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ORIGINAL CONTRIBUTION

Chlorogenic acid attenuates adhesion molecules upregulation in IL-1β-treated endothelial cells

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Abstract
Background Expression of cell adhesion molecules (CAM) on the endothelium and the attachment of monocytes to endothelium may play a major role in the early atherogenic process. Chlorogenic acid is a phenolic compound present in coffee, apples, pears, berries, almonds, artichokes, and aubergines. Previous studies have indicated that CA possesses antioxidant activity in vitro.
Aim We investigated the effects of chlorogenic acid and probucol on monocyte-like adhesion, adhesion molecule expression, NF-κB translocation and ROS production in IL-1β-induced human umbilical vein endothelial cells (HUVECs).
Results According to the results of the MTT assay, we chose 25 and 50 μmol/L to perform the experiments. Chlorogenic acid dose-dependently suppressed IL-1β-induced mRNA expression of vascular cell adhesion molecule-1, intercellular cell adhesion molecule-1 and endothelial cell selectin. Chlorogenic acid also suppressed the IL-1β-induced production of ROS. We also observed that chlorogenic acid attenuated or blocked IL-1β-induced nuclear translocation of nuclear factor-κB subunits p50 and p65, which in turn attenuated CAM expression at the transcription level. Furthermore, chlorogenic acid significantly reduced the adhesion of human monocyte cells (U937) to IL-1β-treated HUVECs in a dose–response manner. These results are similar to that of probucol.
Conclusions We conclude that chlorogenic acid exhibit anti-inflammatory effects in HUVECs by inhibition of U937 monocyte-like adhesion, adhesion molecule expression, NF-κB translocation, and ROS production. The anti-inflammatory activity of chlorogenic acid in HUVECs suggests that chlorogenic acid could be useful in the prevention of atherosclerosis.

Keywords Atherosclerosis · Cell adhesion molecules · Chlorogenic acid · Nuclear factor-κB · Reactive oxygen species

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Introduction

The adhesion of circulating monocytes to the vascular endothelium is a critical early event in the development of atherosclerosis [16]. Many adhesion molecules have been identified on endothelial cells, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular cell adhesion molecule-1 (ICAM-1), and endothelial cell selectin (E-selectin) [9]. The increased expression of adhesion molecules on endothelial cells may lead to further recruitment of monocytes to atherosclerotic sites [27, 35]. Pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α and interleukin (IL)-1, commonly found in atherosclerotic lesions, may induce the activation of chemotactic factors, other cytokines, and cell adhesion molecules, all of which contribute to the inflammatory process [33].

NF-κB represents a group of structurally related and evolutionarily conserved proteins. In mammals, NF-κB consists of five members: Rel (c-Rel), Rel A (p65), Rel B,

NF-κB1 (p50), and NF-κB2 (p52) [13]. Previous studies have indicated that NF-κB/Rel transcription factors may play important roles in the development of atherosclerosis [8, 31]. In the cytoplasm, inactive NF-κB exists as a heterodimeric complex of subunits p50 and p65 that binds to the cytoplasmic protein IκB [31]. Upon activation, IκB 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 [7]. NF-κB is activated by a multitude of stimuli, including inflammatory cytokines and reactive oxygen intermediates [7, 25], which are abundant in atherosclerotic lesions [2]. NF-κB proteins have also been shown to regulate the expression of a variety of genes in atherosclerotic lesions, including those that encode TNF-α, IL-1β, VCAM-1, and ICAM-1 [1]. It is well established that dietary polyphenolic compounds play a significant role in the prevention of atherosclerosis and cardiovascular disease (CVD) [11, 12]. The potential mechanisms of actions in the prevention against the development of atherosclerosis are modulation of serum lipids and influencing the immune and inflammatory processes associated with the development of this disease. Previous studies have indicated that antioxidant, such as vitamin E and flavonoids in tea, may exert their effects by modulating the expression of cytokines and adhesion molecules and the interaction between immune cells and endothelial cells [15, 22, 24]. Chlorogenic acid is a phenolic compound present in fruits and vegetables, including blueberries, strawberries, prunes, apples, grapes, apricots, coffee, and carrots [17, 26, 28]. Previous studies have shown that chlorogenic acid possesses free radical scavenging and metal ion chelating abilities, and that it may inhibit LDL and DNA oxidative damage [17, 19, 32]. In clinical trials, probucol has been shown to have hypocholesterolemic, antioxidative, and antiatherosclerotic effects [4]. Therefore, the present study was designed to examine the effect of chlorogenic acid on monocyte adhesion to cultured human umbilical vein endothelial cells (HUVECs) and the expression of adhesion molecules (VACM-1, ICAM-1, and E-selectin) and to elucidate its possible mechanism of action.

