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

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**Abstract**

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

## Introduction

Advanced glycation end products (AGE) are stable end products of the Maillard reaction. The Maillard reaction was first described by Louis-Camille Maillard in 1912.[1] Reducing sugars such as glucose react non-enzymatically with amino groups of proteins through a series of reactions including Schiff's base formation, Amadori rearrangement, dehydration, condensation, and crosslinking to yield irreversible AGE.[2] In diabetes, AGE have been implicated in the development of diabetic vascular complications.[3]

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

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

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

## Experimental

### Food

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

### Column chromatography

Liquid foods were filtered through a 0.22-μm filter (Millipore). Soy sauce was applied to a column (2 x 7cm) of cosmocil 75C18-OPN (Nacalai Tesque, Japan) equilibrated with $H_2O$ for desalting. The column was washed extensively with water. The bound material was eluted with 100% methanol/0.1% TFA. The filtrates of coffee, cola, red wine, and the desalted soy sauce were used as total crude preparations. All preparations were freeze-dried and the resultant lyophilized powder was fractionated. Size fractionation was performed using a column (5 mL) of PD-10 (GE Healthcare) equilibrated with $H_2O$. Total crude preparations were applied to the column and separated into pass-through fractions and incorporated fractions; these were named HMW fractions and LMW fractions, respectively. The LMW fraction of soy sauce was further applied to a column of cosmocil 75C18-OPN equilibrated with $H_2O$. The column was washed extensively with $H_2O$. The bound material was eluted by stepwise elution with $H_2O$, 20% methanol, 50% methanol, 100% methanol and 100% methanol/0.1% TFA. The eluates were freeze-dried and the lyophilized powder was used in subsequent experiments. Endotoxin was not detected in the preparations and the fractions when tested with Limulus HS-test Wako (Wako Pure Chemical Industries, Osaka, Japan).

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

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

**AGE assays**

$N^{\varepsilon}$-carboxymethyl lysine (CML) was determined with the CML ELISA kit (CycLex, Nagano, Japan). Fluorescence was measured with a TriStar LB941 multireader (Berthold Technologies, Bad Wildbad, Germany). Samples were excited at 355 nm and emission was recorded at 460 nm.

**Luciferase reporter assay**

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

glyceraldehyde-derived AGE-BSA[22] in the presence or absence of food-derived fractions for 4 h. Luciferase activity was determined with a Luciferase Assay System (Promega) and measured in a luminometer (Fluoroskan Ascent FL; Labotal Scientific Equipment Ltd., Abn Gosh, Israel).

**Plate binding assay**

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

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

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

**Sucrose density gradient centrifugation and western blotting**

Lipid rafts were isolated essentially according to the detergent extraction method described by Mitsuda *et al.*[23] The same cell line used for the luciferase-reporter assay, the C6 glioma cells, was plated at a density of 1 x $10^6$/10 cm-dish and cultured to 90% confluence. After washing each well with 0.1%FBS/DMEM, AGE-BSA were added with or without the soy sauce LMF-4 fraction. After 20 h incubation, the cell layer was washed with cold PBS, and the cells were collected, suspended in 1 mL of a buffer containing 1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/mL aprotinin, and disrupted by 5 rounds of 30 sec sonication. Samples were placed on the bottom of Ultra-Clear centrifuge tubes (Beckman Instruments) and mixed with an equal volume of 80% (w/v)

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

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

**Statistical analysis**

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

## Results

*RAGE-dependent NF-κB reporter assay*

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

*LMF-4 inhibited RAGE trafficking to lipid rafts*

We then sought to determine how the LMF fractions halt AGE-RAGE activity using LMF-4, which showed higher inhibitory activity of MCP-1 secretion than LMF-5. Since lipid rafts have recently been reported to be involved in receptor trafficking[26] and signal transduction[27], we investigated the relationship between RAGE and lipid rafts. As shown in Fig. 7, when the C6 cells were treated with non-glycated BSA, RAGE was recovered in the fractions near the bottom. After exposure to AGE-BSA, RAGE moved to the less dense fractions to which GM-1, the marker of lipid rafts, sedimented, indicating that ligand binding to RAGE induced RAGE trafficking to lipid rafts. However, coexistence of LMF-4 completely inhibited RAGE movement to the lipid raft fractions.

## Discussion

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

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

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

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

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

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

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

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

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

ascribed to polyphenol. Resveratrol, a natural polyphenol found in red wine, attenuates NF-κB activation and reduces RAGE expression.[35]

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

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**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 |

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

**Table 2** Determinations of CML concentrations in LMW and HMW 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 |

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

**Figure legends**

Figure 1

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

Figure 2

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

Figure 3

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

Figure 4

RAGE antagonistic activities of subfractions of the Japanese soy sauce LMW fraction. AGE-BSA, 50 μg/mL glyceraldehyde-derived AGE-BSA; BSA, 50 μg/mL non-glycated BSA. #, $p < 0.01$ (*vs.* BSA);**, $p < 0.01$ (*vs.* AGE-BSA); *, $p < 0.05$ (*vs.* AGE-BSA) (n = 3).

Figure 5

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

Figure 6

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

Figure 7

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

Supplemental Experimental

Cytotoxicity Assay

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

Legend to supplemental Figure

Supplemental Fig. 1

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

BSA, 50 μg/mL non-glycated BSA.

