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Epigallocatechin-gallate stimulates NF-E2-related factor and heme oxygenase-1 via caveolin-1 displacement

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Abstract

Flavonoids, such as the tea catechin epigallocatechin-gallate (EGCG), can protect against atherosclerosis by decreasing vascular endothelial cell inflammation. Heme oxygenase-1 (HO-1) is an enzyme that plays an important role in vascular physiology, and its induction may provide protection against atherosclerosis. Heme oxygenase-1 can be compartmentalized in caveolae in endothelial cells. Caveolae are plasma microdomains important in vesicular transport and the regulation of signaling pathways associated with the pathology of vascular diseases. We hypothesize that caveolae play a role in the uptake and transport of EGCG and mechanisms associated with the anti-inflammatory properties of this flavonoid. To test this hypothesis, we explored the effect of EGCG on the induction of NF-E2-related factor (Nrf2) and HO-1 in endothelial cells with or without functional caveolae. Treatment with EGCG activated Nrf2 and increased HO-1 expression and cellular production of bilirubin. In addition, EGCG rapidly accumulated in caveolae, which was associated with caveolin-1 displacement from the plasma membrane towards the cytosol. Similar to EGCG treatment, silencing of caveolin-1 by siRNA technique also resulted in up-regulation of Nrf2, HO-1 and bilirubin production. These data suggest that EGCG-induced caveolin-1 displacement may reduce endothelial inflammation.
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Keywords: EGCG; Nrf2; Caveolae; Endothelial cells; Atherosclerosis

1. Introduction

Diets high in polyphenols (e.g., flavonoids) are associated with a reduced risk of chronic diseases, such as cardiovascular diseases, by affecting molecular mechanisms involved in the initiation and progression of these diseases [1]. Flavonoids constitute a subclass of bioactive compounds rich in fruits and vegetables, soy food, legumes, tea and cocoa [2]. Green tea consumption has been shown to be significantly greater in healthy subjects compared to those with coronary artery disease, suggesting that green tea might be protective against coronary atherosclerosis [3]. Catechins are the major constituents of the polyphenols in green tea, and the most abundant catechin in green tea is epigallocatechin-3-*O*-gallate (EGCG). Even though the consumption of flavonoids such as EGCG is known to improve endothelial cell function and thus reduce cardiovascular risk [4], protective mechanisms are not clear but may be linked to caveolae signaling.

There is increasing evidence that caveolae play a critical role in the pathology of vascular diseases and that the lack of the caveolin-1 gene may provide protection against the development of atherosclerosis [5]. Caveolae are plasma-membrane domains that are highly enriched in cholesterol and sphingolipids. One of the functions attributed to endothelial caveolae is their ability to transfer molecules from the lumen of blood vessels to the subendothelial space [6]. Like clathrin-mediated endocytosis, internalization through caveolae involves complex signaling [7]. Caveolin-1 is a major scaffolding protein constituent of caveolae that participates in vesicular trafficking and signal transduction, and caveolin-1 can cycle between the plasma membrane and several intracellular compartments. Caveolae are particularly abundant in endothelial cells, where they are believed to play a major role in the regulation of endothelial vesicular trafficking as well as the uptake of lipids and related lipophilic compounds possibly including bioactive food components such as polyphenols by means of endocytosis [8,9]. For example, it has been reported that caveolin-1 modulates cellular sensitivity to resveratrol through the enhancement of its internalization and trafficking [10].

Protective mechanisms of flavonoids may include up-regulation of heme oxygenase-1 (HO-1) [11], an enzyme localized to plasma membrane caveolae [12]. Heme oxygenase-1 is an inducible enzyme which catalyzes the oxidative degradation of heme by degrading heme to iron, carbon monoxide and biliverdin, with the latter being quickly reduced to bilirubin. The heme oxygenase system is an

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important regulator of endothelial cell integrity and oxidative stress, and dysfunctional HO-1 signaling may be a proatherogenic event. In fact, the end products of HO-1 metabolism, such as bilirubin, can exert potent antioxidant and anti-inflammatory actions within the vascular system, including endothelial cells [13] Heme oxygenase-1 also can be up-regulated by EGCG [11], and we have recently demonstrated that EGCG-mediated protection against tumor necrosis factor-α (TNF-α)-induced monocyte chemoattractant protein-1 (MCP-1) expression is HO-1 dependent [14].

