RESEARCH ARTICLE

Dietary phenolic acids attenuate multiple stages of protein glycation and high-glucose-stimulated proinflammatory IL-1β activation by interfering with chromatin remodeling and transcription in monocytes

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This study examined the effects of dietary phenolic acids on individual stages of protein glycation and utilized monocyte cultures to assess whether these phytochemicals modulate the activation of proinflammatory cytokine under high glucose (HG, 15 mmol/L) conditions mimicking diabetes. *In vitro* glycation assays showed that a number of phenolic acids exerted inhibitory effects on the glycation reaction and its subsequent crosslinking. Phenolic acids, especially methoxyphenolic acids, prevented increase in both levels of the interleukin-1β (IL-1β) and oxidative stress caused by HG. The effect appeared to be mediated by modulation of the protein kinase C/nuclear factor-κB axis. Chromatin immunoprecipitation demonstrated for the first time that HG increased the recruitment of nuclear factor-κB p65 and CREB-binding protein to the IL-1β promoter. Interestingly, HG also increased histone acetylation and methylation within the IL-1β promoter and decreased histone deacetylase activities in monocytes, thus facilitating chromatin remodeling and transcription. Such inappropriate inflammatory responses were found to be controlled effectively by treatment with methoxyphenolic compounds. In conclusion, this study suggests that phenolic acids could exert their anti-inflammatory activities as antiglycation agents and as modifiers of signaling pathways. It provides evidence for a novel mechanism by which phenolics supplementation might have additional protective effects against diabetic complications.

Keywords:
Chromatin remodeling / Hyperglycemia / Interleukin-1β / Oxidative stress / Phenolic acid

1 Introduction

In patients with diabetes, the glycotoxin concept is an attractive hypothesis to explain at least part of the adverse effects of elevated levels of glucose and advanced glycation endproducts (AGEs). Acute exposure to hyperglycemia induces reversible oxidative stress and activation of circulating monocytes by modulating intracellular signaling

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Abbreviations: δ-Glu, δ-gluconolactone; **AGE,** advanced glycation endproduct; **AG,** aminoguanidine; **CA,** caffeic acid; **CBP,** CREB-binding protein; **CHA,** chlorogenic acid; **ChIP,** chromatin immunoprecipitation; **CML,** N^{ε}-(carboxymethyl)lysine; **DCF-DA,** 5-(and-6)-carboxy-2′,7′-dichlorodihydro-fluorescein diacetate; **ERK1/2,** extracellular signal-regulated kinase; **FA,** ferrulic acid; **FBS,** fetal bovine serum; **GA,** gallic acid; **GE,** gentisic acid; **G.K. peptide,** *N*-acetyl-glycyl-lysine methyl ester; **HAT,** histone acetyltransferase; **HBA,** hydroxybenzoic acids; **HCA,** hydroxycinnamic acids; **HDAC,** histone deacetylase; **HG,** high glucose; **HH3,** histone H3; **HH4,** histone H4; **IL-1β,** interleukin-1β; **JNK,** c-jun N-terminal kinase; **MAPK,** mitogen-activated protein kinase; **NF-κB,** nuclear factor-κB; **NG,** normal glucose; **PBMC,** peripheral blood mononuclear cell; **PCA,** protocatechuic acid; **PKC,** protein kinase C; **RAGE,** receptor for AGE; **ROS,** reactive oxygen species; **SPA,** sinapic acid; **SRA,** syringic acid; **THP-1 cells,** human THP-1 monocytic cells; **TSA,** trichostatin A; **VA,** vanillic acid

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pathways. This is followed by irreversible modifications to cellular proteins and to the vascular matrix during chronic hyperglycemia [1, 2]. The imbalance between the generation of hyperglycemia-dependent reactive oxygen species (ROS) and antioxidant defenses may further aggravate micro- and macrovasculature dysfunction in diabetes. Several mechanisms appear to be involved, such as non-enzymatic glycation, the formation of AGEs, and an increase in diacylglycerol that can activate protein kinase C (PKC) [3]. In addition, glucose can auto-oxidize to form hydrogen peroxide and ketoaldehydes in the presence of transition metal ions [4, 5], subsequently accelerating AGEs formation and oxidative DNA damage [6]. It is thus likely that hyperglycemia causes vascular pathologies through multiple mechanisms and pathways.

Clinical and experimental observations have revealed the central mechanistic relevance of several cytokine and chemokine networks in atherosclerosis [7]. Both type 1 and type 2 diabetes induce significant formation of AGEs and lipid peroxidation products, which are specifically recognized by inflammatory cells in atherosclerotic plaques and induce the release of proinflammatory cytokines [1, 8]. In the early pathogenesis of atherosclerosis, monocytes may be activated by hyperglycemia, AGEs, and/or other oxidant factors. These activated cells can adhere to the surface of damaged endothelial cells and then migrate into the subendothelial space and differentiate into macrophages, where they secrete a variety of proinflammatory cytokines *in situ*. Natarajan and coworkers [9, 10] have reported that high glucose (HG) and AGEs are able to stimulate the production of significant amounts of superoxide anion (O_2^-) and proinflammatory mediators. These inflammatory responses can modulate the activity of proteins such as PKC and/or the mitogen-activated protein kinases (MAPKs). These, in turn, control the activation of the transcription factor (nuclear factor-κB) NF-κB, thereby influencing the synthesis and expression of inflammatory factors. Antioxidants such as α-tocopherol [11], flavonoids [12], or AGEs inhibitors [13] inhibit the aforementioned production of ROS and cytokines and thus postpone inflammation.

Since glycotoxins contribute to diabetic pathology through an increase in oxidant stress, a supplement of antioxidants in response to protein glycation should be a theoretical strategy for preventing diabetic complications [3, 14]. This hypothesis is supported by clinical studies, which indicated that the development of type II diabetes might be reduced by antioxidant supplements [15]. Phenolic acids, especially hydroxybenzoic acids (HBA) and hydroxycinnamic acids (HCA), are plant secondary metabolites that are commonly found in plant-derived foodstuffs. Many studies have suggested that these plant phenolics can relieve oxidative damage and protect from photooxidation. Previously, our group reported that phenolic acids showed biological activity of protection against inflammatory responses and oxidative damage *in vivo* and *in vitro* [16, 17]. In addition, vanillic acid (VA) was found to exert an inhibitory effect on reactive carbonyl species-mediated intracellular AGEs formation in neurocytes [18].

Although the antioxidant properties of phenolic acids have been well documented, little is known about either their antiglycation potential or the molecular basis and nuclear chromatin events involved in their inhibition of elevated inflammatory responses during diabetes-associated hyperglycemia. Thus, the aim of this study was to identify the candidate inhibitor of protein glycation by using several *in vitro* screening models. Meanwhile, since circulating monocytes are continuously exposed to hyperglycemic conditions in diabetes. Vascular cells, especially monocytes, can be activated by elevated levels of glucose, which contribute to inflammatory cytokine-dependent networks in the blood stream by oxidative stress, production of proinflammatory cytokines, response to these potent cell activators, and cytokine-mediated interactions with cells within the vessel wall, such as endothelial and smooth muscle cells. Thus, vascular cells are important in orchestrating these inflammatory effects. In this study, we examined whether dietary phenolic acids influence hyperglycemia-driven inflammatory responses in monocytes. We observed that methoxyphenolic acids effectively prevented proinflammatory interleukin-1β (IL-1β) cytokine activation under hyperglycemic conditions. Notably, our results demonstrated that these phenolic compounds, especially methoxyphenolic acids, decreased *in vivo* recruitment of NF-κB p65 and CREB-binding protein (CBP), as well as decreasing histone acetylation and methylation at the promoter of IL-1β, and consequently interfered with chromatin remodeling and transcription.

2 Materials and methods

2.1 Materials

HBA: *p*-hydroxybenzoic acid (*p*-HA), gallic acid (GA), gentisic acid (GE), protocatechuic acid (PCA), syringic acid (SRA), VA; HCA: caffeic acid (CA), chlorogenic acid (CHA), *m*-coumaric acid, *p*-coumaric acid, ferrulic acid (FA), sinapic acid (SPA), and *N*-acetyl-glycyl-lysine methyl ester (G.K.) peptide, aminoguanidine (AG), BSA (fraction V, essentially fatty acid free), D-glusose, δ-gluconolactone (δ-Glu), and ribose were purchased from Sigma Chemical (St. Louis, MO, USA). Anti-N^{ε}-carboxymethyllysine (CML) mAb (6D12) was purchased from Trans Genic (Kumamoto, Japan). Antibodies to extracellular signal-regulated kinase 1/2 (ERK1/2), phospho-ERK1/2, p38, phospho-p38 (pp38), PKC, p47phox, and β-actin were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-p65, anti-CREB binding protein (CBP), anti-acetyl-histone H3 (acetyl-HH3, recognizes Lys 9 and 14), anti-acetyl-histone H4 (acetyl-HH4, recognizes Lys 4, 7, 11, and 15), and anti-methylated HH3 at Arg 17 antibodies (methyl-HH3R17) were obtained from Upstate Biotechnology (Lake Placid, NY, USA).

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Pharmacological inhibitors specific for p38 MAPK (SB2035800), ERK1/2 (PD98059), c-jun N-terminal kinase 1/2 (JNK1/2) (SP600125), PKC (GF109203X), NADPH oxidase (apocynin), and NF-κB (BAY117085) were obtained from Biosource (Camarillo, CA, USA). Trizol™ RNA isolation kit was obtained from Life Technologies (Rockville, MD, USA). All the chemicals and solvents used were of analytical grade.

