Role of caveolin-1 in EGCG-mediated protection against linoleic-acid-induced endothelial cell activation

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Received 15 October 2007; received in revised form 29 January 2008; accepted 6 February 2008

Abstract

Flavonoids can protect against inflammatory diseases such as atherosclerosis by decreasing vascular endothelial cell activation. Plasma microdomains called caveolae may be critical in regulating endothelial activation. Caveolae are particularly abundant in endothelial cells and play a major role in endothelial trafficking and the regulation of signaling pathways associated with the pathology of vascular diseases. We hypothesize that flavonoids can down-regulate endothelial inflammatory parameters by modulating caveolae-regulated cell signaling. We focused on the role of caveolae and its major protein, caveolin-1, in mechanisms of linoleic-acid-induced endothelial cell activation and protection by the catechin epigallocatechin-3-gallate (EGCG). Exposure to linoleic acid for 6 h induced expression of both caveolin-1 and cyclooxygenase (COX)-2. Pretreatment with EGCG blocked fatty-acid-induced caveolin-1 and COX-2 expression in a time- and concentration-dependent manner. Similar results were observed with nuclear factor-kappa B DNA binding activity, which was also reduced by caveolin-1 silencing. Exposure to linoleic acid rapidly increased phosphorylation of several kinases, including p38 MAPK, extracellular signal regulated kinase 1/2 (ERK1/2) and amino kinase terminal (Akt), with maximal induction at about 10 min. Inhibitors of ERK1/2 and Akt down-regulated the linoleic-acid-induced increase in COX-2 protein, which also occurred after pretreatment with EGCG. Caveolin-1 silencing blocked linoleic-acid-induced phosphorylation of ERK1/2 and protein expression of COX-2, suggesting that specific MAPK signaling is caveolae dependent. Our data provide evidence that caveolae may play a critical role in regulating vascular endothelial cell activation and protection by flavonoids such as EGCG.

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Keywords: Flavonoids; EGCG; Protection; Linoleic acid; Inflammation; Endothelial cell; Caveolae; Cell signaling

1. Introduction

The lining of blood vessels is protected by the endothelium, and dysfunction of endothelial cells is a critical underlying cause of the initiation of cardiovascular diseases [1]. In addition to endothelial barrier dysfunction, another functional change leading to atherosclerosis is the activation of the endothelium by proinflammatory mediators that regulate the vascular entry of leukocytes. Cyclooxygenase (COX) inhibitors can be applied to reduce inflammation, to relieve pain or to prevent atherothrombotic complications in cardiovascular diseases [2]. The inducible isoform of COX-2 plays a role in inflammation and is constitutively expressed in tissues such as the kidney or vascular endothelium [3].

There is clear evidence that hypertriglyceridemia is an independent risk factor of cardiovascular diseases such as atherosclerosis [4,5]. Even though diets high in omega-6 fatty acids may lead to a decrease in serum cholesterol [6], replacing saturated with unsaturated omega-6-rich lipids
may not be desirable because of their ability to easily oxidize. High intake of linoleic-acid-rich oils or fats will lead to an increase in cellular oxidative stress, which has been implicated in most chronic diseases. Omega-6 fatty acids, especially linoleic acid, can cause endothelial cell dysfunction as well as potentiate tumor necrosis factor-α-mediated endothelial injury [7]. We have recently demonstrated that both the extracellular signal regulated kinase (ERK1/2) and phosphoinositide-3 kinase/aminο kinase terminal (Akt) signaling pathways can contribute to the effect of linoleic acid on nuclear factor-kappa B (NF-κB)-dependent transcription and endothelial cell activation [8].

There is much evidence suggesting that diets high in various nutrients and phytochemicals (e.g., flavonoids) are associated with a reduced risk of chronic diseases, such as cardiovascular diseases and cancer, by affecting molecular mechanisms involved in the initiation and progression of these diseases [9,10]. Flavonoids constitute a subclass of bioactive compounds rich in fruits and vegetables, soy food, legumes, tea and cocoa [11]. Many flavonoids are composed of a polyphenol structure (i.e., several hydroxyl groups on aromatic rings), and these polyphenols are often classified according to structural similarities [12]. Examples of flavonoids include flavonols (e.g., quercetin), isoflavones (e.g., genistein), flavonones (e.g., hesperetin) and flavan-3-ols (e.g., catechins). Many of these bioactive food components are lipophilic, suggesting that they may have a possible interaction with membrane domains or cellular lipid components such as caveolae.

