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The proglycation effect of caffeic acid leads to the elevation of oxidative stress and inflammation in monocytes, macrophages and vascular endothelial cells

Chi-Hao Wu, Hsiao-Wen Huang[1], Jer-An Lin, Shang-Ming Huang, Gow-Chin Yen*

Department of Food Science and Biotechnology, National Chung Hsing University, 250 Kuokuang Road, Taichung 40227, Taiwan

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Abstract

In this study, the effects of phenolic acids [caffeic acid (CA), ferulic acid, *m*-coumaric acid, and chlorogenic acid] on methylglyoxal (MG)-induced protein glycation were investigated in vitro. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and advanced glycation end products (AGEs)-specific fluorescence showed that MG-mediated protein modification was enhanced dose-dependently by CA ($P<.05$), whereas α-lipoic acid, glutathione and EDTA inhibited these changes. Electron paramagnetic resonance spectra showed that CA increased reactive oxygen species (ROS) production during glycation, suggesting the proglycation mechanism of CA is associated with its pro-oxidative properties. Additionally, fetal bovine serum (FBS) was utilized as the source of target proteins for evaluating the effects of CA in cells. Differential glycation of FBS samples was performed by incubating FBS with MG, CA or aminoguanidine (AG, an AGE inhibitor). FBS incubated with MG and CA (MG/CA-FBS) evoked the greatest deleterious responses, as follows: (1) inducing proinflammatory tumor necrosis factor (TNF)-α and interleukin-1β expression and ROS production in monocytic THP-1 cells, (2) stimulating TNF-α secretion in RAW 264.7 macrophages and (3) causing oxidative DNA damage and inducing the expression of receptor for AGEs (RAGE), intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 in human umbilical vein endothelial cells. Furthermore, adhesion and transendothelial migration of monocytes were also significantly increased by MG/CA-FBS treatment compared to MG-FBS ($P<.05$). In conclusion, our data show that CA exhibits pro-oxidative and pro-glycative effects during the glycation process, suggesting a detrimental role for CA under high-glycotoxin conditions.
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Keywords: Advanced glycation end products; Caffeic acid; Methylglyoxal; Pro-glycation; Oxidative stress; Inflammation

1. Introduction

Diabetes mellitus is a complicated disease involving systematic metabolic dysfunction. Glycation, the nonenzymatic reaction of reducing sugars with amino groups, is increased in hyperglycemic physiological environments, leading to an acceleration of the formation of advanced glycation endproducts (AGEs). AGEs are crucial factors in evoking diabetic complications: they can crosslink with proteins, lipids and nucleic acids, influencing their structures and functions. Previous studies have indicated that microvascular complications of diabetes, such as neuropathy [1,2], nephropathy [3,4] and retinopathy [5,6] and macrovascular diseases, such as coronary disease and atherosclerosis, are highly related to the accumulation of AGEs [7,8]. Decreasing or inhibiting the synthesis of AGEs might be effective in delaying and preventing the development and progression of diabetes and its complications.

Natural phenolic compounds are the most abundant antioxidant resources. They are widely distributed in plants and present in considerable amounts in the human diet. Phenolic acids can be classified into two groups according to the structure attached to the phenol ring: hydroxybenzoic acids (HBAs) and hydroxycinnamic acids (HCAs). The HCAs are more common than the HBAs and comprise mainly *m*-coumaric acid (*m*COA), ferulic acid (FA) and caffeic acid (CA). CA and its esterified form, chlorogenic acid (CHA), are generally the most abundant phenolic acids, accounting for between 75% and 100% of the total HCA content in most fruits and existing in high concentrations in coffee [9]. These compounds have aroused considerable interest due to their various biological activities, including antioxidant, anti-apoptotic and anti-inflammatory capacities. Research has indicated that CA can protect against the oxidative modification of low-density lipoproteins and may have the potential to lower coronary disease [10]. However, the literature concerning the role of phenolic acids in the formation of AGEs remains unclear and even conflicting.

On the other hand, many studies have revealed that CA, the most abundant phenolic acid, has pro-oxidant activity in the presence of Cu (II) and induces the oxidation of low-density lipoproteins (LDLs) and lipid peroxidation, further causing oxidative DNA damage [11–13]. These studies suggested that phenolic acids may exert negative effects that demand further study. The purpose of this study was to investigate the effects of dietary phenolic acids on protein glycation and to elucidate the role of phenolic acids in AGE-induced biological damage.

* Corresponding author. Tel.: +1 886 4 2287 9755; fax: +1 886 4 2285 4378.
E-mail addresses: gcyen@nchu.edu.tw, gcyen4020@gmail.com (G.-C. Yen).
[1] Contributed equally as first author.

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2. Materials and Methods

2.1. Chemicals

Aminoguanidine hemisulfate (AG), *D*-(+)-glucose, caffeic acid (CA), *m*COA, FA, CHA, methylglyoxal (MG), glyoxal solution (GO), fatty acid-free bovine serum albumin (BSA), (±)-α-lipoic acid (α-LA), glutathione (GSH), *N*-*tert*-butyl-α-phenylnitrone (PBN), 2,7-dichlorofluorescin diacetate (DCF-DA), copper (II) sulfate pentahydrate and iron (II) sulfate heptahydrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tri-Isolation Reagent was purchased from MoBioPlus (Taipei, Taiwan). Coomassie Brilliantblue G 250 was purchased from Merck (Whitehouse Station, NJ, USA). Fetal bovine serum (FBS), RPMI 1640 glucose free medium, Dulbecco's modified Eagle's medium (DMEM), SuperScript III First-Strand Synthesis SuperMix for qRT-PCR kit, SYBR safe DNA gel stain were purchased from Invitrogen (Carlsbad, CA, USA). Disodium dihydrogen EDTA was purchased from Showa chemical (Tokyo, Japan). Medium 200 and low serum growth supplement were purchased from Cascade Biologics (Portland, OR, USA). polymerase chain reaction (PCR) Master Mix 2X Kit, 6X DNA Loading Dye Solution were purchased from Fermentas (Glen Burnie, MD, USA). Mouse TNF-α ELISA Ready-SET-Go was purchased from eBioscience (San Diego, CA, USA). Transwell Permeable Support was purchased from Corning (Lowell, MA, USA).

2.2. In vitro glycation of BSA by MG

In order to investigate the effects of phenolic acids on protein glycation, levels of glycation were measurement according the method of Wu and Yen [14]. Briefly, BSA (0.4 mg/ml) was incubated with MG in the presence or absence of a phenolic acid (CA, FA, *m*COA and CHA), AG, antioxidants (α-LA and GSH), metal ions (Cu^{2+} and Fe^{2+}) and/or the metal-chelating agent EDTA under sterile conditions in 0.1 M sodium phosphate buffer (pH 7.4) at 37°C for 7 days. After incubation, samples were repeatedly filtered though PM-10 ultrafiltration membranes (Amicon) using 20 mM phosphate buffer (pH 7.4) and further desalted with Fast Desalting Column H10/10 (Amersham Pharmacia Biotech, Uppsala, Sweden). The effect of MG modification on the cross-linking and aggregation of BSA was investigated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions using a 4% stacking and 10% separating gel. Total protein was visualized by Coomassie blue stain. The AGE-specific fluorescence of the samples was measured at the excitation and emission maxima of 330 and 410 nm, respectively, versus an unincubated blank containing the MG with BSA proteins.

