RESEARCH ARTICLE

Epigallocatechin gallate reduces vascular inflammation in *db/db* mice possibly through an NF-κB-mediated mechanism

Pon V. Anandh Babu, Hongwei Si * and Dongmin Liu*

Department of Human Nutrition, Foods and Exercise, College of Agriculture and Life Sciences, Virginia Tech, Blacksburg, VA, USA

Scope: Hyperglycemia-induced vascular inflammation resulting in the adhesion of monocytes to endothelium is a key event in the pathogenesis of atherosclerosis in diabetes. We investigated whether epigallocatechin gallate (EGCG), a major catechin found in green tea, reduces vascular inflammation in diabetes.

Methods and results: Human aortic endothelial cells (HAEC) were pretreated with green tea catechins before the addition of high glucose (25 mM) for 72 h. EGCG at physiologically achievable concentration (1 μM) significantly inhibited high glucose induced adhesion of monocytes to HAEC both in static and under flow conditions. EGCG also reduced nuclear factor κB (NF-κB) regulated transcriptional activity in ECs. Six-week-old diabetic *db/db* mice were fed a diet containing 0% or 0.1% EGCG for 8 weeks. ECs were isolated from aortic vessels of *db/db*, *db/db*-EGCG, and control *db/+* mice. EGCG supplementation greatly suppressed diabetes-increased monocytes adhesion to ECs, which is associated with reduced circulating levels of chemokines, and reduced secretions of chemokines and adhesion molecules by aortic ECs from *db/db*-EGCG mice. EGCG treatment reduced nuclear translocation of NF-κB p65 in aortic vessels, decreased blood pressure and serum concentrations of cholesterol and triglycerides in *db/db*-EGCG mice.

Conclusion: EGCG may have a direct protective effect against vascular inflammation in diabetes.

1 Introduction

Diabetes is a major risk factor for cardiovascular disease such as atherosclerosis, which accounts for the largest number of all deaths among American diabetic patients [1]. Inflammation-triggered endothelial activation is one of the key early events in the pathogenesis of atherosclerosis that leads to the adhesion of monocytes to the endothelium followed by their transmigration into the subendothelial space [2, 3]. In diabetes, hyperglycemia-induced vascular inflam-

mation and the subsequent endothelial dysfunction play a pivotal role in the development of atherosclerosis [1]. Indeed, enhanced monocyte–endothelial interactions are reported in diabetic animal models [4], diabetic patients [5], and in endothelial cells (ECs) exposed to high glucose in vitro [4]. High glucose can trigger several intracellular signaling events that ultimately upregulate the expression of a number

Correspondence: Dr. Dongmin Liu, Department of Human Nutrition, Foods and Exercise, College of Agriculture and Life Sciences, Virginia Tech, Blacksburg, VA 24060, USA
E-mail: doliu@vt.edu
Fax: +1-540-231-3916

Abbreviations: BAEC, bovine aortic endothelial cells; **DiO-Ac-LDL**, 3,3′-dioctadecyloxacarbocyanine perchlorate labeled acety-

lated low density lipoprotein; **ECs**, endothelial cells; **EC**, epicatechin; **ECG**, epicatechin gallate; **EGC**, epigallocatechin; **EGCG**, epigallocatechin gallate; **E-selectin**, endothelial-leukocyte adhesion molecule-1; **FBS**, fetal bovine serum; **HAEC**, human aortic endothelial cells; **HG**, high glucose; **ICAM-1**, intercellular adhesion molecule-1; **MAEC**, mouse aortic endothelial cells; **MCP-1**, monocyte chemotactic protein-1; **NF-κB**, nuclear factor κB; **TNF-α**, tumor necrosis factor α; **VCAM-1**, vascular adhesion molecule-1

*Current address: Department of Family and Consumer Sciences, Tennessee State University, Nashville, TN 37209, USA.

Mol. Nutr. Food Res. 2012, *56*, 1424–1432

1425

of pro-inflammatory chemokines such as interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1), and adhesion molecules such as vascular adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1(ICAM-1), and endothelial-leukocyte adhesion molecule-1 (E-selectin). The chemokines and adhesion molecules then induce monocyte adhesion to ECs, and subsequent transendothelial migration in the vessels [3, 4, 6, 7]. Nuclear factor κB (NF-κB) plays a major role in governing the vascular inflammatory process by directly upregulating these chemokines and adhesion molecules [8]. The most abundant form of NF-κB is a p50/p65 heterodimer in which p65 contains the transcriptional activation domain. NF-κB activation may arise from the increased nuclear translocation of the p65 subunit and high glucose was shown to increase nuclear levels of p65 [9]. Thus, attenuation of hyperglycemia-induced NF-κB activation could be a novel molecular target for the treatment or prevention of diabetic vascular inflammation.

Epigallocatechin gallate (EGCG) is a polyphenolic compound abundant in green tea and a number of studies reported the vasculoprotective effects of EGCG [10, 11]. Animal studies showed that EGCG improves endothelial function and reduces blood pressure in hypertensive rats [10]. A human study demonstrated that EGCG can reverse endothelial dysfunction and improve brachial artery flow-mediated dilation in patients with coronary artery disease [12]. The vascular beneficial effects of green tea and EGCG are often explained by their presumably antioxidative and hypolipidemic effects, although emerging evidence shows that catechins may exert vascular effects through other mechanisms [13]. Moreover, most of the reported studies used EGCG at doses that are far beyond the physiologically achievable levels (0.6–1.8 μM) in both humans and animals through dietary ingestion [13, 14]. Therefore, the biological relevance of these findings is largely unclear. Further, studies on the preventive effect of green tea or EGCG in diabetic vascular inflammation are very limited. In the present study, we investigated the role of EGCG at physiologically relevant concentrations in the prevention of high glucose induced monocyte–EC interaction ex vivo and further examined the effect of dietary intake of EGCG on diabetes-caused vascular inflammation in vivo.