Materials and 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, Life Technologies GmbH, Karlsruhe, Germany) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. All experiments were performed with HUVEC from passages one to three.

DPPH radical scavenging and inhibition of LDL oxidation

The DPPH radical-scavenging activities of chlorogenic acid and probucol were determined according to previous study [37]. LDL ($1.019 < d < 1.063$) was isolated using micro-ultracentrifugation in NaBr–NaCl solution as previously described [23]. LDL oxidation was initiated by adding $CuSO_4$ to a final concentration of 10 μmol/L [36]. The concentration of conjugated dienes in the supernatant was determined by absorption at 234 nm. The IC50 values indicated the amount of sample necessary to decrease the absorbance of DPPH or LDL oxidation by 50%).

Cell viability assay using MTT

Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [6].

Estimation of the production of reactive oxygen species (ROS)

The production of intracellular 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. [3]. The formation of 2′7′- dichlorofluorescein (DCF) was determined by flow cytometry. The excitation wavelength was 488 nm, and 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 VACM-1, ICAM-1, and E-selectin expression

The real-time PCR assay for adhesion molecules was performed according to the method reported by Li [21]. Total cellular RNA was isolated from samples (HUVECs) using the Trizol reagent according to the manufacturer's instructions (Gibco BRL). RT reactions were carried out for each RNA sample in thin-welled PCR tubes using the First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Each reaction tube contained 4.2 μg of total RNA in a volume of 21 μg containing 1× RT buffer, 5.5 mM $MgCl_2$, 500 μM of each dNTP, 2.5 μM of oligo-$d(T)_{12–18}$ primers, 40 U/μL RNase inhibitor, 2 U/μL *E coli* RNaseH, and 50 U/μL of SuperScript II reverse transcriptase. The RT reaction was carried out at 65 °C for 5 min, 42 °C for 50 min and 70 °C for 15 min. The following primers were used:

	Forward primer	Reverse primer
IL-1β	5′-AGATGATAAGCCCACTCTACA-3′	5′-ACATTCAGCACAGGACTCTC-3′
VCAM-1	5′-AAGCGGAGACAGGAGACAC-3′	5′-TGGCAGGTATTATTAAGGAGGATG-3′
ICAM-1	5′-TGGTTCACAGGTTCAGATTAC-3′	5′-GACAAGAGGACAAGGCATAGC-3′
E-selectin	5′-TGTGAGATGCGATGCTGTC-3′	5′-AACCTCTTCTGTCCATTGTCC-3′
GAPDH	5′-CCCACTCCTCCACCTTTG-3′	5′-CTTCCTCTTGTGCTCTTGC-3′

Each tube contained 1 μL of each RT product (200 ng total RNA), 5.5 mM $MgCl_2$, 400 μM dNTP, 500 nM primer (forward and reverse), 0.005 U/μL iTaq DNA polymerase, and 20 nM SYBR Green I in a total volume of 25 μL. Amplification conditions were 3 min at 95 °C for activation, then 40 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 Ct and Mt values were obtained during each reaction. The relative quantification was calculated based on its $2^{-\Delta Ct}$ value. $\Delta Ct = Ct$ (sample) $-$ Ct (control).

