Ellagic acid inhibits IL-1β-induced cell adhesion molecule expression in human umbilical vein endothelial cells

Ya-Mei Yu*, Zhi-Hong Wang1, Chung-Hsien Liu2 and Chin-Seng Chen3

1Department of Nutrition, China Medical University, 91, Hsueh-Shih Road, Taichung, Taiwan
2School of Medicine, Chung Shan Medical University, 110, Sec 1, Chien-kuo N Road, Taichung, Taiwan
3Department of Biotechnology, Chang Jung Christian University, 396 Chang Jung Road, Sec 1, Kway Jen, Tainan, Taiwan

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Expression of cell adhesion molecules by endothelium and the attachment of monocytes to endothelium may play a major role in atherosclerosis. Ellagic acid (EA) is a phenolic compound found in fruits and nuts including raspberries, strawberries, grapes and walnuts. Previous studies have indicated that EA possesses antioxidant activity in vitro. In the present study, we investigated the effects of EA on the formation of intracellular reactive oxygen species, the translocation of NFκB and expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 and endothelial leucocyte adhesion molecule (E-selectin) induced by IL-1β in human umbilical vein endothelial cells (HUVEC). We found that EA significantly reduced the binding of human monocytic cell line, U937, to IL-1β-treated HUVEC. The production of reactive oxygen species by IL-1β at 100 ng/ml was dose-dependently suppressed by EA. Supplementation with increasing doses of EA up to 50 μmol/l was most effective in inhibiting the expression of VCAM-1 and E-selectin. Furthermore, the inhibition of IL-1β-induced adhesion molecule expression by EA was manifested by the suppression of nuclear translocation of p65 and p50. In conclusion, EA inhibits IL-1β-induced nuclear translocation of p65 and p50, thereby suppressing the expression of VCAM-1 and E-selectin, resulting in decreased monocyte adhesion. Thus, EA has anti-inflammatory properties and may play an important role in the prevention of atherosclerosis.

Ellagic acid: Cell adhesion molecule: NFκB: Reactive oxygen species: Atherosclerosis

Activation of the vascular endothelium, increased adhesion of mononuclear cells to the injured endothelial layer, and their subsequent extravasations into the vessel wall are initial events in atherogenesis. Endothelial cells recruit leucocytes by expressing adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and endothelial leucocyte adhesion molecules (E-selectin; Cybulsky & Gimbrone, 1991). Several inflammatory cytokines including IL-1, TNF and interferon produced by activated monocytes and macrophages may stimulate the endothelium to up-regulate genes encoding chemokines, other cytokines and adhesion molecules (Ross, 1999).

Previous studies have indicated that NF-κB/Rel transcription factors may play an important role in the development of atherosclerosis (Collins, 1993; Qvarnstrom et al. 1994). Activation of NF-κB by inflammatory stimuli has been demonstrated in cultures of endothelial cells using electrophoretic mobility shift assays (Collins, 1993). A variety of genes induced in the atherosclerotic lesion have been shown to be regulated by NF-κB proteins, including the genes encoding TNF-α (Baeuerle & Henkel, 1994), IL-1B (Hiscott et al. 1993), VCAM-1 (Neish et al. 1992) and ICAM-1 (Poston et al. 1992).

