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

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Abstract

Flavonoids have been suggested to protect against atherosclerosis via their antioxidant 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–induced endothelial cell activation and protection by the catechin epigallocatechin gallate (EGCG). Pretreatment with EGCG inhibited the secretion of monocyte chemoattractant protein–1 and the activation of activator protein–1 in porcine aortic endothelial cells stimulated with tumor necrosis factor–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 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 activator protein–1 signaling.

1. Introduction

Inflammation plays an important role in the development of atherosclerosis [1]. Tumor necrosis factor–α (TNF-α), a proinflammatory cytokine, can induce the activation of the vascular endothelium, including up-regulation of monocyte chemotactic protein–1 (MCP-1) [2]. Monocyte chemotactic protein–1 is a chemoattractant and can be secreted by endothelial cells, vascular smooth muscle cells, and macrophages [3]. Monocyte chemotactic protein–1 messenger RNA (mRNA) expression is significantly increased in macrophage-rich atherosclerotic plaques [4], and elevated MCP-1 serum levels are considered a marker of inflammation in coronary artery disease patients [5]. Thus, in the present study, MCP-1 was used as an inflammation target to evaluate endothelial function modulated by diet-derived flavonoids, such as epigallocatechin gallate (EGCG).

Evidence suggests that diets high in various nutrients and phytochemicals (eg, 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 [6,7]. Flavonoids constitute a subclass of bioactive compounds rich in fruits and vegetables, soy food, legumes, tea, and cocoa [8]. Many flavonoids are composed of a polyphenol structure, and these polyphenols are often classified according to structural similarities [9]. Examples of flavonoids include flavonols, isoflavones, flavonones, and flanan-3-ols (eg, catechins). Epidemiologic studies have shown that green tea rich in catechins may be protective against coronary atherosclerosis [10]. In fact, green tea consumption is usually higher in healthy subjects compared with those with coronary artery disease [11], suggesting that green tea and its polyphenols,

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for example, catechins, can attenuate risk factors associated with the pathology of atherosclerosis [12]. The majority of catechins in green tea include EGCG, which has been shown to improve endothelial function and to induce anti-inflammatory vascular events.

Mechanisms of flavonoid-induced protection of the vasculature may include up-regulation of heme oxygenase–1 (HO-1) [13], an inducible enzyme in the degradation of heme to iron, carbon monoxide, and biliverdin, with the latter being quickly reduced to bilirubin [14]. Besides the function of removing heme, the metabolic products of HO-1 have been recognized recently to play important roles in vascular diseases [15]. For example, prooxidative and proinflammatory defenses are reduced during HO-1 deficiency [15]. There are 3 isoforms of heme oxygenases [16,17]. Whereas HO-1 is structurally different from HO-2 and HO-3, the latter two are very similar (90% homology). Of the 3 isozymes, HO-1 is believed to be the only inducible form [17]. Heme oxygenase–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 [18]. Bilirubin, a product of HO-1–mediated heme degradation, can protect lipid membranes against oxidation as efficiently as α-tocopherol and β-carotene [19]. In contrast, inhibition of bilirubin production by biliverdin reductase small interfering RNA (siRNA) has been shown to increase reactive oxygen species levels in primary neuronal cultures [20]. In general, clinical studies suggest that elevated levels of circulatory bilirubin provide protection against atherosclerosis and coronary artery disease [21,22].

The heme oxygenase system is an important regulator of endothelial cell integrity and oxidative stress [23], and dysfunctional HO-1 signaling may be proatherogenic. Thus, a major objective of the current study was to explore the role of HO-1 in mechanisms of EGCG-mediated protection of the vascular endothelium. Our data demonstrate that EGCG can exhibit anti-inflammatory properties via induction of HO-1 and AP-1 signaling.

2. Materials and methods

2.1. Materials

Tumor necrosis factor–α, bilirubin, and anti–β-actin antibody were obtained from Sigma-Aldrich (St Louis, MO), and EGCG (>98% pure) was purchased from Cayman Chemical (Ann Arbor, MI). Cobalt protoporphyrin (CoPP) was purchased from Frontier Scientific (Logan, UT); and HO-1 antibody, from Abcam (Cambridge, MA). Bilirubin was dissolved in 0.2 N NaOH, neutralized to pH 7.4 using 1 N HCl, and used fresh.