There is increasing evidence that caveolae play a critical role in the pathology of atherosclerosis [13] and that the lack of the caveolin-1 gene may provide protection against the development of atherosclerosis [14]. 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 [15], possibly including bioactive food components such as flavonoids. There is evidence that fatty acids can alter localization and function of caveolae-associated signaling proteins in mouse colonic mucosa [16]. Caveolins have also been reported to colocalize with COX, suggesting that caveolins play a role in regulating the function of this enzyme [17,18]. Besides their role in cellular uptake of lipophilic substances, caveolae house an array of cell signaling molecules involved in endothelial cell dysfunction and inflammation [13].

Little is known about the involvement of caveolae in gene regulation by bioactive compounds. In hypertensive rats, quercetin prevented up-regulation of endothelial nitric oxide synthase expression [19], an enzyme associated with caveolae and involved in vascular tone regulation. Genistein has been shown to decrease the expression of caveolin-1 in ovariectomized rat hearts [20]. Furthermore, the internalization of caveolae can be suppressed by tyrosine kinase inhibitors such as staurosporine [21]. Flavonoids have been described to inhibit these kinases [22,23], which suggests that these bioactive compounds can modulate the function of caveolae.

A major objective of the current study was to explore specific mechanisms involved in anti-inflammatory properties of bioactive compounds like flavonoids within the vascular endothelium. We hypothesized that the regulation of signaling pathways induced by bioactive compounds is associated within caveolae and linked to caveolae function and associated gene inductions. Our data provide evidence that linoleic-acid-induced inflammatory parameters (e.g., COX-2 expression) can be down-regulated by bioactive food components such as epigallocatechin-3-gallate (EGCG) and that these metabolic events are linked to caveolae signaling.

2. Materials and methods

2.1. Materials

Linoleic acid (>99% pure) was obtained from Nu-Chek Prep (Elysian, MN), and EGCG (>98% pure) was purchased from Cayman Chemical (Ann Arbor, MI). Inhibitors LY294002, PD98059 and SB203580 as well as antibodies used for immunoblotting including anti-Akt, anti-phospho-Akt, anti-ERK1/2, anti-phospho-ERK1/2, anti-p38, anti-phospho-p38 and anti-rabbit Ig horseradish-peroxidase-linked antibodies were obtained from Cell Signaling Technology (Danvers, MA). Anti-caveolin-1 antibody was obtained from Affinity BioReagents (Golden, CO). Anti-COX-2 and anti-p65 NF-κB antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin antibody was purchased from Sigma (St. Louis, MO). Supplies and reagents for SDS-PAGE were purchased from Bio-Rad (Hercules, CA).

2.2. Cell culture

EAhy926 cells were a gift from Dr. C.S. Edgell (University of North Carolina). The EAhy926 line was derived by fusing human umbilical vein endothelial cells with the permanent human cell line A549 [24]. The culture medium consisted of Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA), 10% fetal bovine serum (FBS; HyClone, Logan, UT) and antibiotics. Cell cultures were grown until confluent and then synchronized by maintaining in 1% serum for 16 h before treatment for various time periods. Experimental media contained 5% FBS and were supplemented with EGCG for 12 h, followed by linoleic acid at the final concentration of 90 μM, for 6 h. Optimal experimental conditions, such as concentration of linoleic acid (90 μM) and time exposure (6 h), to activate endothelial cells were established previously [25]. Experimental media were enriched with linoleic acid as described earlier [25].

2.3. Caveolin-1 siRNA and transfection

The caveolin-1 gene silencer was designed according to previously described methods [26]. Cells were transfected with control small interfering RNA (siRNA) or caveolin-1
siRNA at a final concentration of 80 nM using GeneSilencer (Genlantis, San Diego, CA) with Optimem I medium (Invitrogen). Cells were incubated with transfection mixtures for 4 h and then replaced with 10% serum medium. Cells were synchronized overnight after 48 h transfection, pretreated with EGCG for 12 h and then treated with linoleic acid or vehicle.