Measurement of NF-κB activity

For analysis of NF-κB activity, a TransAM NF-κB Family Kit was used (Active Motif, Rixensart, Belgium). Nuclear extracts were prepared from HUVECs as described by Dschietzig et al. [10]. Briefly, nuclear extracts were incubated in 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 adding the probe. The wells were then washed and incubated with the primary antibodies for p65, p50, for 60 min. After incubation with a horseradish peroxidase-conjugated secondary antibody, a substrate was added to produce a blue color for quantitation by 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

HUVECs (2×10^5) were distributed into 6-well plates and allowed to reach confluence. They were then incubated for 18 h with medium supplemented with chlorogenic acid at concentrations of 25 and 50 μmol/L, followed by incubation for 6 h with 10 ng/mL of IL-1β in the continued presence of CA. U-937, originally derived from a human histiocytic lymphoma, grown in RPMI-1640 medium (Gibco, NY, USA) containing 10% FBS were labeled for 30 min at 37 °C with calcein AM (10 nM, Molecular Probe) (Invitrogen, Carlsbad, CA, USA). Labeled U937 cells (1×10^6) were added to each HUVEC-containing well and incubated for 1 h. Then, adherent U937 cells were determined by a fluorescence plate reader at an excitation wavelength of 485 nm and emission at 530 nm.

Statistics

Results are presented as means ± SD. Statistical significance was checked by one-way ANOVA. Duncan's Multiple Range test was used to analyze significant effects. Differences were considered significant at $P < 0.05$.

Results

Antioxidative capacity of chlorogenic acid in vitro

Free radical scavenging effect was determined using the free radical generator DPPH (2,2-diphenyl-1-picrylhydrazyl) and the IC_{50} of DPPH assay was 36.3 ± 2.7 μmol/L. In the inhibition of LDL oxidation assay, LDL were oxidized using the classical copper-induced LDL autooxidation and the IC_{50} of inhibition of LDL oxidation was 3.1 ± 0.4 μmol/L.

Concentrations of chlorogenic acid and probucol for HUVECs

Cell viability was assayed by the MTT test. After 24 h incubation with 0, 25, 50, 100, 150, 200, 300 μmol/L chlorogenic acid and probucol, cell viability were, respectively, 100.0 ± 0.9 and 100 ± 0.1, 100.2 ± 0.7 and 103.9 ± 5.4, 98.1 ± 3.6 and 112.0 ± 6.9, 99.8 ± 2.0 and 109.2 ± 12.6, 104.0 ± 2.8 and 108.4 ± 7.9, 98.9 ± 3.2 and 93.6 ± 1.5, 82.7 ± 7.8 and 74.8 ± 8.4%. The two highest concentrations caused a significant reduction in cell viability. Therefore, according to the results of the MTT assay, we chose 25 and 50 μmol/L of chlorogenic acid and probucol to do the experiments.

Chlorogenic acid inhibits IL-1β-induced ROS production in HUVECs

Figure 1a and b shows the effect of CA and probucol on IL-1β-induced ROS production. The production of ROS decreased (−89 and −95%) after addition of 25 or 50 μmol/L CA.

Chlorogenic acid inhibits IL-1β-induced cell surface expression of VACM-1, ICAM-1 and E-selectin in HUVECs

The effects of CA and probucol on IL-1β-induced VCAM-1, ICAM-1, and E-selectin expression by HUVECs were studied by pretreating HUVECs for 18 h with 25 or