2 Materials and methods

2.1 Materials

Primary human aortic endothelial cells (HAEC), bovine aortic endothelial cells (BAEC), endothelial growth supplements EGM2, and DMEM medium were purchased from Lonza (Walkersville, MD); THP-1 monocytes were purchased from ATCC (Manassas, VA) and WEHI78/24 mouse monocytes were provided by Dr. Judith A Berliner (UCLA). Fetal bovine serum (FBS), other cell culture supplements, transfection reagent lipofectamine, calcein-AM, and M199 and Opti-Mem media were from Invitrogen (Grand Island, NY); protein as-

say kits were from Bio-Rad (Hercules, CA); NF-κB promoter linked to firefly luciferase reporter gene was from Clontech (Mountain View, CA); dual luciferase reporter assay system was from Promega (Madison, WI); EGCG (for animal study) was from Taiyo International (Minneapolis, MN). ELISA kits for mouse MCP-1/JE, KC, VCAM-1, and ICAM-1 were from R&D Systems (Minneapolis, MN); fluorescent probe −3,3′-dioctadecyloxacarbocyanine perchlorate labeled acetylated low-density lipoprotein (DiO-Ac-LDL) from Biomedical Technologies Inc (Stoughton, MA); dispase was from BD Biosciences (San Jose, CA); nuclear extraction kit was from Epigentek (Farmingdale, NY); NF-κB p65 and β-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); chemiluminescence reagent was from Thermo Scientific (Waltham, MA); protease and phosphatase inhibitor cocktails, epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), and other general chemicals were from Sigma (St. Louis, MO). Stock solution of 20 mM EGCG in DMSO was stored at −80°C before use.

2.2 Cell culture

HAEC were cultured in M199 medium containing 2% FBS and endothelial growth supplements EGM2 at 37°C in a 5% CO_2/95% air environment and passages 4–6 were used in all experiments. THP-1 cells were cultured in RPMI-1640 medium containing 10% FBS. WEHI78/24 cells, a mouse monocyte cell line, were cultured in DMEM medium containing 10% FBS.

2.3 Monocyte adhesion assay

The determination of monocyte adhesion to ECs was conducted using THP-1 cells as described previously [15] with few modifications. In brief, HAEC were grown to confluence in 96-well plates and treated with EGCG or other structurally related catechins such as EC, ECG, and EGC for 1 h before addition of high glucose (25 mM) for 72 h. Calcein-AM labeled THP-1 cells were then added to HAEC and incubated for 1 h and the monolayer was gently washed with PBS to remove the unbound monocytes. The adhered monocytes were determined by measuring the fluorescence using a FLX800 multi-detection microplate reader (Bio-Tek Instruments, Inc) at excitation and emission wavelengths of 496 and 520 nm, respectively. Parallel experiments were conducted with 20 mM mannitol as osmotic control.

2.4 Monocyte adhesion assay under flow conditions

An automated microfluidic system, Bioflux 200 (Fluxion Biosciences, CA, USA), was used to monitor live EC–monocyte adhesion under flow condition, which simulates physiological conditions by precisely controlling shear flow. HAEC monolayers were grown on fibronectin-coated microfluidic

channels that were integrated into a 24-well plate. The cells were incubated with normal (5.5 mM) or high glucose (25 mM) for 72 h. To determine the EGCG effect, cells were preperfused with 1 μmol/L EGCG for 1 h at 1 dyn/cm² before addition of high glucose (25 mM). Seventy two hours later, THP-1 cells were then perfused into channels for 5 min at 3 dyn/cm², followed by 5 min at 1.5 dyn/cm². Images (20 fields per channel) were captured using a Nikon microscope under flow and the adhered immune cells were enumerated and the adhesion was calculated as number of cells/ field.

2.5 NF-κB transcriptional activity assay

BAEC were used for the transfection study, because it is difficult to yield adequate and consistent transfection efficiency for this construct in HAEC. BAEC plated in 24-well plate were cotransfected with 2 μg of NF-κB promoter-luciferase vector and pRL reporter control plasmid using Lipofectamine transfection reagent. Twenty-four hours after transfection, BAEC were treated with EGCG for 1 h before addition of high glucose (25 mM) for 72 h. Treated cells were then washed with PBS and lysed in reporter lysis reagent. Luciferase activity, normalized to pRL activity in the cell extracts, was determined by using the dual luciferase assay system.

2.6 Mice

Five-week-old male diabetic (*db/db*; B6.Cg-m+/+Lepr^db) mice were obtained from Jackson Laboratory. This is a widely used type 2 diabetic animal model that spontaneously develops metabolic features, including vascular complications similar to those observed in humans with diabetes. Age matched *db/+* mice were used as the controls. Mice were housed in micro-isolator cages in a pathogen-free facility. After 1-week acclimation, the diabetic mice were divided into two groups *db/db* and *db/db*-EGCG with 20 mice per group and fed AIN-93G rodent diet (Dyet, Inc.) or this basal diet containing 1 g EGCG/kg diet. Based on allometric scaling, this EGCG dose is equivalent to consumption of two to three cups of green tea (containing 2 g tea leaves/cup) for an average person requiring 8374 kJ (2000 kcal)/day [16]. After 8 weeks of treatment, the mice were killed after feed-deprived for 12 h. Blood samples were collected into chilled tubes and centrifuged and serum was frozen at −80°C for the analysis. All experimental protocols (08–053-HNFE) were approved by the Institutional Animal Care and Use Committee at Virginia Tech.

