Effects of flavonoids on the expression of the pro-inflammatory response in human monocytes induced by ligation of the receptor for AGEs

Shang-Ming Huang, Chi-Hao Wu and Gow-Chin Yen

Department of Food Science and Biotechnology, National Chung Hsing University, Taichung, Taiwan

Increasing evidence has shown advanced glycation end products (AGEs) receptor ligation (RAGE) to be an important part of complex interactions of the oxidative stress and pro-inflammatory responses. In this study, flavonoids were used to monitor the protective effects against the oxidative damage and inflammation mediated by AGEs in human monocytes. S100B (RAGE ligand) treatment in human THP-1 monocytic cells (THP-1) significantly increased gene expression of the pro-inflammatory cytokines TNF-α and IL-1β; chemokines MCP-1 and IP-10; adhesion factors platelet endothelial cell adhesion molecule (PECAM-1) and β2-integrin; and pro-inflammatory cyclooxygenase-2 (COX-2). S100B treatment with quercetin and catechin in THP-1 cells had inhibitory effects on the expression of pro-inflammatory genes and protein levels. Quercetin and catechin could regulate S100B-activated oxidant stress-sensitive pathways through blocking p47phox protein expression. Treatment with quercetin and catechin could eliminate reactive oxygen species (ROS) to reduce oxidative stress stimulated by S100B in THP-1 cells. Quercetin and catechin also showed different regulatory abilities on mitogen-activated protein kinase (MAPK) signaling pathways by inhibiting protein expression in S100B-stimulated inflammatory responses in THP-1 cells. This study suggests that quercetin and catechin may be of benefit for diabetic vascular complications due to its antioxidant abilities against AGE-mediated oxidative stress through oxidative stress-sensitive and oxidative stress-responsive signaling pathways, which lead to inflammation in human monocytes.

Keywords: AGEs / Catechin / Cytokine / Oxidative stress / Quercetin

Received: May 21, 2006; revised: August 1, 2006; accepted: August 1, 2006

1 Introduction

Glucose and other reducing sugars can react nonenzymatically with the amino groups of proteins, lipids, and nucleic acids through a series of reactions forming reversible Schiff base and Amadori compounds [1]. These early glycation products undergo further complex reactions to become irreversibly crosslinked, heterogeneous fluorescent derivatives termed so advanced glycation end products (AGEs) [2, 3]. Glycation is concentration-dependent in the early, rather than later, stages of the Maillard reaction and is, therefore, enhanced in diabetes. Physical glycation is a major source of ROS and reactive carbonyl species (RCS) that are generated by both oxidative (glycoxidative) and nonoxidative pathways [2, 4]. In addition, glucose itself can auto-oxidize to accelerate the formation of AGEs, leading to oxidative damage [5]. AGEs are known to accumulate in various tissues at accelerated rates in the diabetic condition [6] and are implicated in the development of diabetic vascular complications [7]. *In vitro* work has shown that ligation of the advanced glycation end products receptor (RAGE) is part of the complex interactions within oxidative stress and vascular damage, particularly in atherosclerosis [8] and in the accelerated vascular damage that occurs in diabetes [7, 9].

Several AGEs receptors have been identified, including macrophage scavenger receptor Types I and II and the receptor for AGEs (RAGE), and are expressed on a wide range of cells [10, 11]. The best characterized AGEs receptor is RAGE, which is a multiligand member of the Ig superfamily of cell surface molecules with a diverse repertoire of ligands [10]. These ligands include products of

Correspondence: Dr. Gow-Chin Yen, Department of Food Science and Biotechnology, National Chung Hsing University, 250 Kuokuang Road, Taichung 40227, Taiwan
E-mail: gcyen@nchu.edu.tw
Fax: +886-4-22854378

Abbreviation: AGEs, advanced glycation end products; **COX-2,** cyclooxygenase-2; **DCFH,** 2′,7′-dichlorofluorescein; **HG,** high glucose; **NG,** normal glucose; **MAPK,** mitogen-activated protein kinase; **PECAM-1,** platelet endothelial cell adhesion molecule; **RAGE,** receptor for AGEs; **ROS,** reactive oxygen species; **THP-1,** human THP-1 monocytic cells

nonenzymatic glycation, AGEs, members of the S100/calgranulin family of pro-inflammatory mediators, and β-sheet fibrillar structures such as amyloid and amphoterin [7, 11]. S100B, a member of the S100 family of EF-hand Ca^{2+}-binding proteins, is a peptide produced mostly by astroglial cells of the central nervous system with multiple autocrine and paracrine effects, and is also produced by neurons and glias. S100B signals through RAGE and can produce key pro-inflammatory mediators in endothelium, mononuclear phagocytes, and lymphocytes [12]. S100B now serves as a valuable tool in the study of RAGE signaling and the diabetic inflammatory condition.

The broad consequences of the RAGE–ligand interaction for cellular properties are emphasized by the spectrum of signaling mechanisms. One such consequence of this interaction can lead to the generation of ROS. Other consequences include the production of growth factors and cytokines, chronic inflammatory responses, and cellular and vascular dysfunction associated with diabetic complications [13, 14]. Studies with inflammatory cells such as human monocytes have demonstrated that stimulated diabetic conditions *in vitro* such as high glucose (HG) culture conditions [15, 16] or treatment with AGEs [7, 10–12] lead to oxidative stress, and further induce pro-inflammatory cytokines and related genes *via* activation of specific signaling pathways and transcription factors. This process could then result in mononuclear phagocyte migration and activation, endothelial expression of vascular cell adhesion molecule-1, and increase monolayer permeability [17]. Further evidence for the role of AGEs in diabetes, showing that AGEs can augment inflammatory reponses by up-regulating COX-2 *via* RAGE and multiple signaling pathways, thereby lead to monocyte activation and vascular cell dysfunction [18].

In general, diabetes is associated with atherosclerotic and inflammatory diseases. The adhesion of monocytes to endothelium followed by transmigration into the subendothelial space is one of the early key events in the pathogenesis of atherosclerosis [15]. These processes are further aggravated by hyperglycemia and AGEs, leading to cardiovascular complications in diabetes mellitus. Therapeutic interventions for reducing AGEs formation should target AGEs formation by reducing crosslinking [19]. Other strategies could include receptor inhibition of AGEs to block signaling, which can suppress the inflammatory response in murine models, the diabetic condition and its complications.