Supplemental Fig. 2

Effects of mixtures of HMW and LMW fractions from Japanese soy sauce, coffee, and red wine on RAGE signaling. RAGE signaling was assayed with human RAGE-expressing, NF-κB-promoter-luciferase reporter gene-carrying rat C6 glioma cells as described in the Experimental section. (A) Equal amounts (0.5 mg/mL each) HMW and LMW fractions from soy sauce, coffee and red wine were combined and used for the assay. (B) Soy sauce-derived HMW and LMW fractions were combined at the indicated ratio and used for the assay. AGE-BSA, 50 μg/mL glyceraldehyde-derived AGE-BSA; BSA, 50 μg/mL non-glycated BSA. #, $p < 0.01$ (*vs.* BSA);**, $p < 0.01$ (*vs.* AGE-BSA) (n = 3).

Fig.1

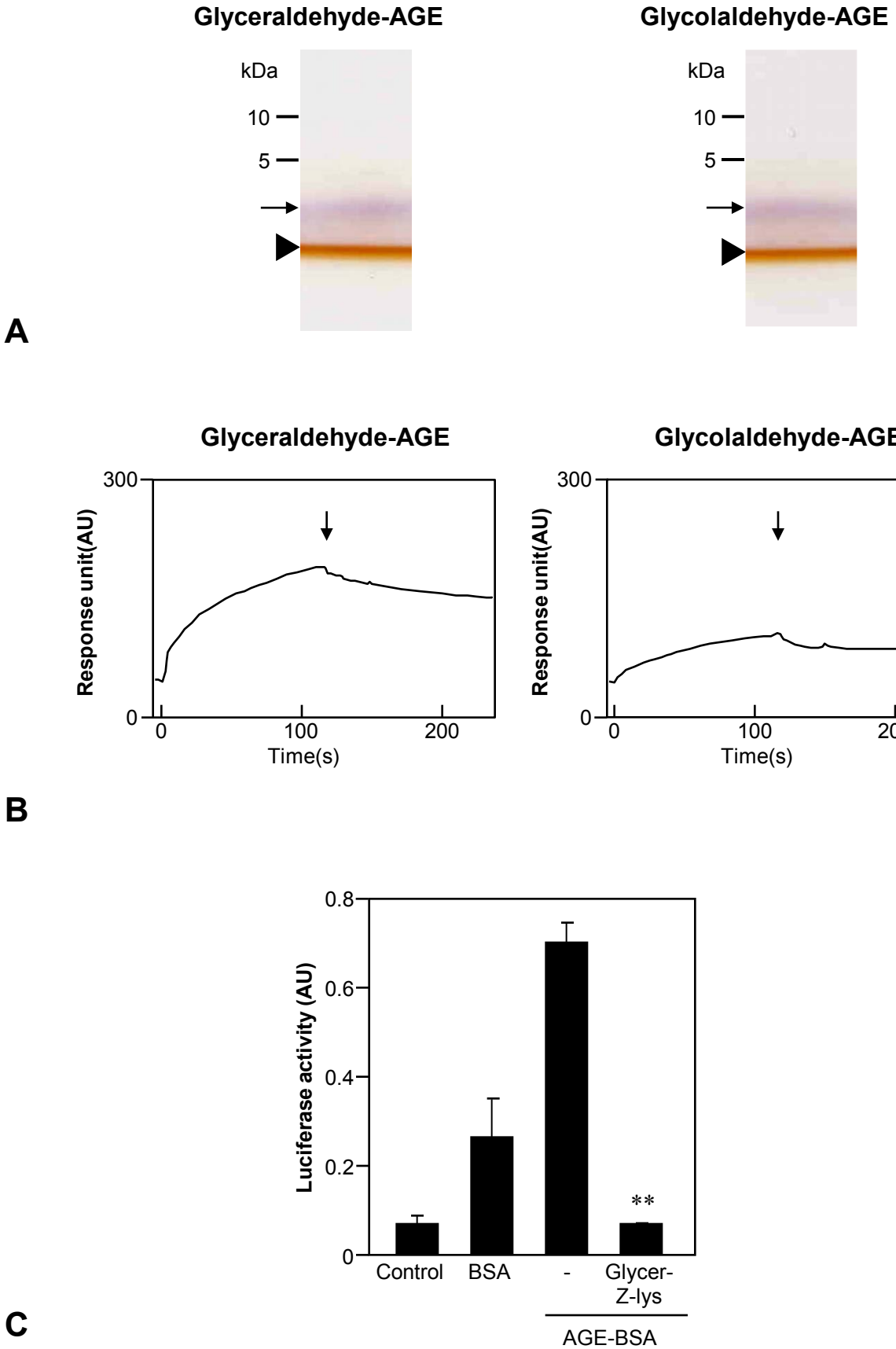
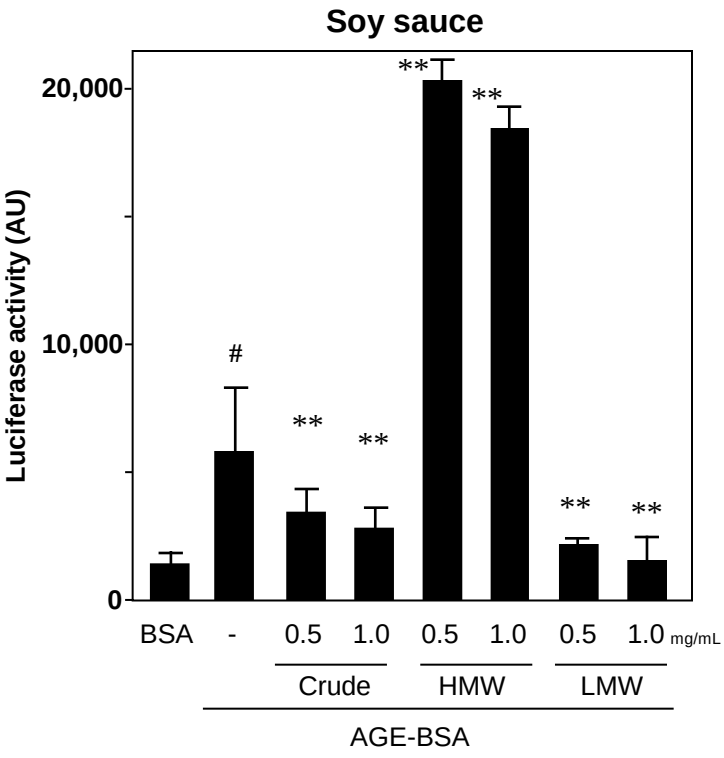
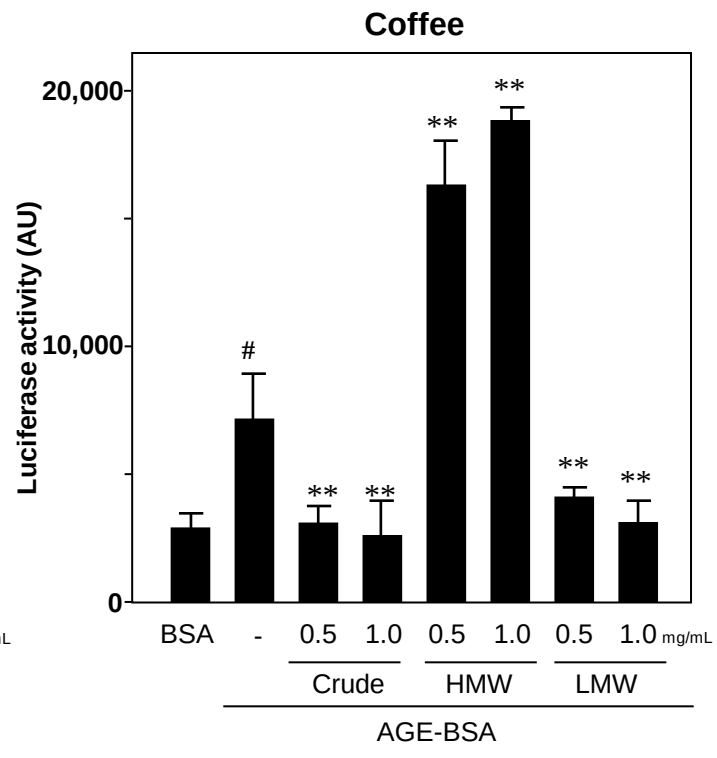