Recent genome-wide analysis demonstrated that the transcription factor NF-E2-related factor (Nrf2) can regulate numerous genes that are involved in the cytoprotective response against oxidative stress [15]. For example, the induction of the antioxidant gene HO-1 in vascular cells is associated with nuclear translocation of Nrf2 and subsequent transactivation of an antioxidant response element in the promoter region of HO-1 [16]. There is evidence that flavonoids like EGCG can induce HO-1 via activation of Nrf2 [11,17]. Since HO-1 is an enzyme localized to plasma membrane caveolae [12], we were interested in the possible involvement of functional caveolae in EGCG-mediated stimulation of Nrf2 and HO-1. We recently reported a role of caveolin-1 in EGCG-mediated protection against linoleic acid-induced endothelial cell activation [9], suggesting that caveolae may provide a regulatory platform of our observed effects of EGCG on stimulation of Nrf2 and HO-1. In the current study, we provide evidence that EGCG stimulates Nrf2 and HO-1 via alteration in caveolae function associated with caveolin-1 displacement.

2. Materials and methods

2.1. Cell culture and experimental media

Primary endothelial cells were isolated from porcine aortic arteries and cultured as previously described [18]. The basic culture medium consisted of medium 199 (M-199) (catalog no. 31100-035; GIBCO Laboratories, NY, USA) containing 10% (vol/vol) fetal bovine serum (FBS; HyClone Laboratories, UT, USA). Experimental media contained 5% FBS and were supplemented with EGCG (Cayman Chemicals, Ann Arbor, MI, USA; purity >98%). The EGCG was dissolved in dimethyl sulfoxide (DMSO); at a final concentration less than 0.1%, DMSO did not affect cell viability.

2.2. Detergent-free purification of caveolae-rich membrane domains

Caveolae-rich membrane domains were isolated as previously reported [19], with minor modifications. Briefly, cells were plated in 150-mm plates. After treatment, cells were washed with phosphate-buffered saline (PBS) and lysed with 2 ml of ice-cold morpholine ethanesulfonic acid (MES)-buffered saline (MBS; 25 mM MES [pH 6.5], 150 mM NaCl) containing 500 mM Na_2CO_3. Following homogenization and sonication, homogenates were adjusted to 45% sucrose by addition of 2 ml of 90% sucrose prepared in MBS and placed at the bottom of an ultracentrifuge tube, and overlaid with a discontinuous sucrose gradient. Subsequently, 5 ml of 35% sucrose and then 3 ml of 5% sucrose were added. The gradient samples were then centrifuged at 39,000 rpm (260,000g) for 16 h using a SW41 rotor (Beckman Instruments, Palo Alto, CA, USA). Twelve 1-ml fractions were collected, and aliquots of each fraction were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting to assess caveolin-1.

2.3. Immunoblot analysis of HO-1, caveolin-1 and Nrf2 protein expression

Cells were treated with either vehicle (0.1% DMSO) or EGCG (30 μM) followed by immunoblot analysis of HO-1 protein expression. To detect the effect of EGCG and caveolin-1 gene silencing on Nrf2 accumulation in nucleus, both nuclear and cytosol protein extracts were prepared from endothelial cells. Western blots were visualized using the appropriate horseradish peroxidase-conjugated secondary antibodies followed by ECL immunoblotting detection reagents (Amersham Biosciences, Buckinghamshire, England).

2.4. Caveolin-1 siRNA and transfection

The caveolin-1 gene silencer was designed by Dharmacon (Lafayette, CO, USA) according to previously described methods [20]. The sequence of the control gene silencer was 5′-AAAGAGCGACTTTACACACdTdT-3′; caveolin-1 gene silencers were 5′-CCAGAAGGGACACACAGUUdTdT-3′ and 5′-CAUCUACAAGCCCAACAACdT dT-3′. Cells were transfected with control siRNA or a mixture of two siRNAs targeted against caveolin-1 (40 nM each) using GeneSilencer (Genlantis, San Diego, CA, USA) with Optimem I medium (Invitrogen, Carlsbad, CA, USA). Cells were incubated with transfection mixtures for 4 h and then replaced with 10% serum medium. Cells were synchronized overnight after 48 h of transfection and then treated with EGCG.

2.5. Electrophoretic mobility shift assays of Nrf2–antioxidant response element–DNA binding

Nuclear extracts were prepared from endothelial cells. Synthetic 5′-biotinylated complementary oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA, USA). Nuclear extracts were incubated at room temperature for 20 min with biotin-labeled oligonucleotide probes containing antioxidant response element (ARE), the enhancer DNA element for HO-1(5′ bio-AGATTTTGCTGAGTCAC-CAGTCCC-3′). Gel mobility shift assay was performed to demonstrate the shifted DNA–protein complexes Nrf2–ARE using a LightShift chemiluminescent EMSA kit (Pierce, Rockford, IL, USA). Reactions using 200-fold molar excess of unlabeled oligonucleotide probes were performed to demonstrate the specificity of the shifted DNA–protein complexes for Nrf2. To further examine the presence of Nrf2 protein in the retarded bands, EMSA was performed and followed by Western blotting (shift-Western) using an antibody against Nrf2.

