ARTICLE
Journal of Cellular Biochemistry 112:2953–2965 (2011)

Journal of Cellular Biochemistry

Phytoestrogen Calycosin-7-*O*-β-D-Glucopyranoside Ameliorates Advanced Glycation End Products-Induced HUVEC Damage

Youhua Xu,[1] Liang Feng,[1] Shanshan Wang,[1] Quan Zhu,[1,2*] Jing Lin,[3] Chihan Lou,[1] Ping Xiang,[1] Bao He,[2] Zhaoguang Zheng,[1,2] Dan Tang,[1,2] and Guoying Zuo[3**]

[1] *State Key Laboratory of Quality Research in Chinese Medicine, Faculty of Chinese Medicine, Macau University of Science and Technology, Avenida Wai Long, Taipa, Macau*

[2] *Institute of Consun Co. for Chinese Medicine in Kidney Diseases, Guangdong Consun Pharmaceutical Group, Dongpeng Road 71, Guangzhou 510530, P. R. China*

[3] *Research Center for Natural Medicines, Kunming General Hospital, PLA, 212 Da Guan Road, Kunming 650032, P. R. China*

ABSTRACT

Vasculopathy including endothelial cell (EC) apoptosis and inflammation contributes to the high incidence of stroke and myocardial infarction in diabetic patients. The aim of the present study was to investigate the effect of calycosin-7-*O*-β-D-glucopyranoside (CG), a phytoestrogen, on advanced glycation end products (AGEs)-induced HUVEC damage. We observed that CG can significantly ameliorate AGEs-induced HUVEC oxidative stress and apoptosis. The ratio of SOD/MDA was significantly increased to the normal level by CG pretreatment. CG preincubation dramatically increased anti-apoptotic Bcl-2 while decreased pro-apoptotic Bax and Bad expressions as detected by immunocytochemistry. Moreover, CG ameliorated macrophage migration and adhesion to HUVEC; the monocyte chemotactic protein-1 and interleukin-6 levels in the culture supernatant were dramatically reduced by CG as determined by ELISA; the expressions of inflammatory proteins including ICAM-1, TGF-β1, and RAGE in both protein and mRNA levels were significantly reduced to the normal level by CG pretreatment as determined by immunocytochemistry and real-time RT-PCR. The intracellular investigation suggests that CG can reverse AGEs-activated ERK1/2 and NF-κB phosphorylation, in which estrogen receptors were involved in. Our results strongly indicate that CG can modulate EC dysfunction by ameliorating AGEs-induced cell apoptosis and inflammation. J. Cell. Biochem. 112: 2953–2965, 2011.

KEY WORDS: APOPTOSIS; CALYCOSIN-7-*O*-β-D-GLUCOPYRANOSIDE; DIABETES; ENDOTHELIAL CELL; INFLAMMATION

Diabetic nephropathy is a major complication of diabetes and a leading cause of end-stage renal failure. None of the currently available pharmacological interventions for diabetic nephropathy can completely forestall or reverse the progression of the disease. Several mechanisms have been postulated for the development of diabetic nephropathy, in which vascular endothelial

Abbreviations: AGEs; advanced glycation end products; AO; acridine orange; BSA; bovine serum albumin; CG; calycosin-7-*O*-β-D-glucopyranoside; EB; ethidium bromide; EC; endothelial cell; ER; estrogen receptor; ERK1/2; extracellular-signal regulated kinase 1/2; FBS; fetal bovine serum; HUVEC; human umbilical vein endothelial cell; ICAM-1; intercellular adhesion molecule-1; ICI; 182780; IL-6; interleukin-6; MAPKs; mitogen-activated protein kinases; MCP-1; monocyte chemotactic protein-1; MDA; malonaldehyde; NF-κB; nuclear factor-kappa B; OS; oxidative stress; PBS; phosphate-buffered saline; PD; PD98059; RAGE; receptor for advanced glycation end products; SOD; superoxidase dismutase; TGF-β1; transforming growth factor beta1.

Conflict of interest: The authors have no financial conflict of interest.

Grant sponsor: Science and Technology Development Fund of Macau; Grant number: FDCT: No.050; Grant sponsor: Macau University of Science and Technology Foundation; Grant sponsor: Guangdong Consun Group.

*Correspondence to: Quan Zhu, Institute of Consun Co. for Chinese Medicine in Kidney Diseases, Guangdong Consun Pharmaceutical Group, Dongpeng Road 71, Guangzhou 510530, P. R. China. E-mail: qzhu40@yahoo.com.cn

**Correspondence to: Guoying Zuo, Research Center for Natural Medicines, Kunming General Hospital, PLA, 212 Da Guan Road, Kunming 650032, P. R. China. E-mail: zuoguoying@263.net

Received 15 March 2011; Accepted 26 May 2011 • DOI 10.1002/jcb.23212 • © 2011 Wiley-Liss, Inc.

Published online 6 June 2011 in Wiley Online Library (wileyonlinelibrary.com).

cell (EC) damage has been demonstrated to play pivotal role in the development of vascular lesions in patients with renal dysfunction [Rabelink et al., 2010]. There is a recent report indicates that individuals with chronic kidney disease are more likely to die of cardiovascular causes than progress to end-stage renal disease [Rifkin and Sarnak, 2010].

As well known, oxidative stress (OS) plays pivotal role in the progression of diabetes [Quagliaro et al., 2005]. As one of the hallmarks of diabetes, OS per se can induce free radical damage to the DNA and finally cause cell apoptosis; furthermore, OS may up-regulate inflammatory proteins expression and exaggerate local inflammation status. Studies indicated that EC apoptosis and inflammation are of great significance in the progression of diabetic nephropathy, in which apoptosis may cause the loss of EC and lead to the vascular dysfunction [Bonetti et al., 2003], and inflammation may contribute to the loss of the non-adhesive property of the endothelium [Orasanu and Plutzky, 2009]. It is observed that the vascular inflammation with monocyte/macrophage adhesion within the vasculature is an early change in experimental diabetes [Orasanu and Plutzky, 2009].

Among factors which may lead to EC apoptosis and inflammation, advanced glycation end products (AGEs) have been recognized as one of the most important accomplices. AGEs, as the oxidative products, may induce OS, promote cell apoptosis, and finally induce both micro- and macro-vascular disease in diabetic patients [Lal et al., 2002; Orasanu and Plutzky, 2009; Dhar et al., 2011]. Furthermore, ligand binding to receptor for AGEs (RAGE) may trigger the activation inflammation-related cell signaling pathways such as mitogen-activated protein kinases (MAPKs) [Lander et al., 1997] and nuclear factor-kappa B (NF-κB). Therefore, exploring ways to ameliorate or reverse AGEs-mediated cell damage is of great significance.

Previously, we found that calycosin-7-*O*-β-D-glucopyranoside (CG), a phytoestrogen, has anti-apoptotic against AGEs-induced EC apoptosis [Tang et al., 2011]. Tang and colleagues demonstrated that a homologue of CG, calycosin, could bind with estrogen receptor (ER) and promote EC proliferation [Tang et al., 2010]. Choi et al. reported that CG could alleviate inflammation in rabbit osteoarthritis (OA) model [Choi et al., 2007]. Recently, we found that calycosin could ameliorate AGEs-induced inflammation in HUVEC (article in revision). The present study was designed to investigate the effect of CG on AGEs-induced EC damage; the potential mechanism was also studied.

MATERIALS AND METHODS

MATERIALS

Calycosin-7-*O*-β-D-glucopyranoside (CG, Fig. 1) was supplied by National Institutes for Food and Drug Control of China (Beijing, China). Transforming growth factor beta1 (TGF-β1), RAGE, p-ERK1/2, and p-NF-κB p65 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The ER antagonist, ICI182780 (ICI), and ERK1/2 inhibitor, PD98059 (PD), were obtained from Sigma (St. Louis, MO). Monocyte chemotactic protein-1 (MCP-1) and interleukin-6 (IL-6) ELISA detection kits, polyclonal intercellular adhesion molecule-1 (ICAM-1), Bad, Bax, and Bcl-2 antibodies were purchased from Boster (Wuhan, China); the SOD and MDA detection kits were from Jiancheng (Nanjing, China). All other reagents used were derived from commercial sources.

PREPARATION OF AGEs

Albumin-derived AGEs were prepared by incubating bovine serum albumin (BSA) with 50 mM D-glucose under sterile conditions in 5% CO_2/95% air at 37°C for 12 weeks as described [Ge et al., 2005]. Unincorporated glucoses were then removed by dialysis overnight against 0.01 M phosphate-buffer solution (PBS). Unmodified BSA was incubated under the same conditions without glucose as control. AGEs were stored at −20°C until use.

CELL CULTURE

Human umbilical vein endothelial cells (HUVECs) were obtained from American Type Culture Collection (ATCC; Manassas, VA) and maintained in low-glucose DMEM medium (Gibco) with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin in a cell incubator. Macrophages were freshly collected from mice abdomen before use as we previously described [Xu et al., 2010].