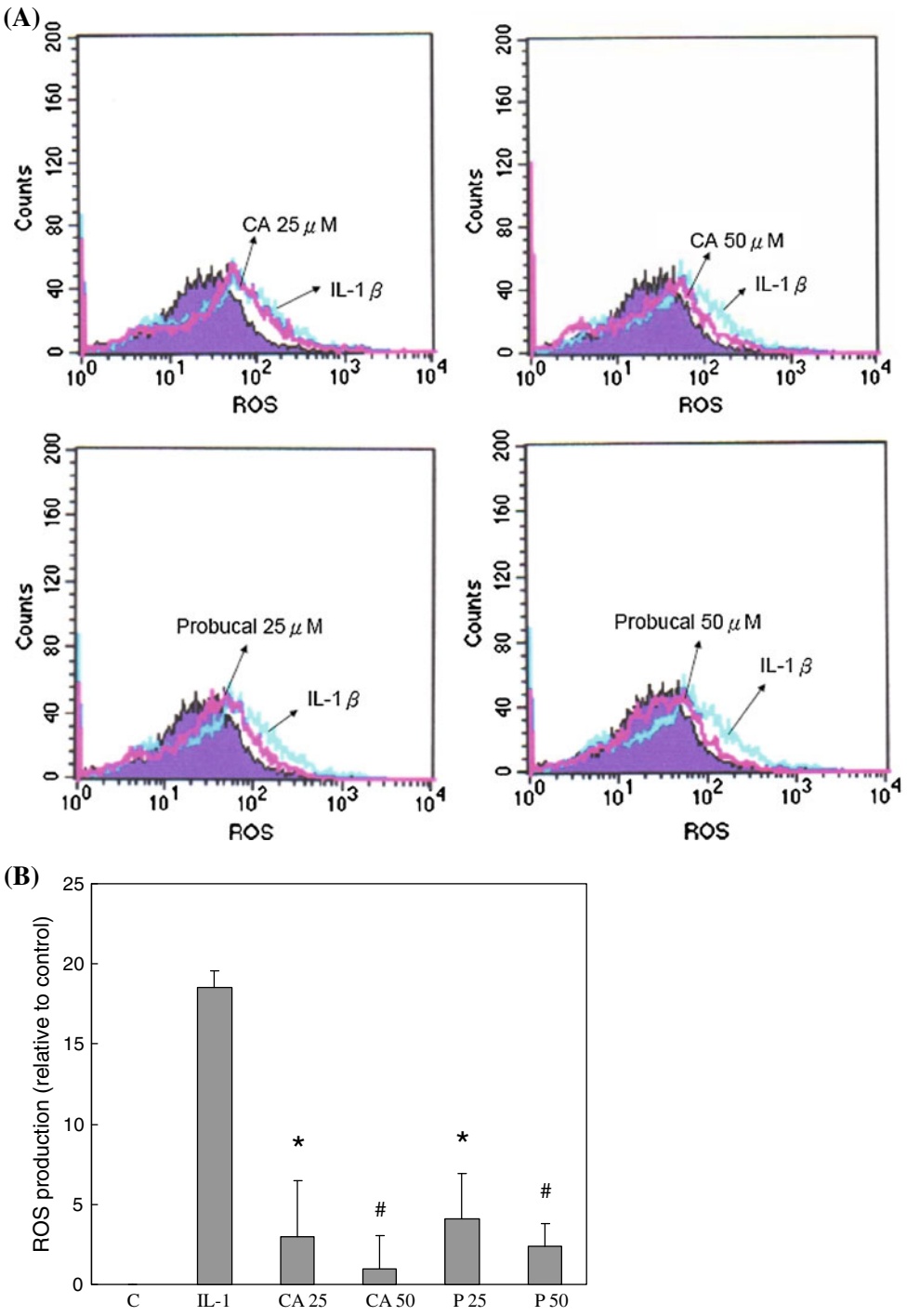

Fig. 1 Effect of chlorogenic acid and probucol on IL-1β-induced ROS production in HUVECs. HUVECs were stimulated with IL-1β(10 ng/mL) after pre-incubation with 25, 50 μmol/L chlorogenic acid or probucol for 18 h. HUVECs were labeled with H_2O_2-sensitive fluorescent probe and the fluorescence was detected by flow cytometry (**a**). Mean ROS production was expressed as relative to control (control = 1). Values are mean ± SD (**b**). *C* Control, *IL-1* Interleukin-1, *CA* Chlorogenic acid, *P* probucol. *$p < 0.05$ group CA25 and 50 and P25 and 50 versus group IL-1, $n = 5$