2.2 The effects of phenolic acids on protein glycation and crosslinking (an estimation of antiglycation)

2.2.1 Hemoglobin-δ-Glu assay

Evaluation of early stage of protein glycation was determined with a δ-Glu assay [19]. This method is specifically designed for the investigation of inhibitors on Amadori products formation as evidenced by lowering HbA_{1C} levels. Briefly, samples were prepared by mixing 200 μL of fresh human blood with 40 μL of PBS (pH 7.4) as the negative control or 40 μL of δ-Glu (50 mM) as the δ-Glu control. The test samples each contained 200 μL of blood plus 40 μL of δ-Glu and 10 μL of the indicated phenolic acids at a final concentration of 1 mM. After incubation at 37°C for 16 h, the percentage of glycated hemoglobin present was determined using a dedicated ion-exchange HPLC system (BIORAD DIASTAT). The blood samples were analyzed in triplicate. The percent inhibition of HbA_{1C} formation was calculated as [(HbA_{1C} of the δ-Glu control−HbA_{1C} of the test group)/(HbA_{1C} of the δ-Glu control−HbA_{1C} of the negative control)] × 100. AG was used at a final concentration of 10 mM as a positive control [20].

2.2.2 BSA-glucose assay

The assay evaluated the ability of the specified phenolic acids to inhibit glucose-mediated protein glycation, and the development of fluorescence of BSA was measurement according to the method of Rahbar *et al.* [19] and Wu and Yen [21]. Briefly, BSA (50 mg/mL), as a model protein for non-enzymatic glycation, was incubated with 0.8 M glucose at 37°C for 7 days in 1.5 M phosphate buffer (pH 7.4) under sterile conditions. In certain experiments, the indicated phenolic acids were added to the model system at a concentration of 1 mM. The fluorescence of the samples was measured at the excitation and emission wavelengths of 330 and 410 nm, respectively, *versus* an unincubated blank containing the protein, glucose, and the inhibitors. The percent inhibition of AGE formation was calculated as [1−(fluorescence of the test group/fluorescence of the control group)] × 100. AG, an inhibitor of AGEs formation [20], was used as a positive control.

2.2.3 G.K. peptide-ribose assay

This test was used to evaluate the ability of phenolic acids to inhibit the crosslinking of G.K. peptide (late glycation products) in the presence of ribose using the method described by Nagaraj *et al.* [22] and Rahbar *et al.* [19]. G.K. peptide (80 mg/mL) was incubated with 0.8 M ribose under sterile conditions in 0.5 M sodium phosphate buffer (pH 7.4) at 37°C for 24 h. The phenolic acids were added to a final concentration of 1 mM. At the end of the incubation period, samples were analyzed for specific fluorescence (excitation, 340 nm; emission, 420 nm). The percent inhibition by different concentrations of inhibitor was calculated as described above.

2.3 Cell culture and treatments

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 glucose-free medium 1640 (Gibco BRL, Grand Island, NY, 11879-020) supplemented with 10% fetal bovine serum (FBS), HEPES (10 mM), streptomycin/penicillin (100 μg/mL/100 U/mL), 50 μM β-mercaptoethanol, and either 5.5 mM D-glucose (normal glucose, NG) or 15 mM D-glucose (HG) in 5% CO_2 at 37°C for 48 or 72 h. Phenolic acids (prepared in DMSO) were added to cells with both the NG and HG medium. Control cells received vehicle only (<0.1% DMSO). The cell viability was determined by a Trypan blue dye exclusion assay.

2.4 RNA preparation and RT-PCR

Total RNA was prepared from NG- or HG-treated THP-1 cells (1×10^6 cells/mL) with a Trizol RNA isolation kit (Rockville) as described in the manufacturer's manual. The primers used to amplify IL-1β cDNA were forward (5′-CTCTCTCACCTCTCCTACTCAC-3′) and reverse (5′-ACACTGCTACTTCTTGCCCC-3′); and those used for β-actin were forward (5′-ACAAAACCTAACTTGCGCAG-3′) and reverse (5′-TCCTGTAACAACGCATCTCA-3′). From each sample, cDNA corresponding to 1 μg of RNA was reverse-transcribed, using 200 U of Superscript II reverse transcriptase, 20 U of RNase inhibitor, 0.6 mM dNTP, and 0.5 μg/μL oligo(dT). PCR analyses were performed on the cDNA preparations to detect IL-1β and β-actin (as an internal standard) gene expression using a P × 2 Thermal cycler (Thermal Electron, Madison, WI, USA). The reactions were performed in a volume of 50 μL containing (final concentrations) 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 2 U of Taq DNA polymerase, and 50 pmol of both the 5′ and 3′ primers. After initial denaturation for 2 min at 95°C, 29–35 cycles of amplification (the

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annealing temperature for IL-1β was 56°C) were performed, followed by 10 min of final extension at 72°C.

2.5 Analysis of PCR products

A 10-μL aliquot from each PCR reaction was electrophoresed on a 1.8% agarose gel containing 0.2 μg/mL ethidium bromide. The gel was then photographed under UV transillumination. For quantification, the PCR bands on the photograph of the gel were scanned using a densitometer linked to a computer analysis system. The results were expressed as fold stimulation over HG after normalizing the gene signal relative to the corresponding β-actin from each sample.

2.6 Western blotting

The protein fractions were isolated from THP-1 cells (1×10^6 cells/mL) after the treatment with individual phenolic acid for 48 or 72 h. The total proteins were extracted by the Total Protein Extraction Kit (Millipore, Bedford, MA, USA), and the cytosolic, nuclear, and membrane fraction proteins were extracted by Compartmental Protein Extraction Kit (Millipore) following the manufacturer's instructions, respectively. Protein concentration was measured by Bradford assay using BSA as a standard. Total protein and compartmental protein extracts (20–50 μg of protein) were separated on 8% SDS-PAGE for PKC detection, and 12% SDS-PAGE for CML (6D12) protein detection, and then transferred to Immobilon polyvinylidene difluoride membrane (Millipore) with transfer buffer composed of 25 mM Tris-HCl (pH 8.9), 192 mM glycine, and 20% methanol. The membrane was blocked in StartingBlockTM Blocking Buffers (Pierce, Rockford, IL, USA) for 15 min at room temperature and then incubated overnight at 4°C with indicated primary antibodies (1:1000 dilutions). After hybridization with primary antibodies, the membrane was washed with TBS containing Tween-20 three times, incubated with HRP-labeled secondary antibody for 45 min at room temperature, and washed with TBS containing Tween-20 three times. Final detection was performed with ECL (Enhance Chemiluminescence) Western blotting reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK). The relative expression of proteins was quantified densitometrically using the software LabWorks 4.5 (Cambridge, UK) and calculated according to the reference bands of loading control.

2.7 Cyotokine ELISA assay

THP-1 cells were incubated in six-well tissue culture plates in RPMI 1640 medium with 0.2% BSA. Cells were treated with individual phenolic acids under HG conditions for 72 h. The supernatant was then harvested and assayed for IL-1β protein secretion using a specific ELISA kit according to the manufacturer's instructions (Pierce Endogen, Rockford, IL, USA). The pure medium (without cells) was incubated under the same conditions and was used as a blank control for the ELISA.

2.8 Intracellular ROS production assay

Intracellular ROS generation was detected using a fluorescent probe, 5-(and-6)-carboxy-2′,7′-dichlorodihydro-fluorescein diacetate (DCF-DA, Molecular Probes, Eugene, OR, USA). DCF-DA readily diffuses through the cell membrane and is enzymatically hydrolyzed by intracellular esterases to form non-fluorescent DCFH, which is then rapidly oxidized to form highly fluorescent DCF in the presence of ROS. The DCF fluorescence intensity is believed to parallel the amount of ROS formed intracellularly. At the end of incubation, cells (10^6 cells/mL) were collected and resuspended in PBS. An aliquot of the suspension (195 μL) was loaded into a 96-well plate, and then 5 μL of DCF-DA was added (final concentration 20 μM). The DCF fluorescence intensity was detected at different time intervals using a FLUOstar galaxy fluorescence plate reader (BMG Labtechnologies, Offenburg, Germany) with an excitation wavelength at 485 nm and emission wavelength at 530 nm.

2.9 Determination of the levels of extracellular glycation

The levels of extracellular glycation have been determined by affinity chromatography on phenylboronate columns, as described by the manufacturer (Pierce Chemicals, Rockford, IL, USA), using A280 absorption spectroscopy to monitor the elution. Aliquots of FBS were incubated with 5.5 mmol/L (NG) or 30 mmol/L (HG) glucose at 37°C for 72 h. The reaction mixture was then dialyzed extensively against 150 mM NaCl to remove unreacted glucose. Further aliquots of FBS were processed in the same way, but without glucose (control FBS). The loading amount of experimental FBS into affinity column was 1 mg of proteins. The AGEs formation in experimental FBS was analyzed for AGEs-related fluorescence (Ex_{360} and Em_{415}).