It is well established that dietary polyphenolic compounds play significant roles in the prevention of atherosclerosis and CVD (Gaziano et al. 1992; Gey et al. 1993). Polyphenolic compounds affect the development of atherosclerosis not only through modulation of serum lipids but also by influencing the immune and inflammatory processes associated with the development of this disease. Previous studies have indicated that polyphenolic compounds such as vitamin E or tea flavonoid may exert their effects through modulation of cytokines, adhesion molecules and interaction of immune cells with endothelial cells (Martin et al. 1997; Islam et al. 1998; Ludwig et al. 2004). Ellagic acid (EA) is a phenolic compound found in fruits including grape juice (10.2 mg/100 g), grape wine (5.6 mg/100 g), blackberries (0.9 mg/100 g), blackberries (42.4 mg/100 g), raspberries (17.9 mg/100 g) and strawberries (19.8 mg/100 g) (de Ancos et al. 2000; Sellappan et al. 2002; Mertens-Talcott et al. 2003). Previous studies have indicated that EA scavenges both oxygen and hydroxyl radicals, and inhibits lipid peroxidation (Cozzi et al. 1995; Laranjinha et al. 1996; Iino et al. 2001). In our laboratory, we found that EA reduced oxidative stress and atherosclerosis in a hyperlipidaemic rabbit model (Yu et al. 2005). Therefore, the present study was designed to examine the effect of EA on monocyte adhesion to cultured human endothelial cells and the
expression of adhesion molecules (VCAM-1, ICAM-1 and E-selectin) and to elucidate its possible mechanism.

**Methods**

**Cell culture**

Human umbilical vein endothelial cells (HUVEC) were isolated by collagenase type II (Biochrom KG, Berlin, Germany) digestion of human umbilical veins by standard techniques and cultured in EC medium (MCDB 131; Gibco-BRL, LifeTechnologies GmbH, Karlsruhe, Germany) at 37°C in a humidified atmosphere of 5% CO2 and 95% air as described previously (Stangl et al. 2001). All experiments were performed with HUVEC from passages one to three. HUVEC were seeded at 1 x 10^4 cells/well in ninety-six-well plates. After 3 d, the medium was replaced by fresh EC medium before treatment.

**Cell viability assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide**

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Chen et al. 2002). The principle of this assay is that mitochondria dehydrogenase in viable cells reduces MTT to a blue formazan. Briefly, cells were grown in ninety-six-well plates and incubated with various concentrations of EA (which was dissolved in dimethyl sulphoxide) for 24 h; 100 µl MTT (0·5 mg/ml) were then added to each well and incubation continued at 37°C for an additional 4 h. The medium was then carefully removed, so as not to disturb the formazan crystals which had formed. Dimethyl sulphoxide (100 µl) which solubilizes formazan crystals, was added to each well and the absorbance of the solubilized blue formazan was read at 530 nm (reaction) and 690 nm (background) using a DIAS Microplate Reader (Dynex Technologies, Chantilly, VA, USA). The reduction in optical density caused by EA was used as a measurement of cell viability, normalized to cells incubated in control medium, which were considered 100% viable.

**Estimation of the production of reactive oxygen species**

The production of intracellular reactive oxygen species (ROS) induced by IL-1β was estimated by a fluorometric assay using 2',7'-dichlorofluorescein-diacetate (DCFH-DA) as a probe according to the method reported by Bass et al. (1983). HUVEC (1 x 10^5) were incubated with IL-1B and EA, suspended in PBS containing 2% fetal calf serum, and then incubated again with 5 mM of 2',7'-dichlorofluorescein-diacetate for 30 min at 37°C. The formation of 2',7'-dichlorofluorescein was determined by flow cytometry. The excitation wavelength was 488 nm, and green fluorescence collected through a 530 nm band-pass filter was measured on a logarithmic scale. The formation of ROS was expressed as relative fluorescence intensity.

**Real-time PCR for vascular cell adhesion molecule-1, intercellular adhesion molecule-1 and endothelial leucocyte adhesion molecule**