2.3. Determination of free radical generation by electron paramagnetic resonance

Electron paramagnetic resonance (EPR) spectra were measured on glycated protein samples (BSA-MG modification) with a reaction mixture containing 0.2 mM PBN. The EPR spectra of PBN-OOH adducts (superoxide adducts of PBN) were recorded on a Brüker EMX-10/12 spectrometer (Ettlingen, Germany) equipped with WINEPR SimFonia software (Brüker Analytische Messtechnik, Karlsruhe, Germany) under the following conditions: microwave power, 20 mW; frequency, 9.774 GHz; modulation amplitude, 1.60 G; modulation frequency, 100 kHz; time constant, 163.84 ms; sweep time, 167.772 s; receiver gain, 1.00×10^{-5}; sweep width, 100 G; field center, 3480 G. No EPR signals were detected in any of the reagents used in EPR analysis. All spectra were recorded at room temperature.

2.4. Preparation of glycated FBS

Fetal bovine serum (FBS) was utilized as the target proteins for evaluating the damaging effect of CA in the cells. Different glycated FBS samples were prepared by incubating FBS with 25 mM MG in the presence or absence of 2.5 mM CA or 50 mM AG under sterile conditions in 0.1 M sodium phosphate buffer (pH 7.4) at 37°C for 9 days. At the end of the incubation period, FBS samples were dialyzed by Cellu Sep T-series dialysis membranes (Membrane Filtration Products, Seguin, TX, USA) to remove unincorporated MG and CA. Glycated FBS samples were filter-sterilized by passing through a 0.2-μm filter and stored at −20°C.

2.5. Cell culture

The human THP-1 monocytic cell line (THP-1 cells) was obtained from the Bioresource Collection and Research Center (BCRC 60430, Food Industry Research and Development Institute, Hsin Chu, Taiwan) and cultured in RPMI 1640 medium supplemented with 10% FBS, 10 mM HEPES, 1% streptomycin/penicillin, sodium pyruvate and 5.5 mM *D*-glucose (normal glucose, NG) at 37°C in a 5% CO_2 incubator. The murine macrophage cell line RAW 264.7 was obtained from BCRC and cultured in DMEM supplemented with 10% FBS and 1% streptomycin/penicillin at 37°C in 5% CO_2. Human umbilical vein endothelial cells (HUVEC) were obtained from the Cascade Biologics and cultured in Medium 200 with 10 ng/ml epidermal growth factor, 3 ng/ml basic fibroblast growth factor, 10 mg/ml heparin and 1% streptomycin/penicillin at 37°C in 5% CO_2.

2.6. Intracellular ROS content measurement

Intracellular ROS was estimated using DCF-DA, similar to those previously described [15]. THP-1 cells were incubated with medium containing 10% normal FBS or 10% glycated FBS for 48 h. At the end of the incubation period, cells were collected and washed with PBS and then resuspended with PBS contained 10 μM DCF-DA for 30 min. Cells were observed using a microscope (IX71, Olympus, Osaka, Japan), and images were captured by ImagePro (Media Cybernetics, Bethesda, MD, USA).

2.7. RNA extraction and RT-PCR

Monocytic THP-1 cells were incubated with medium containing 10% normal FBS or 10% glycated FBS for 48 h. HUVEC were incubated with medium containing 10% normal FBS or 10% glycated FBS for 24 h. After Treatment 1, cells were washed with PBS, and RNA was extracted with Tri-Isolation Reagent (MoBioPlus). One microgram of RNA was reverse-transcribed to cDNA using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR kit (Invitrogen). PCR analyses were performed to detect tumor necrosis factor (TNF)-α and interleukin (IL)-1β gene expression in THP-1 cells, and receptor for AGEs (RAGE), vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1(ICAM-1) gene expression in HUVEC using the PCR Master Mix 2X kit (Fermentas, Glen Burnie, MD, USA). The qRT-PCR primers were as follows: TNF-α: forward 5′-CCAAACGATGTTGTACCCGA-3′; reverse 5′-CAGTTGGAGGAGAGACGGTA-3′. IL-1β: forward 5′-CTCTCTCACCTCTCCTACTCAC-3′; reverse 5′-ACACTGCTACTTCTTGCCCC-3′. RAGE: forward 5′-CACCTTCTCCTGTAGCTTCA-3′; reverse 5′-TGCCACAAGATGACCCCAA-3′. VCAM-1: forward 5′-ATGACATGCTTGAGCCAGG-3′; reverse 5′-GTGTCTCCTTCTTTGACACT-3′. ICAM-1: forward 5′-CAGTGACCATCTACAGCTTTCCGG-3′; reverse 5′-GCTGCTACCACAGTGATGATGACAA-3′. 18s: forward 5′-TTGGAGGGCAAGTCTGGTG-3′; reverse 5′-CCGCTCCCAAGATCCAACTA-3′. The annealing temperatures for TNF-α, IL-1β and 18 s was 59°C; for RAGE was 56°C; for VCAM-1 and ICAM-1 were 51°C and 64°C, respectively. The RT-PCR product was electrophoresed on a 1.8% agarose gel, and the signal was detected using SYBR DNA staining. The gel was photographed under ultraviolet transillumination. The results are expressed relative to the corresponding 18s signal from each sample.

2.8. Cytokine ELISA assays

RAW 264.7 macrophages were incubated with medium containing 10% normal FBS or 10% glycated FBS in six-well tissue plates for 24 h. The supernatant conditioned medium was then harvested and assayed for TNF-α secretion using a specific ELISA kit according to the manufacturer's instructions.

2.9. Alkaline single-cell gel electrophoresis (comet assay)

The comet assay was performed under alkaline conditions, similar to those previously described [16]. Briefly, HUVEC were incubated with medium containing 10% normal FBS or 10% glycated FBS for 72 h and then harvested by mild trypsinization and centrifugation at 1200×*g* at 25°C for 6 min. Further, cells were suspended in 1% low-melting-point agarose and immediately pipetted on a frosted glass microscope slide precoated with a layer of 1% normal melting agarose. After applying a third layer of 1% low-melting agarose, the slides were immersed in cold lysing solution (10 mM Tris, 1% *N*-lauroylsarcosine sodium, 100 mM Na_2EDTA, 2.5 M NaCl, 1% DMSO, pH 10) for at least 1 h at 4°C. The microscope slides were than placed in an electrophoresis tank, and the DNA was allowed to unwinding for 20 min in freshly prepared alkaline electrophoresis buffer (0.3M NaOH and 1 mM Na_2EDTA). Electrophoresis was conducted at 4°C for 20 min at 25V and 300 mA. The slides were then neutralized with Tris buffer (0.4 M, pH 7.5) and stained with ethidium bromide (20 μg/ml). The slides were observed using a fluorescent microscope (IX71, Olympus, Osaka, Japan), and images were captured by ImagePro (Media Cybernetics, Bethesda, MD, USA). The degree of DNA damage was scored by the percentage of tail DNA [% tail DNA=tail/(head+tail)×100%].

2.10. Cell migration assay

To measure the effect of glycated FBS on the transmigration of monocytes to endothelial cells, HUVEC were incubated with medium containing 10% normal FBS or 10% glycated FBS for 24 h in 24-well plates, and then an insert (Transwell Permeable Support, Corning, Lowell, MA, USA) was added to each well. THP-1 cells (3×10^5 cells/insert) starved with serum-free medium were subsequently added to the insert and incubated for 48 h at 37°C. Cell suspensions of THP-1 cells in the insert and in the bottom of the wells were collected and counted under the microscope. Cells in the bottom of the wells were transmigrated THP-1 cells. THP-1 cells were immobilized on the permeable membrane of the insert with methanol for 30 min and then stained with giemsa for 1 h. The permeable membrane of the insert was observed using a microscope (IX71, Olympus, Osaka, Japan). Images were captured by ImagePro (Media Cybernetics, Bethesda, MD, USA).