2.2. Cell culture and experimental media

Endothelial cells were isolated from porcine aortic arteries and cultured as previously described [24]. 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) (catalog no. 31100-035; GIBCO Laboratories, Grand Island, NY) containing 10% (vol/vol) fetal bovine serum (HyClone Laboratories, Logan, 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% fetal bovine serum and were supplemented with various concentrations of EGCG (Cayman Chemical, 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 determined before the current study to understand protective properties of EGCG in our cell culture model. For example, we found that the lowest dose of EGCG that protected against TNF-α–induced inflammatory markers was 30 μmol/L. Thus, 30 μmol/L of EGCG was used in the current study. Others have reported similar concentrations of EGCG to decrease inflammatory markers in culture endothelial cells [25-27]. Epigallocatechin gallate was dissolved in dimethyl sulfoxide (DMSO); at a final concentration of 0.1%, DMSO did not affect cell viability.

2.3. HO-1 siRNA and transfection

The HO-1 gene silencer was designed by Applied Biosystems (Foster City, CA). The sequences of HO-1 gene silencer were 5′-GCAUCUUUCCCAACCAAGAtt-3′ (sense) and 5′-UCUUGGUUGGGAAAGAUGCca-3′ (antisense). The sequences of the control gene silencer were 5′-AAAGAGCGACUUUACACACdTdT-3′ (sense) and 5′-GUGUGUAAAGUCGCUCUUUdTdT-3′ (antisense). Cells were transfected with control siRNA or HO-1 siRNA at a final concentration of 80 nmol/L using GeneSilencer (Genlantis, San Diego, CA) with Opti-MEM I medium (Invitrogen, Carlsbad, CA). Cells were incubated with transfection mixtures for 4 hours, followed by replacement with 10% serum medium. Cells were synchronized overnight after 48-hour transfection, pretreated with EGCG, and subsequently treated with TNF-α or vehicle.

2.4. Immunoblotting

Cells were treated with either vehicle (0.1% DMSO) or EGCG (30 μmol/L) followed by exposure to TNF-α (2 ng/mL) for immunoblot analysis of HO-1 activation. Cell protein was extracted as described before [28]. Equal amounts of protein (20 μg) were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (12% acrylamide) and transferred to nitrocellulose membranes. The membrane was blocked for 1 hour at room temperature with 5% nonfat milk in Tris-buffered saline (TBS, pH 7.6) containing 0.05% Tween-20, and then washed with TBS-Tween. Membranes were incubated overnight with the primary antibody (1000-fold diluted in TBS-Tween contain-

ing 5% bovine serum albumin) at 4°C and for 1 hour with horseradish peroxidase–conjugated secondary antibody (~5000-fold diluted) at room temperature. Bands were visualized using the appropriate horseradish peroxidase–conjugated secondary antibodies followed by ECL immunoblotting detection reagents (Amersham Biosciences, Buckinghamshire, England).

2.5. Electrophoretic mobility shift assays of AP-1–DNA binding

Nuclear extracts from endothelial cells were prepared as previously described [29]. Synthetic 5′-biotinylated complementary oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Nuclear extracts were incubated at room temperature for 20 minutes 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 LightShift chemiluminescent electrophoretic mobility shift assay (EMSA) kit (Pierce, Rockford, IL) [30].

2.6. Real-time reverse-transcription polymerase chain reaction

Total RNA was extracted using TRIzol Reagent (Invitrogen) 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 polymerase chain reaction (PCR) products were then assessed by real-time PCR using 7300 Real-Time PCR System (Applied Biosystems). Samples were mixed with SYBR Green Master Mix (Applied Biosystems), 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); and the sequences were as follows: MCP-1: 5′-CGGCTGATGAGCTACAGAAGAGT-3′ (sense) and 5′-GCTTGGGTTCTGCACAGATCT-3′ (antisense); HO-1: 5′-AGGTCACCCGAGAAGGCTTT-3′ (sense) and 5′-TAGACCGGGTTCTCCTTGTTGT-3′ (antisense). The house keeping gene was β-actin: 5′-TCATCACCATCGGCAACG-3′ (sense) and 5′-TTCCTGATGTCCACGTCG-3′ (antisense).