2.10 Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed using the EZ-ChIP™ Chromatin Immunoprecipitation Kit (Millipore) following the manufacturer's instructions. Briefly, THP-1 cells at a cell concentration of 1×10^7/mL were cross-linked with 1% formaldehyde for 30–60 min, washed twice with cold PBS, resuspended in SDS Lysis Buffer (containing 1X Protease Inhibitor Cocktail II), and sheared with four to five sets of 10-s pulses on wet ice using a Misonix sonicator ultrasonic processor (model XL2020) set to 30% of maximum power. This gave the

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appropriate length DNA fragments (200–1000 bp). This was followed by centrifugation at 12 000 × *g* for 10 min to remove insoluble material. One-tenth of the total lysate was used for total genomic DNA as "Input DNA" control. Dilution buffer (900 μL) containing Protease Inhibitor Cocktail II was added to each tube containing 100 μL of sheared, crosslinked chromatin. This was followed by immunoclearing with 60 μL of Protein G Agarose for 1 h at 4°C. Immunoprecipitation was performed overnight at 4°C with 5–10 μg each of the specified antibodies. Precipitates were washed by resuspending the beads in 1 mL each of the following cold buffers: Low Salt Immune Complex Wash Buffer, High Salt Immune Complex Wash Buffer, and LiCl Immune Complex Wash Buffer. Precipitates were then washed twice with TE Buffer and extracted twice with 1% SDS containing 0.1 M $NaHCO_3$. Elutes were then pooled (total volume = 200 μL) and heated at 65°C for 4–5 h to reverse the crosslinks of the protein/DNA complexes to free the DNA. DNA fragments were purified with Spin Columns. For the PCR, 2 μL of the DNA sample was used. PCR primers correspond to sequences within the promoter regions as follows: IL-1β, forward (5′-cactcttc-cactccctcc-3′) and reverse (5′-agcctcaaacccttcctc-3′).

2.11 Statistical analysis

Each experiment was performed in triplicate. The results are expressed as the means ± SD. Statistical comparisons were made by one-way ANOVA, followed by a Duncan multiple-comparison test. Differences were considered significant when the *p*-values were <0.05.

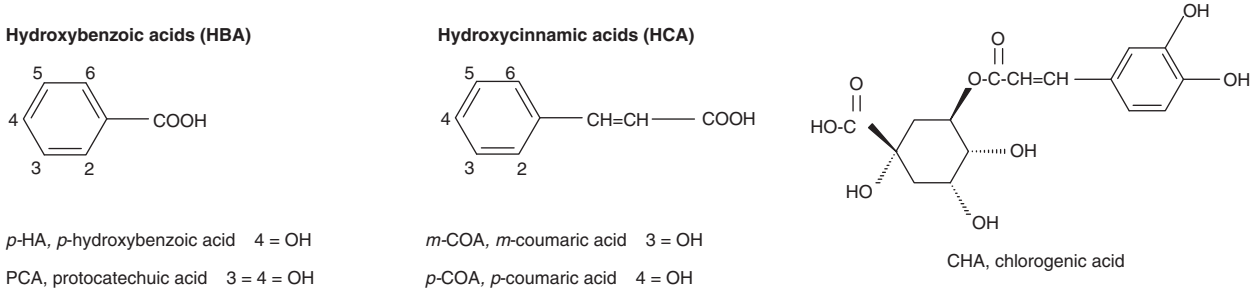
3 Results

3.1 Effect of phenolic acids on individual stages of protein glycation

The results are presented in two categories, HBA and HCA, and the chemical structures of these phenolic acids are shown in Fig. 1. Three assays were used to evaluate the antiglycation effects of these phenolics including: (i) the δ-Glu assay based on inhibition of the Amadori products (HbA_{1c} levels) generated during early stage of protein glycation; (ii) the BSA-glucose assay for AGEs-specific fluorescence generated in the course of glycation; and (iii) the G.K. peptide-ribose assay on inhibition of late glycation products and protein-AGEs crosslinking. In the δ-Glu assay, co-incubation of human hemoglobin with δ-Glu increased the glycated hemoglobin (HbA_{1C}) twofold over the baseline control (5.1%). Both HBA and HCA have moderate inhibitory effects on HbA_{1C} formation (Fig. 2, left panel). For the BSA-glucose assay, BSA was chosen as the model protein, and glucose was utilized as a glycating agent. The products were measured for their intrinsic fluorescence after an incubation period of seven days. As shown in Fig. 2 (middle panel), the various phenolic acid treatments had differing levels of AGEs-specific fluorescence, depending on their inhibitory potencies. The HBA compounds GA (41.9%) and VA (57.5%) and the HCA compounds CHA (37.0%) and FA (31.5%) had the most significant inhibitory activity on glycation ($p<0.05$), indicating that these phenolic acids were effective in the prevention of glucose-mediated protein modification. In the G.K. peptide-ribose assay, a synthetic peptide (G.K. peptide) containing a lysine residue was incubated with ribose for 24 h. This procedure is expected to generate peptides with AGEs that dimerize through lysine–lysine crosslinking [22], which increases the formation of late glycation products [19]. The results (Fig. 2, right panel) show that CHA (60.9%) and the methoxylated phenolic acids such as VA (38.5%), SRA (63.1%), FA (50.3%), and SPA (62.1%) were found to be potent inhibitors of both AGEs formation and the subsequent crosslinking of proteins.

3.2 Effect of phenolic acids on HG-induced IL-1β expression in monocytes

Human monocytic THP-1 cells were incubated in either normoglycemic culture (5.5 mmol/L, NG) or hyperglycemic



p-HA, *p*-hydroxybenzoic acid 4 = OH

PCA, protocatechuic acid 3 = 4 = OH

GE, gentisic acid 3 = 6 = OH

GA, gallic acid 3 = 4 = 5 = OH

VA, vanillic acid 3 = OCH_3, 4 = OH

SRA, syringic acid 3 = 5 = OCH_3, 4 = OH

m-COA, *m*-coumaric acid 3 = OH

p-COA, *p*-coumaric acid 4 = OH

CA, caffeic acid 3 = 4 = OH

FA, ferulic acid 3 = OCH_3, 4 = OH

SPA, sinapic acid 3 = 5 = OCH_3, 4 = OH

Figure 1. Chemical structures of phenolic acids. HBA, hydroxybenzoic acids; HCA, hydroxycinnamic acids.

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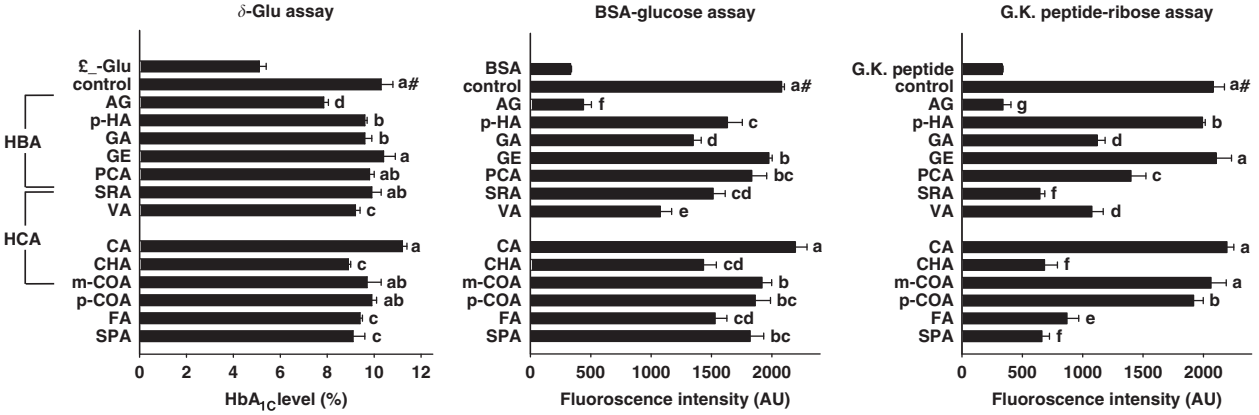

Figure 2. Effects of phenolic acids (HBA and HCA) on individual stages of protein glycation as determined by the development of fluorescence of glycated proteins using the δ-Glu assay (early stage, left panel), BSA-glucose assay (middle panel), and G.K. peptide-ribose assay (late stage, right panel). AG was used as a positive control. Phenolic acids were added at 1 mM final concentrations. The fluorescence of each sample was measured at excitation 330 nm and emission 420 nm *versus* an unincubated blank containing the protein, glycating agents, and inhibitors. Data are the means ± SD for $n = 6$. ♯, $p < 0.01$ compared with δ-Glu, BSA, or G.K. peptide alone (unglycated). Groups with different-letter superscripts are significantly different from each other in individual assays ($p < 0.05$).

culture (15 mmol/L, HG), either with or without phenolic acids, for 72 h. Total RNA was extracted, and the relative changes in mRNA levels in the phenolic acid-treated cells under HG conditions were calculated as fold inductions over HG-treated cells alone (1.0) after normalization to the β-actin level (used as an internal standard). Under both NG and HG conditions, THP-1 monocytes treated with 10–50 μM concentrations of the individual phenolic acids showed no signs of cytotoxicity (cell viability > 95%, data not shown), as determined by Trypan blue dye exclusion tests with a light microscope.

Among the HBA group, SRA and VA exhibited the most potent inhibitory effects on HG-induced IL-1β mRNA expression and protein secretion, followed by PCA and GA, but the GE and *p*-HA groups remained unchanged compared to HG controls ($p > 0.05$, Fig. 3A). Among the HCA group, CHA, FA, and SPA also showed a significant reduction in the mRNA level of IL-1β (Fig. 3B, left panel). This was similar to the pattern of inhibition of IL-1β secretion (Fig. 3B, right panel). Our data indicate that phenolic acids can reduce both IL-1β gene expression and protein levels. Additionally, the addition of 9.5 mM mannitol to the NG culture did not result in increased IL-1β secretion (data not shown), suggesting that the glucose-induced increases were not an osmotic effect. Having also observed that AG, a well-known glycation inhibitor, exhibited suppression of the corresponding proinflammatory cytokine as well (Supporting Information Fig. S1). Overall, the phenolic acid treatments done at 50 μM were capable of abolishing significant protein release of IL-1β, and so all subsequent experiments involving phenolic compounds were performed using a concentration of 50 μM.