Glyceraldehyde-AGE
Glycolaldehyde-AGE
kDa
10
5
A
Glyceraldehyde-AGE
Glycolaldehyde-AGE
Response unit(AU)
300
0
0
100
200
Time(s)
B
Luciferase activity (AU)
0.8
0.6
0.4
0.2
0
**
Control
BSA
-
Glycer-Z-lys
AGE-BSA
C

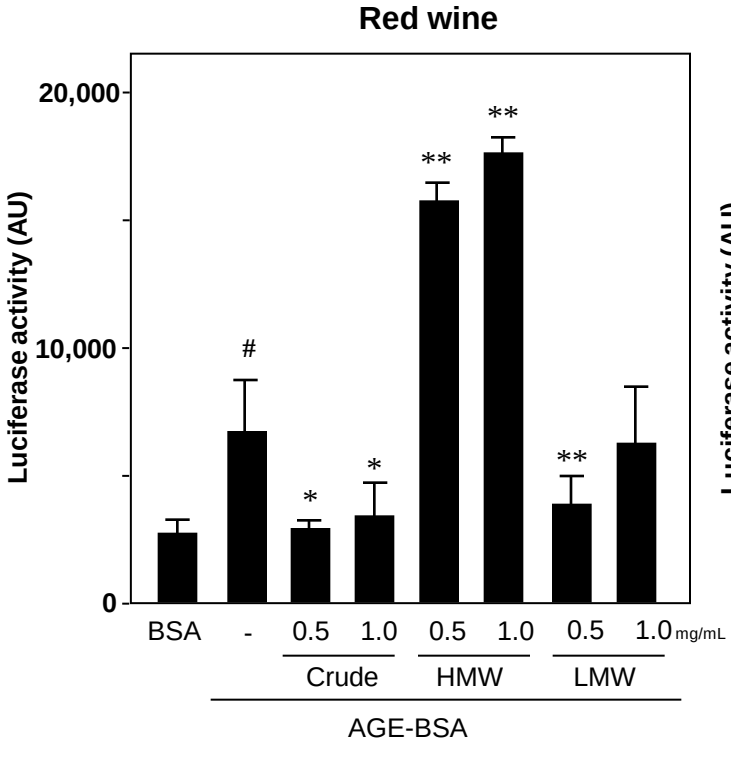
Fig.2


Soy sauce
Luciferase activity (AU)
20,000
10,000
0
#
**
**
**
**
**
**
BSA
-
0.5
1.0
0.5
1.0
0.5
1.0
mg/mL
Crude
HMW
LMW
AGE-BSA