2.7. 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 $BaCl_2 \cdot 2H_2O$/probe 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, 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 mmol/$L^{-1}$$cm^{-1}$[31].

2.8. 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, Kaysville, UT), centrifuged at 4°C to remove cellular debris, and then stored at −80 °C. The MCP-1 levels were assessed using an MCP-1–specific enzyme immunoassay (BD Biosciences, San Jose, CA) following the manufacturer's protocol with minor modification. A microplate spectrophotometer, SpectraMaxPro M2 (Molecular Devices), was used to read the plate at 450 nm.

2.9. 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 were 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 phosphorothioate-bonded oligonucleotides. The ODNs were annealed for 1 hour, while the temperature descended from 80°C to 25°C. DNA derived from endothelial cells was precomplexed with the PLUS reagents (Life Technologies, Rockville, MD) at room temperature for 15 minutes. The precomplexed DNA was combined with diluted LipofectAMINE reagent (Life Technologies), mixed, and incubated for 15 minutes at room temperature. While complexes were forming, medium with serum-free transfection medium was replaced. Afterward, DNAPLUS LipofectAMINE reagent complex was added to each well containing fresh medium; and cells were incubated for 5 hours. After incubation, complete medium with serum was added. After transfection for 48 hours, cell extracts were prepared for real-time PCR analysis [32].

2.10. Statistical analysis

Values are reported as mean ± standard error of the mean (SEM) of at least 3 independent groups. Data were analyzed using Sigma Stat software (Jandel, San Rafael, CA). One-way analysis of variance followed by post hoc least significant difference pairwise multiple comparison procedure was used for statistical analysis of the original data. A statistical probability of $P < .05$ was considered significant.

3. Results

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

Cells were treated with vehicle, EGCG, or 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 pretreatment with either EGCG or the potent HO-1 inducer CoPP (Fig. 1). Most importantly, both CoPP and EGCG significantly induced HO-1 levels in endothelial cells (Fig. 2). In contrast, exposure to TNF-α neither induced HO-1 expression nor affected HO-1 expression induced by both CoPP and EGCG (Fig. 2).

3.2. HO-1 silencing prevents EGCG-mediated protection against TNF-α–induced MCP-1 up-regulation

Small interfering RNA was used 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 (Fig. 3A). Subsequently, cells were pretreated with EGCG, followed by exposure to TNF-α. Tumor necrosis factor–α markedly increased MCP-1 mRNA and protein expression, independent of HO-1 silencing (Fig. 3B, C). In control siRNA cells, pretreatment with EGCG markedly inhibited the MCP-1 up-regulation induced by exposure to TNF-α. In contrast, HO-1 silencing reversed the inhibitory effects of EGCG both at the message and protein level (Fig. 3B, C).

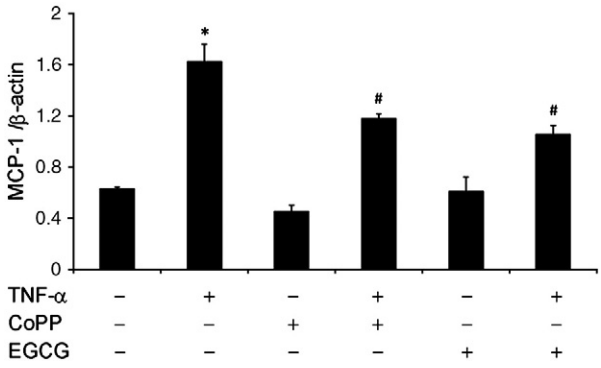

Fig. 1. Epigallocatechin gallate and CoPP inhibit TNF-α–induced MCP-1 expression. Cells were pretreated with vehicle (0.1% DMSO), EGCG (30 μmol/L), or CoPP (10 μmol/L) for 2 hours, followed by exposure to TNF-α (2 ng/mL) for an additional 6 hours. The MCP-1 and β-actin mRNA expressions were measured by real-time PCR. Results shown represent the mean ± SEM; n = 3. Experiments were repeated a minimum of 3 times. *Significantly different compared with vehicle control. #Significantly different compared with cultures treated only with TNF-α.