3.3 Effect of phenolic acids on HG-induced oxidative stress in monocytes

We next examined whether phenolic acids inhibit the formation of free radicals and AGEs in THP-1 cells under HG conditions. Anti-CML (the main glycoxidative AGEs *in vivo*) antibody (6D12) and DCF-DA staining were used to detect the intracellular formation of ROS and AGEs. Figures 4A and B, upper panel, show a representative immunoblot probed with antibodies to CML. The same blot was stripped and re-probed with β-actin antibody to serve as a loading control, as shown in the lower panel. THP-1 monocytes cultured under HG conditions showed a striking increase in the basal levels of CML (Fig. 4A, lanes 1 and 2) and ROS (Fig. 4B, bars 1 and 2) formation compared with the NG control ($p < 0.05$). Furthermore, the production of ROS and AGEs caused by HG were differently inhibited by the treatment of phenolic acids ($p < 0.05$, Fig. 4). Generally, methoxyphenolic acids such as SRA, VA, FA, and SPA tended to have a significantly greater inhibitory effect against ROS and AGEs formation among the compounds tested.

Since AGEs induction of ROS generation and NF-κB-dependent gene expression is known to be mediated by ligand engagement of the receptor for AGE (RAGE). The levels of extracellular glycation were analyzed by affinity chromatography on phenylboronate column, using A_{280} absorption spectroscopy and fluorescence spectroscopy (Ex_{360} and Em_{415}). FBS was incubated with NG or HG at 37°C for 72 h similarly to the cell cultured conditions. The loading amount of experimental FBS into affinity column was 1 mg of protein. The yield of protein eluted from the phenylboronate column in HG-incubated FBS was 73 ± 29 μg (eluted/total = 7.3%). Similar analysis of control (non-glucose) or NG-incubated FBS

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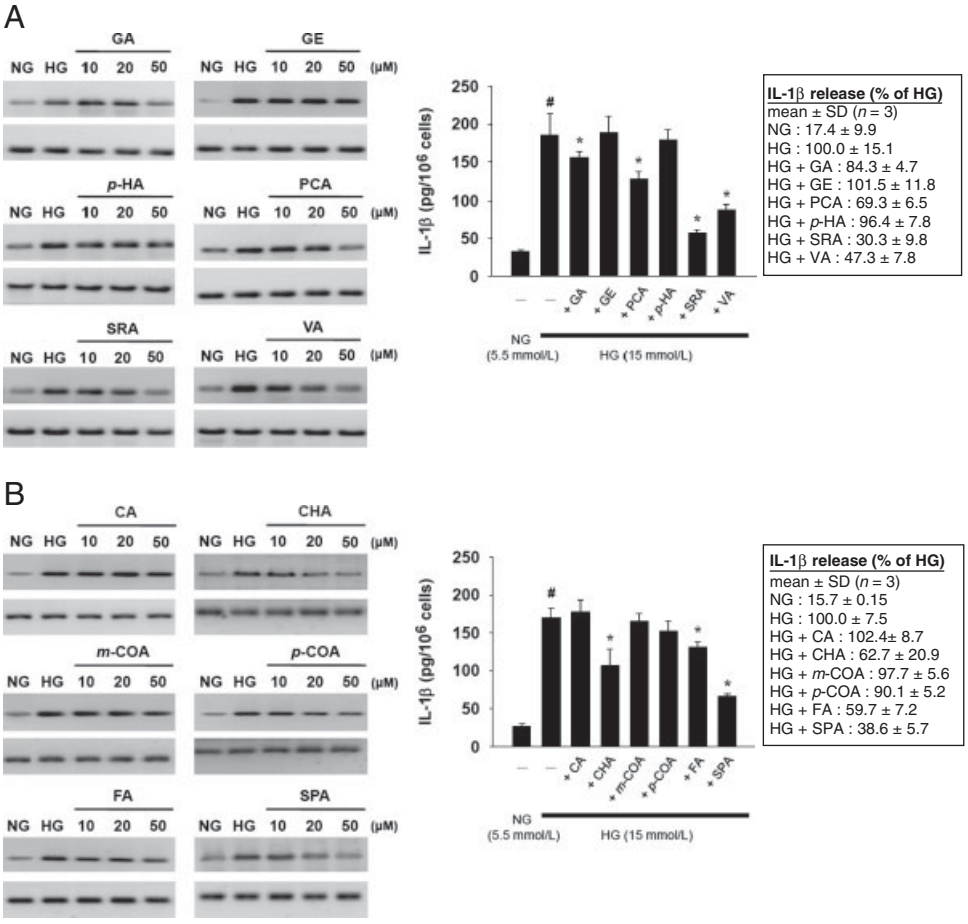

Figure 3. Effects of HBA (A) and HCA (B) on HG-induced mRNA expression and protein secretion of IL-1β in human monocytic cells. THP-1 cells were cultured in HG (15 mmol/L) with or without the indicated phenolic acids for 72 h. After treatment, relative RT-PCRs were performed with total RNA isolated from these cells using gene-specific primers for IL-1β. PCR products were analyzed with 1.8% agarose gel electrophoresis (A and B, left panel). The bar graph shows IL-1β release in the conditioned medium as measured by ELISA (A and B, right panel). Data are the means ± SD from three independent experiments, and asterisks denote significant differences. ♯, $p<0.05$ *versus* NG control. *, $p<0.05$ *versus* HG control. HG, high glucose; NG, normal glucose.

showed that about 5% of protein bound to the phenylboronate column (Supporting Information Fig. S2). However, there was no statistically significant ($p>0.05$) between NG- and HG-incubated FBS with respect to glycation levels. The result indicated that the occurrence of glycated protein in conditioned media was rare and the possible participation of RAGE might be excluded.

3.4 Identification of signaling pathways involved in HG-induced IL-1β secretion in monocytes

To determine which signaling mechanism is responsible for the upregulation of IL-1β expression, we examined the effects of pharmacological inhibitors on IL-1β secretion in THP-1 monocytes under HG conditions. Cells were exposed to the indicated inhibitors, and the cytokine levels in the conditioned media were measured by ELISA. As evidenced in Fig. 5A, HG-induced IL-1β secretion was significantly abrogated by inhibitors specific for SB203580, PD98059, GF109203X, apocynin and BAY117085 ($p<0.05$), whereas treatment with SP600125 (a specific inhibitor of JNK) did not affect these events ($p>0.05$). Moreover, the phosphorylation of the MAPKs p38 and ERK, which normally indicates activation, were decreased by a PKC inhibitor treatment in a dose-dependent manner (Fig. 5B, $p<0.05$). These data demonstrated that one important pathway for HG-induced IL-1β release is PKC signaling.

3.5 Effect of phenolic acids on HG-induced PKC and p47phox activation in monocytes

The activation of PKC and/or PKC-driven p47phox by hyperglycemia is implicated in the elevated levels of oxidative stress [1, 11] and inflammatory responses [9, 10, 23]. Figure 6 shows that phenolic acid treatment inhibited PKC translocation to membranes in the following order of potency: SRA > VA > PCA > GA for HBA (Fig. 6A, top panel); and SPA > CHA > FA > *p*-coumaric acid > *m*-coumaric acid for HCA (Fig. 6B, top panel). However, no significant effects were found in GE, *p*-HA, or CA-treated cells ($p>0.05$). A similar pattern was also found in the inhibition of p47phox translocation by these phenolic acids (Figs. 6A and B, lower panel). Among the phenolic acids tested, the greatest inhibition of protein translocation was observed under treatment with the methoxyphenolic compounds.

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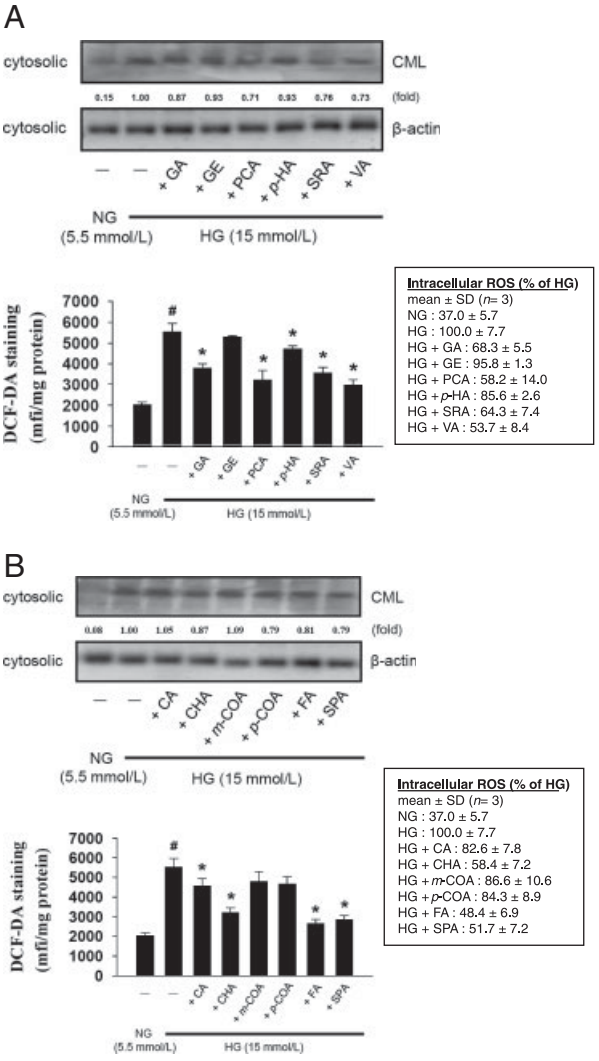

Figure 4. Effects of HBA (A) and HCA (B) on HG-induced oxidative stress in human monocytic cells. THP-1 cells were cultured in HG (15 mmol/L) with or without the indicated phenolic acids for 72 h. Whole-cell lysates were subjected to Western blot analysis using either an anti-CML antibody (6D12) or an anti-β-actin antibody as loading control (A and B, upper panel). The extent of protein expression was expressed as multiples of HG alone. ROS production was quantified using mean fluorescent intensities with the fluorescent probe DCF-DA (A and B, bottom panel) as described in the Section 2. Data shown are means ± SD from three independent experiments, and asterisks denote significant differences: ♯, $p<0.05$ *versus* NG control. *$p<0.05$ *versus* HG control. MFI, mean fluorescent intensities.