2.11. Statistical analysis

All data are expressed as means±S.D. One-way analysis of variance was used to evaluate the difference between multiple groups. If significance was observed between groups, Duncan's test was used to compare the means of two specific groups. $P<.05$ was considered significant.

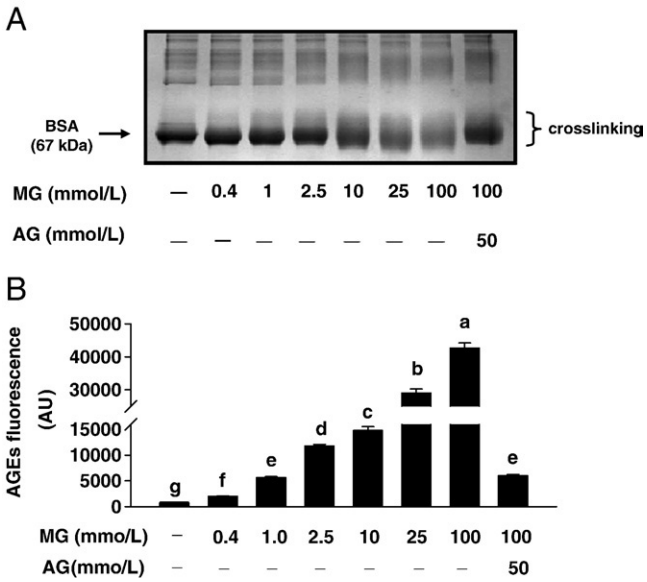

Fig. 1. MG dose-dependently induced protein modification as revealed by (A) SDS-PAGE and (B) AGEs-related fluorescence. BSA (0.4 mg/ml) served as the model protein. It was incubated with MG at indicated concentrations at 37°C for 7 days in the presence or absence of aminoguanidine (AG, 50 mM). (A) SDS-PAGE was carried out in 4–20% Tris–HCl gels. Each lane was loaded with 10 μg of protein, and bands were detected by Coomassie blue staining. (B) AGEs-related fluorescence intensity (arbitrary units, AU) was measured at Ex 355 nm and Em 405 nm. Results are means±S.D. from three independent experiments. Groups with different superscripts were significantly different ($P<.05$).

3. Results

3.1. Glycative modification of proteins by methylglyoxal

In this experiment, the effect of phenolic acids on the glycation of proteins was investigated using the glycative modification of BSA with the reactive carbonyl species MG as a model. BSA is the target protein of glycation by MG. MG modifies BSA by glycation when they are incubated together, causing BSA to lose the integrity of its original conformation and generating glycation-mediated protein crosslinking and aggregation. When analyzed by SDS-PAGE, the original 67-kDa BSA band displayed a shift with a smear. Preliminary data showed that MG caused a marked smear of the target protein band in a concentration-dependent manner (0.4–100 mM) (Fig. 1A). Meanwhile, fluorescence analysis revealed that MG led to AGEs-specific fluorescence of the glycated protein (Fig. 1B). AG, a known AGEs inhibitor, was used as a negative control and significantly inhibited the MG-mediated glycative modification of BSA (Fig. 1).

3.2. Effects of phenolic acids on MG-mediated protein glycation

Based on the above results, various phenolic acids, such as CA, FA, *m*COA and CHA, were selected for the investigation of their effects on the glycation of BSA by MG. As shown in Fig. 2, FA, *m*COA and CHA had no effect on MG-mediated BSA glycation. However, CA markedly enhanced this glycation, an effect that increased with increasing CA concentration (0.1–2.5 mM). It was also demonstrated, as shown in Fig. 3, that when MG or glyoxal (GO) was used as a glycation factor, CA remarkably enhanced protein glycation mediated by these

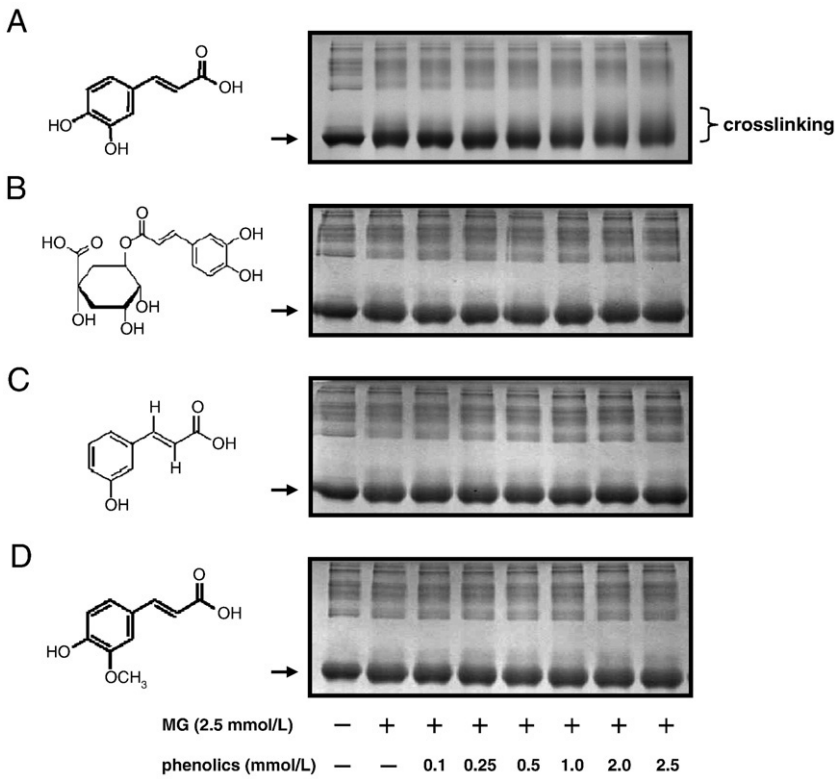

Fig. 2. Effects of (A) CA, (B) CHA, (C) *m*COA and (D) FA on MG-induced protein modification. BSA (0.4 mg/ml) was incubated with MG at 37°C for 7 days as described in Fig. 1 with varying amounts of phenolic acids. SDS-PAGE was carried out in 4–20% Tris-HCl gels. The arrow indicates the usual position of BSA (67 kDa).