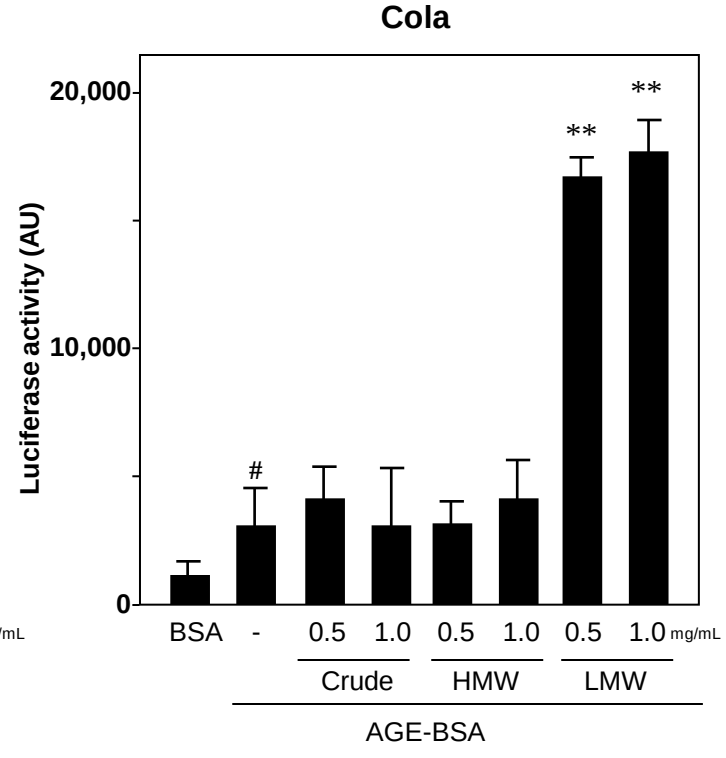
A


Coffee
Luciferase activity (AU)
20,000
10,000
0
#
**
**
**
**
**
**
BSA
-
0.5
1.0
0.5
1.0
0.5
1.0
mg/mL
Crude
HMW
LMW
AGE-BSA

B


Red wine
Luciferase activity (AU)
20,000
10,000
0
#
*
*
**
**
**
BSA
-
0.5
1.0
0.5
1.0
0.5
1.0
mg/mL
Crude
HMW
LMW
AGE-BSA

C


Cola
Luciferase activity (AU)
20,000
10,000
0
#
**
**
BSA
-
0.5
1.0
0.5
1.0
0.5
1.0
mg/mL
Crude
HMW
LMW
AGE-BSA