2.6. Quantification of EGCG by liquid chromatography/tandem mass spectrometry

After treatment with EGCG, cells were washed twice in cold PBS. Cells were then scraped with lysis buffer. Protein concentrations were measured by the Bradford method. Samples were then diluted in 2 ml cold PBS containing 100 μl 20% ascorbic acid solution and 50 pmol internal standards (ethyl gallate). Cell suspensions or aqueous samples were diluted with 4 ml ethyl acetate, vortexed and then centrifuged for 10 min at 3500 rpm. Organic layers were removed and evaporated to dryness under a gentle N_2 stream in a water bath. Dry extracts were reconstituted with 50:50 water:acetonitrile and injected into liquid chromatography/tandem mass spectrometry (LC/MS/MS). The mass spectrometer included an Applied Biosystems/MDS SCIEX 4000-Qtrap hybrid, linear ion trap and triple quadrupole MS (Applied Biosystems, Foster City, CA, USA), and the liquid chromatograph was a Prominence UFLC from Shimadzu Corp. (Columbia, MD, USA). Polyphenols were separated using an Eclipse XDB C8, 5-μM, 4.6×150-mm (Agilent) column.

2.7. Measurement of bilirubin production

Bilirubin was measured as previously described [14]. Briefly, after plating and gene silencing, cells were treated with EGCG or vehicle, and all further manipulations were carried out in a dark room. Absorbance of the organic layer containing bilirubin was measured at 450 nm with a reference wavelength at 600 nm using a SpectraMaxPro M2 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The quantity of bilirubin produced was calculated using a molar extinction coefficient of bilirubin dissolved in benzene, with the molar extinction coefficient being e^{450}=27.3 $mmol^{-1}$ L cm^{-1} [21].

2.8. Statistical analysis

Values are reported as mean±standard error of the mean (S.E.M.) of at least three independent groups. Data were analyzed using Sigma Stat software (Jandel Corp., San Rafael, CA, USA). One-way analysis of variance followed by post hoc least significant difference pairwise multiple comparison procedure was used for statistical analysis of the original data. A statistical probability of $P<.05$ was considered significant.

3. Results

3.1. EGCG treatment stimulates caveolin-1 displacement

We tested the hypothesis that EGCG can co-localize with caveolae and displace caveolin-1. Caveolae-enriched fractions were isolated using a detergent-free sucrose density gradient centrifugation method, followed by protein analysis using Western blot. In control cultures, and as expected, caveolin-1 protein was most enriched in fractions 4 and 5 (Fig. 1). In contrast, treatment with EGCG induced displacement of caveolin-1 in endothelial cells. At 0.5 h and 1 h, caveolin-1 translocated from fractions 4 and 5 toward fractions 6 to 8, and after 2 h, caveolin-1 recycled back mainly in plasma membrane lipid rafts (fractions 4 and 5). These data suggest that exposure to EGCG leads to caveolin-1 displacement and that caveolae may transport EGCG from the plasma membrane toward the cytosol or intracellular compartments.

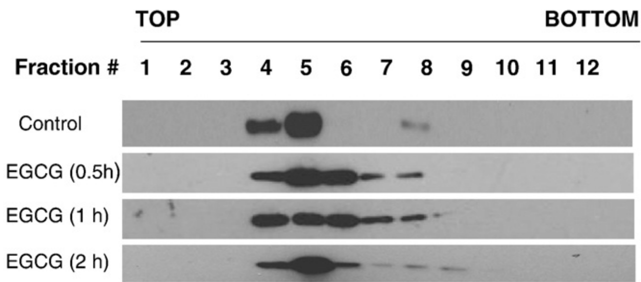

Fig. 1. EGCG treatment stimulates caveolin-1 displacement. Endothelial cells were treated with vehicle (0.1% DMSO) or EGCG (30 μM) for 0.5–2 h. Caveolae-enriched fractions were isolated by detergent-free sucrose gradient centrifugation method. The expression of caveolin-1 was then measured by Western blot. Fractions 1 to 3 contain 5% sucrose, fractions 4 to 8 contain 35% sucrose and fractions 9 to 12 contain 45% sucrose. Result shown represents one of three independent experiments.

