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Research Article

Naturally occurring flavonoids attenuate high glucose-induced expression of proinflammatory cytokines in human monocytic THP-1 cells

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Activation of circulating monocytes by hyperglycemia is bound to play a role in inflammatory and atherosclerosis. In this study, we examined whether flavonoids (catechin, EGCG, luteolin, quercetin, rutin) – phytochemicals that may possible belong to a new class of advanced glycation end products (AGEs) inhibitors – can attenuate high glucose (15 mmol/L, HG)-induced inflammation in human monocytes. Our results show that all flavonoids significantly inhibited HG-induced expression of proinflammatory genes and proteins, including TNF-α, interleukin-1β (IL-1β), and cyclooxygenase (COX)-2, at a concentration of 20 μM. Flavonoids also prevented oxidative stress in activated monocytes, as demonstrated by their inhibitory effects on intracellular reactive oxygen species (ROS) and N^{ε}-(carboxymethyl)lysine formation caused by HG. These inhibitory effects may involve inhibition of nuclear factor-κB activation and may be supported by downregulation of the following: i) PKC-dependent NADPH oxidase pathway; ii) phosphorylation of p38 mitogen-activated protein kinase and extracellular signal-regulated protein kinase, and iii) mRNA expression of receptor of AGEs. In addition, we found for the first time that lower levels of Bcl-2 protein under HG conditions could be countered by the action of flavonoids. Our data suggest that, along with their antioxidant activities, flavonoids possess anti-inflammatory properties and might therefore have additional protective effects against glycotoxin-related inflammation.

Keywords: Advanced glycation endproducts / CML / Flavonoids / Glucose / Oxidative stress

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1 Introduction

Growing evidence indicates that chronic and acute exposure hyperglycemia is the primary casual factor in the development of cardiovascular complications. Elevated levels of glucose in the blood stream induce a large number of alterations at the cellular level of vascular tissue. This includes monocyte activation and functional damage to endothelial cells, both of which potentially accelerate the atherosclerotic process [1]. Diabetes is usually accompanied by increased production of reactive oxygen species (ROS) and impaired antioxidant defenses. Animal and human studies have elucidated several major mechanisms that explain the adverse effects observed in the hyperglycemia, including glucose autooxidation, nonenzymatic glycosylation, formation of advanced glycation endproducts (AGEs), and diacylglycerol (DAG)–protein kinase C (PKC) pathway activation, *etc*. [2, 3]. It is very likely that hyperglycemia causes vascular pathologies by multiple mechanisms and pathways.

With our increased understanding of atherosclerosis as a chronic inflammatory process, there has been increased interest in inflammatory activity at the level of the vascula-

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Abbreviation: AGEs, advanced glycation end products; **CML,** N^{ε}-(carboxymethyl)lysine; **COX-1/2,** cyclooxygenase-1/2; **DCF-DA,** 5-(and-6)-carboxy-2′,7′-dichlorodihydro-fluorescein diacetate; **HG,** high glucose; **IL-1β**, interleukin-1β; **MAPK,** mitogen-activated protein kinase; **NG,** normal glucose; **NF-κB,** nuclear factor-κB; **PKC,** protein kinase C; **RAGE,** receptor for AGEs; **ROS,** reactive oxygen species; **THP-1 cells,** human THP-1 monocytic cells; **TNF-α**, turmor necrosis factor-α

ture. Clinical and experimental observations have demonstrated the central mechanistic relevance of several cytokine and chemokine networks in atherosclerotic processes [4]. Both type I and type II diabetes demonstrate a significant formation of AGEs and lipid peroxidation products, which are specifically recognized by inflammatory cells in atherosclerotic plaques, and induce the release of inflammatory cytokines [5, 6]. In the early pathogenesis of atherosclerosis, monocytes can be activated by hyperglycemia, AGEs, or other oxidant factors. These activated cells would adhere to the surface of damaged endothelial cells and then migrate into the sub-endothelial space and differentiate into macrophages, where they secrete a variety of proinflammatory cytokines in situ. Finally, the uptake of oxLDL *via* scavenger receptors leads to foam cells and fatty streak formation, resulting in atheroma [7]. Natarajan and co-workers [8, 9] have pointed out that HG and AGEs could generate large amounts of superoxide anion ($O_2^{\bullet -}$) and proinflammatory cytokines such as TNF-α, interleukin-1β (IL-1β), as well as chemokines including MCP-1 and IP-10, *etc*. These adverse effects were found to be related to the modulation of signaling molecules such as PKC, p47phox, and/or mitogen-activated protein kinases (MAPKs), through oxidant stress-dependent or independent pathways. These, in turn, control the activation of transcription factor nuclear factor-κB (NF-κB), influencing the synthesis and expression of inflammatory factors. Antioxidants, such as α-tocopherol [10] and *N*-acetylcystein [9], or AGEs inhibitors [11] inhibit the aforementioned formation of ROS and cytokines, hence postponing inflammation.

Flavonoids have multiple physiological activities including the prevention of cancer, degenerative disease, acute, and chronic inflammation, and immunological regulation. These effects are believed to derive from the antioxidant properties of the related flavonoids [12]. The polyphenolic structure of most of these compounds enables them not only to scavenge radicals but also to function as metal chelators [12]. Given the importance of oxidative stress and glycoxidative stress in the pathological mechanisms of hyperglycemia, taking a supplement of antioxidants to inhibit inflammatory response and AGEs accumulation may be a beneficial strategy for prevent diabetic complications [2, 13]. Our recent studies have shown that flavonoids significantly inhibit different stages of protein glycation *in vitro* [14]. They inhibited a certain degree of S100B (RAGE ligand)-induced expression of proinflammatory cytokines/chemokines genes [15]. Flavonoids also have a protective effect on reactive dicarbonyl species (RCS)-induced glycation and apoptosis of neurocytes [16].