The real-time PCR assay for adhesion molecules was conducted according to the method reported by Li & Wang (2002). Total cellular RNA was isolated from samples (HUVEC) using the Trizol reagent according to the manufacturer's instructions (Gibco BRL). RT reactions were carried out for each RNA sample in thin-walled PCR tubes using the First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Each reaction tube contained 4·2 µg total RNA in a volume of 21 µl containing 1× RT buffer, 5·5 mM MgCl₂, 500 µmol/l of each dNTP, 2·5 µmol/l of oligo-d(T)₁₂₋₁₈ primers, 40 U/µl RNase inhibitor, 2 U/µl Escherichia coli RNaseH and 50 U/µl of SuperScript II RT. RT reaction was carried out at 65°C for 5 min, 42°C for 50 min and 70°C for 15 min. The RT reaction mixture was then placed at 4°C for immediate PCR amplification or stored at −20°C for later use. Real-time PCR was performed in optical real-time PCR tubes. The following primers were used: VCAM-1: forward 5′-AACCCAGCACAGAGACAC-3′, reverse 5′-TGCGACGTTATATTAAGAGAGATG-3′; ICAM-1: forward 5′-TGTTCCACAGGTTCAGATTAC-3′, reverse 5′-GACAAGAGGACAAGGATAGC-3′; E-selectin: forward 5′-TGTTGAGATGCGATGCTGTC-3′, reverse 5′-AACTCTCTGTCGATTGTC-3′; glyceraldehyde-3-phosphate dehydrogenase: forward 5′-CCCAGCTTTTCACCGT-3′, reverse 5′-CTTCCCTGTTGCCTTG-3′.

Each tube contained 1 µl of each RT product (200 ng total RNA), 5·5 mM MgCl₂, 400 µmol/dNTP, 500 µmol/l primer (forward and reverse), 0·005 U/µl iTaq DNA polymerase and 20 mmol/l SYBR Green I in a total volume of 25 µl. Amplification conditions were 3 min at 95°C for activation, then run for forty cycles at 95°C for 15 s and 60°C for 1 min. All reactions were performed in the Bio-Rad iCycle Sequence Detection System using the iCycle V3.1 program. The threshold cycle (Ct) and melting point (M) were obtained during each reaction. The relative quantification was calculated based on its 2^(-ΔCt) value, ΔCt = Ct (sample) – Ct (control).

**Measurement of NF-κB activity**

Nuclear extracts were prepared as described previously (Dschietzig et al. 2001). Bradford reagent determined protein concentrations. For analysis of NF-κB activity, a TransAM NF-κB Family kit was used (Active Motif, Rixensart, Belgium). In this assay, ninety-six-well plates were coated with an oligonucleotide containing the consensus binding sequence for NF-κB 5′-GGGACTTTCC-3′. Specific primary antibodies included in the kit detected the binding of NF-κB family transcription factors to their consensus sequence. Experiments were analysed by an ELISA-based assay. A total of 100 ng nuclear extract was used in each experiment and processed according to the manufacturer’s protocol. Briefly, nuclear extracts were incubated with the oligonucleotide-coated wells for 60 min. Where indicated a competitor for NF-κB binding (NF-κB wild-type consensus oligonucleotide) was added in molar excess prior to the probe. The wells were then washed and incubated with the primary antibodies for p65, p50, c-Rel, p52 and RelB for 60 min. After incubation with a horseradish peroxidase-conjugated secondary antibody, a substrate was added to produce blue colour and then quantified for a standard ELISA reader. The absorbance was read at 450 nm and the blanks were subtracted from all measurements. The data presented are the result of three independent experiments.
Monocyte-endothelial cell adhesion

HUVEC (2 x 10^5) were distributed into six-well plates and allowed to reach confluence. They were then incubated for 18 h with medium supplemented with EA at concentrations of 25 and 50 μmol/l according to the MTT test, followed by incubation for 6 h with 10 ng/ml IL-1β in the continued presence of EA. U937 cells, originally derived from a human histiocytic lymphoma and used for the monocyte-endothelial cell adhesion assay, were grown in RPMI-1640 medium (Gibco, New York, USA) containing 10% fetal bovine serum and subcultured at a 1:5 ratio three times per week, labelled for 30 min at 37°C with calcein AM (10 nmol/l; Molecular Probe; Invitrogen) in RPMI-1640 medium and washed with PBS to remove free dye, and then resuspended in 10% M-199 medium. Labelled U937 cells (1 x 10^6) were added to each HUVEC-containing well and incubated for 1 h. Non-adherent cells were removed by two gentle washes with PBS. Then, adherent U937 cells were determined by a fluorescence plate reader at an excitation wavelength of 485 nm and emission at 530 nm; HUVEC cell monolayers served as the blank.

