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PROTECTION AGAINST ENDOTHELIAL INFLAMMATION BY GREEN TEA FLAVONOIDS

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ABSTRACT OF DISSERTATION

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The Graduate School
University of Kentucky
2010

PROTECTION AGAINST ENDOTHELIAL INFLAMMATION BY GREEN TEA FLAVONOIDS

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
Graduate Center for Nutritional Sciences
at the University of Kentucky

By

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Lexington, Kentucky

Advisor: Dr. Bernhard Hennig, Professor of Nutrition and Toxicology

Lexington, Kentucky

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Endothelial inflammation is a pivotal early event in the development of atherosclerosis. Long term exposure to cardiovascular risk factors will ultimately exhaust those protective anti-inflammatory factors such as the heme oxygenase (HO) system. The HO system plays a critical role in cellular and tissue self-defense against oxidative stress and inflammation. Caveolae are membrane domains and 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 flavonoids. Research in this dissertation addresses the role of HO-1 and caveolae on dietary flavonoid epigallocatechin gallate (EGCG) mediated protection against pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) and linoleic acid-induced activation of endothelial cells. The data support the hypothesis that EGCG protects against TNF-α-induced monocyte recruitment and adhesion partially through the induction of HO-1 and bilirubin. The observed anti-inflammatory effects of EGCG are mimicked by the HO-1 inducer cobalt protoporphyrin (CoPP) and abolished by HO-1 gene silencing. Nrf2 is the major transcription factor of phase II antioxidant enzymes including HO-1. Results clearly show that EGCG-induced HO-1 expression and subsequent bilirubin productions are dependent on functional Nrf2. EGCG also can down-regulate the base-line level of caveolin-1. Furthermore, silencing of the caveolin-1 gene can markedly down-regulate linoleic acid-induced COX-2 and MCP-1, indicating that caveolae may be a critical platform regulating inflammatory signaling pathways. Similar to EGCG treatment, silencing of caveolin-1 can also result in the activation of Nrf2, up-regulation of HO-1 and bilirubin. This may be one of the mechanisms to explain the protection effect of caveolin-1 gene silencing against endothelial inflammation. Moreover, EGCG rapidly accumulates in caveolae, which is associated with caveolin-1 displacement from the plasma membrane towards the cytosol. Caveolin-1 gene silencing can significantly reduce the uptake of EGCG in endothelial cells within 30 min. These data suggest that caveolae may play a role in the uptake and transport of EGCG in endothelial cells. These studies provide a

novel target through which EGCG functions to protect against inflammatory diseases such as atherosclerosis.

KEYWORDS: epigallocatechin gallate (EGCG), heme oxygenase-1 (HO-1), caveolae, linoleic acid, endothelial cells

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PROTECTION AGAINST ENDOTHELIAL INFLAMMATION BY GREEN TEA FLAVONOIDS

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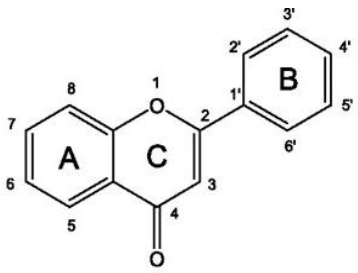
Chapter One: Introduction

Green tea (Camellia sinensis) catechins and vascular function

Polyphenols characterized by aromatic rings bearing one or more hydroxyl substitutions are secondary metabolites of a wide variety of plants, including fruits, vegetables, and legumes. Polyphenols also are enriched in plant-derived beverages, such as tea and wine [4, 5]. Polyphenols are divided into more than ten different classes depending on their chemical structures. The most widely distributed plant phenolics are flavonoids. Flavonoids are composed of two benzene rings, A-ring and B-ring, and joined by a three-carbon chain to form a closed heterocyclic C-ring. According to the degree of oxidation in the C-ring, flavonoids are divided into six major groups: flavones (e.g.,luteolin, apigenin), flavonols (e.g.,myricetin, quercetin), catechins (e.g.,epicatechin, gallocatechin), flavanones (e.g., naringenin, hesperidin), anthocyanidins (e.g.,cyanidin, pelargonidin), and isoflavones (e.g.,genistein, daidzein) [3]. So far, more than 5000 different flavonoids have been reported. They provide much of the color and flavor to vegetables and fruits. Numerous studies focus on flavonoids' abilities to protect against oxidative stress and inflammation in disease processes. There are only a few estimations of the daily intake of dietary flavonoids available. It has been reported that the average daily intake of flavonoids in the United States is between 1 to 1.1g/day [6]. Higher intake of flavonoids is associated with a reduced risk for coronary artery disease [7-10]. However, there are also some studies which have found no significant association after elevated consumption of flavonoids [11]. Flavonoids have been of interest due to their bioactive effects *in vitro*. Some of them are anti-inflammatory, anti-proliferative, and anti-estrogenic agents; others are enzymatic activity modulators [5].



Flavonoids

Tea is one of the most popular beverages in the world. Green tea (Camellia sinensis) originates from Asia. It differs from black tea by the minimal fermentation of tea leaves during production. Green tea is rich in polyphenols, about 80% of which are catechins. They are epicatechin (EC), epigallocatechin (EGC), epicatechin-3-gallate

(ECG), and epigallocatechin-3-gallate (EGCG). The most beneficial effects of green tea are attributed to catechins. Flavonoids account for up to 30% of the dry weight of green tea. EGCG is the most abundant and most bioactive catechin in green tea. A cup of green tea contains 20-150 mg EGCG [12]. Green tea catechins are absorbed intestinally, with peak absorption occurring at 1.5-2.5 h after consumption. The bioavailability of catechins is low because of the poor absorption, and rapid metabolism. Only 2% of consumed catechins appear in the plasma [13]. However, the biological activities of some green tea catechin metabolites have been shown to have similar or even higher activity than the parent compounds [14, 15].

Epidemiological studies have provided a clear link between the consumption of green tea and the prevention of cardiovascular diseases. Imai showed that increased green tea consumption can reduce total cholesterol, triacylglyceride and atherogenic index [16]. A recent study of 76,979 Japanese adults found that green tea consumption can decrease death from cardiovascular disease [17] . There are only a few chronic human intervention studies in the area of green tea and vascular function. Recently, Widlansky *et al.* [18] specifically investigated the effects of isolated EGCG. They found that vascular function was significantly increased after acute but not chronic supplementation. Conversely, Kim *et al.* [19] found a significant improvement in flow-mediated dilation (FMD) –assessed vascular reactivity after 14 d consumption of green tea. Similar conclusions were also found by Tinahones *et al.* [20]. Lee *et al.* [21] conducted a study that showed a significant reduction in soluble P-selectin and oxidized LDL after the consumption of 600 ml green tea for 28 d in 20 smokers. Numerous animal and cell-culture models also indicate that green tea catechins have a positive effect on endothelial and overall vascular functions.

In recent years, the mechanisms underlying the impact of green tea catechins on vascular function have been the focus of much research. A number of plausible molecular mechanisms have been proposed. A report by Nanjo *et al.* [22] suggested that EGCG is the most effective catechin for free radical scavenging. Paquay *et al.* [23] showed that EGCG could scavenge peroxynitrite, which can proceed to oxidize LDL or up-regulate pro-inflammatory cytokines and adhesion molecules. Similarly, EGCG inhibited the cytokine induction of VCAM-1 in HUVEC [24]. Pretreatment of human aortic

endothelial cells with catechin metabolites extracted from the plasma of catechin treated rats significantly inhibited monocyte adhesion [25], suggesting that the metabolites of catechins also have strong anti-inflammatory properties. The inhibition of endothelial exocytosis and P-selectin cell-surface expression by EGCG has also been reported [26]. Moreover, catechins may down-regulate NADPH oxidase activity, reducing the production of superoxide, and protecting NO from peroxynitrite formation [27]. Mizugaki *et al.* [28] reported that prostacyclin (PGI_2), a potent vasodilator, was dose-dependently induced by the addition of EGCG in cultured bovine endothelial cells. Moreover, EGCG reduced the level of asymmetric dimethylarginine (ADMA), which plays a crucial role in endothelial dysfunction and coronary artery disease [29]. Actis-Goretta *et al.* [30] demonstrated that green tea can significantly inhibit the activity of angiotensin-converting enzyme (ACE), which is an important mediator in blood pressure regulation.

Endothelial cell dysfunction, inflammation and atherosclerosis

Cardiovascular disease (CVD) is America's leading killer for both men and women among all racial groups. Almost 1 million Americans die of CVD each year. CVD is responsible for more than 30 percent of all deaths in the United States. Almost 75% of all deaths from cardiovascular disease are due to myocardial infarction or stroke caused by atherosclerosis. Atherosclerosis is a chronic inflammatory disease developing in the arterial wall in response to various injurious stimuli and resulting in excessive inflammatory reactions. Endothelial cells (ECs) are involved in all stages of atherogenesis and their dysfunction initiates plaques formation [31, 32].

The endothelium separates the plasma and the underlying tissue, and its constitutive properties are involved in a large array of functions. Under physiological conditions, ECs regulate vasoconstriction and vasodilation, and hence the control of blood pressure. The endothelium acts as a selective barrier between the vessel lumen and underlying tissue, monitoring the transport of materials and plasma molecules into and out of the bloodstream. Excessive increases in permeability of the endothelial layer may lead to tissue edema. In addition, ECs play a crucial role in regulating cholesterol and lipid homeostasis, signal transduction, inflammation and haemostasis [33, 34]. Under

pathological conditions, endothelial dysfunction is a biomarker for vascular diseases and is often regarded as a key early event in the development of atherosclerotic plaques. Impaired endothelial function is often seen in patients with coronary artery disease, diabetes mellitus, hypertension, and hypercholesterolemia.

A pivotal early event in the development of atherosclerosis is the activation of ECs by persistent dyslipidemia generated ROS, which has been increasingly recognized as a common denominator that induces most cardiovascular diseases. Hydrogen peroxide and superoxide anions are two of the most important biological ROS in cardiovascular systems. Excessive ROS production stimulates various endothelial events, including induction of inflammatory gene, monocyte adhesion, platelet aggregation, and impaired endothelium-dependent relaxation [35]. ROS production is positively regulated by many cytokines whose expressions are induced in activated ECs. These cytokines include tumor necrosis factor-α (TNF-α), interleukins (IL), angiotensin II, vascular endothelial growth factor and CAMs: intercellular adhesion molecule-1 (ICAM), vascular cell adhesion molecule-1 (VCAM-1), E and P selectin and chemokines, such as the monocyte chemoattractant protein-1 (MCP-1). Lipid infusion also stimulates the production of ROS and inflammation [31]. NF-κB is an inducible transcription factor which is activated by several cytokines and growth factors found in atherosclerotic lesions, such as MCP-1, TNF-α, and IL-1β. Evidence suggests that oxidative stress stimulates the translocation of NF-κB from the cytoplasm to the nucleus [36]. AP-1 is another important transcription factor influenced by the cellular redox state, and induced by H_2O_2, LDL, and oxLDL, to regulate vascular inflammatory genes, such as MCP-1and ICAM-1 [37].

Nitric oxide regulates vascular tone and also has anti-atherogenic effects on EC and platelets [38]. NO bioactivity is significantly decreased in atherosclerosis due to diminished eNOS expression and activity, eNOS uncoupling, and impaired NO-regulated cell signaling [39]. Endothelial regulated vascular tone is dependent on the synthesis and release of vasodilators, such as NO, prostacyclin and EDHF, of vasoconstrictors, such as endothelin-1(ET-1), angiotensin II, and prostanoids. Increased plasma levels of remnant-like lipoprotein particles (PLp), which are highly atherogenic, may impair endothelial functions through eNOS [40]. Endothelial CD36 is a scavenger receptor (SR) that binds to oxLDL, trombospondin, and long-chain fatty acids. LOX-1 which mediates the action

of oxLDL in the vascular wall is also detected in EC in early atherosclerosis, and it is induced by ROS, ET-1, TNF-α, and inflammatory molecules [41].

In the early stage of atherosclerosis, modified lipoproteins accumulate in the intima, the endothelial cell lining plaques take up modified lipoproteins, which are either degraded or exocytosed into the lumen. Also, EC-SR non-regulated uptake of the modified lipoproteins results in the accumulation of some large lipid droplets within the EC [31, 42]. Later, local inflammatory regulators, cytokines, ROS and oxLDL induce EC synthesis of MMP, and finally trigger EC apoptosis [43]. In more advanced stages of atherosclerosis, formation of fibrous plaques and lesions will ultimately lead to an acute clinical event by plaque rapture and thrombosis [44, 45].

Overall, the inflammatory reaction in endothelial cells is regulated by complex interactions. Long time exposure to cardiovascular risk factors will ultimately exhaust those protective anti-inflammatory factors in EC. Since the initial stage of atherosclerosis is the critical time point when nutrition (e.g. flavonoids) modulation could have the most efficient effects, the research in the current dissertation focuses on the early events of atherosclerosis, including the role of caveolae and heme oxygenase-1 (HO-1) on dietary flavonoid EGCG-mediated protection against pro-inflammatory cytokine tumor necrosis factor (TNF)-α or linoleic acid-induced activation of endothelial cells.

Caveolae and atherosclerosis

Caveolae are 50-100 nm omega-shaped cell membrane invaginations present in almost all cell types in cardiovascular systems [46]. They have been widely studied since their initial description by electron microscopy in 1950. Caveolae are a subcategory of lipid rafts and are enriched in structural proteins called caveolins. Three caveolin homologues exist, caveolin-1, -2, and -3, with molecular weights of about 24 kDa. Caveolin-1 and -3 are necessary for the formation of caveolae structure, while caveolin-2 is dispensable. Endothelial cells, adipocytes, pneumocytes, and fibroblasts have the highest levels of caveolin-1 and -2 [47, 48], while caveolin-3 expression is restricted to smooth and striated muscle cells [49]. Since caveolae are highly enriched in endothelial cells, cavolin-1-deficient mice showed several defects that correlated with endothelial cells [50]. Studies show that caveolae play an important role in the regulation of

endothelial cell function. The most frequently studied area focused on the ability of caveolae to mediate endothelial nitric oxide synthase (eNOS) and their role in the regulation of angiogenesis. Researchers found that caveolin-1-deficient mice displayed increased eNOS activity, pulmonary dysfunction, and cardiomyopathy [51-53]. The fatty acid composition of caveolae in endothelial cells has been documented by Gafencu *et al*. [54]. They found that palmitic acid, palmitoleic, stearic acid, and oleic acid are most prevalent and compose 80% of total fatty acids; and linoleic acid is about 8 to 10% of total fatty acids. Ma *et al.* [55] found that omega-3 fatty acids altered the function of proteins in caveolae by reducing both caveolin-1 and cholesterol in mice colons. Li *et al*. [56] reported that eicosapentaenoic acid (EPA) induced caveolin-1 translocation from caveolae towards cytosol in endothelial cells.

One of the aspects of caveolae that makes them an interesting research subject is the fact that they are believed to harbor a subset of important signal transduction molecules and receptors. Several G proteins and G protein-coupled receptors (GPCRs) [57], tyrosine kinase-coupled receptors [58], such as epidermal growth factor receptors, vascular endothelial growth factor receptors and transforming growth factor receptors, have been reported to reside in caveolae. eNOS is another important caveolae-associated signaling enzyme. Caveolin-1 inhibits eNOS activity [59]. Increased eNOS activity has been observed by several groups in caveolin-1 deficient mice. Moreover, increased plasma levels of NO oxidation products, nitrite and nitrate were documented [52, 60]. PKC is also dynamically associated with caveolae [61, 62]. The scaffolding domain of caveolae which is responsible for molecular interactions inhibit PKC activity [63] and vasoconstriction [64]. In some arteries of caveolin-1 deficient mice, PKC-regulated vasoconstriction is increased [65]. Although caveolae play a critical role in several signaling pathways, surprisingly, the mice lacking caveolae are both viable and fertile [52]. However, there are still some phenotypes which involve heart, lungs and blood vessel change, e.g., increased thickness of the left ventricular wall [51], increased fibrosis in many organs including the heart [66], and pulmonary arterial hypertension [67].

In endothelial cells, another important function of caveolae is their ability to transfer molecules from the lumen of blood vessels to the sub-endothelial space, a process called transcytosis. Three different pathways exist: receptor-mediated

transcytosis, fluid-phase transcytosis, and paracellular transfer. Caveolae are involved in the first two pathways. For example, caveolae can transport albumin [68], insulin [69], low-density lipoproteins (LDL), modified LDL [68, 70], and chemokines [71]. These transcytosis systems play a critical role in protection and regulation of homeostasis, and specific and targeted delivery of molecules. As is well known, the transcytosis of LDL across endothelial cells is an important event in the development of atherosclerosis. Although it has been studied for more than three decades, the specificity of the pathway is still not very clear as contradictory results showed presence or absence of a specific receptor of LDL [72]. Some receptors, such as CD36, SR-B1 and the LDL receptor may be the candidate receptors. Since the accumulation of modified LDL in the sub-endothelial space is the initial step in the development of atherosclerosis, the elimination of this transcytosis would therefore be associated with reduced atherosclerotic lesions. Studies have shown that the absence of caveolae in apolipoprotein-E/caveolin-1 double knockout mice reduced fatty streak lesion formation by about 70% compared with apo-E-deficient mice [73]. Since the transcytosis process seriously affects endothelial permeability, many studies have focused on the pathways regulated by caveolin-1 involved in the regulation of endothelial permeability. It has been shown that eNOS and NF-κB are involved in these signaling pathways. Increased activity of eNOS in caveolin-1 deficient mice displayed increased vascular permeability, shown by increased albumin transportation [73].

Besides transcytosis, caveolae also play an important role in endocytosis. Unlike clathrin-mediated endocytosis, internalization through caveolae involves complex signaling [74]. Caveolin-1, the major scaffolding protein constituent of caveolae, participates in vesicular trafficking and signal transduction. And caveolin-1 can cycle between the plasma membrane and several intracellular compartments [75]. 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 flavonoids by means of endocytosis [76, 77].

Little is known about the involvement of caveolae in gene regulation by bioactive compounds. Genistein has been shown to decrease the expression of caveolin-1

in ovariectomized rat hearts [78]. Furthermore, our lab found that quercetin could decrease caveolin-1 protein expression in endothelial cells [79]. All these results suggest that these bioactive compounds can modulate the function of caveolae. Recently, it has been reported that the laminin receptor which resides in lipid rafts as a cell surface receptor could form laminin receptor-EGCG complex and link EGCG action to MAPK pathways [80, 81]. These findings suggest that lipid rafts may be associated with the function of EGCG. Caveolae, a subcategory of lipid rafts, may also provide an active platform for optimal tuning of signaling responses. The dynamic regulation of signaling transduction through recruitment of EGCG targeting caveolae may result in reprogramming of cellular responses.

Heme oxygenase-1, nuclear factor-E2-related factor 2 (Nrf2) and cardiovascular diseases

Heme oxygease-1 (HO-1) is an inducible isoform of the rate-controlling enzyme degrading heme to carbon monoxide (CO), ferrous (Fe^{2+}) and biliverdin, the latter being subsequently converted into bilirubin. Heme is the prosthetic group of several enzymes which play critical roles in the regulation of endothelial function. These enzymes include nitric oxide synthase (NOS), cytochrome P450, monooxygenase, cyclooxygenase (COX) and catalase [82]. Excess heme catalyzes the formation of reactive oxygen species, which cause endothelial dysfunction. Therefore, the HO system may be recognized as a protector of endothelial cell integrity. It has been reported that HO systems have three isoenzymes HO-1, -2, and -3. HO-2 is the constitutive isoform. HO-3 is only expressed in rats. HO-1 is the only inducible isoform which can be up-regulated by a wide spectrum of inducers, including endotoxin, heavy metals, oxidants, and hypoxia and pharmaceutical agents, such as aspirin, statins, probucol, losatan and resveratrol [1]. These products have both physiological and pathological functions. CO is not an antioxidant, but it could induce antioxidant genes. CO scavenges superoxide [83], and increases glutathione level [84]. Furthermore, CO induces cGMP level, leading to vasorelaxation [85]. Bilirubin has strong anti-oxidant properties which have been shown to scavenge reactive oxygen species, inhibit NADPH oxidase activity [86], preserve endothelial integrity, in part by increasing the bioavailability of NO required for endothelial integrity [87]. Bilirubin also

inhibits oxidation of low density lipoprotein [88]. Therefore, HO-1 derived bilirubin plays an important role in cellular and tissue self-defense against oxidative stress. Higher serum bilirubin levels are associated with a decreased risk for coronary artery disease [89]. Induction of CO and bilirubin has shown promise in protecting against oxidative stress, whereas HO-1 deficient mice showed accelerated atherosclerotic lesion formation, vein-graft disease and elevated blood pressure [90]. Moreover, it has been shown by several laboratories that HO-1 up-regulation is cytoprotective in atherosclerosis [91], diabetes [83], lung injury [92], and occlusive vascular disease [93].

HO-1 is highly expressed in the endothelium and foam cells of atherosclerotic lesions [94]. In vascular endothelial cells, smooth muscle cells, and macrophages, HO-1 can be induced by oxLDL, a major determinant in the pathogenesis of atherosclerosis [95]. HO-1 expression and activity is high throughout the development of atherosclerosis, from an early fatty streak to an advanced complex atherosclerotic lesion in human aortic endothelial cells and smooth muscle cells. It has been reported in LDL receptor deficient mice or Watanabe heritable hyperlipidemic rabbits that the induction of HO-1 could reduce atherosclerotic lesion size [96, 97]. Moreover, HO-1 knockout mice with an apolipoprotein E deficient background showed accelerated atherosclerotic lesions compared with apolipoprotein E deficient mice [98]. Induction of HO-1 by pharmaceutical agents or adenovirus-mediated gene transfer reduces atherosclerosis in murine models [91]. Endothelial dysfunction is a well established response to cardiovascular risk factors, and precedes the development of atherosclerosis. Endothelial activation caused by oxLDL and TNF-α is critical in the development of atherosclerosis. HO-1 induction in endothelial cells attenuates inflammatory mediator production, and reverses oxLDL and TNF-α-impaired-vasodilatory responses [99]. CO inhibits the lipopolysaccharide-induced expression of pro-inflammatory cytokines, such as IL-1β, TNF-α, and increases the expression of anti-inflammatory cytokine IL-10 in both endothelial and macrophages [100]. CO induces sGC and cGMP levels, which regulate blood pressure and vascular contractility [101].

The expression of HO-1 may be regulated by NF-E2-related factor 2 (Nrf2), a leucine zipper transcription factor. Nrf2 plays an essential role in the up-regulation of phase II anti-oxidant genes, including HO-1 [102]. Its activity is in part regulated by an

Nrf2 inhibitory protein called Kelch-like ECH-associated protein 1 (Keap1). Keap1 suppresses the transcriptional activity of Nrf2 by specifically binding to its evolutionarily conserved amino-terminal in the cytoplasm. When electrophiles or other reactive species activate cells, Keap1 dissociates with Nrf2, releases Nrf2 and allows this transcription factor to traverse from the cytoplasm into the nucleus. Once migrated to the nucleus, Nrf2 forms heterodimers with small Maf proteins and subsequently binds to the cis-acting antioxidant response element (ARE). This leads to the transcriptional activation of a number of genes that encode for the phase II detoxifying or antioxidant enzymes, such as NQO1, GST, GCL, and HO-1 [103].

It has been reported that the induction of endogenous anti-oxidative enzymes by tea flavonoids contributes to their anti-oxidative effect. Wu *et al.* found that EGCG protection against oxidative stress by the induction of HO-1 in human endothelial cells is PI3K/Akt-dependent [104]. The aim of this dissertation project was to understand whether the induction of HO-1 by EGCG in endothelial cells correlated with its protective effects and whether direct inhibition of HO-1 will diminish EGCG-mediated protection against TNF-α–induced endothelial activation.

TNF-α induces endothelial dysfunction

TNF-α, one of the most potent pro-inflammatory cytokines [105], is highly expressed throughout the full spectrum of atherosclerotic development [106]. TNF-α is a critical player in inflammation through stimulating the expression of adhesion molecules on endothelium and decreasing endothelial NO generation thereby inducing endothelial dysfunction [107]. Accumulating evidence suggests that TNF-α is important in the disruption of macrovascular and microvascular circulation by modulating advanced glycation end-products (AGEs), receptor for AGEs (RAGE), lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) and NF-κB regulated signaling pathways [108]. TNF-α stimulates superoxide production in endothelial cells through NADPH oxidase [109], XO [110], NOS [111] and other pathways. The production of ROS stimulates a cytokine cascade which induces the expression of TNF-α [112]. TNF-α also affects and mediates lipid metabolism. TNF-α levels are associated with the concentration of very

low density lipoprotein (VLDL), triacylglycerol, and cholesterol and negatively with high density lipoprotein (HDL) in hyperlipidaemia patients [113]. In the current study, endothelial cells were stimulated with TNF-α to induce inflammation.

Linoleic acid induces endothelial inflammation

Linoleic acid (LA, 18:2n-6) is the primary essential fatty acid and represents the basis of the omega-6 family. Linoleic acid is enriched in safflower oil, sunflower oil, corn oil, peanut oil and cotton seed oils, accounting for more than half of the total fatty acid in these oils [114]. Diets high in omega-6 fatty acids are associated with increased inflammation and thus an increased risk for cardiovascular diseases. Linoleic acid, the major omega-6 fatty acid in the American diet, is recognized to be pro-inflammatory and pro-atherogenic since it favors oxidative modification of LDL cholesterol, and increases platelet aggregation [115]. Although diets high in omega-6 fatty acids may decrease serum cholesterol [116], it is not a good choice to replace saturated with unsaturated omega-6 fatty acid because they are easily oxidized. High intake of linoleic acid–rich oils or fats causes cellular oxidative stress and induces an inflammatory response [117], which initiates the development of endothelial dysfunction and atherosclerosis. It has been reported that linoleic acid induced oxidative stress, increased intracellular calcium levels [118] , and diminished nitric oxide synthase activity [119] in cultured endothelial cells. People demonstrated that linoleic acid could potentiate TNF-α–mediated endothelial injury [120]. Our lab has reported that both the extracellular signal regulated kinase (ERK1/2) and phosphoinositide-3 kinase/amino kinase terminal signaling pathways contribute to the effect of linoleic acid on NF-κB-mediated endothelial cell activation [121]. Linoleic acid is proatherogenic by inducing arterial smooth muscle cell proliferation [122]. Moreover, linoleic acid derived from the hydrolysis of triglyceride-rich lipoproteins caused endothelial injury [120]. The current hypothesis is that linoleic acid-induced signaling pathways can be affected by bioactive food components such as epigallocatechin-3-gallate (EGCG), and that these metabolic events are linked to caveolae signaling.

Hypothesis and significance of the current study

Previous studies have demonstrated that increasing levels of circulating cytokines such as TNF-α can induce endothelial activation and promote an inflammatory response [121, 123]. Epidemiological studies have provided a clear link between the consumption of green tea and the prevention against cardiovascular diseases. A number of plausible molecular mechanisms using the green tea extracts EGCG have been proposed. However, the role of caveolae on the protection effects of EGCG against endothelial cell activation has not been explored. It is possible that caveolae play a role in the uptake and transport of EGCG, and that EGCG can alter the membrane lipid rafts (caveolae) environment, therefore affecting the connected cell signaling transduction and gene expression. This dissertation investigated the regulation of signaling pathways induced by bioactive compounds within caveolae and linked to caveolae function and associated gene inductions. Specifically, it was elucidated how dietary omega-6 fatty acid-induced signaling pathways are regulated by bioactive food components such as EGCG and the subsequent events linked to caveolae signaling.

This dissertation also describes the role of HO-1 in EGCG-mediated protection against TNF-α-induced endothelial activation. The heme oxygenase system is an important regulator of endothelial cell integrity and oxidative stress. HO-1 deficient mice showed accelerated atherosclerotic lesion formation, vein-graft disease and elevated blood pressure [90]. Moreover, HO-1 up-regulation is cytoprotective in atherosclerosis [91]. Thus, a major objective of the current study was to explore the role of HO-1 on mechanisms of EGCG-mediated protection of the vascular endothelium. The hypothesis tested was that EGCG can exhibit anti-inflammatory properties partially via induction of HO-1 and subsequent AP-1 signaling.

Induction of Nrf2-ARE signaling provides a cellular self-defense against a variety of electrophilic compounds, reactive toxicants and oxidant induced cell stress [124]. To further explore the hypothesis that caveolae play a role in the uptake and transport of EGCG and the association with the activation of Nrf2/HO-1 cellular self-defense system, EGCG levels in caveolae-enriched fractions were quantified, and the effect of EGCG on the activation of Nrf2 and HO-1 with or without functional caveolae was explored. It was

hypothesized that EGCG can stimulate caveolin-1 displacement from lipid microdomains towards the cytosol, thereby altering their functionality and subsequent downstream signaling. These investigations may provide a novel target through which EGCG functions to protect against inflammatory diseases such as atherosclerosis.

In summary, this dissertation describes and elucidates caveolae and HO-1 associated mechanisms of inflammatory diseases and protection by bioactive food components with antioxidant and anti-inflammatory properties such as the green tea catechin EGCG.