To date, studies of the contribution of protein glycation (Maillard reaction *in vivo*) to diseases have primarily focused on its relationship to diabetes and diabetes-related complications [13, 17]. However, there is no relevant information for natural phytochemicals that occur in food, and in particular, we do not understand whether they can offer protective effects to guard against glycotoxin-induced damage. According to several studies of hyperglycemia-mediated inflammatory response [6, 8–10, 18, 19], there exists a well-established cell model to investigate HG/AGEs-induced expression of inflammatory molecules in human monocytic THP-1 cells. Figure 1 shows the proposed signaling pathways by which HG/AGEs-induced inflammatory molecules operate in THP-1 cells *via* increased production of ROS and activation of PKC/p47phox, phosphorylation of MAPKs, and NF-κB. Moreover, for the first time, we have observed a new potential mechanism presented by flavonoids to modulate the inflammatory responses under HG conditions such as the upregulation of Bcl-2 protein as well as the inhibition of RAGE gene expression. Unlike the LPS/macrophage model, using HG as an inducing agent for inflammatory response would better reflect the pathophysiological conditions of diabetic patients. Therefore, we used this model to investigate the effects of flavonoids on HG-induced proinflammatory cytokines in THP-1 cells and their possible molecular mechanisms.

2 Materials and methods

2.1 Chemicals

D-glucose, mannitol, catechin, epigallocatechin-3-gallate (EGCG), luteolin, quercetin, rutin, were purchased from Sigma Chemical (St. Louis, MO, USA). RPMI-1640 glucose free medium and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Penicllin-streptomycin solution, and sodium pyruvate solution were purchased from Hyclone (Logan, UT, USA). Anti-N^{ε}-carboxymethyllysine (CML) antibody (6D12) was purchased from Trans Genic Inc. (Kumamoto, Japan). Antibodies to ERK1/2, phospho-ERK1/2, p38, phospho-p38, PKC, p47phox, NF-κB subunit p65, IκBα, and β-actin were obtained from cell signaling technology (Beverly, MA, USA). Anti-Bcl-2 antibodies were obtained from Pharmingen (San Diego, CA, USA). Anti-epidermal growth factor receptor (EGFR), and anti-histone H1 antibody were obtained from Santa cruz biotechnology (Santa Cruz, CA, USA). Trizol™ RNA isolation kit was obtained from life technologies (Rockville, MD, USA); and primers for RT-PCR, dNTP, reverse transcriptase, and Taq polymerase were obtained from Gibco BRL (Cergy Pontoise, France). All other chemicals used were of the highest purity available.

2.2 Cell culture and treatments

The human THP-1 monocytic cells (THP-1) line 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, 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 a 5% CO_2 incubator at 37°C for 72 h. Flavonoids (final concentration of 20 μM; prepared in DMSO) were added to cells with NG or HG media. Control cells received vehicle only (<0.1% DMSO). Flavonoids or the corresponding vehicle were re-added every 24 h. The cell viability test was determined by trypan blue assay.

2.3 RNA preparation and RT-PCR

Total RNA was prepared from NG- or HG-treated THP-1 cells (1×10^6 cells/mL) by Trizol RNA isolation kit (Rockville, MD, USA) as described in the manufacturer's manual. The primer sequences were: TNF-α (forward 5′-CCAAACGATGTTGTACCCGA-3′, reverse 5′-CAGTTGGAGGAGAGACGGTA-3′); IL-1β (forward 5′-CTCTCTCACC-TCTCCTACTCAC-3′, reverse 5′-ACACTGCTACTTCTTGCCCC-3′); RAGE (forward 5′-AGAGGAGAGGAAGGCCCCAGA-3′, reverse 5′-GGCAAGGTGGGGTTATACAGG-3′), and β-actin (forward 5′-ACAAAACCTAACTTGCGCAG-3′, reverse 5′-TCCTGTAACAACGCATC-TCA-3′). Briefly, from each sample, cDNA corresponding to 0.05 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 of oligo(dT). PCR analyses were performed on the aliquots of the cDNA preparations to detect TNF-α, IL-1β, RAGE, and β-actin (as an internal standard) gene expression using the FailSafe PCR system (Epicenter Technologies, 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 $MnCl_2$, 0.2 mM dNTP, 2 U of Taq DNA polymerase, and 50 pmol of 5′ and 3′ primers. After initial denaturation for 2 min at 95°C, 29–35 cycles of amplification (the annealing temperature for TNF-α was 59°C; for IL-1β and RAGE was 56°C) were performed, followed by a 10 min final extension at 72°C.

2.4 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 NG after normalizing the gene signal, relative to the corresponding β-actin signal from each sample.

2.5 Western blotting

The protein fractions were isolated from human monocytes THP-1 cells (1×10^6 cells/mL) after the treatment with 20 μM of individual flavonoids for 72 h. Briefly, 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, Bedford, MA, USA) following the manufacturer's instructions, respectively. Protein concentration was measured by Bradford assay with BSA as a standard. Total protein and compartmental protein extracts (20–50 μg of protein) were separated on 8% SDS-polyacrylamide minigels for PKC, p47phox, COX-2, and COX-1 detection, and 12% SDS polyacrylamide minigels for CML (6D12) and MAPKs family (p38 MAPK and ERK1/2) protein detection, and then transferred to Immobilon polyvinylidene difluoride membrane (PVDF; Millipore, Bedford, MA, USA) with transfer buffer composed of 25 mM Tris-HCl (pH 8.9), 192 mM glycine, and 20% methanol. The membrane was blocked in StartingBlock™ 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 Tris buffered saline containing Tween-20 (TBST) three times, incubated with HRP-labeled secondary antibody for 45 min at room temperature, and washed with TBST 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.

2.6 Cyotokine ELISA assays

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

2.7 Intracellular ROS production assay

Intracellular ROS generation was detected using fluorescent probe, 5-(and-6)-Carboxy-2′,7′-dichlorodihydro-fluorescein diacetate (DCF-DA). DCF-DA readily diffuses through the cell membrane and is enzymatically hydrolyzed by intracellular esterases to form nonfluorescent 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 with 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.

2.8 Statistical analysis

Each experiment was performed in triplicate and repeated three times. The results are expressed as means ± SD. Statistical comparisons were made by one-way analysis of variance (ANOVA), followed by a Duncan multiple-comparison test. Differences were considered significant when the p values were < 0.5.

3 Results

3.1 Effects of flavonoids on HG-induced proinflammatory cytokine activation

In this study, human monocytic THP-1 cells were cultured in normal glucose (5.5 mmol/L, NG) or 15 mmol/L, HG, media with or without flavonoids for 72 h. Total RNA was extracted, and gene expression for TNF-α and IL-1β was performed using RT-PCR. The relative changes in mRNA levels in flavonoid-treated cells under HG conditions were calculated as fold inductions over HG-treated cells alone (1.0) after normalization to the β-actin level (internal standard). Under neither NG nor HG conditions, flavonoids (20 μM) showed no cytotoxicity on THP-1 cells (cell viability > 95%, data not shown).