2.7 Measurements of blood pressure, body composition, and serum lipids

At the end of treatment period, blood pressure was determined using the Kent CODA 2 blood pressure system [17]. Body composition was determined by using LF90 Minispec Time Domain Nuclear Magnetic Resonance Spectrometer (Bruker Optics Inc., MA, USA). Serum total cholesterol, HDL-cholesterol, and triglycerides levels were measured using PTS CardioChek blood analysis meters (Maria Stein Inc., OH, USA). Serum levels of JE/MCP-1, KC, ICAM-1, and VCAM-1 were measured by using ELISA kits according to the manufacturer's instructions.

2.8 Isolation of mouse aortic endothelial cells

Mouse aortic endothelial cells (MAEC) from control *db/+* and *db/db* mice were harvested under sterile conditions as previously described [4]. Briefly, the aorta was excised and cleansed of periadventitial fat. The aorta was cut into rings and the aortic ring pieces were placed onto Matrigel. The pieces were then incubated in DMEM containing 1% penicillin-streptomycin, 15% FBS, 180 μg/mL heparin, and 20 μg/mL EC growth supplement [4, 18]. The aortic explants were removed once cell outgrowth was observed and ECs were allowed to grow until they reaches confluence. The cells were then passaged using dispase and cultured for 2 days in DMEM medium containing D-valine to eliminate fibroblast contamination. ECs were then cultured in DMEM medium without D-valine until confluence and these cells exhibit a cobblestone-like morphology with contact-inhibited growth at confluence [19]. The purity of ECs was verified by DiO-Ac-LDL uptake. The passages 2–3 were used for experiments.

2.9 Mouse monocyte adhesion assay

For adhesion assay, MAEC were cultured to confluence in 96-well plates. Calcein-AM labeled WEHI 78/24 cells were then added to MAEC and cell adhesion was determined as describe above. As a positive control, MAEC were incubated with 10 μg/L tumor necrosis factor α (TNF-α) for 6 h before adhesion assay.

2.10 Measurements of chemokines and adhesion molecules

JE/MCP-1, KC, and soluble forms of ICAM-1 (sICAM-1) and VCAM-1 (sVCAM-1) in the serum and cell culture supernatants were measured by ELISA kits according the manufacturer's instructions.

2.11 Analysis of NF-κB activation in mouse aortic vessels

Total and nuclear NF-κB p65 in mouse aortic vessels was analyzed by using Western blot. The nuclei were extracted from the aortas using Nuclear Extraction Kit and nuclear proteins were subjected to immunoblot and membranes were probed with antibody against NF-κB p65. Immunoreactive proteins were detected using chemiluminescence reagent

and the band densities were determined using Genetools software (Syngene). Proteins extracted from aortic vessels were used to measure total p65. NF-κB p65 expression levels were normalized to β-actin contents in the same sample.

2.12 Statistics

The data were derived from at least three independent experiments for ex vivo experiments and six mice in each group for animal studies. All data were analyzed with one-way analysis of variance (ANOVA) using SPSS/10 software. Values are expressed as the mean ± SEM. Treatment differences were subjected to Tukey's multiple comparison tests, where $p < 0.05$ was considered significantly different.

3 Results

3.1 EGCG inhibits high glucose induced adhesion of monocytes to ECs and this effect of EGCG is structure specific

Exposure of HAEC to high glucose significantly stimulated adhesion of monocytes to ECs compared with control, both in static condition and under flow. Pretreatment with EGCG as low as 1 μM concentration suppressed high glucose induced adhesion of monocytes to HAEC in static condition (Fig. 1A). As ECs under flow may have different functional characteristics from cells cultured in static condition, we further determined whether high glucose induces monocyte adhesion to ECs under flow condition using a shear flow imaging system, which creates an environment simulating physiological flow conditions in the vessels. High glucose induced three-fold increase in EC–monocyte interaction as compared to control. Consistent with the results of static assay, 1 μM EGCG significantly inhibited high glucose induced EC–monocyte interaction under flow (Fig. 1B and Supporting Information Fig. S1). While EGCG suppressed EC inflammation induced by high glucose, other structurally related catechins including EC, EGC, and ECG failed to exert such an effect (Fig. 1C), suggesting the unique effect of EGCG on vascular inflammation that is not shared by other structurally related catechin compounds. D-Mannitol, which was used as osmotic control, did not alter monocyte adhesion to HAEC (data not shown).

3.2 EGCG suppresses high glucose induced NF-κB activity in ECs

Monocyte adhesion to endothelium is mediated through chemokines and adhesion molecules that are critically upregulated by NF-κB and hence we determined NF-κB transcriptional activity. Exposure of BAEC to high glucose potently increased luciferase activity, indicating the induction of NF-κB-regulated gene expression (Fig. 2). However, coincu-
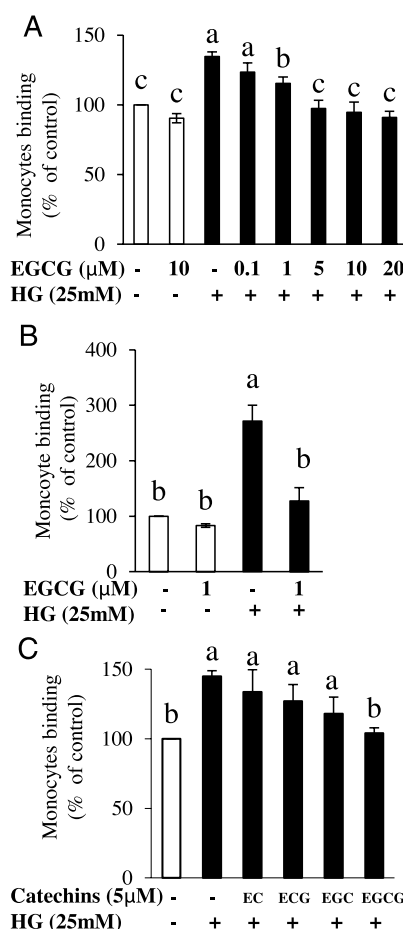