D

Fig.3

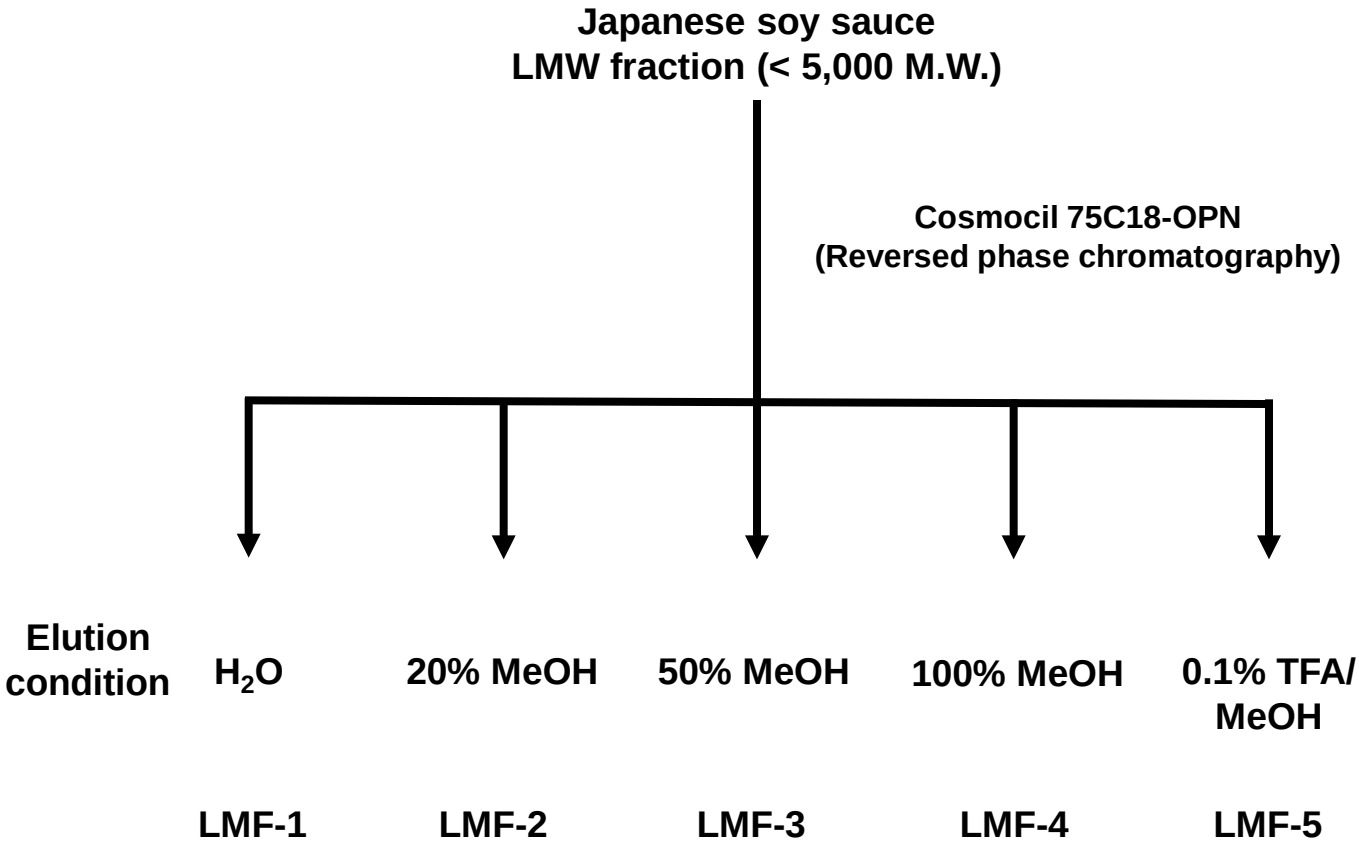

Japanese soy sauce
LMW fraction (< 5,000 M.W.)
Cosmocil 75C18-OPN
(Reversed phase chromatography)
Elution condition
H2O
20% MeOH
50% MeOH
100% MeOH
0.1% TFA/ MeOH
LMF-1
LMF-2
LMF-3
LMF-4
LMF-5

Fig.4

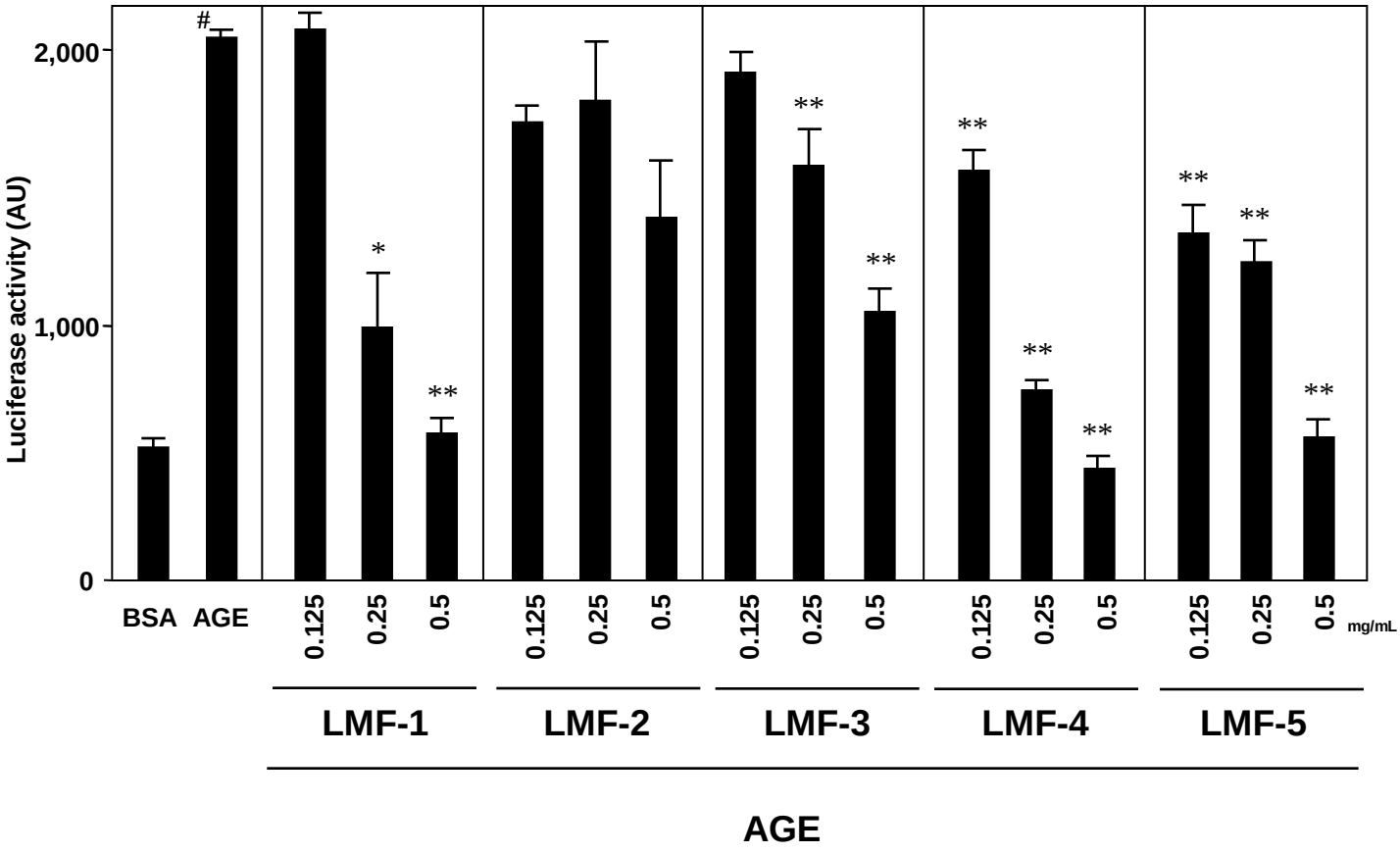

Luciferase activity (AU)
2,000
1,000
0
#
*
**
BSA
AGE
0.125
0.25
0.5
mg/mL
LMF-1
LMF-2
LMF-3
LMF-4
LMF-5
AGE