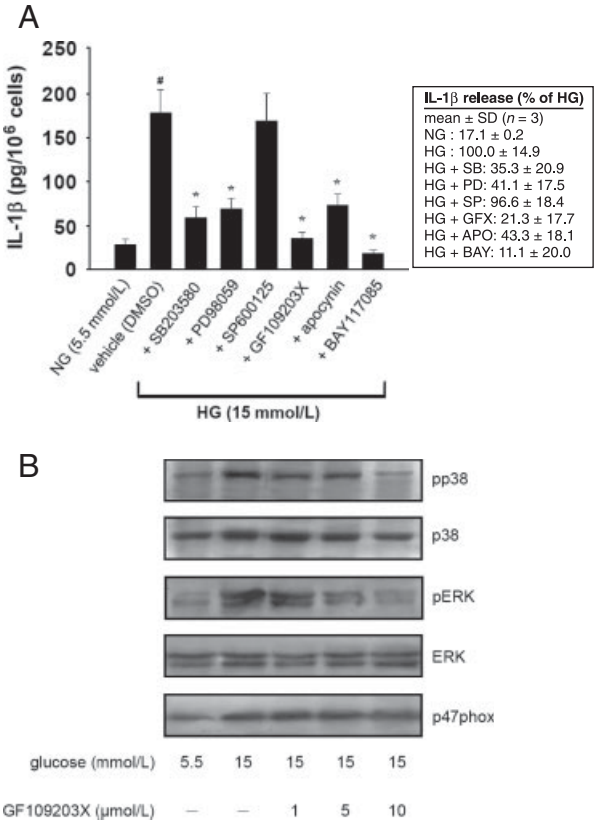

Figure 5. Effects of specific inhibitors on IL-1β protein secretion (A) and MAPK phosphorylation (B) in human monocytic cells under HG conditions. (A) THP-1 cells were pretreated with or without the inhibitors of the MAPK p38 (SB203580, SB, 5 μmol/L), ERK1/2 (PD98059, PD, 40 μmol/L), JNK1/2 (SP600125, SP, 20 μmol/L), PKC (GF109203X, GFX, 10 μmol/L), NADPH oxidase (apocynin, APO, 30 μmol/L), or NF-κB (BAY117085, BAY, 10 μmol/L), in NG medium for 1–3 h, and then cultured under HG conditions for a further 72 h. Secreted IL-1β in the conditioned media was measured by ELISA. (B) The dose-dependent effect of a PKC inhibitor on p38 MAPK and ERK1/2 phosphorylation in THP-1 cells under HG conditions. Cells were pretreated with increasing concentrations of GFX (1–10 μmol/L) as described in (A). After treatment, whole-cell lysates were subjected to Western blot analysis using phosphospecific antibodies to p38 MAPK or ERK, then stripped and reprobed with their non-phosphospecific antibodies to show equal loading. Results shown are means ± SD from three independent experiments, and asterisks denote significant differences. ♯, $p<0.05$ *versus* NG control. *$p<0.05$ *versus* HG control (vehicle).

3.6 HG increased the recruitment of NF-κB p65 subunit and the coactivator CBP to the IL-1β gene promoter and simultaneously inhibited histone deacetylase (HDAC) activities in monocytes

Diabetic stimuli such as hyperglycemia [9, 10, 24] and/or AGEs [8] can induce the expression of inflammatory genes by transcriptional mechanisms. Thus, it is important to know how DNA-binding proteins affect the functioning of any particular gene and to identify which particular protein binds to a specific gene sequence *in vivo*. We used ChIP assays to show that HG increased the binding of the NF-κB p65 subunit to the promoter of IL-1β gene in THP-1 monocytes (Fig. 7A). Very little or no binding behavior was found in either NG conditions or in NG without the addition of p65 antibody (Fig. 7A, lanes 1 and 2), indicating the specificity of the glucose effect. In all cases, there was no

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change in the amplification of input DNA (loading control) (Fig. 7A, lower panel). Furthermore, the HG-induced recruitment of chromatin remodeling factors to the IL-1β promoter was assayed with antibodies to CBP, acetyl-HH3, acetyl-HH4, and methyl-HH3R17. Figure 7B shows that (i) HG increased the recruitment of CBP to the IL-1β promoter and that this appeared to be sustained even at 72 h (the top panel); (ii) acetylation of HH3 occurred as early as 24 h after HG treatment, reached a plateau at an incubation period of 48 h and decreased after 72 h (the second panel); and (iii) similar results were also obtained with acetylation of HH4 (the third panel) and methylation of HH3 at Arg 17 (the fourth panel). The lowest panel shows that the input DNA was not altered by HG. Our results indicate that HG stimuli could induce histone acetylation as well as methylation at specific Lys and Arg residues within the IL-1β gene. These provide novel evidence that a close relationship exists between proinflammatory gene activation and acetylated and/or methylated histone accumulation

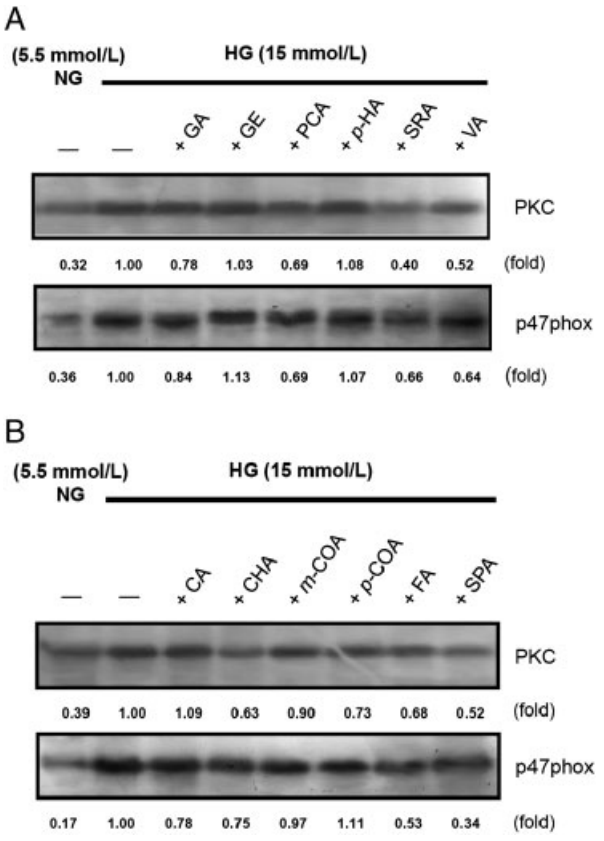

Figure 6. Effects of HBA (A) and HCA (B) on HG-induced PKC and p47phox protein translocation in THP-1 cells. Membrane fractions of cell lysates were prepared after treatment with or without the indicated phenolic acids for 48 h under HG conditions. Proteins separated by SDS-PAGE electrophoresis were immunoblotted and probed with anti-PKC or anti-p47phox antibody. The extent of protein expression was quantified and expressed as multiples of HG alone. Results shown are representative of three independent experiments.