Increasing evidence indicates that AGEs are one of the most important mechanisms of diabetic complication. There has been much work done to elaborate the etiology, prevention, and treatment of diabetes-related complications. In theory, the strategy for preventing diabetic complications is to use antioxidants to inhibit oxidative stress and AGEs toxicity in diabetic patients. In the literature, the prevention of biological glycation damage is still remained to block the formation of AGEs [20]. Previously, we found that inhibitory capability of flavonoids against protein glycation was remarkably related to the scavenging free radicals derived from glycoxidation [21]. The inhibitory mechanism of flavonoids against glycation was due to their antioxidant properties. However, data concerning the effects of naturally occurring antioxidants on AGEs accumulation leading to oxidative damage and pro-inflammatory responses in diabetes is unclear. The objective of this study was to investigate the inhibitory effects of flavonoids, quercetin and catechin on S100B-induced pro-inflammatory responses and the molecular mechanisms involved in human monocytes.

2 Materials and methods

2.1 Chemicals

S100B protein (bovine brain), quercetin, catechin, D-glucose, mannitol, ribose, and RNase 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). L-Glutamine, penicllin–streptomycin solution, and sodium pyruvate solution were from Hyclone (Logan, UT, USA). 6-Carboxy-2′,7′-dichlorofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes (Eugene, OR, USA). Anti-ERK1/2, anti-phospho-ERK1/2, anti-JNK, anti-phospho-JNK, anti-p38, anti-phospho-p38, anti-PI3K, and anti-β-actin antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-Bcl-2 antibodies were obtained from Pharmingen (San Diego, 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, Food Industry Research and Development Institute, Hsin Chu, Taiwan). Cells were grown in RPMI 1640 medium, supplemented with 10% FBS, glutamine (2 mM), HEPES (10 mM), streptomycin/penicillin (100 mg/mL/100 U/mL), 50 μM β-mercaptoethanol, and 5.5 mM D-glucose (normal glucose; NG) at 37°C, in a humidified atmosphere of 95% air and 5% CO_2. For diabetic conditions, cells were cocultured with 6.5 μg/mL

S100B protein and treated with querctin or catechin, as indicated. In our preliminary test, the cell viability of THP-1 cells was tested using the MTT assay and was not significantly affected when incubated with the indicated flavonoids up to a concentration of 50 μM.

2.3 RNA preparation and RT-PCR

THP-1 cells (2×10^6 cells/mL) in 10 mL of medium containing 5.5 mM (NG) with 6.5 μg/mL S100B and the indicated flavonoids were cultured in 10 cm culturing dishes for various time intervals. Cellular RNA was extracted with a Trizol RNA isolation kit (Rockville, MD, USA) as described in the manufacturer's manual. The forward and reverse primers for pro-inflammatory cytokine TNF-α were 5′-CCAAACGATGTTGTACCCGA-3′ and 5′-CAGTTGGAGGAGAGACGGTA-3′. The 5′ and 3′ primers for pro-inflammatory cytokine IL-1β were 5′-CTCTCTCACCTCTCCTACTCAC-3′ and 5′-ACACTGCTACTTCTTGCCCC-3′. The 5′ and 3′ primers for chemokine MCP-1 were 5′-CAAACTGAAGCTCGCACTC-3′ and 5′-CATTTCCACAATAATATTTTAG-3′. The 5′ and 3′ primers for chemokine IP-10 were 5′-TGAAAAAGAAGGGTGAGAAGAG-3′ and 5′-GGAAGATGGGAAAGGTGAGG-3′. The 5′ and 3′ primers for adhesion factor platelet endothelial cell adhesion molecule (PECAM-1) were 5′-AGGAAAGAAGGACACAGAGAC-3′ and 5′-ATGGATTTAAGAACCGGCAG-3′. The 5′ and 3′ primers for integrin factor β2–integrin were 5′-AAAAACATCCAGCCCATCTTC-3′ and 5′-ATCTGCACGCCATCACAGTC-3′. The 5′ and 3′ primers for COX-2 were 5′-ATCTACCCTCCTCAAGTCCC-3′ and 5′-TACCAGAAGGGCAGGATACA-3′. The 5′ and 3′ primers for housekeeping gene β-actin were 5′-ACAAAACCTAACTTGCGCAG-3′ and 5′-TCCTGTAACAACGCATCTCA-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) 12–18. PCR analyses were performed on the aliquots of the cDNA preparations to detect TNF-α, IL-1β, MCP-1, IP-10, PECAM-1, β2-integrin, COX-2, 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 (at 95°C for 1 min, 56–59°C for 1 min, and 72°C for 1.5 min) 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 blot analysis

The cytosolic proteins were isolated from human monocytes THP-1 cells (1×10^6 cells/mL) after the treatment with 20 and 50 μM of quercetin and catechin for 2 h. The total proteins were extracted by adding 500 μL of lysis buffer (50 mM Tris-HCl, pH 8.0; 1 mM NaF; 150 mM NaCl; 1 mM EGTA; 1 mM phenylmethanesulfonyl fluoride; 1% NP-40; and 10 μg/mL leupeptin) to the cell pellets on ice for 30 min, followed by centrifugation at $10\,000 \times g$ for 30 min at 4°C. The cytosolic fraction (supernatant) proteins were measured by Bradford assay [22] with BSA as a standard. Total cytosolic extracts (250 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 mitogen-activated protein kinases (MAPKs) family and β-actin 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 5% BSA solution for 1 h 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 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 β-actin.

2.6 Cyotokine ELISA assays

THP-1 cells (2×10^6 cells/mL) were incubated in six-well tissue culture plates in RPMI 1640 medium with 0.2% BSA. Cells were cotreated with S100B and quercetin or catechin for 12 h. The supernatant conditioned medium

was then harvested and assayed for TNF-α and IL-1β levels 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 were estimated by using a fluorescent probe, DCFH-DA. DCFH-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 DCFH-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 were 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 Inhibitory effects of quercetin and catechin on the expression of pro-inflammatory cytokines

As AGEs accumulate under the diabetic condition and contribute to the progression of diabetic inflammation, pro-inflammatory cytokines such as TNF-α and IL-1β are believed to play an important role in pro-inflammation. In this study, we evaluated the effects of quercetin and catechin on the expression of pro-inflammatory cytokines induced by ligation of RAGE by S100B protein in THP-1 cells. As shown in Fig. 1, S100B treatment of THP-1 cells for 4 h led to significantly increased expression of TNF-α and IL-1β mRNA ($p < 0.05$). However, significant inhibitory effects on the expression of TNF-α mRNA in THP-1 cells were observed with the treatment of 20 and 50 μM of quercetin and catechin ($p < 0.05$). In addition, the treatments with quercetin and catechin significantly decreased the expression of IL-1β mRNA levels in THP-1 cells ($p < 0.05$).