Fig.5

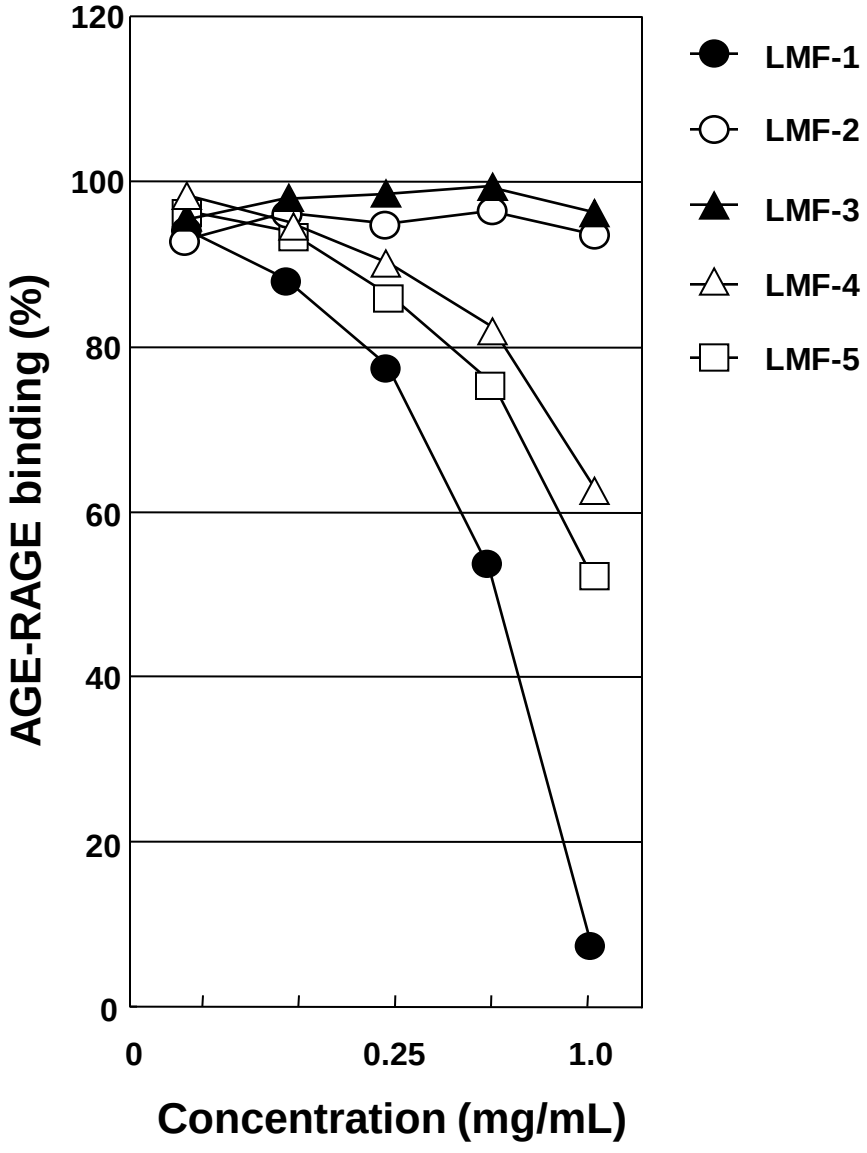

120
100
80
60
40
20
0
AGE-RAGE binding (%)
0
0.25
1.0
Concentration (mg/mL)
LMF-1
LMF-2
LMF-3
LMF-4
LMF-5