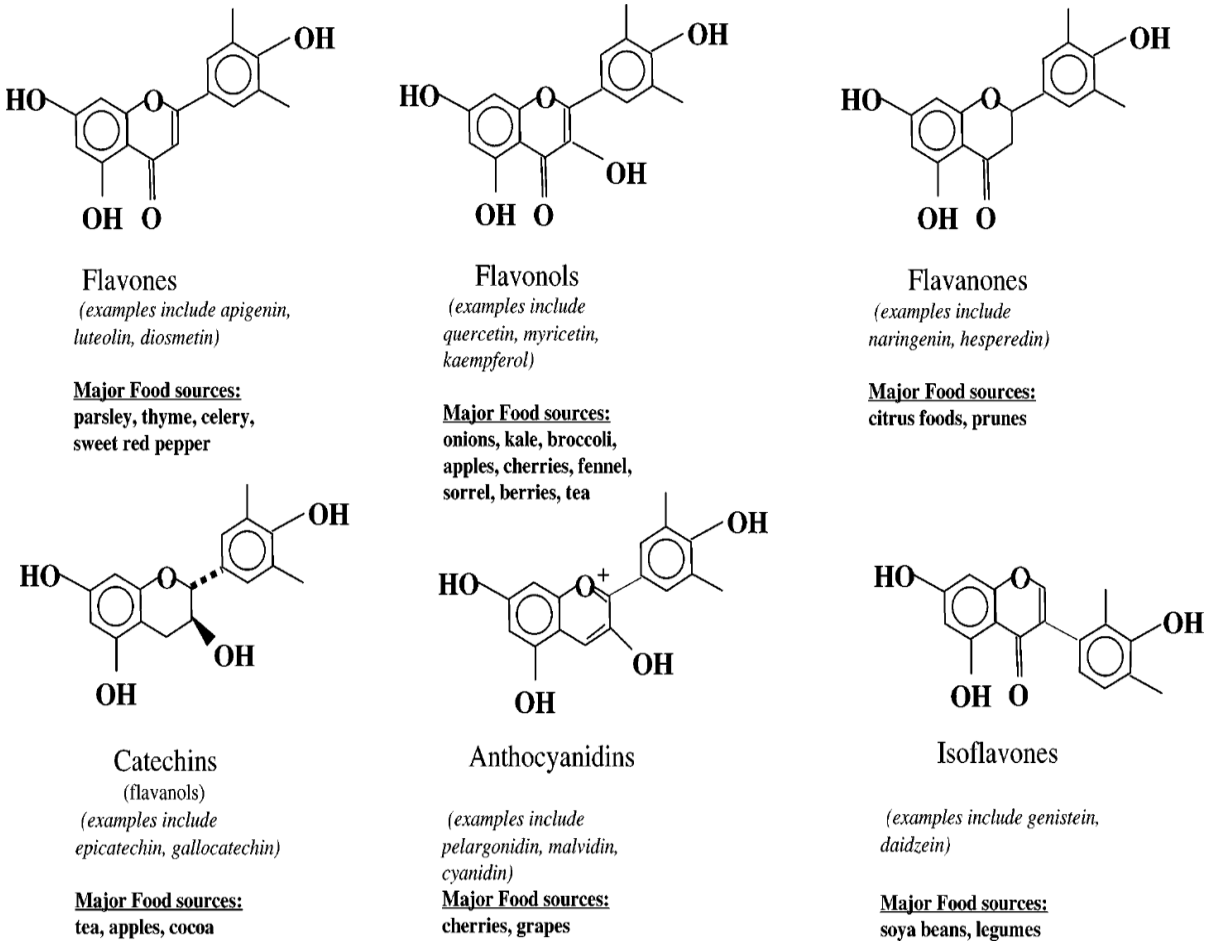


Figure 1-1 Classification of flavonoids. Adapted from Ross *et al* [3]

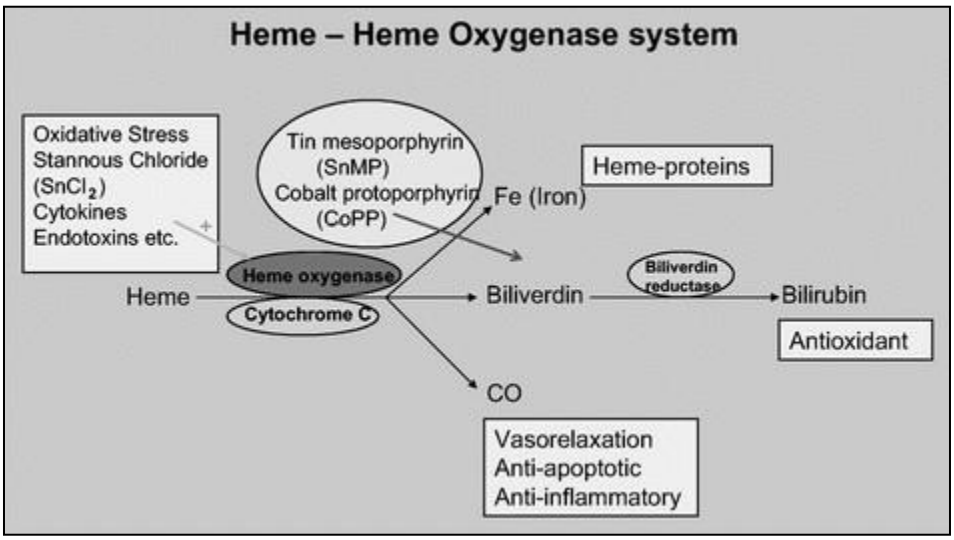


Figure 1-2 The heme oxygenase (HO) system, including the metabolism of heme and effects of HO on its final products. Adapted from Peterson *et al* [1]

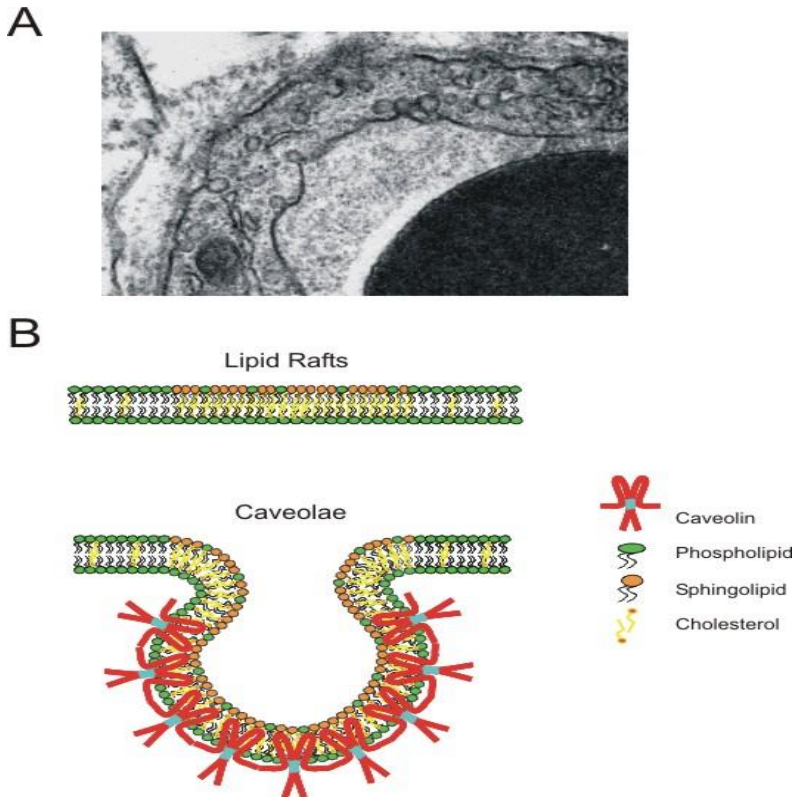


Figure 1-3 Caveolae and lipid rafts. Adapted from Galbiati *et al* [2]
A) Electron micrograph of an endothelial cell showing caveolae
B) The biochemical composition of lipid rafts and caveolae

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

This work has been published in the Journal of Nutritional Biochemistry (Zheng et al., J Nutr Biochem 2009 Mar; 20 (3):202-9).

Synopsis

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 EGCG. Exposure to linoleic acid for 6 h induced both caveolin-1 and COX-2 expression. 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 NF-κB DNA binding activity, which was also reduced by caveolin-1 silencing. Exposure to linoleic acid rapidly increased phosphorylation of several kinases, including p38 MAPK, ERK1/2, and 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.

Introduction

Endothelium locates between the plasma and the underlying tissue and its constitutive properties are involved in a large array of functions. 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 [123]. Under pathological conditions, endothelial dysfunction is a biomarker for vascular diseases and is often regarded as a key early event in the development of atherosclerotic plaques. In addition to endothelial barrier dysfunction, another functional change leading to atherosclerosis is the activation of the endothelium by pro-inflammatory mediators that regulate the vascular entry of leukocytes. COX-2 is an inducible isoform of cyclooxygenase, which converts arachidonic acid to prostaglandin. It plays a crucial role in inflammation. The expression of COX-2 is low in endothelial cells, but it could be up-regulated by pro-inflammatory reagents [125]. Cyclooxygenase inhibitors can be applied to reduce inflammation, to relieve from pain, or to prevent atherothrombotic complications in cardiovascular diseases [126].

Hypertriglyceridemia is an independent risk factor of cardiovascular diseases such as atherosclerosis [127, 128]. Dietary balance of long-chain fatty acids may influence processes involving leukocyte-endothelium interactions [129]. Diets high in omega-6 fatty acids have been shown to increase the risk of cardiovascular diseases. Linoleic acid belongs to omega-6 fatty acid family. It is abundant in many vegetable oils, especially safflower and sunflower oils. 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. Even though diets high in omega-6 fatty acids may lead to a decrease in serum cholesterol [116], replacing saturated with unsaturated omega-6 rich lipids may not be desirable because of their ability to easily oxidize. Omega-6 fatty acids, and especially linoleic acid can cause endothelial cell dysfunction as well as potentiate tumor necrosis factor-α (TNF-α)-mediated endothelial injury [120]. Previous studies have shown that linoleic acid potentiated TNF-α–mediated pro-inflammatory responses in endothelial cells [130]. Linoleic acid may further be atherogenic by causing activation of nuclear factor-kappa B (NF-κB) which in turn induces the expression of E-selectin and VCAM-1

[120, 131]. We have recently demonstrated that both the extracellular signal regulated kinase (ERK1/2) and phosphoinositide-3 kinase/ amino kinase terminal (PI3K/Akt) signaling pathways can contribute to the effect of linoleic acid on NF-κB-dependent transcription and endothelial cell activation [121].

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 [132, 133]. Polyphenols comprise a diverse group of compounds that are secondary metabolites of plants, characterized by aromatic rings bearing one or more hydroxyl substitutions [4]. Flavonoids are a kind of polyphenols which rich in fruits and vegetables, soy food, legumes, tea and cocoa [134]. Flavonoids are composed of two benzene ring, A-ring and B-ring, and joined by a three -carbon- chain to form a closed C-ring [135]. Examples of flavonoids include flavonols (e.g., quercetin), isoflavones (e.g., genistein), flavonones (e.g., hesperetin), and flanan-3-ols (e.g., catechins). Flavonoids are extensively studied for their antioxidant and anti-inflammatory abilities. However, various studies have shown that flavonoids can be cardioprotective as antioxidants, but also via antioxidant independent mechanisms [136]. Many of these bioactive food components are lipophilic, suggesting they may have a possible interaction with membrane domains or cellular lipid components such caveolae.

Caveolae have been recognized a specialized form of lipid raft [137]. But certain structure proteins have been found to preferentially partition into lipid rafts or caveolae but not both [138]. The localization caveolin proteins to caveolae distinguishes these membrane domains from lipid rafts [139]. There are three gene family members of caveolin: caveolin-1, -2, and -3. Cholesterol and glycosphingolipids are essential components of caveolae. There is increasing evidence that caveolae play a critical role in the pathology of atherosclerosis [140] and that the lack of the caveolin-1 gene may provide protection against the development of atherosclerosis [141]. 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 [76], 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 [56]. Caveolins also have been reported to co-localize with cyclooxygenase, suggesting that caveolins play a role in regulating the function of this enzyme [142, 143]. Besides their role in cellular uptake of lipophilic substances, caveolae house an array of cell signaling molecules, and numerous genes involved in endothelial cell dysfunction and inflammation are associated with caveolae [140].

There is few study focused on the role of caveolae in gene regulation by bioactive compounds. Genistein has been shown to decrease the expression of caveolin-1 in ovariectomized rat hearts [78]. Our laboratory recently found that co-treatment with quercetin reduced PCB77 induced caveolin-1 protein expression [79]. Furthermore, the internalization of caveolae can be suppressed by tyrosine kinase inhibitors such as staurosporine [144]. Flavonoids have been described to inhibit these kinases [145, 146], which suggests that these bioactive compounds can modulate the function of caveolae.

The current study focused on specific mechanisms involved in anti-inflammatory properties of bioactive compounds like flavonoids within the vascular endothelium. We hypothesized that caveolae may play a role in the regulation of signaling pathways induced by linoleic acid and protection by bioactive compounds. Our data provide evidence that linoleic acid-induced signaling pathways can be down-regulated by bioactive food components such as epigallocatechin-3-gallate (EGCG) and that these metabolic events are linked to caveolae signaling.

Materials and Methods

Materials

Epigallocatechin gallate (EGCG, >98% pure), was obtained from Cayman Chemical (Ann Arbor, MI). Linoleic acid (>99% pure), was obtained from Nu-Chek Prep (Elysian, MN). Anti-caveolin-1 was obtained from Affinity BioReagents (Golden, MO). Anti-COX-2 and anti-p65 NF-κB were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin was purchased from Sigma (Saint Louis, MO). Inhibitors LY294002, PD98059, and SB 203580 as well as antibodies used for immunobloting

including anti-Akt, anti-phospho Akt, anti-ERK, anti-phospho ERK, anti-p38, anti-phospho p38, and anti-rabbit Ig horseradish peroxidase linked were obtained from Cell Signaling Technology (Danvers, MA). Supplies and reagents for SDS-PAGE were purchased from Bio-Rad (Hercules, CA).

Cell culture

EAhy 926 cells were a gift from Dr. C. S. Edgell (University of North Carolina). The EAhy 926 line was derived by fusing human umbilical vein endothelial cells with the permanent human cell line A549 [147]. The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM) (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 times periods. Experimental media contained 5% FBS and was supplemented with EGCG for 12 h, and followed by linoleic acid at the final concentration of 90 μM, for 6 h. Preparation of experimental media with linoleic acid was performed as described previously [148]. It has been demonstrated [149] that the molar ratio of free fatty acid to albumin is the main factor controlling free fatty acid availability to tissues. Plasma albumin concentration is about 600 μM, and total free fatty acid is between180 and 2000 μM. In another word, the range of ratios of fatty acid to albumin is 0.3 to 4. In my study, 90 μM fatty acids were enriched in 5% media with which the albumin concentration is about 60 μM, thus the ratio of free fatty acids to albumin is within physiological range.

Caveolin-1 siRNA and transfection

The caveolin-1 gene silencer was designed according to previously described methods [150]. The caveolin-1 gene silencer was designed by Dharmacon (Lafayette Colorado). The sequences of caveolin-1 gene silencer were 5'- CCAGAAGGGACA CACAGUUdTdT-3' (sense), 5'- AACUGUGUGUCCCUUCUGGdTdT-3' (anti-sense). The sequences of the control gene silencer were 5'-AAAGAGCGACUUUACACAC

dTdT-3' (sense), 5'-GUGUGUAAAGUCGCUCUUUdTdT -3' (anti-sense). Cells were transfected with control siRNA or caveolin-1 siRNA at a final concentration of 80 nM using GeneSilencer (Genlantis, San Diego, CA) with Optimem I medium (Invitrogen, Carlsbad, CA). Cells were incubated with transfection mixtures for 4 h and then replaced with 10% serum medium. Cells were synchronized overnight after 48 h transfection, pre-treated with EGCG for 12 h, and then treated with linoleic acid or vehicle.

Immunoblot analysis of caveolin-1, COX-2, phospho-Akt, ERK, and p38 MAPK protein expression

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 phospho-Akt, -ERK, and -p38, cells were treated Akt, ERK, and p38 inhibitors (LY294002, PD98059, and SB 203580) for 1 h, followed by linoleic acid (90 μM, 10 min), or vehicle (0.1% DMSO). Cell protein was extracted as described before [121]. Equal amounts of protein (30 μg) were fractionated by SDS-PAGE (12% acrylamide) and transferred to nitrocellulose membranes using a Bio-Rad immunoblot transfer apparatus (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. The nonspecific sites on the membrane were blocked 1 h at room temperature with 5% nonfat dry milk in Trisbuffered saline (TBS, pH 7.6) containing 0.05% Tween-20, and then washed with TBS-Tween. Membranes were incubated overnight with the primary antibody (~1,000-fold diluted in TBST containing 5% bovine serum albumin) at 4 ℃ and for 1 h with HRP-conjugated secondary antibody (~5,000-fold diluted) at room temperature. Bands were visualized using the appropriate horseradish peroxidase-conjugated secondary antibodies followed by ECL immunoblotting detection reagents (Amersham Biosci, UK). Detection and quantitative analysis were performed using a digitizing system (UN-SCAN-IT, Silk Scientific Corporation).

Electrophoretic mobility shift assays of NF-kB DNA binding

Nuclear extracts from endothelial cells were prepared as previously described [151]. Binding reactions were performed in a 20 μL volume containing 6 μg of nuclear protein extracts. 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' -AGTTGAGGGGACTTTCCCAG GC-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) [152]. To further exam the presence of p65 NF-κB protein in the retarded bands, EMSA followed by Western blotting (shift-Western) using an antibody against p65 NF-κB was performed.

Measurement of mRNA levels of MCP-1 and caveolin-1 by real-time reverse-transcription polymerase chain reaction (Real time-PCR)

Total RNA was extracted using TRIzol Reagent (Invitrogen, NY), according to the manufacturer's protocol. Reverse transcription 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, CA). Real-time PCR samples were mixed with SYBR Green Master Mix (Applied Biosystems CA), and MCP-1, 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 CA), and the sequences were as followed: MCP-1, 5'-CGGCTGATGAGCTACAGAAGAGT-3'(sense);5'-GCTTGGGTTCTCACAGATC T-3' (anti-sense), Caveolin-1, 5'-TCAACCGCGACCCTAAACAC-3'(sense); 5'- CCTT CCAAATGCCGTCAAAA-3'(anti-sense); The house keeping gene was 18S (sense, 5'-TCG GAACTGAGGCCATGATT-3', antisense, 5'-TTTCGCTCTGGTCCGTCTTG-3').

Statistical analysis

Values are reported as mean ± standard error of the mean (SEM) of at least three independent groups. Data were analyzed using Sigma Stat software (Jandel Corp., Wan Rafael, CA). One way ANOVA followed by post hoc least significant difference (LSD)'s [153] pairwise multiple comparison procedure were used for statistical analysis of the original data. A statistical probability of $p < 0.05$ was considered significant. * indicates significant difference from control group. # Significantly different compared to cultures treated only with Linoleic Acid.

Results

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 [141]. Thus, we tested the possibility that EGCG can down-regulated base-line levels of caveolin-1. Indeed, pretreatment of endothelial cells with increasing concentrations of EGCG down-regulated base-line levels of caveolin-1. Protein expression of caveolin-1 reached the lowest values in cultures exposed to 5 and 20-30 μM EGCG (Figure 2-1).

EGCG protects against linoleic acid-induced caveolin-1 expression

Exposing endothelial cells for 6 h to linoleic acid (90 μM) markedly induced caveolin-1 (Figure 2-2A) protein expression. To test whether EGCG can down-regulate the fatty acid-mediated induction of caveolin-1, endothelial cells were pretreated 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 affect.

To assess the time-dependent protective effects of EGCG, endothelial cultures were pretreated 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 (Figure 2-2B).

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 (Figure 2-3A). Pretreatment with 10 to 30 μM EGCG for 12 h markedly attenuated the fatty acid-induced COX-2 protein expression (Figure 2-3A). 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 (Figure 2-3B).

ERK1/2 and Akt but not p38 MAPK are involved in linoleic acid-induced upregulation 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 Figure 2-4, linoleic acid rapidly increased activation of all kinases in a time-dependent manner, with maximum phosphorylation at 5 min for p-p38 MAPK (Figure 2-4A), 10 min for p-ERK1/2 (Figure 2-4B), and 10 min for p-Akt (Figure 2-4C). In order to determine which of these kinases is involved in linoleic acid-mediated COX-2 expression, endothelial cells were pretreated 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 Figure 2-5, LY294002 and PD98059, but not SB203580, effectively blocked linoleic acid-induced activation of COX-2.

Caveolin-1 silencing mimics the protective effects of EGCG on linoleic acid-induced ERK1/2 phosphorylation, COX-2 and MCP-1 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 small interfering RNA 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 (Figure 2-6A). Most importantly, caveolin-1 silencing significantly protected against linoleic acid-induced phosphorylation of ERK1/2 (Figure 2-6A); however, it did not affect linoleic acid-mediated phosphorylation of Akt (data not shown). Pretreatment with EGCG blocked linoleic acid-mediated phosphorylation of ERK1/2 (Figure 2-6A). Similar to the ERK1/2 phosphorylation data, pretreatment with caveolin-1 silencing effectively blocked linoleic acid-mediated induction of COX-2 (Figure 2-6B) and MCP-1 (Figure 2-6C).

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 pre-treated with EGCG (Figure 2-7). In addition, caveolin-1 silencing mimicked the effects of EGCG and significantly decreased the linoleic acid-induced activation of NF-κB (Figure 2-7).

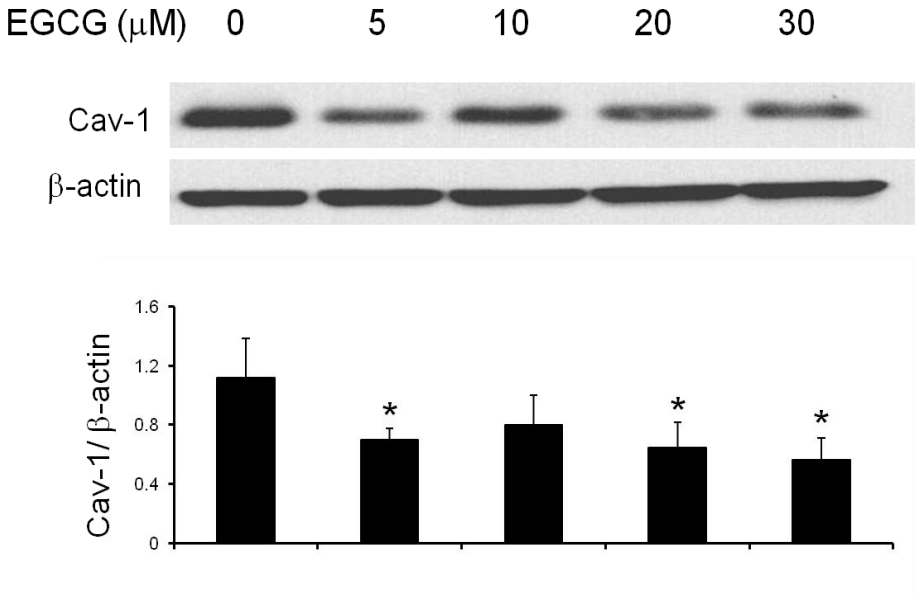


Figure 2-1 EGCG decreases caveolin-1 levels in endothelial cells.
Cells were treated with either vehicle (0.1% DMSO) or EGCG (0-30 μM) for 12 h before determining caveolin-1 (Cav-1) expression by Western blot analysis. The Western blot shown represents one of three experiments. Results shown represent the mean±S.E.M. of three independent experiments. *Significantly different compared to control cultures.

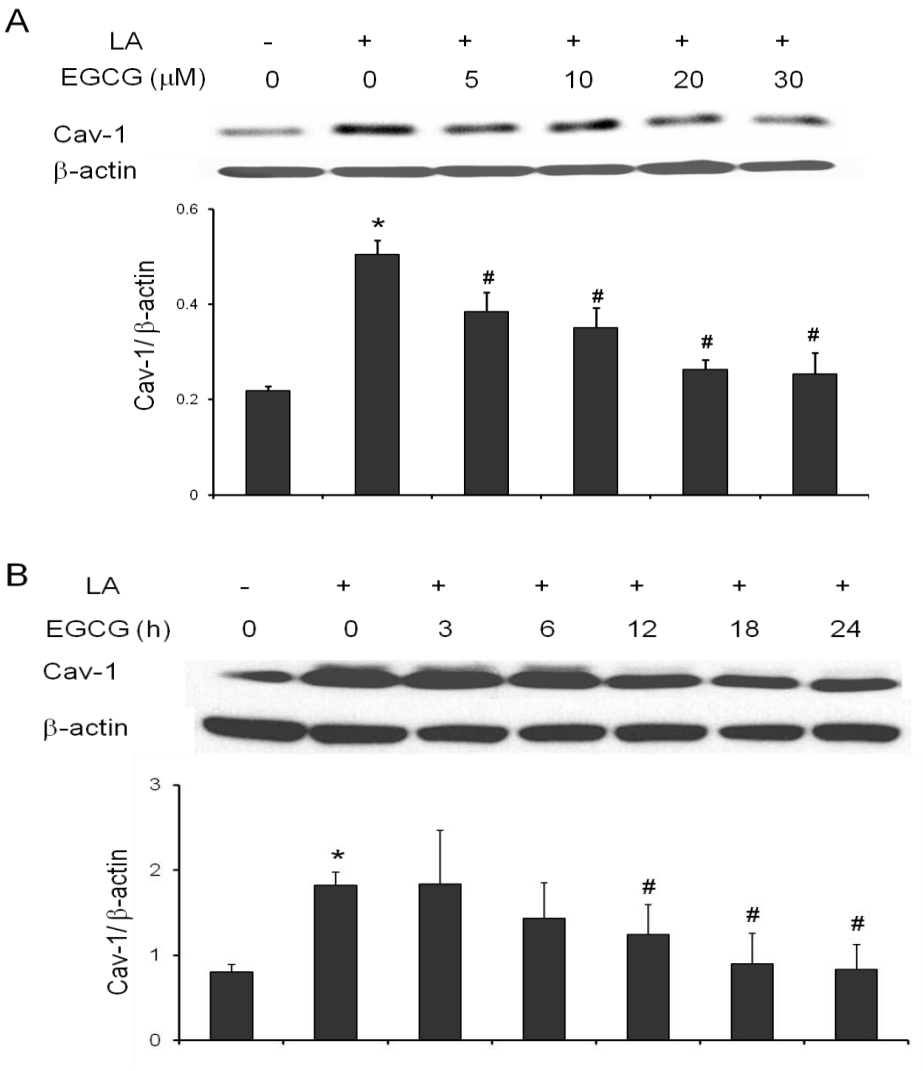


Figure 2-2 EGCG protects against linoleic-acid-induced caveolin-1 expression.
Cells were pretreated with either vehicle (0.1% DMSO) or EGCG (0-30 μM) for 12 h, followed by exposure to linoleic acid (LA, 90 μM) for an additional 6 h (A). To assess the time-dependent protective effect of EGCG (B), we pretreated some cultures for up to 24 h with EGCG (20 μM) before exposure to LA for an additional 6 h. Caveolin-1 (Cav-1) protein was determined by Western blot analysis. Each Western blot shown represents one of three experiments. Results shown 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.

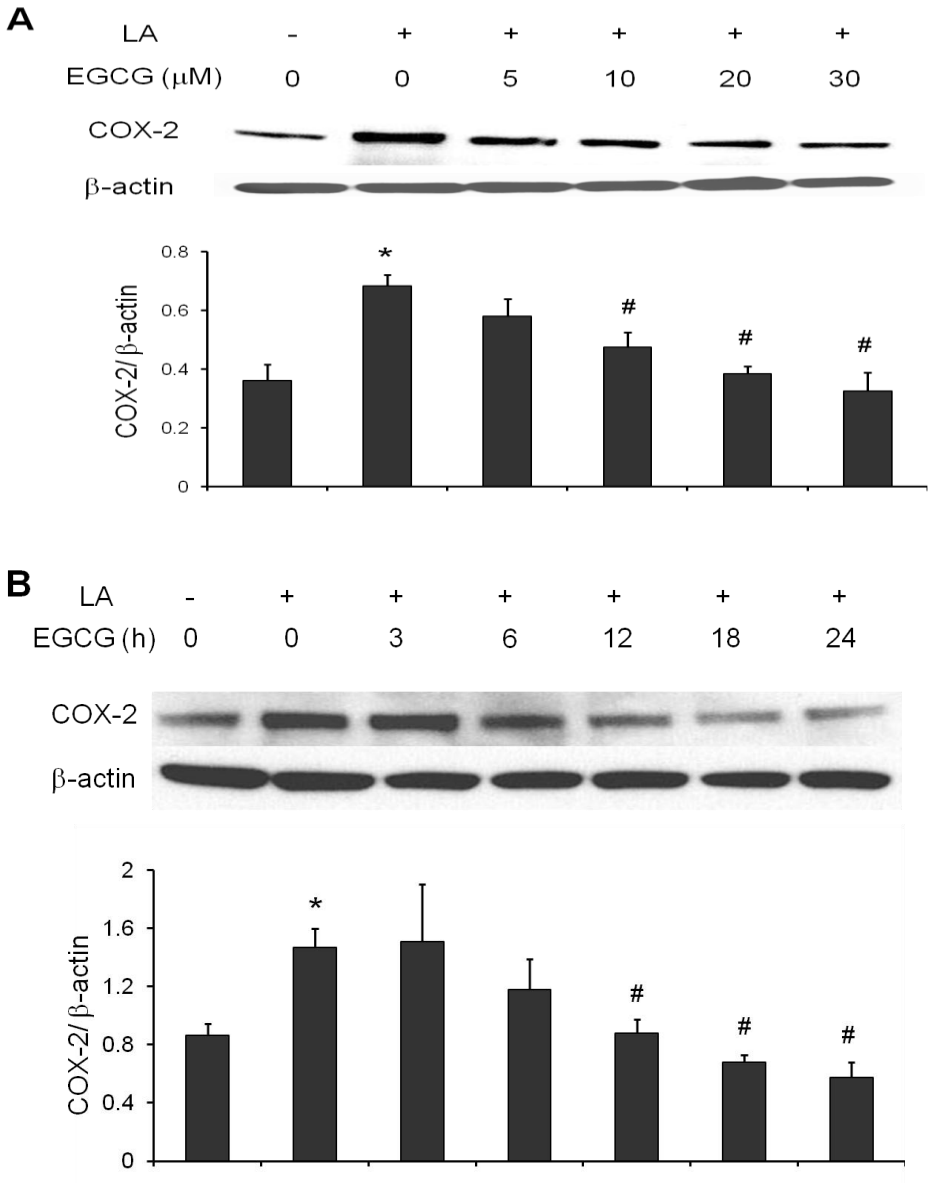


Figure 2-3 EGCG attenuates linoleic-acid-mediated up-regulation of COX-2.
Cells were pretreated with either vehicle (0.1% DMSO) or EGCG (0-30 μM) for 12 h, followed by exposure to linoleic acid (LA, 90 μM) for an additional 6 h (A). Some cultures were pretreated for up to 24 h with EGCG (20 μM) before exposure to LA for an additional 6 h (B). COX-2 protein expression was determined by Western blot analysis. Each Western blot shown represents one of three experiments. Results shown 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.

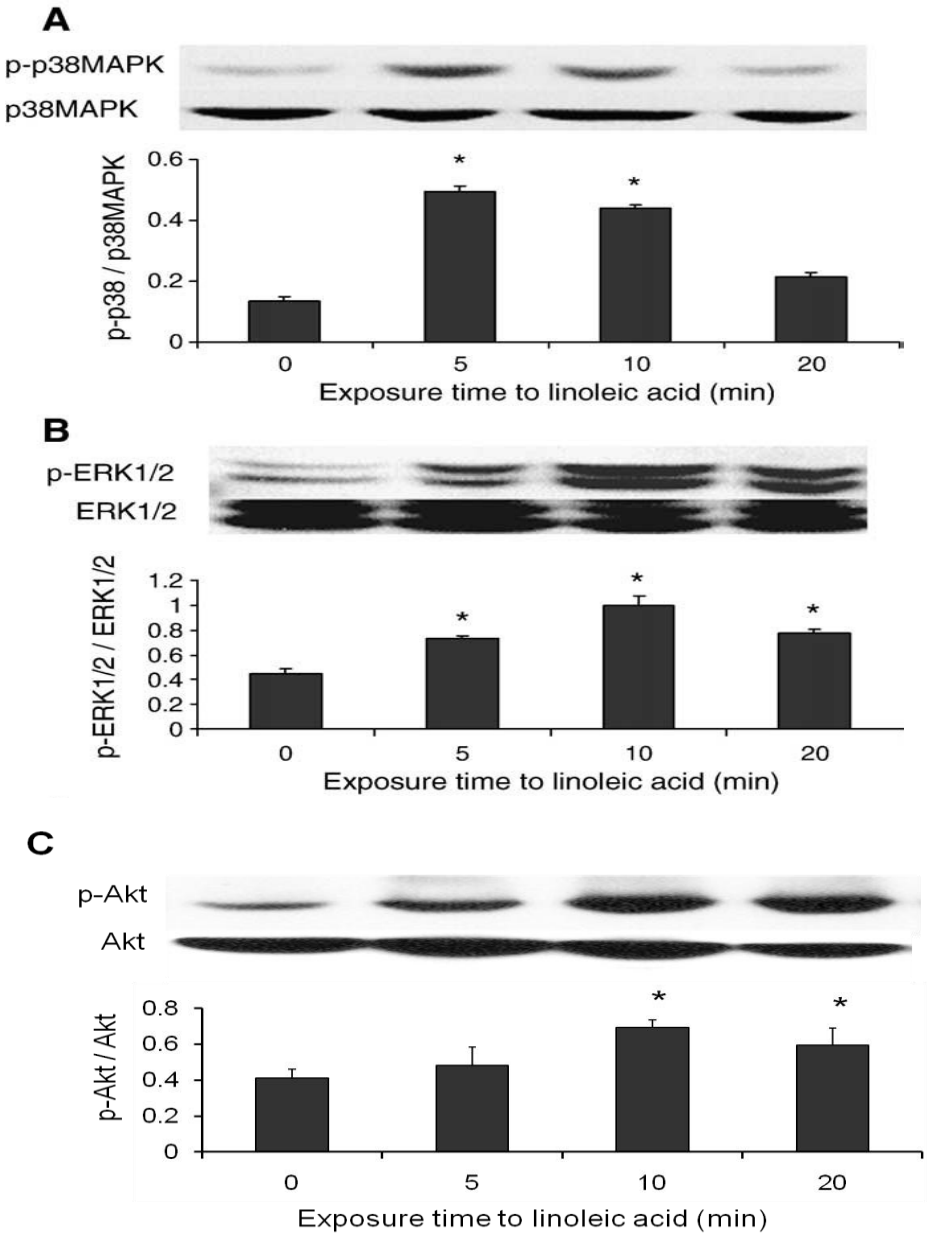


Figure 2-4 Linoleic acid activates p38 MAPK, Akt and ERK1/2 signaling in vascular endothelial cells. Cells were exposed to linoleic acid (90 μM) for 5, 10 or 20 min. Total p38 MAPK, Akt or ERK1/2 and phosphorylated p38 MAPK, Akt or ERK1/2 was detected by Western blot using specific antibodies. The Western blots shown for each phosphorylated kinase represent one of three experiments. Results shown represent the mean ±S.E.M. of three independent experiments. *Significantly different compared to control cultures.