Figure 1. HAEC were pretreated with catechins for 1 h before the addition of high glucose (25 mM) for 72 h and cell adhesion was measured using fluorescent-labeled THP-1 monocytes. (A) EGCG inhibited high glucose induced monocyte adhesion to ECs in static condition. Values are mean ± SEM, $n = 4$ (means of quadruplicates). (B) EGCG inhibited high glucose stimulated adhesion of monocytes to ECs under shear flow control. Values are mean ± SEM, $n = 3$ (means of 20 fields). Please also see supplemental online material for the images. (C) The effect of EGCG on high glucose induced monocyte adhesion to ECs is structure specific. Values are mean ± SEM, $n = 3$ (means of quadruplicates). EC, epicatechin; ECG, epicatechin gallate; EGC, epigallocatechin; EGCG, epigallocatechin gallate; HG, high glucose. Means without a common letter differ, $p < 0.05$.

bation with EGCG normalized high glucose induced NF-κB activation, suggesting that EGCG may inhibit inflammation through suppressing NF-κB activity (Fig. 2).

3.3 Effects of dietary intake of EGCG on metabolic variables

EGCG treatment to *db/db* mice has no effect on food intake, body composition, blood glucose, and HDL cholesterol levels,
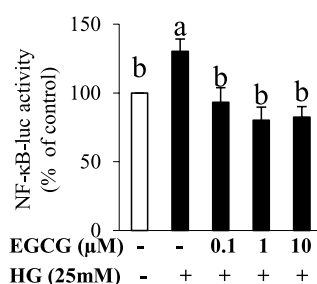
Figure 2. BAEC were cotransfected with NF-κB promoter-luciferase vector and pRL reporter control plasmid. Twenty four hours after transfection, BAEC were treated with EGCG for 1 h before addition of high glucose (25 mM) for 72 h. Luciferase activity, normalized to pRL activity in the cell extracts, was determined. EGCG inhibited high glucose induced NF-κB-regulated gene transcription in BAEC. Values are mean ± SEM, $n = 3$ (means of duplicates). EGCG, epigallocatechin gallate; HG, high glucose. Means without a common letter differ, $p < 0.05$.

but it reduced animal body weight. Serum concentrations of total cholesterol and triglyceride were significantly greater in *db/db* mice than those in control mice. Dietary intake of EGCG suppressed total cholesterol and triglyceride in *db/db* mice (Supporting Information Table S1). Both systolic and diastolic blood pressures were significantly higher in *db/db* mice than those in the controls. However, intake of EGCG normalized systolic and diastolic blood pressures in *db/db* mice (Supporting Information Table S1).

3.4 Dietary supplementation of EGCG suppresses vascular inflammation in *db/db* mice

We further assessed whether EGCG has the potential to prevent vascular inflammation in diabetes in vivo. Mouse monocyte WEHI 78/24 cells had significantly higher binding to ECs isolated from diabetic *db/db* mice than those from control mice (Fig. 3A), indicating that the vessels in diabetic mice are activated and inflammatory [4]. However, dietary supplementation of EGCG reversed this adverse effect (Fig. 3A). MCP-1 and IL-8 are essential for firm adhesion of monocyte to ECs and subsequent transmigration into vascular tissue. The serum concentrations of MCP-1/JE and KC, the mouse homologues of human MCP-1 and IL-8, respectively, were significantly greater in *db/db* mice than those in control mice (Fig. 3B), similar to those observed in diabetic patients with vascular diseases [20]. Dietary ingestion of EGCG greatly suppressed the diabetes-induced increase in circulating concentrations of MCP-1/JE and KC in *db/db* mice (Fig. 3B). While the concentrations of serum sICAM-1 and sVCAM-1 were nonsignificantly increased in *db/db* mice, EGCG treatment significantly reduced the levels of sICAM-1 and nonsignificantly reduced sVCAM-1 in *db/db* mice (Fig. 3C). The concentrations of MCP-1/JE, KC, VCAM-1, and ICAM-1 were significantly higher in culture medium of ECs isolated from *db/db*
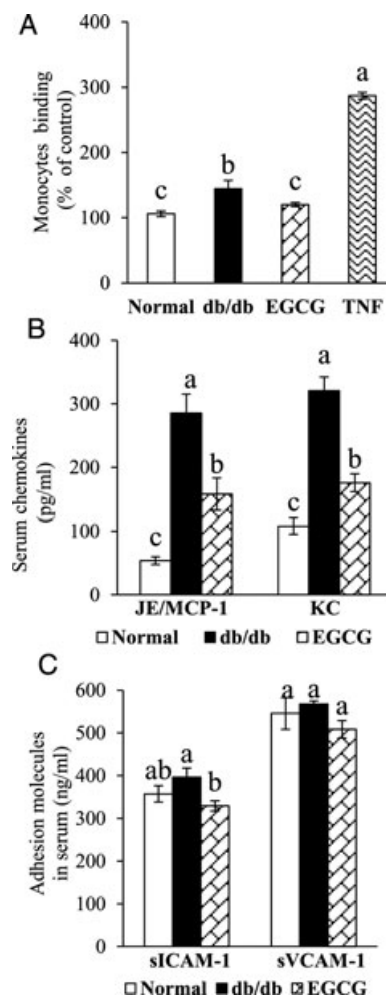