As shown in Fig. 2A, luteolin exhibited the most powerful inhibitory effect on HG-induced TNF-α mRNA expression (100%), followed by EGCG (80%), rutin (73%), quercetin (67%), and catechin (32%). Expression of the IL-1β gene was also effectively inhibited by individual flavonoids, and a similar trend of inhibition was observed in the order luteolin > EGCG > rutin > quercetin > catechin (Fig. 2B). To examine whether the decrease in expression of TNF-α and IL-1β genes due to flavonoid treatment observed in the RT-PCR experiment above could result in reduced protein secretion, THP-1 cells were treated with or without flavonoids and the cytokine levels in supernatants were assayed using ELISA. As shown in Fig. 2C, HG led to almost a 7- and 20-fold increase in TNF-α and IL-1β secretion as compared to NG ($p < 0.05$). These increases were inhibited by treatment with individual flavonoids, similar to their inhibitory activity in respect of mRNA expression. Our data indicate that flavonoids can reduce TNF-α and IL-1β mRNA expression as well as secreted protein levels. In addition, adding 9.5 mM mannitol to NG did not result in increased TNF-α and IL-1β secretion, suggesting that the glucose-induced increases were not an osmotic effect (data not shown).

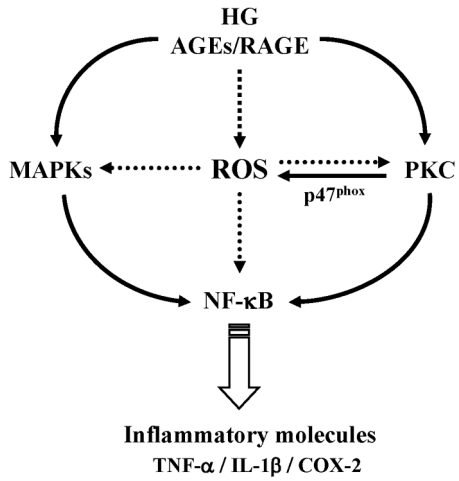

Figure 1. Proposed mechanisms for the pathway by which HG/AGEs-induced inflammatory molecules are expressed in THP-1 cells *via* increased production of ROS and activation of PKC-dependent NADPH oxidase, phosphorylation of MAPKs, and NF-κB. Solid line, oxidative stress-independent pathway; dotted line, oxidative stress-dependent pathway.

3.2 Effects of flavonoids on HG-induced COX-2 protein expression

COX-2 is induced by HG [9] and AGEs [18] *in vitro* and is expressed at a high rate at inflammation sites *in vivo* [20]. Figure 3 shows that THP-1 cells cultured under HG conditions for 72 h exhibited an approximately four-fold increase in COX-2 protein as compared to NG ($p < 0.05$, middle panel, lanes 1–2), whereas the COX-1 protein was unaffected ($p > 0.05$, upper panel, lanes 1–2). Luteolin, rutin, EGCG and quercetin inhibited HG-induced COX-2 protein expression with variable success (Fig. 3). Catechin shows only a slight effect on COX-2 protein (5%) that did not reach statistical significance ($p > 0.05$), whereas luteolin, EGCG, quercetin and rutin inhibited COX-2 protein expression by approximately 30–64% at 20 μM ($p < 0.05$).

3.3 Effects of flavonoids on HG-induced CML formation and oxidative stress in monocytes

AGEs have been reported to alter the redox status of cells through the overproduction of ROS, leading to diabetic complications. To examine whether flavonoids inhibit the formation of free radicals and oxidative-dependent AGEs in the context of human monocytic cells under HG conditions, anti-CML (main glycoxidative AGEs) monoclonal antibody (6D12) and DCF-DA staining were used to measure the intracellular accumulation of AGEs and ROS. The results show that cells cultured under HG conditions showed a significant 16.7 and 1.8 fold increase of CML

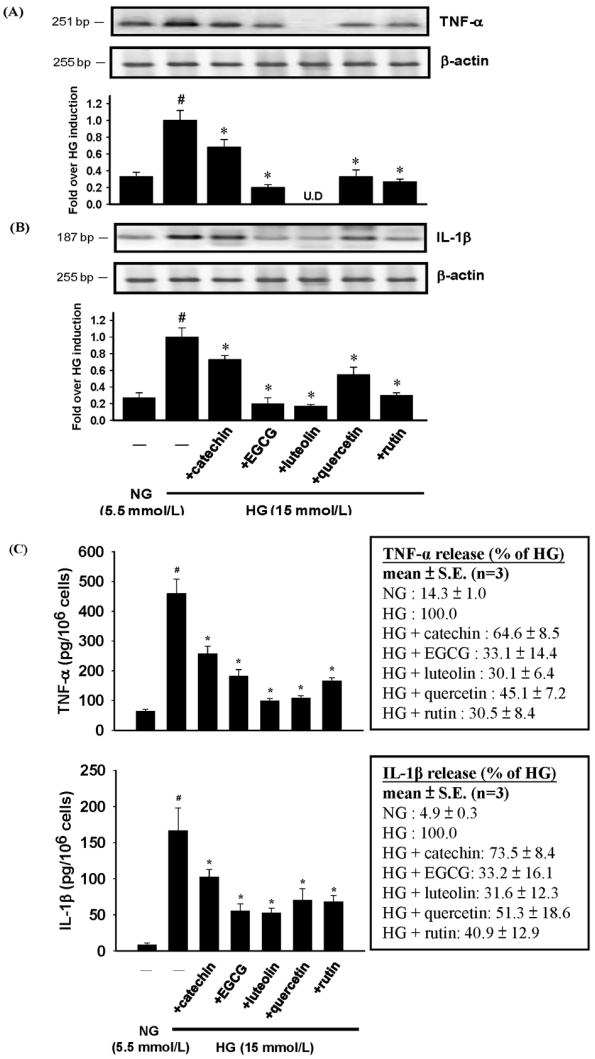

Figure 2. Effects of flavonoids on HG-induced cytokines TNF-α and IL-1β activation. (A) and (B): Cells were cultured in HG (15 mmol/L) with or without flavonoids (20 μM) for 72 h. Total RNA was isolated and analyzed for TNF-α and IL-1β mRNA expression by RT-PCR. The upper panel of (A) and (B) indicated an original band; the lower panel shows the results of densitometric analyses. The intensity of each gene-specific band was normalized to internal controls (β-actin) and the results expressed as a multiple of HG induction. (C): Cells treated under the same conditions were assayed for TNF-α and IL-1β production by ELISA as described in Materials and Methods. All appropriate controls and standards as specified by the manufacturer were used, and the data is expressed as picograms of TNF-α and IL-1β secretion by a million cells. Values shown are mean ± SD from three independent experiments. #, $p < 0.05$ compared with NG alone. *, $p < 0.05$ compared with HG alone. U.D., undetectable.