The effects of quercetin and catechin on the induction of protein levels of TNF-α and IL-1β pro-inflammatory cytokine in THP cells stimulated by S100B were further determined. Figure 2 shows similar results by RT-PCR analyses where S100B (6.5 μg/mL) treatment led to a significant increase in TNF-α and IL-1β protein levels in THP-1 cells ($p < 0.05$). The treatment of these cells with 20 and 50 μM

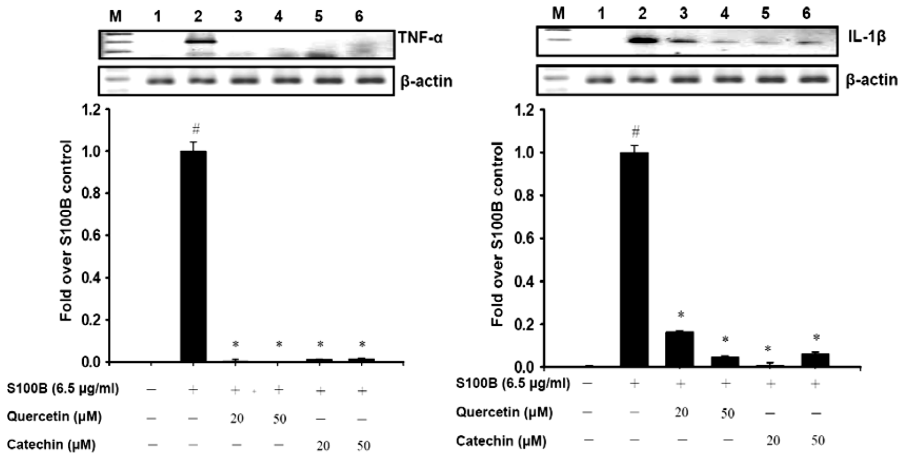

Figure 1. Effects of quercetin and catechin on the expression of pro-inflammatory TNF-α and IL-1β mRNA in THP-1 cells induced by S100B. Values are means ± SD of three experiments. Cells were cocultured with the indicated flavonoids (20 and 50 μM) and S100B protein (6.5 μg/mL) for 4 h. The figure shows ethidium bromide-stained agarose gels of RT-PCR products: 1, NG; 2, S100B treatment; 3, S100B plus quercetin (20 μM); 4, S100B plus quercetin (50 μM); 5, S100B plus catechin (20 μM); 6, S100B plus catechin (50 μM). # $p < 0.05$ *vs.* vehicle control. * $p < 0.05$ *vs.* S100B treatment only.

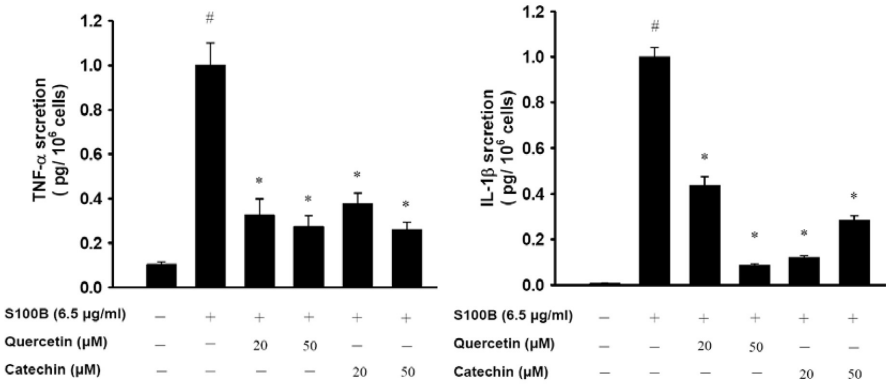

Figure 2. Effects of quercetin and catechin on TNF-α and IL-1α secretion in THP-1 cells induced by S100B. Values are means ± SD of three experiments. All appropriate controls and standards, as specified by the manufacturer were used, and the data are expressed as picograms TNF- secreted by 1 million cells. # $p < 0.05$ *vs.* vehicle control. * $p < 0.05$ *vs.* S100B treatment only.

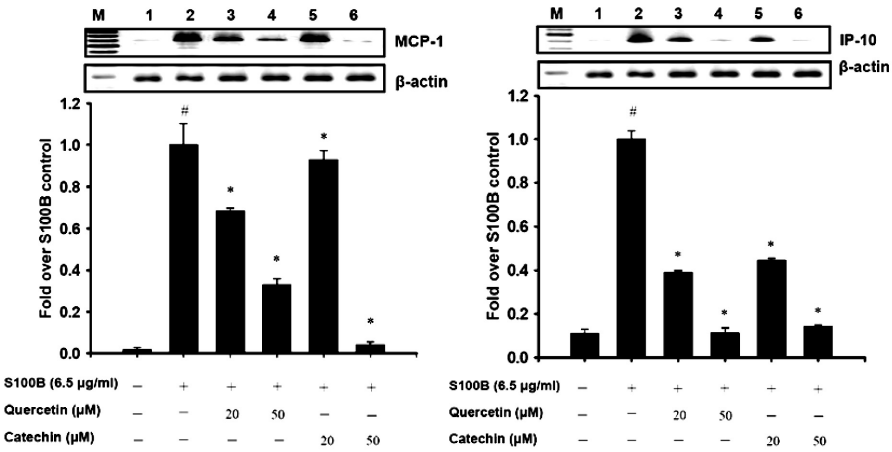

Figure 3. Effects of quercetin and catechin on the expression of pro-inflammatory MCP-1 and IP-10 mRNA in THP-1 cells induced by S100B. Values are means ± SD of three experiments. Cells were cocultured with the indicated flavonoids (20 and 50 μM) and S100B protein (6.5 μg/mL) for 4 h. The figure shows ethidium bromide-stained agarose gels of RT-PCR products: 1, NG; 2, S100B treatment; 3, S100B plus quercetin (20 μM); 4, S100B plus quercetin (50 μM); 5, S100B plus catechin (20 μM); 6, S100B plus catechin (50 μM). # $p < 0.05$ *vs.* vehicle control. * $p < 0.05$ *vs.* S100B treatment only.

of quercetin and catechin resulted in significant inhibitory effects ($p < 0.05$).

3.2 Inhibitory effects of quercetin and catechin on the expression of chemokines and adhesion genes

Chemokines, such as monocyte chemoattractant protein-1 (MCP-1) and inducible protein-10 (IP-10), play an important role in monocyte chemotaxis and diabetic vascular complications, including atherosclerosis. Thus, the effects of quercetin and catechin on the expression of chemokine genes in THP-1 cells induced by S100B were evaluated. As shown in Fig. 3, S100B treatment of THP-1 cells for 4 h led to significantly increased expression of MCP-1 and IP-10 mRNA ($p < 0.05$). These results indicated that the induction of MCP-1 mRNA expression by S100B was significantly blocked by the addition of quercetin and catechin ($p < 0.05$). Furthermore, Fig. 3 indicated that IP-10 mRNA expression induced by S100B was also significantly blocked by the addition of quercetin and catechin ($p < 0.05$).