Figure 3. EGCG treatment reduced (A) adhesion of monocytes to ECs, (B) serum levels of MCP-1/JE and KC, and (C) serum levels of soluble adhesion molecules sICAM-1 and cVCAM-1in diabetic *db/db* mice. Values are mean ± SEM, $n = 6$ (means of quadruplicates) for (A) and $n = 8$ (means of duplicate) for (B) and (C). Means without a common letter differ, $p < 0.05$.

mice than those in control mice. Dietary ingestion of EGCG greatly suppressed the amounts of MCP-1/JE, KC, sVCAM-1, and sICAM-1 in the culture medium of ECs isolated from EGCG-treated *db/db* mice (Fig. 4A and B).

3.5 EGCG treatment reduces NF-κB nuclear translocation in aorta of *db/db* mice

NF-κB p65 nuclear translocation was significantly greater in the aorta of *db/db* mice than that of control mice, which was normalized by dietary supplementation of EGCG (Fig. 5A). However, no significant changes were detected in total NF-κB p65 protein expression (Fig. 5B).

*Mol. Nutr. Food Res.* 2012, *56*, 1424–1432

1429
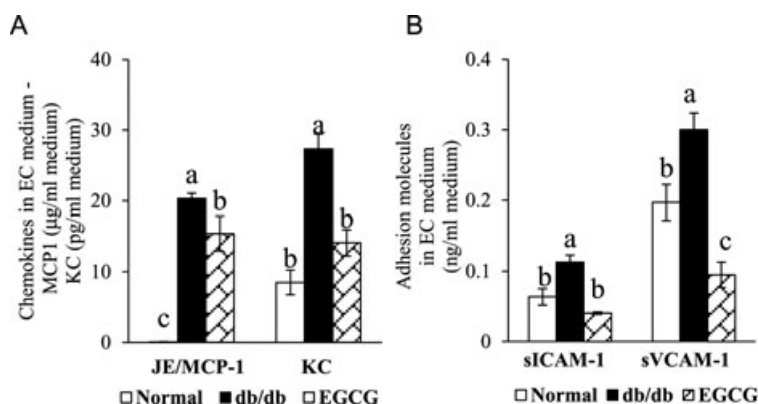
Figure 4. Dietary EGCG reduced the secretion of (A) chemokines and (B) adhesion molecules from aortic ECs of diabetic *db/db* mice. Values are mean ± SEM, $n = 6$ (means of duplicate). Means without a common letter differ, $p < 0.05$.

4 Discussion

The main finding of the present study is that EGCG at a concentration (1 μM) achievable in humans consuming green tea beverage can suppress high glucose triggered EC inflammation ex vivo and reduce vascular inflammation in diabetic *db/db* mice. Hyperglycemia plays a significant role in atherogenesis and epidemiological studies have shown that diabetes is an independent risk factor for atherosclerosis-associated morbidity [1]. Endothelial activation and increased monocyte–EC interaction are the key early events in triggering vascular inflammation in diabetes [3]. Monocytes that firmly adhere to vessel wall transmigrate cross endothelial barrier. In subendothelial space, monocytes then transform into macrophages and lipid-rich foam cells, which ultimately lead to the development of atherosclerotic lesions [21]. Indeed, monocytes are the primary inflammatory cell type that has been localized to human atherosclerotic plaques [4] and atherosclerosis fail to develop in models where monocytes are depleted [21]. Thus, protection of monocytes from adhesion to endothelium could be an useful strategy for the prevention of atherosclerosis. Previous studies demonstrate the vasculoprotective effect of green tea and EGCG [11, 13] but the underlying mechanism remains elusive and the protective role of green tea in diabetic vascular inflammation is unknown. In the present study, our findings suggest that EGCG may be a naturally occurring low-cost agent for prevention of diabetes-caused vascular inflammation and atherosclerosis.