(Fig. 4A, lanes 1–2) and ROS (Fig. 4B, lanes 1–2) formation compared to NG ($p < 0.05$), which were inhibited by the addition of flavonoids to different extents ($p < 0.05$) (Fig. 4). Regarding CML formation, the extent of inhibition

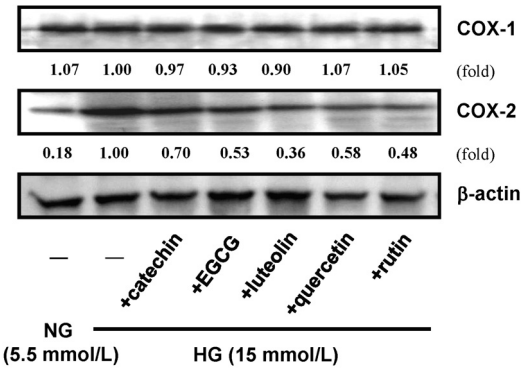

Figure 3. Effects of flavonoids on HG-induced COX-2 protein expression in THP-1 cells. Whole-cell lysates were prepared after treatment with or without indicated flavonoids (20 μM) for 72 h under high glucose conditions. Proteins separated by SDS-PAGE electrophoresis were immunoblotted and probed with anti-COX-1 antibody, anti-COX-2 antibody, or anti-β-actin antibody as an internal control. To ascertain that the total level of each protein did not change, western blots were probed with anti-PKC antibody (top panel), stripped and reprobed with anti-p47phox antibody (middle panel), then stripped and reprobed with anti-β-actin antibody (bottom panel) to show equal loading. Protein expression was quantified using densitometry with LabWorks 4.5 (Upland, CA) commercial software, and was expressed as multiples of HG. Results shown are representative of the three independent experiments.

by luteolin, rutin, EGCG, quercetin, and catechin was 77, 61, 53, 34, and 7%, respectively ($p < 0.05$, Fig. 4A). These five flavonoids also similarly inhibited HG-induced intracellular ROS formation (Fig. 4B).

3.4 Effects of flavonoids on HG-induced PKC and p47phox protein expression

Several studies have shown that $O_2^{\bullet -}$ release from human monocytes was mediated *via* activation of protein kinase C (PKC) [5, 10]. To this end, we tested the expression of PKC and p47phox proteins in THP-1 cells under HG conditions. Immunoblots of total PKC in membranes showed a significant increase in PKC translocation to the membranes in HG conditions compared to NG ($p < 0.05$), whereas addition of flavonoids inhibited the PKC translocation to membranes with potency as follows: luteolin (54%) > rutin (50%) > EGCG (43%) > quercetin (31%) > catechin (20%) (Fig. 5, top panel). The effect of HG on p47phox translocation to the membrane was investigated. p47phox translocation was also significantly increased under HG conditions as compared with NG ($p < 0.05$, bottom panel, lanes 1–2), whereas in flavonoids-treated cells, it was significantly reduced, similar to their inhibition of the PKC protein (Fig. 5, bottom panel). These results suggest that catechin has a lower impact on intracellular ROS formation. This may be partly due to less efficient downregulation of the PKC cascade.

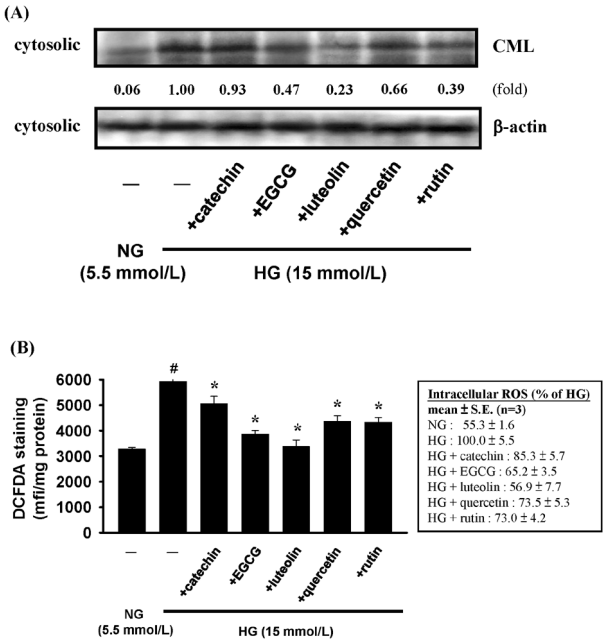

Figure 4. Effects of flavonoids on HG-induced CML formation and ROS release in THP-1 cells. Cells were cultured in HG (15 mmol/L) with or without flavonoids (20 μM) for 72 h. (A): Whole-cell lysates were subjected to western blot analysis using an anti-CML antibody (6D12), or anti-β-actin antibody as an internal control. Extent of protein expression was expressed as multiples of HG alone. (B): ROS production was quantified using mean fluorescent intensities with the fluorescent probe DCF-DA (10 μmol/L) as described in Materials and Methods. Values shown are mean ± SD from three independent experiments. #, $p < 0.05$ compared with NG alone. *, $p < 0.05$ compared with HG alone. mfi, mean fluorescent intensities.