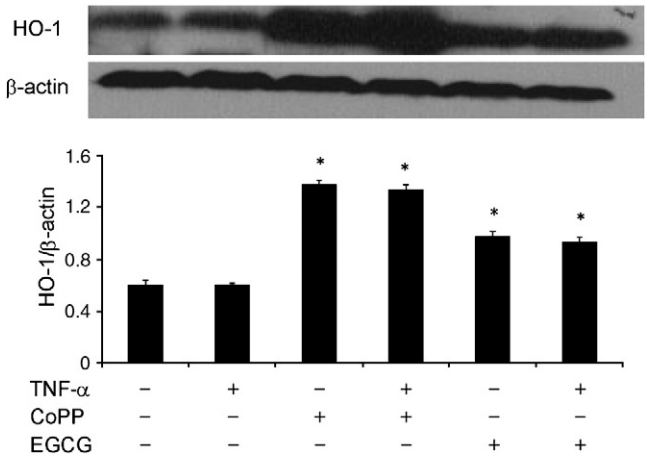

Fig. 2. Epigallocatechin gallate and CoPP induce HO-1 levels in endothelial cells. Cells were treated with vehicle (0.1% DMSO), EGCG (30 μmol/L), or CoPP (10 μmol/L) in the presence or absence of TNF-α (2 ng/mL) for 4 hours before determining HO-1 expression by Western blot analysis. The Western blot shown represents 1 of 3 experiments. Densitometry results shown in parallel represent the mean ± SEM; n = 3. Experiments were repeated a minimum of 3 times. *Significantly different compared with control cultures.

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

Heme oxygenase–1 has potent anti-inflammatory effects, which may be exerted through the generation of bilirubin [33]. 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 (Fig. 4A, B). Furthermore, supplemental bilirubin markedly quenched the TNF-α–stimulated induction of MCP-1, with maximal effects at 5 μmol/L supplemental bilirubin (Fig. 4C). As expected, HO-1 silencing abolished not only 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 (Fig. 4A, B).

3.4. 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). The AP-1 decoy ODN partially inhibited TNF-α–induced MCP-1 mRNA expression (Fig. 5A), 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 (Fig. 5B). Furthermore, the protective effects of EGCG through AP-1 signaling appear to be dependent on functional HO-1.