In our study, exposure of ECs to high glucose significantly increased the adhesion of monocytes to ECs and EGCG (1 μM) suppressed this high glucose induced inflammation both in static condition and under flow. The mean plasma EGCG level can reach 1 μM in humans drinking green tea [22], suggesting that this finding may have an important physiological relevance. While the exact structural features of EGCG responsible for this action is unknown, this unique effect of EGCG at physiological concentration could be due to the presence of the galloyl moiety and hydroxyl group at the 3′ position on its molecule. Indeed, it has been reported that these structural features of EGCG play a major role in its anti-inflammatory properties [23]. Unless stimulated, ECs do not adhere to monocytes under normal conditions. Therefore, the significant increase in monocyte adhesion to *db/db* ECs suggest that ECs of diabetic mice are preactivated to interact with monocytes [4]. However, dietary supplementation of EGCG normalized diabetes-induced binding of monocytes to aortic ECs. Given aforementioned role of increased interaction of monocytes–ECs in the pathogenesis of atherosclerosis, this
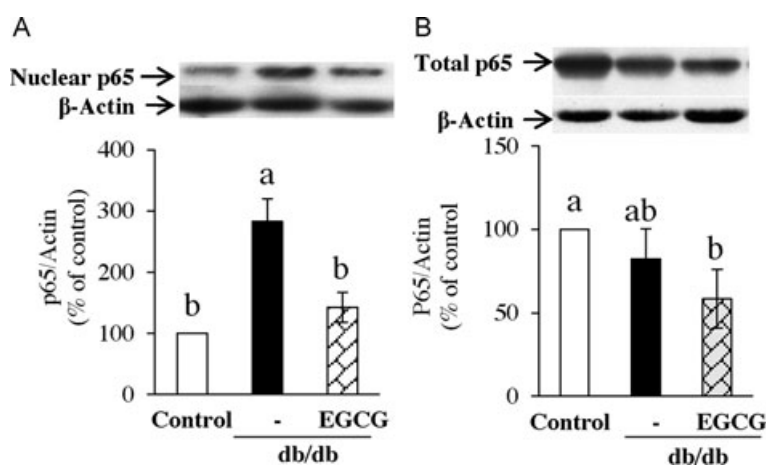
Figure 5. (A) p65 nuclear translocation increased in aorta of *db/db* mice and EGCG supplementation greatly suppressed this translocation in *db/db* mice. (B) Total NF-κB p65 protein expression in aortic vessels of the mice. Values are mean ± SE, $n = 5$ (means of duplicate). Means without a common letter differ, $p < 0.05$.

may provide an explanation for a previous study showing that dietary intake of EGCG can reduce atherosclerotic lesions in ApoE-knockout mice [24].

Chemokines such as MCP-1 and IL-8 are implicated in inflammatory responses and the pathogenesis of atherosclerosis. They are essential for monocyte rolling, firm adhesion to ECs, and their subsequent transmigration into vascular tissue [25]. Indeed, MCP-1 and IL-8 are found in human atheroma and mice lacking receptors for these chemokines are less susceptible to atherosclerosis and have fewer monocytes in vascular lesions [25]. Mice do not express IL-8, but KC is considered to be the chemokine that is most closely related to human IL-8 [4]. KC triggers monocyte arrest in carotid arteries with early atherosclerotic lesions [26]. High glucose activates aortic ECs to produce IL-8 and thereby accelerates monocytes–ECs interaction, which may be a primary link between hyperglycemia and the mechanism leading to atherosclerotic plaque formation [7]. We found that serum MCP-1/JE and KC levels are significantly increased in *db/db* mice compared to those in controls, but dietary ingestion of EGCG greatly suppressed diabetes-caused increase in circulating MCP-1 and KC in *db/db* mice. Consistently, the secretion of these chemokines by aortic ECs isolated from EGCG-treated *db/db* mice was also significantly lower as compared with *db/db* mice. These suggest the anti-inflammatory effect of EGCG given the critical role of these chemokines in vascular inflammation.

Chemokines accelerate the increased expression of adhesion molecules that play a pivotal role in attracting, binding, and transmigration of monocytes into sites of inflammation [27]. Indeed, the adhesion molecules such as VCAM-1, ICAM-1, and E-selectin are elevated in type 2 diabetic patients with atherosclerosis [28]. ICAM-1 and VCAM-1 are widely expressed in different cell types. In addition to endothelium, VCAM-1 is expressed on a variety of cell types including fibroblasts, macrophages, and dendritic cells, while ICAM-1 can be released from ECs, epithelial cells, dendritic cells, lymphocytes, monocytes, eosinophils, keratinocytes, liver cells, and fibroblasts [29]. Hence, the circulating adhesion molecules could be produced largely from sources other than ECs. In this study, we observed that the secretion of ICAM-1 and VCAM-1 from ECs were significantly increased in diabetic *db/db* mice compared with normal mice, which was reversed by treatment with EGCG. However, the circulating levels of these adhesion molecules were not altered in *db/db* mice and the level of sICAM-1 was moderately reduced in EGCG-treated *db/db* mice. These results suggest that ECs are primarily activated in these diabetic mice, and that EGCG may primarily target vascular ECs for exerting this anti-inflammatory action, which are consistent with our ex vivo finding that EGCG suppresses hyperglycemia-induced inflammation of HAEC.

Proinflammatory chemokines and adhesion molecules are directly regulated by the activation of NF-κB [8]. Hyperglycemia enhances the activation of NF-κB that then induces the expression of inflammatory genes encoding a number of mediators of atherogenesis such as inflammatory chemokines and adhesion molecules [1]. In unstimulated cells, NF-κB exists in an inactive form sequestered in the cytoplasm and exposure of cells to inflammatory stimuli results in rapid translocation of NF-κB into the nucleus. In the nucleus, p50/p65 dimers bind to the promoters of NF-κB-dependent inflammatory genes and regulate variety of genes that are induced in atherosclerosis, including genes encoding VCAM-1, ICAM-1 [9], and IL-8 [30]. NF-κB activation and nuclear localization of p65 were detected in human atherosclerotic lesions in vascular smooth muscle cells, macrophages, and ECs [31]. High glucose was shown to increase p65 nuclear translocation and its activation [9, 32]. Indeed, activation of NF-κB has been suggested to participate in diabetes-caused vascular complications [32]. In the present study, EGCG suppressed high glucose induced NF-κB activation in vascular ECs. Further, dietary supplementation of EGCG greatly suppressed the increased p65 nuclear translocation without altering protein expression of total p65 in the aortic vessels of *db/db* mice. To our knowledge, we are the first to show the increased p65 nucleus translocation in the aortic vessel of *db/db* mice, which was prevented by EGCG treatment.