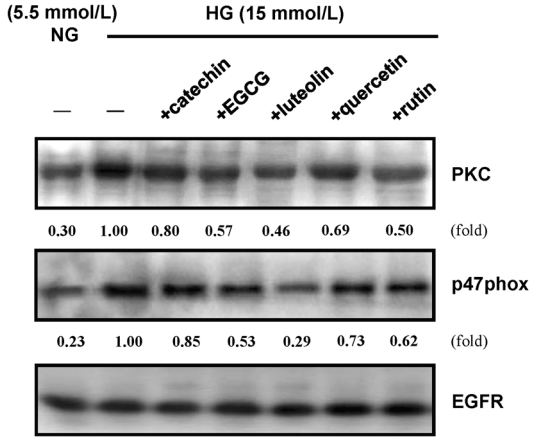

Figure 5. Effects of flavonoids on HG-induced PKC and p47phox protein expression in THP-1 cells. Membrane fractions of cell lysates were prepared after treatment with or without indicated flavonoids (20 μM) for 72 h under HG (15 mmol/L) conditions. Proteins separated by SDS-PAGE electrophoresis were immunoblotted and probed with anti-PKC antibody, anti-p47phox antibody, or anti-EGFR antibody as an internal control. To ascertain that the total level of each protein did not change, western blots were probed with anti-PKC antibody (top panel), stripped and reprobed with anti-p47phox antibody (bottom panel) to show equal loading. Extent of protein expression was quantified as described in Fig. 4, and was expressed as multiples of HG. Results shown are representative of three independent experiments.

3.5 Effects of flavonoids on HG-induced phosphorylation of p38 MAPK and ERK1/2

HG and diabetes have been shown to specifically activate stress-responsive MAPKs pathways (p38 and ERK1/2) *via* ROS intermediated in human monocytes [8, 19]. Western blotting showed that total p38 MAPK and ERK1/2 protein levels did not differ significantly between groups whether under NG or HG conditions ($p < 0.05$, Fig. 6), and with or without the addition of flavonoids in THP-1 cells ($p < 0.05$, Fig. 6). This showed that p38 MAPK and ERK1/2 protein expression were not influenced by glucose concentrations and flavonoids. Furthermore, using total p38 MAPK and ERK1/2 as an internal standard, we compared the changes of phospho-p38 (pp38) MAPK and phospho-ERK1/2 (pERK1/2) proteins. Fig. 6 shows that, compared to NG conditions, HG significantly induced pp38 MAPK and pERK1/2 protein expression ($p < 0.05$). As for pp38 MAPK, luteolin and rutin had the greatest inhibition ability, followed by quercetin, catechin, and EGCG; whereas for pERK1/2, luteolin also exhibited the best inhibitory effect, followed by EGCG, catechin, rutin, and then quercetin.

3.6 Effects of time and glucose concentrations on the expression of Bcl-2 protein under HG conditions: Induction of flavonoids

Expression of apoptotic Bcl-2 protein in aortic endothelial cells could decrease intracellular AGEs and lipid peroxide formation caused by HG [21]. In view of the importance of oxidative stress in monocytic activation, we proposed that the upregulation of Bcl-2 protein may be a beneficial strategy for preventing adverse effects under HG conditions. THP-1 cells were cultured in NG or HG conditions, respectively, for 24, 48, and 72 h. Bcl-2 protein levels were measured using western blots. Figure 7A shows that THP-1 cells incubated in NG media exhibited stable Bcl-2 expression at selected time points, showing that Bcl-2 could remain constant under normal physiological glucose concentrations. In respect of HG incubation, the expression of Bcl-2 protein in THP-1 cells is significant and maximal at 24 h, it declined and stabilized at 48 h, and it was completely eliminated after 72 h. These results suggest that persistent HG culturing of monocytes may lead to degradation of Bcl-2 protein, resulting in increased oxidative stress and progression of glycoxidation. Based on the above results, we further investigated whether flavonoids inhibit Bcl-2 degradation caused by HG. Figure 7B shows that the repression of Bcl-2 caused by HG was counteracted most significantly by rutin (6.05 fold) and catechins (catechin, 7.01 fold; EGCG, 6.28 fold).

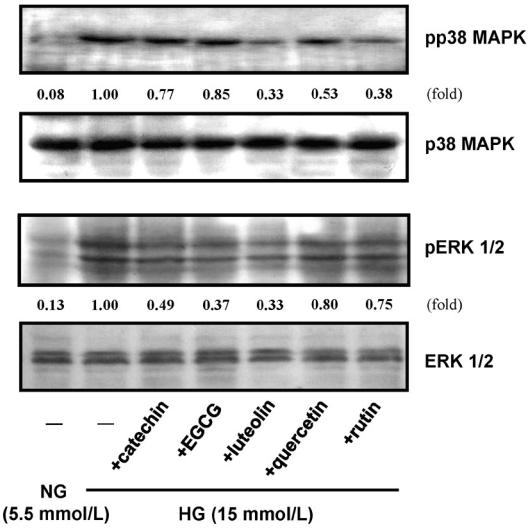

Figure 6. Effects of flavonoids on HG-induced phosphorylations of p38 and ERK MAPK in THP-1 cells. Whole-cell lysates were prepared after treatment with or without indicated flavonoids (20 μM) for 72 h under HG (15 mmol/L) conditions, and analyzed by immunoblot analysis using phosphor-specific antibodies to p38 and ERK1/2, or to total p38 MAPK and ERK1/2 (non-phospho-specific form) as an internal control. To ascertain that the total level of each MAPK did not change, blots were stripped and reprobed with the antibodies raised against the corresponding phosphorylation-independent MAPK for equal loading. Extent of protein expression was quantified as described in Fig. 4, and was expressed as multiples of HG alone. Results shown are representative of three independent experiments.