Statistics

Results are presented as means and standard deviations. Statistical significance was determined by one-way ANOVA. Differences were considered significant at P<0.05.

Results

Concentrations of ellagic acid for human umbilical vein endothelial cells

Cell viability was assayed by the MTT test. After 24 h incubation with 10, 25, 50, 75, 100 and 150 μmol/l EA, cell viability was 125.2 (sd 4.9), 122.3 (sd 4.4), 106.7 (sd 2.7), 91.3 (sd 1.8), 75.8 (sd 1.4) and 78.4 (sd 1.4) %, respectively, of control levels, the three highest concentrations causing a significant reduction in cell viability. Therefore, according to the MTT test we chose 25 and 50 μmol/l to do all the experiments.

Ellagic acid inhibits IL-1β-induced reactive oxygen species production in human umbilical vein endothelial cells

Fig. 1(A) shows the results of ROS production induced by IL-1β. The production of ROS decreased after addition of 25 and 50 μmol/l EA (Fig. 1(B, C)).

Ellagic acid inhibits IL-1β-induced cell surface expression of vascular cell adhesion molecule-1 and endothelial leukocyte adhesion molecule but not expression of intercellular adhesion molecule-1 in human umbilical vein endothelial cells

The effects of EA on IL-1β-induced VCAM-1, ICAM-1 and E-selectin expression by HUVEC were studied by pretreating HUVEC for 18 h with 25 or 50 μmol/l EA before addition of 10 ng/ml IL-1β. This resulted in reduced cell surface expression of VCAM-1 and E-selectin, but had no effect on cell surface expression of ICAM-1 (Fig. 2(A–C)).

Ellagic acid attenuates activation of NF-κB expression and nuclear translocation of NF-κB p65 and p50 in IL-1β-stimulated human umbilical vein endothelial cells

To examine whether the inhibitory effect of EA on the cytokine-induced expression of adhesion molecules is mediated via NF-κB, we measured the nuclear translocation of p65 and p50 protein of the NF-κB family of transcription factors. Incubation of IL-1β (10 ng/ml) for 6 h induced the nuclear translocation of p65 and p50 (Fig. 3(A, B)). Preincubation of HUVEC with 50 μmol/l EA prior to IL-1β stimulation did significantly prevent the nuclear translocation of p65 and p50 (Fig. 3(A, B)).
Ellagic acid inhibits adhesion of U937 cells to IL-1β-stimulated human umbilical vein endothelial cells

To explore the effects of EA on endothelial cell leucocyte interactions, we examined the adhesion of U937 cells to cytokine-activated HUVEC. Control confluent HUVEC showed minimal binding to U937 cells, but adhesion increased when the HUVEC were treated with IL-1β (Fig. 4(A, B)). Pretreatment of HUVEC with 50 μmol/l EA reduced the number of U937 cells adhering to IL-1β-stimulated HUVEC (Fig. 4(A, B)).