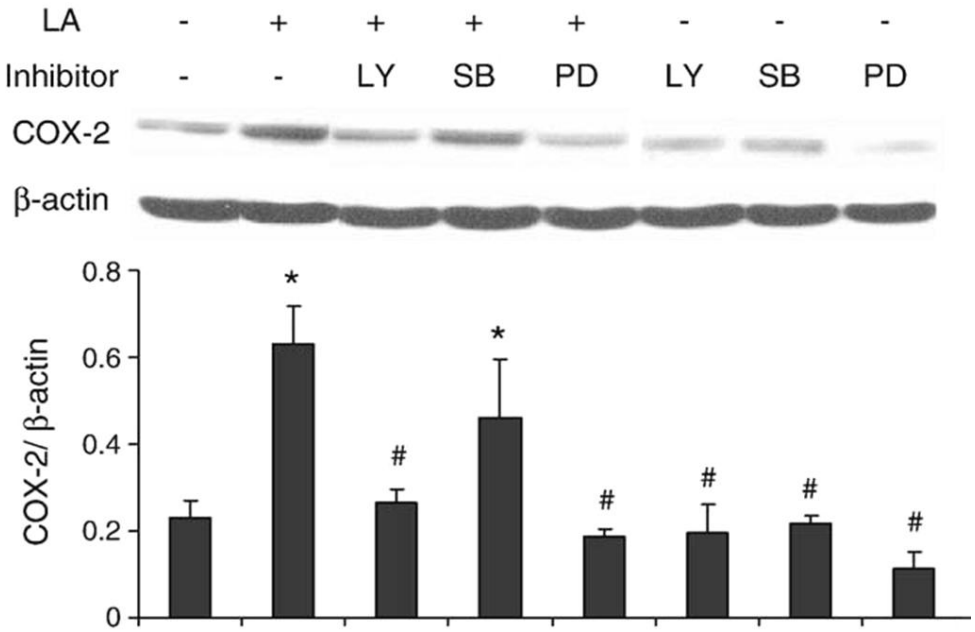


Figure 2-5 ERK1/2 and Akt but not p38 MAPK are involved in linoleic-acid induced up-regulation of COX-2. Endothelial cells were pretreated with or without the Akt inhibitor LY294002 (LY, 10 μM for 1 h), the p38 MAPK inhibitor SB203580 (SB, 10 μM for 1 h) or the ERK1/2 inhibitor PD98059 (PD, 20 μM for 1 h), followed by exposure to linoleic acid (LA, 90 μM) for 6 h. Activation of COX-2 was determined by Western blot analysis. The Western blot shown represents one of three experiments. Results shown represent the mean ±S.E.M. of three independent experiments. *Significantly different compared to control cultures. #Significantly different compared to cultures treated only with linoleic acid.

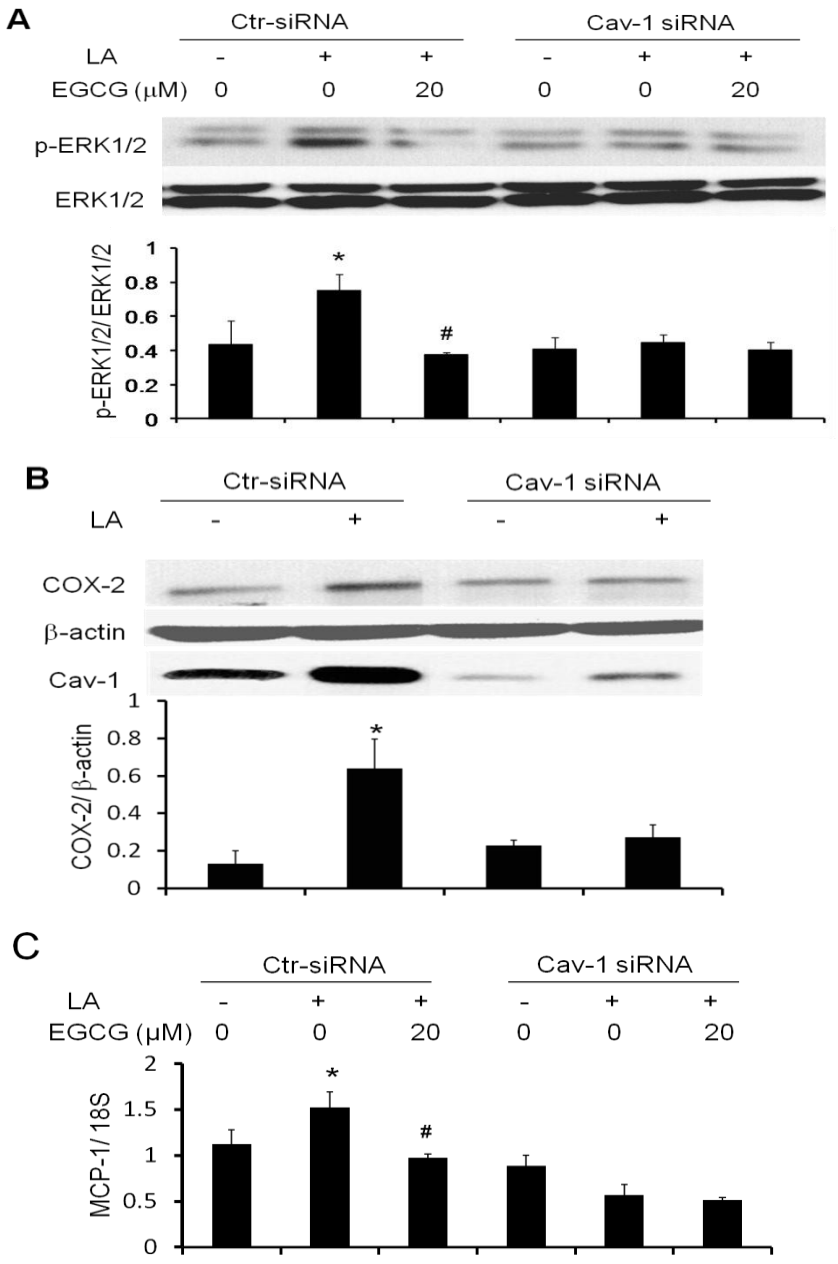


Figure 2-6 Caveolin-1 silencing mimics the protective effects of EGCG on linoleic-acid-induced ERK1/2 phosphorylation (A) and activation of COX-2 (B) and MCP-1 (C). Endothelial cells were transfected with siRNA for caveolin-1 (Cav-1 siRNA) or with control siRNA (Ctr-siRNA) and treated with EGCG (20 μM) for 12 h, followed by exposure to linoleic acid (LA, 90 μM) for 10 min (A) or 6 h (B and C). Cell lysates were probed with caveolin-1, COX-2 and β-actin or with anti-p-ERK1/2 and anti-ERK1/2. Protein expression was determined by Western blot analysis. MCP-1 and 18S mRNA expressions were measured by real-time PCR. Results shown represent the mean±S.E.M. of three independent experiments. *Significantly different compared to control cultures. #Significantly different compared to cultures treated only with LA (Ctr-siRNA).

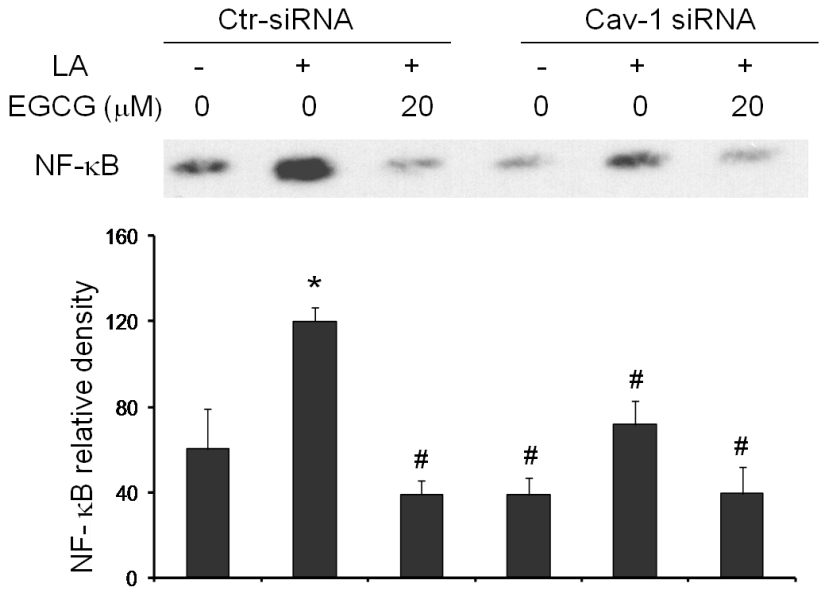


Figure 2-7 Both EGCG and caveolin-1 silencing reduce linoleic-acid-induced NF-κB DNA binding. Endothelial cells were transfected with siRNA for caveolin-1 (Cav-1 siRNA) or with control siRNA (Ctr-siRNA) and treated with EGCG (20 μM) for 12 h, followed by exposure to linoleic acid (LA, 90 μM) for 3 h. Electrophoretic mobility shift assay for NF-κB was performed with nuclear proteins extracted from endothelial cells. Results shown 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).

Discussion

Flavonoids are naturally occurring polyphenolic compounds found in numerous fruits, vegetables, and specific beverages, such as tea, grape juice, and wine. Flavonoids have been reported to have anti-inflammatory, and antioxidant, and anti-angiogenic activities. Some of them are known to provide protection against inflammatory diseases such as atherosclerosis [134]. In regard to tea consumption, studies have reported up to a 58% reduction in cardiovascular risk for individuals consuming the highest compared to the lowest amount of tea [8]. 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 is considered an early event in the etiology of cardiovascular diseases such as the pathology of atherosclerosis [123]. Endothelial cells are constantly exposed to blood components, including food-derived lipids, toxicans, 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. Endothelial cells play a critical role in the regulation of vascular tone and other important processes which contribute to normal blood vessel function and integrity [154].

Increased exposure of the endothelium to free fatty acid anions, metabolic events known to activate endothelial cells [155, 156]. In the current study, endothelial cells were activated with linoleic acid to mimic a postprandial hyperlipidemic state. We have previously demonstrated that endothelial cell exposure to fatty acids, and especially linoleic acid, markedly induced an endothelial inflammatory response [157]. We also have demonstrated that the ERK1/2 signaling pathway can contribute to the effect of linoleic acid on NF-κB-dependent transcription [121]. In the current study, inhibitors of ERK 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.

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 [120]. Gafencu *et al* [54]

investigated fatty acid composition of caveolae from endothelial cells and indicated that linoleic acid was about 8 to 10% of total fatty acids. Caveolae are important for organizing and regulating the molecular interactions of multiple signaling pathways. My results showed that linoleic acid induced both caveolin-1 and COX-2, which is significant because of the link between caveolins (caveolae) and the pathology of atherosclerosis [140]. Caveolin-1 has been reported to co-localize with interleukin-1beta-induced COX-2 [156], 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 [158], suggesting that our fatty acid data may mimic an *in vivo* response by activating COX-2.

Protective molecular mechanisms of flavonoids against endothelial activation and inflammation are not well understood. EGCG, the most abundant and effective flavonoid of green tea, has been shown to inhibit the expression of COX-2 and the production of prostaglandin E_2 [156, 159]. 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. Pretreatment with EGCG alone down-regulated baseline levels of the caveolin-1 at 12 h. 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 ERK and Akt. Others also have reported EGCG-mediated down-regulation of MAPK pathways such as ERK and decreased COX-2 activity in cancer cell lines [159, 160] and in human vascular smooth much cells [161]. MAPKs such as ERK are known to regulate NF-κB [162], and there is evidence that a decrease in COX-2 by EGCG may be through inhibition of NF-κB [163]. Indeed, our data show that EGCG can decrease linoleic acid-induce 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. Further studies are needed in animal models to examine the effects of EGCG on inflammatory genes.

EGCG was unstable in cell culture medium. EGCG is quickly oxidized, dimerized and metabolized. Some of the metabolites of EGCG have similar or even higher anti-oxidant and anti-inflammatory effects. The instability of EGCG is primarily

due to its quick auto-oxidation. Several factors, such as pH, temperature, oxygen levels, antioxidant levels, metal ions, concentrations of EGCG could affect the stability of EGCG [164]. In our study, to prevent the quick auto-oxidation of EGCG, we flushed EGCG extensively with N_2 gas in stock solutions, and prepare fresh EGCG each time before doing the experiments.

In summary, we provide evidence in the current study that EGCG-mediated down-regulation of 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 [76]. We provide novel data, demonstrating that silencing of the caveolin-1 gene can markedly down-regulate linoleic acid-induced phosphorylation of ERK, MCP-1 and COX-2, suggesting that specific MAPK signaling is caveolae-dependent. Data from the current study strongly support our hypothesis that membrane domains called caveolae are a critical platform regulating inflammatory signaling pathways that can be modulated by the interaction of bioactive compounds such as flavonoids as well as the cellular lipid milieu. Because caveolae and caveolins have been implicated in several human diseases and in particular vascular diseases, our data may have implications in understanding caveolae-associated mechanisms of inflammatory diseases and protection by bioactive food components with antioxidant and anti-inflammatory properties such plant-derived flavonoids. This may in part explain the potent protective properties of EGCG against inflammatory diseases such as atherosclerosis.

Chapter Three: Epigallocatechin gallate-mediated protection against tumor necrosis factor-α-induced monocyte chemoattractant protein-1 expression is heme oxygenase-1 dependent

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Synopsis

Flavonoids have been suggested to protect against atherosclerosis by their anti-oxidant and anti- inflammatory properties. Heme oxygenase-1 (HO-1) is an enzyme that plays an important role in the vascular system, and its induction may provide a protective role against atherosclerosis. We hypothesize that flavonoids can down-regulate endothelial inflammatory parameters by modulating HO-1-regulated cell signaling. We focused on the role of HO-1 and its major metabolic product, bilirubin, on mechanisms of tumor necrosis factor-α (TNF-α)-induced endothelial cell activation and protection by the catechin epigallocatechin-gallate (EGCG). Pre-treatment with EGCG inhibited the secretion of monocyte chemoattractant protein (MCP)-1 and the activation of activator protein-1 (AP-1) in porcine aortic endothelial cells stimulated with TNF-α. Moreover, EGCG up-regulated the expression of HO-1 and further induced the secretion of bilirubin. The observed anti-inflammatory effects of EGCG were mimicked by the HO-1 inducer cobalt protoporphyrin (CoPP) and abolished by HO-1 gene silencing. These data suggest that the protective properties of flavonoids, such as EGCG, against endothelial inflammation may be regulated in part though induction of HO-1 and subsequent AP-1 signaling.

Introduction

Atherosclerosis depends critically on altered behavior of the intrinsic cells of the artery wall, in particular the endothelial cells. Inflammation plays an important role in the development of endothelial dysfunction and atherosclerosis [165]. TNF-α is one of the most potent pro-inflammatory cytokines [105, 166] which is highly expressed throughout the full spectrum of atherosclerotic development [106]. TNF-α can induce the activation of the vascular endothelium, including up-regulation of monocyte chemotactic protein-1 (MCP-1) [167]. MCP-1 is a member of the CC chemokines and can be secreted by monocytes, endothelial cells, vascular smooth muscle cells and macrophages [168]. A bulk of evidence suggests that the inflammatory response in vascular injury involves recruitment and activation of monocytes through activation of the MCP-1 [44], a potent chemotactic factor for monocytes. Atherosclerosis is indeed a chronic inflammatory disease initiated by monocyte adhesion to activated endothelial cells [169]. In both human and experimental animal models, expression of MCP-1 have been found to be markedly elevated in atherosclerotic lesions [170], and elevated MCP-1 serum levels are considered a marker of inflammation in coronary artery disease patients [171]. Thus, in the current study MCP-1 was used as an inflammation target to evaluate endothelial function modulated by epigallocatechin-gallate (EGCG), the most abundant flavonoid which is rich in green tea.

A bulk of evidence supports that the dietary intake of polyphenols - particularly of flavonoids might be able to exert some beneficial vascular effects and reduce the risk for cardiovascular morbidity and mortality. Flavonoids constitute a subclass of bioactive compounds rich in fruits and vegetables, soy food, legumes, tea and cocoa [134]. Many flavonoids are composed of a polyphenol structure, and these polyphenols are often classified according to structural similarities [135]. Examples of flavonoids include flavonols, isoflavones, flavonones, and flanan-3-ols (e.g., catechins). The review of epidemiological and mechanistic studies supports the role of flavonoids, particularly tea catechins, in protecting the cardiovascular system against cardiovascular disease. Evidence suggests that habitual daily consumption of green tea is associated with a reduced risk of coronary artery diseases, by affecting molecular mechanisms involved in

the initiation and progression of these diseases [132, 133]. The majority of catechins in green tea include EGCG, which has been shown to improve endothelial function and to induce anti-inflammatory vascular events. There is strong evidence from multiple studies that flavonoid-containing beverages and foods have such an effect. For example, Hodgson et al showed a beneficial effect of tea consumption on endothelial function in otherwise healthy subjects with cardiovascular risk factors [172]. Other investigators have observed favorable vascular effects of black and green tea [173, 174]. There are two subclasses of flavonoids in tea, the flavanols and flavonols [175] . Flavanols are found in monomer as well as polymer form. Monomer flavanols found in tea include catechin, epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG) whereas polymer forms found in tea include theaflavins and thearubigins. Some recent studies have showed the effects of specific catechins on endothelial function. Widlansky et al demonstrated an improvement in brachial artery flow-mediated dilation following acute administration of EGCG [18].

The up-regulation of hemeoxygenase (HO-1) by polyphenols may be one of the mechanisms to explain their protection effects on vasculature inflammatory disease, such as atherosclerosis [104]. HO-1 is an inducible rate-limiting enzyme degrading heme to iron, carbon monoxide, and biliverdin, with the latter being quickly reduced to bilirubin [176]. Besides the function of removing heme, the metabolic products of HO-1 have been recognized recently to play important roles in vascular diseases [177]. For example, proxidative and pro-inflammatory defenses are reduced during HO-1 deficiency [177]. There are three isoforms of heme oxygenases [178, 179]. Of the three isozymes, HO-2 is the constitutive form of heme oxygeanse system, HO-3 only exist in rat, and HO-1 is believed to be the only inducible form [179]. HO-1 is considered a protective, stress-response enzyme, and its basal expression can be significantly up-regulated by a wide variety of stimuli including heme, heavy metals, hydrogen peroxide, growth factors, as well as some antioxidants [180]. Bilirubin, a product of HO-1-mediated heme degradation, can protect lipid membranes against oxidation as efficiently as α-tocopherol and β-carotene [181]. In contrast, inhibition of bilirubin production by biliverdin reductase siRNA has been shown to increase ROS levels in primary neuronal cultures [182]. HO-1 deficient mice showed reduced stress defense and elevated blood pressure.

Clinical studies suggest that higher level of circulatory bilirubin is associated with reduced risks for coronary artery diseases [183, 184]. The heme oxygenase system is an important regulator of endothelial cell integrity and oxidative stress [185], and dysfunctional HO-1 signaling may be pro-atherogenic.

Thus, a major objective of the present study was to explore the role of HO-1 in mechanisms of EGCG-mediated protection of the vascular endothelium dysfunction. In our current study, we investigated both the effects of pharmacological concentrations of EGCG on the expression of HO-1 in endothelial cells and the involvement of specific transcription factor-dependent pathways in this process. Our results support the hypothesis that EGCG can exhibit anti-inflammatory properties through induction of HO-1 and subsequent AP-1 signaling.

Materials and Methods

Materials

Epigallocatechin gallate (EGCG, purity >98%) was purchased from Cayman Chemical (Ann Arbor, MI). TNF-α, bilirubin and anti-β-actin antibody were obtained from Sigma (Sigma-Aldrich, MO). Bilirubin was dissolved in 0.2 N NaOH, neutralized to pH 7.4 using 1 N HCl, and used fresh. Cobalt protoporphyrin was purchased from Frontier Scientific (Logan, Utah). HO-1 antibody was got from Abcam (Cambridge, MA).

Cell culture and experimental media

Endothelial cells were isolated from porcine aortic arteries and cultured as previously described [186]. Arteries obtained during routine slaughter were donated from the College of Agriculture, University of Kentucky. The basic culture medium consisted of medium 199 (M-199) (Cat. No. 31100-035; GIBCO Laboratories, NY) containing 10% (v/v) fetal bovine serum (FBS, HyClone Laboratories, UT). Cell cultures were grown until confluent, and then synchronized by maintaining in 1% serum

overnight before exposure to various experimental settings. Experimental media contained 5% FBS and were supplemented with various concentrations of EGCG (Cayman Chemicals, Ann Arbor, MI; purity >98%) and/or TNF-α (at a final concentration of 2 ng/ml). Time and concentration data of optimal protection of EGCG against endothelial inflammation were conducted prior to the current study to understand protective properties of EGCG in our cell culture model. For example, we found that the lowest dose of EGCG which protected against TNF-α-induced inflammatory markers was 30 μM. Thus, 30 μM of EGCG was used in the current study. Others have reported similar concentrations of EGCG to decrease inflammatory markers in culture endothelial cells [24, 187, 188]. EGCG was dissolved in DMSO; at a final concentration less than 0.1%, DMSO did not affect cell viability.

HO-1 siRNA and transfection

The HO-1 gene silencer was designed by Applied Biosystems (Lincoln Centre Drive Foster City, CA). The sequences of HO-1 gene silencer were 5'-GCAUCUUUC CCAACCAAGAtt-3' (sense), 5'-UCUUGGUUGGGAAAGAUGCca-3' (anti-sense). The sequences of the control gene silencer were 5'-AAAGAGCGACUU UACACACdTdT-3' (sense), 5'-GUGUGUAAAGUCGCUCUUUdTdT-3' (anti-sense). Cells were transfected with control siRNA or HO-1 siRNA at a final concentration of 80 nM using GeneSilencer (Genlantis, San Diego, CA) with Optimem I medium (Invitrogen, Carlsbad, CA). Cells were incubated with transfection mixtures for 4 h, followed by replacement with 10% serum medium. Cells were synchronized overnight after 48 h transfection, pre-treated with EGCG, and subsequently treated with TNF-α or vehicle.

Immunoblotting

Cells were treated with either vehicle (0.1% DMSO) or EGCG (30 μM) followed by exposure to TNF-α (2 ng/ml) for immunoblot analysis of HO-1 activation. Cell protein was extracted as described before [121]. 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 (1,000-fold diluted in TBST containing 5% bovine serum albumin) at 4 ℃ and for 1 h with HRP-conjugated secondary antibody (~5,000-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).

Electrophoretic mobility shift assays of AP-1-DNA binding

Nuclear extracts from endothelial cells were prepared as previously described [151]. 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 AP-1 (5'bio-CGCTTGATGACTCAGCCGGAA-3'). Gel mobility shift assay was performed to demonstrate the shifted DNA-protein complexes for AP-1 using a LightShiftTM chemiluminescent EMSA kit (Pierce, Rockford, IL). Reactions using 200-fold molar excess of unlabeled oligonucletide probes were performed to demonstrate the specificity of the shifted DNA-protein complexes for AP-1. [152].

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

Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. Reverse transcription 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, CA). Samples were mixed with SYBR Green Master Mix

(Applied Biosystems CA), and MCP-1, HO-1 or β-actin specific primers. The sequences for porcine HO-1, MCP-1, and β-actin gene were designed by Primer Express Software 3.0 for real-time PCR (Applied Biosystems CA), and the sequences were as followed: MCP-1,5'-CGGCTGATGAGCTACAGAAGAGT-3'(sense);5'-GCTTGGGTTCTCACA GATCT-3' (anti-sense), HO-1, 5'-AGGTCACCCGAGAAGGCTTT-3'(sense), 5'-TAGA CCGGGTTCTCCTTGTTGT-3'(anti-sense). The house keeping gene was β-actin, 5'-TCATCACCATCGGCAACG-3'(sense), 5'-TTCCTGATGTCCACGTCG-3'(anti-sense).

Measurement of bilirubin production

After plating, cells were treated with TNF-α and EGCG or vehicle. All further manipulations were carried out in a dark room. After incubation, 0.5 ml of each culture supernatant was collected and 250 mg $BaCl_2$. $2H_2O$ was added. After vortexing, 0.75 ml benzene was added; then, tubes were vortexed vigorously leading to the formation of a relatively stable milky-white emulsion. After centrifugation (13000g, 30min), the upper benzene layer was collected and the absorbance was measured at 450 nm with a reference wavelength at 600 nm using a SpectraMaxPro M2 spectrophotometer (Molecular Devices, Sunnyvale, CA). In a separate tube, 0.5 ml of fresh culture medium was processed in the same way and the benzene layer was collected and used as a blank. 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$mM^{-1}cm^{-1}$ [189].

MCP-1 determination

Cells were seeded in 6 or 24 well microplates and grown to confluence. After treatment, supernatants of cell cultures were collected into microcentrifuge tubes (Isc BioExpress, UT), centrifuged at 4 ℃ to remove cellular debris and then stored at -80 ℃. MCP-1 levels were assessed using a MCP-1-specific enzyme immunoassay [190] (BD Biosciences, CA) following the manufacturer's protocol with minor modification. A

microplate spectrophotometer SpectraMaxPro M2 (Molecular Devices Corporation, CA) was used to read the plate at 450 nm.

Transfection of antisense oligodeoxynucleotides into porcine endothelial cells

The sequences of the phosphorothioate double-stranded antisense oligodeoxynucleotides (ODNs) against the AP-1 binding site used in this study was AP-1 decoy ODN, 5'-AGCTTGTGAGTCAGAAGCT-3'and AP-1 mismatched ODN, 5'-AGCTTGAATCTCAGAAGCT-3'. The double-stranded ODNs were prepared from complementary single-stranded phosphorothiolate-bonded oligonucleotides. The ODNs were annealed for 1 h, while the temperature descended from 80 to 25 ℃. DNA derived from endothelial cells was precomplexed with the PLUS reagents (Life Technologies, Rockville, MD) at room temperature for 15 min. The pre-complexed DNA was combined with diluted LipofectAMINE reagent (Life Technologies), mixed and incubated for 15 min at room temperature. While complexes were forming, medium with serum-free transfection medium were replaced. Then DNAPLUS Lipofect AMINE reagent complex was added to each well containing fresh medium, and cells were incubated for 5 h. After incubation, complete medium with serum was added. After transfection for 48 h, cell extracts were prepared for Real-time PCR analysis [191].

Monocyte adhesion assay

Human THP- 1 monocytes were activated with TNF-α (10 min) and loaded with the fluorescent probe calcein (Molecular Probes, Carlsbad, CA). Porcine aortic endothelial cells were treated with EGCG and TNF-α. Monocytes were added to treated endothelial cell monolayers and incubated (30 min), allowing for monocyte adhesion. Unbound monocytes were washed away, and the monolayer was fixed with 1% glutaraldehyde. Attached fluorescent monocytes were counted using a fluorescent microscope (Olympus IX70, Center Valley, PA).

Statistical analysis

Values are reported as mean ± standard error of the mean (SEM) of at least three independent groups. Data were analyzed using Sigma Stat software (Jandel Corp., Wan Rafael, CA). One way ANOVA followed by post hoc least significant difference (LSD)'s [153] pairwise multiple comparison procedure were used for statistical analysis of the original data. A statistical probability of $p < 0.05$ was considered significant.

Results

Both EGCG and CoPP induce HO-1 and inhibit TNF-α-induced MCP-1 expression

Cells were treated either with vehicle, EGCG, or cobalt protoporphyrin (CoPP) in the presence or absence of TNF-α before determining MCP-1 mRNA and HO-1 protein expression. Exposure to TNF-α markedly induced MCP-1 mRNA expression, which was significantly reduced by pre-treatment with wither EGCG or the potent HO-1 inducer CoPP (Figure 3-1). Most importantly, both CoPP and EGCG significantly induced HO-1 levels in endothelial cells (Figure 3-2). In contrast, exposure to TNF-α neither induced HO-1 expression nor affected HO-1 expression induced by both CoPP and EGCG (Figure 3-2).

HO-1 silencing prevents EGCG-mediated protection against TNF-α-induced MCP-1 up-regulation and monocyte adhesion

Small interfering RNA was utilized to specifically silence HO-1 expression in endothelial cells. Cells were transfected with siRNA for HO-1 (HO-1 siRNA) or with control siRNA (Ctr-siRNA), and silencing of HO-1 was significant, as shown by diminished levels of HO-1 protein (Figure 3-3A). Subsequently, cells were pre-treated with EGCG, followed by exposure to TNF-α. TNF-α markedly increased MCP-1 mRNA and protein expression, independent of HO-1 silencing (Figures 3-3B and C). In control siRNA cells, pre-treatment with EGCG markedly inhibited MCP-1 up-regulation,

and monocyte adhesion induced by exposure to TNF-α. In contrast, HO-1 silencing reversed the inhibitory effects of EGCG both at the message and protein level (Figure 3-3B and C). And similarly, the absence of HO-1 also diminished the protection effects of EGCG against monocyte adhesion (figure 3-4).

EGCG induces bilirubin levels in endothelial cells, and supplemental bilirubin reduces TNF-α-induced up-regulation of MCP-1, and monocyte adhesion

HO-1 has potent anti-inflammatory effects, which may be exerted through the generation of bilirubin [192]. Therefore, we tested whether this enzymatic product of HO-1 could mediate the potential protective effects in TNF-α-stimulated endothelial cells. In addition to up-regulating HO-1, both CoPP and EGCG also significantly induced the secretion of bilirubin (Figures 3-5A and 3-5B). Furthermore, supplemental bilirubin markedly quenched the TNF-α-stimulated induction of MCP-1, and monocyte adhesion (Figure 3-7A and 3-7B). As expected, HO-1 silencing not only abolished the induction of HO-1, but also the cellular ability for the production of bilirubin as induced by EGCG and CoPP in Ctr-siRNA cells (Figures 3-5A and 3-5B).