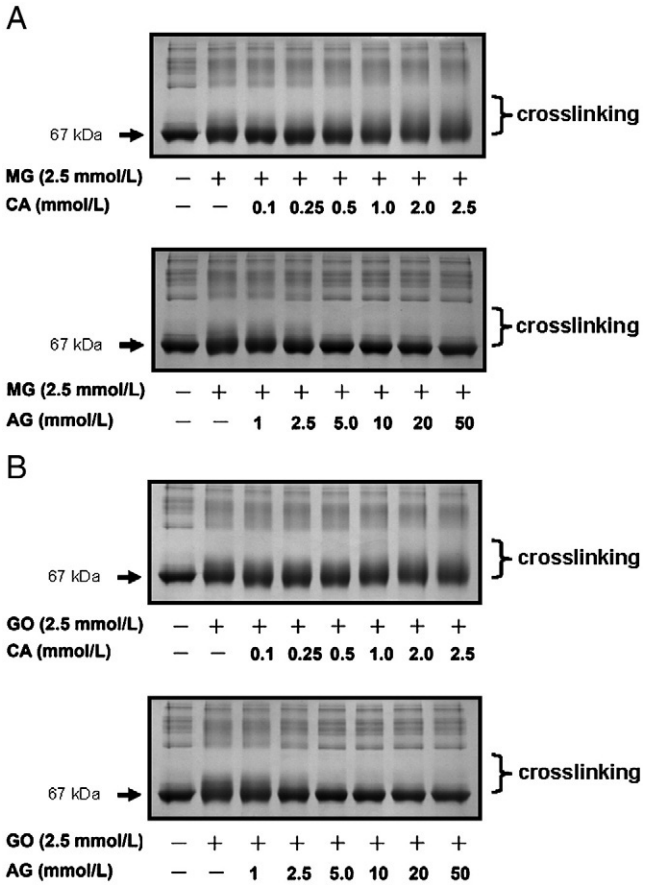

Fig. 3. Dose dependency of the effects of CA and AG on reactive carbonyl species-mediated protein modifications. The modulation of (A) MG- or (B) GO-mediated protein modification was revealed by SDS-PAGE. BSA (0.4 mg/ml) was incubated with (A) 2.5 mM MG or (B) 2.5 mM GO under sterile conditions for 7 days with CA (upper panel) or AG (lower panel).

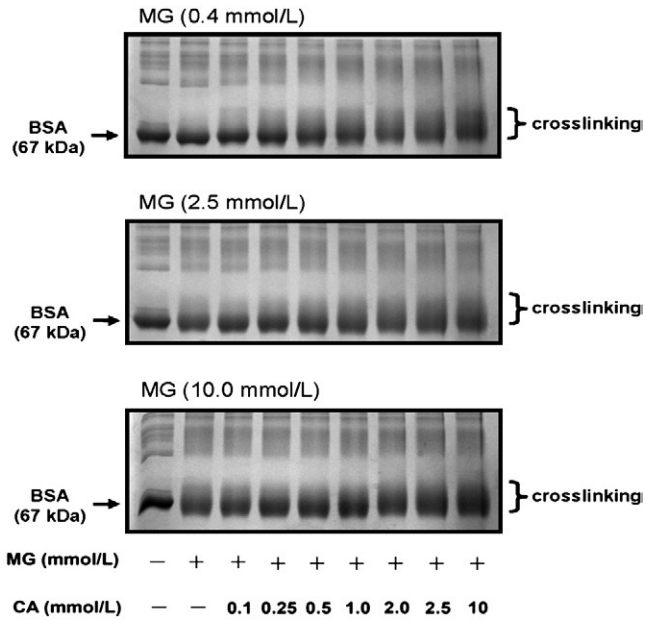

Fig. 4. Pro-glycation effect of CA in the presence of different concentrations of MG. The modulation of MG-mediated protein modification was revealed by SDS-PAGE. BSA (0.4 mg/ml) was incubated with MG under sterile conditions for 7 days with CA.

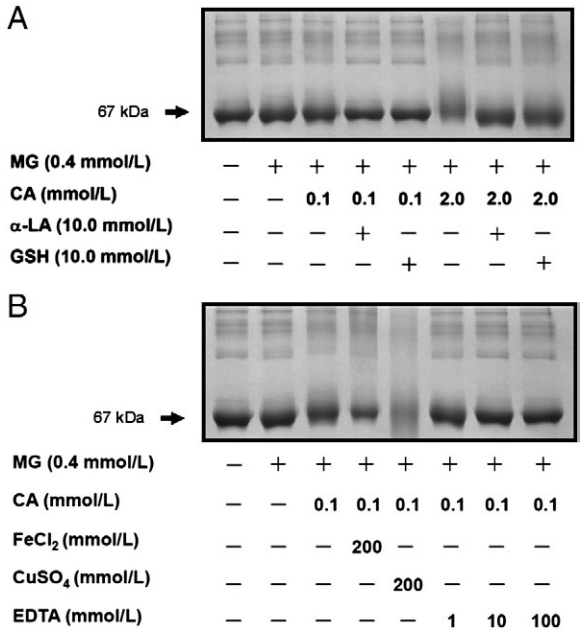

Fig. 5. Effects of antioxidants (A) and metal ions (B) on methylglyoxal-induced protein modification in the presence of CA. BSA (0.4 mg/ml) was incubated with MG and caffeic acid (CA) for 7 days in the absence or presence of the indicated concentration of α-LA, GSH, $FeCl_2$, $CuSO_4$ and EDTA. SDS-PAGE was carried out in 4–20% Tris-HCl gels. The arrow indicates the usual position of BSA (67 kDa).

reactive carbonyl species in a concentration-dependent manner. Furthermore, the pro-glycation phenomenon of CA did not change with MG concentration (0.4–10 mM) (Fig. 4), indicating that the pro-glycation effect of CA is not limited to an individual reaction at a specific concentration.

3.3. Effects of anti-oxidants and a metal ion chelator on MG/CA-mediated protein glycation

To study the roles of free radical generation and metal ions in the pro-glycation effect of CA, antioxidants and the metal chelator EDTA were added to the glycation reactions. The pro-glycation effect of CA at 0.1 mM or 2.0 mM was inhibited by the antioxidants α-LA and GSH (Fig. 5A). On the other hand, Cu^{2+} and Fe^{2+} promoted the pro-glycation effect of CA. Furthermore, the addition of the metal chelator EDTA dose-dependently inhibited these modified effects of metal ions (Fig. 5B). These results indicate that the pro-glycation effect of CA is related to its pro-oxidative property and depends upon metal ions.

3.4. Pro-oxidative effect of CA

To examine whether the pro-glycation effect of CA is linked to its pro-oxidative activity, the generation of free radicals during glycation was analyzed by electron paramagnetic resonance (EPR) using PBN as a free radical trapping agent. In this experiment, H_2O_2/Fe^{2+} (Fenton reaction) was used as a positive control for the generation of free radicals and PBN alone as the blank. As shown in the EPR spectra in Fig. 6, MG or CA alone led to the generation of ROS. However, CA promoted the generation of free radicals caused by MG. The addition of α-LA and GSH inhibited the pro-oxidative effect of CA during glycation.

3.5. Pro-atherosclerotic effects of glycated FBS proteins

In this experiment, FBS was used as a source of target proteins for glycation. MG, CA or AG was added to FBS in the reaction system and