Some genes like PECAM-1 play an important role in the adhesion of monocytes to human aortic endothelia cells (HAECs) in the progression of diabetic complications such as atherosclerosis. Therefore, the effects of quercetin and catechin on the expression of adhesion gene PECAM-1, induced by S100B in THP-1 cells, were investigated. As shown in Fig. 4, the results indicated that S100B significantly induced PECAM-1 mRNA expression after 4 h of incubation ($p < 0.05$). However, S100B-induced THP-1

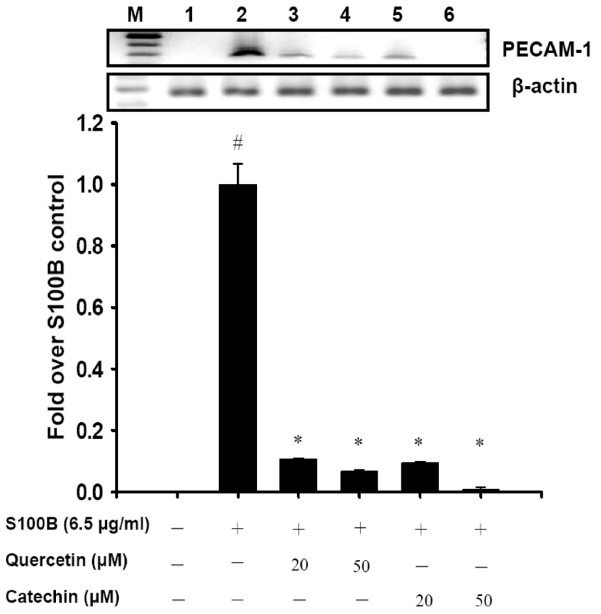

Figure 4. Effects of quercetin and catechin on the expression of adhesion PECAM-1 mRNA in THP-1 cells induced by S100B. Values are means ±SD of three experiments. Cells were treated with the indicated flavonoids (20 and 50 μM) in NG (5.5 mM glucose) or S100B medium for 4 h. The figure shows ethidium bromide-stained agarose gels of RT-PCR products: 1, NG; 2, S100B treatment; 3, S100B plus quercetin (20 μM); 4, S100B plus quercetin (50 μM); 5, S100B plus catechin (20 μM); 6, S100B plus catechin (50 μM). # $p < 0.05$ *vs.* vehicle control. * $p < 0.05$ *vs.* S100B treatment only.

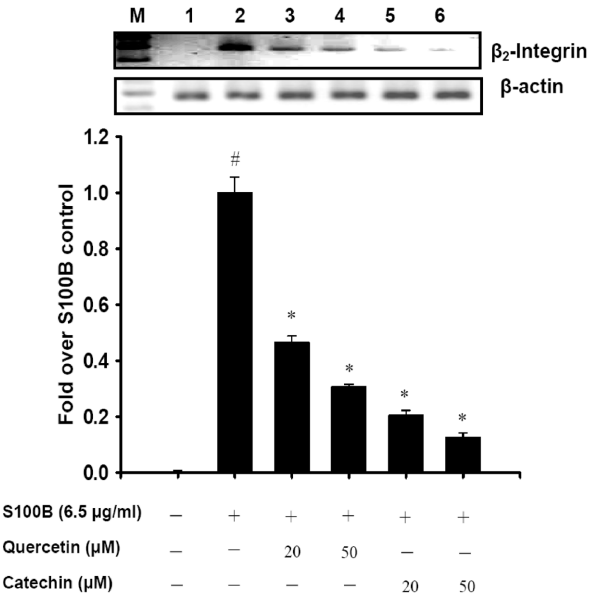

Figure 5. Effects of quercetin and catechin on the expression of β_2-Integrin mRNA in THP-1 cells induced by S100B. Values are means ± SD of three experiments. Cells were treated with the indicated flavonoids (20 and 50 μM) in NG (5.5 mM glucose) or S100B medium for 4 h. The figure shows ethidium bromide-stained agarose gels of RT-PCR products: 1, NG; 2, S100B treatment; 3, S100B plus quercetin (20 μM); 4, S100B plus quercetin (50 μM); 5, S100B plus catechin (20 μM); 6, S100B plus catechin (50 μM). # $p < 0.05$ *vs.* vehicle control. * $p < 0.05$ *vs.* S100B treatment only.

PECAM-1 mRNA expression was significantly inhibited by the addition of quercetin and catechin.

3.3 Inhibitory effects of quercetin and catechin on the expression of integrin genes and cyclooxygenase-2 (COX-2)

As illustrated in Fig. 5, β_2-integrin mRNA was markedly induced by S100B in THP-1 cells at 4 h ($p < 0.05$). These results showed that β_2-integrin mRNA expression was significantly ($p < 0.05$) blocked by 20 and 50 μM of quercetin and catechin in a dose-dependent manner. These results indicated that quercetin and catechin could suppress expression of β_2-integrin mRNA, which is relevant to monocyte activation.

AGEs can augment inflammatory responses by up-regulating COX-2 *via* RAGE and multiple signaling pathways, thereby leading to monocyte activation and vascular cell dysfunction. Thus, the effects of quercetin and catechin on the expression of COX-2 mRNA induced by S100B in THP-1 cells were investigated. As shown in Fig. 6, RT-PCR data clearly showed a significant induction of COX-2 mRNA in S100B-treated THP-1 cells ($p < 0.05$). S100B-induced COX-2 mRNA expression was transcriptionally inhibited by quercetin and catechin in a dose-dependent manner ($p < 0.05$). Thus, quercetin and catechin might exhibit the potential to modulate inflammation by inhibition of COX-2 transcription. COX-2 protein expression induced by S100B in THP-1 cells was also reduced by the addition of quercetin and catechin (Fig. 7).