CELL VIABILITY ASSAY

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Roche Molecular Biochemicals, Laval, PQ, Canada) assay was performed to evaluate cell viability. Briefly, cells in exponential growth were seeded in 96-well flat-bottomed plates (Corning Costar Corporation, Cambridge, MA) and incubated overnight at 37°C. The cells were then treated with drugs for 48 h. The culture supernatant was collected for SOD/MDA detection. Then 100 μl of MTT (final concentration of 0.5 mg/ml) were added to each well and incubated at 37°C further for 4 h. The formazan crystals were dissolved in DMSO. The absorbance was determined with a plate reader (Molecular Devices Corporation, Sunnyvale, CA) at 550 nm. Absorbance values were normalized to the values obtained for the control cells to determine percentage survival.

To observe the morphological changes of apoptosis, cells were stained with acridine orange/ethidium bromide (AO/EB). HUVEC cells were grown on glass coverslips in six-well plates. At 80% confluence, cells were treated with drugs for 48 h. Then the cells were washed with PBS for one time and incubated with AO/EB working solution (100 μg/ml AO and 100 μg/ml EB in PBS). Then cells were observed under a fluorescence microscope. The cytoplasm and nucleus of normal cells were stained with bright green, whereas the apoptotic cells exhibited with red.

SOD/MDA DETERMINATION

HUVECs were treated with drugs for 48 h, and the culture supernatant was collected as described above. SOD and MDA levels were determined by SOD/MDA detection kits within 6 h according to the manufacturer's protocol.

ELISA ASSAY

HUVECs were treated with BSA (200 μg/ml), AGEs (200 μg/ml) [Ge et al., 2005], CG (10^{-8} M) + AGEs (200 μg/ml), or ICI182780 (ICI, 10^{-5} M) + CG (10^{-8} M) + AGEs (200 μg/ml) as indicated for 48 h and the culture supernatant was collected. MCP-1 and IL-6

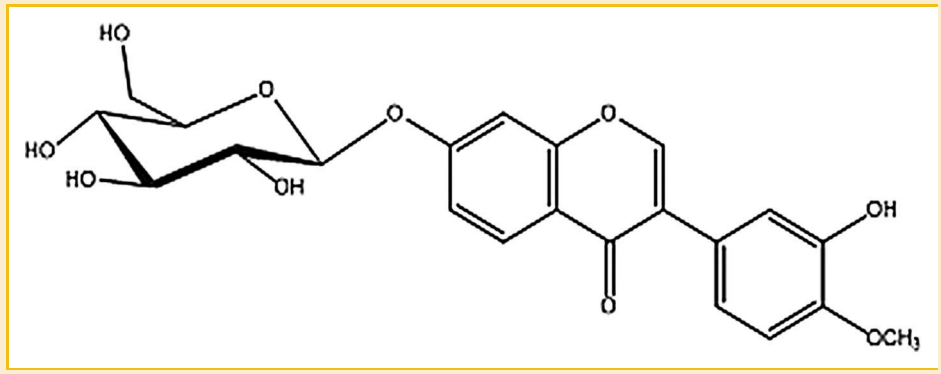

Fig. 1. The chemical structure of calycosin-7-*O*-β-D-glucopyranoside.

levels were immediately determined according to the manufacturer's protocol.

HUVEC-MACROPHAGE CO-CULTURE

To investigate the effect of CG on modulating EC inflammation in vitro, a HUVEC-macrophage co-culture system was applied as we previously reported [Xu et al., 2010]. In general, HUVECs were seeded on the glass coverslips in 24-well cell culture plate and incubated with BSA (200 μg/ml), AGEs (200 μg/ml) [Ge et al., 2005], or CG (10^{-8} M) + AGEs (200 μg/ml) for 24 h. Then the plates were inserted with Transwell inserts (5.0 μm pore size polycarbonate membrane, Corning Costar, Cambridge, MA); the macrophages were plated into the inserts and co-cultured with HUVECs for further 24 h. In the end, macrophages in the upper compartment were gently wiped out and the microporous membranes were stained with 10% crystal violet; after washing with PBS, the membranes were carefully clipped with razor blade (for microscopy observation) or washed with 30% glacial acetic acid (for macrophage counting determination at the wavelength of 580 nm).

HUVECs seeded on the coverslips in the lower compartment were performed with detection of macrophage adhesion and Immunocytochemistry assay of TGF-β1 expression.

IMMUNOCYTOCHEMISTRY AND IMMUNOFLUORESCENCE ANALYSIS

HUVECs were grown on glass coverslips in 24-well cell culture plates. For immunocytochemistry assay, the cells were incubated with drugs as indicated for 48 h and fixed with fresh 4% paraformaldehyde. The cells were then treated with primary antibodies (ICAM-1 (1:200), TGF-β1 (1:400), or RAGE (1:200)) followed by a sequential incubation with biotin-conjugated secondary antibodies and StreptAvidin–Biotin-enzyme Complex (SABC) or SABC-Alkaline Phosphatase (SABC-AP, for RAGE detection). Color was developed by 3,3′-diaminobenzidine (DAB) or 5-bromo-4-chloro-3-indolyl phosphate/nitrotetrazolium Blue chloride (BCIP/NBT, for RAGE detection); positive staining was indicated with brown or dark blue (for RAGE) deposits. The cells were counterstained with hematoxylin or nuclear fast red (for RAGE).

For immunofluorescence observation, HUVECs were permeabilized with 0.2% Triton X-100 on ice before 5% BSA blockade. The cells were incubated with p-ERK1/2 (1:50) or p-NF-κB p65 (1:50) antibodies in a humid chamber at 4°C overnight. Then cells were incubated with TRITC-conjugated secondary antibody at dark for 2 h. After that, the cells were mounted on a slide and visualized under fluorescence microscope.

In immunocytochemistry and immunofluorescence assay, the relative protein expression, and protein phosphorylation status were measured using the Image-pro Plus software (Media Cybernetics, MD). The relative protein expression was defined as a ratio of positively stained area to the total area under the microscope, and the relative protein phosphorylation status was defined as the mean fluorescence density of the total area. In negative controls, primary antibody was replaced with PBS.

REAL-TIME RT-PCR ANALYSIS

After 24 h drugs incubation as indicated, total RNAs were extracted from HUVECs by Trizol (Invitrogen, Carlsbad, CA) and reverse-transcribed using M-MLV RTase (Promega). For PCR reaction, a SYBR Green Q-PCR Mix was applied as per the manufacture's instructions. Primer sequences for TGF-β1, ICAM-1, and RAGE were synthesized as previous report [Xu et al., 2010] and were shown in Table I. The fluorescence threshold value was calculated to produce threshold cycle (Ct) value for each sample. The calculation of relative change in mRNA was performed using the delta–delta method, which corrections for the housekeeping gene β-actin.

STATISTICAL ANALYSIS

Data were expressed as means ± SD unless otherwise indicated. Statistical significance regarding multigroup comparisons was determined with SPSS 13.0 by one-way ANOVA. Differences were considered to be statistically significant at a P-value of <0.05.

RESULTS

CG INCREASED CELL ANTIOXIDANT ABILITY

It has been well established that AGEs-induced OS plays important role in vascular dysfunction [Quagliaro et al., 2005]. In the present study, we found that (Fig. 2) the anti-OS index, SOD/MDA was

TABLE I. A Summary of the Real-Time RT-PCR Primer Sequences Used to Amplify TGF-β1, ICAM-1 and RAGE

		Sequences	Gene bank accession numbers
TGF-β1	Sense	5′-GCCCTCCTACCTTTTGC-3′	NM_000660
	Antisense	5′-AGGCGTCAGCACCAGTAG-3′	
ICAM-1	Sense	5′-GTGGTAGCAGCCGCAGT-3′	NM_000201
	Antisense	5′-TTCGGTTTCATGGGGGT-3′	
RAGE	Sense	5′-CTGGTGCTGAAGTGTAAGGG-3′	NM_001136
	Antisense	5′-GAAGAGGGAGCCGTTGG-3′	

dramatically decreased in HUVEC incubated with AGEs compared with that incubated with BSA ($P < 0.05$). To assess if CG can protect HUVEC from OS damage, cells were pretreated with 10^{-9}–10^{-7} M of CG. As depicted in Figure 2, CG concentration-dependently reversed AGEs-decreased cell anti-oxidative ability to the normal level.

CG REVERSED AGEs-INDUCED CELL APOPTOSIS

It has been demonstrated that AGEs, as the oxidative products, can induce OS and finally promote cell apoptosis [Lal et al., 2002]. As depicted in Figure 3, 200 μg/ml of AGEs induced HUVEC apoptosis by 12.4 ± 3.0% as detected by MTT method ($P < 0.05$, vs. BSA). While pretreatment with CG ameliorated the apoptosis in a concentration-dependent manner, in which CG at the concentration of 10^{-8} and 10^{-7} M was shown with significant anti-apoptotic effect as compared with AGEs ($P < 0.05$, vs. AGEs).