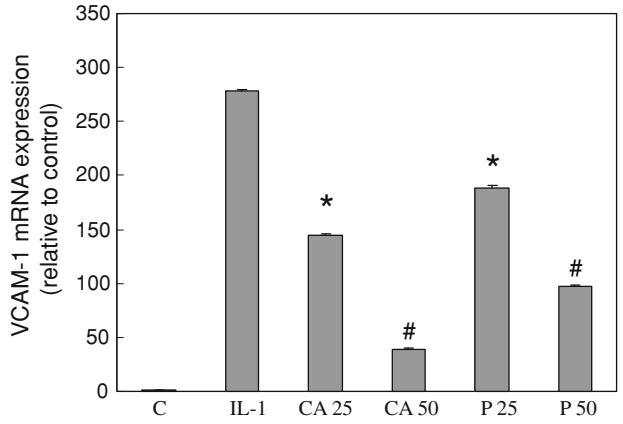

Fig. 2 Effect of chlorogenic acid on VCAM-1 expression in HUVECs. HUVECs were pretreated with 25 or 50 μmol/L CA or probucol for 18 h, and induced by IL-1β(10 ng/mL) for 6 h. The mRNA expression of VCAM-1 was measured by real-time PCR. Mean VCAM-1 expression was expressed as relative to control (control = 1). *C* Control, *IL-1* Interleukin-1, *CA* Chlorogenic acid, *P* probucol. *$p < 0.05$ group CA25, 50 and P25, 50 versus group IL-1, #$p < 0.05$ group CA 50 and P50 versus group CA25 and P25, $n = 5$

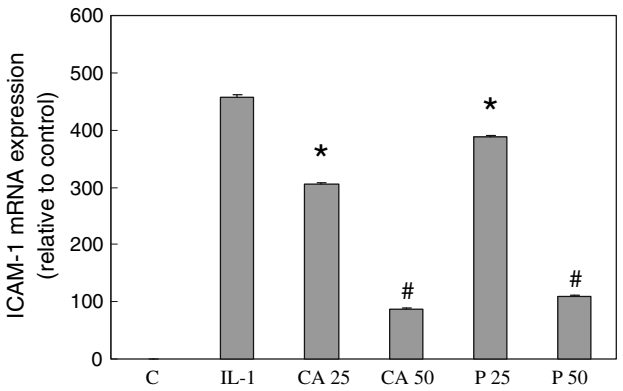

Fig. 3 Effect of chlorogenic acid on ICAM-1 expression in HUVECs. HUVECs were pretreated with 25, 50 μmol/L CA or probucol for 18 h, and induced by IL-1β (10 ng/ml) for 6 h. The mRNA expression of ICAM-1 was measured by real-time PCR. Mean ICAM-1 expression was expressed as relative to control (control = 1). *C* Control, *IL-1* Interleukin-1, *CA* Chlorogenic acid, *P* probucol. *$p < 0.05$ group CA25, 50 and P25, 50 versus group IL-1, #$p < 0.05$ group CA 50 and P50 versus group CA25 and P25, $n = 5$

50 μmol/L CA or probucol before addition of 10 ng/mL IL-1β. CA (25 and 50 μmol/L) caused significantly decreases in cell surface expression of VCAM-1 (−47.8 and −86%), ICAM-1 (−33 and −81%) and E-selectin (−12.9 and −81%). CA was more potent than probucol in inhibiting the expression of adhesion molecules (Figs. 2, 3, 4).

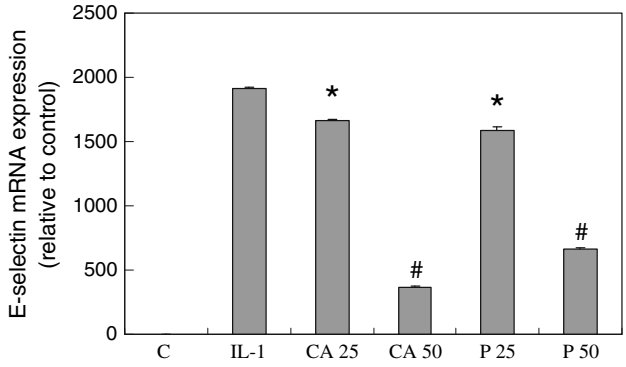

Fig. 4 Effect of chlorogenic acid on E-selectin expression in HUVECs. HUVECs were pretreated with 25, 50 μmol/L CA or probucol for 18 h, and induced by IL-1β (10 ng/mL) for 6 h. The mRNA expression of E-selectin was measured by real-time PCR. Mean E-selectin expression was expressed as relative to control (control = 1). *C* Control, *IL-1* Interleukin-1, *CA* Chlorogenic acid, *P* probucol. *$p < 0.05$ group CA25, 50 and P25, 50 versus group IL-1, #$p < 0.05$ group CA 50 and P50 versus group CA25 and P25, $n = 5$

