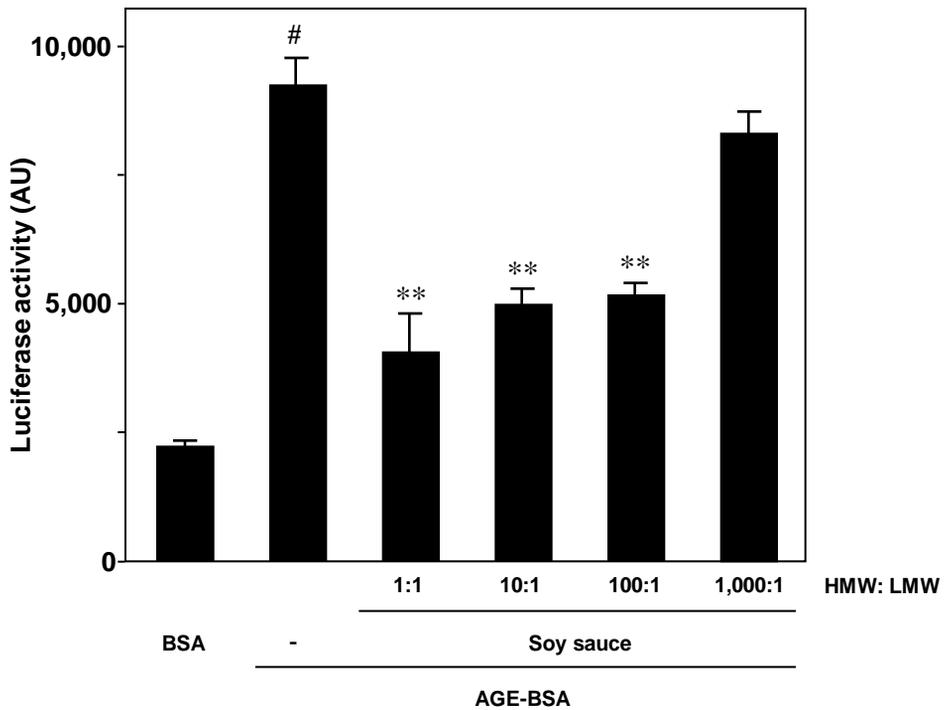


B

Supplemental Fig. 2



A



B