Fig.6

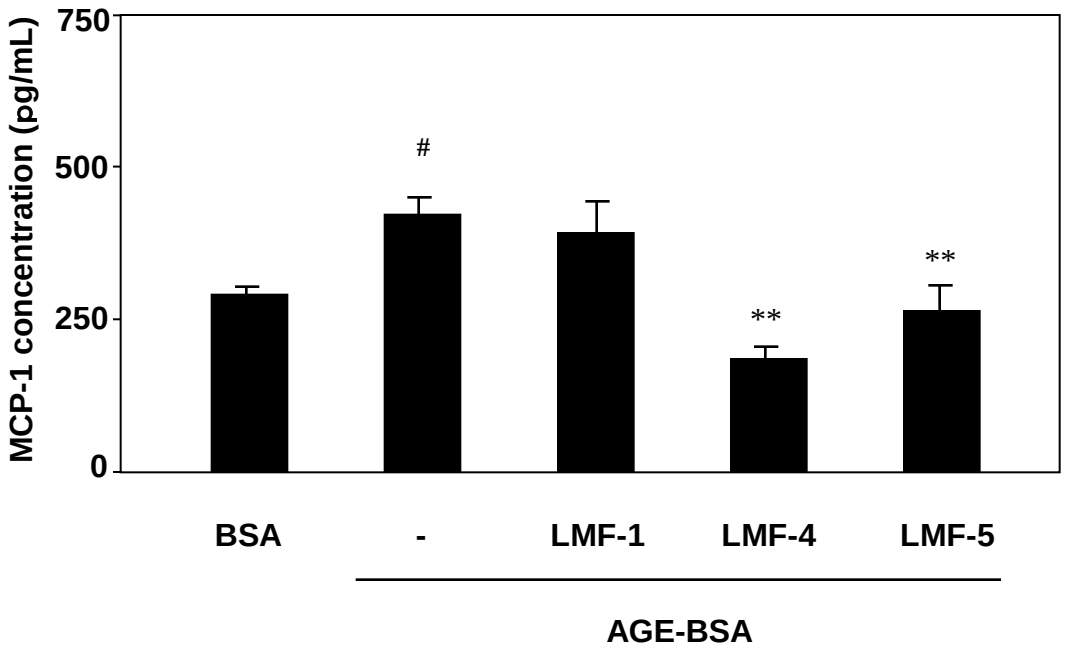
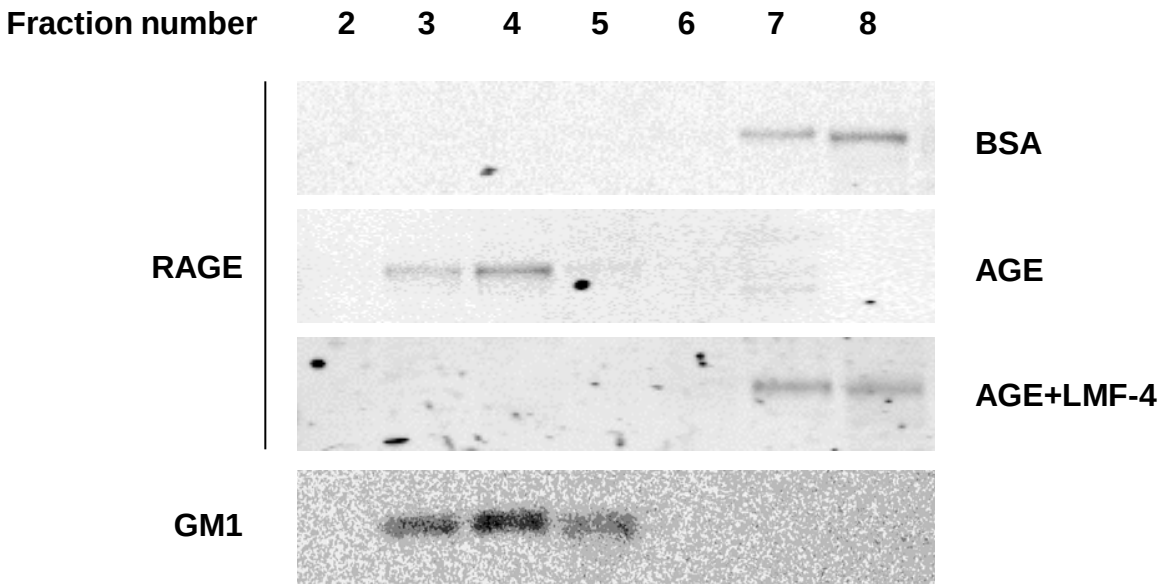
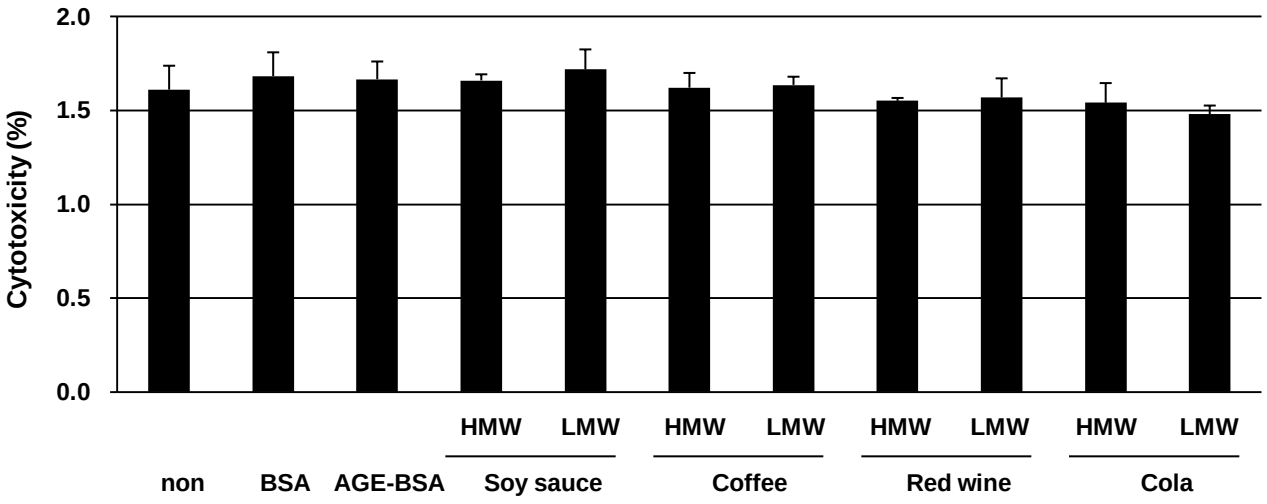

MCP-1 concentration (pg/mL)
750
500
250
0
#
**
**
BSA
-
LMF-1
LMF-4
LMF-5
AGE-BSA