The effect of EGCG on DNA binding of AP-1 and subsequent MCP-1 induction is dependent on functional HO-1

To assess whether activation of AP-1 is implicated in TNF-α-induced MCP-1 gene transcription, cells were stimulated with TNF-α after transfection with AP-1 decoy ODN (an inhibitor of AP-1) and AP-1 mismatched ODN (negative control). AP-1 decoy ODN partially inhibited TNF-α-induced MCP-1 mRNA expression (Figure 3-6A), suggesting that AP-1 is involved in TNF-α-induced MCP-1 production. Similar to its ability to quench TNF-α-mediated induction of MCP-1, supplemental bilirubin also significantly reduced AP-1 DNA binding activity (Figure 3-6B). Furthermore, the protective effects of EGCG through AP-1 signaling appear to be dependent on functional HO-1. Pre-treatment with EGCG markedly suppressed AP-1 DNA-binding activity (Figure 3-6C). HO-1 siRNA transfection not only blocked EGCG-induced production of

bilirubin (Figure 3-5B), but it also reversed the inhibitory effect of EGCG against TNF-α-induced AP-1 activation (Figure 3-6C).

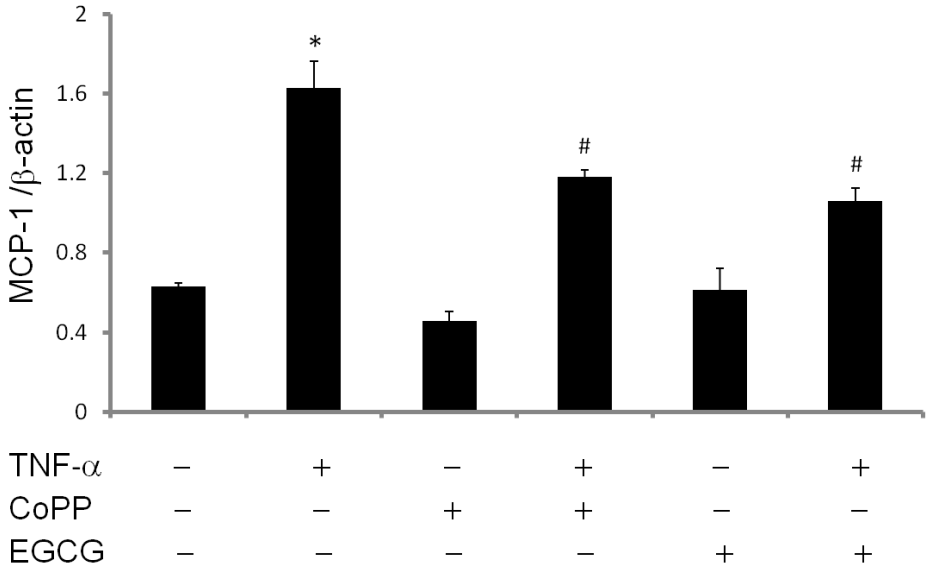

Figure 3-1 EGCG and CoPP inhibit TNF-α-induced MCP-1 expression. Cells were pretreated with either vehicle (0.1% DMSO), EGCG (30 μM) or CoPP (10 μM) for 2 h, followed by exposure to TNF-α (2 ng/ml) for an additional 6 h. MCP-1and β-actin mRNA expressions were measured by real time-PCR. Results shown represent the mean ± SEM, n=3; *Significantly different compared to vehicle control. #Significantly different compared to cultures treated only with TNF-α.

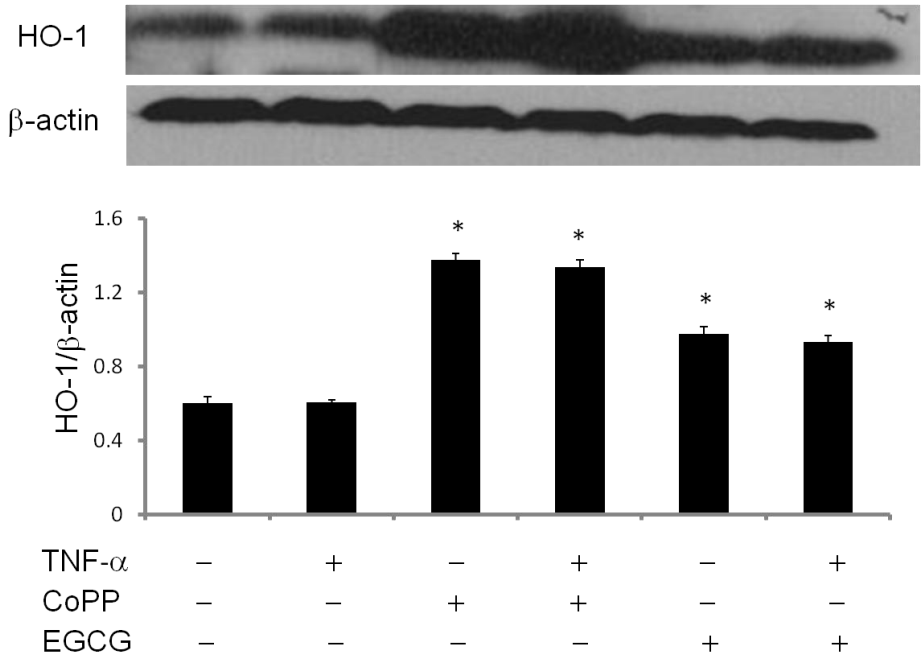

Figure 3-2 EGCG and CoPP induce HO-1 levels in endothelial cells. Cells were treated with either vehicle (0.1% DMSO), EGCG (30 μM), or CoPP (10 μM) in the presence or absence of TNF-α (2 ng/ml) for 4 h before determining HO-1expression by western blot analysis. The western blot shown represents one of three experiments. Results shown represent the mean ± SEM, n=3; *Significantly different compared to control cultures.

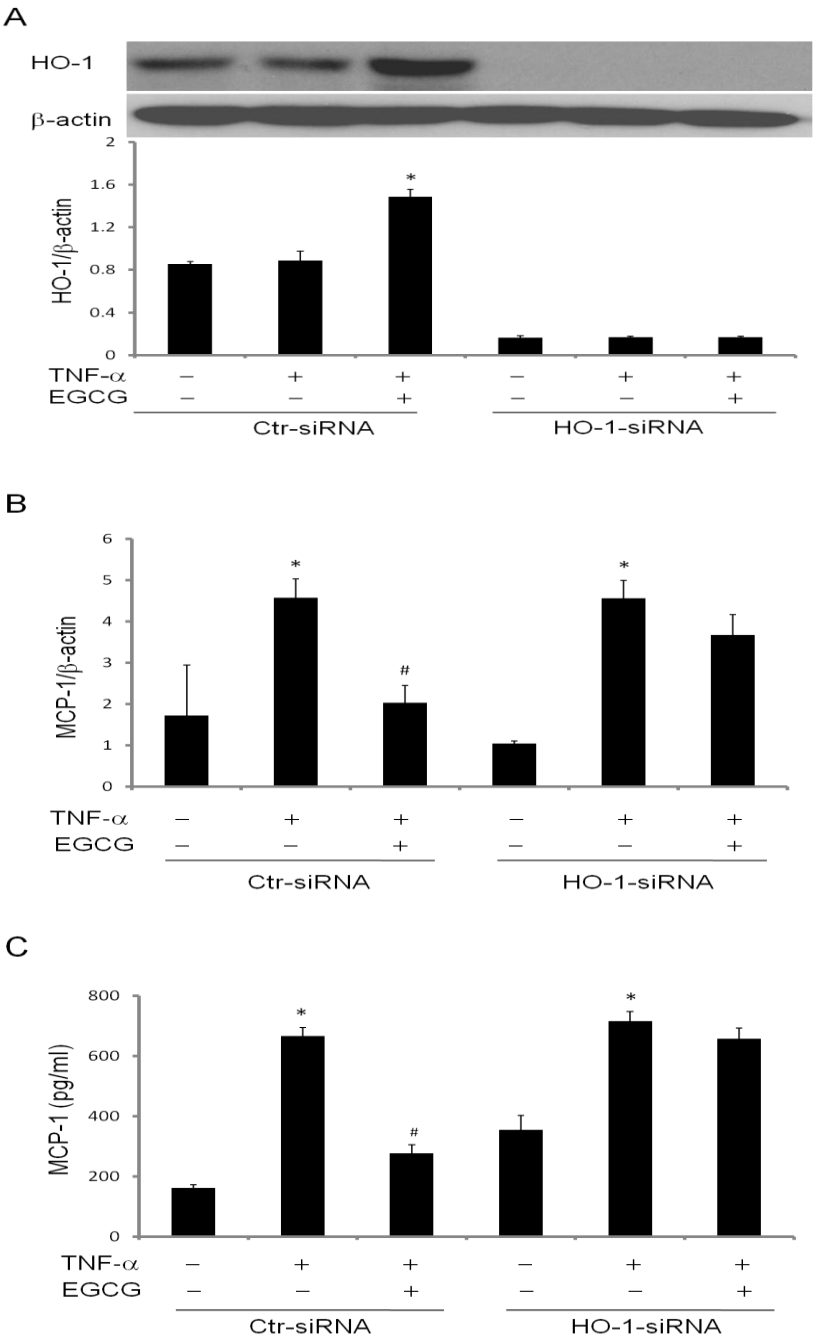

Figure 3-3 HO-1 silencing prevents EGCG-mediated protection against TNF-α-induced up-regulation of MCP-1. Endothelial cells were transfected with siRNA for HO-1 (HO-1 siRNA) or with control siRNA (Ctr-siRNA) and treated with EGCG (30 μM) for 2 h, followed by exposure to TNF-α (2 ng/ml) for 6 h. Figures 3-3A demonstrates successful silencing. MCP-1 mRNA expressions were measured by real time-PCR (Figure 3-3B). Media were collected, and MCP-1 levels were measured by enzyme immunoassay (Figure 3-3C). Results shown represent the mean ± SEM, n=3; *Significantly different compared to respective control cultures. #Significantly different compared to cultures treated only with TNF-α (Ctr-siRNA).

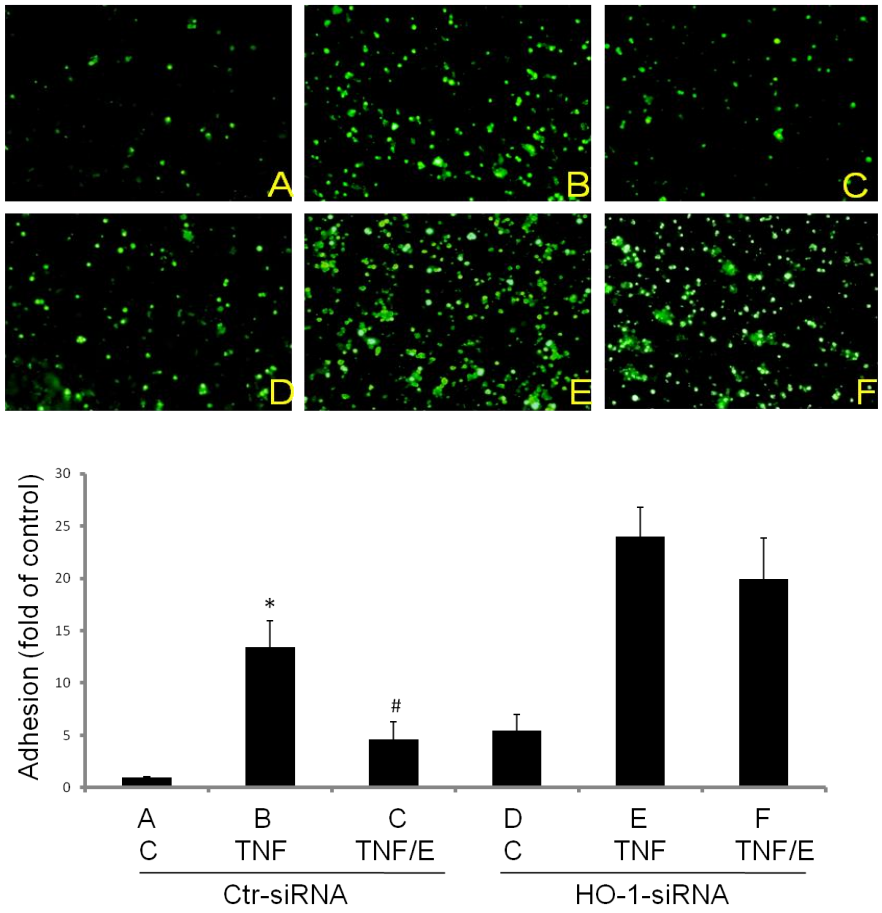

Figure 3-4 HO-1 silencing prevents EGCG-mediated protection against TNF-α-induced monocyte adhesion. Endothelial cells were transfected with siRNA for HO-1 (HO-1 siRNA) or with control siRNA (Ctr-siRNA). Human THP-1 monocytes were loaded with the fluorescent probe calcein. Endothelial cells were pre-treated with EGCG (30 μM) for 2 h and followed by the exposure to TNF-α (2 ng/ml) for 6 h. Monocytes were added to treated endothelial cell monolayers and incubated allowing for monocyte adhesion. Attached fluorescent monocytes were counted using a fluorescent microscope. Results shown represent the mean ± SEM, n=3; *Significantly different compared to control cultures. #Significantly different compared to cultures treated only with TNF-α (Ctr-siRNA).

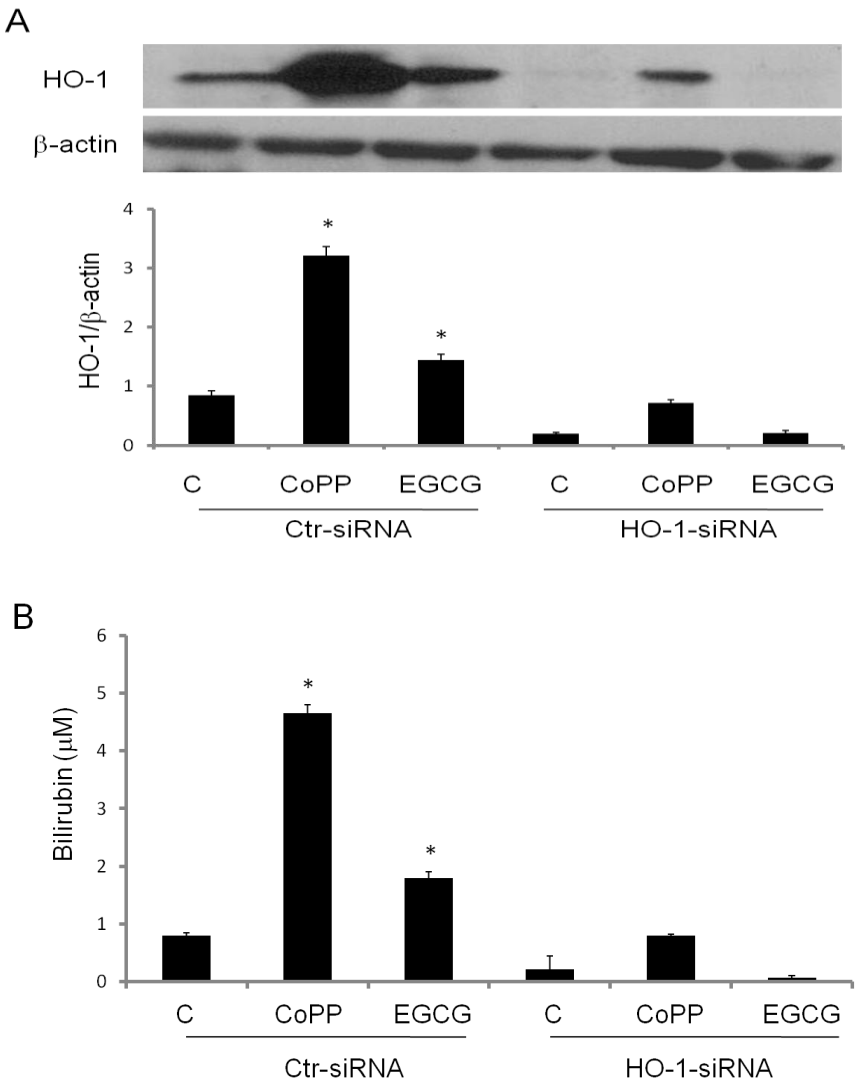

Figure 3-5 EGCG induces bilirubin levels in endothelial cells. Endothelial cells were transfected with siRNA for HO-1 (HO-1 siRNA) or with control siRNA (Ctr-siRNA) and cells were treated with either vehicle (0.1% DMSO), EGCG (30 μM), or CoPP (10 μM) for 4 h before determining HO-1 and bilirubin production (3-5A and B). Results shown represent the mean ± SEM, n=3; *Significantly different compared to control cultures.

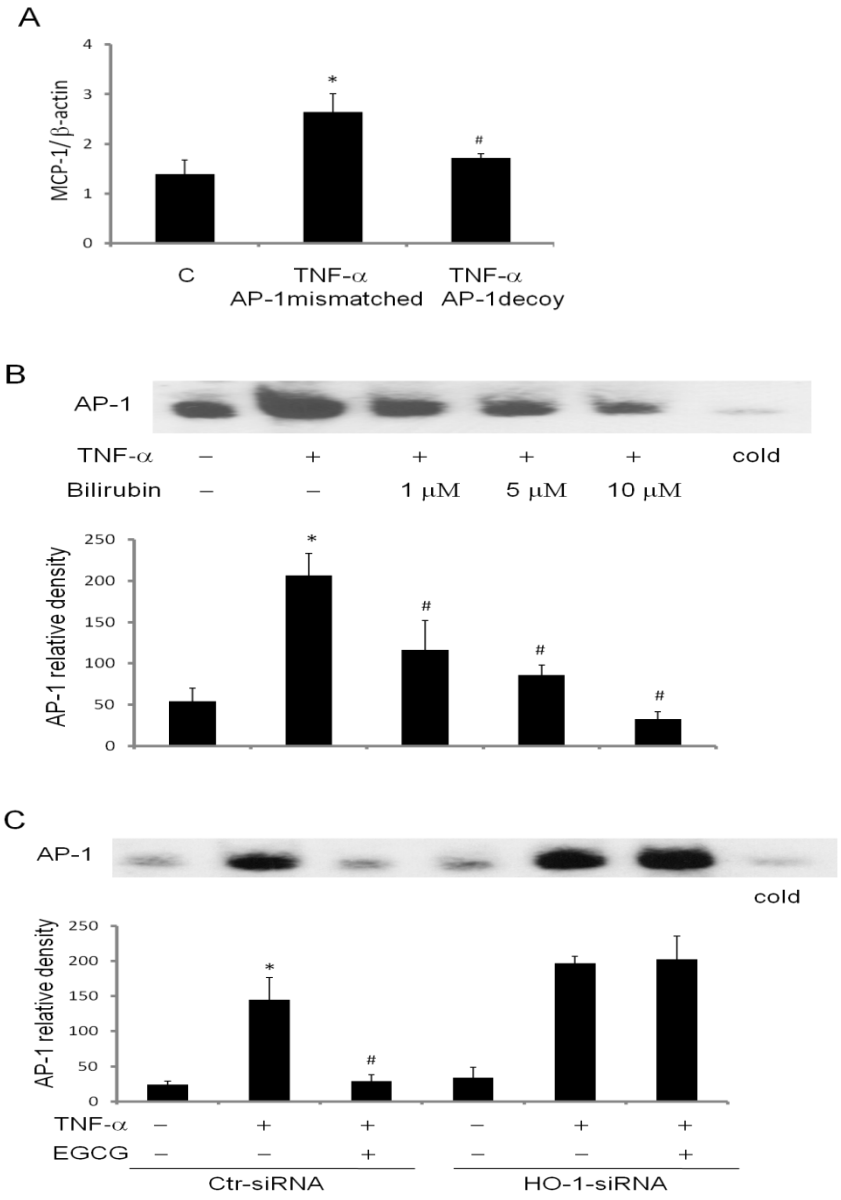

Figure 3-6 The effect of EGCG on DNA binding of AP-1 and subsequent MCP-1 induction is dependent on functional HO-1. Endothelial cells were stimulated with TNF-α for 4 h after transfection of AP-1 decoy ODN and mismatched-ODN. Subsequently, MCP-1 mRNA levels were measured (Figure 3-6A). Cells also were pretreated with either vehicle (H_2O) or bilirubin (0-10 μM) for 2 h, followed by exposure to TNF-α for an additional 4 h. Electrophoretic mobility shift assay for AP-1 was performed with nuclear proteins (Figure 3-6B). In separate experiments, endothelial cells were transfected with siRNA for HO-1 (HO-1 siRNA) or with control siRNA (Ctr-siRNA) and treated with EGCG for 2 h, followed by exposure to TNF-α for 4 h. Electrophoretic mobility shift assay for AP-1 was performed with nuclear proteins (Figure 3-6C). Results show represent the mean ± SEM, n=3; *Significantly different compared to vehicle control. #Significantly different compared to cultures treated only with TNF-α (Ctr-siRNA).

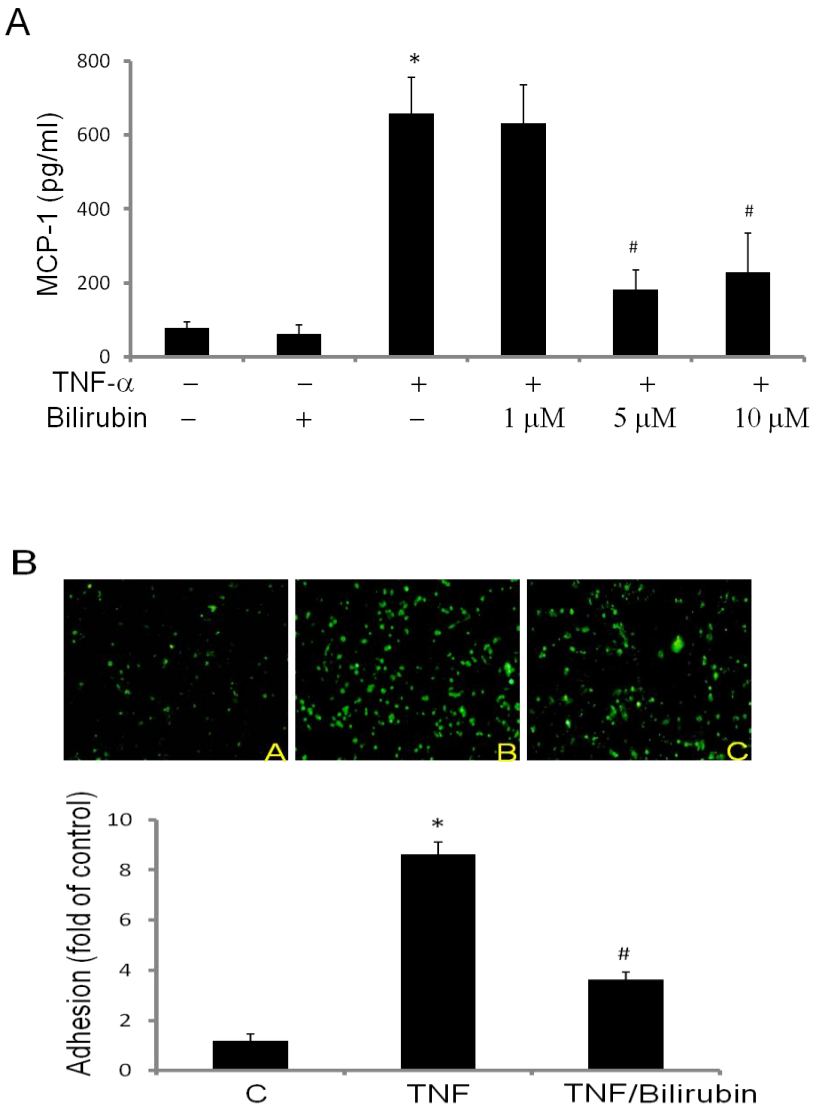

Figure 3-7 Bilirubin inhibits TNF-α-induced MCP-1 expression and monocyte adhesion. Cells were pretreated with bilirubin (1-10 μM), followed by exposure to TNF-α (2 ng/ml) for 6 h. MCP-1 (Figure 3-7A) and monocyte adhesion (Figure 3-7B) were measured. Results shown represent the mean ± SEM, n=3; *Significantly different compared to vehicle control. #Significantly different compared to cultures treated only with TNF-α.

Discussion

Flavonoids have anti-inflammatory properties and thus can provide protection against inflammatory diseases such as atherosclerosis [134]. EGCG, the most abundant catechins derived from green tea, have various bioactive properties associated with antioxidant, anti-angiogenesis, and anti-inflammatory functions, which all are relevant to the prevention and treatment of cardiovascular diseases [193]. 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 is considered an early event in the etiology of cardiovascular diseases such as the pathology of atherosclerosis [123]. Studies suggest that EGCG, the major polyphenolic constituent in green tea, has anti-inflammatory effects, such as inhibition of cytokine-induced VCAM-1 expression [24]. However, detailed mechanisms of the anti-inflammatory properties of EGCG associated with endothelial cell function are not well defined.

In the present study, we used TNF-α, a major pro-inflammatory cytokine stimulate cells to induce endothelial inflammation. Our data suggest that pre-treatment with EGCG inhibited TNF-α-induced superoxide production and endothelial secretion of MCP-1, a critical chemokine responsible for the recruitment of monocytes to the intima [194]. Oxidative stress refers to disrupted redox equilibrium between the production of free radicals and the ability of cells to protect against damage caused by these molecules. Moreover, EGCG also inhibited the activation of TNF-α-induced AP-1, which has been recognized as one of the required transcription factors for MCP-1 gene induction besides NF-κB [195, 196]. In the current study, the DNA-binding activity of AP-1 increased after TNF-α stimulation along with an increase in MCP-1 mRNA. We also found that transfection of an AP-1 decoy ODN into the cells down-regulated the TNF-α-induced MCP-1 mRNA levels. Thus, TNF-α induced MCP-1 production in our cell culture system was partially dependent on AP-1 activation. In addition to AP-1, TNF-α also induced NF-κB DNA binding. However, EGCG only inhibited TNF-α-induced AP-1 activation, with little effect on NF-κB DNA binding. Others also have reported that EGCG did not influence TNF-α-stimulated NF-κB in human endothelial cells [24], suggesting that EGCG more specifically alters AP-1 signaling in endothelial cells.

HO-1 over-expression can inhibit pathological activities including inflammation, vascular proliferation, and chronic transplant rejection [197, 198]. It has been reported that overexpression of the HO-1 protein inhibits lipopolysaccharide-induced iNOS expression and NO production [199, 200]. Furthermore, the HO-1 protein is essential for the anti-inflammatory effects of IL-10 and 15-deoxy-delta 12, 14-prostaglandin J2 [201, 202]. Our data support the hypothesis that EGCG can provide protection against endothelial inflammation through induction of HO-1 gene expression. In fact, the observed anti-inflammatory effects of EGCG were mimicked by the HO-1 inducer cobalt protoporphyrin (CoPP), and abolished by HO-1 siRNA transfection. In the current study, treatment with CoPP, inhibited TNF-α-induced MCP-1 up-regulation, suggesting that HO-1 may be an important target in endothelial cells against inflammation and the further development of atherosclerosis. We investigated the relationship between HO-1, ROS production, AP-1 activation, and monocyte adhesion in TNF-α-stimulated endothelial cells. Cells were pre-treated with EGCG and then exposed to TNF-α. Our data suggest that the EGCG-mediated HO-1 protein expression interferes with TNF-α-induced superoxide production and AP-1 activation. Moreover, blocking of HO-1 expression by transfection with siRNA reversed the suppressive effects of EGCG in terms of AP-1 translocation and MCP-1 upregulation in TNF-α-treated endothelial cells.

Although the molecular targets of HO-1 are not fully clear, our study demonstrates that HO-1-mediated signaling is a critical mechanisms during the reduction of endothelial inflammation by EGCG. In addition, our data also suggest that HO-1 acts upstream of AP-1 activity, supporting a previous study demonstrating that HO-1 expression has a direct effect on the activation of the proinflammatory AP-1 pathway [203]. EGCG could induce HO-1 indirectly through other signaling mechanisms, such as via induction of NO, because it has been reported that phenolics can increase NO [204, 205] and that NO can induce HO-1 [180, 206]. To further investigate the role of HO-1 in EGCG-mediated protection against endothelial cell activation, we explored effects of bilirubin, a specific enzymatic metabolite of HO-1. Serum bilirubin levels are inversely associated with insulin resistance and other complications of metabolic syndrome [207], and exogenous administration of bilirubin can promote endothelial cell survival [208]. In the current study, EGCG significantly induced bilirubin levels in endothelial cultures,

which was blocked in HO-1-silenced cells. Supplemental bilirubin also significantly inhibited the expression of MCP-1 and monocyte adhesion in a concentration-dependent manner. Moreover, similar to EGCG, supplemental bilirubin inhibited the TNF-α-stimulated AP-1 activation.

Taken together, our data support the hypothesis that the protection effects of EGCG against TNF-α-induced DNA binding of AP-1 and subsequent MCP-1 induction, monocyte adhesion are dependent on functional HO-1. Furthermore, data from the current study also strongly support our hypothesis that HO-1 expression and bilirubin secretion induced by flavonoids such as EGCG can inhibit TNF-α-stimulated MCP-1 up-regulation, and AP-1 activation associated with vascular endothelial inflammation. This may in part explain the potent protective properties of EGCG against inflammatory diseases such as atherosclerosis. Our findings add to the growing body of evidence for the beneficial effects of green tea consumption on improving cardiovascular health.

Chapter Four: EGCG stimulates Nrf2 and heme oxygenase-1 via caveolin-1 displacement

This work has been submitted for publication (Zheng et al., Atherosclerosis, 2010).

Synopsis

Flavonoids, such as the tea catechin epigallocatechin-gallate (EGCG), can protect against atherosclerosis by decreasing vascular endothelial cell inflammation. Plasma microdomains called caveolae are important in vesicular transport and the regulation of signaling pathways associated with vascular diseases. We hypothesized 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 measured EGCG levels in caveolae-enriched domains and caveolin-1 gene silencing cells. We also explored the effects of EGCG on the induction of Nrf2 and HO-1 in endothelial cells with or without functional caveolae. EGCG rapidly accumulated in caveolae, which was associated with caveolin-1 displacement from the plasma membrane towards the cytosol. Furthermore, caveolin-1 gene silencing significantly reduces the uptake of EGCG in endothelial cells within 30 min. Treatment with EGCG activated Nrf2 and increased HO-1 expression and cellular production of bilirubin. Similar to EGCG treatment, silencing of caveolin-1 by siRNA technique also resulted in upregulation of Nrf2, HO-1 and bilirubin production. These data suggest that caveolae may play a role in the uptake and transport of EGCG. EGCG-mediated displacement of caveolin-1 may associate with the activation of Nrf2/HO-1 pathway.

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 [132, 133]. Flavonoids constitute a subclass of bioactive compounds rich in fruits and vegetables, soy food, legumes, tea and cocoa [134]. Green tea consumption has been shown to be significantly greater in healthy subjects compared to those with coronary artery disease [209], suggesting that green tea might be protective against coronary atherosclerosis [210]. 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 are known to improve endothelial cell function, and thus reduce cardiovascular risk [211], 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 [212]. 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 sub-endothelial space [73]. Unlike clathrin-mediated endocytosis, internalization through caveolae involves complex signaling [74]. 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 [75]. 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 flavonoids by means of endocytosis [76, 77].

Protective mechanisms of flavonoids may include upregulation of heme oxygenase-1 (HO-1) [104], an enzyme localized to plasma membrane caveolae [213]. HO-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 [176]. The heme oxygenase system is an important regulator of endothelial cell integrity and oxidative stress [185], and dysfunctional HO-1 signaling may be a pro-atherogenic event. HO-1 can be upregulated by EGCG [104], and we have recently demonstrated that EGCG-mediated protection against TNF-α induced MCP-1 expression is HO-1 dependent [214].