2.4. Immunoblotting

Cells were treated with either vehicle (0.1% DMSO) or EGCG (0–40 μM), followed by linoleic acid (90 μM) for immunoblot analysis of caveolin-1 and COX-2 protein activation. Treatment with DMSO (vehicle) alone for up to 48 h did not affect the expression of both caveolin-1 and COX-2 (data not shown). To analyze the expression of linoleic-acid-induced phospho-Akt, -ERK1/2 and -p38, we treated cells with linoleic acid (90 μM) for 20 min or vehicle (0.1% DMSO). To analyze the effects of MAPK inhibitors on linoleic-acid-induced COX-2 expression, we treated cells with Akt, ERK1/2 and p38 inhibitors (LY294002, PD98059 and SB203580, respectively) for 1 h, followed by the treatment of linoleic acid (90 μM, 6 h) or vehicle (0.1% DMSO). Cell protein was extracted as described before [8]. Equal amounts of protein (20 μg) were fractionated by SDS-PAGE (12% acrylamide) and transferred to nitrocellulose membranes. The membrane was blocked 1 h at room temperature with 5% nonfat milk in Tris-buffered saline (TBS; pH 7.6) containing 0.05% Tween-20 and then washed with TBS-Tween. Membranes were incubated overnight with the primary antibody (1000-fold diluted in TBST containing 5% bovine serum albumin) at 4°C and for 1 h with HRP-conjugated secondary antibody (~5000-fold diluted) at room temperature. Bands were visualized using the appropriate horseradish-peroxidase-conjugated secondary antibodies followed by ECL immunoblotting detection reagents (Amersham Biosciences, Buckinghamshire, England).

2.5. Electrophoretic mobility shift assays of NF-κB DNA binding

Nuclear extracts from endothelial cells were prepared as previously described [27]. Synthetic 5′-biotinylated complementary oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Nuclear extracts were incubated at room temperature for 20 min with biotin-labeled oligonucleotide probes containing the enhancer DNA element for NF-κB (5′ AGTTGAGGGGACTTTCCGCAGGC 3′). Gel mobility shift assay was performed to demonstrate the shifted DNA–protein complexes for NF-κB using a LightShiftTM chemiluminescent EMSA kit (Pierce, Rockford, IL) [28].

2.6. Real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted using TRIzol Reagent (Invitrogen), according to the manufacturer’s protocol. RT was performed using the AMV Reverse Transcription System (Promega, Madison, WI). The levels of mRNAs and the PCR products were then assessed by real-time PCR using 7300 Real-Time PCR System (Applied Biosystems, California). Samples were mixed with SYBR Green Master Mix (Applied Biosystems) and caveolin-1- or 18S-specific primers. The sequences for human caveolin-1 gene were designed by Primer Express Software 3.0 for real-time PCR (Applied Biosystems) and were as follows: sense, 5′ TCAACCGCGACCCTAAACAC 3′; antisense, 5′ CCTTCAATGCCCATTTG 3′. The housekeeping gene was 18S (sense, 5′ TCAGAAGCAGGCTGATT 3′; antisense, 5′ TGGCGCTCTGGTCCGTC 3′).

2.7. 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). One-way ANOVA followed by post hoc least significant difference’s pairwise multiple comparison procedure were used for statistical analysis of the original data. A statistical probability of P<.05 was considered significant.

3. Results

3.1. EGCG decreases caveolin-1 levels in endothelial cells

There is evidence that the lack of the caveolin-1 gene may provide protection against the development of atherosclerosis.
Thus, we tested the possibility that EGCG can down-regulate baseline levels of caveolin-1. Indeed, pretreatment of endothelial cells with increasing concentrations of EGCG down-regulated baseline levels of caveolin-1. A maximum decrease in caveolin-1 mRNA occurred in cells treated with 20 μM EGCG (Fig. 1A). Protein expression of caveolin-1, however, reached the lowest values in cultures exposed to 5 and 20–30 μM EGCG (Fig. 1B).

### 3.2. EGCG protects against linoleic-acid-induced caveolin-1 expression

Exposing endothelial cells for 6 h to linoleic acid (90 μM) markedly induced caveolin-1 (Fig. 2A) protein expression. To test whether EGCG can down-regulate the fatty-acid-mediated induction of caveolin-1, we pretreated endothelial cells with EGCG for 12 h, followed by exposure to linoleic acid. A concentration-dependent protective effect of EGCG against linoleic-acid-induced caveolin-1 was observed, with a 20-μM EGCG pretreatment completely blocking the fatty acid effect.

To assess the time-dependent protective effects of EGCG, we pretreated endothelial cultures for up to 24 h with EGCG (20 μM), before exposure to linoleic acid for an additional 6 h. The fatty-acid-mediated induction of caveolin-1 was completely blocked when cultures were pretreated with EGCG for 12 h (Fig. 2B).

### 3.3. EGCG attenuates linoleic-acid-mediated up-regulation of COX-2

Similar to the caveolin-1 data, exposing endothelial cells to linoleic acid markedly induced COX-2 protein expression (Fig. 3A). Pretreatment with 10 to 30 μM EGCG for 12 h markedly attenuated the fatty-acid-induced COX-2 protein expression.
expression (Fig. 3A). Similar to the caveolin-1 data, the linoleic-acid-induced induction of COX-2 was time dependent and completely blocked after a minimum of 12 h pre-exposure to EGCG (Fig. 3B).