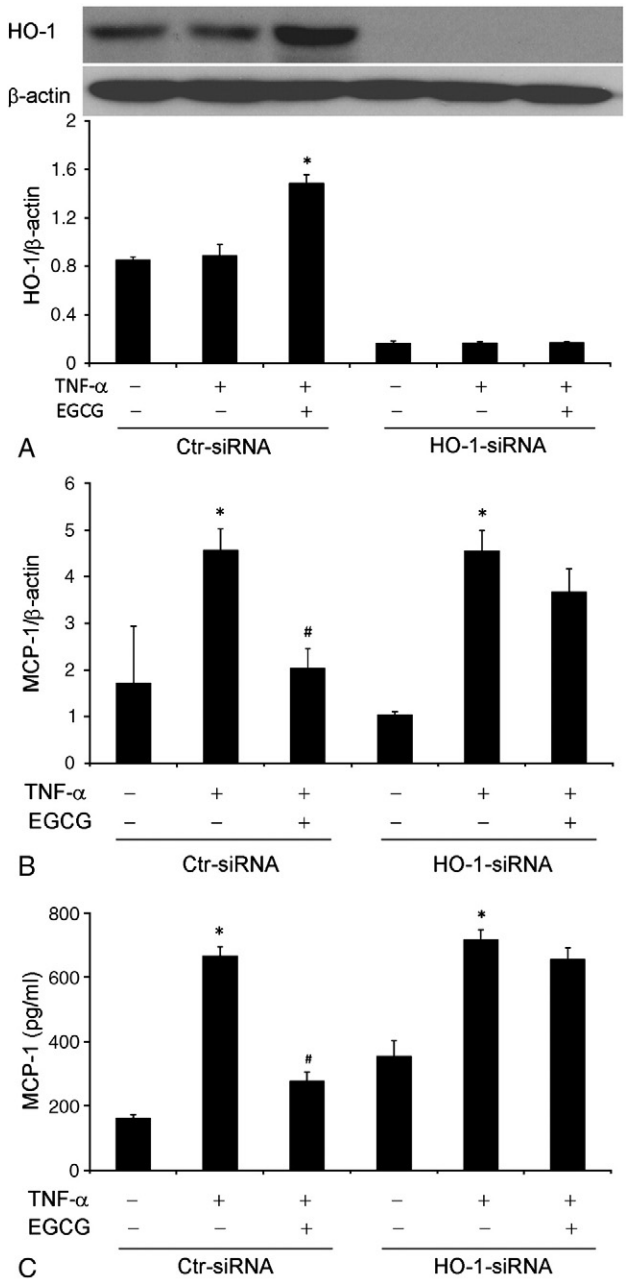

Fig. 3. Heme oxygenase–1 silencing prevents EGCG-mediated protection against TNF-α–induced up-regulation of MCP-1. Endothelial cells were transfected with HO-1 siRNA or with Ctr-siRNA and treated with EGCG (30 μmol/L) for 2 hours, followed by exposure to TNF-α (2 ng/mL) for 6 hours. Successful silencing (A). The MCP-1 mRNA expression was measured by real-time PCR (B). Media were collected, and MCP-1 levels were measured by enzyme immunoassay (C). Results shown represent the mean ± SEM; n = 3. Experiments were repeated a minimum of 3 times. *Significantly different compared with respective control cultures. #Significantly different compared with cultures treated only with TNF-α (Ctr-siRNA).

Pretreatment with EGCG markedly suppressed AP-1 DNA-binding activity (Fig. 5C). Heme oxygenase–1 siRNA transfection not only blocked EGCG-induced production of bilirubin (Fig. 4B), but it also reversed the inhibitory effect of EGCG against TNF-α–induced AP-1 activation (Fig. 5C).

4. Discussion

There is evidence that flavonoids have anti-inflammatory properties and thus can provide protection against inflammatory diseases such as atherosclerosis [8]. More specifically, catechins derived from green tea have various bioactive properties associated with antioxidant, antiangio-