To observe the morphological changes of the cells, HUVECs were stained with AO/EB solution and observed under a fluorescence microscope. As shown in Figure 4, cells treated with BSA (200 μg/ml) were stained with bright green, and no obvious red-staining was observed; while AGEs (200 μg/ml) treatment obviously increased the intracellular red staining (Fig. 4b), indicating more cell apoptosis appeared. As expected, pretreatment with 10^{-8} M of CG obviously reduced the red staining.

CG MODULATED BCL-2 FAMILY PROTEIN EXPRESSION IN AGEs-ACTIVATED HUVEC

Bcl-2 family proteins, including pro-apoptotic Bax and Bad and anti-apoptotic Bcl-2, have been demonstrated to play pivotal role in modulating cell apoptosis [Li et al., 1998]. In order to clarify if Bcl-2 family proteins were involved in CG-modulated cell function, their expressions were determined by immunocytochemistry. As shown in Figure 5A,B, AGEs dramatically increased Bad and Bax while decreased the anti-apoptotic Bcl-2 expressions and pre-incubation with CG significantly reversed AGEs' effect.

To evaluate the overall effect of CG on Bcl-2 family proteins, the ratio of Bcl-2/Bad was statistically calculated. As depicted in Figure 5B, pre-incubation with CG strikingly elevated the ratio as compared with AGEs; what is more importantly is that the ratio was even higher than BSA group ($P < 0.01$, vs. AGEs; $P < 0.05$, vs. BSA). It indicates that CG can reverse AGEs-induced cell apoptosis and may have cell proliferation effect.

CG AMELIORATED AGEs-INDUCED MACROPHAGE INFILTRATION TO HUVEC

It has been demonstrated that OS can induce cell inflammation status. Inflammatory cell infiltration within vasculature has been demonstrated to be an early change in experimental diabetes [Orasanu and Plutzky, 2009]. Choi and colleagues found that CG could alleviate inflammation in rabbit OA model [Choi et al., 2007].

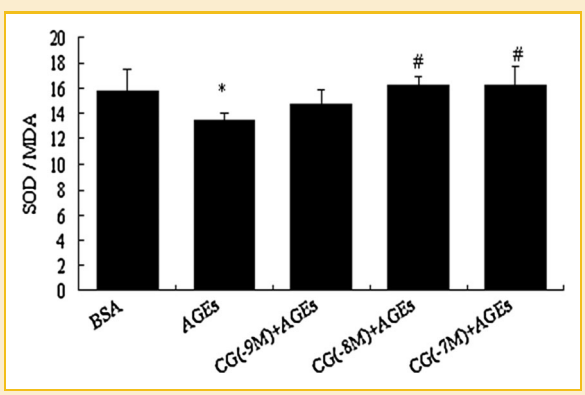

Fig. 2. CG increased HUVEC anti-oxidative ability. HUVECs were incubated with 200 μg/ml BSA, 200 μg/ml AGEs, or 10^{-9}–10^{-7} M CG + 200 μg/ml AGEs for 48 h. The culture supernatant was collected for SOD and MDA determination. Ratio of SOD/MDA was obtained to evaluate cell anti-oxidative ability. Data was expressed as means ± SD. The experiment was repeated for three times. $^{*}P < 0.05$, versus BSA; $^{\#}P < 0.05$, versus AGEs.

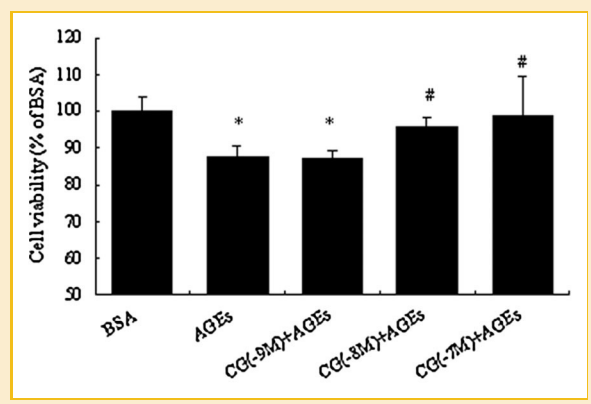

Fig. 3. The viability of HUVEC was increased after treatment with CG before AGEs. HUVECs were incubated with 200 μg/ml BSA, 200 μg/ml AGEs, or 10^{-9}–10^{-7} M CG + 200 μg/ml AGEs for 48 h and the MTT assay was performed to determine the cell viability. The formazan resolved by DMSO was calculated by microplate spectrophotometer at 580 nm. Results were expressed as means ± SD. $^{*}P < 0.05$, versus BSA; $^{\#}P < 0.05$, versus AGEs.

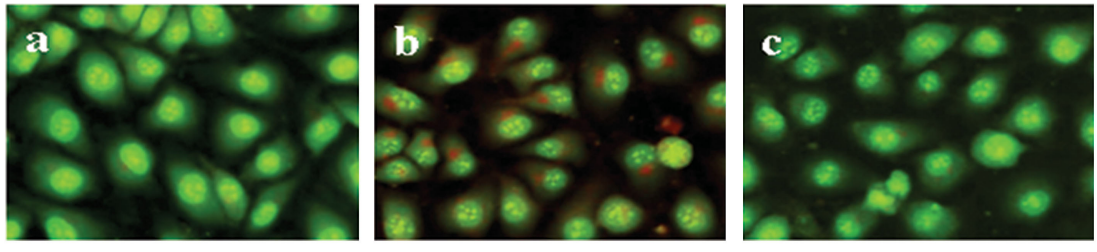

Fig. 4. CG protected HUVECs from AGEs-induced apoptosis. HUVECs were incubated with 200 μg/ml BSA, 200 μg/ml AGEs, or 10^{-8} M CG + 200 μg/ml AGEs for 48 h and HUVECs were stained with AO/EB solution. The red staining indicates cell apoptosis status. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

Previously, we (article in revision) and others [Chen et al., 2008] have observed a homologous compound of CG, calycosin, has significant effect against AGEs-induced EC inflammation. We postulate that CG may also have effect against AGEs-induced cell inflammation. To this end, a HUVEC-macrophage co-culture system was established as described in Material and Methods section.

As shown in Figure 6A,B, the amount of macrophage migration through Transwell membrane was strikingly increased in HUVECs incubated with AGEs in the lower compartment of the co-culture system as compared with BSA group ($P < 0.01$); while in cells pretreated with CG (Fig. 6A-d), the macrophage migration was significantly reduced to the normal level ($P < 0.01$, vs. AGEs; $P = 0.540$, vs. BSA). Interestingly, it was observed in the present study that in cells treated with CG alone (Fig. 6A-c,B), the macrophage migration was even lower than that of BSA group ($P < 0.05$, vs. BSA), indicating CG may have cell protective effect at normal condition.

To further observe if CG pretreatment can attenuate macrophage adhesion to HUVECs in the lower compartment, immunocytochemistry assay was carried out. As shown in Figure 5A, AGEs-induced more macrophage adhesion to HUVECs as compared with BSA. In parallel, preincubation with CG before AGEs obviously attenuated inflammatory cell adhesion.

INFLAMMATORY PROTEINS WERE INVOLVED IN CG-MODULATED INFLAMMATION

To explore the mechanism in CG-modulated macrophage infiltration, some inflammatory protein levels were determined. It has been well defined that MCP-1 plays pivotal role in the inflammation and inflammatory cell infiltration [Takada et al., 2010] and IL-6 is an important modulator in the inflammation and diabetic nephropathy [Navarro-González and Mora-Fernández, 2008]. To investigate the effects of CG on these two cytokines, their levels were determined by ELISA. As depicted in Figure 6C, AGEs dramatically increased MCP-1 and IL-6 levels in the culture supernatant ($P < 0.05$, vs. BSA), and pretreatment with CG before AGEs significantly decreased MCP-1 ($P < 0.01$) and IL-6 ($P < 0.05$) as compared with AGEs alone; what's more interesting is that CG downregulated MCP-1 to the level even lower than that of BSA group ($P < 0.05$). As CG is a phytoestrogen, to further investigate the role of ER in CG-modulated effects, the cells were pretreated with a non-selective ER inhibitor, ICI. Expectedly, ICI significantly reversed effects of CG on MCP-1 levels ($P < 0.01$, vs. CG + AGEs; $P < 0.01$, vs. BSA); though no significance was observed in IL-6 level after ER inhibition, the concentration of IL-6 was increased on ICI pretreatment (from 1529.71 ± 66.02 pg/ml in CG + AGEs group to 1566.65 ± 80.63 pg/ml in ICI + CG + AGEs group).

As well known, adhesion molecule ICAM-1 plays pivotal role in mediating inflammatory cell adhesion and TGF-β1 participates in AGEs-induced inflammation [Lu et al., 2007; Xu and Li, 2009]. By immunocytochemistry, we found that AGEs dramatically increased ICAM-1 and TGF-β1 expression (Fig. 7A,B), and pretreatment with CG reduced their expression to the normal level. In line with protein expression, CG pre-incubation reversed AGEs' effect on ICAM-1 and TGF-β1 mRNA expression (Fig. 7C, $P < 0.01$, vs. AGEs).