3.4. ERK1/2 and Akt but not p38 MAPK are involved in linoleic-acid-induced up-regulation of COX-2

The p38 MAPK, Akt and/or ERK1/2 pathways may regulate COX-2 expression. Therefore, endothelial cells were exposed to linoleic acid, and the levels of phosphorylated p38 MAPK (p-p38), Akt (p-Akt) and ERK1/2 (p-ERK1/2) were assessed by Western blotting. As indicated in Fig. 4, linoleic acid rapidly increased activation of all kinases in a time-dependent manner, with maximum phosphorylation at 5 min for p-p38 MAPK (Fig. 4A), 5 min for p-ERK1/2 (Fig. 4B) and 10 min for p-Akt (Fig. 4C). In order to determine which of these kinases is involved in linoleic-acid-mediated COX-2 expression, we pretreated endothelial cells for 1 h with specific pharmacological inhibitors of individual kinases, such as LY294002 for Akt, SB203580 for p38 MAPK and PD98059 for ERK1/2. Subsequently, cells were incubated with or without 90 μM linoleic acid for 6 h. As indicated in Fig. 5, LY294002 and PD98059, but not SB203580, effectively blocked linoleic-acid-induced activation of COX-2. Treatment with inhibitors alone had no effect on activation of COX-2 or expression of caveolin-1 (data not shown).

3.5. Caveolin-1 silencing mimics the protective effects of EGCG on linoleic-acid-induced ERK1/2 phosphorylation and COX-2 expression

 Knowing that the ERK1/2 and Akt pathways are involved in linoleic-acid-mediated induction of COX-2, we next determined the role of caveolin-1 and EGCG in activation of these signaling cascades. In these experiments, we utilized siRNA to specifically silence caveolin-1. This procedure reduced caveolin-1 expression by ~80% as compared with control cells without changing β-actin and total ERK1/2 levels (Fig. 6A). Most importantly, caveolin-1 silencing significantly protected against linoleic-acid-induced phosphorylation of ERK1/2 (Fig. 6A); however, it did not affect linoleic-acid-mediated phosphorylation of Akt (data not shown).
shown). Pretreatment with EGCG blocked linoleic-acid-mediated phosphorylation of both ERK1/2 (Fig. 6A) and Akt (data not shown). Similar to the ERK1/2 phosphorylation data, both pretreatment with EGCG and caveolin-1 silencing effectively blocked linoleic-acid-mediated induction of COX-2 (Fig. 6B).

### 3.6. Caveolin-1 silencing reduces linoleic-acid-induced NF-κB DNA binding

NF-κB is a transcriptional regulator of COX-2 induction. Therefore, our studies were completed by determination of the role of EGCG and caveolin-1 in linoleic-acid-induced activation of NF-κB. Linoleic acid significantly increased NF-κB DNA binding activity, which was blocked when cells were pretreated with EGCG (Fig. 7). In addition, caveolin-1 silencing mimicked the effects of EGCG and significantly decreased the linoleic-acid-induced activation of NF-κB (Fig. 7).

### 4. Discussion

Bioactive compounds such as flavonoids are known to have anti-inflammatory properties and to provide protection against inflammatory diseases such as atherosclerosis [11]. Endothelial cells line the inner layer of blood vessels and play a critical role in the overall dynamics of vascular physiology. Activation and subsequent dysfunction of the endothelium are considered early events in the etiology of cardiovascular diseases such as the pathology of atherosclerosis [1]. Endothelial cells are constantly exposed to blood components, including food-derived lipids, toxicants and so forth, and are thus highly susceptible to insult and activation, leading to inflammatory interactions with cytokines and increased uptake of activated leukocytes into the vasculature.

In the current study, endothelial cells were activated with linoleic acid to mimic a postprandial hyperlipidemic state.
Lipoproteins [7]. Lipoprotein-lipase-mediated hydrolysis of triglyceride-rich particles exposed to significant levels of free fatty acids derived from hypertriglyceridemia, and the vascular endothelium can be activated COX-2. High-fat diets contribute to [32], suggesting that our fatty acid data may mimic an in vivo situation. Caveolin-1 expression in aorta of diet-induced obese rats evidenced suggests that high-fat diets can up-regulate COX-2 induction on functional caveolae. Recent studies are needed in animal models to examine the effects of EGCG on inflammatory genes.