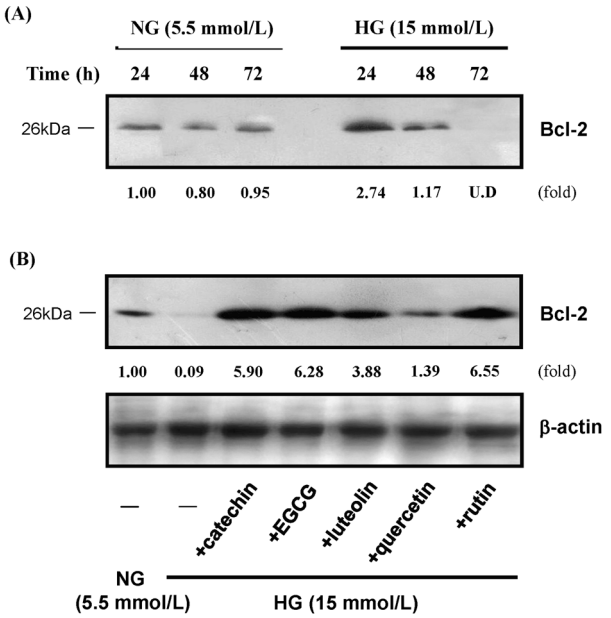

Figure 7. Effects of flavonoids on the degradation of Bcl-2 protein in THP-1 cells induced by HG. (A): To investigate time course and glucose effect on Bcl-2 protein degradation, THP-1 cells were cultured under NG (5.5 mmol/L) or HG (15 mmol/L) conditions for 24, 48, and 72 h, respectively. Whole-cell lysates were subjected to western blot analysis using an anti-Bcl-2 antibody. The basal Bcl-2 protein level of THP-1 cells cultured under NG conditions at 24 h was set to 1.0, and the relative changes in Bcl-2 protein expression were expressed as multiples of NG control data. (B): THP-1 cells were cultured in HG (15 mmol/L) with or without flavonoids (20 μM) for 72 h, and Bcl-2 levels were analyzed by western blot (upper panel). The same membrane was stripped and reprobed with anti-β-actin antibody to show equal loading (lower panel). Extent of protein expression was quantified as described in Fig. 4, and expressed as multiples of NG data (24 h). Results shown are representative of three independent experiments.

This study found that flavonoids stimulated the expression of Bcl-2 protein under HG conditions. Our results may indicate a protective mechanism of flavonoids that is worthy of further investigation.

3.7 Effects of flavonoids on the HG-induced p65 subunit of NF-κB translocation and IκBα degradation

HG-induced proinflammatory cytokine gene expression was found to involve the IκB/NF-κB pathway [9]. The effects of flavonoids on HG-induced NF-κB activation were examined. Incubation of THP-1 cells in HG media led to a rapid loss of IκBα (Fig. 8, top panel, lanes 1–2) from the cytoplasm and increased the levels of NF-κB transcriptionally active subunit p65 in the nuclear fractions (Fig. 8, bottom panel, lanes 1–2). All flavonoids stabilized IκBα degradation induced by HG with different extents (Fig. 8, top panel). Similarly, the treatment of flavonoids prevented translocations of NF-κB p65 to the nucleus, with luteolin, EGCG, and rutin being the most efficient compounds (Fig. 8, >40% inhibition). This phenomenon was consistent with the inhibition of downstream inflammatory factors (TNF-α/IL-1β/COX-2). The data described above suggest that the anti-inflammatory effects of flavonoids on activated monocytes could be due to their modulation of NF-κB activation events.

3.8 Effects of HG on expression of RAGE: Inhibition of flavonoids

Studies have shown that the binding of AGEs to membrane RAGE can lead to activation of multiple signal transduction events, proinflammatory gene expression, and ROS overproduction [11, 17]. This study found that THP-1 cells cultured under HG conditions for 72 h show an approximate 2.8-fold increase in mRNA expression of RAGE as compared to NG. ($p < 0.05$, Fig. 9A). Luteolin, rutin, and quercetin are the three most efficient inhibitors, allowing only for minimal HG/AGEs-induced monocytic inflammation (Fig. 9B).

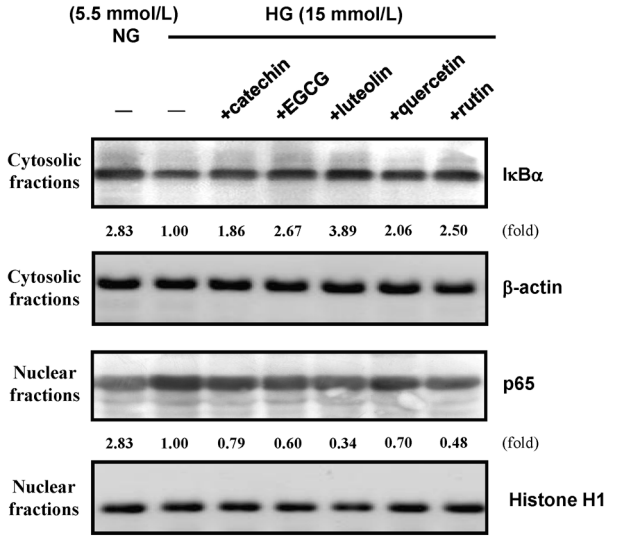

Figure 8. Effects of flavonoids on HG-induced NF-κB subunit p65 translocation and IκB protein degradation in THP-1 cells. Nuclear and cytosolic extracts were prepared after treatment with or without indicated flavonoids (20 μM) for 72 h under HG conditions. Nuclear proteins were immunoblotted and probed with anti-p65, or anti-Histone H1 antibody as an internal control. Cytosolic proteins were immunoblotted and probed with anti-IκBα antibody, or anti-β-actin antibody as an internal control. The same membrane was stripped and reprobed with anti-Histone H1 or anti-β-actin antibody to show equal loading. Extent of protein expression was quantified as described in Fig. 4, and expressed as multiples of HG data. Results shown are representative of three independent experiments.

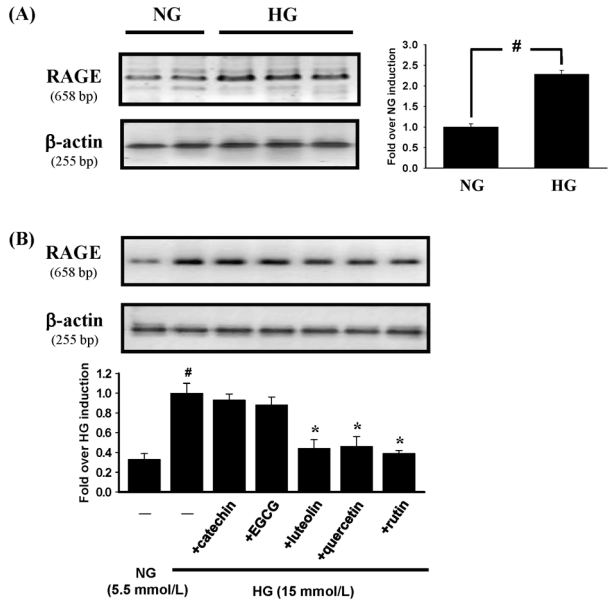

Figure 9. Effects of flavonoids on HG-induced receptor of AGEs (RAGE) activation in THP-1 cells. (A): To investigate glucose-mediated effects on RAGE mRNA expression, cells were cultured in NG (5.5 mmol/L) or HG (15 mmol/L) for 72 h, respectively. Total RNA was isolated and analyzed for RAGE mRNA expression using RT-PCR. The basal RAGE mRNA level for THP-1 cells cultured under NG condition was set to 1.0, and the relative changes in RAGE mRNA were expressed as multiples of NG control data. (B): THP-1 cells were cultured in HG with or without flavonoids (20 μM) for 72 h, and RAGE mRNA levels were analyzed as described in (A), and expressed as multiples of NG control data. Results shown are representative of three independent experiments. #, $p < 0.05$ compared with NG alone. *, $p < 0.05$ compared with HG control.