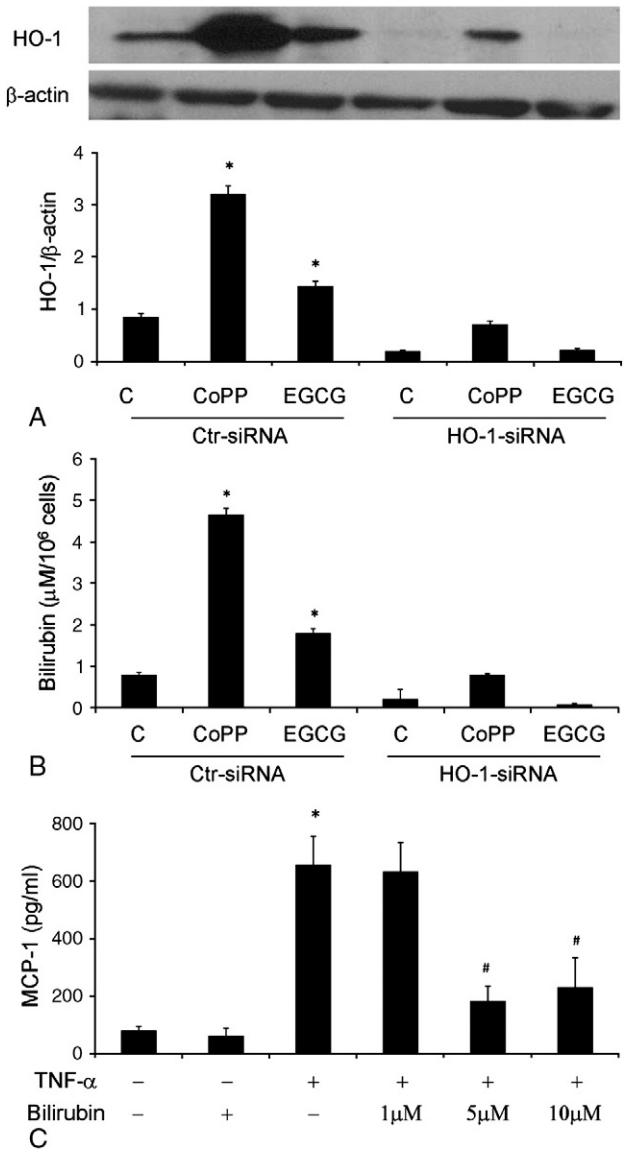

Fig. 4. Epigallocatechin gallate induces bilirubin levels in endothelial cells, and supplemental bilirubin reduces TNF-α–induced up-regulation of MCP-1. Endothelial cells were transfected with HO-1 siRNA or with Ctr-siRNA; and cells were treated with vehicle (0.1% DMSO), EGCG (30 μmol/L), or CoPP (10 μmol/L) for 4 hours before determining HO-1 and bilirubin production (A and B). In separate experiments, cells were pretreated with either vehicle (H_2O) or bilirubin (0-10 μmol/L) for 2 hours, followed by exposure to TNF-α (2 ng/mL) for an additional 4 hours. Media were collected, and MCP-1 levels were measured by enzyme immunoassay (C). Results shown represent the mean ± SEM; n = 3. Experiments were repeated a minimum of 3 times. *Significantly different compared with control cultures. #Significantly different compared with cultures treated only with TNF-α.