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 [215]. For example, the induction of the antioxidant gene HO-1 is associated with nuclear translocation of Nrf2 and subsequent transactivation of an antioxidant response element in the promoter region of HO-1 [216]. There is evidence that flavonoids like EGCG can induce HO-1 via activation of Nrf2 [104, 217]. Since HO-1 is an enzyme localized to plasma membrane caveolae [213], 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 [77], suggesting that caveolae may provide a regulatory platform of our observed effects of EGCG on stimulation of Nrf2 and HO-1.

Materials and Methods

Cell culture and experimental media

Primary endothelial cells were isolated from porcine aortic arteries and cultured as previously described [186]. The basic culture medium consisted of medium 199 (M-199) (Cat. No. 31100-035; GIBCO Laboratories, NY) containing 10% (v/v) fetal bovine serum (FBS, HyClone Laboratories, UT). Experimental media contained 5% FBS and were supplemented with EGCG (Cayman Chemicals, Ann Arbor, MI; purity >98%). EGCG was dissolved in DMSO; at a final concentration less than 0.1%, DMSO did not affect cell viability.

Detergent- free purification of caveolae-rich membrane domains

Caveolae-rich membrane domains were isolated as previously reported [218], with minor mortifications. Briefly, cells were plated in 150 mm plates. After treatment, cells were washed with PBS and lysed with 2 ml of ice-cold MES-buffered saline [MBS; 25mM MES (morpholineethanesulfonic acid, 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 (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). Twelve 1 ml fractions were collected, and aliquots of each fraction were subjected to SDS-PAGE and immunoblotting to assess caveolin-1.

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 activation. To detect the effect of EGCG and caveolin-1 gene silencing on Nrf2 accumulation in nucleus, both nuclear and cytosol protein extracts from endothelial cells were prepared as previously described [151]. Western blots were visualized using the appropriate horseradish peroxidase-conjugated secondary antibodies followed by ECL immunoblotting detection reagents (Amersham Biosciences, Buckinghamshire, England).

Caveolin-1 siRNA and transfection

The caveolin-1 gene silencer was designed by Dharmacon (Lafayette Colorado) according to previously described methods [150]. The sequences of caveolin-1 gene silencer were 5'-CCAGAAGGGACACACAGUUdTdT-3' (sense), 5'-AACUGUGUGU CCCUUCUGGdTdT-3' (anti-sense). The sequences of the control gene silencer were 5’-AAAGAGCGACUUUACACACdTdT-3' (sense), 5’-GUGUGUAAAGUCGCUCUUU dTdT-3' (anti-sense). Cells were transfected with control siRNA or caveolin-1 siRNA at

a final concentration of 80 nM using GeneSilencer (Genlantis, San Diego, CA) with Optimem I medium (Invitrogen, Carlsbad, CA). Cells were incubated with transfection mixtures for 4 h and then replaced with 10% serum medium. Cells were synchronized overnight after 48 h transfection, and then treated with EGCG.

Electrophoretic mobility shift assays of Nrf2-ARE-DNA binding

Nuclear extracts from endothelial cells were prepared as previously described [151]. 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 ARE, the enhancer DNA element for HO-1 (5' bio-AGATTTTGCTGAGTCACCAGTCCC-3') [219]. Gel mobility shift assay was performed to demonstrate the shifted DNA-protein complexes Nrf2-ARE using a LightShiftTM chemiluminescent EMSA kit (Pierce, Rockford, IL) [152]. Reactions using 200-fold molar excess of unlabeled oligonucletide 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.

Quantification of EGCG by LC/MS/MS

After treatment with EGCG, cells were washed twice in cold PBS. Then cells were scraped 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 3,500 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 LC/MS/MS. The mass spectrometer included an Applied Biosystems/MDS SCIEX 4000-Qtrap hybrid, linear ion trap, triple quadrupole MS (Applied Biosystems, Foster City, CA), and the liquid chromatograph was a Prominence UFLC from Shimadzu Corp., Columbia, MD.

Polyphenols were separated using an Eclipse XDB C8, 5 μM, 4.6 X 150 mm (Agilent) column.

Measurement of bilirubin production

After plating, cells were treated with TNF-α and EGCG or vehicle. All further manipulations were carried out in a dark room. After incubation, 0.5 ml of each culture supernatant was collected and 250 mg $BaCl_2$. $2H_2O$ was added. After vortexing, 0.75 ml benzene was added; then, tubes were vortexed vigorously leading to the formation of a relatively stable milky-white emulsion. After centrifugation (30 min at 13,000g), the upper benzene layer was collected and the absorbance was measured at 450 nm with a reference wavelength at 600 nm using a SpectraMaxPro M2 spectrophotometer (Molecular Devices, Sunnyvale, CA). In a separate tube, 0.5 ml of fresh culture medium was processed in the same way and the benzene layer was collected and used as a blank. 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$mM^{-1}cm^{-1}$ [189].

Statistical analysis

Values are reported as mean ± standard error of the mean (SEM) of at least three independent groups. Data were analyzed using Sigma Stat software (Jandel Corp., Wan Rafael, CA). One way ANOVA followed by post hoc least significant difference (LSD)'s pairwise multiple comparison procedure were used for statistical analysis of the original data. A statistical probability of $P< 0.05$ was considered significant.

Results

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 (Figure 4-1). 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.

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 (Figure 4-2A). Cell-associated levels were maximal at 30 min and then declined in parallel with observed media levels (Figure 4-2B). These results confirmed that EGCG is easily auto-oxidized and quickly metabolized [164]. Results in Figure 4-2C demonstrate that endothelial cells can become enriched with EGCG in a dose-dependent manner.

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 non-caveolae fractions after treatment with EGCG for 5, 10, 20 or 40 min. EGCG levels showed in the figures were normalized by the amount of proteins in caveolae-rich domains and non-caveolae fractions respectively. EGCG markedly accumulated in caveolae-rich membrane domains at 10 min (Figure 4-3A), with a subsequent relocation into the non-caveolae cell fractions (Figure 4-3B).

Caveolae silencing reduces cellular EGCG uptake

To explore a potential role of caveolae in EGCG uptake, cells were transfected with caveolin-1 specific siRNAs [153]. Compared to cells with functional caveolae, cellular levels of EGCG at 30 min were significantly decreased after silencing caveolin-1 (Figure 4-4). This trend continued but was not significant at 1 h after EGCG exposure.

Cavelin-1 silencing induces upregulation of HO-1 and bilirubin production

HO-1 has potent anti-inflammatory effects, which may be exerted through the generation of bilirubin [192]. 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 (Figure 4-5A). Similar to the HO-1 data, both EGCG and caveolin-1 gene silencing significantly induced the cellular secretion of bilirubin (Figure 4-5B).

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 [220]. Activation of Nrf2 results in increased accumulation of Nrf2 in the nucleus [221]. 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 Figure 4-6A, 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 unaffected by EGCG treatment or caveolin-1 silencing (Figure 4-6B). Since Nrf2 activates transcription activities of its genes through

binding specifically to the ARE found in the promoters of target genes [222, 223], 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 both in EGCG treated or caveolin-1 silenced cells (Figure 4-6C, there were two retarded bands, Western Blotting with Nrf2 antibody following the EMSA assay demonstrated the upper band was Nrf2-ARE complex).

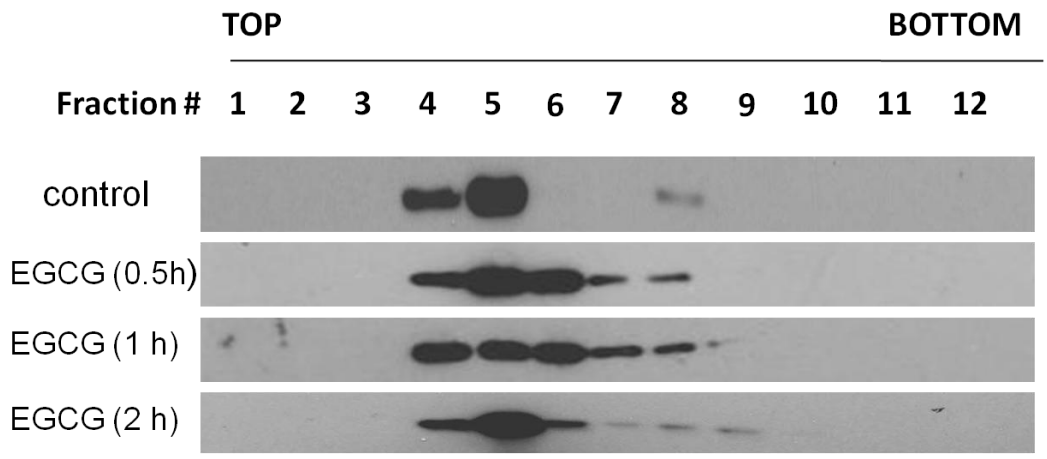

Figure 4-1 EGCG treatment stimulates caveolin-1 translocation. Endothelial cells were treated with vehicle (0.1% DMSO) or EGCG (30 μM) for 0.5-2 h. Caveolae enriched fractions were isolated by detergent-free sucrose gradient centrifugation method. Then the expression of caveolin-1 was measured by Western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

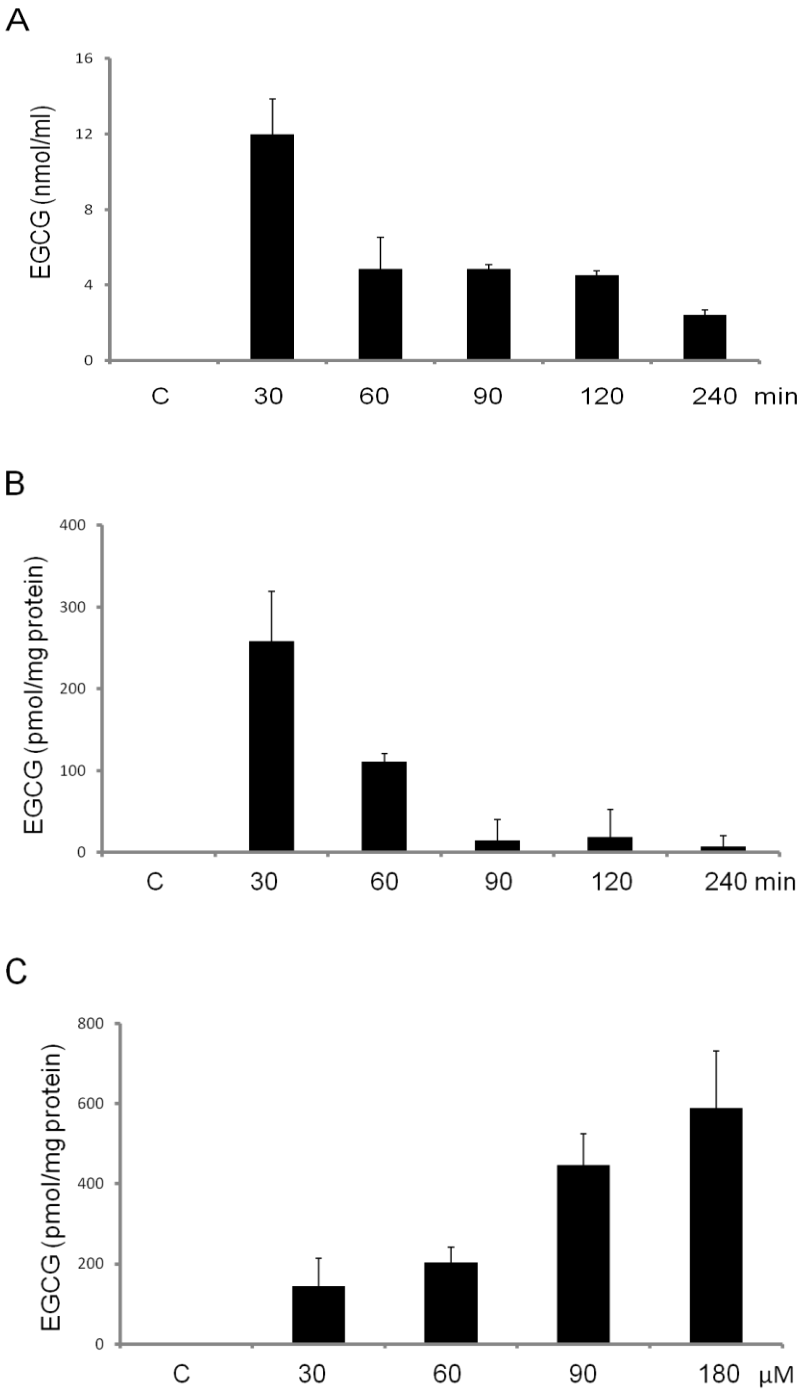

Figure 4-2 EGCG quantification in endothelial cells was performed by LC/MS/MS analysis. Endothelial cells were treated with EGCG (30 μM) for 0.5-4 h, and EGCG levels were measure by LC/MS/MS in the media (Figure 4-2A) and cells (Figure 4-2B). In separate experiments, endothelial cells were treated with increasing concentrations of EGCG (0-180 μM) for 1 h, before measuring cellular levels of EGCG (Figure 4-2C). Results shown represent the mean ± SEM of three independent experiments.

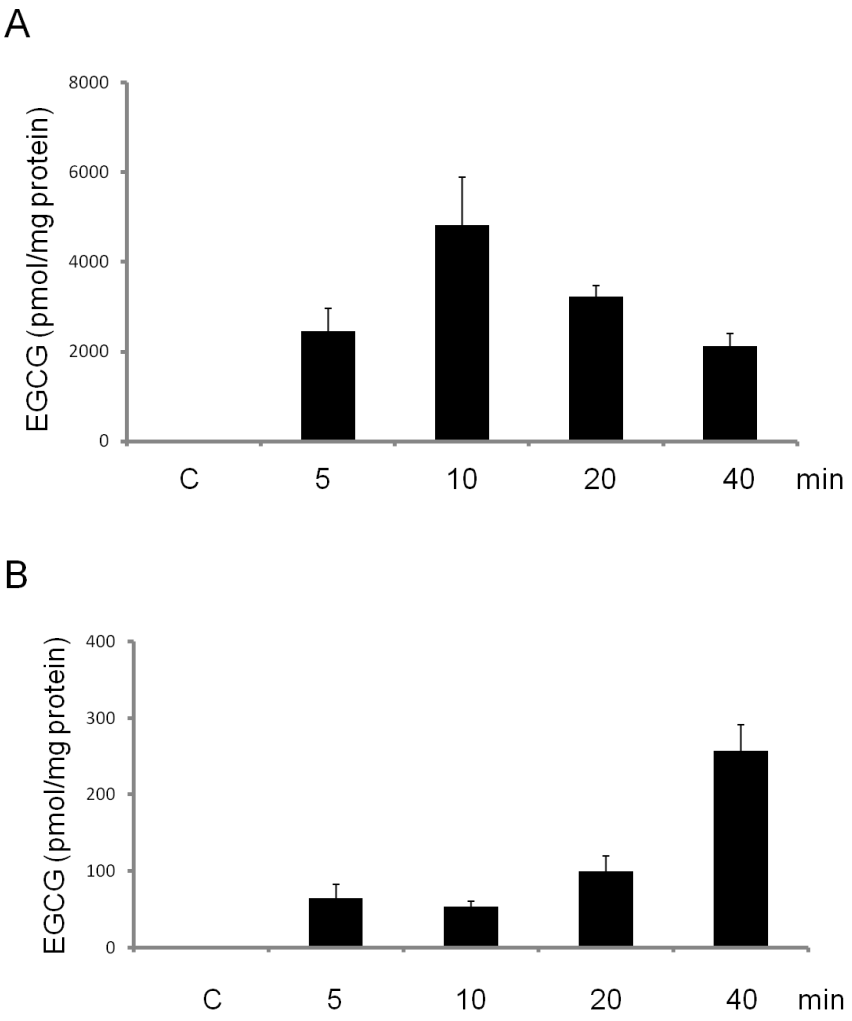

Figure 4-3 EGCG accumulates in caveolae-enriched fractions. Endothelial cells were treated with EGCG (30 μM) for 5-40 min. Caveolae were isolated by detergent-free sucrose density gradient centrifugation method. EGCG levels in caveolae-rich domains (Figure 4-3A) and non-caveolae fractions (Figure 4-3B) were measured by LC/MS/MS. EGCG levels shown in the figure were normalized against protein levels in caveolae or non-caveolae fractions, respectively. Results shown represent the mean ± SEM of three independent experiments.

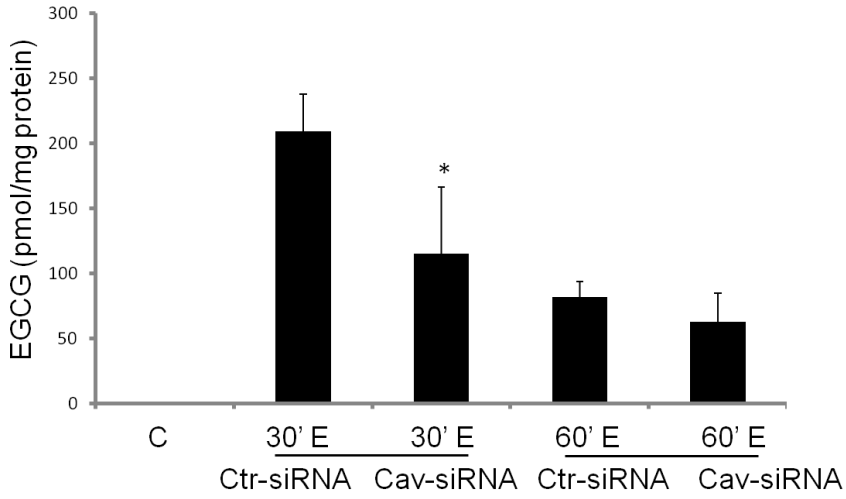

Figure 4-4 Caveolae take up EGCG in endothelial cells. Endothelial cells were transfected with siRNA for caveolin-1 (Cav-1 siRNA) or with control siRNA (Ctr-siRNA) and treated with EGCG (30 μM) for 30 min and 1 h. EGCG levels in the whole cells were measured by LC/MS/MS. Results shown represent the mean ± SEM of five independent experiments.

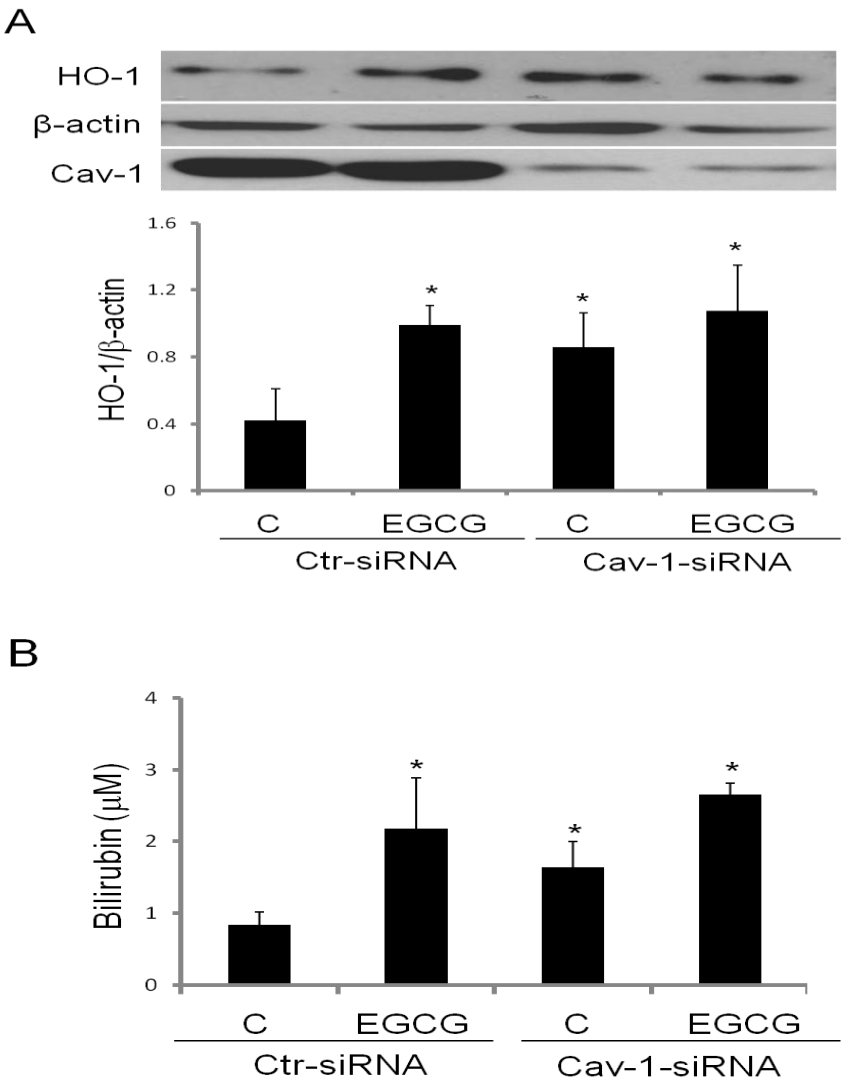

Figure 4-5 Cavelin-1 silencing induces upregulation of HO-1 and bilirubin production. Endothelial cells were transfected with siRNA for caveolin-1 (Cav-1 siRNA) or with control siRNA (Ctr- siRNA) and then treated with vehicle (0.1% DMSO) or EGCG (30 μM) for 6 h, before determining HO-1 protein expression (Figure 4-5A) and bilirubin production (Figure 4-5B). Results shown represent the mean ± SEM of three independent experiments. *Significantly different compared to control cultures.

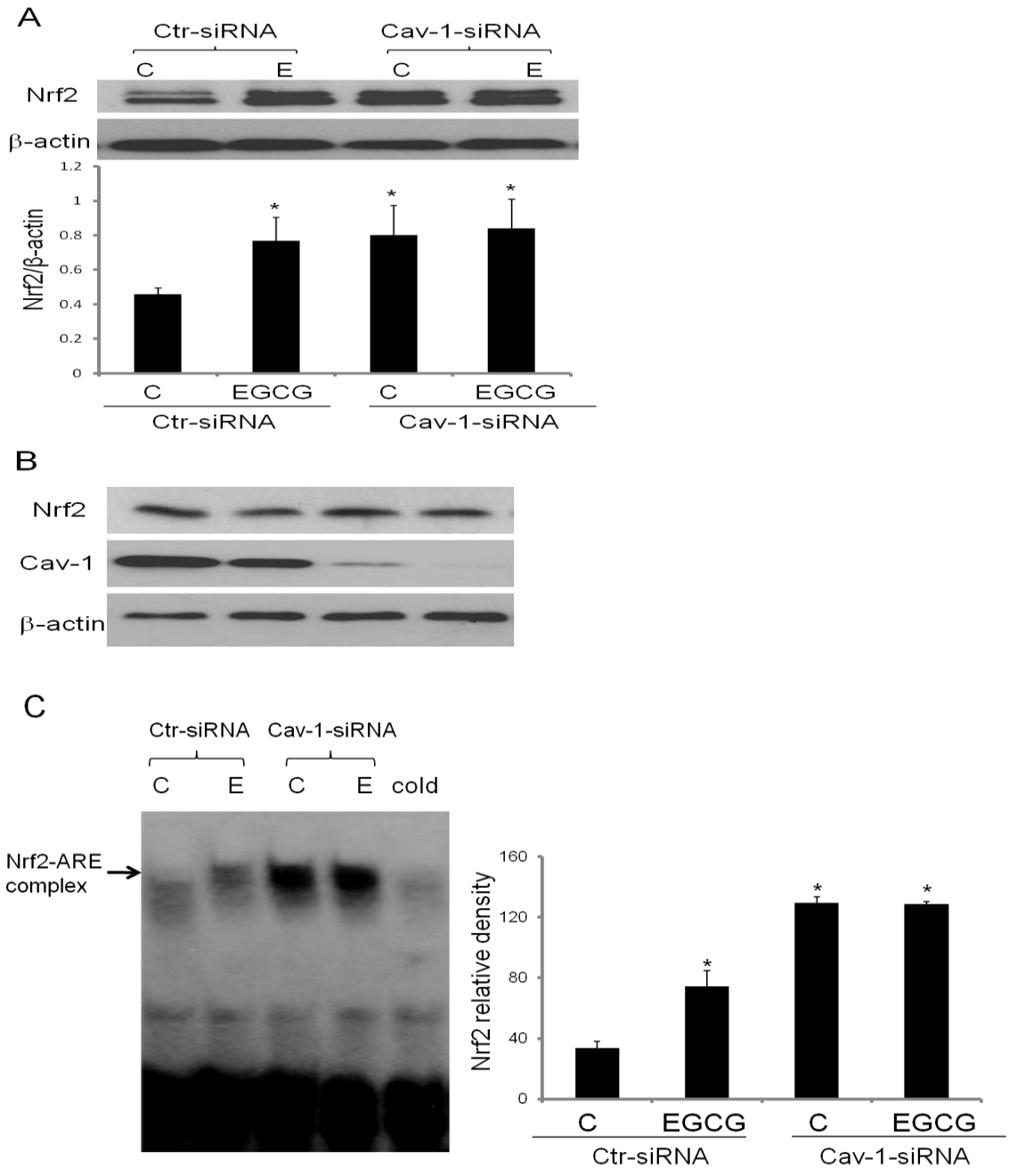

Figure 4-6 Caveolin-1 silencing increases both nuclear accumulation of Nrf2 and Nrf2-ARE binding. Endothelial cells were transfected with siRNA for caveolin-1 (Cav-1 siRNA) or with control siRNA (Ctr- siRNA) and then treated with vehicle (0.1% DMSO) or EGCG (30 μM) for 3 h, before determining Nrf2 protein expression both in nucleus (Figure 4-6A) and cytosol (Figure 4-6B). Electrophoretic mobility shift assay for Nrf2-ARE binding was performed with nuclear proteins (Figure 4-6C). 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 ± SEM of three independent experiments. *Significantly different compared to control cultures.

Discussion

There is evidence that catechins derived from green tea, such as EGCG, have antioxidant, anti-inflammatory and anti-angiogenesis properties and thus can provide protection against inflammatory diseases such as atherosclerosis [224]. 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 recently recognized as signal transduction hubs [225, 226], and may be involved in the selective cellular uptake of plasma-derived material [227, 228] and possibly resveratrol [229] 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 (LamR), and that it further alters the composition of membrane domains as well as changes in some signaling pathways [230]. 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 kinases pathway [80, 231]. 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 non-caveolae 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.

Caveolae usually have a specific lipid composition, which appears to be required for the specific functional relevance of caveolae-associated proteins [232, 233]. In line with these functional characteristics, caveolae can provide a regulatory platform for proinflammatory signaling associated with vascular diseases such as atherosclerosis [234]. For example, enriching endothelial cells with docosahexaenoic acid can affect caveolae-

associated nitric oxide synthase activity [235], a process which may be linked to caveolae-mediated endocytosis [236]. We also found previously that pretreatment with EGCG can block fatty acid-induced caveolin-1 expression in a time and concentration dependent manner [77]. 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 may be 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 monocyte chemoattractant protein-1 expression is HO-1 dependent [214], 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 [237]. HO-1 over-expression can inhibit pathological activities, including inflammation, vascular proliferation, and chronic transplant rejection [197, 198]. It has been reported that over-expression of the HO-1 protein inhibits lipopolysaccharide-induced iNOS expression [199, 200]. Furthermore, the HO-1 protein is essential for the anti-inflammatory effects of IL-10 and 15-deoxy-delta 12, 14-prostaglandin J2 [201, 202].

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 anti-oxidant genes, including HO-1 [102]. Once migrated to the nucleus, Nrf2 forms heterodimers with small Maf proteins and subsequently binds to the cis-acting antioxidant response element (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 [103]. 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.

EGCG can increase the nitric oxide level in endothelial cells [238]. Furthermore, Ramirez-Sanchez *et al* demonstrated that (-)-epicatechin treatment induced eNOS uncoupling from caveolin-1, thus activating eNOS function [239]. Thus, there is a possibility that our observed EGCG-mediated upregulation of Nrf2 is regulated in part through caveolae-dependent eNOS/NO signaling [240]. It is well known that caveolin-1 is a negative regulator for eNOS activity [241]. Thus, EGCG-activated caveolin-1 displacement may lead to its disassociation with eNOS, leading to NO production. Moreover, NO has been found to increase HO-1 induction in various cell types [242]. It has also been reported that NO can stimulate Nrf2 translocation and activation in the vascular endothelium [243-245]. It is not clear how flavonoids like EGCG enter the cell to modulate gene expression; however, some endocytotic process might be involved [74]. In fact, it has been demonstrated that caveolae-mediated endocytosis can play a critical role of in regulating eNOS activation in endothelial cells [236]. More studies are needed to fully understand the link between protective properties of dietary flavonoids such as EGCG and caveolae-mediate-cellular signaling.

In summary, we provide evidence that caveolae play a role in the uptake and transport of EGCG and mechanisms may associate with the anti-inflammatory properties of this flavonoid. Functional caveolae may be a prerequisite for EGCG to cause caveolin-1 displacement and to initiate the protective signaling cascade.

Chapter Five: Summary

Conclusion

Endothelial dysfunction is a critical early event in the development of atherosclerosis. The inflammatory reaction in endothelial cells is regulated by complex interactions. Long term exposure to cardiovascular risk factors will ultimately exhaust those protective anti-inflammatory factors. Through increased expression of adhesion molecules, activated endothelial cells stimulate monocytes attachment and migration into sub-endothelial space, where the monocytes differentiate into macrophages and by taking up modified lipids further become foam cells. Smooth muscle cells proliferate and migrate from tunica media to intima responding to cytokines secreted by damaged endothelial cells. Finally, these inflammatory events cause the formation of a fibrous cap covering the fatty streak, an accumulation of lipid-laden cells in the sub-endothelial spaces. In more advanced stages of atherosclerosis, formation of fibrous plaque and lesions will ultimately lead to an acute clinical event caused by plaque rapture and thrombosis [44, 45]. Since the initial stage of atherosclerosis is the critical time point when nutrition (e.g. flavonoids) modulation could have the most beneficial effects, the research in this dissertation focused on the early events of atherosclerosis, including the role of caveolae and heme oxygenase-1 (HO-1) on dietary flavonoid EGCG-mediated protection against pro-inflammatory cytokine tumor necrosis factor (TNF)-α or linoleic acid-induced activation of endothelial cells.

One major link of the three chapters of this dissertation is the bioactive green tea flavonoid EGCG. Chapter Two focused on caveolae-associated mechanisms of inflammatory diseases and protection by EGCG. Chapter Three focused on the role of HO-1 in EGCG-mediated protection against endothelial inflammation. Since it has been reported that deletion of caveolin-1 protects against oxidative lung injury via up-regulation of HO-1, and caveolin-1 may interact with and modulate HO-1 activity in endothelial cells [213, 246], Chapter Four focused on the correlations between caveolae and HO-1 and their roles in EGCG-mediated anti-inflammation effects.