Chlorogenic acid attenuates activation of NF-κB expression and nuclear translocation of NF-κB subunits p50 and p65 in IL-1β-stimulated HUVECs

To examine whether the inhibitory effect of CA and probucol on the cytokine-induced expression of adhesion molecules is mediated via NF-κB, we measured the nuclear translocation of two protein members of the NF-κB family of transcription factors. Incubation of HUVECs with IL-1β (10 ng/mL) for 6 h induced the nuclear translocation of p50 and p65. Preincubation of HUVECs with CA and probucol prior to IL-1β stimulation significantly prevented the nuclear translocation of p50 and p65. (Fig. 5a, b).

Chlorogenic acid inhibits binding of U937 cells to IL-1β-stimulated HUVECS

To explore the effects of CA and probucol on endothelial cell-leukocyte interactions, we examined the adhesion of U937 cells to cytokine-activated HUVECs. Control confluent HUVECs showed minimal binding to U937 cells, but adhesion substantially increased when the HUVECs were treated with IL-1β. Pretreatment with CA (25 and 50 μmol/L) reduced the adhesion (−32.8 and 60%) of U937 cells to IL-1β-stimulated HUVECs (Fig. 6a, b).

Discussion

In this study, we found that CA significantly reduced the binding of human monocyte cells, U937, to IL-1β-treated

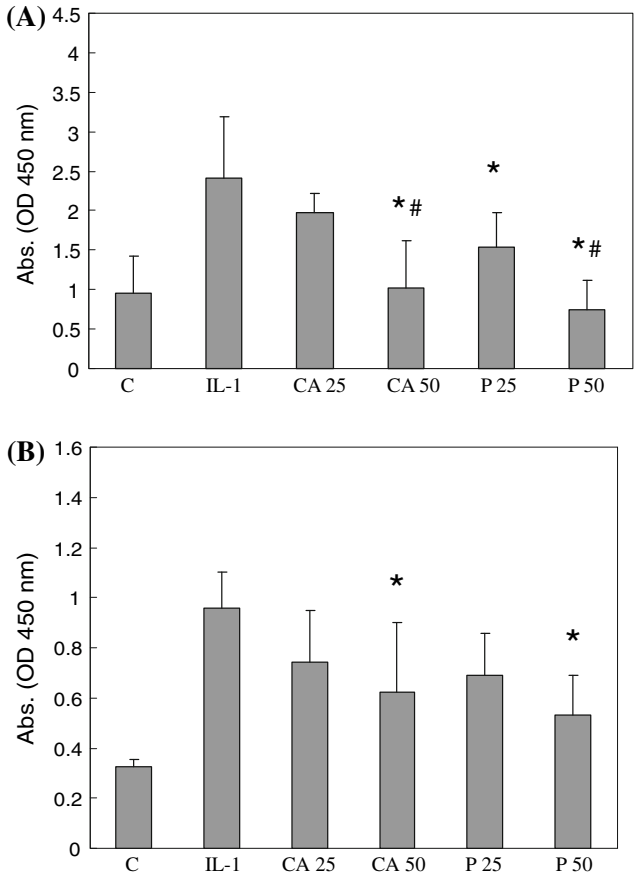

Fig. 5 Effect of chlorogenic acid on IL-1β-induced activation of NF-κB. HUVECs were pretreated with 25, 50 µmol/L CA or probucol for 18 h, and induced by IL-1β(10 ng/ml) for 6 h. Activated NF-κB was assessed in terms of the amount of NF-κB subunits P50 (**a**), P65 (**b**) contained in DNA binding complexes extracted from the nuclei isolated from HUVECs and expressed as absorbance (OD 450). Values are mean ± SD, $n = 3$. *C* Control, *IL-1* Interleukin-1, *CA* Chlorogenic acid, *P* probucol. *$p < 0.05$ group CA 50 and P25, 50 versus group IL-1, #$p < 0.05$ group CA 50 and P50 versus group CA25 and P25, $n = 5$

HUVECs. CA suppressed IL-1β-induced mRNA expression of VCAM-1, ICAM-1 and E-selectin. Studies show that CAM plays a critical role in the initiation of atherosclerosis [26, 33].