Hypertension is prevalent in patients with type 2 diabetes [33], and we assessed whether EGCG can prevent the inflammation impaired vascular function in these diabetic mouse models. Similar to observations in humans, there were significant increases in systolic and diastolic blood pressures in *db/db* mice. Interestingly, dietary EGCG normalized blood pressures, suggesting that EGCG can ameliorate vascular function in diabetes. However, whether this EGCG effect is a direct result of its anti-inflammatory effect on vasculature remains to be investigated. In the present study, EGCG treatment did not alter blood glucose levels, although it moderately reduced body weight and improved lipid profiles in EGCG supplemented *db/db* mice. These results are inconsistent with data from a previous study showing that dietary EGCG supplementation can improve blood glucose and triglycerides in diabetic *db/db* mice [34]. This discrepancy could be due to the relative low dose used in the present study (0.1% EGCG in diet) as compared to the doses used in this previous study (0.25%, 0.5%, and 1% EGCG in diet). Because the bioavailability of EGCG in rodents is low [34], it is possible that administration of 0.1% EGCG in the diet cannot achieve efficacious plasma concentrations for exerting an antidiabetic effect as observed with higher doses in *db/db* mice [34]. Based on allometric scaling conversion [16], the dose of 1 g EGCG/kg diet used in the present animal study is equivalent to two to three cups of green tea (assuming a 200-mL cup and 200 mg of EGCG per cup) per day for a person requiring 8374 kJ (2000 kcal)/day [16]. However, it is questionable whether similar blood EGCG concentrations can be achieved in humans and mice exposed to the same dose of EGCG, as EGCG may be less bioavailable in humans as compared to mice [35].

Taken together, dietary supplementation of EGCG counteracts several adverse effects associated with vascular inflammation and atherosclerosis, including increased adhesion

Mol. Nutr. Food Res. 2012, *56*, 1424–1432

1431

of ECs to monocytes, enhanced circulating chemokines, increased secretion of chemokines and adhesion molecules by ECs, as well as hypertension of diabetic *db/db* mice. However, whether EGCG can prevent or ameliorate the pathogenesis of atherosclerosis remains to be determined. In addition, the cellular and molecular mechanism by which EGCG protects diabetic vasculature from chronic inflammation needs to be further defined.

5 References

[1] Beckman, J. A., Creager, M. A., Libby, P., Diabetes and atherosclerosis: epidemiology, pathophysiology, and management. *JAMA* 2002, *287*, 2570–2581.

[2] Ross, R., Atherosclerosis–an inflammatory disease. *N. Engl. J. Med.* 1999, *340*, 115–126.

[3] Potenza, M. A., Gagliardi, S., Nacci, C., Carratu, M. R. et al., Endothelial dysfunction in diabetes: from mechanisms to therapeutic targets. *Curr. Med. Chem.* 2009, *16*, 94–112.

[4] Srinivasan, S., Bolick, D. T., Hatley, M. E., Natarajan, R. et al., Glucose regulates interleukin-8 production in aortic endothelial cells through activation of the p38 mitogen-activated protein kinase pathway in diabetes. *J. Biol. Chem.* 2004, *279*, 31930–31936.

[5] Kunt, T., Forst, T., Fruh, B., Flohr, T. et al., Binding of monocytes from normolipidemic hyperglycemic patients with type 1 diabetes to endothelial cells is increased in vitro. *Exp. Clin. Endocrinol. Diabetes* 1999, *107*, 252–256.

[6] Morigi, M., Angioletti, S., Imberti, B., Donadelli, R. et al., Leukocyte-endothelial interaction is augmented by high glucose concentrations and hyperglycemia in a NF-kB-dependent fashion. *J. Clin. Invest.* 1998, *101*, 1905–1915.

[7] Srinivasan, S., Yeh, M., Danziger, E. C., Hatley, M. E. et al., Glucose regulates monocyte adhesion through endothelial production of interleukin-8. *Circ. Res.* 2003, *92*, 371–377.

[8] Savoia, C., Schiffrin, E. L., Vascular inflammation in hypertension and diabetes: molecular mechanisms and therapeutic interventions. *Clin. Sci. (Lond)* 2007, *112*, 375–384.

[9] Yerneni, K. K., Bai, W., Khan, B. V., Medford, R. M. et al., Hyperglycemia-induced activation of nuclear transcription factor kappaB in vascular smooth muscle cells. *Diabetes* 1999, *48*, 855–864.

[10] Potenza, M. A., Marasciulo, F. L., Tarquinio, M., Tiravanti, E. et al., EGCG, a green tea polyphenol, improves endothelial function and insulin sensitivity, reduces blood pressure, and protects against myocardial I/R injury in SHR. *Am. J. Physiol. Endocrinol. Metab.* 2007, *292*, E1378–E1387.

[11] Wolfram, S., Effects of green tea and EGCG on cardiovascular and metabolic health. *J. Am. Coll. Nutr.* 2007, *26*, 373S–388S.

[12] Widlansky, M. E., Hamburg, N. M., Anter, E., Holbrook, M. et al., Acute EGCG supplementation reverses endothelial dysfunction in patients with coronary artery disease. *J. Am. Coll. Nutr.* 2007, *26*, 95–102.

[13] Babu, P. V., Liu, D., Green tea catechins and cardiovascular health: an update. *Curr. Med. Chem.* 2008, *15*, 1840–1850.

[14] Lambert, J. D., Lee, M. J., Diamond, L., Ju, J. et al., Dose-dependent levels of epigallocatechin-3-gallate in human colon cancer cells and mouse plasma and tissues. *Drug Metab. Dispos.* 2006, *34*, 8–11.