4 Discussion

Hyperglycemia induced inflammation, employing macrophages/monocytes, innate cytokines or innate receptors all seem to play a causal role in the early stage of atherogenesis. Among the inflammatory pathways, the cytokines are central players. Several cellular models which mimic hyperglycemic conditions further support the suggestion that cytokines and oxidative stress may contribute to atherosclerosis [8 – 10]. Vascular cells, especially monocytes, can be activated by elevated levels of glucose which contribute to inflammatory cytokine-dependent networks in the blood stream by: i) oxidative stress; ii) production of proinflammatory cytokines; iii) response to these potent cell activators, and iv) 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.

Our study showed that pathologically high glucose could stimulate the activation of THP-1 monocytes, promote TNF-α and IL-1β mRNA expression and protein secretion, and then selectively induce inflammatory COX-2 protein. Given that there is a paucity of data examining the effects of flavonoids on HG-induced monocyte activation, this study selected five natural flavonoids with anti-oxidation [12] and anti-glycation [14] characteristics, making use of the HG/THP-1 model (Fig. 1) to investigate their effects on proinflammatory cytokines in the context of exploring possible mechanisms. Our results reveal that flavonoids can regulate hyperglycemic inflammation by attenuating TNF-α and IL-1β genes at the transcription level as well as by modulating protein secretion. Moreover, luteolin, EGCG, quercetin, and rutin exhibited significant inhibitory effects on intracellular CML and ROS formation under HG, with the exception of catechin which showed weaker activity (Fig. 4). ROS acted as a key intermediate that was directly involved in the HG-mediated inflammation pathway [6, 8 – 11]. Our data may be interpreted as intensity differences for reactions between proinflammatory cytokines and individual flavonoids. The anti-inflammatory mechanism of flavonoid action under HG conditions may be closely related to their antioxidant characteristics.

Poor glycemic control in diabetes provides advantageous conditions for glycation, also known as the Maillard reaction [3, 17]. Acute hyperglycemia may lead to intracellular

formation of CML and activation of MAPKs in peripheral monocytes [22]. CML is a dominant AGEs [23] as well as a biomarker of oxidative stress *in vivo* [24], which can accumulate in tissue proteins and contribute to the progression of inflammation [18, 24]. Numerous animal experiments [25] have shown that AGEs content increases within a few weeks after the animals were rendered diabetic and that this increase was systemic, occurring in the kidneys, skin, and vascular tissue. Endogenous AGEs are formed in both extracellular (plasma and tissue extracellular matrix) and intracellular compartments (proteins, DNA); the latter seems to exhibit a much higher concentration than plasma [26]. Cellular interactions with AGEs proteins are known to induce several biological responses, including both ROS generation and induction of inflammatory-related cytokines [18, 24]. These responses are thought to be mediated by AGEs receptors (RAGE) [13, 17]. In addition, AGEs have an apparent synergistic effect on HG-induced monocyte activation [27]. This study used an anti-AGEs monoclonal antibody (6D12) to detect CML formation in THP-1 cells using western blots. Our results showed that after 72 h HG culture, intracellular CML content was significantly higher than that in the NG group ($p < 0.05$). Among the five flavonoids, luteolin and rutin best inhibited CML formation (Fig. 4A). In our previous study [14], of all the flavonoids, luteolin had markedly anti-glycation activity toward various glycated agents such as glucose, methylglyoxal and cross-linked G. K. peptide. Even the luteolin 6-C-(6"-O-trans-caffeoylglucoside (glycosylated form of luteolin) has been shown to have an AGEs-inhibitory effect [28]. It seems plausible that flavonoids may act as anti-glycated agents to decrease ROS and AGEs formation during glycation, and thus attenuate HG-induced inflammation.

Regarding chemical structure, the position and numbers of flavonoid hydroxy groups seem to be related to degree of anti-inflammatory activity. Ueda *et al*. [29] pointed out that flavonoids with four hydroxylations at position 5, 7, 3′, and 4′ had the greatest inhibitory effect on TNF-α secretion. Comalada *et al*. [30] also showed that the presence of double bonds at C2-C3 and the position of the ring B at 2′ are necessary structures for a strong anti-inflammatory flavonoid. The five flavonoids with different structural characteristics tested in this study: flavanols (catechin, EGCG), flavone (luteolin), and flavonols (quercetin, rutin), all have a common anti-inflammatory structures as described above. Our work has shown that certain flavonoids, especially flavone, express their anti-inflammatory activity at least in part by modulation of proinflammatory genes and protein levels. We observed inhibition of several pivotal cytokines, for which luteolin is the most effective, and catechin is the least. We thus proposed that the 4-keto group on C ring of the flavonoids seems to be necessary for the highest anti-inflammatory activity; however, a hydroxyl group at C-3 was nonessential. Generally, aglycon is no more effective than its glycoside.

Kanková and Sebeková [31] have pointed out that hyperglycemia might act through i) the mitochondrial respiratory chain; ii) NADPH oxidase activation and iii) formation of AGEs, to allow for increased production of ROS. In addition, glucose itself can auto-oxidize to form $O_2^{\bullet -}$, OH, and H_2O_2 in the presence of transition metal ions (Fe^{3+}, Cu^{2+}) [2, 3, 32], and subsequently accelerate the formation of AGEs. Similarly, the important mechanistic finding by Venugopal *et al*. [10] indicated that NADPH oxidase is activated *via* PKC by translocating p47phox to membranes under HG conditions, resulting in increased $O_2^{\bullet -}$ release, whereas α-tocopherol supplementation significantly inhibited these changes. Specific inhibitors combining PKC with p38 MAPK, or combining antioxidant with PKC inhibitor, block 90% of HG-induced expression of the proinflammatory genes [8]. Rosiglitazone [33] could inhibit HG-induced oxidative stress, also through the regulation of the PKC-dependent NADPH oxidase pathway. Consistent with the results of Figs. 4 and 5, we proposed that the inhibitory effects of flavonoids on HG-induced ROS formation may be exerted *via* inhibition of PKC activation, as flavonoids, especially luteolin, EGCG, and rutin, significantly suppressed p47phox membrane translocation.