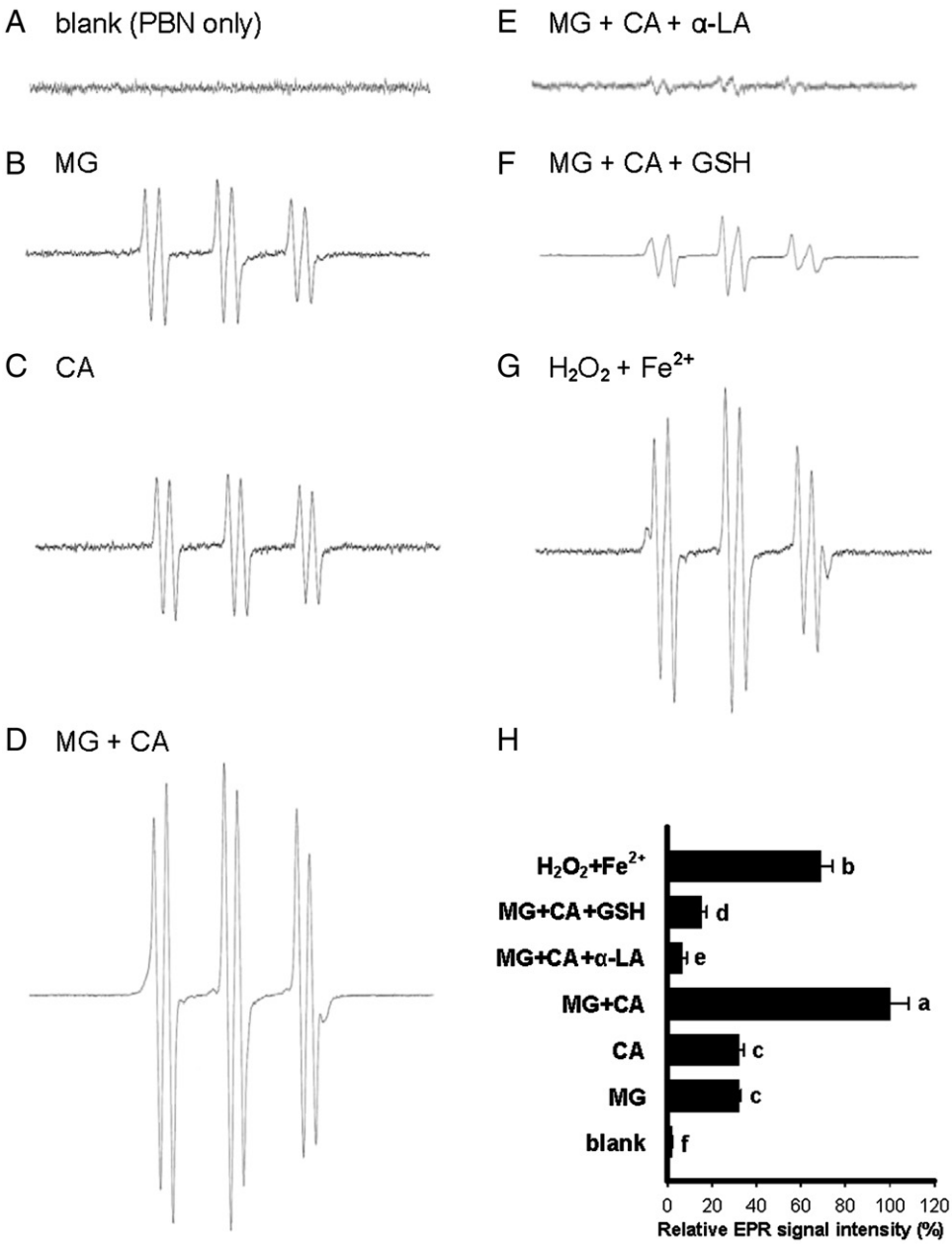

Fig. 6. EPR spectra of radical adducts detected in the reaction mixtures containing MG, CA and the indicated antioxidants in potassium phosphate buffer. Reaction and EPR conditions are described in Materials and Methods. PBN (0.2 mM) served as the free radical trapping agent. (A) Blank (PBN only). (B) MG (0.4 mM). (C) CA (0.1 mM). (D) MG (0.4 mM) and CA (0.1 mM). (E) MG (0.4 mM), CA (0.1 mM) and α-LA (10 mM). (F) MG (0.4 mM), CA (0.1 mM) and GSH (10 mM). (G) H_2O_2 (2.5 mM) and Fe^{2+} (2.5 mM). (H) The graph represents quantitative data of relative EPR signal intensity (%) as estimated by WINEPR SimFonia software (Version 1.25). Results are means±S.D. from three independent experiments. Groups with different superscripts were significantly different ($P<.05$).

incubated at 37°C for 9 days under sterile conditions. At the end of the reaction, free unbound substances were removed by dialysis. Then, endotoxins were removed using a PD-10 column. Finally, the FBS reaction mixture was passed through a 0.22-μm sterile filter to obtain the differential levels of glycated FBS. These glycated FBS samples were then added individually to cell culture media at a final concentration of 10% (v/v). The adverse effects of these glycated FBS proteins, including pro-oxidation, proinflammation and chemotactic migration, were compared in both vascular and circulating cells, such as monocytes, macrophages and human umbilical vein endothelial cells (HUVEC). The negative biological effects caused by the pro-glycation activity of CA on FBS were verified using cell models as described below.

The analysis of proinflammatory mediators showed that the FBS prepared by incubation with both MG and CA (MG/CA-FBS) significantly induced IL-1β gene expression in THP-1 monocytes (Fig. 7A) and TNF-α secretion from RAW 264.7 macrophages (Fig. 7B). When AG was added to the FBS glycation reaction, it reduced the activation of these cytokines. The results from oxidative injury measurements show that among three differentially glycated FBS samples, MG/CA-FBS induced the highest level of intracellular ROS (Fig. 8A) and caused the most severe oxidative DNA damage in HUVEC (Fig. 8B). MG/CA-FBS significantly induced the expression of genes encoding the AGEs-specific receptor RAGE and cytokines ICAM-1 and VCAM-1 after 24 h in HUVEC (Fig. 9). This effect was further confirmed in a Transwell migration assay. The results in

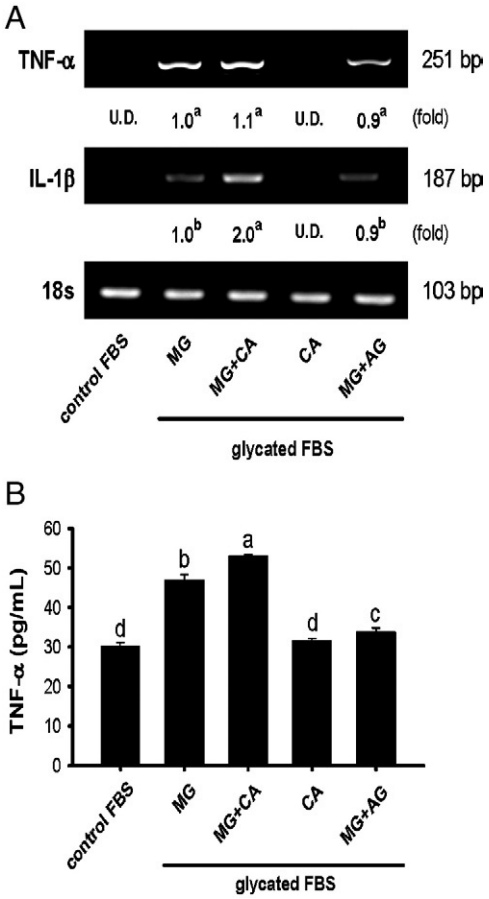

Fig. 7. Activation of proinflammatory cytokines in (A) monocytes and (B) macrophages induced by differential glycated FBS samples. (A) Monocytic THP-1 cells were incubated with normal-glucose (NG, 5.5 mM) medium supplemented either with 10% non-glycated FBS (control FBS) or glycated FBS samples for 48 h. Total RNA was isolated and analyzed for TNF-a and IL-1β mRNA expression by RT-PCR. The intensity of each gene-specific band was normalized to internal controls (18s rRNA) and the results expressed as multiples of MG-FBS induction. (B) RAW 264.7 cells treated under the same conditions were assayed for TNF-α production by ELISA as described in Materials and Methods. All appropriate controls and standards as specified by the manufacturer were used, and the data are expressed as picograms of TNF-α secretion by one million cells. Values shown are means±S.D. from three independent experiments. Groups with different superscripts were significantly different ($P<.05$). U.D., undetectable.