![Fig. 7. Both EGCG and caveolin-1 silencing reduce linoleic-acid-induced NF-κB DNA binding. Endothelial cells were transfected with siRNA for caveolin-1 (Cav-1 siRNA) or with control siRNA (Ctr-siRNA) and treated with EGCG (20 μM) for 12 h, and then exposed to linoleic acid (LA, 90 μM) for 3 h. Electrophoretic mobility shift assay for NF-κB was performed with nuclear proteins extracted from endothelial cells. The Western blot shown represents one of three experiments. Densitometry results shown in parallel represent the mean±S.E.M. of three independent experiments. *Significantly different compared to vehicle control. #Significantly different compared to cultures treated only with LA (Ctr-siRNA).](image)

High-fat or high-energy diets can lead to hypertriglyceridemia and increased exposure of the endothelium to free fatty acid anions, metabolic events known to activate endothelial cells [29,30]. We have previously demonstrated that endothelial cell exposure to fatty acids, especially linoleic acid, markedly induced an endothelial inflammatory response [31]. We also have demonstrated that the ERK1/2 signaling pathway can contribute to the effect of linoleic acid on NF-κB-dependent transcription [8]. In the current study, inhibitors of ERK1/2 and Akt down-regulated the linoleic-acid-induced increase in COX-2 protein, demonstrating the involvement of MAPK signaling in our model of endothelial cell activation.

Most interestingly, linoleic acid induced both caveolin-1 and COX-2, which is significant because of the link between caveolins (caveolae) and the pathology of atherosclerosis [13]. Caveolin-1 has been reported to colocalize with interleukin-1β-induced COX-2 [30], suggesting the dependence of COX-2 induction on functional caveolae. Recent evidence suggests that high-fat diets can up-regulate caveolin-1 expression in aorta of diet-induced obese rats [32], suggesting that our fatty acid data may mimic an in vivo response by activating COX-2. High-fat diets contribute to hypertriglyceridemia, and the vascular endothelium can be exposed to significant levels of free fatty acids derived from lipoprotein-lipase-mediated hydrolysis of triglyceride-rich lipoproteins [7].

Protective mechanisms of plant-derived bioactive compounds (e.g., flavonoids) against endothelial activation and inflammation are not well understood. EGCG, the most abundant and effective polyphenol of green tea, has been shown to inhibit the expression of COX-2 and the production of prostaglandin E2 [30,33]. In the current study, pretreatment with EGCG blocked both fatty-acid-induced COX-2 and caveolin-1 protein expression in a time- and concentration-dependent manner. We also found that EGCG can down-regulate baseline levels of the caveolin-1 gene at both the mRNA and protein level. These data suggest that the anti-inflammatory properties of EGCG may reside at or be initiated at the cellular level of caveolae and associated signaling molecules. In fact, down-regulation of COX-2 by EGCG was mimicked by selective inhibitors of kinases such as ERK1/2 and Akt. Others also have reported EGCG-mediated down-regulation of MAPK pathways such as ERK1/2 and decreased COX-2 activity in cancer cell lines [33,34] and in human vascular smooth muscle cells [35]. MAPKs such as ERK1/2 are known to regulate NF-κB [36], and there is evidence that a decrease in COX-2 by EGCG may be through inhibition of NF-κB [37]. Indeed, our data show that EGCG can decrease linoleic-acid-induced activation of NF-κB. Most importantly, our data suggest that caveolae may provide a critical signaling platform for both induction and protection of inflammatory genes. EGCG concentrations utilized in our cell culture model system were relatively high compared to plasma levels attainable through diets [38]. Thus, further studies are needed in animal models to examine the effects of EGCG on inflammatory genes.

We provide evidence in the current study that EGCG-mediated down-regulation of both NF-κB and COX-2 is dependent on functional caveolin-1, the main structural protein of caveolae. This is significant because caveolae are highly expressed in endothelial cells [15]. We provide novel data that demonstrate that silencing of the caveolin-1 gene can markedly down-regulate linoleic-acid-induced phosphorylation of ERK1/2, NF-κB and COX-2, suggesting that specific MAPK signaling is caveolae dependent. The specificity for ERK signaling may be due to the colocalization of Ras, a small GTPase upstream of ERK, with caveolae [39–41].

In summary, data from the current study strongly support the hypothesis that caveolae are involved in regulation of inflammatory signaling pathways in vascular endothelial cells. In addition, these signaling mechanisms can be modulated by the interaction of bioactive compounds, such as EGCG, with the cellular lipid milieu.

Acknowledgments

This research was supported in part by grants from NIEHS, NIH (P42ES07380) and the University of Kentucky AES.
References