RAGE was known to mediate AGEs-induced cell damage [Goldin et al., 2006]. As shown in Figure 7, CG pretreatment dramatically ameliorated AGEs-induced RAGE expression at both protein and mRNA levels.

CG MODULATED EC INFLAMMATION VIA ERK1/2-NF-κB PATHWAY

ERK1/2 and NF-κB phosphorylation and nuclear translocation have been demonstrated to be key down-stream signals in AGEs-induced inflammation [Bianchi et al., 2011]. To further characterize the role of ERK1/2 and NF-κB in CG-modulated inflammation, their activation status was assayed by immunofluorescence. As shown in Figure 8A, AGEs induced ERK1/2 and NF-κB phosphorylation to a peak between 10 and 15 min in HUVEC; thereafter, their phosphorylation was obviously decreased. To observe the effect of CG on ERK1/2 and NF-κB activation, the cells were preincubated with CG before AGEs. As expected, AGEs-induced ERK1/2 (Fig. 8B,D-a) and NF-κB (Fig. 8C,D-b) activation was significantly inhibited by CG preincubation to the normal level.

A homolog of CG, calycosin, has been reported to competitively bind with ERs [Tang et al., 2010]. We postulate that CG, as a phytoestrogen, may also function via interaction with ERs. To this end, the cells were pretreated with a non-selective ERs inhibitor, ICI, before CG and AGEs. As observed, blockade of ERs obviously inhibited CG's effect on modulating ERK1/2 (Fig. 8B,D-a) and NF-κB (Fig. 8C,D-b) phosphorylation and nuclear translocation. To further investigate if AGEs-induced NF-κB activation was via ERK1/2 pathway, the cells were preincubated with a selective ERK1/2 antagonist, PD98059, before AGEs. As expected, NF-κB phosphorylation and nuclear translocation were decreased by ERK1/2

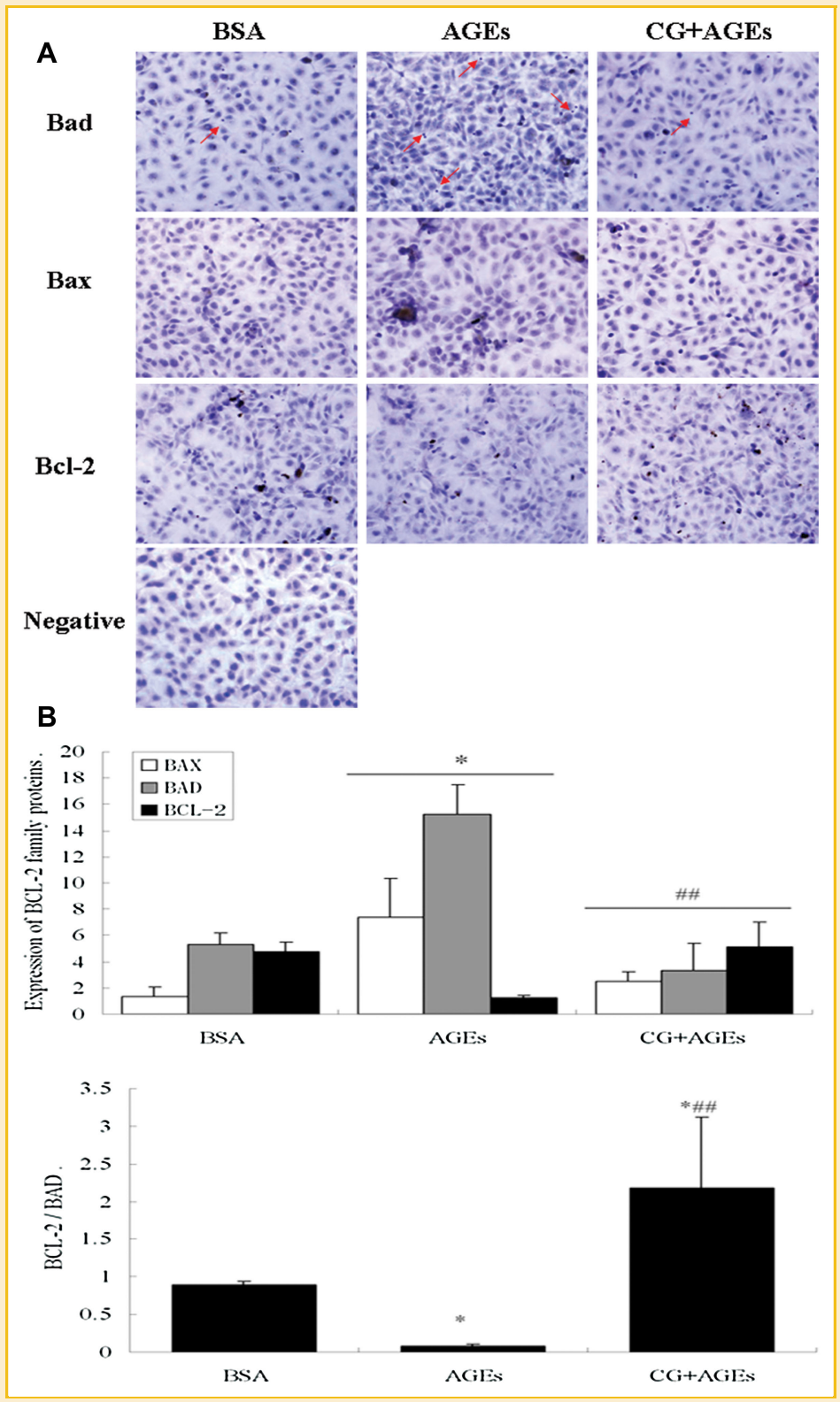

Fig. 5. CG modulated Bcl-2 family proteins. HUVECs were treated with 200 μg/ml BSA, 200 μg/ml AGEs, or 10^{-8} M CG + 200 μg/ml AGEs for 48 h and Bcl-2 family proteins were detected. A: CG incubation increased Bcl-2 while decreased Bad and Bax expression and macrophage adhesion. Bcl-2 family protein expression was detected by immunocytochemistry SABC method. The cells were counterstained with hematoxylin and the positive expression appeared brown due to DAB colorimetric reaction. The red arrows show macrophage adhesion to HUVEC. Primary antibody was replaced with PBS in negative control. (Magnification, 200×). B: The positive staining was quantitated by Image-Pro Plus software and the difference between groups was compared. $^{*}P < 0.05$, versus BSA; $^{\#\#}P < 0.01$, versus AGEs. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