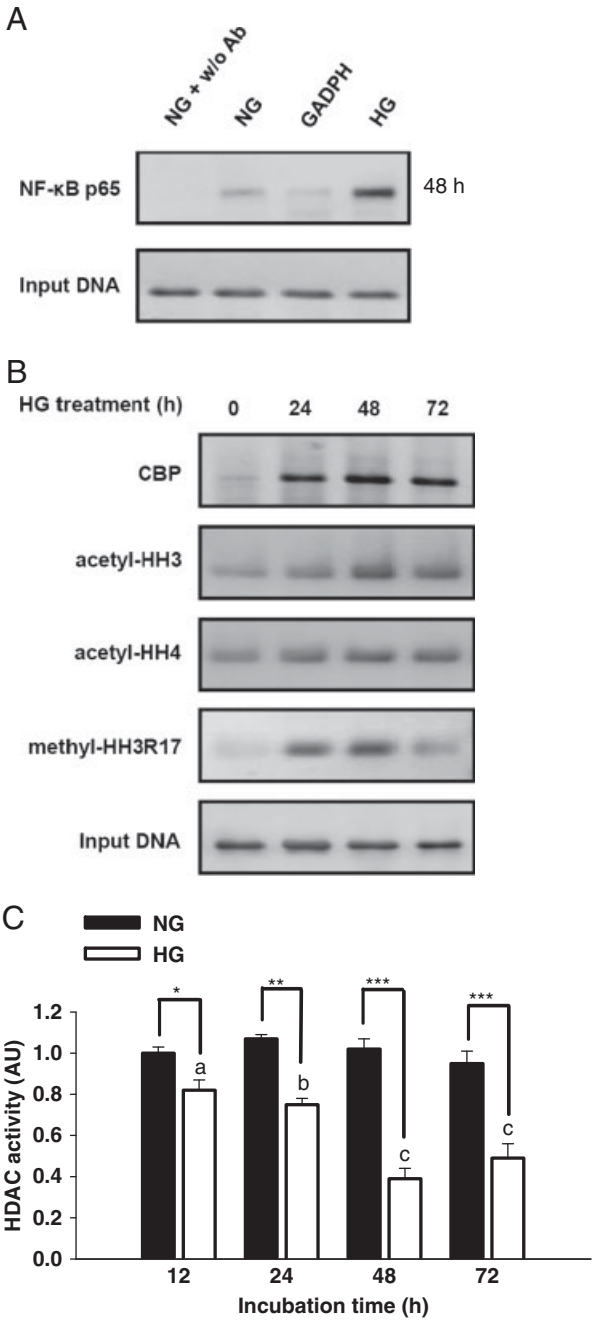

Figure 7. Time course HG-induced the recruitment of NF-κB p65 (A), CBP (B), and histone acetylation and methylation (B) at the promoter of IL-1β gene and inhibits HDAC activities (C). Cells were cultured under NG or HG conditions for various time periods. Crosslinked chromatin samples were subjected to ChIP assays using antibodies specific to p65, CBP, acetyl-HH3, acetyl-HH4, and HH3R17Me. PCRs were then performed with the immunoprecipitated DNA to amplify the IL-1β or GADPH promoters as indicated in Section 2. The lower panel was amplification of input DNA prior to immunoprecipitation. Results shown are representative of three independent experiments. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, statistical difference from each other. Groups with different-letter superscripts (a–c) are significantly different between distinct time points of HG treatment ($p<0.05$). Ab, antibody; w/o, without.

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under hyperglycemic conditions. Additionally, HG exerted time-dependent inhibition of the activities of HDAC (Fig. 7C), and NG conditions had no impact on this enzymatic activity at the same time intervals ($p<0.05$).

3.7 Effect of phenolic acids on HG-induced p65 recruitment and HH3 acetylation and methylation at the IL-1β promoter in monocytes

To clarify the molecular transcription mechanisms and nuclear chromatin remodeling events exerted by phenolic acids for their anti-inflammatory activities, selected phenolic acids were examined using ChIP assays. An inhibitor of HDAC, trichostatin A (TSA), was also included as a reference drug [24]. The results show that the recruitment of p65, which is normally involved in HG-stimulated IL-1β promoter activation, was enhanced significantly by TSA (Fig. 8, lane 2). As for the phenolic acid treatments, the methoxyphenolic acids such as SRA, VA, FA, and SPA were the most effective compounds in the inhibition of HG-induced p65 recruitment (Fig. 8A, >67% inhibition). Conversely, hydroxyphenolic acids such as GA and PCA have a lesser impact on these events (<31% inhibition), and CA did not reach significance at concentration tested ($p>0.05$). Additional experiments shown in Fig. 8B revealed that these methoxyphenolic compounds were actively abrogated the amounts of acetylated Lys residues and methylated Arg residues of HH3 within IL-1β promoter under HG conditions. Phenolic acids that have double methoxy moieties exert stronger inhibition than compounds with single moieties.

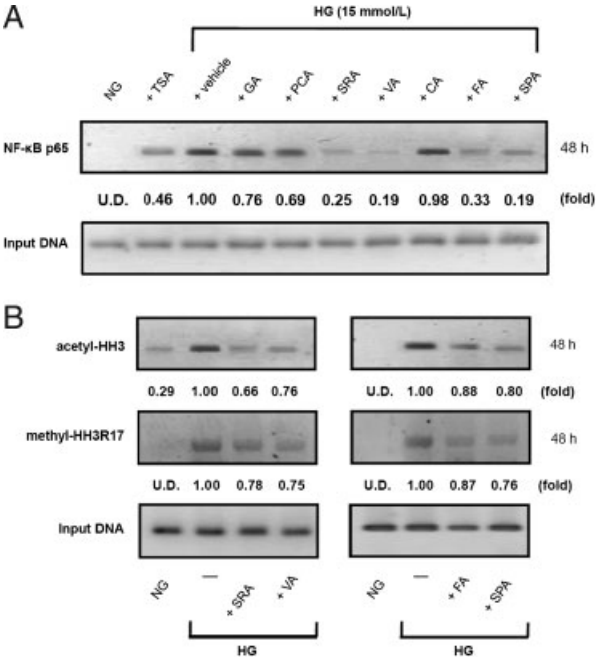

Figure 8. Effects of phenolic acids on HG-induced NF-κB p65 recruitment (A) and histone acetylation and methylation (B) at the promoter of the IL-1β gene. THP-1 cells cultured under HG conditions were treated with or without phenolic compounds or TSA for 48 h. ChIP assays were performed with specific Abs as indicated in Fig. 7. The extent of chromatin modifications was quantified and expressed as multiples of HG alone. The lower panel was amplification of input DNA prior to immunoprecipitation. Results shown are representative of three independent experiments. U.D., undetectable.

4 Discussion

A more succinct descriptive term for AGEs is glycotoxin, since AGEs are known to be toxic. Clinical evidence and experimental data have shown that AGEs levels are particularly high in people with diabetes, and that elevated levels of glucose accelerate protein glycation, facilitating the formation of AGEs [25]. For example, glycotoxins accumulate in peripheral blood mononuclear cells (PBMCs) faster under hyperglycemia than in normal conditions [2]. We also know that hyperglycemia itself or hyperglycemia-associated glycotoxins play a crucial role in chronic inflammatory processes, which can cause cells to emit signals that trigger overproduction of ROS and inflammatory mediators *via* both RAGE-dependent and RAGE-independent pathways [1, 8–10, 23, 24]. Notably, most of these adverse events appear to be involved in the pathogenesis of diabetic complications such as atherosclerosis.

The main question addressed by this study was whether dietary phenolic acids can attenuate protein glycation and inflammation in response to an HG challenge. Phenolic acids, especially HBA and HCA, are secondary plant products that are commonly found in plant-derived foodstuffs in higher concentrations than polyphenolic flavonoids [26]. Much of earlier studies on phenolic acids have reviewed the antioxidant effects of these compounds. However, it is believed that phenolic acids could also work as modulators of signal transduction pathways to elicit their beneficial effects [27]. In this study, we evaluated the hypothesis that phenolic acids can inhibit glycation and can decrease proinflammatory cytokines under hyperglycemic conditions. Of particular note, the molecular mechanisms and nuclear chromatin events involved were also investigated.

Several lines of evidence support the hypothesis. First, we adopted fluorescence as the parameter to study the effects of 12 phenolic acids on glycation, AGEs formation, and the cross-linking of proteins using several glycation assays because specific fluorescence increases during the process of glycation [28]. Most of phenolic acids inhibited glycation, but some compounds accelerated it (CA and GE). The reason for this acceleration has not been clarified. Work is still in progress in our laboratory to investigate these unexpected effects. Second, this study showed that several phenolic acids were potent inhibitors that act at multiple steps of glycation, including the early stage, as evidenced by the lowering of HbA_{1c} levels in the δ-Glu assay. Certain

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phenolic acids (CHA, FA, VA, and SPA) showed more than 15% inhibition in these separate determinations. In addition, most of these phenolic acids significantly inhibited post-Amadori glycation as shown by the BSA-glucose assay. Also, some of them are anti-crosslinking agents, as evidenced by the G.K. peptide-ribose assays. However, no direct structure-activity relationship could be established to explain the antiglycation actions shown in the assays above.

A possible mechanism by which phenolic acids inhibit the glycation of proteins could be through their antioxidant properties. Glycation is accompanied by the formation of ROS *via* glucose autoxidation and glycated protein [4, 5]. Increasing evidence has shown that supplemental antioxidants (such as vitamin B1, B6, C, E, carnosine, and sodium selenite) inhibit AGEs formation both *in vivo* and *in vitro* [29–31]. Findings from Finotti *et al.* [32] and our own laboratory [21] indicate that the inhibition of ROS produced by the glycation process, as determined by electron spin resonance, was considered to be one of the antiglycation mechanisms of plant flavonoids. Given the link mentioned above between glycation and oxidation, we thus propose that the antiglycation action of phenolic acids could correlate with their abilities to scavenge free radicals. Further studies on other possible mechanisms such as the binding affinity for proteins [33, 34], metal-chelating properties [35], and/or action as scavengers for reactive carbonyls [36] should be conducted.