3.2. EGCG quantification in endothelial cells

To further understand the stability and cell uptake of EGCG by endothelial cells, we quantified EGCG by LC/MS/MS analysis. Cells were exposed to EGCG for up to 4 h. After a peak at 30 min, EGCG levels in the media decreased, with only about 20% of the parent flavonoid remaining at 4 h (Fig. 2A). Cell-associated levels were maximal at 30 min and then declined in parallel with observed media levels (Fig. 2B). These results suggest that EGCG is easily auto-oxidized and quickly metabolized [22]. Results in Fig. 2C demonstrate that endothelial cells can become enriched with EGCG in a dose-dependent manner.

3.3. EGCG accumulates in caveolae-enriched fractions

To further investigate the role of caveolae in the uptake and transport of EGCG, we measured EGCG levels in both caveolae-rich membrane fractions and noncaveolae fractions after treatment with EGCG for 5, 10, 20 or 40 min. The EGCG markedly accumulated in caveolae-rich membrane domains at 10 min (Fig. 3A), with a subsequent relocation into the noncaveolae cell fractions. At 40 min, the uptake of the EGCG into the noncaveolae fractions was increased 1.6-fold compared to the that at the 20-min time point (Fig. 3B).

3.4. Caveolae silencing reduces cellular EGCG uptake

To explore a potential role of caveolae in EGCG uptake, cells were transfected with caveolin-1 specific siRNAs. Compared to cells with functional caveolae, cellular levels of EGCG at 30 min were significantly decreased after silencing caveolin-1 (Fig. 4A). This trend continued but was not significant at 1 h after EGCG exposure. As illustrated in Fig. 4B, caveolin-1 was successfully silenced.

3.5. Cavelin-1 silencing induces up-regulation of HO-1 and bilirubin production

Heme oxygenase-1 has potent anti-inflammatory effects, which may be exerted through the generation of bilirubin. Therefore, we tested the effects of caveolin-1 gene silencing on HO-1 expression and bilirubin production in endothelial cells. The results showed that both EGCG treatment and caveolin-1 gene silencing significantly induced HO-1 levels (Fig. 5A). Similar to the HO-1 data, both EGCG and caveolin-1 gene silencing significantly induced the cellular secretion of bilirubin (Figs. 5B).

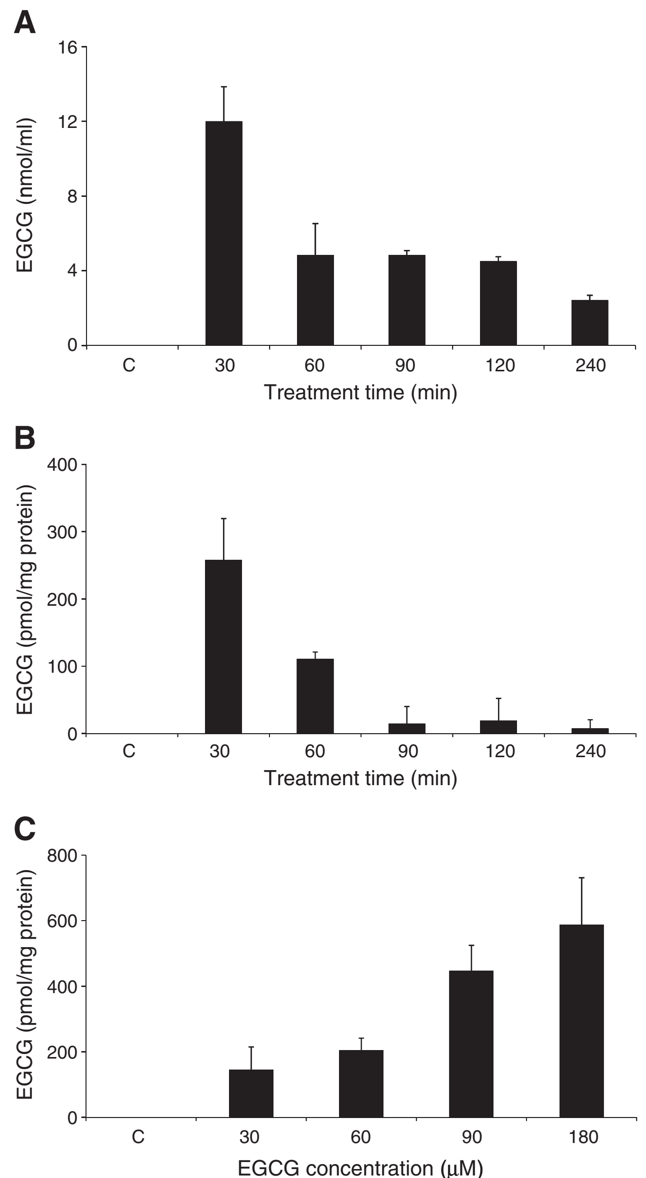

Fig. 2. EGCG quantification in endothelial cells was performed by LC/MS/MS analysis. Endothelial cells were treated with EGCG (30 μM) for up to 240 min, and EGCG levels were measure by LC/MS/MS in the media (A) and cells (B). In separate experiments, endothelial cells were treated with increasing concentrations of EGCG (0–180 μM) for 1h before measuring cellular levels of EGCG (C). Results shown represent the mean±S.E.M. of three independent experiments.