Previous study indicated that consumption of high doses of CA (2 g/day) might increase plasma total homocysteine concentration in humans [29]. Previous study also indicated the absorption of CA was 33 ± 17% in Humans [28]. Therefore, the dose we used in this study (0.05 g/day CA) should not cause the increasing of plasma homocysteine concentration. Previous study also indicated that dietary phenols might metabolize to other compounds which had much lower antioxidant activity [30]. Further studies are needed to investigate the antioxidant activity of CA in vivo.

NF-κB is activated by a multitude of stimuli, including inflammatory cytokines and reactive oxygen intermediates [7, 25]. In the present study, we demonstrated that CA reduced cytokine-induced expression of CAM and prevented cytokine-induced the nuclear translocation of p65 and p50 in endothelial cells. These results suggest that CA might interrupt a signaling cascade involving CAM transcription-mediated activation of NF-κB. Several studies have indicated that ROS are implicated in the activation of NF-κB [25]. The current study shows that IL-1β-induced production of ROS decreased in HUVECs that had been pretreated with CA (Fig. 1a, b). Based on this result, we propose that the inhibitory effect of CA on CAM expression and NF-κB activation might be due to its anti-inflammatory properties.

The inhibition of cytokine-induced CAM expression has also been described for other polyphenolic compounds, such as resveratrol (red wine), oleuropein (olive), and other flavonoids [5, 22, 39]. Our results also showed that chlorogenic acid scavenged DPPH as well as RO• and ROO• radicals which produced from LDL oxidation. And the possible chelating effect of Cu^{++} by chlorogenic acid may also inhibit the LDL oxidation (see "Results"). Since atherosclerosis is a chronic inflammatory disease associated with increased oxidative stress in the vascular endothelium, it is conceivable that the antiatherogenic effects of chlorogenic acid are mainly due to its antioxidant properties.

In this study, we found that IL-1β-induced expression of VCAM, ICAM-1, and E-selectin was significantly reduced in HUVECs pretreated with chlorogenic acid. This effect is similar to that of probucol. In clinical trials, probucol has been shown to have hypocholesterolemic, antioxidative, and anti-atherosclerotic effects [4]. Previous studies also indicated that probucol might regulate some aortic gene expression, such as VCAM-1 [38], preserve endothelium-derived relaxing factor action [18], inhibit HDL-mediated cholesterol efflux [34], increase the urinary excretion of oxidized cholesterol [14] and inhibit the ox-LDL-induced adhesion of monocytes to aortic endothelial cells [20], to slow the progress of atherosclerosis. Therefore, further research is needed to investigate the anti-atherogenic mechanism of chlorogenic acid.

We conclude that chlorogenic acid at 25 and 50 µmol/L inhibits U937 monocyte-like adhesion, adhesion molecule expression, NF-κB translocation, and ROS production in HUVECs. The anti-inflammatory activity of chlorogenic acid in HUVECs suggests that chlorogenic acid could be useful in the prevention of atherosclerosis.