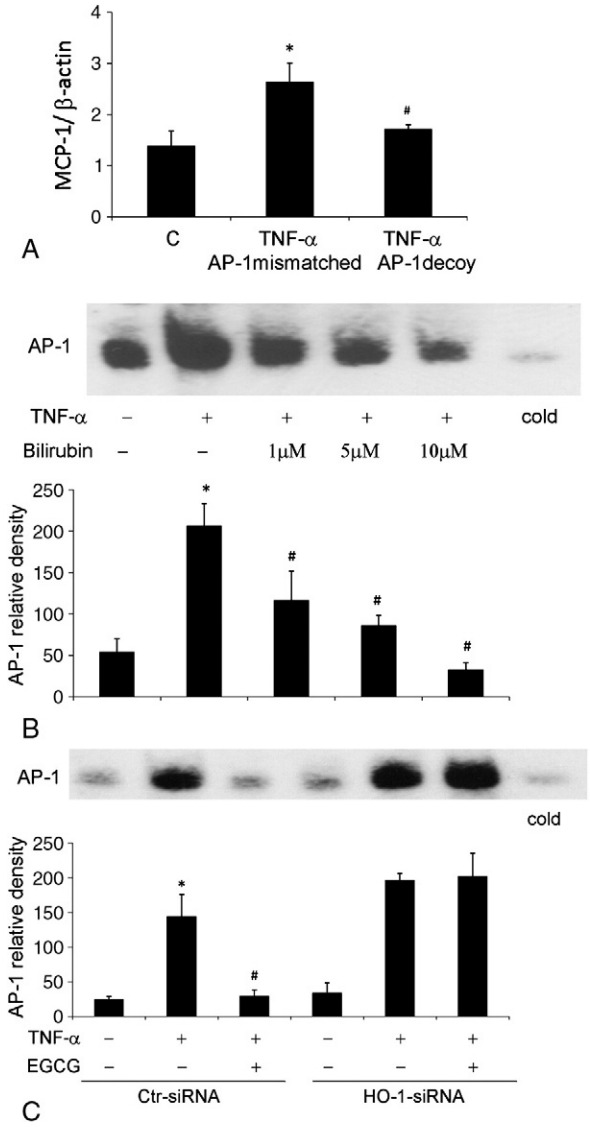

Fig. 5. The effect of EGCG on DNA binding of AP-1 and subsequent MCP-1 induction is dependent on functional HO-1. Endothelial cells were stimulated with TNF-α for 4 hours after transfection of AP-1 decoy ODN and mismatched-ODN. Subsequently, MCP-1 mRNA levels were measured (A). Cells also were pretreated with either vehicle (H_2O) or bilirubin (0-10 μmol/L) for 2 hours, followed by exposure to TNF-α for an additional 4 hours. The EMSA for AP-1 was performed with nuclear proteins extracted from endothelial cells (B). In separate experiments, endothelial cells were transfected with HO-1 siRNA or with Ctr-siRNA and treated with EGCG for 2 hours, followed by exposure to TNF-α for 4 hours. The EMSA for AP-1 was performed with nuclear proteins extracted from endothelial cells (C). The EMSAs shown in panels B and C represent 1 of 3 experiments. Densitometry results shown in parallel represent the mean ± SEM; n = 3. Experiments were repeated a minimum of 3 times. *Significantly different compared with vehicle control. #Significantly different compared with cultures treated only with TNF-α (Ctr-siRNA).

genesis, and anti-inflammatory functions, which all are relevant to the prevention and treatment of cardiovascular diseases [34]. Endothelial cells line the inner layer of blood vessels and play a critical role in the overall dynamics of vascular physiology. Activation and subsequent dysfunction of the endothelium are considered early events in the etiology of cardiovascular diseases such as the pathology of atherosclerosis [35]. Studies suggest that EGCG, the major polyphenolic constituent in green tea, has anti-inflammatory effects, such as inhibition of cytokine-induced vascular cell adhesion molecule–1 expression [27]. However, detailed mechanisms of the anti-inflammatory properties of EGCG associated with endothelial cell function are not well defined. Plasma concentrations after consumption of tea are usually less than 1 μmol/L [36]. Because of large plasma fluctuations in vivo, local concentrations at the surface of the vascular endothelium are not known and could be significantly higher than circulating levels.

In the current study, endothelial cells were stimulated with TNF-α, a major proinflammatory cytokine contributing to endothelial inflammation. We found that pretreatment with EGCG inhibited TNF-α–induced endothelial secretion of MCP-1, a critical chemokine responsible for the recruitment of monocytes to the intima [37]. 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 nuclear factor (NF)–κB [38,39]. 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 (data not shown). Others also have reported that EGCG did not influence TNF-α–stimulated NF-κB in human endothelial cells [27], suggesting that EGCG more specifically alters AP-1 signaling.

We hypothesized 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 CoPP and abolished by HO-1 siRNA transfection. Heme oxygenase–1 overexpression can inhibit pathologic activities including inflammation, vascular proliferation, and chronic transplant rejection [40,41]. It has been reported that overexpression of the HO-1 protein inhibits lipopolysaccharide-induced inducible nitric oxide (NO) synthase expression and NO production [42,43]. Furthermore, the HO-1 protein is essential for the anti-inflammatory effects of interleukin-10 and 15-deoxy-delta 12,14-prostaglandin J2 [44,45]. In the present study, treatment with CoPP inhibited TNF-α–induced MCP-1 up-regulation, suggesting that HO-1 plays an important role as a target in endothelial cells against proinflammatory damage.

Epigallocatechin gallate can induce HO-1 in various cell systems [13,46], but little is known about the link of HO-1 in EGCG-mediated reduction of inflammation. We

investigated the relationship between HO-1 and AP-1 activation in TNF-α–stimulated endothelial cells. Cells were pretreated with EGCG and then exposed to TNF-α. Our data suggest that the EGCG-mediated HO-1 protein expression interferes with TNF-α–induced 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 up-regulation 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 mechanism 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 [47]. Epigallocatechin gallate could induce HO-1 indirectly through other signaling mechanisms, such as via induction of NO, because it has been reported that EGCG can increase NO [48,49] and that NO can induce HO-1 [18,50].

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 [51], and exogenous administration of bilirubin can promote endothelial cell survival [52]. In the current study, EGCG significantly induced bilirubin levels in endothelial cultures; and this effect was blocked in HO-1–silenced cells. Supplemental bilirubin also significantly inhibited the expression of MCP-1 in a concentration-dependent manner. Moreover, similar to EGCG, supplemental bilirubin inhibited the TNF-α–stimulated AP-1 activation.

In summary, our data suggest that TNF-α–induced DNA binding of AP-1 and subsequent MCP-1 induction 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.

Acknowledgment

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