Shanmugam *et al*. [9] have pointed out that inhibition of p38 MAPK partially inhibits the gene expression of TNF-α and IL-1β. At the same time, HG-induced IL-1β protein release is abrogated with the inhibition of ERK1/2 [19]. ROS are increasingly recognized for their ability to control signal transduction *via* activation of MAPKs. It is known that p38 MAPK can be directly regulated by cellular stress and proinflammatory stimuli, and more generally this is assumed to be a classical stress-responsive target system [34]. Figure 6 shows that luteolin can inhibit 67% of MAPKs phosphorylation, being the most potent inhibitor of inflammatory molecules among flavonoids. It is noteworthy that EGCG may inhibit p38 MAPK rather than rutin due to their differential effects on intracellular ROS/CML inhibition (Fig. 4); however, contradictory results were obtained in this study. We therefore posit that the influence of rutin on p38 MAPK might be *via* the ROS-independent pathway, while the signaling molecule p38 MAPK may not be completely regulated by ROS under HG conditions.

Our study for the first time has investigated the effects of glucose concentrations on the anti-apoptotic protein Bcl-2 in THP-1 monocytes at various time points. Our results showed that after culturing THP-1 cells for 72 h under HG conditions, Bcl-2 protein expression was completely blocked (Fig. 5A). We note that intervention flavanols (catechin, EGCG, and rutin) had an obvious promotion effect on Bcl-2 expression (Fig. 5B). Giardino *et al*. [21] have indicated that Bcl-2 expression helps to decrease AGEs formation and lipid peroxidation caused by HG. Such a mechanism would not influence intracellular ROS formation. Simultaneously, Cipollone *et al*. [35] have analyzed the monocytic Bcl-2 gene expression found in microalbuminu-

Table 1. Overview of flavonoid effects on monocytes physiology under HG conditions[a)]

Group	Cytotoxicity (>5% necrosis)	Inflammatory molecules (TNF-α /IL-1β/COX-2)	Oxidant markers (CML/ROS)	PKC cascade (PKC/ p47phox)	pp38 MAPK	pERK1/2 MAPK	Bcl-2	NF-κB (IκBα/p65)	RAGE
Flavone									
luteolin	–	+++	+++	+++	+++	+++	++	+++	+++
Flavonol									
quercetin	–	++	++	++	++	+	+	++	+++
rutin	–	+++	+++	+++	+++	+	+++	+++	+++
Flavanol									
catechin	–	+	+	+	+	++	+++	+	–
EGCG	–	+++	+++	++	+	+++	+++	++	–

a) Inhibition is shown as +/++/+++. Absence of effect is shown as –.

ric diabetic patients, and they concluded that increased oxidative stress and activation of NF-κB in these patients was related to low Bcl-2 expression level. Complementing α-tocopherol effectively inhibited the repression of Bcl-2 and improved inflammation characteristics. On the basis of the anti-glycation nature of flavonoids and their ability to inhibit the accumulation of AGEs or RCS in cells, we first proposed that flavonoids could inhibit the generation of ROS *via* monocyte activation, thereby restricting the downstream inflammatory signal molecules and activation of transcription factor. However, our results showed that the anti-inflammatory activity of flavonoids under HG conditions might involve another unknown mechanism. The differential effects of rutin between the inhibition of intracellular ROS and CML formation were probably related to Bcl-2 (Fig. 4).

NF-κB plays a final deciding role in regulating inflammatory genes. HG can promote IκBα phosphorylation through a series of signaling events, facilitating the translocation of NF-κB subunit p65 into the nucleus and stimulating the expression of inflammatory factors [8]. Type I DM patients with poor glycemic control had their monocytic NF-κB abnormally activated. Intervention antioxidant thioctic acid can decrease NF-κB binding activity [36]. This study demonstrated that flavonoids may promote cytosolic IκBα degradation and inhibit nuclear p65 translocation with their activities in potency order luteolin > rutin > EGCG > quercetin > catechin (Fig. 8). This seems to be correlated with the anti-inflammation characteristics of flavonoid samples. The regulation of flavonoids for upstream signaling such as ROS, PKC/p47phox, p38 MAPK, and ERK1/2 did indeed influence downstream NF-κB activation and inhibited the expression of proinflammatory cytokine genes.

The effect of AGEs is mainly regulated by its membrane receptor RAGE. Some studies have demonstrated that RAGE exists in vascular cells (monocytes/macrophages, smooth muscle cells and endothelial cells) that play a role in atherosclerosis [37]. RAGE is not expressed under normal physiological conditions, but it is expressed when ligands (AGEs) exist or when transcription factors regulating RAGE are activated [38]. Tanji *et al*. [39] have demonstrated RAGE overexpression under the accumulation of AGEs in diabetic patients. In addition, β-amyloid, S100/calgranulins, TNF-α, and oxidative stress can also induce RAGE expression [11, 38]. According to Fig. 9A, THP-1 cells cultured under HG conditions significantly increased RAGE mRNA expression (2.8-fold) ($p < 0.05$). These results indicate that HG culturing led to the production of AGEs and ROS. Compared with flavanols (catechin, EGCG), luteolin, rutin and quercetin exhibited significantly greater inhibition of RAGE mRNA expression (Fig. 9B). As for the influence of flavonoids on RAGE, except for grape seed proanthocyanidin extracts [40], there exist very few references in the published literature. Based on our study, the inhibitory effects of flavonoids on RAGE gene expression seem to be closely related to their anti-inflammation and anti-glycation characteristics.

In conclusion, flavonoids, a group of naturally occurring phytochemicals widely present in the plant kingdom, seem to inhibit the expression of proinflammatory factors in the HG/monocyte model as summasizied in Table 1. They also repress the deleterious effects of AGEs. In our study, we revealed the novel anti-inflammation protective mechanism of flavonoids under HG conditions, namely that they stimulate the anti-apoptotic protein Bcl-2 and that they inhibit RAGE mRNA expression. Complementing antioxidant/anti-glycated flavonoids might be an effective adjuvant strategy for delaying diabetic complications.

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