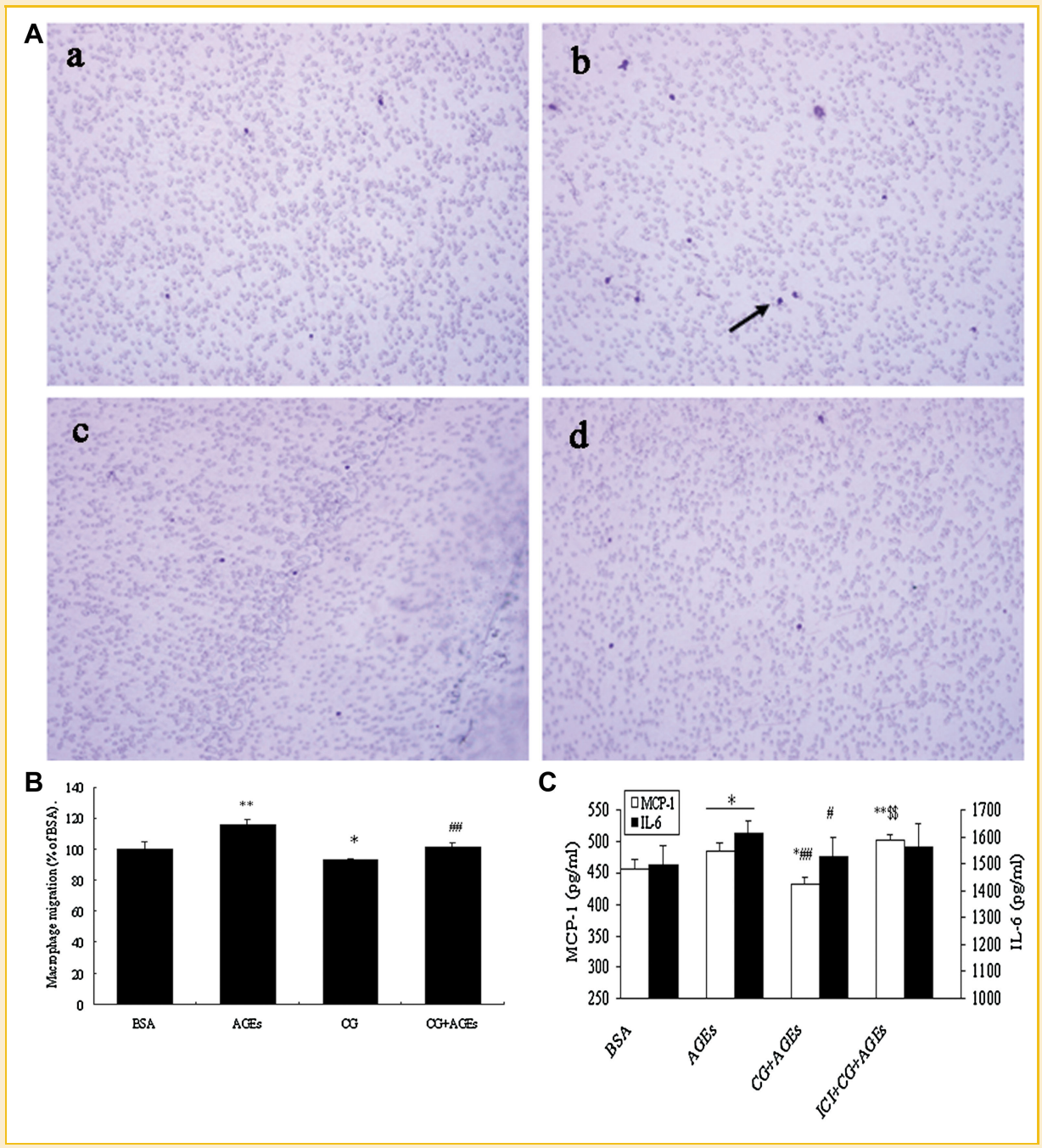

Fig. 6. CG treatment decreased macrophage migration to HUVEC as detected by crystal violet staining (A) or chromatometry (B) and downregulated MCP-1 and IL-6 levels (C). HUVECs at lower compartment were incubated with 200 μg/ml BSA (A-a), 200 μg/ml AGEs (A-b), 10^{-8} M CG (A-c), or 10^{-8} M CG + 200 μg/ml AGEs (A-d) and macrophages migrated through the membrane (indicated by black arrow) were stained by 10% crystal violet and observed under microscope (magnification, 200×). For macrophage counting (B), the crystal violet was washed out by 30% glacial acetic acid and the washing solution was detected at wavelength of 580 nm, the data was expressed as means ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, versus BSA; $^{\#\#}P < 0.01$, versus AGEs. The experiments of macrophage migration assay were repeated for three independent times, and representative pictures were shown. C: HUVECs were treated with BSA (200 μg/ml), AGEs (200 μg/ml), CG (10^{-8} M) + AGEs (200 μg/ml), or ICI (10^{-5} M) + CG (10^{-8} M) + AGEs (200 μg/ml) as indicated for 48 h, MCP-1 and IL-6 levels in the culture supernatant were determined by ELISA method. $^{*}P < 0.05$, $^{**}P < 0.01$, versus BSA; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, versus AGEs; $^{\$\$}P < 0.01$, versus CG + AGEs. The experiment was repeated for six independent times. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

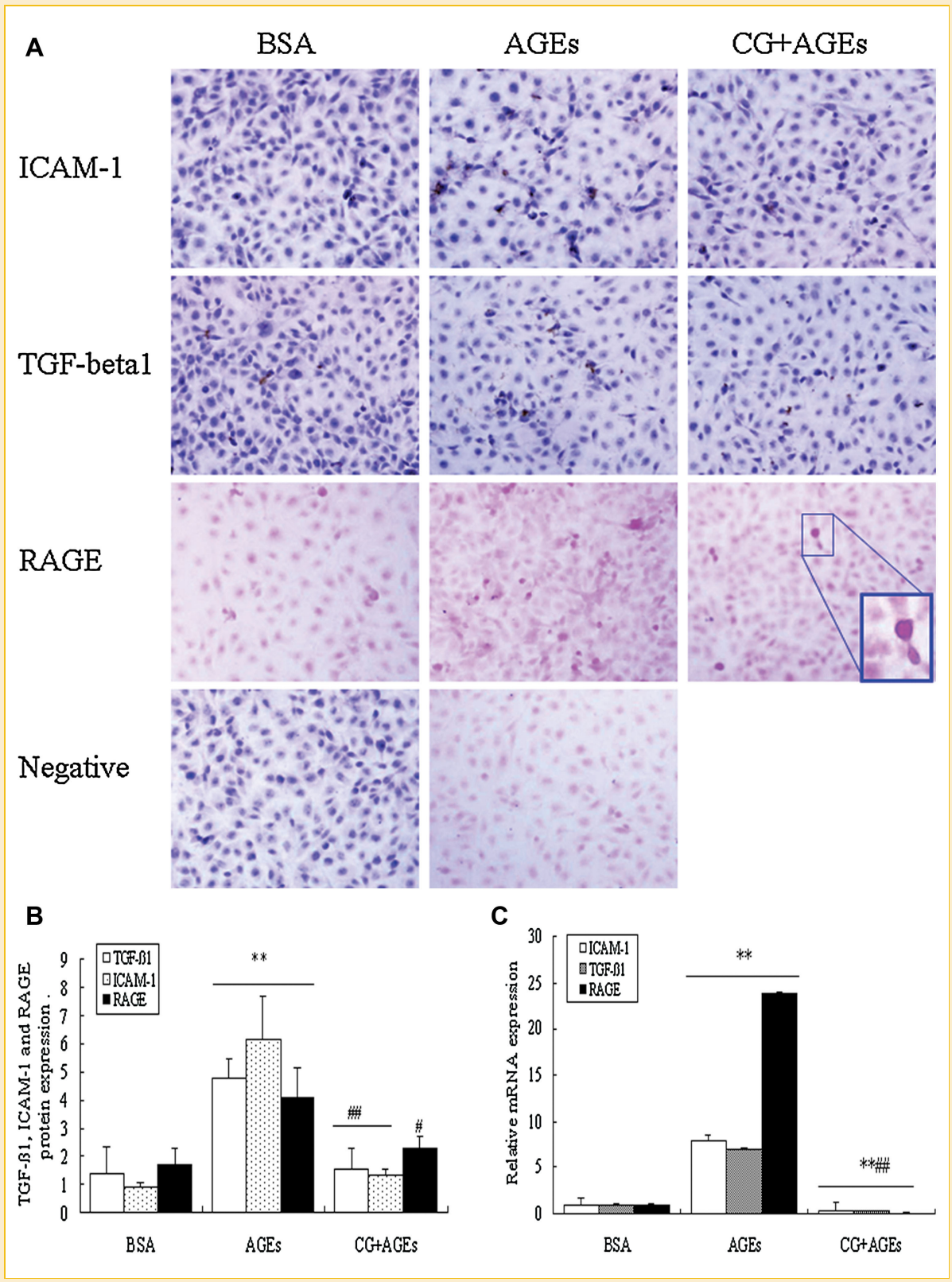

Fig. 7. CG decreased inflammatory proteins over-expression induced by AGEs. A: HUVECs were incubated with 200 μg/ml BSA, 200 μg/ml AGEs, or 10^{-8} M CG + 200 μg/ml AGEs for 48 h, protein expression of ICAM-1, TGF-β1, and RAGE were detected by Immunocytochemistry SABC or SABC-AP (RAGE) method. The cells were counterstained with hematoxylin or fast red (RAGE) and the positive expression appeared brown or dark blue (RAGE) due to DAB or BCIP/NBT (RAGE) colorimetric reaction. Primary antibodies were replaced with PBS as negative control. (Magnification, 200×). B: Protein expression of ICAM-1 and TGF-β1 were determined by Image-pro Plus and the difference among groups was compared. C: The mRNA expression of ICAM-1, TGF-β1, and RAGE were determined by real-time RT-PCR. $^{**}P < 0.01$, versus BSA; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, versus AGEs. The experiments were repeated for three independent times, and representative pictures were shown. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