Linoleic acid, the essential omega-6 fatty acid in the American diet is pro-inflammatory and pro-atherogenic. It favors oxidative modification of LDL cholesterol,

and increases platelet aggregation. Previous research has shown that linoleic acid potentiated TNF-α-induced endothelial cell activation [120]. EGCG, the most abundant bioactive polyphenol of green tea, has been suggested to have an anti-inflammatory function by inhibiting cytokine-induced VCAM-1 expression in HUVEC [24]. EGCG has also been shown to inhibit the expression of COX-2 and the production of prostaglandin E2 [156, 159]. Caveolae, the plasma membrane invaginations, have been implicated in several human diseases and in particular vascular diseases. 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 [76]. In Chapter Four, evidence is provided that caveolae may also regulate vascular trafficking of 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 [56]. Besides the role of vesicular transport, caveolae harbor an array of cell signaling molecules, and numerous enzymes involved in endothelial cell dysfunction and inflammation [140]. Considering these findings led to the hypothesis that caveolae possibly play a role in EGCG mediated protection against endothelial cell activation. The data support the hypothesis that EGCG inhibited linoleic acid-induced endothelial inflammation. The current research provided novel data demonstrating that EGCG down-regulated the base-line level and linoleic acid-induced caveolin-1, the main structural protein of caveolae. The results further suggested that silencing of the caveolin-1 gene expression by small-interference RNA technique markedly down-regulated linoleic acid-induced COX-2 and MCP-1 expression indicating that caveolae may be a critical platform regulating inflammatory signaling pathways that can be modulated by the interaction of bioactive compounds such as flavonoids.

The HO system plays a critical role in cellular and tissue self-defense against oxidative stress and inflammation. HO-1 is the inducible isoform degrading heme to carbon monoxide (CO), ferrous (Fe^{2+}) and biliverdin, the latter being subsequently converted into bilirubin. Excess heme catalyzes the formation of reactive oxygen species, which cause endothelial dysfunction. Therefore, the HO system may be recognized as a protector of endothelial cell integrity. Bilirubin has strong anti-oxidant properties and

thus can scavenge reactive oxygen species, inhibit NADPH oxidase activity [86], and preserve endothelial integrity, in part by increasing the bioavailability of NO [87]. Higher serum bilirubin levels are associated with a reduced risk of coronary artery disease [89]. The dissertation hypothesized that the flavonoid EGCG might down-regulate endothelial inflammatory parameters by modulating HO-1-regulated cell signaling. Thus, a focus of this study was on the role of HO-1 and its major metabolic product, bilirubin, on mechanisms of TNF-α-induced endothelial cell activation and protection by EGCG. The data support the hypothesis that EGCG can protect against TNF-α-induced DNA binding of AP-1, subsequent MCP-1 induction, and monocyte adhesion. Moreover, EGCG up-regulated the expression of HO-1 and further induced the secretion of bilirubin. The observed anti-inflammatory effects of EGCG were mimicked by the HO-1 inducer cobalt protoporphyrin (CoPP) and abolished by HO-1 gene silencing. The data suggested that the protective properties of flavonoids, such as EGCG, against endothelial inflammation might be regulated in part through induction of HO-1 and subsequent AP-1 signaling. This in part explains the protective properties of EGCG against inflammatory diseases such as atherosclerosis.

Caveolin-1 ablation not only prevented linoleic acid-induced endothelial activation, but also protected against environmental toxin PCB77-induced MCP-1 up-regulation in endothelial cells [247]. To further understand the molecular mechanisms underlying the anti-inflammatory effects of caveolin-1 ablation, caveolin-1 was silenced in endothelial cells, and its effect on Nrf2/HO-1 cellular self-defense system was investigated. The results clearly showed that caveolin-1 gene silencing induced HO-1 protein expression, as well as up-regulation of the production of bilirubin. Nrf2 is a redox-sensitive transcription factor. Most genes encoding phase II enzymes, such as some detoxifying or antioxidant enzymes, contain a common DNA sequence called antioxidant response elements (ARE) that are located in their promoter regions. Nrf2 is the major transcription factor that regulates gene expression under the control of ARE. These Nrf2 regulated phase II enzymes play key roles in cellular defense by enhancing the removal of cytotoxic electrophiles or reactive oxygen species [248]. Thus, induction of Nrf2-ARE signaling provides a cellular self-defense against a variety of electrophilic compounds, reactive toxicants and oxidants [124]. This research provided novel data which suggest

that either EGCG or caveolin-1 silencing can induce up-regulation of HO-1 expression and subsequent bilirubin induction, and that these events require functional Nrf2.

One of the functions attributed to endothelial caveolae is their ability to transfer molecules by endocytosis. It has been reported that some polyphenols such as resveratrol can be transported by caveolae [229]. Cavolin-1, the major constituent of caveolae participates in the process of endocytosis. Caveolin-1 constantly recycles between the plasma membrane, cytosol and intracellular compartments. Caveolin-1 could directly bind to some lipophilic compounds, such as some fatty acids, fat-soluble vitamins and polyphenols. Unlike clathrin-mediated endocytosis, internalization by caveolae involves complex signaling transduction [74]. Caveolae are highly enriched in membrane binding proteins, such as H-ras, src-family tyrosine kinases, heterotrimeric G-proteins, eNOS, etc. [139, 249, 250]. Caveolins can bind and functionally regulate (mostly inhibit) these caveolae-localized molecules [251-256]. When caveolin-1 moves away from the plasma membrane, some of these co-localized signaling molecules are released and activated. It was hypothesized that EGCG might cause caveolae displacement and thus modulate those signaling proteins associated with caveolae. The data clearly showed that EGCG rapidly accumulated in caveolae, which was associated with caveolin-1 translocation from plasma membrane towards cytosol. More interestingly, EGCG failed to further activate Nrf2 in caveolin-1 silenced cells. This suggests that EGCG-mediated Nrf2 activation may need caveolae function perhaps by caveolin-1 displacement. It has been reported that caveolae-mediated endocytosis induced eNOS activation in endothelial cells [236]. It is well known that nitric oxide is a strong inducer of Nrf2 and HO-1 [243-245]. Furthermore, EGCG can increase the nitric oxide level in endothelial cells [238]. Thus, the hypothesis is that EGCG-mediated caveolin-1 translocation may activate eNOS, and further activate Nrf2/HO-1. More work is needed to further demonstrate this hypothesis. Taken together, these data suggest that EGCG-induced caveolin-1 displacement may reduce endothelial inflammation. This study provides a novel target through which EGCG functions to protect against inflammatory diseases such as atherosclerosis.

The quantification of EGCG in endothelial cells was also successfully performed by using LC/MS/MS. In most of the *in vitro* experiments which have been published, the cell cultures were treated with 20-100 μM of EGCG or other tea polyphenols. These

concentrations are much higher than those observed in human plasma (plasma concentrations are usually lower than 1 μM) [257]. Sang *et al* [164] reported that EGCG concentration decreased rapidly, with a half-life of approximately 30 min, and that EGCG dimers and other products were formed. Several factors, such as pH, temperature, oxygen levels, antioxidant levels, metal ions and the concentration of EGCG could affect the stability of EGCG. To further understand the amount of EGCG taken up by endothelial cells, in the study, EGCG levels in endothelial cell cultures were measured by LC/MS/MS in the laboratory of Dr. Morris at the University of Kentucky. The results showed that in the culture media, EGCG levels decreased over the course of time. Cell-associated EGCG levels were maximal at 30 minutes and then declined in parallel with the medium levels. These data suggest that EGCG was quickly auto-oxidized and metabolized. Concentration studies also demonstrated that the uptake of EGCG by endothelial cells was increased in a dose- dependent manner.

Taken together, the data provide implications in understanding HO-1 and caveolae-associated mechanisms of inflammatory diseases and protection by bioactive food components with antioxidant and anti-inflammatory properties such as the green tea flavonoid EGCG. The data provided in this dissertation may in part explain the protective properties of EGCG against inflammatory diseases such as atherosclerosis.

Future directions

It is well known that EGCG is quickly metabolized both in cell culture and in the human body. Once absorbed into the intestine, EGCG undergoes glucuronidation, sulfation, and methylation [258]. Research found that in mouse tissues, 55-90% of EGCG is conjugated [259]. Thus, in future studies, it is tempting to set up experiments to identify what is being formed during EGCG metabolism and which metabolites may have similar or even higher anti-oxidant or anti-inflammatory properties. Further research could extract EGCG metabolic products from the serum of animals which have been treated with EGCG and further test the protective effects of these metabolites against endothelial inflammation and atherosclerosis.

The data suggest that caveolin-1 silencing and subsequent up-regulation of HO-1 may involve the transcription factor Nrf2. Also, a direct interaction between HO-1 and caveolin-1 has been established by immunocytochemistry and coimmunoprecipitation [260]. Research also found the location of HO-1 protein in the detergent-resistant membrane fraction, where it appears to be in the caveolae, as caveolin-1 itself is localized to the structure [213]. This dynamic caveolin constantly recycles between the plasma membrane, endosomes, and trans-Golgi network. Caveolae may associate with HO-1 to undergo internalization and recycling [213]. In addition, studies demonstrated that activation of mitogen-activated protein kinases (MAPKs) contributes to the induction of HO-1 [261]. These pathways may affect endothelial cell function and are likely to be activated by substances interacting with or disrupting caveolae. Thus, future studies of a more detailed involvement of the interactions between caveolae and HO-1 in endothelial cells could be tested.

This dissertation provided novel data which suggested EGCG-induced up-regulation of Nrf2/HO-1 might be associated with EGCG-induced caveolin-1 translocation. But the mechanism is not clear. The hypothesis is that EGCG-induced caveolin-1 displacement may cause activation of eNOS, which further activates Nrf2. To further demonstrate this hypothesis, eNOS, p-eNOS protein expressions, eNOS activities, and NO production should be measured. An eNOS inhibitor could be used to demonstrate the role of NO on Nrf2 activation by EGCG. Furthermore, the protein interactions between caveolin-1 and eNOS in the presence or absence of EGCG should also be tested by immunoprecipitation or immunocytochemistry. Moreover, besides eNOS, it is possible that some other caveolae bound signaling proteins (e.g. H-ras, src-family tyrosine kinases) also participate in the caveolae-associated induction of Nrf2/HO-1. It will be interesting to know which caveolae co-localized molecules are involved in the mechanisms underlying the protection effects of EGCG against endothelial inflammation.

After investigating the molecular and cellular mechanisms by which up-regulation of HO-1 may protect against endothelial dysfunction in cultured cells and how flavonoids protect against endothelial dysfunction, studies *in vivo* are needed to confirm this hypothesis. These studies will allow us to determine if HO-1 is necessary for the protective actions of flavonoids against vascular oxidative stress and inflammation, and

whether the crosstalk between caveolae and Nrf2/HO-1 as influenced by EGCG helps to prevent against atherosclerosis *in vivo*. One might hypothesize that flavonoids, such as EGCG, could ameliorate ApoE knockout-induced endothelial dysfunction partially via up-regulation of HO-1. ApoE knockout mice, ApoE-/-/caveolin-1-/- and ApoE-/-/HO-1-/- double knockout mice could be used in future studies and treated with flavonoids or vehicle. Endothelial functions (such as endothelium dependent vasodilation, Ca^{2+} dependent NOS enzyme activity, cGMP and cAMP levels), atherosclerosis lesion areas, oxidative stress levels, and phase II anti-oxidant enzyme activities could be determined throughout these studies.

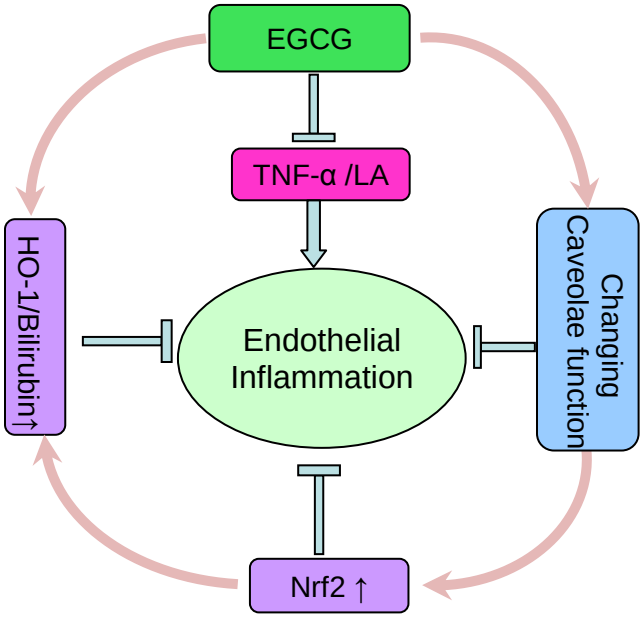

Figure 5-1 Crosstalk between the caveolae and Nrf2/HO-1/bilirubin pathways during EGCG-mediated protection against endothelial inflammation

Appendix Methods

❖ 1. Primary culture of porcine pulmonary artery and aortic endothelial cells

Porcine pulmonary arteries and aortas are obtained from University of Kentucky Agriculture Department.

Cell culture growth media
M199 – total 400 ml
Penn-Strep – 4 ml
L-glutamine – 4 ml
Amino Acids – 4 ml
Fetal Bovine Serum (FBS) – 40 ml (10%)

Hanks
Water – 2 L autoclaved/filtered
KCL – 0.8 g
KH_2PO_4 – 0.12 g
NaCl – 16 g
$NaHCO_3$ – 0.7 g
Na_2HPO_4 * 7 H_2O – 0.18 g
D-glucose – 2 g
Phenol Red – 0.02 g

Reviving the cells (Cells are frozen in high concentrations of DMSO, so it must be thawed in media immediately)

1. Fill 10 ml of 37 ℃ M199 medium (10% FBS) in a 15 ml tube.
2. Take 1 ml of the M199 media and add to cryovial containing the frozen cells, pipette repeatedly and once some of the cells thaw, transfer to the 15 ml tube. Continue until all thawed cells are in suspension.
3. Centrifuge (5 min at 2,500 rpm at 37 ℃)
4. Discard supernatant, resuspend in fresh media, and repeat centrifugation
5. Seed it to T75 flask
6. Change media after 24 hours

Freezing the cells

Cocktail:
FBS - 2 ml
M199 10% FBS – 13 ml
28% DMSO (1.75 ml DMSO + 4.5 ml M199) – 5 ml

1. Trypsinize Cells
2. Spin cells in 10ml of media in 15 ml tube, and centrifuge (2,500 rpm for 5 min)
3. Resuspend pellet in 2 ml freezing cocktail
4. Split into 2 cyrovials (1 ml each)
5. Cover with white foam

6. -20 °C overnight
7. -80 °C overnight
8. Put it into liquid nitrogen

❖ **2. Protein isolation**

a) Whole cell extraction
Lysis buffer (100 ml)
Tris-HCl – 1 M 2 ml (pH ~7.4, final concentration: 20 mM,)
Tris + H_2O up to 50 ml, stir, add HCl (2M), adjust pH, add rest of H_2O
NaCl – 1 M 15 ml (Final concentration150 mM)
Triton X-100 – 10% (v/v) 5 ml (final 0.5% liq)
PMSF – 10 mg/ml (DMSO/EtOH) 1 ml (final 0.1 mg/ml)
NP-40 500 µl (final 0.5% liq)
EDTA (0.1 M) 1 ml (final 1 mM)
Leupeptin (1 mg/ml) DMSO 250 µl (final 2.5 µg/ml)
Pepstatin (1 mg/ml) DMSO 1 ml (final 10 µg/ml)
Na_3VO_4 (sodium orthovanadate) 500 µl (final 1 mM)
Protease inhibitor 200 mM

Make lysis buffer (-) then add proteinase inhibitors just before using lysis buffer

Isolate proteins
1) Wash the cells with ice-cold PBS for 3 times
2) Add 3ml of ice-cold PBS and scrape the cells. Transfer to conical tube
3) Add another 3ml PBS to the plate and collect the remaining cells
4) Pellet it down at 2,500 rpm for 10 min at 4 ℃
5) Pour off supernatant
6) Add 200-300 µl of lysis buffer and vortex it for 30 sec and put on ice for 2 min (5-8 times repeat)
7) Keep on ice and incubate for at least 20 min
8) Vortex and then centrifuge at 12,000 rpm for 15 min at 4 ºC
9) Take the supernatant out and make aliquots of it
10) Freeze in dry ice then store at -80 ºC

Whole cell extraction by sonication
Lysis buffer
Water
Tris HCl (pH 7.5) 20 mM
NaCl 150 mM
EDTA 1 mM
EGTA 1 mM
Triton X-100 1%
Sodium pyrophosphate 2.5 mM
β-glycerophosphate 1 mM

To add just before:
Na_3VO_4 1 mM
Leupeptin 1 µg/ml
PMSF 1 mM

1) Remove media and rinse with cold PBS 2 times
2) Scrape cells in PBS into centrifuge tube on ice
3) Centrifuge 2,500 rpm 10 min at 4 ℃
4) Remove all PBS
5) Add protease inhibitors to lysis buffer
6) Add 100 µl to pellet, put in eppendorf tube
7) Sonicate samples on ice 3x for 5 seconds at 65%/sec
8) Microcentrifuge for 10 min at 14,000 rpm at 4 ℃
9) Transfer supernatant to new tube, store at -80 ℃

b) Nuclear extraction
Buffer A
Water – total 10 ml
HEPES 10 mM pH 7.9
KCl 10 mM
EDTA 0.1 mM
DTT 1 mM
PMSF 0.5 mM

Buffer B
HEPES 20 mM
NaCl 0.4 M
EDTA 1 mM
DTT 1 mM
PMSF 1 mM

1) Wash cells with ice cold PBS for 3 times
2) Add 3ml cold PBS, scrape the cells
3) Centrifuge 10 min at 13,000 rpm at 4 ℃
4) Resuspend in 400 µl of buffer A
5) 15 min incubation on ice
6) Add 25 µl 10% NP-40
7) Wait 5 min until cells are lysed
8) Centrifuge 14,000 rpm for 1 min
9) Resuspend with 25-50 µl of buffer B
10) Nuclei lysed by shaking vigorously at 4 ℃ for 5 min
11) Collect clear supernatant, store at -80 ℃

❖ **3. Western blot**

Preparing sample

1) Dilute sample with water to 1 µg/µl
2) Add 5 µl of sample loading buffer (contains 400 µl 2x SSB and 100 µl DTT, 1 M)
3) Boil at 100 ºC for 6 min
4) Put on ice until loading
5) Load 6 µl of the marker and 24 µl of sample

Preparing the gel

1. Separation gel

1) Prepare or 10 ml/gel
2) Prevent apparatus from leaking
3) After pouring, cover with ethanol ~20 min to solidify
4) Rinse with H_2O for 3 times

2. Stacking gel

1) Put into less than 5 ml/gel
2) Fill up to glass, then insert comb (~1 cm between comb and separation gel)
3) 30 min to solidify

Running the gel

1) Prepare the running buffer (900 ml H_2O + 100 ml of 10x buffer)
2) Put the gel in
3) Load the samples (including marker)
4) Run at 80V until samples get to resolving buffer and then 100 V for 1 to 2 h

Transfer onto membrane

1) Prepare: sponges, holders, ice cold transfer buffer
2) Cut the filter paper and membrane – slightly smaller than the sponges
3) Add transfer buffer in the dish
4) Put into **a.** sponge **b.** filter paper **c.** gel (cut off stacking, cut side to tell orientation) **d.** membrane **e.** filter paper **f.** sponge (roll with tube)
5) Put in apparatus
6) Add buffer
7) Add the box with ice and cover with the whole thing
8) Run at 340mV for 90min

Blocking with milk

1) Prepare 5% (g/100ml) milk in TBST
2) Put membrane in the milk/TBST
3) Shake 30-60 min at room temperature
4) Rinse with TBST
5) Shake in TBST for 5min, 3 times

Binding of the primary antibody
1) Add certain amount of primary antibody in 5% milk/TBST, 2 h incubation at RT or 4 ° C overnight
2) Wash 3x 10 min with TBST

Binding of the secondary antibody
1) Put the secondary antibody into 5% milk/ TBST
2) Shake for 1 h at RT
3) Wash 4 x 15 min with TBST

Visualization
1) Discard the TBST
2) Mix ECL WB Detection Reagents (Amersham Biosci)
3) 1.5 ml of reagent A + 1.5 ml of reagent B
4) Distribute the mixture evenly by pipette on the membrane
5) Let sit for 1-2 minute
6) Dry the membrane gently on a kim wipe
7) Put the membrane into the plastic case in the cassette
8) Put the film inside (Amersham- Hyperfilm)
9) Exposure (time dependent on the signal)
10) Develop the film

❖ **4. Monocyte cell (THP-1) adhesion**

Growth media
RPMI – total 400 ml (Gibco 11875 with L-glutamine)
Glucose (2.5 g/L) – 4 ml of 0.25 g/ml stock
HEPES (10 mM) – 4 ml of 1 M stock
Sodium Pyruvate (1 mM) – 4 ml of 100 mM stock
Penn-Strep (1%) – 4 ml of 100x stock
FBS – 10%
β-mercaptoethanol (50 μM) – 1.75 μl of 14.3 M stock

1) Treat cells 6-8 hrs with compound, TNF-α as a positive control (10 ng/ml)
2) Activate THP-1 with TNF-α (10 ng/ml) for 10 min (wash 3 times)
3) Count 50,000 monocyte cells/well
4) Resuspend in 1.0 ml 1% media
5) Load cells with calcein (3 μl/ml)
6) Incubate 37 ºC for 15 min
7) Spin to get rid of excess calcein, wash with media
8) Spin again, resuspend in 50 μl/well media
9) Aspirate off endothelial treatment media, add 1ml media
10) Add 50 μl monocytes to each well
11) Incubate 37 ℃ for 30 min
12) Gently wash unbound monocytes off with 1% media
13) Add 200 μl 1% Glutaraldehyde (made with PBS containing Ca and Mg)
14) Room Temp for 30 min

15) Wash plate w/ PBS
16) Count using confocal microscope

❖ **5. RNA isolation**

1) Discard treatment media; add 800 µl Trizol to cells
2) Scrape into 1.5 ml microcentrifuge tube, incubate 5 min at RT
3) Add 0.2 ml chloroform, shake tubes vigorously by hand for 15 seconds and incubate them at RT for 5 min
4) Centrifuge at 12,000 g for 15 min at 4 ℃
5) Transfer aqueous phase to a fresh tube
6) Add 0.5 ml isopropyl alcohol to precipitate RNA, incubate samples at RT for 10 min
7) Centrifuge at 12000 g for 10 min at 4 ℃ and then remove supernatant
8) Add 1 ml 75% ethanol to pellet, vortex, and centrifuge at 7,500 g for 5 min
9) Remove ethanol, air dry
10) Dissolve RNA in RNase- free water (~20 µl), Boil at water bath 55 °C for 10 min
11) Store at - 80 ℃

❖ **6. Chemiluminescent EMSA** (LightShift® Chemiluminescent EMSA Kit 20148, **protocol is adapted from Pierce Web Site**)

a). Anneal oligos

1. Spin down the lyophilized oligos and resuspend in TE buffer.
2. Dilute each oligo to 1 pmol/µl in TE.
3. Mix 1 µl of each forward and reverse oligo in 48 µl of TE to a final concentration of 20 fmol/µl.
4. Incubate the oligos at 95 ℃ for 2 min. Switch off the heat block and let it cool down to room temperature.
5. Keep oligos on ice and store at -20 ℃.

b). Prepare and pre-run gel

5X TBE pH 8.3
450 mM Tris
450 mM boric acid
10 mM EDTA

1. Prepare a 4 -6% polyacrylamide gel in 0.5X TBE.
2. Fill with 0.5X TBE to just above the bottom of the wells. Flush wells and pre-electrophorese the gel for 30-60 minutes. Apply 100 V for an 8 x 8 x 0.1 cm gel.
3. Pre-electophoresing.

c). Binding reaction

1. Thaw all binding reaction components, and place them on ice. Avoid excessive warming of DNA probes.
2. Prepare complete sets of 20 µl binding reactions for the Control EBNA System and/or the Test System (Table 1).
3. Incubate binding reactions at room temperature for 20 minutes.

4. Add 5 µl of 5X Loading buffer to each 20 µl binding reaction, pipetting up and down several times to mix. Do not vortex or mix vigorously.

d). Electrophorese binding reactions

1. Switch off current to the electrophoresis gel.
2. Flush the wells and then load 20 µl of each sample onto the polyacrylamide gel.
3. Switch on current (set to 100 V for 8 x 8 x 0.1 cm gel) and electrophorese samples until the bromophenol blue dye has migrated approximately 3/4 down the length of the gel.

e). Electrophoretic transfer of binding reactions to nylon membrane

1. Soak nylon membrane in 0.5X TBE for at least 10 min.
2. Sandwich the gel and nylon membrane in a clean electrophoretic transfer unit. Use 0.5X TBE cooled to ~10 ℃ with a circulating water bath. Use very clean forceps and powder-free gloves, and handle the membrane only at the corners.
3. Transfer at 380 mA (~100V) for 30 min. Typical transfer times are 30-60 min at 380 mA using a standard tank transfer apparatus for mini gels (8 x 8 x 0.1 cm).
4. When the transfer is complete, place the membrane with the bromophenol blue side up on a dry paper towel. Allow buffer on the membrane surface to absorb into the membrane. This will only take a min. Do not let the membrane dry.

f). Cross-link transferred DNA to membrane

Cross-link at 120 mJ/cm2, use a commercial UV-light cross-linker instrument equipped with 254 nm bulbs (45-60 second exposure using the auto cross-link function).

g). Detect biotin-labeled DNA by chemiluminescence

1. Warm the Blocking Buffer and the 4X Wash Buffer to 37-50 ℃ in a water bath.
2. Block the membrane by adding 20 ml of Blocking Buffer and incubate for 15 min with gentle shaking.
3. Prepare conjugate/blocking buffer solution by adding 66.7 µl Stabilized Streptavidin-Horseradish Peroxidase Conjugate to 20 ml Blocking Buffer (1:300 dilution).
4. Decant blocking buffer and replace it with the conjugate/blocking solution. Incubate membrane in the conjugate/blocking buffer solution for 15 min with gentle shaking.
5. Prepare 1X wash solution by adding 40 ml of 4X Wash Buffer to 120 ml water.
6. Transfer membrane to a new container and rinse it with 20 ml wash solution.
7. Wash membrane 4X 5 min each in 20 ml of wash solution with gentle shaking.
8. Transfer membrane to a new container and add 30 ml of Substrate Equilibration Buffer. Incubate membrane for 5 min with gentle shaking.
9. Prepare Substrate Working Solution by adding 6 ml Luminol/Enhancer Solution to 6 ml Stable Peroxide Solution.
10. Remove membrane from the Substrate Equilibration Buffer, carefully blotting an edge of the membrane on a paper towel to remove excess buffer.
11. Pour the Substrate Working Solution onto the membrane so that it completely covers the surface. Incubate membrane in the substrate solution for 5 minutes without shaking.
12. Remove membrane from the Working Solution and blot an edge of the membrane on a paper towel for 2-5 seconds to remove excess buffer.

13. Wrap the moist membrane in plastic wrap.
14. Place the membrane in a film cassette and expose to X-ray film for 2-5 min. Develop the film according to manufacturer's instructions.

Table 1	**Final Amount**
dH2O	
10x Bing Buffer	1X
1μg/μl Poly (dI dC)	50 ng/μl
50% Glycerol	2.50%
1% NP-40	0.05%
100 mM MgCl2	5 mM
Super Sift Antibody	5 μl
Unlabeled Target DNA	4 pmol
Protein Extract	3 μg
Biotin End-labeled Target DNA	20 fmol
Unlabeled EBNA DNA	4 pmol
EBNA Extract	1 Unit
Biotin-EBNA Control DNA	20 pmol

❖ 7. Preparation of fatty acid enriched media

a). Prepare fatty acids stock solution

1. The pure fatty acids are purchased from NuCheck.

2. Get fatty acid from the commercial container, record the weight of fatty acid (X mg), and the molecular weight (MW). Make a 5 ml solution in graduated flask and then calculate the final concentration:

$$N1\ (mM) = \frac{X\ mg \times 1000}{5\ ml \times MW}$$

3. Fill the graduated flask with gas N2 and seal with parafilm.

b). Prepare fatty acids enriched media

1. Calculate how much fatty acid solution in hexane we will need. Suppose the final concentration of fatty acid in media (10 ml) is 20 μM

$V1N1=V2N2$

$X \times N1 = 10 \times 0.02$

$$X\ (\mu l) = \frac{10 \times 0.02}{N1}$$

2. Take a glass tube put NaOH 30 times exceed of fatty acid concentration. Volume of 6 N NaOH:

$$V3 = \frac{FA\ conc. \times 30 \times V_2}{6}$$

Put NaOH (V3) in a culture glass tube, and then add FA (V1).
Carefully blow in the gas N2 for 15 min to 30 min until it totally dried.

3. Wash out fatty acid from glass tubes into the media (V2).
Adjust pH of the media to 7.4 with HCl.

4. Filtration through the syringe filter or bottle filters (0.02 μm), then add to the cells.

❖ 8. Superoxide measurement

1. Grow cells in microplates (24 or 48) well plates. Include a control groups to be used as negative and positive controls.
2. Wash cells at least three times with Hanks buffer to remove serum.
3. Add HEPES containing DHE (20 μmol/L) to microplates. Use 1ml for 12 wells and 0.5 ml for 24 wells and incubate for 30 min. Blank well receives HEPES buffer without DHE probe. Wrap plate in aluminum foil to protect from the light.
4. Wash 2 to 3 times with HEPES buffer.
5. Set plate reader excitation and emission wavelengths 490 / 605 nm.

Light sensitive reactions: protect stocks from light by wrapping in aluminum foil when adding DHE media on cells, switch off the light in the hood.

HEPES buffer (500ml pH 7.4):
NaCl: 4.237g
KCl: 186.4mg
MgCl2: 50.8mg
D-glucose: 901.0mg
HEPES: 1.1915g
CaCl2: 110.25mg

❖ 9. Detergent-free purification of caveolin enriched membrane fractions (Sucrose density gradient)

1. Cells were plated in 150 mm X 25mm plate/treatment. Cells were washed with PBS and lysed with 2 ml of ice-cold MES-buffered saline (MBS; 25mM MES (morpholineethanesulfonic acid, pH 6.5), 150 mM NaCl) containing 500 mM Na_2CO_3.

25 mM MES: 2.4405 g in 500 ml water, adjust PH to 6.5.
Then add 4.383g NaCl into MES, achieve MBS.
Take 50 ml MBS, add 2.65g Na_2CO_3, get 500mM. This is lysis buffer.
90% Sucrose: 45g sucrose and make final volume 50 ml. Heat in water bath to dissolve.
35% Sucrose: 17.5 g sucrose and final volume 50 ml.
5% sucrose: 2.5 g sucrose and final volume 50 ml.

2. Homogenization was carried out with 10 strokes of a loose-fitting Dounce homogenizer. After homogenized, get samples into 15ml cornival tubes for sonication
3. After sonication, centrifuge at 3,000 rpm for 10 min at 4 ℃.
4. Collect supernatant into centrifuge tubes (14 x 89mm), if it's not exactly 2 ml, add more MBS to reach 2 ml.
5. The homogenate was adjusted to 45% sucrose by addition of 2 ml of 50ml 90% sucrose prepared in MBS and placed at the bottom of an ultracentrifuge tube, and overlaid with a discontinuous sucrose gradient.
Add 2 ml 90% sucrose to make the final 45% sucrose, add into tube slowly against walls, then vortex to mix well.
6. Add 5 ml of 35% sucrose. Use 1 ml pipette five times.
7. Add 3 ml of 5% sucrose. Then the tube is pretty full.
8. Measure weight of the tubes, put the one with more volume first on the scale, set to 0. Then put the less volume ones. Achieve 0 with 5% sucrose.
9. Get the rotor (SW 41) from 4 ℃. Centrifuge 39,000 rpm for 18 h.
10. The second day morning, collect fractions into 1.5ml clear tubes. Label 1 to 12, can see a white fraction which is Cav-1 enriched faction. Sometime the 12th fraction has a little bit less or more volume.
11. Run the gel. 15 μl sample+ 3 μl 6x loading buffer.
Run the gel for 12 fractions on each western blot gel.