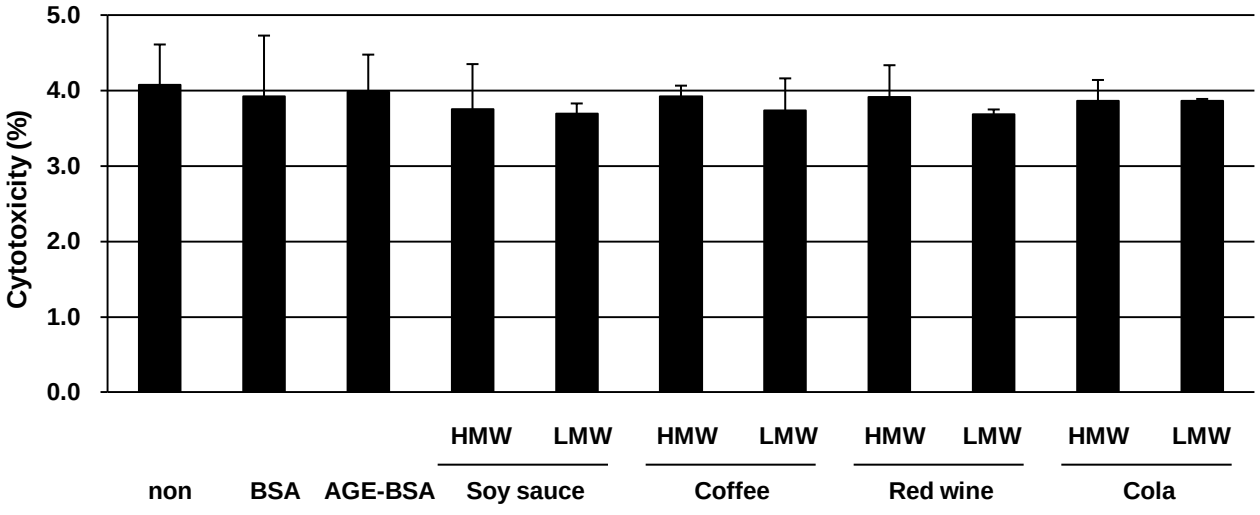
Fig.7



# Supplemental Fig. 1

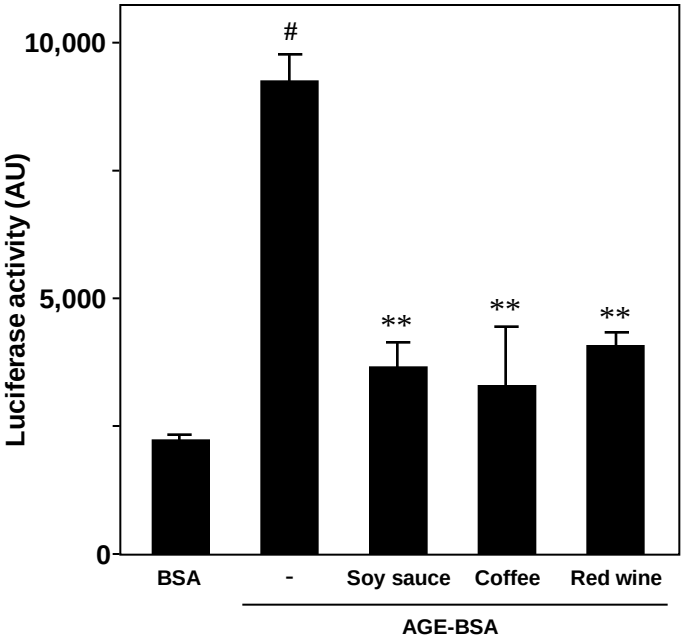



A

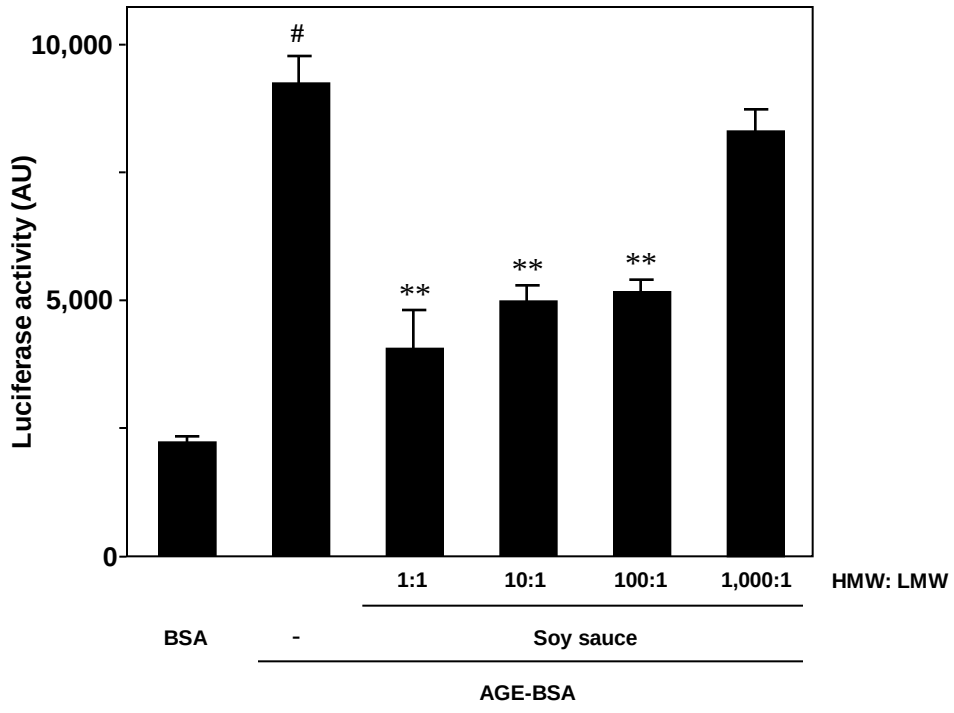



B

# Supplemental Fig. 2




A




B