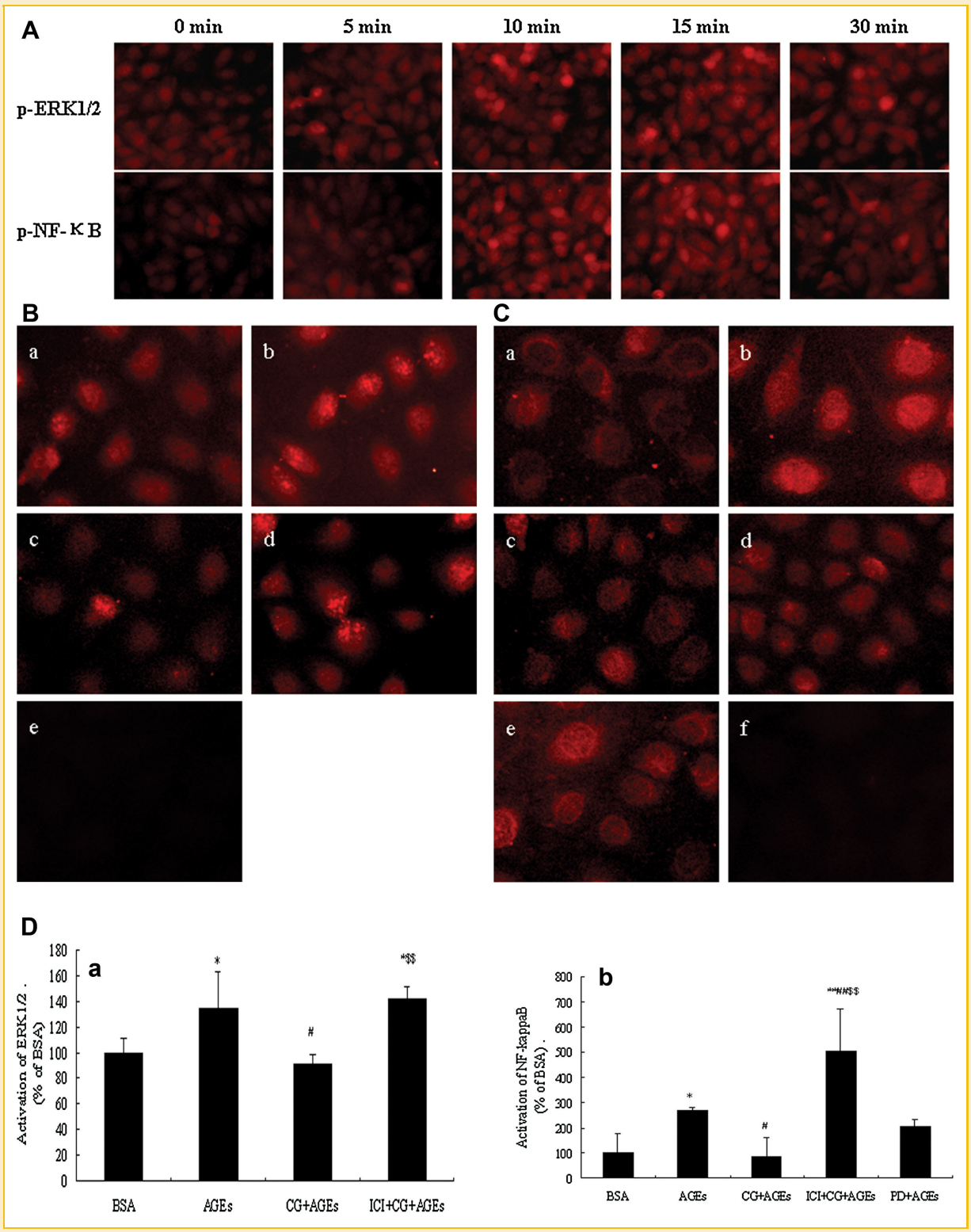

Fig. 8. CG modulated ERK1/2 and NF-κB activation in AGEs-activated HUVECs. A: Cells were incubated with 200 μg/ml AGEs for 0–30 min as indicated, the fluorescence-density of p-ERK1/2 and p-NF-κB was observed by immunofluorescence. B: Cells were treated with 200 μg/ml BSA (B-a), 200 μg/ml AGEs (B-b), 10^{-8} M CG + 200 μg/ml AGEs (B-c), or 10 μM ICI + 10^{-8} M CG + 200 μg/ml AGEs (Bd) for 15 min, and the fluorescence-density of p-ERK1/2 was observed; primary antibody was replaced with PBS in negative control (B-e). (C) Cells were treated with 200 μg/ml BSA (C-a), 200 μg/ml AGEs (C-b), 10^{-8} M CG + 200 μg/ml AGEs (C-c), 10 μM ICI + 10^{-8} M CG + 200 μg/ml AGEs (C-d), or 10 μM PD + 200 μg/ml AGEs (C-e) for 15 min, and the fluorescence-density of p-NF-κB p65 was observed; primary antibody was replaced with PBS in negative control (C-f). The experiment was repeated for at least three independent times and the representative pictures were shown (Magnification: 400×). D: Activation status of ERK1/2 (D-a) and NF-κB (D-b) was quantitatively analyzed by Image-pro Plus, and the difference between groups was compared. $^{*}P < 0.05$, $^{**}P < 0.01$, versus BSA; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, versus AGEs; $^{\$\$}P < 0.01$, versus CG + AGEs. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

blockade (Fig. 8C), although no statistical significance was observed (Fig. 8D-b).

DISCUSSION

Renal dysfunction is very common in patients with diabetes. Several mechanisms have been postulated for the development of diabetic nephropathy, in which vascular EC damage and dysfunction has been demonstrated to play pivotal role [Rabelink et al., 2010]. It is well defined that vascular dysfunction contributes to the high incidence of stroke and myocardial infarction in diabetic patients [Rahman et al., 2007]. AGEs have been widely studied in its key role in promoting vascular dysfunction and diabetes development [Orasanu and Plutzky, 2009]. Previously, we found and reported that calycosin could ameliorate AGEs-induced apoptosis [Tang et al., 2011] and inflammation (article in revision) in EC. In the present study, we reported for the first time that a homolog of calycosin, calycosin-7-*O*-β-D-glucopyranoside (CG), can also protect EC from AGEs-induced apoptosis and inflammation.

Oxidative stress (OS) is a known causative factor in renal damage and is thought to be involved in apoptosis. AGEs, as the oxidative products, can induce OS, promote cell apoptosis, and finally induce both micro- and macro-vascular disease in diabetic patients [Lal et al., 2002; Orasanu and Plutzky, 2009]. Quagliaro and colleagues demonstrated that AGEs can stimulate EC production of superoxide [Quagliaro et al., 2005], which may induce free radical damage to the DNA and finally cause cell apoptosis. SOD and MDA levels have been widely applied in evaluating cell anti-OS ability, in which SOD as an anti-OS factor and MDA represents cell OS damage. To evaluate effect of CG on protecting cells from OS, the ratio of SOD, and MDA, SOD/MDA, was determined to evaluate cell OS damage in general. In line with previous reports that phytoestrogen can protect cells from OS damage [Hsieh et al., 2011], here we observed that CG reversed AGEs-induced OS in HUVECs.

It has been well defined that OS per se can induce DNA damage and finally results in the abnormal EC apoptosis. Apoptosis plays an important role in renal development, physiology, and pathology. Studies indicated that EC apoptosis is of great significance in the progression of diabetic nephropathy, in which it can cause the loss of EC, the reduction of the endothelium non-adhesive property, and the occurrence of the vascular dysfunction [Bonetti et al., 2003]. In the present study, we found that 200 μg/ml of AGEs significantly induced HUVECs apoptosis as compared with control and CG pretreatment protected the cell from AGEs-induced damage.

Much of the recent understanding concerns the molecular regulation of cell survival or death by the Bcl-2 multigene family, including the inhibitors (for example, Bcl-2, Bcl-X_L, and BCL-1) or accelerators of apoptosis (e.g., Bax and Bad). To further investigate the mechanism of CG on ameliorating AGEs-induced cell apoptosis, the expressions of Bcl-2 family proteins were studied. Previously, we have demonstrated that AGEs could induce EC apoptosis, in which Bcl-2 was down-regulated and Bax and Bad were up-regulated [Wang et al., 2011]. Si and colleagues have demonstrated that phytoestrogen genistein protects human vascular ECs against tumor necrosis factor-alpha-induced apoptosis and enhances expression of Bcl-2 protein [Si and Liu, 2009]. In the present study, we firstly found that CG modulated Bcl-2 family protein expression and exhibited an anti-apoptosis property. In fact, the balance of Bcl-2 family proteins per se can protect cell from apoptosis by reducing cell OS. Bcl-2 polypeptides localize to intracellular sites where reactive oxygen species are generated, including membranes of the mitochondria, nuclei, and endoplasmic reticulum, and they have been shown to inhibit apoptosis via an anti-oxidant pathway that prevents cellular damage by reactive oxygen species [Hockenbery et al., 1993].

Because both increased OS and low grade inflammation are hallmarks of diabetes and OS per se can promote the inflammation progression, the effect of CG on modulating the local inflammation status was studied. The macrophages migration and adhesion to EC is a key step in initiating the local inflammation process. Reports indicated that recruitment of monocyte/macrophage and lymphocytes from the peripheral blood to EC is an early and central event in vascular dysfunction development [Orasanu and Plutzky, 2009] and experimental diabetes [Miyamoto et al., 1999]. To investigate the effect of CG on modulating EC inflammation, an in vitro EC-macrophage co-culture system was applied as we previously reported [Xu et al., 2010]. As expected, CG significantly inhibited AGEs-induced macrophage infiltration to EC. This is in line with previous reports. Cheng and colleagues demonstrated that the phytoestrogen can ameliorate IL-1 induced chondrocytes inflammation via NF-κB pathway [Cheng et al., 2010]. It has been well defined that MCP-1 and IL-6 play pivotal role in the inflammatory cell infiltration [Takada et al., 2010] and diabetic nephropathy [Navarro-González and Mora-Fernández, 2008]. In the present study, we observed CG significantly decreased MCP-1 and IL-6 levels via ER. We draw that inflammatory cytokines are of great significance in AGEs-induced EC inflammation, and downregulating their expression may effectively ameliorate the inflammatory cell infiltration and inflammation status.