❖ 10. Small interfering RNA (siRNA)

1) To begin, siRNA concentrations must be determined.
2) Incubate the siRNA mix 5 min at RT
3) Add Gene Silencer mixture to each diluted siRNA
4) Incubate the Transfection Mix 5 min at RT
5) Wash cells 2 times with serum-free OptiMEM
6) Add serum-free OptiMEM to the Transfection Mix
7) Add 1ml of the mixture to each well
8) Add 1ml of 20% FBS OptiMEM to get final 10% FBS concentration 4 hours later
9) Treat the cells 48 hours later

❖ 11. Measurement of bilirubin production

1) All further manipulations were carried out in a dark room.
2) After incubation, 0.5 ml of each culture supernatant was collected and 250 mg $BaCl_2$. $2H_2O$ was added.
3) After vortexing, 0.75 ml benzene was added; then, tubes were vortexed vigorously leading to the formation of a relatively stable milky-white emulsion.
4) After centrifugation, the upper benzene layer was collected and the absorbance was measured at 450 nm with a reference wavelength at 600 nm using a SpectraMaxPro M2 spectrophotometer (Molecular Devices, Sunnyvale, CA).
5) In a separate tube, 0.5 ml of fresh culture medium was processed in the same way and the benzene layer was collected and used as a blank.

6) 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} $=27.3mM^{-1}cm^{-1}$.

❖ 12. MCP-1 determination by ELISA

a) Cells were seeded in 24 or 48 well microplates and grown to confluence.

b) After treatment, supernatants of cell cultures were collected into microcentrifuge tubes (Isc BioExpress, UT), centrifuged at 4 ℃ to remove cellular debris and then stored at -80 ℃.

c) MCP-1 levels were assessed using a MCP-1-specific enzyme immunoassay [190] (BD Biosciences, CA) following the manufacturer's protocol (**adapted from BD web site**, Cat. No. 559017):

1. Add 100 µl standard or sample to each well.
Incubate 2 hours at room temperature.
2. Aspirate and wash 5 times.
3. Add 100 µl prepared Working Detector to each well.
Incubate 1 hour at room temperature.
4. Aspirate and wash/soak 7 times.
5. Add 100 µl Substrate Solution to each well.
Incubate 30 minutes at room temperature.
6. Add 50 µl Stop Solution to each well.
7. A microplate spectrophotometer SpectraMaxPro M2 (Molecular Devices Corporation, CA) was used to read the plate at 450 nm. λ correction 570 nm.

❖ 13. Transfection of antisense oligodeoxynucleotides into porcine endothelial cells

1) The sequences of the phosphorothioate double-stranded antisense oligodeoxynucleotides (ODNs) against the AP-1 binding site used in this study was AP-1 decoy ODN, 5'-AGCTTGTGAGTCAGAAGCT-3'and AP-1 mismatched ODN, 5'-AGCTTGAATCTCAGAAGCT-3'.
2) The double-stranded ODNs were prepared from complementary single-stranded phosphorothiolate-bonded oligonucleotides.
3) The ODNs were annealed for 1 h, while the temperature descended from 80 to 25 ℃.
4) DNA derived from endothelial cells was precomplexed with the PLUS reagents (Life Technologies, Rockville, MD) at room temperature for 15 min.
5) The pre-complexed DNA was combined with diluted LipofectAMINE reagent (Life Technologies), mixed and incubated for 15 min at room temperature.
6) While complexes were forming, medium with serum-free transfection medium were replaced.
7) Then DNAPLUS Lipofect AMINE reagent complex was added to each well containing fresh medium, and cells were incubated for 5 h.
8) After incubation, complete medium with serum was added.
9) After transfection for 48 h, cell extracts were prepared for Real-time PCR analysis.

❖ 14. Extraction of EGCG from cultured cells for LC/MS/MS analysis

1) After removing the media, cells were washed in cold PBS twice.
2) Cells were scraped in 2 ml cold PBS containing 100 µl 20% ascorbic acid solution and 50 pmol internal standards (ethyl gallate).
3) Add cell suspension or aqueous samples up to 2ml volume to 4 ml Ethyl Acetate in 8 ml glass tube.
4) After 10 min vortex mixing, cells were centrifuged at 3000 rpm, 10 min, 25 ℃.
5) Remove upper organic phase to 4ml vial, evaporate to dryness under a gentle N_2 stream in a water bath.

❖ 15. Quantification of EGCG by LC/MS/MS analysis

1) After drying the extracts under nitrogen, they were reconstituted with 50: 50 water: acetonitrile and injected into LC/MS/MS.
2) LC/MS/MS analyses of polyphenols were performed using an ABI 4000-Qtrap hybrid linear ion trap triple quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode.
3) Polyphenols were separated using an Eclipse XDB C8, 5 µM, 4.6 X 150 mm (Agilent) column.
4) The mobile phase consisted of water with 0.1% formic acid as solvent A and acetonitrile with 0.1 % formic acid as solvent B.
5) For the analysis of polyphenols the separation was achieved by starting with 0% solvent B, which was gradually increased to 100% over 10 min, and maintained it at 100% for the last 2 min.
6) The column was equilibrated to initial conditions in 3 min.
7) The flow rate was 0.5 ml/min with a column temperature of 30 ℃.
8) The sample injection volume was 10 µl.
9) The mass spectrometer was operated in the negative electrospray ionization mode with optimal ion source settings determined by synthetic standards of EGCG with a declustering potential of -40 V, entrance potential of -10 V, collision energy of -18 V, collision cell exit potential of -17 V, curtain gas of 20 psi, ion spray voltage of -4500 V, ion source gas1/ gas2 of 40 psi and temperature of 550 ℃.
10) MRM transitions monitored were as follows: 457.1/168.9 and 457.1/124.9 for EGCG; 197.1/168.6 and 197.1/124.6 for ethyl gallate.
11) LC/MS/MS calibrations were obtained with R2 values ≥ 0.998.

❖ 16. Cignal Reporter Assay (Transfection for Dual-Luciferase Reporter Assay)

1) Prepare nucleic acid mixtures in appropriate ratios.
Experimental transfection (Cignal Reporter+ test nucleic acid);
Control transfection (a. Cignal Reporter+ negative control for test nucleic acid; b. Cignal Negative Control+test nucleic acid; c. Cignal Negative Control+negative control for text nucleic acid; d. Cignal Positive Control)
2) Dilute Sure FECT into Opti-MEM

3) Add diluted SureFECT to nucleic acid mixture, incubate at room temperature for 20 min
4) Trypsinize, count and suspend cells to appropriate density
5) Aliquot transfection complexes into wells, immediately seed cells to each well
6) Incubate 37° C for 16 h.
7) Change media to assay media (0.5% FBS)

❖ 17. Dual-Luciferase Reporter Assay

1) Prepare 1X PLB (passive lysis buffer, dilute 5X PLB), LAR II (Luciferase Assay reagent II), and Stop & Glo Reagent (mix 50X Substrate and Stop & Glo Buffer). Thaw the reagent and buffer at room temperature.
2) Remove the medium from the culture dishes and wash twice with PBS. Completely remove PBS before applying PLB.
3) Dispense into each culture well the following volume of 1X PLB.

6 well plate	500ul
12 well plate	250ul
24 well plate	100ul
96 well plate	20ul

4) Place the culture plates on a rocking platform at room temperature for 15 minutes. Meanwhile, place LAR II and Stop & Glo Reagent at room temperature.
5) Transfer the lysate to a tube or directly add 20 µl to a well of 96 well luciferase activity measuring plate.
6) Dispense 20 µl of lysate into luminometer plate.
7) Turn on the luminometer and prime both injectors with LAR II and Stop & Glo Reagent.
8) Program it to perform 2-second premeasurement delay, followed by a 10-second measurement period for each reporter assay.
9) Begin measurement.
10) Wash the injectors once with water and once with air.
11) Store the remaining reagent at -70°C up to 1 month.

References

1. Peterson, S.J., W.H. Frishman, and N.G. Abraham, *Targeting heme oxygenase: therapeutic implications for diseases of the cardiovascular system.* Cardiol Rev, 2009. **17**(3): p. 99-111.
2. Galbiati, F., B. Razani, and M.P. Lisanti, *Emerging themes in lipid rafts and caveolae.* Cell, 2001. **106**(4): p. 403-11.
3. Ross, J.A. and C.M. Kasum, *Dietary flavonoids: bioavailability, metabolic effects, and safety.* Annu Rev Nutr, 2002. **22**: p. 19-34.
4. Rahman, I., S.K. Biswas, and P.A. Kirkham, *Regulation of inflammation and redox signaling by dietary polyphenols.* Biochem Pharmacol, 2006. **72**(11): p. 1439-52.
5. Bravo, L., *Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance.* Nutr Rev, 1998. **56**(11): p. 317-33.
6. Kuhnau, J., *The flavonoids. A class of semi-essential food components: their role in human nutrition.* World Rev Nutr Diet, 1976. **24**: p. 117-91.
7. Dauchet, L., et al., *Fruit and vegetable consumption and risk of coronary heart disease: a meta-analysis of cohort studies.* J Nutr, 2006. **136**(10): p. 2588-93.
8. Hertog, M.G., et al., *Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study.* Lancet, 1993. **342**(8878): p. 1007-11.
9. Knekt, P., et al., *Flavonoid intake and coronary mortality in Finland: a cohort study.* BMJ, 1996. **312**(7029): p. 478-81.
10. Yochum, L., et al., *Dietary flavonoid intake and risk of cardiovascular disease in postmenopausal women.* Am J Epidemiol, 1999. **149**(10): p. 943-9.
11. Rimm, E.B., et al., *Relation between intake of flavonoids and risk for coronary heart disease in male health professionals.* Ann Intern Med, 1996. **125**(5): p. 384-9.
12. Moore, R.J., K.G. Jackson, and A.M. Minihane, *Green tea (Camellia sinensis) catechins and vascular function.* Br J Nutr, 2009. **102**(12): p. 1790-802.
13. Miyazawa, T., *Absorption, metabolism and antioxidative effects of tea catechin in humans.* Biofactors, 2000. **13**(1-4): p. 55-9.
14. Lu, H., et al., *Glucuronides of tea catechins: enzymology of biosynthesis and biological activities.* Drug Metab Dispos, 2003. **31**(4): p. 452-61.
15. Lambert, J.D., et al., *Synthesis and biological activity of the tea catechin metabolites, M4 and M6 and their methoxy-derivatives.* Bioorg Med Chem Lett, 2005. **15**(4): p. 873-6.
16. Imai, K. and K. Nakachi, *Cross sectional study of effects of drinking green tea on cardiovascular and liver diseases.* BMJ, 1995. **310**(6981): p. 693-6.
17. Mineharu, Y., et al., *Coffee, green tea, black tea and oolong tea consumption and risk of mortality from cardiovascular disease in Japanese men and women.* J Epidemiol Community Health, 2009.
18. Widlansky, M.E., et al., *Acute EGCG supplementation reverses endothelial dysfunction in patients with coronary artery disease.* J Am Coll Nutr, 2007. **26**(2): p. 95-102.

19. Kim, W., et al., *Effect of green tea consumption on endothelial function and circulating endothelial progenitor cells in chronic smokers.* Circ J, 2006. **70**(8): p. 1052-7.
20. Tinahones, F.J., et al., *Green tea reduces LDL oxidability and improves vascular function.* J Am Coll Nutr, 2008. **27**(2): p. 209-13.
21. Lee, W., et al., *Long-term effects of green tea ingestion on atherosclerotic biological markers in smokers.* Clin Biochem, 2005. **38**(1): p. 84-7.
22. Nanjo, F., et al., *Radical scavenging activity of tea catechins and their related compounds.* Biosci Biotechnol Biochem, 1999. **63**(9): p. 1621-3.
23. Paquay, J.B., et al., *Protection against nitric oxide toxicity by tea.* J Agric Food Chem, 2000. **48**(11): p. 5768-72.
24. Ludwig, A., et al., *The tea flavonoid epigallocatechin-3-gallate reduces cytokine-induced VCAM-1 expression and monocyte adhesion to endothelial cells.* Biochem Biophys Res Commun, 2004. **316**(3): p. 659-65.
25. Koga, T. and M. Meydani, *Effect of plasma metabolites of (+)-catechin and quercetin on monocyte adhesion to human aortic endothelial cells.* Am J Clin Nutr, 2001. **73**(5): p. 941-8.
26. Yamakuchi, M., et al., *Epigallocatechin gallate inhibits endothelial exocytosis.* Biol Chem, 2008. **389**(7): p. 935-41.
27. Schewe, T., Y. Steffen, and H. Sies, *How do dietary flavanols improve vascular function? A position paper.* Arch Biochem Biophys, 2008. **476**(2): p. 102-6.
28. Mizugaki, M., et al., *Epigallocatechin gallate increase the prostacyclin production of bovine aortic endothelial cells.* Prostaglandins Other Lipid Mediat, 2000. **62**(2): p. 157-64.
29. Tang, W.J., et al., *Epigallocatechin gallate preserves endothelial function by reducing the endogenous nitric oxide synthase inhibitor level.* Can J Physiol Pharmacol, 2006. **84**(2): p. 163-71.
30. Actis-Goretta, L., J.I. Ottaviani, and C.G. Fraga, *Inhibition of angiotensin converting enzyme activity by flavanol-rich foods.* J Agric Food Chem, 2006. **54**(1): p. 229-34.
31. Sima, A.V., C.S. Stancu, and M. Simionescu, *Vascular endothelium in atherosclerosis.* Cell Tissue Res, 2009. **335**(1): p. 191-203.
32. Libby, P., M. Aikawa, and M.K. Jain, *Vascular endothelium and atherosclerosis.* Handb Exp Pharmacol, 2006(176 Pt 2): p. 285-306.
33. Simionescu, M. and F. Antohe, *Functional ultrastructure of the vascular endothelium: changes in various pathologies.* Handb Exp Pharmacol, 2006(176 Pt 1): p. 41-69.
34. Mehta, D. and A.B. Malik, *Signaling mechanisms regulating endothelial permeability.* Physiol Rev, 2006. **86**(1): p. 279-367.
35. Papaharalambus, C.A. and K.K. Griendling, *Basic mechanisms of oxidative stress and reactive oxygen species in cardiovascular injury.* Trends Cardiovasc Med, 2007. **17**(2): p. 48-54.
36. Pennathur, S. and J.W. Heinecke, *Oxidative stress and endothelial dysfunction in vascular disease.* Curr Diab Rep, 2007. **7**(4): p. 257-64.

37. Manea, A., et al., *AP-1-dependent transcriptional regulation of NADPH oxidase in human aortic smooth muscle cells: role of p22phox subunit.* Arterioscler Thromb Vasc Biol, 2008. **28**(5): p. 878-85.
38. Jones, S.P. and R. Bolli, *The ubiquitous role of nitric oxide in cardioprotection.* J Mol Cell Cardiol, 2006. **40**(1): p. 16-23.
39. Braam, B. and M.C. Verhaar, *Understanding eNOS for pharmacological modulation of endothelial function: a translational view.* Curr Pharm Des, 2007. **13**(17): p. 1727-40.
40. Zheng, X.Y., L. Liu, and D.G. Yang, *Interaction between remnant-like lipoprotein particles and adipocytes.* Int J Cardiol, 2009. **133**(1): p. 3-7.
41. Chen, X.P., T.T. Zhang, and G.H. Du, *Lectin-like oxidized low-density lipoprotein receptor-1, a new promising target for the therapy of atherosclerosis?* Cardiovasc Drug Rev, 2007. **25**(2): p. 146-61.
42. Constantinescu, E., et al., *Endothelial cell-derived foam cells fail to express adhesion molecules (ICAM-1 and VCAM-1) for monocytes.* J Submicrosc Cytol Pathol, 2000. **32**(2): p. 195-201.
43. Simionescu, M., *Implications of early structural-functional changes in the endothelium for vascular disease.* Arterioscler Thromb Vasc Biol, 2007. **27**(2): p. 266-74.
44. Glass, C.K. and J.L. Witztum, *Atherosclerosis. the road ahead.* Cell, 2001. **104**(4): p. 503-16.
45. Lusis, A.J., *Atherosclerosis.* Nature, 2000. **407**(6801): p. 233-41.
46. Rahman, A. and K. Sward, *The role of caveolin-1 in cardiovascular regulation.* Acta Physiol (Oxf), 2009. **195**(2): p. 231-45.
47. Scherer, P.E., et al., *Identification, sequence, and expression of caveolin-2 defines a caveolin gene family.* Proc Natl Acad Sci U S A, 1996. **93**(1): p. 131-5.
48. Scherer, P.E., et al., *Cell-type and tissue-specific expression of caveolin-2. Caveolins 1 and 2 co-localize and form a stable hetero-oligomeric complex in vivo.* J Biol Chem, 1997. **272**(46): p. 29337-46.
49. Tang, Z., et al., *Molecular cloning of caveolin-3, a novel member of the caveolin gene family expressed predominantly in muscle.* J Biol Chem, 1996. **271**(4): p. 2255-61.
50. Schubert, W., et al., *Microvascular hyperpermeability in caveolin-1 (-/-) knock-out mice. Treatment with a specific nitric-oxide synthase inhibitor, L-NAME, restores normal microvascular permeability in Cav-1 null mice.* J Biol Chem, 2002. **277**(42): p. 40091-8.
51. Cohen, A.W., et al., *Caveolin-1 null mice develop cardiac hypertrophy with hyperactivation of p42/44 MAP kinase in cardiac fibroblasts.* Am J Physiol Cell Physiol, 2003. **284**(2): p. C457-74.
52. Zhao, Y.Y., et al., *Defects in caveolin-1 cause dilated cardiomyopathy and pulmonary hypertension in knockout mice.* Proc Natl Acad Sci U S A, 2002. **99**(17): p. 11375-80.
53. Zhao, Y.Y., et al., *Persistent eNOS activation secondary to caveolin-1 deficiency induces pulmonary hypertension in mice and humans through PKG nitration.* J Clin Invest, 2009. **119**(7): p. 2009-18.

54. Gafencu, A., et al., *Protein and fatty acid composition of caveolae from apical plasmalemma of aortic endothelial cells.* Cell Tissue Res, 1998. **293**(1): p. 101-10.
55. Li, Q., et al., *Eicosapentaenoic acid modifies lipid composition in caveolae and induces translocation of endothelial nitric oxide synthase.* Biochimie, 2007. **89**(1): p. 169-77.
56. Ma, D.W., et al., *n-3 PUFA alter caveolae lipid composition and resident protein localization in mouse colon.* FASEB J, 2004. **18**(9): p. 1040-2.
57. Patel, H.H., et al., *Increased smooth muscle cell expression of caveolin-1 and caveolae contribute to the pathophysiology of idiopathic pulmonary arterial hypertension.* FASEB J, 2007. **21**(11): p. 2970-9.
58. Albinsson, S. and P. Hellstrand, *Integration of signal pathways for stretch-dependent growth and differentiation in vascular smooth muscle.* Am J Physiol Cell Physiol, 2007. **293**(2): p. C772-82.
59. Michel, J.B., et al., *Reciprocal regulation of endothelial nitric-oxide synthase by Ca2+-calmodulin and caveolin.* J Biol Chem, 1997. **272**(25): p. 15583-6.
60. Drab, M., et al., *Loss of caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice.* Science, 2001. **293**(5539): p. 2449-52.
61. Lisanti, M.P., et al., *Characterization of caveolin-rich membrane domains isolated from an endothelial-rich source: implications for human disease.* J Cell Biol, 1994. **126**(1): p. 111-26.
62. Hill, M.M., et al., *PTRF-Cavin, a conserved cytoplasmic protein required for caveola formation and function.* Cell, 2008. **132**(1): p. 113-24.
63. Oka, N., et al., *Caveolin interaction with protein kinase C. Isoenzyme-dependent regulation of kinase activity by the caveolin scaffolding domain peptide.* J Biol Chem, 1997. **272**(52): p. 33416-21.
64. Je, H.D., et al., *Caveolin-1 regulates contractility in differentiated vascular smooth muscle.* Am J Physiol Heart Circ Physiol, 2004. **286**(1): p. H91-8.
65. Shakirova, Y., et al., *Increased Rho activation and PKC-mediated smooth muscle contractility in the absence of caveolin-1.* Am J Physiol Cell Physiol, 2006. **291**(6): p. C1326-35.
66. Murata, T., et al., *Reexpression of caveolin-1 in endothelium rescues the vascular, cardiac, and pulmonary defects in global caveolin-1 knockout mice.* J Exp Med, 2007. **204**(10): p. 2373-82.
67. Wunderlich, C., et al., *The adverse cardiopulmonary phenotype of caveolin-1 deficient mice is mediated by a dysfunctional endothelium.* J Mol Cell Cardiol, 2008. **44**(5): p. 938-47.
68. Ghitescu, L., et al., *Specific binding sites for albumin restricted to plasmalemmal vesicles of continuous capillary endothelium: receptor-mediated transcytosis.* J Cell Biol, 1986. **102**(4): p. 1304-11.
69. King, G.L. and S.M. Johnson, *Receptor-mediated transport of insulin across endothelial cells.* Science, 1985. **227**(4694): p. 1583-6.
70. Kim, M.J., J. Dawes, and W. Jessup, *Transendothelial transport of modified low-density lipoproteins.* Atherosclerosis, 1994. **108**(1): p. 5-17.
71. Ge, S., et al., *Transcellular transport of CCL2 across brain microvascular endothelial cells.* J Neurochem, 2008. **104**(5): p. 1219-32.

72. Frank, P.G., et al., *Caveolae and caveolin-1: novel potential targets for the treatment of cardiovascular disease.* Curr Pharm Des, 2007. **13**(17): p. 1761-9.
73. Frank, P.G., S. Pavlides, and M.P. Lisanti, *Caveolae and transcytosis in endothelial cells: role in atherosclerosis.* Cell Tissue Res, 2009. **335**(1): p. 41-7.
74. Pelkmans, L. and A. Helenius, *Endocytosis via caveolae.* Traffic, 2002. **3**(5): p. 311-20.
75. Conrad, P.A., et al., *Caveolin cycles between plasma membrane caveolae and the Golgi complex by microtubule-dependent and microtubule-independent steps.* J Cell Biol, 1995. **131**(6 Pt 1): p. 1421-33.
76. Matveev, S., et al., *The role of caveolae and caveolin in vesicle-dependent and vesicle-independent trafficking.* Adv Drug Deliv Rev, 2001. **49**(3): p. 237-50.
77. Zheng, Y., et al., *Role of caveolin-1 in EGCG-mediated protection against linoleic-acid-induced endothelial cell activation.* J Nutr Biochem, 2009. **20**(3): p. 202-9.
78. Tang, Y.B., et al., *Phytoestrogen genistein supplementation increases eNOS and decreases caveolin-1 expression in ovariectomized rat hearts.* Sheng Li Xue Bao, 2005. **57**(3): p. 373-8.
79. Choi, Y.J., et al., *Quercetin blocks caveolae-dependent pro-inflammatory responses induced by co-planar PCBs.* Environ Int, 2009.
80. Khan, N., et al., *Targeting multiple signaling pathways by green tea polyphenol (-)-epigallocatechin-3-gallate.* Cancer Res, 2006. **66**(5): p. 2500-5.
81. Jamieson, K.V., et al., *Crystal structure of the human laminin receptor precursor.* J Biol Chem, 2008. **283**(6): p. 3002-5.
82. Abraham, N.G., et al., *Heme oxygenase: the key to renal function regulation.* Am J Physiol Renal Physiol, 2009. **297**(5): p. F1137-52.
83. Abraham, N.G., et al., *Overexpression of human heme oxygenase-1 attenuates endothelial cell sloughing in experimental diabetes.* Am J Physiol Heart Circ Physiol, 2004. **287**(6): p. H2468-77.
84. Moqattash, S., et al., *Hemopoietic recovery from AZT toxicity with recombinant hemoglobin in a murine model of AIDS.* Acta Haematol, 1994. **92**(4): p. 182-6.
85. Kaide, J., et al., *Vascular CO counterbalances the sensitizing influence of 20-HETE on agonist-induced vasoconstriction.* Hypertension, 2004. **44**(2): p. 210-6.
86. Kwak, J.Y., et al., *Bilirubin inhibits the activation of superoxide-producing NADPH oxidase in a neutrophil cell-free system.* Biochim Biophys Acta, 1991. **1076**(3): p. 369-73.
87. Sedlak, T.W. and S.H. Snyder, *Bilirubin benefits: cellular protection by a biliverdin reductase antioxidant cycle.* Pediatrics, 2004. **113**(6): p. 1776-82.
88. Frei, B., R. Stocker, and B.N. Ames, *Antioxidant defenses and lipid peroxidation in human blood plasma.* Proc Natl Acad Sci U S A, 1988. **85**(24): p. 9748-52.
89. Hill-Kapturczak, N., S.H. Chang, and A. Agarwal, *Heme oxygenase and the kidney.* DNA Cell Biol, 2002. **21**(4): p. 307-21.
90. Perrella, M.A. and S.F. Yet, *Role of heme oxygenase-1 in cardiovascular function.* Curr Pharm Des, 2003. **9**(30): p. 2479-87.
91. Juan, S.H., et al., *Adenovirus-mediated heme oxygenase-1 gene transfer inhibits the development of atherosclerosis in apolipoprotein E-deficient mice.* Circulation, 2001. **104**(13): p. 1519-25.

92. Hashiba, T., et al., *Adenovirus-mediated transfer of heme oxygenase-1 cDNA attenuates severe lung injury induced by the influenza virus in mice.* Gene Ther, 2001. **8**(19): p. 1499-507.
93. Guo, Y., et al., *Administration of a CO-releasing molecule at the time of reperfusion reduces infarct size in vivo.* Am J Physiol Heart Circ Physiol, 2004. **286**(5): p. H1649-53.
94. Wang, L.J., et al., *Expression of heme oxygenase-1 in atherosclerotic lesions.* Am J Pathol, 1998. **152**(3): p. 711-20.
95. Agarwal, A., et al., *Renal tubular epithelial cells mimic endothelial cells upon exposure to oxidized LDL.* Am J Physiol, 1996. **271**(4 Pt 2): p. F814-23.
96. Ishikawa, K., et al., *Heme oxygenase-1 inhibits atherosclerotic lesion formation in ldl-receptor knockout mice.* Circ Res, 2001. **88**(5): p. 506-12.
97. Ishikawa, K., et al., *Heme oxygenase-1 inhibits atherogenesis in Watanabe heritable hyperlipidemic rabbits.* Circulation, 2001. **104**(15): p. 1831-6.
98. Yet, S.F., et al., *Absence of heme oxygenase-1 exacerbates atherosclerotic lesion formation and vascular remodeling.* FASEB J, 2003. **17**(12): p. 1759-61.
99. Kawamura, K., et al., *Bilirubin from heme oxygenase-1 attenuates vascular endothelial activation and dysfunction.* Arterioscler Thromb Vasc Biol, 2005. **25**(1): p. 155-60.
100. Otterbein, L.E., et al., *Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway.* Nat Med, 2000. **6**(4): p. 422-8.
101. Ndisang, J.F., H.E. Tabien, and R. Wang, *Carbon monoxide and hypertension.* J Hypertens, 2004. **22**(6): p. 1057-74.
102. Bloom, D.A. and A.K. Jaiswal, *Phosphorylation of Nrf2 at Ser40 by protein kinase C in response to antioxidants leads to the release of Nrf2 from INrf2, but is not required for Nrf2 stabilization/accumulation in the nucleus and transcriptional activation of antioxidant response element-mediated NAD(P)H:quinone oxidoreductase-1 gene expression.* J Biol Chem, 2003. **278**(45): p. 44675-82.
103. Nguyen, T., et al., *Increased protein stability as a mechanism that enhances Nrf2-mediated transcriptional activation of the antioxidant response element. Degradation of Nrf2 by the 26 S proteasome.* J Biol Chem, 2003. **278**(7): p. 4536-41.
104. Wu, C.C., et al., *Upregulation of heme oxygenase-1 by Epigallocatechin-3-gallate via the phosphatidylinositol 3-kinase/Akt and ERK pathways.* Life Sci, 2006. **78**(25): p. 2889-97.
105. Hakoshima, T. and K. Tomita, *Crystallization and preliminary X-ray investigation reveals that tumor necrosis factor is a compact trimer furnished with 3-fold symmetry.* J Mol Biol, 1988. **201**(2): p. 455-7.
106. Barath, P., et al., *Detection and localization of tumor necrosis factor in human atheroma.* Am J Cardiol, 1990. **65**(5): p. 297-302.
107. Bruunsgaard, H., *Physical activity and modulation of systemic low-level inflammation.* J Leukoc Biol, 2005. **78**(4): p. 819-35.
108. Zhang, H., et al., *Role of TNF-alpha in vascular dysfunction.* Clin Sci (Lond), 2009. **116**(3): p. 219-30.

109. Sorescu, D. and K.K. Griendling, *Reactive oxygen species, mitochondria, and NAD(P)H oxidases in the development and progression of heart failure.* Congest Heart Fail, 2002. **8**(3): p. 132-40.
110. Downey, J.M., et al., *Superoxide dismutase therapy for myocardial ischemia.* Free Radic Res Commun, 1991. **12-13 Pt 2**: p. 703-20.
111. Cai, H. and D.G. Harrison, *Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress.* Circ Res, 2000. **87**(10): p. 840-4.
112. Ye, J., et al., *Inhibition of TNF-alpha gene expression and bioactivity by site-specific transcription factor-binding oligonucleotides.* Am J Physiol Lung Cell Mol Physiol, 2003. **284**(2): p. L386-94.
113. Jovinge, S., et al., *Evidence for a role of tumor necrosis factor alpha in disturbances of triglyceride and glucose metabolism predisposing to coronary heart disease.* Metabolism, 1998. **47**(1): p. 113-8.
114. Sauer, L.A., R.T. Dauchy, and D.E. Blask, *Polyunsaturated fatty acids, melatonin, and cancer prevention.* Biochem Pharmacol, 2001. **61**(12): p. 1455-62.
115. Simopoulos, A.P., *Evolutionary aspects of diet, the omega-6/omega-3 ratio and genetic variation: nutritional implications for chronic diseases.* Biomed Pharmacother, 2006. **60**(9): p. 502-7.
116. Psota, T.L., S.K. Gebauer, and P. Kris-Etherton, *Dietary omega-3 fatty acid intake and cardiovascular risk.* Am J Cardiol, 2006. **98**(4A): p. 3i-18i.
117. Grimble, R.F., *Dietary lipids and the inflammatory response.* Proc Nutr Soc, 1998. **57**(4): p. 535-42.
118. Toborek, M., et al., *Linoleic acid and TNF-alpha cross-amplify oxidative injury and dysfunction of endothelial cells.* J Lipid Res, 1996. **37**(1): p. 123-35.
119. Davda, R.K., et al., *Oleic acid inhibits endothelial nitric oxide synthase by a protein kinase C-independent mechanism.* Hypertension, 1995. **26**(5): p. 764-70.
120. Hennig, B., M. Toborek, and C.J. McClain, *High-energy diets, fatty acids and endothelial cell function: implications for atherosclerosis.* J Am Coll Nutr, 2001. **20**(2 Suppl): p. 97-105.
121. Hennig, B., et al., *Linoleic acid induces proinflammatory events in vascular endothelial cells via activation of PI3K/Akt and ERK1/2 signaling.* J Nutr Biochem, 2006. **17**(11): p. 766-72.
122. Askari, B., et al., *Oleate and linoleate enhance the growth-promoting effects of insulin-like growth factor-I through a phospholipase D-dependent pathway in arterial smooth muscle cells.* J Biol Chem, 2002. **277**(39): p. 36338-44.
123. Ross, R., *Atherosclerosis--an inflammatory disease.* N Engl J Med, 1999. **340**(2): p. 115-26.
124. Kensler, T.W., N. Wakabayashi, and S. Biswal, *Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway.* Annu Rev Pharmacol Toxicol, 2007. **47**: p. 89-116.
125. Cipollone, F. and M.L. Fazia, *Cyclooxygenase-2 inhibition: vascular inflammation and cardiovascular risk.* Curr Atheroscler Rep, 2006. **8**(3): p. 245-51.
126. Sohn, H.Y. and F. Krotz, *Cyclooxygenase inhibition and atherothrombosis.* Curr Drug Targets, 2006. **7**(10): p. 1275-84.