3.4 Effects of quercetin and catechin on intercellular ROS generation

AGEs have been reported to alter the redox status of cells through the overproduction of ROS, leading to diabetic complications. The role of ROS as second messengers for inducing expression of various inflammatory genes in monocytes has been reported recently. In this study, we determined whether S100B protein induced a pro-oxidant environment in THP-1 cells, and estimated the inhibitory effects of quercetin and catechin on ROS production. As shown in Fig. 8, treatment with S100B protein in THP-1 cells after 2 h induced an increase of ROS indicated by DCF fluorescence ($p < 0.05$). However, a significant reduc-

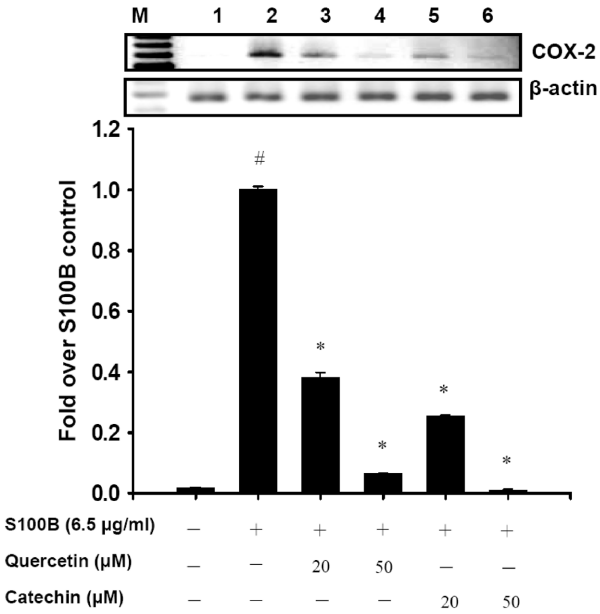

Figure 6. Effects of quercetin and catechin on the expression of COX-2 mRNA in THP-1 cells induced by S100B. Values are means ±SD of three experiments. Cells were treated with the indicated flavonoids (20 and 50 μM) in NG (5.5 mM glucose) or S100B medium for 4 h. The figure shows ethidium bromide-stained agarose gels of RT-PCR products: 1, NG; 2, S100B treatment; 3, S100B plus quercetin (20 μM); 4, S100B plus quercetin (50 μM); 5, S100B plus catechin (20 μM); 6, S100B plus catechin (50 μM). # $p < 0.05$ *vs.* vehicle control. * $p < 0.05$ *vs.* S100B treatment only.

tion of ROS was observed after the treatment with quercetin and catechin ($p < 0.05$).

3.5 Effects of quercetin and catechin on oxidative stress-sensitive protein expression

Increased oxidative stress has been reported both *in vitro* and *in vivo* in diabetic state *via* production of ROS. We further investigated the effects quercetin and catechin on oxidative stress-sensitive protein expression, including p47phox and PKC protein. As shown in Fig. 9, S100B treatment with THP-1 cells significantly elicited the activation of p47phox and PKC protein ($p < 0.05$), while quercetin and catechin showed significant inhibitory effects on the expression of S100B-induced p47phox protein. In contrast, the expression of PKC protein induced by S100B was not significantly inhibited by quercetin and catechin ($p < 0.05$).

3.6 Effects of quercetin and catechin on oxidative stress-responsive MAPK signal pathways

HG and diabetes have been shown to specifically activate MAPK pathways in human monocytes. In addition, the production of inflammatory cytokines such as TNF-α and MCP-1 by activated human monocytic cells was regulated by the MAPK pathway. Thus, the effects of quercetin and catechin on the expression of protein in S100B-activated MAPK signal pathways were further investigated. As shown in Fig. 10, S100B treatment of THP-1 cells elicited the activation of the three MAPK cascades. Significant activation of p38, ERK, and JNK were observed after 2 h of S100B-stimulation ($p < 0.05$). Furthermore, the expression of these MAPKs proteins was further confirmed in THP-1

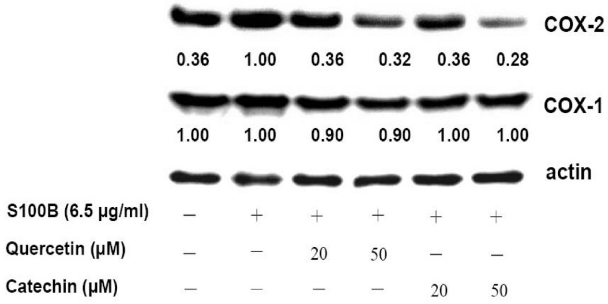

Figure 7. Effects of quercetin and catechin on the expression of COX-2 protein in THP-1 cells induced by S100B. Values are means ± SD of three experiments. Cells were treated with the indicated flavonoids (20 and 50 μM) in NG (5.5 mM glucose) or S100B medium for 4 h. The figure shows ethidium bromide-stained agarose gels of RT-PCR products: 1, NG; 2, S100B treatment; 3, S100B plus quercetin (20 μM); 4, S100B plus quercetin (50 μM); 5, S100B plus catechin (20 μM); 6, S100B plus catechin (50 μM). # $p < 0.05$ *vs.* vehicle control. * $p < 0.05$ *vs.* S100B treatment only.

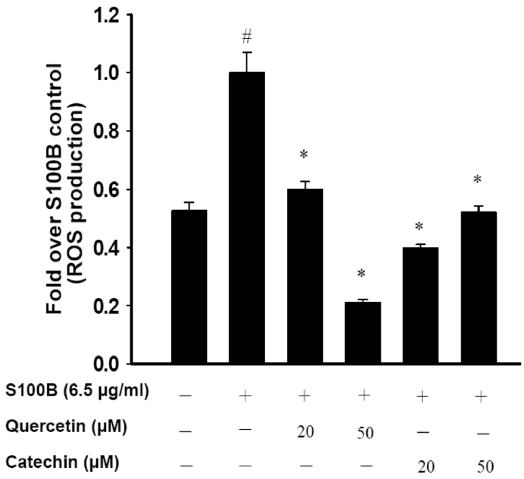

Figure 8. Flow cytometric histogram of DCFH-DA in THP-1 cells induced by S100B. Values are means ±SD of three experiments. Counts: cell number; FL1-H: relative DCFH-DA fluorescence intensity. Column bar graph of mean cell fluorescence for DCFH-DA, evaluate 2 h after S100B treatments. The fluorescence intensities in NG treatment cells are expressed as 100%. # $p < 0.05$ *vs.* vehicle control. * $p < 0.05$ *vs.* S100B treatment only.