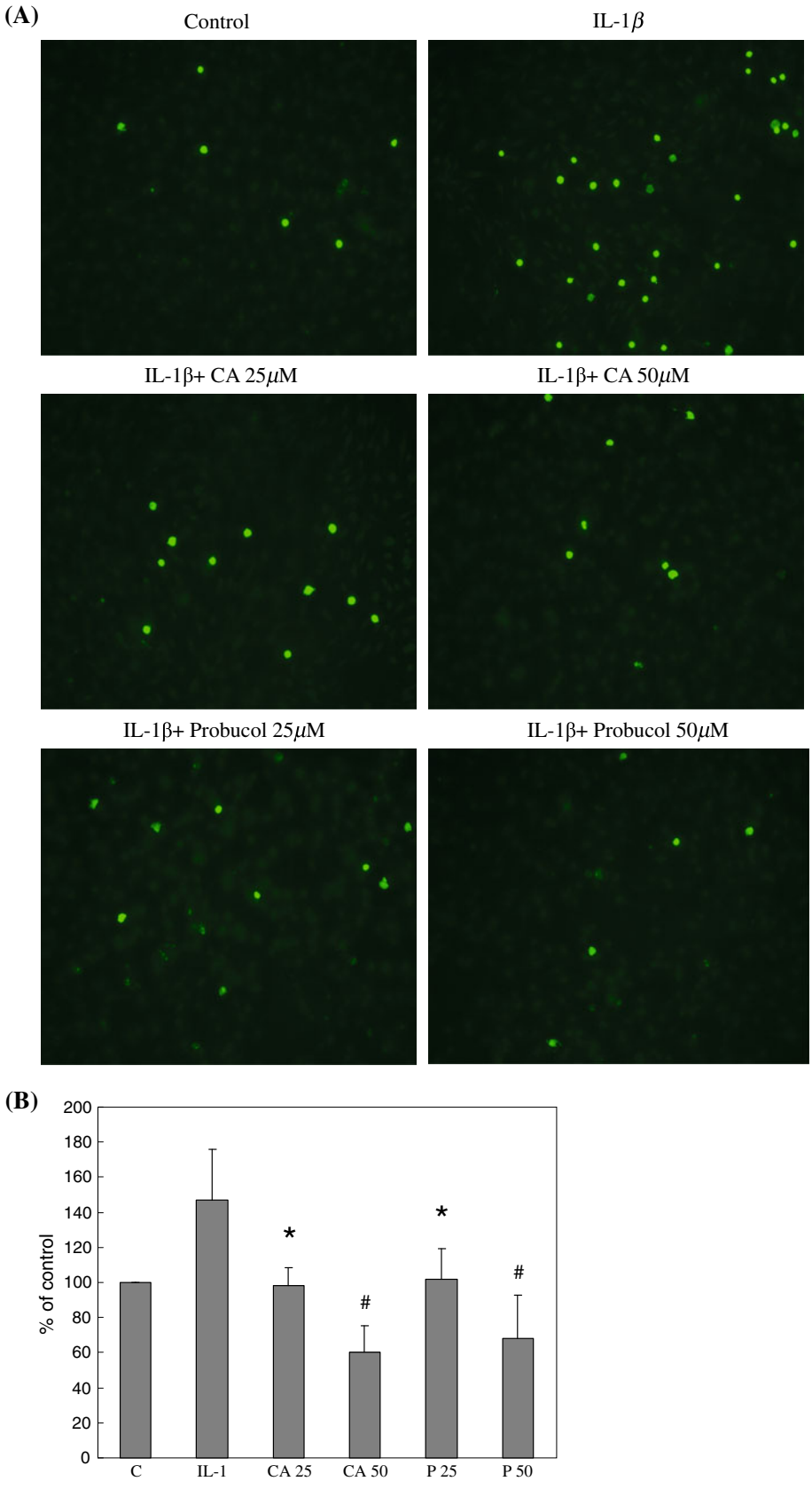

Fig. 6 Effect of chlorogenic acid on U937 monocyte adhesion to IL-1β-activated HUVECs. HUVECs were pretreated with 25, 50 μmol/L CA or probucol for 18 h, induced by IL-1β(10 ng/ml) for 6 h, and cocultured with fluorescence-labeled U-937 for 30 min. **a** Microphotographs were obtained using a fluorescence microscope. **b** Values are mean ± SD and expressed as % of control, $n = 8$. *C* Control, *IL-1* Interleukin-1, *CA* Chlorogenic acid, *P* probucol. $*p < 0.05$ group CA25, 50 and P25, 50 versus group IL-1, $\#p < 0.05$ group CA 50 and P50 versus group CA25 and P25, $n = 5$

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