Adhesion molecules play pivotal in controlling the influx of inflammatory cells to EC. ICAM-1, also known as CD54, is considered as a hallmark in the etiology of atherosclerosis and other vasculitis [Gimbrone, 1999; Xu and Li, 2009]. An increase of soluble form of ICAM-1 has been observed in diabetic patients [Becker et al., 2002] and some scholars even regarded it as a powerful independent predictor of type 2 diabetes in initially healthy people [Meigs et al., 2004]. In the present study, we found AGEs significantly up-regulated the expression of ICAM-1 in both mRNA and protein levels, and CG pretreatment ameliorated ICAM-1 expression to normal level. Chow et al. demonstrated that in ICAM-1-deficient db/db mice the development of renal dysfunction was strikingly attenuated [Chow et al., 2005]. Previous report has demonstrated the phytoestrogen genistein can down-regulate cytokine-induced ICAM-1 expression in human brain microvascular ECs and relieve the inflammation status [Lee and Lee, 2008]. We hypothesize the effect of CG on ameliorating AGEs-induced EC inflammation is at least partially via down-regulating ICAM-1 expression.

TGF-β1, which promotes the synthesis of matrix proteins within the basement membrane of EC at pathological status, has been well known in its pivotal role in diabetic-nephropathy progression.

Publications have shown that TGF-β1 expression at low levels may be effective in aiding repair of injured renal tubular epithelium after ischaemic injury [Basile et al., 1996]; while the maintenance of TGF-β1 expression after repair of renal epithelium is injurious, being associated with activation of fibroblasts and macrophages in the renal interstitium, deposition of collagen, and renal fibrosis [Border and Noble, 1997]. Recently, it is found that TGF-β1 can regulate inflammation by modulating MCP-1 [Qi et al., 2006], IL-1 [Lu et al., 2007] and NF-κB signaling pathways [Yang et al., 2010]. To investigate if CG has effect on TGF-β1, its expression was determined by immunocytochemistry and real-time RT-PCR. As expected, we observed a significant decrement of TGF-β1 expression on pretreatment of CG before AGEs-incubation in HUVEC. Converging with previous reports we presume that TGF-β1 participate in AGEs-induced macrophages migration and adhesion process.

AGEs impair vessel functions via RAGE [Goldin et al., 2006]. On activation, RAGE may mediate AGEs-induced cell damage, including cell apoptosis [Yamagishi et al., 2002] and inflammation [Figarola et al., 2007]. Furthermore, their accumulation within diabetic vasculature may contribute to accelerated atherosclerosis; on the other hand, OS per se contributes to the formation of AGEs that, on interacting with their receptors, produce further up-regulation of free radicals and result in altered gene expression [Zhang et al., 2003]. Tumur et al. reported that OS was increased under inflammatory conditions, and reduction of OS can decrease ICAM-1 and MCP-1 expression [Tumur et al., 2010]. Previously we found a homologue of CG, calycosin, can down-regulate the expression of RAGE in HUVEC (article in revision). In the present study, we found CG could also down-regulate AGEs-induced RAGE over-expression in HUVEC. Experimental evidence and observation strongly suggest RAGE signaling results in profound inflammation [Schmidt et al., 2001] and vascular injury [Zhou et al., 2003]. We hypothesize the down-regulation of RAGE expression may be a key junction that links AGEs-induced cell apoptosis and inflammation.

It has been well defined that OS per se can induce DNA damage and finally results in the abnormal EC apoptosis, thus CG may indirectly modulate Bcl-2 family proteins' expression via enhancing cell anti-OS ability. Furthermore, chronic inflammation can modulate Bcl-2 family proteins expression and induce cell apoptosis [Xiong et al., 2008], thus CG may indirectly downregulate Bax and Bad while upregulate Bcl-2 expression and inhibit HUVEC apoptosis via ameliorating cell inflammation. On the other hand, there was report indicating that phytoestrogen might decrease Bcl-2 while increase Bad and Bad expression and inhibit tumor cell proliferation [Ferenc et al., 2010]. Converging with the discussion above, we hypothesize CG mediated protective effect against HUVEC apoptosis in an indirect way, in which enhancing cell anti-OS ability and inhibiting AGEs-induced cell inflammation play important role.

Most recently, it is reported that ERK1/2 and NF-κB play pivotal role in AGEs-induced cell inflammation [Bianchi et al., 2011]. To further investigate the intracellular mechanism of CG on modulating AGEs-induced EC inflammation, their phosphorylation and nuclear translocation status were examined. As expected, CG pretreatment dramatically attenuated AGEs-induced ERK1/2 and NF-κB phosphorylation and nuclear translocation. This is in line with previous reports that phytoestrogen can significantly inhibit the activation of ERK1/2 [Liao et al., 2008] and NF-κB [Cheng et al., 2010]. Although there is a report indicates a phytoestrogen genistein can activate ERK1/2 [Fu et al., 2010], we presume this discrepancy may due to different disease model. It has been well defined that the activation of ERK1/2 is of great significance in the down-stream NF-κB activation. To illustrate this relationship, the cells (HUVEC) in the present study were firstly treated with ERK1/2 inhibitor, PD98059 (PD), before AGEs. As expected, we found that p-NF-κB p65 nuclear translocation was significantly inhibited by PD.

Most estrogen functions via the interaction with ERs. Previously, there is a report demonstrated a homologue of CG, calycosin, can specifically bind with ERs [Tang et al., 2010]. To investigate if CG modulates AGEs-induced cell damage via ERs, the cells were pretreated with a non-selective ERs antagonist, ICI, before CG and AGEs. As expected, blockade of ERs significantly inhibited the effects of CG on ERK1/2 and NF-κB activation. Converging with the above results and previous reports, we conclude CG modulated AGEs-induced inflammation via ERs-ERK1/2-NF-κB pathways.

In conclusion, we found a phytoestrogen, CG, could ameliorate AGEs-induced HUVEC apoptosis and inflammation. CG dramatically attenuated AGEs-induced cell OS, and significantly increased Bcl-2 while decreased Bax/Bad expression. Furthermore, CG pretreatment decreased EC inflammation status by down-regulating MCP-1, IL-6, ICAM-1, TGF-β1, and RAGE expressions via ERs-ERK1/2-NF-κB pathways. CG may be a potential therapeutic agent on ameliorating diabetic-vasculopathy.

ACKNOWLEDGMENTS

This work was supported by the Science and Technology Development Fund of Macau (FDCT: No.050), Macau University of Science and Technology Foundation and Guangdong Consun Group. We would like to thank Fei Gu from Guangdong Consun Pharmaceutical Group for kind help during the study.

REFERENCES

Basile D, Rovak J, Martin D, Hammerman M. 1996. Increased transforming growth factor-beta 1 expression in regenerating rat renal tubules following ischemic injury. Am J Physiol 270:F500–F509.

Becker A, van Hinsbergh VW, Jager A, Kostense PJ, Dekker JM, Nijpels G, Heine RJ, Bouter LM, Stehouwer CD. 2002. Why is soluble intercellular adhesion molecule-1 related to cardiovascular mortality? Eur J Clin Invest 32:1–8.

Bianchi R, Kastrisianaki E, Giambanco I, Donato R. 2011. S100B protein stimulates microglia migration via RAGE-dependent upregulation of chemokine expression and release. J Biol Chem 286:7214–7226.

Bonetti PO, Lerman LO, Lerman A. 2003. Endothelial dysfunction: A marker of atherosclerotic risk. Arterioscler Thromb Vasc Biol 23:168–175.

Border W, Noble N. 1997. TGF-beta in kidney fibrosis: A target for gene therapy. Kidney Int 51:1388–1396.

Chen HQ, Wang XJ, Jin ZY, Xu XM, Zhao JW, Xie ZJ. 2008. Protective effect of isoflavones from Trifolium pratense on dopaminergic neurons. Neurosci Res 62:123–130.

Cheng CC, Chen YH, Chang WL, Yang SP, Chang DM, Lai JH, Ho LJ. 2010. Phytoestrogen bavachin mediates anti-inflammation targeting Ikappa B

kinase-I kappaB alpha-NF-kappaB signaling pathway in chondrocytes in vitro. Eur J Pharmacol 636:181–188.

Choi SI, Heo TR, Min BH, Cui JH, Choi BH, Park SR. 2007. Alleviation of osteoarthritis by calycosin-7-*O*-beta-D-glucopyranoside (CG) isolated from Astragali radix (AR) in rabbit osteoarthritis (OA) model. Osteoarthritis Cartilage 15:1086–1092.

Chow FY, Nikolic-Paterson DJ, Ozols E, Atkins RC, Tesch GH. 2005. Intercellular adhesion molecule-1 deficiency is protective against nephropathy in type 2 diabetic db/db mice. J Am Soc Nephrol 16:1711–1722.

Dhar A, Dhar I, Jiang B, Desai KM, Wu L. 2011. Chronic methylglyoxal infusion by minipump causes pancreatic {beta}-cell dysfunction and induces type 2 diabetes in Sprague-Dawley rats. Diabetes 60:899–908.