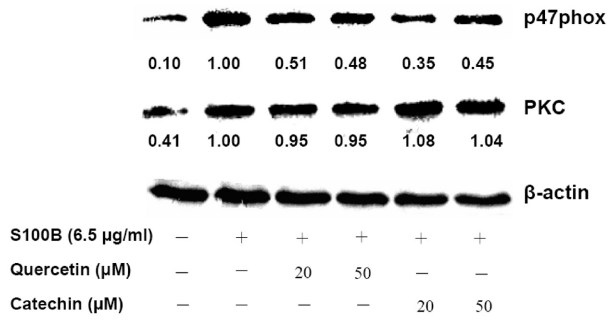

Figure 9. Effects of quercetin and catechin on the expression of p47phox and PKC protein in THP-1 cells induced by S100B. Values are means ± SD of three experiments. Total cell lysates of THP-1 cells were prepared after stimulating with S100B at 2 h. Proteins separated by SDS-PAGE electrophoresis were immunoblotted and probed with antibodies of p47phox, PKC, and β-actin.

cells with antibodies specific for phospho-p38, phospho-ERK, and phospho-JNK. These results indicated that quercetin showed a significant inhibitory effect against S100B-mediated ERK, phospho-ERK; JNK and phospho-JNK activated in THP-1 cells ($p < 0.05$). However, catechin had significant inhibitory effects against S100B-mediated p38, phospho-p38; JNK and phospho-JNK activation in THP-1 cells ($p < 0.05$).

4 Discussion

Hyperglycemia has been implicated in the accelerated vascular complications associated with diabetes [6–9, 23, 24]. Although the mechanisms leading to vascular complications are not fully understood, three pathways have been proposed: activation of protein kinase C isoforms, the polyol pathway, and AGEs formation [6, 25, 26]. Increasing evidence suggests that AGEs are one of the most important mechanisms of diabetic complications. AGEs are products of nonenzymatic glycation/oxidation of protein/lipids that accumulate during natural aging [5, 27], and is also greatly augmented in disorders such as diabetes and diabetic vascular complications, *e.g.*, retinopathy, nephropathy, and macrovascular disease [28]. The process of advanced glycation itself is not harmful, but rather only the products of it are [29]. RAGE (receptor for AGEs) is known to belong to the Ig superfamily of cell surface molecules. Ligands for RAGE such as S100B can produce key pro-inflammatory mediators in endothelium, mononuclear phagocytes, and lymphocytes, serving as valuable tools in the study of RAGE signaling [12]. Moreover, numerous studies have been published that have shown cells exposed to HG [15, 30] or AGEs [31, 32], conditions resulting in significant alterations in the expression of many genes including pro-inflammatory TNF-α, IL-1β, IL-6, platelet-derived growth factor, IGF-1, VCAM-1, and TF. Diabetic animal models [33] have shown that AGEs concentration increased within a few weeks after the animal was rendered diabetic and that this increase was systemic, occurring in the kidneys, skin, and vascular tissue.

AGEs can accumulate under diabetic conditions and contribute to the progression of diabetic inflammation. In the present study, we have demonstrated the effects of quercetin and catechin on the expression of pro-inflammatory cytokines and protein levels, including TNF-α and IL-1β induced by RAGE ligand S100B protein in THP-1 cells. Our results indicate that quercetin and catechin could regulate the diabetic inflammatory condition by decreasing TNF-α and IL-1β mRNA as well as protein expression induced by S100B in THP-1 cells (Figs. 1 and 2). This finding strongly suggests that quercetin and catechin show inhi-

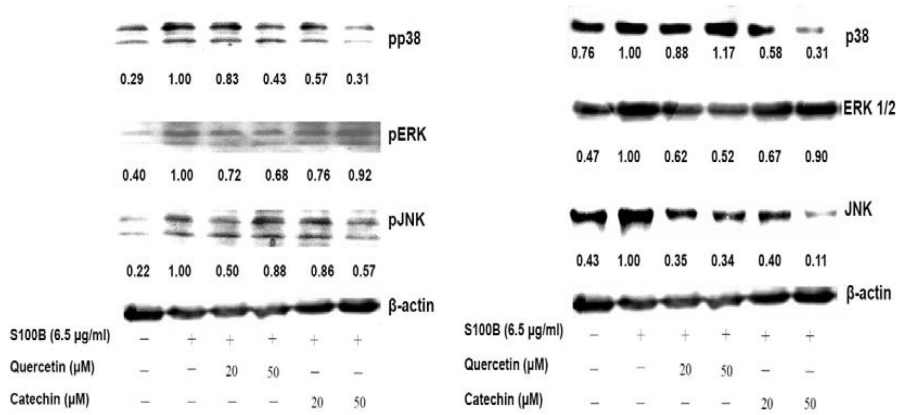

Figure 10. Effects of quercetin and catechin on the protein expression of total and phosphorylated forms of MAPK. Total cell lysates of THP-1 cells were prepared after stimulating with S100B at 2 h. Proteins separated by SDS-PAGE electrophoresis were immunoblotted and probed with antibodies to total and phosphorylated forms of MAPK and β-actin. Data shown are representative of three independent experiments.

bitory activity on pro-inflammatory cytokine expression under diabetic conditions.

It is particularly noteworthy that S100B significantly increased the expression of MCP-1 mRNA (Fig. 3), a chemoattractant member of the chemokine superfamily. MCP-1 is known to play a key role thought to be related to its chemotactic effects responsible for monocyte recruitment in acute and chronic inflammatiory states [34]. *In vitro* and *in vivo* studies with MCP-1 knockout mice have shown its importance in the development of atherosclerosis [17, 35]. We further evaluated the effects of quercetin and catechin on the expression of chemokine genes. The data showed that the effect of S100B on MCP-1 mRNA expression in THP-1 cells was significantly blocked by quercetin and catechin (Fig. 3).

IP-10, a CXC chemokine, is induced in response to INF stimulation and is regulated, at least in part, by NF-κB [36]. IP-10 regulation by HG is also relevant since it is a potent inflammatory chemokine for monocytes and vascular smooth muscle cells [15]. IP-10 has been implicated in islet cell dysfunction, and was recently shown to be increased in diabetic patients [37]. Increased serum concentrations of IP-10/CXCL10 were observed in both newly diagnosed Type I diabetic patients and in subjects at "high risk" of disease, as these concentrations correlated positively with those of IFN-γ [37, 38]. In the present study, S100B treatment of THP-1 cells led to the significantly increased expression of IP-10 mRNA. However, IP-10 mRNA expression induced by S100B protein was also significantly blocked by quercetin and catechin (Fig. 3). Our findings support that quercetin and catechin might have important roles in the regulation of monocyte chemotaxis by blocking the expression of chemokine mRNAs, including MCP-1 and IP-10.