Fig. 10 show that the potential induction of ICAM-1 and VCAM-1 expression after a 24-h incubation of HUVEC with MG/CA-FBS induced THP-1 monocytes in the insert to penetrate the permeable membrane and migrate toward the endothelial cells in the lower compartment.

4. Discussion

Diabetes mellitus is a complex physiological metabolic disorder. Chronic poor control of blood glucose in diabetic patients can facilitate the glycation process and the accumulation of AGEs inside the body, thus triggering complications of diabetes. AGEs can be covalently linked to intracellular proteins, lipids and nucleic acids, affecting their structures and functions. This, in turn, leads to the decline of the overall function of tissues and organs, resulting in pathogenesis and aging of the body [17]. Nakamura et al. [18] have reported that the deposition of AGEs can be seen in macrophages and smooth muscle cells at the lesion sites of atherosclerosis. In addition, AGEs concentration in tissues shows a positive correlation with the severity of atherosclerotic lesions as well as the amount of plasma

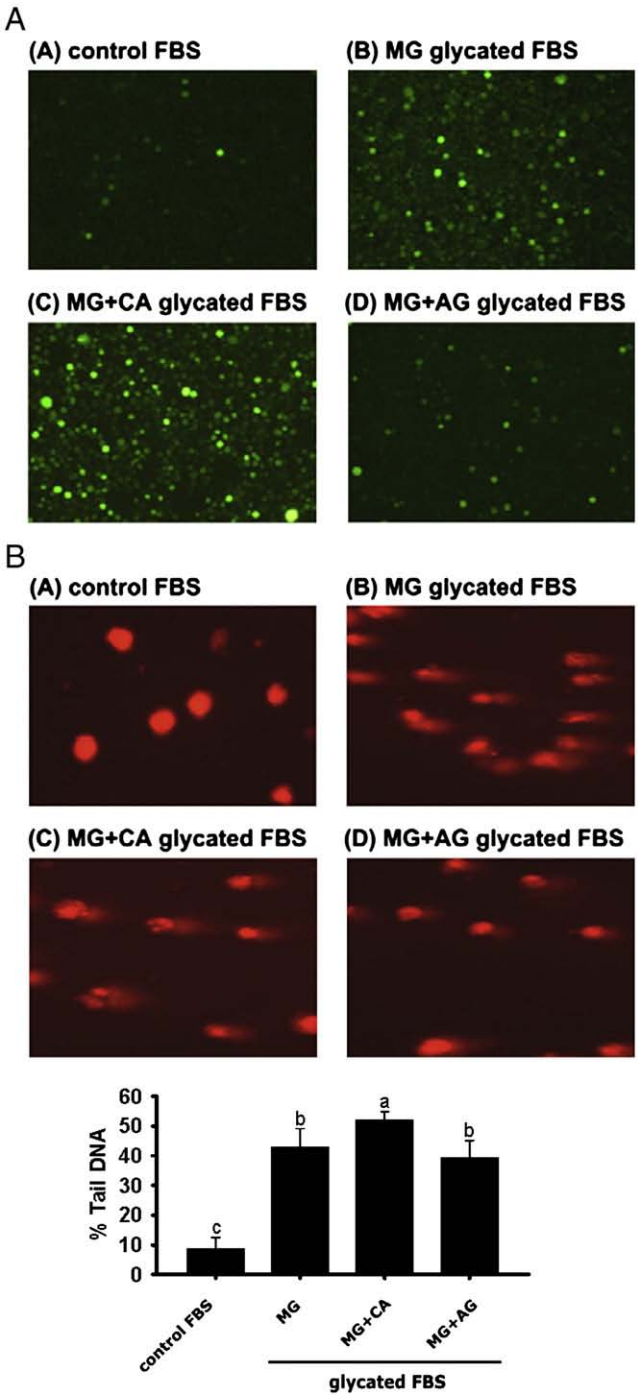

Fig. 8. Induction of oxidative injury by glycated FBS proteins. (A) Effect of glycated FBS on intracellular ROS levels in THP-1 monocytes. Cells were incubated with medium containing 10% normal FBS (A), MG-glycated FBS (B), MG/CA-glycated FBS (C) or MG/AG-glycated FBS (D) for 48 h and stained with DCFH-DA. (B) Effect of glycated FBS on DNA damage in HUVEC. Cells were incubated with medium containing 10% normal FBS or 10% glycated FBS for 72 h. Results are means±S.D. from three independent experiments. Groups with different superscripts were significantly different ($P<.05$).

proteins, lipoproteins and lipids accumulated on the vascular wall [19]. Therefore, AGEs may play an important role in the formation of atherosclerosis.

Natural polyphenols display beneficial antioxidant activities. Countless studies have revealed that polyphenols have multiple positive physiological functions [20–23]. In recent years, studies on

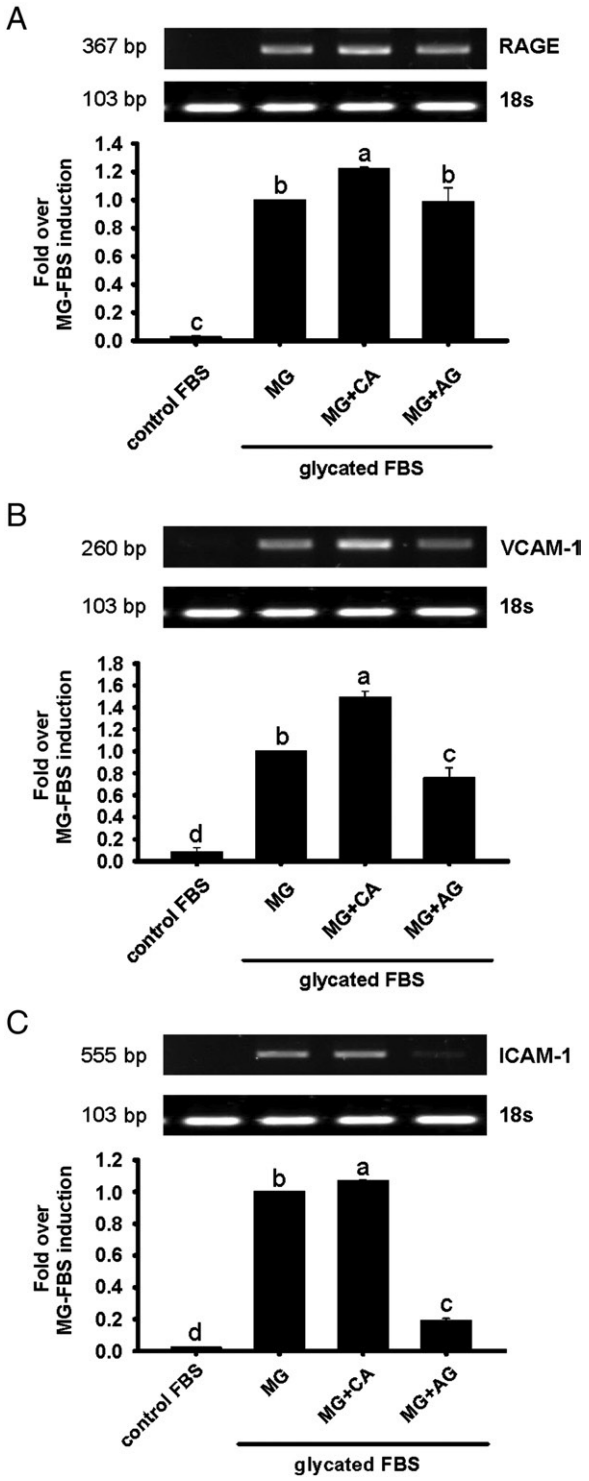

Fig. 9. Effects of glycated FBS on the mRNA expression of RAGE, VCAM-1 and ICAM-1 in HUVEC. Cells were incubated with medium containing 10% normal FBS, MG-glycated FBS, MG/CA-glycated FBS and MG/AG-glycated FBS, respectively, for 24 h. Total RNA was isolated and analyzed for RAGE (A), VCAM-1 (B) and ICAM-1 (C) mRNA expression by RT-PCR. The intensity of each gene-specific band was normalized to internal controls (18s rRNA) and the results expressed as multiples of MG-FBS induction. Results are means±S.D. from three independent experiments. Groups with different superscripts were significantly different ($P<.05$).