127. Austin, M.A., et al., *Cardiovascular disease mortality in familial forms of hypertriglyceridemia: A 20-year prospective study.* Circulation, 2000. **101**(24): p. 2777-82.
128. Malloy, M.J. and J.P. Kane, *A risk factor for atherosclerosis: triglyceride-rich lipoproteins.* Adv Intern Med, 2001. **47**: p. 111-36.
129. De Caterina, R., J.K. Liao, and P. Libby, *Fatty acid modulation of endothelial activation.* Am J Clin Nutr, 2000. **71**(1 Suppl): p. 213S-23S.
130. Toborek, M., et al., *Linoleic acid potentiates TNF-mediated oxidative stress, disruption of calcium homeostasis, and apoptosis of cultured vascular endothelial cells.* J Lipid Res, 1997. **38**(10): p. 2155-67.
131. Meerarani, P., et al., *Zinc protects against apoptosis of endothelial cells induced by linoleic acid and tumor necrosis factor alpha.* Am J Clin Nutr, 2000. **71**(1): p. 81-7.
132. Horia, E. and B.A. Watkins, *Comparison of stearidonic acid and alpha-linolenic acid on PGE2 production and COX-2 protein levels in MDA-MB-231 breast cancer cell cultures.* J Nutr Biochem, 2005. **16**(3): p. 184-92.
133. Munoz-Espada, A.C. and B.A. Watkins, *Cyanidin attenuates PGE2 production and cyclooxygenase-2 expression in LNCaP human prostate cancer cells.* J Nutr Biochem, 2006. **17**(9): p. 589-96.
134. Kris-Etherton, P.M., et al., *Bioactive compounds in nutrition and health-research methodologies for establishing biological function: the antioxidant and anti-inflammatory effects of flavonoids on atherosclerosis.* Annu Rev Nutr, 2004. **24**: p. 511-38.
135. Manach, C., et al., *Polyphenols: food sources and bioavailability.* Am J Clin Nutr, 2004. **79**(5): p. 727-47.
136. Waddington, E., I.B. Puddey, and K.D. Croft, *Red wine polyphenolic compounds inhibit atherosclerosis in apolipoprotein E-deficient mice independently of effects on lipid peroxidation.* Am J Clin Nutr, 2004. **79**(1): p. 54-61.
137. Simons, K. and D. Toomre, *Lipid rafts and signal transduction.* Nat Rev Mol Cell Biol, 2000. **1**(1): p. 31-9.
138. Liu, J., et al., *Organized endothelial cell surface signal transduction in caveolae distinct from glycosylphosphatidylinositol-anchored protein microdomains.* J Biol Chem, 1997. **272**(11): p. 7211-22.
139. Razani, B., S.E. Woodman, and M.P. Lisanti, *Caveolae: from cell biology to animal physiology.* Pharmacol Rev, 2002. **54**(3): p. 431-67.
140. Frank, P.G., et al., *Caveolin, caveolae, and endothelial cell function.* Arterioscler Thromb Vasc Biol, 2003. **23**(7): p. 1161-8.
141. Hoelscher, D.M., et al., *Measuring the prevalence of overweight in Texas schoolchildren.* Am J Public Health, 2004. **94**(6): p. 1002-8.
142. Cha, S.H., et al., *Evidence for cyclooxygenase-1 association with caveolin-1 and -2 in cultured human embryonic kidney (HEK 293) cells.* IUBMB Life, 2004. **56**(4): p. 221-7.
143. Kwak, J.O., et al., *Evidence for cyclooxygenase-2 association with caveolin-3 in primary cultured rat chondrocytes.* J Korean Med Sci, 2006. **21**(1): p. 100-6.
144. Nabi, I.R. and P.U. Le, *Caveolae/raft-dependent endocytosis.* J Cell Biol, 2003. **161**(4): p. 673-7.

145. Pignatelli, P., et al., *Polyphenols enhance platelet nitric oxide by inhibiting protein kinase C-dependent NADPH oxidase activation: effect on platelet recruitment.* Faseb J, 2006. **20**(8): p. 1082-9.
146. Choi, Y.J., et al., *(-)Epigallocatechin gallate and quercetin enhance survival signaling in response to oxidant-induced human endothelial apoptosis.* J Nutr, 2005. **135**(4): p. 707-13.
147. Edgell, C.J., C.C. McDonald, and J.B. Graham, *Permanent cell line expressing human factor VIII-related antigen established by hybridization.* Proc Natl Acad Sci U S A, 1983. **80**(12): p. 3734-7.
148. Toborek, M., et al., *Measurement of inflammatory properties of fatty acids in human endothelial cells.* Methods Enzymol, 2002. **352**: p. 198-219.
149. Spector, A.A., *Lipids, hormones, and atherogenesis. The transport and utilization of free fatty acid.* Ann N Y Acad Sci, 1968. **149**(2): p. 768-83.
150. Repetto, S., et al., *Insulin and IGF-I phosphorylate eNOS in HUVECs by a caveolin-1 dependent mechanism.* Biochem Biophys Res Commun, 2005. **337**(3): p. 849-52.
151. Lim, E.J. and C.W. Kim, *Functional characterization of the promoter region of the chicken elongation factor-2 gene.* Gene, 2007. **386**(1-2): p. 183-90.
152. Sauzeau, V., et al., *RhoA expression is controlled by nitric oxide through cGMP-dependent protein kinase activation.* J Biol Chem, 2003. **278**(11): p. 9472-80.
153. Kilsdonk, E.P., et al., *Cellular cholesterol efflux mediated by cyclodextrins.* J Biol Chem, 1995. **270**(29): p. 17250-6.
154. Drexler, H., et al., *Endothelial function in congestive heart failure.* Am Heart J, 1993. **126**(3 Pt 2): p. 761-4.
155. Hennig, B., et al., *Disruption of endothelial barrier function by lipolytic remnants of triglyceride-rich lipoproteins.* Atherosclerosis, 1992. **95**(2-3): p. 235-47.
156. Moller, D.E. and K.D. Kaufman, *Metabolic syndrome: a clinical and molecular perspective.* Annu Rev Med, 2005. **56**: p. 45-62.
157. Toborek, M., et al., *Unsaturated fatty acids selectively induce an inflammatory environment in human endothelial cells.* Am J Clin Nutr, 2002. **75**(1): p. 119-25.
158. Yang, N., et al., *High-fat diet up-regulates caveolin-1 expression in aorta of diet-induced obese but not in diet-resistant rats.* Cardiovasc Res, 2007.
159. Jeong, Y.I., et al., *(-)-Epigallocatechin gallate suppresses indoleamine 2,3-dioxygenase expression in murine dendritic cells: evidences for the COX-2 and STAT1 as potential targets.* Biochem Biophys Res Commun, 2007. **354**(4): p. 1004-9.
160. Kundu, J.K., et al., *Inhibition of phorbol ester-induced COX-2 expression by epigallocatechin gallate in mouse skin and cultured human mammary epithelial cells.* J Nutr, 2003. **133**(11 Suppl 1): p. 3805S-3810S.
161. Park, J.S., et al., *Epigallocatechin-3-gallate inhibits the PDGF-induced VEGF expression in human vascular smooth muscle cells via blocking PDGF receptor and Erk-1/2.* Int J Oncol, 2006. **29**(5): p. 1247-52.
162. Adhikari, N., et al., *Transcription factor and kinase-mediated signaling in atherosclerosis and vascular injury.* Curr Atheroscler Rep, 2006. **8**(3): p. 252-60.

163. Peng, G., et al., *Green tea polyphenol (-)-epigallocatechin-3-gallate inhibits cyclooxygenase-2 expression in colon carcinogenesis.* Mol Carcinog, 2006. **45**(5): p. 309-19.
164. Sang, S., et al., *Stability of tea polyphenol (-)-epigallocatechin-3-gallate and formation of dimers and epimers under common experimental conditions.* J Agric Food Chem, 2005. **53**(24): p. 9478-84.
165. Blake, G.J. and P.M. Ridker, *Novel clinical markers of vascular wall inflammation.* Circ Res, 2001. **89**(9): p. 763-71.
166. Aggarwal, B.B., et al., *Human tumor necrosis factor. Production, purification, and characterization.* J Biol Chem, 1985. **260**(4): p. 2345-54.
167. Murao, K., et al., *TNF-alpha stimulation of MCP-1 expression is mediated by the Akt/PKB signal transduction pathway in vascular endothelial cells.* Biochem Biophys Res Commun, 2000. **276**(2): p. 791-6.
168. Seino, Y., et al., *Expression of monocyte chemoattractant protein-1 in vascular tissue.* Cytokine, 1995. **7**(6): p. 575-9.
169. Hansson, G.K., *Inflammation, atherosclerosis, and coronary artery disease.* N Engl J Med, 2005. **352**(16): p. 1685-95.
170. Yla-Herttuala, S., et al., *Expression of monocyte chemoattractant protein 1 in macrophage-rich areas of human and rabbit atherosclerotic lesions.* Proc Natl Acad Sci U S A, 1991. **88**(12): p. 5252-6.
171. Martinovic, I., et al., *Elevated monocyte chemoattractant protein-1 serum levels in patients at risk for coronary artery disease.* Circ J, 2005. **69**(12): p. 1484-9.
172. Hodgson, J.M., et al., *Regular ingestion of black tea improves brachial artery vasodilator function.* Clin Sci (Lond), 2002. **102**(2): p. 195-201.
173. Hirata, K., et al., *Black tea increases coronary flow velocity reserve in healthy male subjects.* Am J Cardiol, 2004. **93**(11): p. 1384-8, A6.
174. Nagaya, N., et al., *Green tea reverses endothelial dysfunction in healthy smokers.* Heart, 2004. **90**(12): p. 1485-6.
175. Erdman, J.W., Jr., et al., *Flavonoids and heart health: proceedings of the ILSI North America Flavonoids Workshop, May 31-June 1, 2005, Washington, DC.* J Nutr, 2007. **137**(3 Suppl 1): p. 718S-737S.
176. Tenhunen, R., H.S. Marver, and R. Schmid, *Microsomal heme oxygenase. Characterization of the enzyme.* J Biol Chem, 1969. **244**(23): p. 6388-94.
177. Idriss, N.K., A.D. Blann, and G.Y. Lip, *Hemoxygenase-1 in cardiovascular disease.* J Am Coll Cardiol, 2008. **52**(12): p. 971-8.
178. Li, X., et al., *Amino acids and gaseous signaling.* Amino Acids, 2009. **37**(1): p. 65-78.
179. Wunder, C. and R.F. Potter, *The heme oxygenase system: its role in liver inflammation.* Curr Drug Targets Cardiovasc Haematol Disord, 2003. **3**(3): p. 199-208.
180. Loboda, A., et al., *Heme oxygenase-1 and the vascular bed: from molecular mechanisms to therapeutic opportunities.* Antioxid Redox Signal, 2008. **10**(10): p. 1767-812.
181. Stocker, R., et al., *Bilirubin is an antioxidant of possible physiological importance.* Science, 1987. **235**(4792): p. 1043-6.

182. Baranano, D.E., et al., *Biliverdin reductase: a major physiologic cytoprotectant.* Proc Natl Acad Sci U S A, 2002. **99**(25): p. 16093-8.
183. Mayer, M., *Association of serum bilirubin concentration with risk of coronary artery disease.* Clin Chem, 2000. **46**(11): p. 1723-7.
184. Ollinger, R., et al., *Bilirubin and biliverdin treatment of atherosclerotic diseases.* Cell Cycle, 2007. **6**(1): p. 39-43.
185. Marks, G.S., *Heme oxygenase: the physiological role of one of its metabolites, carbon monoxide and interactions with zinc protoporphyrin, cobalt protoporphyrin and other metalloporphyrins.* Cell Mol Biol (Noisy-le-grand), 1994. **40**(7): p. 863-70.
186. Hennig, B., et al., *Exposure to free fatty acid increases the transfer of albumin across cultured endothelial monolayers.* Arteriosclerosis, 1984. **4**(5): p. 489-97.
187. Lee, A.S., et al., *Epigallocatechin-3-O-gallate decreases tumor necrosis factor-alpha-induced fractalkine expression in endothelial cells by suppressing NF-kappaB.* Cell Physiol Biochem, 2009. **24**(5-6): p. 503-10.
188. Ahn, H.Y., Y. Xu, and S.T. Davidge, *Epigallocatechin-3-O-gallate inhibits TNFalpha-induced monocyte chemotactic protein-1 production from vascular endothelial cells.* Life Sci, 2008. **82**(17-18): p. 964-8.
189. Clark, J.E., et al., *Dynamics of haem oxygenase-1 expression and bilirubin production in cellular protection against oxidative stress.* Biochem J, 2000. **348 Pt 3**: p. 615-9.
190. Aubert, B., et al., *Measurement of CP-violating asymmetries in B0 decays to CP eigenstates.* Phys Rev Lett, 2001. **86**(12): p. 2515-22.
191. Park, S.K., et al., *Dexamethasone regulates AP-1 to repress TNF-alpha induced MCP-1 production in human glomerular endothelial cells.* Nephrol Dial Transplant, 2004. **19**(2): p. 312-9.
192. Otterbein, L.E., et al., *Heme oxygenase-1: unleashing the protective properties of heme.* Trends Immunol, 2003. **24**(8): p. 449-55.
193. Cooper, R., D.J. Morre, and D.M. Morre, *Medicinal benefits of green tea: Part I. Review of noncancer health benefits.* J Altern Complement Med, 2005. **11**(3): p. 521-8.
194. Charo, I.F. and M.B. Taubman, *Chemokines in the pathogenesis of vascular disease.* Circ Res, 2004. **95**(9): p. 858-66.
195. Ruiz-Ortega, M., O. Lorenzo, and J. Egido, *Angiotensin III increases MCP-1 and activates NF-kappaB and AP-1 in cultured mesangial and mononuclear cells.* Kidney Int, 2000. **57**(6): p. 2285-98.
196. Martin, T., et al., *Cytokine induction of monocyte chemoattractant protein-1 gene expression in human endothelial cells depends on the cooperative action of NF-kappa B and AP-1.* Eur J Immunol, 1997. **27**(5): p. 1091-7.
197. Duckers, H.J., et al., *Heme oxygenase-1 protects against vascular constriction and proliferation.* Nat Med, 2001. **7**(6): p. 693-8.
198. Chauveau, C., et al., *Gene transfer of heme oxygenase-1 and carbon monoxide delivery inhibit chronic rejection.* Am J Transplant, 2002. **2**(7): p. 581-92.
199. Lin, H.Y., et al., *Inhibition of lipopolysaccharide-induced nitric oxide production by flavonoids in RAW264.7 macrophages involves heme oxygenase-1.* Biochem Pharmacol, 2003. **66**(9): p. 1821-32.

200. Lin, H.Y., S.C. Shen, and Y.C. Chen, *Anti-inflammatory effect of heme oxygenase 1: glycosylation and nitric oxide inhibition in macrophages.* J Cell Physiol, 2005. **202**(2): p. 579-90.
201. Lee, T.S. and L.Y. Chau, *Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice.* Nat Med, 2002. **8**(3): p. 240-6.
202. Lee, T.S., H.L. Tsai, and L.Y. Chau, *Induction of heme oxygenase-1 expression in murine macrophages is essential for the anti-inflammatory effect of low dose 15-deoxy-Delta 12,14-prostaglandin J2.* J Biol Chem, 2003. **278**(21): p. 19325-30.
203. Yeh, C.H., et al., *HO-1 Activation Can Attenuate Cardiomyocytic Apoptosis via Inhibition of NF-kappaB and AP-1 Translocation Following Cardiac Global Ischemia and Reperfusion.* J Surg Res, 2008.
204. Lorenz, M., et al., *Green and black tea are equally potent stimuli of NO production and vasodilation: new insights into tea ingredients involved.* Basic Res Cardiol, 2009. **104**(1): p. 100-10.
205. Kim, J.A., et al., *Epigallocatechin gallate, a green tea polyphenol, mediates NO-dependent vasodilation using signaling pathways in vascular endothelium requiring reactive oxygen species and Fyn.* J Biol Chem, 2007. **282**(18): p. 13736-45.
206. Sawle, P., et al., *Homocysteine attenuates endothelial haem oxygenase-1 induction by nitric oxide (NO) and hypoxia.* FEBS Lett, 2001. **508**(3): p. 403-6.
207. Lin, L.Y., et al., *Serum bilirubin is inversely associated with insulin resistance and metabolic syndrome among children and adolescents.* Atherosclerosis, 2009. **203**(2): p. 563-8.
208. Wei, Y., et al., *Hypochlorous acid-induced heme oxygenase-1 gene expression promotes human endothelial cell survival.* Am J Physiol Cell Physiol, 2009. **297**(4): p. C907-15.
209. Sano, J., et al., *Effects of green tea intake on the development of coronary artery disease.* Circ J, 2004. **68**(7): p. 665-70.
210. Sasazuki, S., et al., *Relation between green tea consumption and the severity of coronary atherosclerosis among Japanese men and women.* Ann Epidemiol, 2000. **10**(6): p. 401-8.
211. Shenouda, S.M. and J.A. Vita, *Effects of flavonoid-containing beverages and EGCG on endothelial function.* J Am Coll Nutr, 2007. **26**(4): p. 366S-372S.
212. Frank, P.G., et al., *Genetic ablation of caveolin-1 confers protection against atherosclerosis.* Arterioscler Thromb Vasc Biol, 2004. **24**(1): p. 98-105.
213. Kim, H.P., et al., *Caveolae compartmentalization of heme oxygenase-1 in endothelial cells.* FASEB J, 2004. **18**(10): p. 1080-9.
214. Zheng, Y., M. Toborek, and B. Hennig, *Epigallocatechin gallate-mediated protection against tumor necrosis factor-alpha-induced monocyte chemoattractant protein-1 expression is heme oxygenase-1 dependent.* Metabolism.
215. Itoh, K., J. Mimura, and M. Yamamoto, *Discovery of the negative regulator of Nrf2, Keap1: A historical overview.* Antioxid Redox Signal.
216. Siow, R.C., T. Ishii, and G.E. Mann, *Modulation of antioxidant gene expression by 4-hydroxynonenal: atheroprotective role of the Nrf2/ARE transcription pathway.* Redox Rep, 2007. **12**(1): p. 11-5.

217. Romeo, L., et al., *The major green tea polyphenol, (-)-epigallocatechin-3-gallate, induces heme oxygenase in rat neurons and acts as an effective neuroprotective agent against oxidative stress.* J Am Coll Nutr, 2009. **28 Suppl**: p. 492S-499S.
218. Lim, E.J., et al., *Coplanar polychlorinated biphenyl-induced CYP1A1 is regulated through caveolae signaling in vascular endothelial cells.* Chem Biol Interact, 2008. **176**(2-3): p. 71-8.
219. Hsieh, C.Y., et al., *Regulation of shear-induced nuclear translocation of the Nrf2 transcription factor in endothelial cells.* J Biomed Sci, 2009. **16**: p. 12.
220. Dinkova-Kostova, A.T., et al., *Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants.* Proc Natl Acad Sci U S A, 2002. **99**(18): p. 11908-13.
221. Kobayashi, M. and M. Yamamoto, *Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation.* Antioxid Redox Signal, 2005. **7**(3-4): p. 385-94.
222. Kobayashi, A., et al., *Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1.* Mol Cell Biol, 2006. **26**(1): p. 221-9.
223. Lee, J.S. and Y.J. Surh, *Nrf2 as a novel molecular target for chemoprevention.* Cancer Lett, 2005. **224**(2): p. 171-84.
224. Ramesh, E., P. Geraldine, and P.A. Thomas, *Regulatory effect of epigallocatechin gallate on the expression of C-reactive protein and other inflammatory markers in an experimental model of atherosclerosis.* Chem Biol Interact, 2010. **183**(1): p. 125-32.
225. Patra, S.K. and S. Bettuzzi, *Epigenetic DNA-methylation regulation of genes coding for lipid raft-associated components: a role for raft proteins in cell transformation and cancer progression (review).* Oncol Rep, 2007. **17**(6): p. 1279-90.
226. Adachi, S., et al., *The inhibitory effect of (-)-epigallocatechin gallate on activation of the epidermal growth factor receptor is associated with altered lipid order in HT29 colon cancer cells.* Cancer Res, 2007. **67**(13): p. 6493-501.
227. Schnitzer, J.E., J. Allard, and P. Oh, *NEM inhibits transcytosis, endocytosis, and capillary permeability: implication of caveolae fusion in endothelia.* Am J Physiol, 1995. **268**(1 Pt 2): p. H48-55.
228. Meijering, B.D., et al., *Ultrasound and microbubble-targeted delivery of macromolecules is regulated by induction of endocytosis and pore formation.* Circ Res, 2009. **104**(5): p. 679-87.
229. Yang, H.L., et al., *Caveolin-1 enhances resveratrol-mediated cytotoxicity and transport in a hepatocellular carcinoma model.* J Transl Med, 2009. **7**: p. 22.
230. Patra, S.K., et al., *Molecular targets of (-)-epigallocatechin-3-gallate (EGCG): specificity and interaction with membrane lipid rafts.* J Physiol Pharmacol, 2008. **59 Suppl 9**: p. 217-35.
231. Patra, S.K., *Dissecting lipid raft facilitated cell signaling pathways in cancer.* Biochim Biophys Acta, 2008. **1785**(2): p. 182-206.
232. Li, X.A., W.V. Everson, and E.J. Smart, *Caveolae, lipid rafts, and vascular disease.* Trends Cardiovasc Med, 2005. **15**(3): p. 92-6.
233. Fielding, C.J. and P.E. Fielding, *Cholesterol and caveolae: structural and functional relationships.* Biochim Biophys Acta, 2000. **1529**(1-3): p. 210-22.

234. Majkova, Z., M. Toborek, and B. Hennig, *The role of caveolae in endothelial cell dysfunction with a focus on nutrition and environmental toxicants.* J Cell Mol Med.
235. Li, Q., et al., *Docosahexaenoic acid affects endothelial nitric oxide synthase in caveolae.* Arch Biochem Biophys, 2007. **466**(2): p. 250-9.
236. Maniatis, N.A., et al., *Novel mechanism of endothelial nitric oxide synthase activation mediated by caveolae internalization in endothelial cells.* Circ Res, 2006. **99**(8): p. 870-7.
237. Chung, H.T., H.O. Pae, and Y.N. Cha, *Role of heme oxygenase-1 in vascular disease.* Curr Pharm Des, 2008. **14**(5): p. 422-8.
238. Appeldoorn, M.M., et al., *Some phenolic compounds increase the nitric oxide level in endothelial cells in vitro.* J Agric Food Chem, 2009. **57**(17): p. 7693-9.
239. Ramirez-Sanchez, I., et al., *(-)-Epicatechin Activation of Endothelial Cell Endothelial Nitric Oxide Synthase, Nitric Oxide, and Related Signaling Pathways.* Hypertension.
240. Mann, G.E., et al., *Targeting the redox sensitive Nrf2-Keap1 defense pathway in cardiovascular disease: protection afforded by dietary isoflavones.* Curr Opin Pharmacol, 2009. **9**(2): p. 139-45.
241. Xu, Y., et al., *Caveolae and endothelial dysfunction: filling the caves in cardiovascular disease.* Eur J Pharmacol, 2008. **585**(2-3): p. 256-60.
242. Pae, H.O., et al., *Roles of heme oxygenase-1 in the antiproliferative and antiapoptotic effects of nitric oxide on Jurkat T cells.* Mol Pharmacol, 2004. **66**(1): p. 122-8.
243. Buckley, B.J., Z.M. Marshall, and A.R. Whorton, *Nitric oxide stimulates Nrf2 nuclear translocation in vascular endothelium.* Biochem Biophys Res Commun, 2003. **307**(4): p. 973-9.
244. Park, E.Y. and S.G. Kim, *NO signaling in ARE-mediated gene expression.* Methods Enzymol, 2005. **396**: p. 341-9.
245. Mann, G.E., et al., *Activation of endothelial nitric oxide synthase by dietary isoflavones: role of NO in Nrf2-mediated antioxidant gene expression.* Cardiovasc Res, 2007. **75**(2): p. 261-74.
246. Jin, Y., et al., *Deletion of caveolin-1 protects against oxidative lung injury via up-regulation of heme oxygenase-1.* Am J Respir Cell Mol Biol, 2008. **39**(2): p. 171-9.
247. Majkova, Z., et al., *Up-regulation of endothelial monocyte chemoattractant protein-1 by coplanar PCB77 is caveolin-1-dependent.* Toxicol Appl Pharmacol, 2009. **237**(1): p. 1-7.
248. Lee, J.M. and J.A. Johnson, *An important role of Nrf2-ARE pathway in the cellular defense mechanism.* J Biochem Mol Biol, 2004. **37**(2): p. 139-43.
249. Lisanti, M.P., et al., *Caveolae, caveolin and caveolin-rich membrane domains: a signalling hypothesis.* Trends Cell Biol, 1994. **4**(7): p. 231-5.
250. Smart, E.J., et al., *Caveolins, liquid-ordered domains, and signal transduction.* Mol Cell Biol, 1999. **19**(11): p. 7289-304.
251. Feron, O., et al., *Endothelial nitric oxide synthase targeting to caveolae. Specific interactions with caveolin isoforms in cardiac myocytes and endothelial cells.* J Biol Chem, 1996. **271**(37): p. 22810-4.

252. Garcia-Cardena, G., et al., *Targeting of nitric oxide synthase to endothelial cell caveolae via palmitoylation: implications for nitric oxide signaling.* Proc Natl Acad Sci U S A, 1996. **93**(13): p. 6448-53.
253. Li, S., et al., *Baculovirus-based expression of mammalian caveolin in Sf21 insect cells. A model system for the biochemical and morphological study of caveolae biogenesis.* J Biol Chem, 1996. **271**(45): p. 28647-54.
254. Song, K.S., et al., *Co-purification and direct interaction of Ras with caveolin, an integral membrane protein of caveolae microdomains. Detergent-free purification of caveolae microdomains.* J Biol Chem, 1996. **271**(16): p. 9690-7.
255. Song, K.S., et al., *Targeting of a G alpha subunit (Gi1 alpha) and c-Src tyrosine kinase to caveolae membranes: clarifying the role of N-myristoylation.* Cell Mol Biol (Noisy-le-grand), 1997. **43**(3): p. 293-303.
256. Razani, B. and M.P. Lisanti, *Caveolins and caveolae: molecular and functional relationships.* Exp Cell Res, 2001. **271**(1): p. 36-44.
257. Johnson, M.K. and G. Loo, *Effects of epigallocatechin gallate and quercetin on oxidative damage to cellular DNA.* Mutat Res, 2000. **459**(3): p. 211-8.
258. Manach, C. and J.L. Donovan, *Pharmacokinetics and metabolism of dietary flavonoids in humans.* Free Radic Res, 2004. **38**(8): p. 771-85.
259. Henning, S.M., et al., *Tea polyphenols and theaflavins are present in prostate tissue of humans and mice after green and black tea consumption.* J Nutr, 2006. **136**(7): p. 1839-43.
260. Jung, N.H., et al., *Evidence for heme oxygenase-1 association with caveolin-1 and -2 in mouse mesangial cells.* IUBMB Life, 2003. **55**(9): p. 525-32.
261. Kietzmann, T., A. Samoylenko, and S. Immenschuh, *Transcriptional regulation of heme oxygenase-1 gene expression by MAP kinases of the JNK and p38 pathways in primary cultures of rat hepatocytes.* J Biol Chem, 2003. **278**(20): p. 17927-36.

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1. EGCG-mediated protection against TNF-α-induced MCP-1 expression is heme oxygenase 1 dependent. Zheng Y, Toborek M, Hennig B. Metabolism 2010 Oct;59(10):1528-35. Epub 2010 Jul 2
2. Role of caveolin-1 in EGCG-mediated protection against linoleic acid-induced endothelial cell activation. Zheng Y, Lim EJ, Wang L, Smart EJ, Toborek M, Hennig B. J Nutr Biochem 2009 Mar;20(3):202-9. Epub 2008 Jul 24
3. Effects of the extracts of Cajanus cajan L. on cell functions in human osteoblast-like TE85 cells and the derivation of osteoclast-like cells. Zheng Y, Yang J, Chen DH, Sun L. Acta Pharmaceutica sinica. 2007 Apr;42(4):386-91

4. The effects of the stilbene extracts from Cajanus Cajan L. on ovariectomy-induced bone loss in rats. Zheng Y, Yang J, Chen DH, Sun L, Acta Pharmaceutica sinica, 2007, May; 42(5):562-5.
5. Progress in the primate model of Alzheimers disease, Zheng Y, Sun L, Chinese Pharmacological Bulletin, 2005, June, vol.21, No 6, 649-652

Presentation:

1. The roles of caveolin-1 and heme oxygenase-1 in EGCG-mediated protection against TNF-α-induced endothelial inflammation, Yuanyuan Zheng, Joseph Layne, Michal Toborek, Bernhard Hennig (Abstract at the Experimental Biology 2010 meeting, April, 2010, Anaheim, California)
2. Flavonoids protect against endothelial dysfunction by induction of heme oxygenase-1, Yuanyuan Zheng, Elizabeth Oesterling, Michal Toborek, Bernhard Hennig (Abstract at the Experimental Biology 2009 meeting, April, 2009, New Orleans, LA)
3. Caveolae play a role in flavonoid protection against linoleic acid-induced endothelial cell activartion, Yuanyuan Zheng, Eric Smart, Michal Toborek, Bernhard Hennig (oral presentation at American College of Nutrition' s 49th Annual Meeting: "Advances in clinical nutrition", Oct, 2008, Arlington, Virginia)
4. Caveolae play a role in EGCG-mediated protection against linoleic acid-induced endothelial cell activation, Yuanyuan Zheng, Eum Jin Lim, Michal Toborek, Bernhard Hennig (Abstract at The Experimental Biology 2008 meeting, April, 2008, San Diego, California)
5. PCB-77 induced adhesion molecule expression in primary endothelial cells is mediated through caveolae signaling, Yeah Jung Choi, Eun jin, Lim, Yuanyuan Zheng, et al, (Abstract at The Experimental Biology 2008 meeting, April, 2008, San Diego, California)
6. Omega-3 and omega-6 fatty acids can differentially modulate signaling involved in prostaglandin synthesis Lei Wang, Eun-Jin Lim, Yuanyuan Zheng, Michal Toborek, Bernhard Hennig. (Abstract at the Experimental Biology 2007 meeting, April, 2007, Washington DC)
7. Extract of Pigeon pea promotes bone formation in HOS TE85 cell line and in OVX rats, Yuanyuan Zheng, Jing Yang, Lan Sun, Dihua Chen. (Abstract published in "2005 National Pharmacology Conference and Chinese Young Researchers' Symposium on Pharmacology", Xi'an, China)