Genes like PECAM-1 play an important role in the adhesion of monocytes to HAECs, in the progression of diabetic complications such as atherosclerosis [39]. We have noted significantly increased expression of PECAM-1 mRNA in THP-1 cells after S100B treatment (Fig. 4). However, S100B-induced THP-1 PECAM-1 mRNA expression was significantly inhibited by catechin (Fig. 4). Furthermore, RT-PCR analysis indicated that β2-integrin mRNA expression was significantly blocked by quercetin and catechin (Fig. 5). Diabetes is an established risk factor for the premature development of atherosclerosis [6]. Some previous studies have shown that patients with Type 2 diabetes exhibited increased circulating levels of serum adhesion molecules, and monocytes isolated from these subjects showed enhanced adhesion to endothelial cells. We found that HG conditions lead to significant induction of cytokines, such as TNF-α and IL-1β and their receptors; chemokines, such as MCP-1 and IP-10, PECAM-1; integrins, such as β2-integrin; and other genes, many of which were regulated by NF-κB [15]. However, our data further suggest that quercetin and catechin could block key genes relevant to monocyte activation and decrease the recruitment of monocytes into the intima of the arterial wall mediated through the expression of MCP-1, IP-10, PECAM-1, and β2-integrin.

COX-2 and its pro-inflammatory products, which are induced by oxidized lipids, have been implicated in the pathogenesis of atherosclerosis [40]. COX-2 has been indicated to promote the formation of early atherosclerotic lesions in low density lipoprotein receptor-deficient mice [41]. It has also been shown to play an important role in islet dysfunction related to the development of Type 1 diabetes [42]. Several reports have demonstrated that COX-2 is a highly inducible gene. It is particularly responsive to growth factors and mediators of inflammation such as TNF-α, IL-1β, IL-6, and LPS. AGE–RAGE interaction has been implicated in inflammatory responses in diabetes. AGEs can augment inflammatory responses by up-regulating COX-2 *via* RAGE and multiple signaling pathways, thereby leading to monocyte activation and vascular cell dysfunction [18]. In the present study, we have shown the effects of quercetin and catechin on the expression of COX-2 mRNA and protein levels induced by S100B. S100B-induced COX-2 mRNA and protein expression were significantly inhibited by quercetin and catechin (Figs. 6 and 7).

The formation and accumulation of AGEs adducts in various tissues are known to be a function of age and glycemia, and are able to generate reactive oxygen intermediates and induce cellular activation [7]. The progression of AGEs activation of signal-transducing RAGE has been linked to a pro-inflammatory condition in diabetes. Involvement of the mechanism includes reactive oxygen species (ROS), protein kinase C, and the MAPK family in AGE-mediated NF-κB transcription activation [6]. Furthermore, recent studies have reported that the involvement of PKC, oxidative stress, and p38 MAPK pathways were also activated by HG concentrations in THP-1 cells [15]. In our previous experiment (data not shown), we also demonstrated the effects of flavonoids on the expression of IκB nuclear protein induced by S100B in THP-1 monocytes. The data showed that S100B promoted IκB protein translocation into the nuclear of THP-1 cells. However, IκB nuclear translocation was reduced by flavonoid treatment in S100B-stimulated THP-1 cells. Production of inflammatory cytokines such as TNF-α and MCP-1 by activated human monocytic cells was regulated by the MAPK pathways. In the present study, we further investigated the effects of flavonoid on the expression of S100B stimulated molecule mechanisms of either oxidative stress-sensitive or oxidative stress-responsive MAPK signal pathways in THP-1 cells. As shown in Fig. 9, S100B treatment with THP-1 cells significantly elicited the activation of p47phox and PKC protein, while quercetin

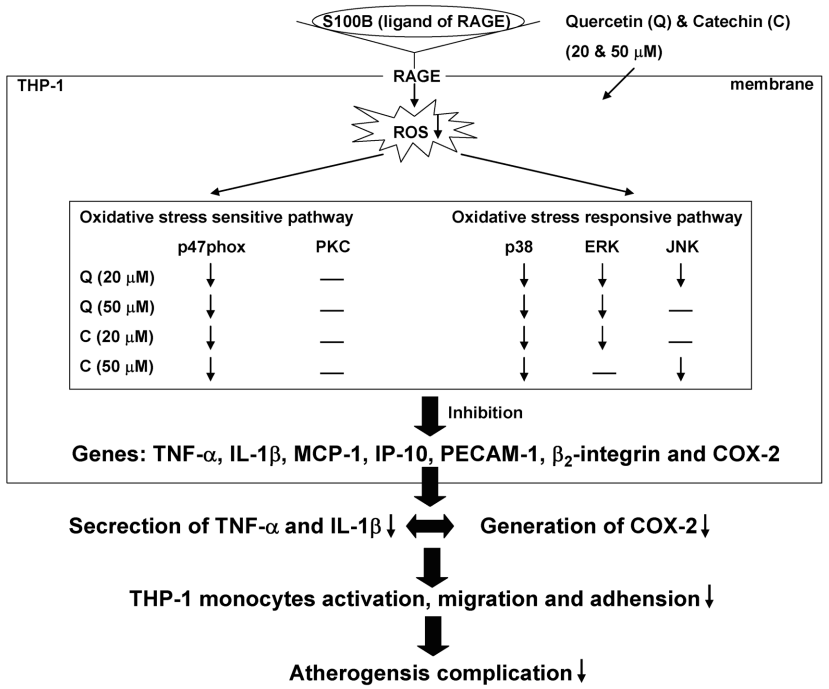

Figure 11. Regulation of pro-inflammatory reponses in THP-1 human monocytes by ligation of the receptor for advanced glycation end products (RAGE): Inhibitory effects by flavonoids and molecular mechanisms.

and catechin showed significant inhibitory effects on the expression of S100B-induced p47phox protein. In contrast, the expression of PKC protein induced by S100B was not significantly inhibited by quercetin and catechin. Thus, quercetin and catechin might directly regulate the expression of p47phox protein in oxidative stress-sensitive pathway to reduce superoxide production induced by S100B. The data indicated that S100B increased the activities of proteins of oxidative stress-sensitive pathways, including p47phox and PKC (Fig. 9). S100B also increased the activities of proteins of the oxidative stress-responsive signal pathways, including p38, ERK1/2, and JNK MAPK as indicated by the increased levels of the phosphorylated forms of MAPK (pp38, pERK1/2, and pJNK) (Fig. 10). Treatment of quercetin and catechin with S100B in THP-1 cells could regulate the expression of proteins of oxidative stress-sensitive and oxidative stress-responsive signal pathways (Figs. 9 and 10). We also found that S100B treatment led to a significant increase in ROS levels (Fig. 8). This meant that treatment with quercetin and catechin could eliminate ROS to reduce oxidative stress stimulated by S100B in THP-1 cells.