3.6. Caveolin-1 silencing increases both nuclear accumulation of Nrf2 and Nrf2–ARE binding

It has been suggested that the regulation of Nrf2 transcriptional activation of phase II antioxidant enzymes (e.g., HO-1) relies on subcellular distribution rather than induction of this transcription factor through *de novo* synthesis [23]. Activation of Nrf2 results in increased accumulation of Nrf2 in the nucleus. Thus, we determined nuclear levels of Nrf2 in both control and EGCG-treated cells with or without functional caveolae to investigate whether induction of HO-1 is associated with nuclear translocation of Nrf2. As shown by immunoblot analysis in Fig. 6A, Nrf2 protein levels in the nucleus significantly increased in cells treated with EGCG. Caveolin-1 silencing independently increased Nrf2, with no additive effects due to cellular exposure to EGCG. Nrf2 protein in the cytosol was

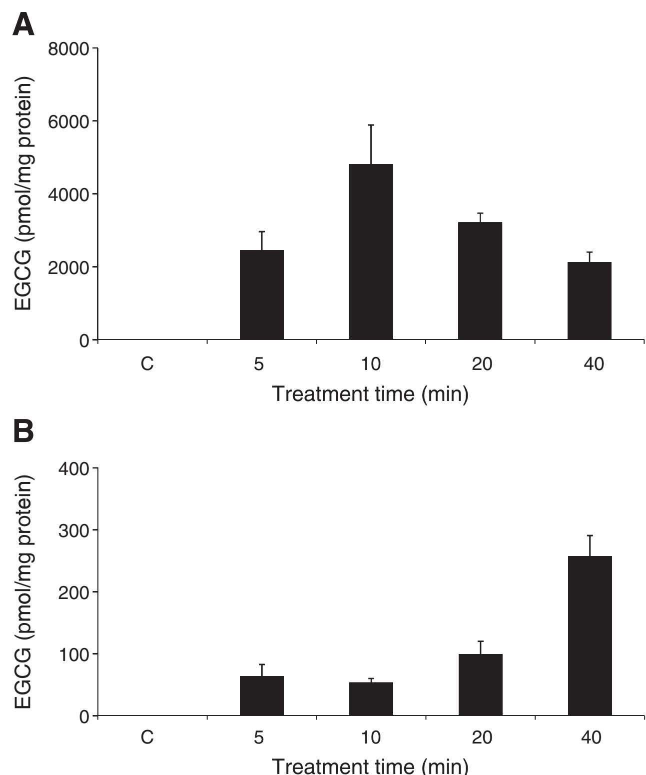

Fig. 3. EGCG accumulates in caveolae-enriched fractions. Endothelial cells were treated with EGCG (30 μM) for 5–40 min. Caveolae were isolated by detergent-free sucrose density gradient centrifugation method. EGCG levels in caveolae-rich domains (A) and noncaveolae fractions (B) were measured by LC/MS/MS. EGCG levels shown in the figure were normalized against protein levels in caveolae or noncaveolae fractions, respectively. Results shown represent the mean±S.E.M. of three independent experiments.

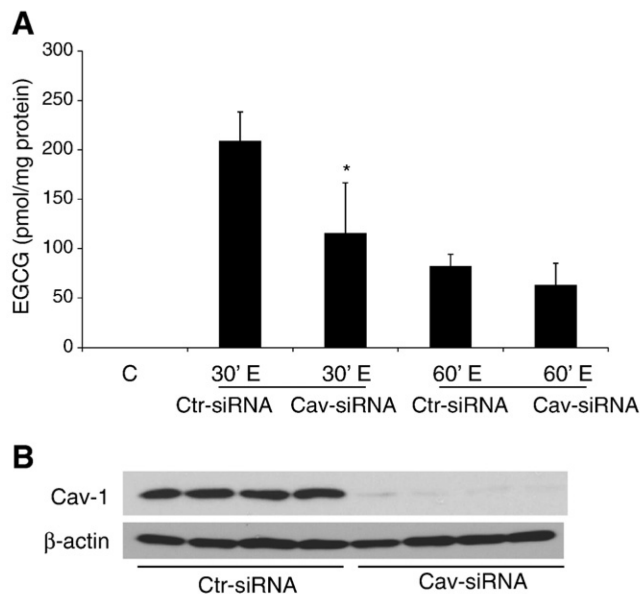

Fig. 4. Caveolae silencing reduces cellular EGCG uptake. Endothelial cells were transfected with siRNA for caveolin-1 (Cav-1 siRNA) or with control siRNA (Ctr− siRNA) and treated with EGCG (30 μM) for 30 min and 1 h. EGCG levels in whole cells were measured by LC/MS/MS (A). Results shown represent the mean±S.E.M. of three independent experiments. B shows successful silencing of caveolin-1.