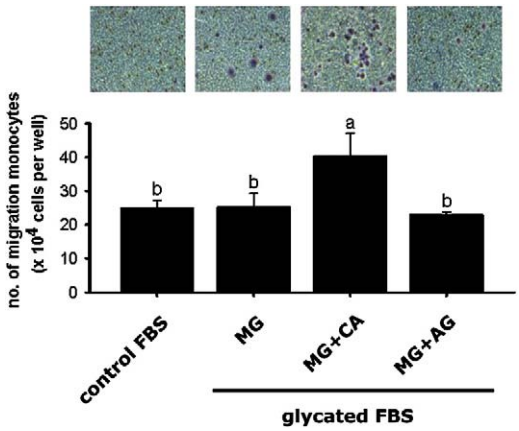

Fig. 10. Effects of glycated FBS proteins on the transmigration of monocytes toward endothelial cells. HUVEC were incubated with medium containing 10% normal FBS or 10% glycated FBS sample for 24 h in 24-well plates. The THP-1 cell suspensions were subsequently added into insert (Transwell Permeable Support) and incubated another 48 h. Upper figures show monocyte morphology on the permeable membrane of the insert. Suspensions of migrated monocytes in the bottom of the wells, which were transmigrated THP-1 cells, were collected and counted by microscope. Results are means±S.D. from three independent experiments. Groups with different superscripts were significantly different ($P<.05$).

polyphenols and their functions in glycation and the inhibition of AGEs production have also drawn broad attention. During the previous screening of a series of polyphenolic compounds in our laboratory, we found that several flavonoids exhibited the potential to inhibit the production of AGEs. Among them, luteolin, rutin and epigallocatechin gallate displayed the strongest anti-glycation effects by inhibiting protein glycation at different stages [14]. However, interestingly, a few phenolic acids showed significant pro-glycation properties instead of inhibiting protein glycation. Based on this observation, the present study used MG to induce protein glycation, using BSA and FBS as the target proteins to investigate the effects of multiple phenolic acids, such as CA, FA, *m*COA and CHA, on glycation. The detrimental effects of the pro-glycation activity of phenolic acids were also studied at the cellular level in monocytes, macrophages and vascular endothelial cells, which are all important in the pathogenesis of atherosclerosis. Notably, the present study provides some insights into the negative effects of phenolic acids on protein glycation, the induction of oxidative stress and inflammatory responses.

It is well known that serum albumin is the most abundant protein in plasma and functions as a versatile transporter with normal concentrations lying between 35–50 g/L [24]. Epidemiological studies have established that approximately 6–10% of the albumin in normal human serum is modified by nonenzymatic glycation [25]. However, this proportion typically increases between two- to three-fold in diabetic patients [26]. Based on the concept that alteration of the structure of albumin can result in impairments of its biological properties, BSA was therefore utilized as the source of target proteins for evaluating the effects of phenolic acids on glycation. The results from the present study show that FA, *m*COA and CHA had no effect on MG-induced protein glycation (Fig. 2). However, CA significantly promoted protein glycation induced by MG (Figs. 2–4). Lee et al. [27] have observed that CA isolated from *Phellinus linteus* does not inhibit glycation in a model mimicking product formation during the early, intermediate and late stages of glycation. The current study further confirmed that CA not only was unable to inhibit glycation but that is also plays a role as catalyzer during glycation. The EPR spectrum further indicated that CA promoted the generation of free radicals during glycation (Fig. 6). It has been reported that CA serves as a pro-oxidant in the presence of metal ions, causing oxidation of LDLs and peroxidation of lipids, as well as oxidative DNA damage [12,13]. Sakihama et al. [28] showed that transition metals, such as iron and copper, catalyze the oxidation-reduction cycle of phenolic compounds in the presence of oxygen, resulting in the production of ROS

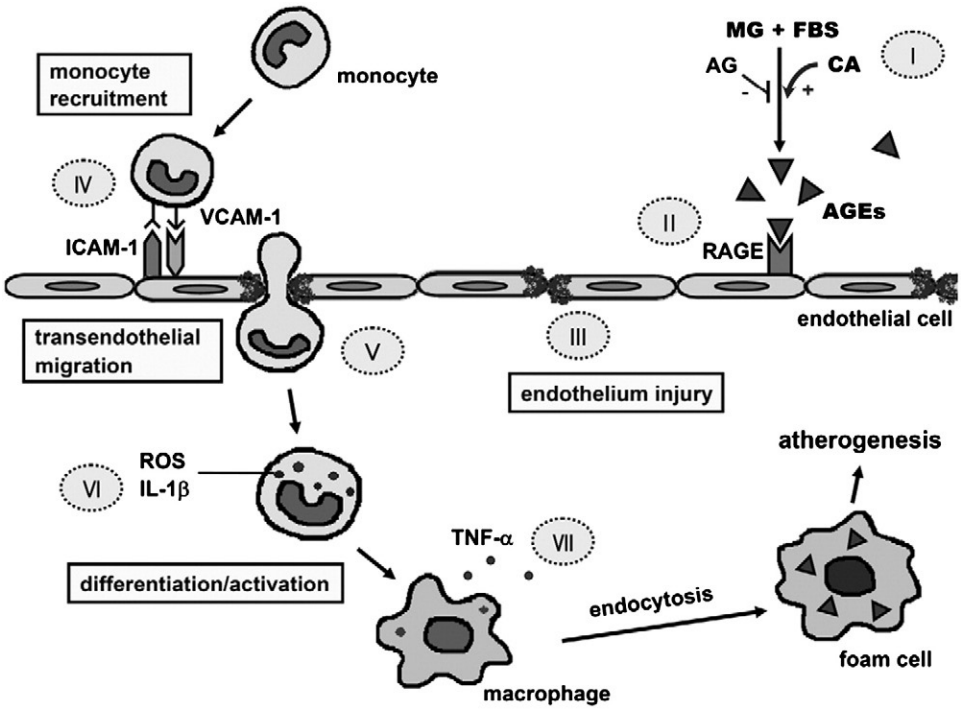

Fig. 11. CA enhances methylglyoxal-induced protein glycation and causes oxidative and inflammatory injury of monocytes, macrophages and vascular endothelial cells: proposed mechanisms in the pathogenesis of atherosclerosis. Circled letter (I) represents CA promoting protein glycation, whereas the intervention of AGE inhibitors, antioxidants or metal chelator inhibits this pro-glycation effect. The interaction of between AGEs and RAGE (II) ensues to modulate several steps of atherosclerosis, including endothelial injury (III), increasing the expression of ICAM-1 and VCAM-1 and resultant adhesion (IV), transendothelial migration of monocytes to the endothelium (V) and production of ROS and inflammatory mediators in monocytes (VI) and macrophages (VII). MG, methylglyoxal.

to damage DNA, lipids and other molecules. Therefore, we speculate that the enhanced effects of CA on protein glycation and the production of free radicals induced by MG may be related to its pro-oxidative property.