In conclusion, the present study shows that S100B could trigger a strong inflammatory response, leading to monocyte and inflammatory gene activation relevant to the pathogenesis of diabetes complications. As summarized in Fig. 11, our results are the first evidence that treatment with the naturally occurring flavonoids, quercetin, and catechin can inhibit the diabetic inflammatory condition by decreasing pro-inflammatory cytokine: TNF-α and IL-1β; chemokine: MCP-1 and IP-10; adhesion factor: PECAM-1; β_2-integrin, and COX-2 expression induced by S100B in THP-1 cells. Moreover, quercetin and catechin possess potent antioxidant abilities that may be able to regulate diabetic pro-inflammatory responses *via* the regulation of oxidative stress through oxidative stress-sensitive and oxidative stress-responsive signaling pathways.

This research work was partially supported by the National Science Council (NSC94-2313-B005-001), the Republic of China.

5 References

[1] Baisier, W. M., Labuza, T. P., *J. Agric. Food Chem*. 1992, *40*, 707 – 713.

[2] Chellan, P., Nagaraj, R. H., *Arch. Biochem. Biophys.* 1999, *368*, 98 – 104.

[3] Monnier, V. M., *J. Gerontol.* 1990, *45*, 105 – 111.

[4] Bonnefont-Rousselot, D., *Curr. Opin. Clin. Nutr. Metab. Care* 2002, *5*, 561 – 568.

[5] Brownlee, M., *Annu. Rev. Med.* 1995, *46*, 813 – 820.

[6] Brownlee, M., *Nature* 2001, *414*, 813 – 820.

[7] Vlassara, H., Palace, M. R., *J. Int. Med.* 2002, *251*, 87 – 101.

[8] Mullarkey, C. J., Edelstein, D., Brownlee, M., *Biochem. Biophys. Res. Commun.* 1990, *173*, 932 – 939.

[9] Ruderman, N., Williamson, J. R., Brownlee, M., *FASEB. J.* 1992, *6*, 2905 – 2914.

[10] Singh, R., Barden, A., Mori, T., Beilin, L., *Diabetologia* 2001, *44*, 129 – 146.

[11] Park, I. H., Yeon, I. S., Youn, J. H., Choi, J. E. *et al.*, *Mol. Immuno.* 2004, *40*, 1203 – 1211.

[12] Arolt, V., Peter, M., Erfurth, A., Wiesmann, M. *et al.*, *Eur. Neuropsychopharm.* 2003, *13*, 235 – 239.

[13] Lander, H. M., Tauras, J. M., Ogiste, J. S., Hori, O. *et al.*, *J. Biol. Chem.* 1997, *272*, 17810 – 17814.

[14] Yan, S. D., Schmidt, A. M., Anderson, G. M., *J. Biol. Chem.* 1944, *269*, 9889 – 9897.

[15] Shanmugam, N., Reddy, M. A., Guha, M., Natarajan, R., *Diabetes* 2003, *52*, 1256 – 1264.

[16] Guha, M., Bai, W., Nadler, J. L., Natarajan, R., *J. Biol. Chem*. 2000, *275*, 17728 – 17739.

[17] Jiang, Y., Beller, D. I., Frendl, G., Graves, D. T., *J. Immunol.* 1992, *148*, 2423 – 2428.

[18] Shanmugam, N., Kim, Y. S., Lanting, L., Natarajan, R., *J. Biol. Chem.* 2003, *278*, 34834 – 34844.

[19] Bierhaus, A., Hofmann, M. A., Ziegler, R., Naworth, P. P., *Cardiovasc. Res*. 1998, *38*, 586 – 600.

[20] Bonnefont-Rousselot, D., *J. Soc. Biol*. 2001, *195*, 391 – 398.

[21] Wu, C. H., Yen, G. C., *J. Agric. Food Chem.* 2005, *53*, 3167 – 3173.

[22] Bradford, M. M., *Anal. Biohcem.* 1976, *72*, 248 – 254.

[23] Lyons, T. J., Mrcp, M. D., *Am. J. Cardiol*. 1993, *71*, 26 – 31.

[24] Singleton, J. R., Smith, A. G., Russell, J. W., Feldman, E. L., *Diabetes* 2003, *52*, 2867 – 2873.

[25] Lee, A. Y., Chung, S. S., *FASEB J.* 1999, *13*, 23 – 30.

[26] Nishikawa, T., Edelstein, D., Du, X. L., Yamagishi, S.-I. *et al.*, *Nature* 2000, *404*, 787 – 790.

[27] Wolff, S. P., Jiang, Z. Y., Hunt, J. V., *Free Radic. Biol. Med.* 1991, *10*, 339 – 352.

[28] Takenaka, K., Yamagishi, S., Matsui, T., Nakamura, K., Imaizumi, T., *Curr. Neurovasc. Res*. 2006, *3*, 73 – 77.

[29] Kennedy, L., Baynes, J. W., *Diabetologia* 1984, *26*, 93 – 98.

[30] Galassetti, P. R., Iwanaga, K., Pontello, A. M., Zaldivar, F. P. *et al.*, *Am. J. Physiol. Endocrinol. Metab.* 2005, *290*, E833 – 839.

[31] Schmidt, A. M., Hori, O., Chen, J. X., Li, J. F. *et al.*, *J. Clin. Invest.* 1995, *96*, 1395 – 1403.

[32] Bierhaus, A., Illmer, T., Kasper, M., Luther, T. *et al.*, *Circulation* 1997, *96*, 2262 – 2271.

[33] Baynes, J. W., Thorpe, S. R., *Diabetes* 1999, *48*, 1 – 9.

[34] Gu, L., Tseng, S. C., Rollins, B. J., *Chem. Immunol*. 1999, *72*, 7 – 29.

[35] Rollins, B. J., *Mol. Med. Today.* 1996, *2*, 198 – 204.

[36] Marx, N., Mach, F., Sauty, A., Leung, J. H. *et al.*, *J. Immunol*. 2000, *164*, 6503 – 6508.

[37] Shimada, A., Morimoto, S. A., Kodama, K., Suzuki, R. *et al.*, *Diabetes Care* 2001, *24*, 510 – 515.

[38] Tomura, K., Narumi, S., *Int. J. Mol. Med.* 1999, *3*, 477 – 484.

[39] Gong, N., Chatterjee, S., *Mol. Cell Biochem*. 2003, *253*, 151 – 158.

[40] Inoue, H., Umesono, K., Nishimori, T., Hirata, T., Tanabe, T., *Biochem. Bioph. Res. Commun.* 1999, *254*, 292 – 298.

[41] Burleigh, M. E., Babaev, V. R., Yancey, P. G., Major, A. S. *et al.*, *J. Mol. Cell Cardiol*. 2005, *39*, 443 – 452.

[42] Tabatabaie, T., Vasquez-Weldon, A., Moore, D. R., Kotake, Y., *Diabetes* 2003, *52*, 1994 – 1999.