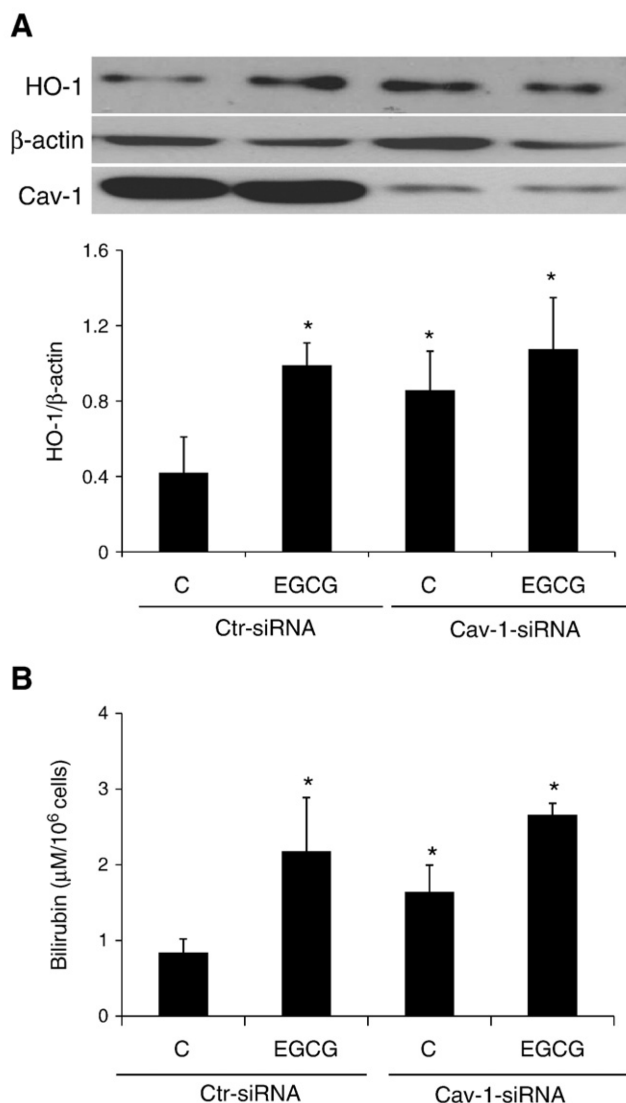

Fig. 5. Cavelin-1 silencing induces up-regulation of HO-1 and bilirubin production. Endothelial cells were transfected with siRNA for caveolin-1 (Cav-1 siRNA) or with control siRNA (Ctr− siRNA) and then treated with vehicle (0.1% DMSO) or EGCG (30 μM) for 6 h before determining HO-1 protein expression (A) and bilirubin production (B). Results shown represent the mean±S.E.M. of three independent experiments. *Significantly different compared to control cultures.

unaffected by EGCG treatment or caveolin-1 silencing (Fig. 6B). Since Nrf2 activates transcription activities of its genes through binding specifically to the ARE found in the promoters of target genes, we also studied the effects of EGCG on Nrf2–ARE binding. In agreement with the observed nuclear accumulation of Nrf2, Nrf2–ARE specific HO-1 promoter binding was also significantly enhanced in both EGCG-treated and caveolin-1-silenced cells (Fig. 6C).

4. Discussion

There is evidence that catechins derived from green tea, such as EGCG, have antioxidant, anti-inflammatory and antiangiogenesis properties and thus can provide protection against inflammatory diseases such as atherosclerosis [24]. In the current study, we provide evidence that the vascular antioxidant and anti-inflammatory properties of EGCG may be regulated through the interaction of this flavonoid with caveolae. Lipid rafts, and especially caveolae, have been