Ferenc P, Solár P, Kleban J, Mikes J, Fedorocko P. 2010. Down-regulation of Bcl-2 and Akt induced by combination of photoactivated hypericin and genistein in human breast cancer cells. J Photochem Photobiol B 98:25–34.

Figarola JL, Shanmugam N, Natarajan R, Rahbar S. 2007. Anti-inflammatory effects of the advanced glycation end product inhibitor LR-90 in human monocytes. Diabetes 56:647–655.

Fu Z, Zhang W, Zhen W, Lum H, Nadler J, Bassaganya-Riera J, Jia Z, Wang Y, Misra H, Liu D. 2010. Genistein induces pancreatic beta-cell proliferation through activation of multiple signaling pathways and prevents insulin-deficient diabetes in mice. Endocrinology 151:3026–3037.

Ge J, Jia Q, Liang C, Luo Y, Huang D, Sun A, Wang K, Zou Y, Chen H. 2005. Advanced glycosylation end products might promote atherosclerosis through inducing the immune maturation of dendritic cells. Arterioscler Thromb Vasc Biol 25:2157–2163.

Gimbrone MA, Jr. 1999. Endothelial dysfunction, hemodynamic forces, and atherosclerosis. Thromb Haemost 82:722–726.

Goldin A, Beckman JA, Schmidt AM, Creager MA. 2006. Advanced glycation end products: Sparking the development of diabetic vascular injury. Circulation 114:597–605.

Hockenbery D, Oltvai Z, Yin X-M, Milliman C, Korsmeyer S. 1993. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell 75:241–251.

Hsieh HM, Wu WM, Hu ML. 2011. Genistein attenuates D-galactose-induced oxidative damage through decreased reactive oxygen species and NF-κB binding activity in neuronal PC12 cells. Life Sci 88:82–88.

Lal MA, Brismar H, Eklöf AC, Aperia A. 2002. Role of oxidative stress in advanced glycation end product-induced mesangial cell activation. Kidney Int 61:2006–2014.

Lander HM, Tauras JM, Ogiste JS, Hori O, Moss RA, Schmidt AM. 1997. Activation of the receptor for advanced glycation endproducts triggers a MAP kinase pathway regulated by oxidant stress. J Biol Chem 272:17810–17814.

Lee YW, Lee WH. 2008. Protective effects of genistein on proinflammatory pathways in human brain microvascular endothelial cells. J Nutr Biochem 19:819–825.

Li F, Ambrosini G, Chu EY, Plescia J, Tognin S, Marchisio PC, Altieri DC. 1998. Control of apoptosis and mitotic spindle checkpoint by surviving. Nature 396:580–584.

Liao QC, Li YL, Qin YF, Quarles LD, Xu KK, Li R, Zhou HH, Xiao ZS. 2008. Inhibition of adipocyte differentiation by phytoestrogen genistein through a potential downregulation of extracellular signal-regulated kinases 1/2 activity. J Cell Biochem 104:1853–1864.

Lu T, Tian L, Han Y, Vogelbaum M, Stark GR. 2007. Dose-dependent cross-talk between the transforming growth factor-beta and interleukin-1 signaling pathways. Proc Natl Acad Sci USA 104:4365–4370.

Meigs JB, Hu FB, Rifai N, Manson JE. 2004. Biomarkers of endothelial dysfunction and risk of type 2 diabetes mellitus. JAMA 291:1978–1986.

Miyamoto K, Khosrof S, Bursell SE, Rohan R, Murata T, Clermont AC, Aiello LP, Ogura Y, Adamis AP. 1999. Prevention of leukostasis and vascular leakage in streptozotocin-induced diabetic retinopathy via intercellular adhesion molecule-1 inhibition. Proc Natl Acad Sci USA 96:10836–10841.

Navarro-González JF, Mora-Fernández C. 2008. The role of inflammatory cytokines in diabetic nephropathy. J Am Soc Nephrol 19:433–442.

Orasanu G, Plutzky J. 2009. The pathologic continuum of diabetic vascular disease. J Am Coll Cardiol 53:S35–S42.

Qi W, Chen X, Polhill TS, Sumual S, Twigg S, Gilbert RE, Pollock CA. 2006. TGF-beta1 induces IL-8 and MCP-1 through a connective tissue growth factor-independent pathway. Am J Physiol Renal Physiol 290:F703–F709.

Quagliaro L, Piconi L, Assaloni R, Da Ros R, Maier A, Zuodar G, Ceriello A. 2005. Intermittent high glucose enhances ICAM-1, VCAM-1 and E-selectin expression in human umbilical vein endothelial cells in culture: The distinct role of protein kinase C and mitochondrial superoxide production. Atherosclerosis 183:259–267.

Rabelink TJ, de Boer HC, van Zonneveld AJ. 2010. Endothelial activation and circulating markers of endothelial activation in kidney disease. Nat Rev Nephrol 6:404–414.

Rahman S, Rahman T, Ismail AA, Rashid AR. 2007. Diabetes-associated macrovasculopathy: Pathophysiology and pathogenesis. Diabetes Obes Metab 9:767–780.

Rifkin DE, Sarnak MJ. 2010. Chronic kidney disease: Absence of evidence or evidence of absence? ACS treatment in CKD. Nat Rev Nephrol 6:449–450.

Schmidt AM, Yan SD, Yan SF, Stern DM. 2001. The multiligand receptor RAGE as a progression factor amplifying immune and inflammatory responses. J Clin Invest 108:949–955.

Si H, Liu D. 2009. Isoflavone genistein protects human vascular endothelial cells against tumor necrosis factor-alpha-induced apoptosis through the p38beta mitogen-activated protein kinase. Apoptosis 14:66–76.

Takada Y, Hisamatsu T, Kamada N, Kitazume MT, Honda H, Oshima Y, Saito R, Takayama T, Kobayashi T, Chinen H, Mikami Y, Kanai T, Okamoto S, Hibi T. 2010. Monocyte chemoattractant protein-1 contributes to gut homeostasis and intestinal inflammation by composition of IL-10-producing regulatory macrophage subset. J Immunol 184:2671–2676.

Tang JY, Li S, Li ZH, Zhang ZJ, Hu G, Cheang LC, Alex D, Hoi MP, Kwan YW, Chan SW, Leung GP, Lee SM. 2010. Calycosin promotes angiogenesis involving estrogen receptor and mitogen-activated protein kinase (MAPK) signaling pathway in zebrafish and HUVEC. PLoS One 5:e11822.

Tang D, He B, Zheng ZG, Wang RS, Gu F, Duan TT, Cheng HQ, Zhu Q. 2011. Inhibitory effects of two major isoflavonoids in radix astragali on high glucose-induced mesangial cells proliferation and AGEs-induced endothelial cells apoptosis. Planta Med 77:729–732.

Tumur Z, Shimizu H, Enomoto A, Miyazaki H, Niwa T. 2010. Indoxyl sulfate upregulates expression of ICAM-1 and MCP-1 by oxidative stress-induced NF-kappaB activation. Am J Nephrol 31:435–441.

Wang S, Xu Y, Feng L, Zhu Q, He B. 2011. A PKC-beta inhibitor prompts the HUVECs apoptosis-induced by advanced glycation end products. Pharmazie (in press).

Xiong Z, Song J, Yan Y, Huang Y, Cowan A, Wang H, Yang XF. 2008. Higher expression of Bax in regulatory T cells increases vascular inflammation. Front Biosci 13:7143–7155.

Xu Y, Li S. 2009. Blockade of ICAM-1: A novel way of vasculitis treatment. Biochem Biophys Res Commun 381:459–461.

Xu Y, Wang S, Feng L, Zhu Q, Xiang P, He B. 2010. Blockade of PKC-beta protects HUVEC from advanced glycation end products induced inflammation. Int Immunopharmacol 10:1552–1559.

Yamagishi S, Inagaki Y, Okamoto T, Amano S, Koga K, Takeuchi M, Makita Z. 2002. Advanced glycation end product-induced apoptosis and overexpression of vascular endothelial growth factor and monocyte chemoattractant protein-1 in human-cultured mesangial cells. J Biol Chem 277:20309–20315.

Yang L, Pang Y, Moses HL. 2010. TGF-beta and immune cells: An important regulatory axis in the tumor microenvironment and progression. Trends Immunol 31:220–227.

Zhang L, Zalewski A, Liu Y, Mazurek T, Cowan S, Martin JL, Hofmann SM, Vlassara H, Shi Y. 2003. Diabetes-induced oxidative stress and low-grade inflammation in porcine coronary arteries. Circulation 108:472–478.

Zhou Z, Wang K, Penn MS, Marso SP, Lauer MA, Forudi F, Zhou X, Qu W, Lu Y, Stern DM, Schmidt AM, Lincoff AM, Topol EJ. 2003. Receptor for AGE (RAGE) mediates neointimal formation in response to arterial injury. Circulation 107:2238–2243.