Recently, we have found that naturally occurring flavonoids play a protective role against HG-induced proinflammatory cytokines expression by downregulating NF-κB-related genes [12]. Although there is a growing body of evidence that inhibition of NF-κB might be a novel therapeutic target for preventing hyperglycemia-driven inflammatory responses in animal and cultured cell models [2, 9, 10, 23, 24], the effects of dietary phenolic acids on monocyte activation remain to be elucidated. In this study, we focused on HG-stimulated monocytes, in which a large number of NF-κB-responsive genes, including IL-1β, are enhanced. Interestingly, we observed that VA, SRA, FA, and SPA were the most potent phenolics tested for inhibiting HG-induced IL-1β expression (Fig. 3). The greater the number of methoxy groups linked to the aromatic ring, the greater is the anti-inflammatory activity of the analyzed compound. It was suggested that the anti-inflammatory activities of these phenolic compounds might be attributed to their methoxy groups rather than hydroxyl groups, in agreement with other studies [37–39]. Additionally, the superior ability of methylated derivatives of polyphenols to exert anti-inflammatory actions might relate to their lipophilic properties and increased uptake through the cell membrane as seen in flavonoid compounds [40].

CML is a major antigenic AGEs structure correlate with the severity of complication in diabetic patients [2]. ROS generated in oxidative stress can in turn accelerate the AGEs formation [3]. It is established that hydroxyl radical [41] or peroxynitrite [42] mediates CML formation from Amadori product. Thrope and Bayne [43] have also proposed that CML as an indicator of ongoing oxidative damage. Furthermore, both experimental and clinical studies have shown a tendency to consider that CML concentrations reflect intracellular oxidative stress [44–46]. Thus, progress in determination of glycoxidation product CML should provide insight into the levels of oxidative stress in cell biology. In this context, the formation of CML was assayed as a marker indicating generation of intracellular oxidative stress concomitant with the detection of intracellular ROS in response to HG stimuli in this study.

Our study showed that pathologically HG could stimulate the intracellular formation of ROS and CML. These oxidative parameters were found to be suppressed by monocytes treated with phenolic acids, especially methoxyphenolic compounds (Fig. 4). Brownlee and colleagues [47, 48] demonstrated that CML accumulation was much rapidly inside endothelial cells than they do on extracellular proteins, increasing over tenfold after 168 h of incubation in HG-containing medium. Hammes *et al.* [49] have also detected increased levels of CML in T lymphocytes of diabetic patients. Interestingly, a human study by Schiekofer and coworkers showed that acute exposure to hyperglycemia conditions (10 mmol/L of glucose) over 2 h can cause significant formation of CML in PBMCs by 173% in non-diabetic volunteers compared to their euglycemic counterparts (5 mmol/L of glucose) [2]. Similarly, hyperglycemia-mediated increase of CML content was also seen in *ex vivo*-isolated PBMCs stimulated with 10 mmol/L glucose for 2 h [2]. These findings may support and provide evidence for our observation that monocytic cells cultured under HG conditions (15 mmol/L) for consecutive 72 h could lead to an increase of intracellular CML content.

On the other hand, Dasu *et al.* [50] reported that high concentrations of glucose led to inflammatory mediator release from monocytes, which can be interferenced with MAPKs, PKC, and NADPH oxidase. Furthermore, the underlying mechanism was suggested to be an increase in the activation of the PKC-dependent NF-κB cascade. Similar results were obtained in this study with a pathophysiological concentration of glucose (15 mM). Of note, the decrease in IL-1β release from monocytes caused by phenolic acids seems to operate *via* the abolishment of oxidative stress and the PKC-associated signaling cascade, a process that we believe mediates NF-κB-dependent gene expression. Indeed, we show for the first time that HG evoked IL-1β production, which was associated with increased p65 and CBP recruitment to the IL-1β promoter (Fig. 7). It is known that transcriptional initiation of many genes requires changes in higher order chromatin surrounding the promoter [24, 51, 52]. The particular pattern of modification determines whether a gene is in an active or inactive conformation [53]. Chromatin remodeling usually includes histone phosphorylation, acetylation, and methylation, accompanied by nucleosome disassembly. It has become clear that both nuclear acetylation controlled by histone acetyltransferase

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and HDAC [24] and methylation controlled by several site-specific methyltransferases [51, 52] are critical events during diabetic and inflammatory conditions. CBP has intrinsic histone acetyltransferase properties and is able to acetylate both histones and inflammation-responsive transcription factors. Among the histone modifications, we have observed that the following were associated with IL-1β gene activation under HG conditions: (i) acetylation of HH3 at Lys 9 and/or Lys 14; (ii) acetylation of HH4 at Lys 4, Lys 7, Lys 11 and/or Lys 15; and (iii) methylation of HH3 at Arg 17. However, despite HG induction of nuclear NF-κB and CBP in monocytic cells, treatment with phenolic acids prevented the recruitment of p65 to its site on the promoter. Furthermore, methoxyphenolic acids interfered with HG-induced histone modifications surrounding the IL-1β promoter, providing an explanation for the decreased ability to recruit the transcription factor NF-κB. Thus, we propose that the inhibition by phenolic acids of PKC signaling might affect NF-κB-responsive gene expression *via* modulating CBP recruitment and histone modifications at the chromatin. The importance of the contribution of PKC to facilitating NF-κB-dependent transcription by colocalizing with coactivators might be similar to the finding reported by Clarke *et al.* [54].

In conclusion, these results show that dietary phenolic acids can target the histone modification machinery to prevent proinflammatory mediator transcription, and they provide a likely explanation for the widespread suppressive effects of the natural occurring polyphenols on inflammatory responses induced by HG and other stimuli. These new findings, coupled with past results, lay the groundwork for future *in vivo* investigations that will examine additional protective effects against diabetic complications.

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These authors have declared no conflict of interest.

5 References

[1] Esposito, K., Nappo, F., Marfella, R., Giugliano, G. *et al.*, Inflammatory cytokine concentrations are acutely increased by hyperglycemia in humans: role of oxidative stress. *Circulation* 2002, *106*, 2067–2072.

[2] Schiekofer, S., Andrassy, M., Chen, J., Rudofsky, G. *et al.*, Acute hyperglycemia causes intracellular formation of CML and activation of ras, p42/44 MAPK, and nuclear factor kappaB in PBMCs. *Diabetes* 2003, *52*, 621–633.

[3] Bonnefont-Rousselot, D., Glucose and reactive oxygen species. *Curr. Opin. Clin. Nutr. Metab. Care* 2002, *5*, 561–568.

[4] Jiang, Z. Y., Woollard, A. C., Wolff, S. P., Hydrogen peroxide production during experimental protein glycation. *FEBS Lett.* 1990, *268*, 69–71.

[5] Wolff, S. P., Jiang, Z. Y., Hunt, J. V., Protein glycation and oxidative stress in diabetes mellitus and ageing. *Free Radic. Biol. Med.* 1991, *10*, 339–352.

[6] Shimoi, K., Okitsu, A., Green, M. H., Lowe, J. E. *et al.*, Oxidative DNA damage induced by high glucose and its suppression in human umbilical vein endothelial cells. *Mutat. Res.* 2001, *480–481*, 371–378.

[7] Patel, S., Celermajer, D. S., Bao, S., Atherosclerosis-underlying inflammatory mechanisms and clinical implications. *Int. J. Biochem. Cell Biol.* 2008, *40*, 576–580.

[8] Shanmugam, N., Kim, Y. S., Lanting, L., Natarajan, R., Regulation of cyclooxygenase-2 expression in monocytes by ligation of the receptor for advanced glycation end products. *J. Biol. Chem.* 2003, *278*, 34834–34844.

[9] Guha, M., Bai, W., Nadler, J. L., Natarajan, R., Molecular mechanisms of tumor necrosis factor alpha gene expression in monocytic cells via hyperglycemia-induced oxidant stress-dependent and -independent pathways. *J. Biol. Chem.* 2000, *275*, 17728–17739.

[10] Shanmugam, N., Reddy, M. A., Guha, M., Natarajan, R., High glucose-induced expression of proinflammatory cytokine and chemokine genes in monocytic cells. *Diabetes* 2003, *52*, 1256–1264.

[11] Venugopal, S. K., Devaraj, S., Yang, T., Jialal, I., Alpha-tocopherol decreases superoxide anion release in human monocytes under hyperglycemic conditions via inhibition of protein kinase C-alpha. *Diabetes* 2002, *51*, 3049–3054.

[12] Wu, C. H., Wu, C. F., Huang, H. W., Jao, Y. C. *et al.*, Naturally occurring flavonoids attenuate high glucose-induced expression of proinflammatory cytokines in human monocytic THP-1 cells. *Mol. Nutr. Food Res.* 2009, *53*, 984–995.

[13] Figarola, J. L., Shanmugam, N., Natarajan, R., Rahbar, S., Anti-inflammatory effects of the advanced glycation end product inhibitor LR-90 in human monocytes. *Diabetes* 2007, *56*, 647–655.

[14] Rahimi, R., Nikfar, S., Larijani, B., Abdollahi, M., A review on the role of antioxidants in the management of diabetes and its complications. *Biomed. Pharmacother.* 2005, *59*, 365–373.

[15] Montonen, J., Knekt, P., Jarvinen, R., Reunanen, A., Dietary antioxidant intake and risk of type 2 diabetes. *Diabetes Care* 2004, *27*, 362–366.

[16] Yeh, C. T., Yen, G. C., Induction of hepatic antioxidant enzymes by phenolic acids in rats is accompanied by increased levels of multidrug resistance-associated protein 3 mRNA expression. *J. Nutr.* 2006, *136*, 11–15.

[17] Huang, S. M., Chuang, H. C., Wu, C. H., Yen, G. C., Cytoprotective effects of phenolic acids on methylglyoxal-induced apoptosis in Neuro-2A cells. *Mol. Nutr. Food Res.* 2008, *52*, 940–949.