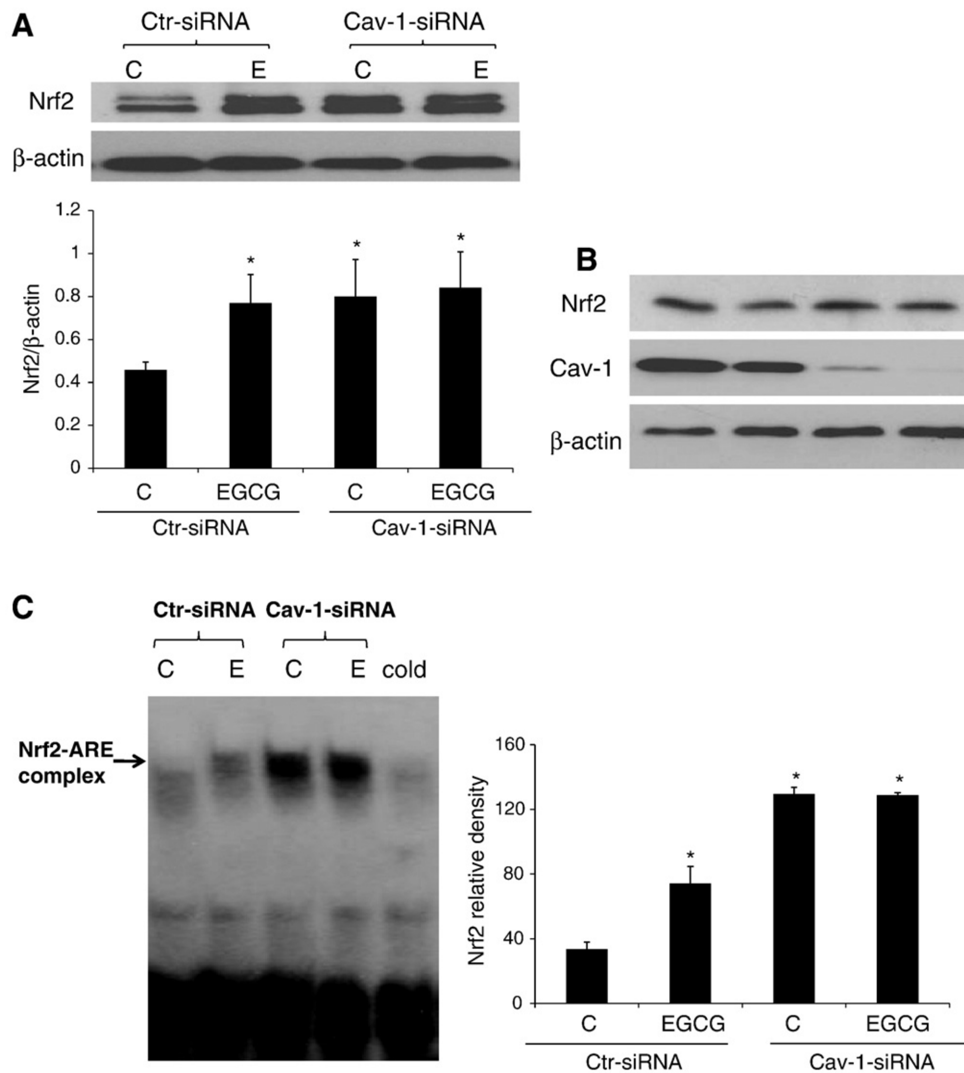

Fig. 6. Caveolin-1 silencing increases both nuclear accumulation of Nrf2 and Nrf2–ARE binding. Endothelial cells were transfected with siRNA for caveolin-1 (Cav-1 siRNA) or with control siRNA (Ctr- siRNA) and then treated with vehicle (0.1% DMSO) or EGCG (30 μM) for 3 h before determining Nrf2 protein expression in both nucleus (A) and cytosol (B). Electrophoretic mobility shift assay for Nrf2–ARE binding was performed with nuclear proteins extracted from these endothelial cells (C). Following the EMSA assay, Western blotting with the Nrf2 antibody demonstrated the upper band to be the Nrf2–ARE complex. Results shown represent the mean±S.E.M. of three independent experiments. *Significantly different compared to control cultures.

recently recognized as signal transduction hubs and may be involved in the selective cellular uptake of plasma-derived material [25] and possibly resveratrol [10] and other polyphenols such as EGCG.