[15] Rajesh, M., Mukhopadhyay, P., Batkai, S., Hasko, G. et al., Cannabidiol attenuates high glucose-induced endothelial cell inflammatory response and barrier disruption. *Am. J. Physiol. Heart Circ. Physiol.* 2007, *293*, H610–H619.

[16] Bose, M., Lambert, J. D., Ju, J., Reuhl, K. R. et al., The major green tea polyphenol, (−)-epigallocatechin-3-gallate, inhibits obesity, metabolic syndrome, and fatty liver disease in high-fat-fed mice. *J. Nutr.* 2008, *138*, 1677–1683.

[17] Si, H., Liu, D., Genistein, a soy phytoestrogen, upregulates the expression of human endothelial nitric oxide synthase and lowers blood pressure in spontaneously hypertensive rats. *J. Nutr.* 2008, *138*, 297–304.

[18] Srinivasan, S., Hatley, M. E., Reilly, K. B., Danziger, E. C. et al., Modulation of PPARalpha expression and inflammatory interleukin-6 production by chronic glucose increases monocyte/endothelial adhesion. *Arterioscler. Thromb. Vasc. Biol.* 2004, *24*, 851–857.

[19] Nishiyama, T., Mishima, K., Ide, F., Yamada, K. et al., Functional analysis of an established mouse vascular endothelial cell line. *J. Vascul. Res.* 2007, *44*, 138–148.

[20] de Lemos, J. A., Morrow, D. A., Sabatine, M. S., Murphy, S. A. et al., Association between plasma levels of monocyte chemoattractant protein-1 and long-term clinical outcomes in patients with acute coronary syndromes. *Circulation* 2003, *107*, 690–695.

[21] Pittet, M. J., Swirski, F. K., Monocytes link atherosclerosis and cancer. *Eur. J. Immunol.* 2011, *41*, 2519–2522.

[22] Lee, M. J., Maliakal, P., Chen, L., Meng, X. et al., Pharmacokinetics of tea catechins after ingestion of green tea and (−)-epigallocatechin-3-gallate by humans: formation of different metabolites and individual variability. *Cancer Epidemiol. Biomarkers Prev.* 2002, *11*, 1025–1032.

[23] Andriamanalijaona, R., Kypriotou, M., Bauge, C., Renard, E. et al., Comparative effects of 2 antioxidants, selenomethionine and epigallocatechin-gallate, on catabolic and anabolic gene expression of articular chondrocytes. *J. Rheumatol.* 2005, *32*, 1958–1967.

[24] Chyu, K. Y., Babbidge, S. M., Zhao, X., Dandillaya, R. et al., Differential effects of green tea-derived catechin on developing versus established atherosclerosis in apolipoprotein E-null mice. *Circulation* 2004, *109*, 2448–2453.

1432   P. V. A. Babu et al.

Mol. Nutr. Food Res. 2012, *56*, 1424–1432

[25] Gerszten, R. E., Garcia-Zepeda, E. A., Lim, Y. C., Yoshida, M. et al., MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. *Nature* 1999, *398*, 718–723.

[26] Huo, Y., Weber, C., Forlow, S. B., Sperandio, M. et al., The chemokine KC, but not monocyte chemoattractant protein-1, triggers monocyte arrest on early atherosclerotic endothelium. *J. Clin. Invest.* 2001, *108*, 1307–1314.

[27] Martin, J., Collot-Teixeira, S., McGregor, L., McGregor, J. L., The dialogue between endothelial cells and monocytes/macrophages in vascular syndromes. *Curr. Pharm. Des.* 2007, *13*, 1751–1759.

[28] Otsuki, M., Hashimoto, K., Morimoto, Y., Kishimoto, T. et al., Circulating vascular cell adhesion molecule-1 (VCAM-1) in atherosclerotic NIDDM patients. *Diabetes* 1997, *46*, 2096–2101.

[29] Golias, C., Tsoutsi, E., Matziridis, A., Makridis, P. et al., Review. Leukocyte and endothelial cell adhesion molecules in inflammation focusing on inflammatory heart disease. *In Vivo* 2007, *21*, 757–769.

[30] Bartchewsky, W., Jr., Martini, M. R., Masiero, M., Squassoni, A. C. et al., Effect of Helicobacter pylori infection on IL-8, IL-1beta and COX-2 expression in patients with chronic gastritis and gastric cancer. *Scand. J. Gastroenterol.* 2009, *44*, 153–161.

[31] Brand, K., Page, S., Rogler, G., Bartsch, A. et al., Activated transcription factor nuclear factor-kappa B is present in the atherosclerotic lesion. *J. Clin. Invest* .1996, *97*, 1715–1722.

[32] Bierhaus, A., Schiekofer, S., Schwaninger, M., Andrassy, M. et al., Diabetes-associated sustained activation of the transcription factor nuclear factor-kappaB. *Diabetes* 2001, *50*, 2792–2808.

[33] Sowers, J. R., Epstein, M., Frohlich, E. D., Diabetes, hypertension, and cardiovascular disease: an update. *Hypertension* 2001, *37*, 1053–1059.

[34] Wolfram, S., Raederstorff, D., Preller, M., Wang, Y. et al., Epigallocatechin gallate supplementation alleviates diabetes in rodents. *J. Nutr.* 2006, *136*, 2512–2518.

[35] Lambert, J. D., Hong, J., Kim, D. H., Mishin, V. M. et al., Piperine enhances the bioavailability of the tea polyphenol (−)-epigallocatechin-3-gallate in mice. *J. Nutr.* 2004, *134*, 1948–1952.