[18] Huang, S. M., Hsu, C. L., Chuang, H. C., Shih, P. H. *et al.*, Inhibitory effect of vanillic acid on methylglyoxal-mediated glycation in apoptotic Neuro-2A cells. *Neurotoxicology* 2008, *29*, 1016–1022.

[19] Rahbar, S., Yerneni, K. K., Scott, S., Gonzales, N. *et al.*, Novel inhibitors of advanced glycation endproducts (part II). *Mol. Cell Biol. Res. Commun.* 2000, *3*, 360–366.

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[20] Thornalley, P. J., Use of aminoguanidine (Pimagedine) to prevent the formation of advanced glycation endproducts. *Arch. Biochem. Biophys.* 2003, *419*, 31–40.

[21] Wu, C. H., Yen, G. C., Inhibitory effect of naturally occurring flavonoids on the formation of advanced glycation endproducts. *J. Agric. Food Chem.* 2005, *53*, 3167–3173.

[22] Nagaraj, R. H., Shipanova, I. N., Faust, F. M., Protein cross-linking by the Maillard reaction. Isolation, characterization, and *in vivo* detection of a lysine-lysine cross-link derived from methylglyoxal. *J. Biol. Chem.* 1996, *271*, 19338–19345.

[23] Devaraj, S., Venugopal, S. K., Singh, U., Jialal, I., Hyperglycemia induces monocytic release of interleukin-6 *via* induction of protein kinase C-α and -β. *Diabetes* 2005, *54*, 85–91.

[24] Miao, F., Gonzalo, I. G., Lanting, L., Natarajan, R., *In vivo* chromatin remodeling events leading to inflammatory gene transcription under diabetic conditions. *J. Biol. Chem.* 2004, *279*, 18091–18097.

[25] Yamagishi, S., Nakamura, K., Matsui, T., Ueda, S., Noda, Y. *et al.*, Inhibitors of advanced glycation end products (AGEs): potential utility for the treatment of cardiovascular disease. *Cardiovasc. Ther.* 2008, *26*, 50–58.

[26] Choudhury, R., Srai, S. K., Debnam, E., Rice-Evans, C. A., Urinary excretion of hydroxycinnamates and flavonoids after oral and intravenous administration. *Free Radic. Biol. Med.* 1999, *27*, 278–286.

[27] Santangelo, C., Varì, R., Scazzocchio, B., Di Benedetto, R. *et al.*, Polyphenols, intracellular signalling and inflammation. *Ann. Ist. Super. Sanita* 2007, *43*, 394–405.

[28] Brownlee, M., Advanced protein glycosylation in diabetes and aging. *Annu. Rev. Med.* 1995, *46*, 223–234.

[29] Booth, A. A., Khalifah, R. G., Hudson, B. G., Thiamine pyrophosphate and pyridoxamine inhibit the formation of antigenic advanced glycation end-products: comparison with aminoguanidine. *Biochem. Biophys. Res. Commun.* 1996, *220*, 113–119.

[30] Vinson, J. A., Howard, T. B., III, Inhibition of protein glycation and advanced glycation end products by ascorbic acid and other vitamins and nutrients. *J. Nutr. Biochem.* 1996, *7*, 659–663.

[31] Krone, C. A., Ely, J. T., Ascorbic acid, glycation, glycohemoglobin and aging. *Med. Hypotheses* 2004, *62*, 275–279.

[32] Finotti, P., Pagetta, A., Ashton, T., The oxidative mechanism of heparin interferes with radical production by glucose and reduces the degree of glycooxidative modifications on human serum albumin. *Eur. J. Biochem.* 2001, *268*, 2193–2200.

[33] Soares, S., Mateus, N., Freitas, V., Interaction of different polyphenols with bovine serum albumin (BSA) and human salivary alpha-amylase (HSA) by fluorescence quenching. *J. Agric. Food Chem.* 2007, *55*, 6726–6735.

[34] Wu, C. H., Lin, J. A., Hsieh, W. C., Yen, G. C., LDL-bound flavonoids increase the resistance of LDL to oxidation and glycation under pathophysiological concentrations of glucose *in vitro*. *J. Agric. Food Chem.* 2009, *57*, 5058–5064.

[35] Price, D. L., Rhett, P. M., Thorpe, S. R., Baynes, J. W., Chelating activity of advanced glycation end-product inhibitors. *J. Biol. Chem.* 2001, *276*, 48967–48972.

[36] Lo, C. Y., Li, S., Tan, D., Pan, M. H. *et al.*, Trapping reactions of reactive carbonyl species with tea polyphenols in simulated physiological conditions. *Mol. Nutr. Food Res.* 2006, *50*, 1118–1128.

[37] Choi, S. Y., Ko, H. C., Ko, S. Y., Hwang, J. H. *et al.*, Correlation between flavonoid content and the NO production inhibitory activity of peel extracts from various citrus fruits. *Biol. Pharm. Bull.* 2007, *30*, 772–778.

[38] Ohtani, H., Ikegawa, T., Honda, Y., Kohyama, N. *et al.*, Effects of various methoxyflavones on vincristine uptake and multidrug resistance to vincristine in P-gp-overexpressing K562/ADM cells. *Pharm. Res.* 2007, *24*, 1936–1943.

[39] Yang, Y. T., Weng, C. J., Ho, C. T., Yen, G. C., Resveratrol analog-3,5,4′-trimethoxy-trans-stilbene inhibits invasion of human lung adenocarcinoma cells by suppressing the MAPK pathway and decreasing matrix metalloproteinase-2 expression. *Mol. Nutr. Food Res.* 2009, *53*, 407–416.

[40] Rothwell, J. A., Day, A. J., Morgan, M. R., Experimental determination of octanol-water partition coefficients of quercetin and related flavonoids. *J. Agric. Food Chem.* 2005, *53*, 4355–4360.

[41] Nagai, R., Ikeda, K., Higashi, T., Sano, H. *et al.*, Hydroxyl radical mediates N^{ε}-(carboxymethyl)lysine formation from Amadori product. *Biochem. Biophys. Res. Commun.* 1997, *234*, 167–172.

[42] Nagai, R., Unno, Y., Hayashi, M. C., Masuda, S. *et al.*, Peroxynitrite induces formation of N(epsilon)-(carboxymethyl)lysine by the cleavage of Amadori product and generation of glucosone and glyoxal from glucose: novel pathways for protein modification by peroxynitrite. *Diabetes* 2002, *51*, 2833–2839.

[43] Thrope, S. R., Baynes, J. W., CML: a brief history. *Int. Congress Ser.* 2002, *1245*, 91–99.

[44] Schleicher, E., Weigert, C., Rohrbach, H., Nerlich, A. *et al.*, Role of glucoxidation and lipid oxidation in the development of atherosclerosis. *Ann. NY Acad. Sci.* 2005, *1043*, 343–354.

[45] Shaw, J. N., Baynes, J. W., Thorpe, S. R., N epsilon-(carboxymethyl)lysine (CML) as a biomarker of oxidative stress in long-lived tissue proteins. *Methods Mol. Biol.* 2002. *186*, 129–137.

[46] Ahmed, K. A., Muniandy, S., Ikram, S. I., Role of N-(carboxymethyl)lysine in the development of ischemic heart disease in type 2 diabetes mellitus. *J. Clin. Biochem. Nutr.* 2007, *41*, 97–105.

[47] Giardino, I., Edelstein, D., Brownlee, M., Nonenzymatic glycosylation *in vitro* and in bovine endothelial cells alters basic fibroblast growth factor activity. A model for intracellular glycosylation in diabetes. *J. Clin. Invest.* 1994, *94*, 110–117.

[48] Shinohara, M., Thornalley, P. J., Giardino, I., Beisswenger, P. *et al.*, Overexpression of glyoxalase-I in bovine endothelial cells inhibits intracellular advanced glycation endproduct formation and prevents hyperglycemia-induced

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increases in macromolecular endocytosis. *J. Clin. Invest.* 1998, *101*, 1142–1147.

[49] Hammes, H. P., Brownlee, M., Lin, J., Schleicher, E., Bretzel, R. G., Diabetic retinopathy risk correlates with intracellular concentrations of the glycoxidation product Nepsilon-(carboxymethyl) lysine independently of glycohaemoglobin concentrations. *Diabetologia* 1999, *42*, 603–607.

[50] Dasu, M. R., Devaraj, S., Jialal, I., High glucose induces IL-1β expression in human monocytes: mechanistic insights. *Am. J. Physiol. Endocrinol. Metab.* 2007, *293*, E337–E346.

[51] Miao, F., Li, S., Chavez, V., Lanting, L. *et al.*, Coactivator-associated arginine methyltransferase-1 enhances nuclear factor-kappaB-mediated gene transcription through methylation of histone H3 at arginine 17. *Mol. Endocrinol.* 2006, *20*, 1562–1573.

[52] Li, Y., Reddy, M. A., Miao, F., Shanmugam, N. *et al.*, Role of the histone H3 lysine 4 methyltransferase, SET7/9, in the regulation of NF-kappaB-dependent inflammatory genes. Relevance to diabetes and inflammation. *J. Biol. Chem.* 2008, *283*, 26771–26781.

[53] Berger, S. L., The complex language of chromatin regulation during transcription. *Nature* 2007, *447*, 407–412.

[54] Clarke, D. L., Sutcliffe, A., Deacon, K., Bradbury, D. *et al.*, PKCbetaII augments NF-kappaB-dependent transcription at the CCL11 promoter via p300/CBP-associated factor recruitment and histone H4 acetylation. *J. Immunol.* 2008, *181*, 3503–3514.

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