Discussion

An early stage in atherosclerosis is the adhesion of monocytes to the arterial wall, followed by their infiltration and differentiation into macrophages. This key stage is mediated by the interaction of monocytes with adhesion molecules expressed by endothelial cells. In the present study, we found that 50 μmol/l EA treatment (50 μmol/l EA is equivalent to the dietary intake of approximately 200 g blackberries or 350 g strawberries; Walgren et al. 1998; Mertens-Talcott et al. 2003; Whitley et al. 2003) effectively blocked VCAM-1 and E-selectin expression in IL-1β-induced HUVEC. It significantly reduced the binding of human monocytic cell line U937 to IL-1β-induced HUVEC. Previous studies also...
showed that other polyphenolic compounds, such as vitamin E (40 μmol/l), probucol (50 μmol/l) or tea flavonoid (60 μmol/l epigallocatechin-3-gallate), reduce cytokine-induced adhesion molecule expression and monocyte adhesion to endothelial cells (Islam et al. 1998; Zapolska-Downar et al. 2001; Ludwig et al. 2004). In the present study, EA reduced cytokine-induced expression of VCAM-1 and E-selectin but not ICAM-1. A similar result was seen when HUVEC were pretreated with probucol; probucol reduced IL-1β-induced VCAM-1 surface protein and mRNA expression, but not ICAM-1 expression (Zapolska-Downar et al. 2001). Previous studies indicated that VCAM-1, but not ICAM-1, plays a critical role in the initiation of atherosclerosis (Cybulsky et al. 2001). VCAM-1 is expressed in vascular lesions in early atherosclerosis and has been found to be elevated in serum from patients with early atherosclerosis, suggesting that this adhesion protein is one of the key molecules involved in the atherogenic process (Cybulsky & Gimbrone, 1991; Rohde et al. 1998).

The NF-κB family controls the expression of genes involved in the inflammation and immune response (Baeuerle, 1991). In the cytoplasm, inactive NF-κB exists as a heterodi-
meric complex of subunits p50 and p65 that binds to a cytoplasmic protein, IkB (Baeuerle & Henkel, 1994). Upon activation, IkB is rapidly degraded, and the p50/p65 heterodimer is translocated from the cytoplasm into the nucleus where the dimer interacts with regulatory κB elements in promoters and enhancers, thereby controlling gene transcription (Baeuerle & Baltimore, 1988; Grilli et al. 1993; Chenbg et al. 1994). NF-κB is activated by a multitude of stimuli, including inflammatory cytokines and reactive oxygen intermediates (Baeuerle & Baltimore, 1988; Grilli et al. 1993; Chenbg et al. 1994; Muller et al. 1997), which are activated in atherosclerotic lesions (Brand et al. 1996; Barnes & Karin, 1997; D’Acquisto et al. 2002). In the present study, we demonstrated that EA reduced cytokine-induced expression of VCAM-1 and E-selectin and prevented the nuclear translocation of p65 and p50 in endothelial cells. The present results suggest that the inhibitory mechanisms of EA might interrupt a signalling cascade involving VCAM transcription-mediated activation of NF-κB.

Several studies have indicated that ROS are implicated in the activation of NF-κB (Muller et al. 1997). The current study shows that the ROS production stimulated by IL-1β was decreased by EA pretreatment (Fig. 2A–C). Based on the present result, we propose that the inhibitory effect of EA on VCAM-1 expression and NF-κB activation may be due to its antioxidant properties and that it may act by directly scavenging free radicals. In one of our previous studies, we found that EA is approximately 2–3-fold more potent than Trolox in antioxidative ability. Our previous results showed that it scavenged α-α-diphenol-β-pircrylhydrazyl (DPPH), alkoxyl radical (RO') and peroxyl radical (ROO') and inhibited LDL oxidation (Yu et al. 2005). Since atherosclerosis is a chronic inflammatory disease associated with increased oxidative stress in the vascular endothelium, it would be conceivable that the anti-atherogenic effects of EA might due to its antioxidative properties. The inhibition of cytokine-induced VCAM-1 expression has been described for other substances with antioxidant properties such as tea flavonoid epigallocatechin-3-gallate, probucol, magnolol, protocatechuic aldehyde and other flavonoids (Zapolska-Downar et al. 2001; Chen et al. 2002; Ludwig et al. 2004; Zhou et al. 2005).

In conclusion, EA inhibits IL-1β-induced VCAM-1 and E-selectin expression in HUVEC through a mechanism that involves NF-κB. It reduces the binding of human monocytic cell line U937 to IL-1β-induced HUVEC, which might be due to its antioxidant properties.

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