It has been reported that EGCG is taken up by some lipid raft proteins in the membrane, such as laminin receptor, and that it further alters the composition of membrane domains as well as changes in some signaling pathways [26]. Besides specific interactions with some genes and proteins, another important pathway that may function in EGCG action is direct targeting on lipid rafts. Structure and composition alterations of the lipid raft by polyphenols can dramatically affect signaling pathways, such as MAP kinase pathways [27]. Induction of MAP kinases is critical in the regulation of inflammatory pathways, and inhibition of MAP kinase signaling may in part explain the anti-inflammatory effects of polyphenols [28]. Our LC/MS/MS results suggest that EGCG can concentrate in the caveolae fractions before further cellular redistribution. In fact, following cellular exposure, EGCG quickly enriched in caveolae membrane domains, with a subsequent relocation to noncaveolae fractions. This suggests that caveolae may play a role in the uptake and transport of EGCG from plasma membrane lipid rafts towards the cytosol in endothelial cells. To confirm the role of caveolae in the uptake of EGCG, endothelial cells were silenced with caveolin-1 siRNA. Results showed that caveolin-1 gene silencing significantly decreased cellular uptake of EGCG at 30 min, i.e., at a time point when EGCG was markedly enriched in the caveolae fractions of normal endothelial cells. Thus, these data suggest that EGCG uptake into endothelial cells may be in part dependent on functional caveolae.

Caveolae usually have a specific lipid composition, which appears to be required for the specific functional relevance of caveolae-associated proteins [29]. In line with these functional characteristics, caveolae can provide a regulatory platform for proinflammatory signaling associated with vascular diseases such as atherosclerosis [30]. For example, enriching endothelial cells with docosahexaenoic acid can affect caveolae-associated nitric oxide synthase activity [31], a process which may be linked to caveolae-mediated endocytosis [32]. We also found previously that pretreatment with EGCG can block fatty acid-induced caveolin-1 expression in a time- and concentration-dependent manner [9]. In the current study, we provide evidence that EGCG can stimulate caveolin-1 displacement from the plasma membrane towards the cytosol. Most importantly,

the ability of EGCG to displace caveolin-1 was associated with its ability to activate Nrf2 and to increase HO-1 expression and cellular production of bilirubin.

We have reported recently that EGCG-mediated protection against TNF-α-induced MCP-1 expression is HO-1 dependent [14], and we now provide data which demonstrate that caveolae play an important role in the uptake and transport of EGCG, as well as protection against endothelial inflammation through the induction of Nrf2-dependent HO-1. Our results clearly show that, in addition to EGCG, caveolin-1 gene silencing can induce HO-1 protein expression and thus up-regulate the production of bilirubin. Both HO-1 and bilirubin have been reported to play critical roles in cellular and tissue defenses against oxidative stress and inflammation [33]. Heme oxygenase-1 overexpression can inhibit pathological activities, including inflammation, vascular proliferation and chronic transplant rejection. It has been reported that overexpression of the HO-1 protein inhibits lipopolysaccharide-induced iNOS expression [34]. Furthermore, the HO-1 protein is essential for the anti-inflammatory effects of interleukin-10 and 15-deoxy-delta 12, 14-prostaglandin J2 [35].

Since Nrf2 is the major transcription factor of HO-1, we investigated the role of Nrf2 in EGCG or caveolin-1 gene silencing mediated activation of HO-1. Nrf2 is a leucine zipper transcription factor which plays an essential role in the up-regulation of phase II antioxidant genes, including HO-1. Once migrated to the nucleus, Nrf2 forms heterodimers with small Maf proteins and subsequently binds to the *cis*-acting ARE. This leads to the transcriptional activation of a number of genes that encode the phase II detoxifying or antioxidant enzymes, such as NQO1, GST, GCL and HO-1 [36]. In the current study, EGCG treatment significantly induced nuclear accumulation of Nrf2 as well as enhancement of Nrf2-ARE binding at the HO-1 promoter site. In contrast, in caveolin-1-silenced cells, both accumulation of Nrf2 as well as enhancement of Nrf2-ARE binding at the HO-1 promoter site were already elevated in untreated cells, with EGCG having no additional effect on stimulation of Nrf2. This suggests that caveolin-1 displacement within functional caveolae may be associated with EGCG-mediated induction of Nrf2 in endothelial cells. To support this hypothesis, it was shown that other nutrients can contribute to caveolin-1 displacement. For example, Li et al [37] found that displacement of caveolin-1 from the plasma membrane by treatment with EPA or DHA [31] is an important mechanism in the prevention against atherosclerosis.

In summary, we provide evidence that caveolae play a role in the uptake and transport of EGCG and mechanisms, which may be associated with the anti-inflammatory properties of this flavonoid. Displacement of caveolin-1 may be necessary for EGCG to initiate the protective signaling cascade. Similar to EGCG treatment, silencing of caveolin-1 by siRNA also resulted in up-regulation of Nrf2, HO-1 and bilirubin production. These data suggest that EGCG-induced caveolin-1 displacement, and subsequent alteration in caveolae function, may be necessary for the activation of Nrf2/HO-1 cellular protection system.

Acknowledgments

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