Natural phenolic acids are widely present in the diet, serving as the most abundant source of antioxidants. They can be divided into two major groups: HBAs and HCAs. Manach et al. [9] reported that the occurrence of HCAs, including *m*COA, FA, CA and CHA, is generally higher than that of HBAs. Among the HCAs selected in the current study, only CA promoted glycation. Other HCAs, such as FA, *m*COA and CHA, have a similar structure as CA, with slightly different substitution groups at different positions. Sakihama et al. [28] and Zheng et al. [13] reported that CA with *ortho*-dihydroxyl groups can dissociate into phenoxide in a reaction system in the presence of copper ions. Phenoxide chelates Cu^{2+} and reduces to Cu^{+} and a semiquinone radical anion through intramolecular electron transfer. The generated semiquinone radical anion, in turn, reacts with oxygen and undergoes electron transfer to form *ortho*-quinone and superoxide anion (O_2^-). This O_2^- can further react with Cu^{+} to produce H_2O_2 and then undergo the Fenton reaction to generate OH·leading to oxidative damage. Sakihama et al. [28] demonstrated that phenolic acids with *ortho*-dihydroxyl groups can dissociate and form phenoxyl radicals. Their pro-oxidative property is correlated with their structural resonance stability, the presence of double bonds on their side chains and the esterification of their side chains. Based on these studies, it is now known that phenolic acids with *ortho*-dihydroxyl groups exhibit pro-oxidative activity. In addition, phenolic compounds with no double bonds or esterified groups have higher pro-oxidative activity. According to these reports, it is speculated that among HCAs, CA exhibits a pro-oxidative property due to the special structure of its *ortho*-dihydroxyl group, which in turn leads to its pro-glycation effect. When the antioxidants α-LA and GSH were introduced into the reactions of this study, the pro-oxidative activity of CA was suppressed (Fig. 6). Similarly, the addition of α-LA and GSH also significantly inhibited the pro-glycation effect of CA (Fig. 5). These results suggest that oxidative stress and glycation are concurrent. The pro-oxidative activity of CA may facilitate the progress of the glycation reaction, thereby enhancing the glycative effect of MG and promoting the production of AGEs.

Atherosclerosis is a chronic inflammatory response. Many different cell types are involved in its pathogenesis. Among these cell types, monocytes, macrophages and vascular endothelial cells interact with each other to cause prolonged inflammation and amplify damage, which play a key role in the pathogenesis of atherosclerosis. Klein et al. [29] demonstrated that the glycated LDL level in the macrophages of diabetic patients is significantly higher than that of healthy people, indicating their potential to become foam cells. Sims et al. [19] also showed that the AGEs concentration in tissues has a positive correlation with the severity of damage caused by atherosclerosis. The results from these studies indicate that the accumulation of AGEs in diabetes patients plays a critical role in the pathogenesis of atherosclerosis.

In the present study, FBS was chosen as a glycation model. FBS is a required supplement for general cell culture. It is enriched in lipids, carbohydrates, electrolytes and many proteins, such as albumin, hormones, growth factors and enzymes. It is usually added to the cell culture medium as 5–10% of the final volume. In the present study, MG, CA and AG were added to FBS and incubated at 37°C for 9 days to prepare FBS samples with different degrees of glycation. These FBS samples included control FBS, MG-FBS, MG/CA-FBS and MG/AG-FBS. These FBS samples were added to the cultures of monocytes, macrophages and vascular endothelial cells to investigate their effects on inflammatory mediators expression, oxidative damage and monocyte chemotactic migration. The results show that incubation

of monocytes with glycated FBS induced the expression of TNF-α and IL-1β (Fig. 7A). The expression of IL-1β in the MG/CA-FBS group was significantly higher than in the MG-FBS group, which may have been due to the pro-glycation effect of CA. Moreover, the intracellular levels of ROS in the MG/CA-FBS group showed a significant increasing tendency, and the intracellular ROS production was also higher than in the MG-FBS group (Fig. 8). Basta et al. [7] showed that the binding of AGEs to RAGE on the surface of monocytes induced the intracellular generation of ROS, which can activate NF-κB and regulate the release of proinflammatory cytokines, to induce the transformation of monocytes into macrophages. Similarly, the secretion of TNF-α was induced in the RAW 264.7 cells incubated with glycated FBS and was more significant in the MG/CA-FBS group, with a 1.13-fold higher production of TNF-α than the MG-FBS group (Fig. 7B). Moreover, with the addition of AG in the MG/AG-FBS group, the inhibition of glycation alleviated the proinflammatory responses and reduced the release of TNF-α (Fig. 7). These results suggest that the pro-glycation effect of CA could induce cellular oxidative stress and inflammatory responses.

Previous studies have shown that inflammatory factors can induce the expression of cell adhesion molecules, such as VCAM-1 and ICAM-1, which recruit monocytes from the peripheral blood circulation to adhere to the arterial wall, with subsequent infiltration into the inner layer of the artery and differentiation into macrophages. This is a key event in the pathogenesis of atherosclerosis [30]. We used the transwell model to mimic monocyte migration to the vascular endothelium and confirmed that MG/CA-FBS could induce the expression of RAGE and adhesion molecules and promote the migration of monocytes into the endothelial cell layer (Figs. 9 and 10). We hypothesized that the pro-glycation effect of CA could accelerate the production of AGEs, whose binding to RAGE can induce DNA damage in vascular endothelial cells and the expression of adhesion molecules, thereby promoting the migration of monocytes. When stimulated by AGEs, monocytes and vascular endothelial cells can release large amounts of inflammatory factors and increase cellular oxidative stress. Macrophages can also release inflammatory mediators upon stimulation by AGEs. This vicious cycle between inflammation and oxidation caused by glycated products may explain the pathogenesis and progression of atherosclerosis in humans. The proposed mechanism is outlined in Fig. 11.

It has been reported that oxidative stress is highly correlated with the production of AGEs. Sandu et al. [31] showed that the plasma of diabetic animals on high-AGEs and high-fat diets contains high levels of AGEs and the oxidative biomarkers 8-isoprostane compared to normal animals. Yamabe et al. [32] also observed an elevated level of thiobarbituric acid–reactive substances in diabetic rats, indicating increased peroxidation of lipids. Furthermore, the increased level of AGEs induces the expression of RAGE in kidney tissue. In addition, Gupta et al. [33] found that type 2 diabetics have elevated oxidative stress, which decreases the level of GSH and increases the levels of malondialdehyde and AGEs in their serum. The results from these studies indicate that AGEs production and the increase of oxidative stress might occur in a vicious circle. The present study also confirms that the antioxidants α-LA and GSH could inhibit the pro-oxidative property of CA, thereby suppressing its pro-glycation effect.

CA is the most abundant phenolic acid in the human diet. The properties of CA described here are different from the previous opinions about phenolic acids, which were considered to have beneficial physiological activity [34]. Our findings provide a different viewpoint for the understanding of the physiological functions of phenolic acids. Based on the observations, the pro-oxidative property of CA could promote glycation and induce the expression of factors related to atherosclerosis. CA might play a negative role in the induction of chronic complications of diabetes by glycation and/or AGEs. Its in vivo effect remains to be further investigated, which is currently in progress.

Acknowledgments

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