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RESEARCH ARTICLE

Curcumin inhibits TNFα-induced lectin-like oxidised LDL receptor-1 (LOX-1) expression and suppresses the inflammatory response in human umbilical vein endothelial cells (HUVECs) by an antioxidant mechanism

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Abstract

In this study, the anti-oxidative activities of 70% ethanol extract from *Curcuma aromatica* Salisb. (CAS) and curcumin (CUR) were studied. The CAS extracts and CUR were both found to have a potent scavenging activity against the reactive species tested, as well as an inhibitory effect on LDL oxidation. Cultured human umbilical vein endothelial cells (HUVECs) were stimulated with tumour necrosis factor α (TNFα), expression of intracellular reactive oxygen species (ROS), nitric oxide (NO), endothelial nitric oxide synthase (eNOS), lectin-like oxidised LDL receptor-1 (LOX-1), adhesion molecules, inhibitory kappa Bα (IκBα) and nuclear factor kappa B (NFκB) were measured. In HUVECs stimulated with TNFα, CUR significantly suppressed expression of the intracellular ROS, LOX-1 and adhesion molecules, degradation of IκBα and translocation of NFκB, while inducing production of NO by phosphorylation of eNOS ($p < 0.05$). In conclusion, CAS and CUR may modulate lipoprotein composition and attenuate oxidative stress by elevated antioxidant processes.

Keywords: Curcuma aromatica *Salisb.; curcumin; reactive oxygen species; reactive nitrogen species; antioxidant*

Introduction

Oxidative stress, which is defined as an imbalance between the production of reactive oxygen species (ROS) or reactive nitrogen species (RNS) and antioxidant defence, is considered to be an important pathogenic factor in degenerative diseases such as cardiovascular dysfunctions, atherosclerosis, inflammation, carcinogenesis, drug toxicity, reperfusion injury and neurodegenerative diseases [1]. The antioxidative system evolved not only to eliminate ROS and RNS, but also to adjust the cellular redox state and enable redox signal transduction, however, these systems are not completely efficient. Antioxidants also inhibit the oxidative modification of LDL. Oxidised low-density lipoprotein (Ox-LDL) is a primary constituent of atherosclerotic lesions, which may promote endothelium dysfunction by increasing the production of endothelium-derived ROS and enhancing the expression of adhesion molecules on the endothelium via the lectin-like oxidised LDL receptor-1 (LOX-1) [2,3], therefore, if antioxidant nutrients have the ability to inhibit the formation of atherosclerotic lesions they may be useful for the prevention and treatment of atherosclerotic cardiovascular disease (CVD). Also, Miyake et al. [4] reported that the nuclear factor kappa B (NFκB) decoy oligodeoxynucleotides significantly inhibited neointimal hyperplasia in the rabbit vein graft model, supporting the promotive role of NFκB in blood vessel formation. Therefore, NFκB plays a critical role in activating endothelial cells by an anti-inflammatory signaling pathway during atherosclerosis.

Curcuma aromatica Salisb. (CAS) is widely used as condiment, drug, cosmetic, flavour and in the food industry. It has been used in Korean traditional herbal medicine for the treatment of menstrual pain, severe stomach ache and inflammation of the joints. Turmeric constituents include the three curcuminoids: curcumin, demethoxycu1rcumin, and

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bisdemethoxycurcumin, as well as volatile oils and resins. A number of pharmacological activities including anti-cancer [5], anti-virus [6] and anti-microbial properties [7] have been attributed to curcumin (CUR). In addition, it has been reported that CUR has an inhibitory effect on angiogenesis related to proliferation of cancer cells [8], protective effects on neurotoxicity [9] and allergic encephalomyelitis [10]. Recently, CUR has received attention as a chemopreventive agent due to its anti-inflammatory abilities [11]. However, to date studies concerning anti-atherosclerotic properties by the antioxidant mechanisms of CAS and CUR haven't been conducted.

This study was conducted to evaluate the *in vitro* scavenging activity and inhibitory effect of LDL oxidation of pro-oxidant reactive species in response to treatment with CAS and CUR using biological and non-biological oxidants. In addition to investigate the suppressive effect of CUR on oxidative stress and the inflammatory, atherosclerotic response through the antioxidative mechanism in TNFα-stimulated human umbilical vein endothelial cells (HUVECs).

Materials and methods

Chemicals

Curcumin, Trolox, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS), sodium carbonate (Na_2CO_3), sodium chloride (NaCl), potassium chloride (KCl), Folin-Ciocalteu's phenol reagent, human low-density lipoprotein (LDL), naphthyl ethylenediamine dihydrochloride (NED), ethanol (E), hexane (H), dichloromethane (DCM), ethyl acetate (EA) and n-butanol (B) were purchased from Merck (Merck KGaA, Darmstadt). Gallic acid (GA), potassium persulphate, ascorbic acid (AA), butylated hydroxytoluene (BHT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), potassium ferricyanide ($K3[Fe(CN)_6$), nitro blue tetrazolium (NBT), ethylenediaminetetraacetic acid (EDTA), hypoxanthine, xanthine oxidase, sodium phosphate monobasic (NaH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), hydrogen peroxide (H_2O_2), ferrous sulphate ($FeSO_4$), 4,5-diaminofluorescein (DAF-2), dimethyl sulphoxide (DMSO), 3-morpholinosydnonimine hydrochloride (SIN-1), diethylenetriaminepentaacetic acid (DTPA), copper (II) sulphate ($CuSO_4$), tris base, trichloroacetic acid (TCA), and 2-thiobarbituric acid (TBA) were purchased from Sigma (St Louis, MO). Dihydrorhodamine 123 (DHR 123) and 6-carboxy-2',7'-dichlorofluorescein diacetate (DCFH-DA) were obtained from Molecular Probes (Eugene, OR). Agarose and Coomassie brilliant blue R-250 were purchase from Promega (Madison, WI). Peroxynitrite was obtained from Cayman Chemical (Ann Arbor, MI). The CellTiter96® colorimetric assay kit was purchased from Promega (Madison, WI) and the NucBuster™ protein extraction kit was purchased from Novagen (Darmstadt). The protein extraction solution was purchased from Intron Biotechnology (Gyeonggi-do, Korea). The polyvinylidene fluoride membrane and ECL kit were obtained from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire). The human MCP-1 ELISA kit was purchased from Endogen (Thermo Fishen Scientific, Rockford, IL). The anti-LOX-1 anti body and TNFα were obtained from R&D Systems (Madison, WI). The anti-VCAM-1, anti-ICAM-1, anti-IκB-α, anti-NFκB p65, goat anti-rabbit IgG-HRP, goat anti-mouse IgG-HRP and donkey anti-goat IgG antibodies were purchased from Santa Cruz Biotechology (Santa Cruz, CA). The anti-β-actin, anti-eNOS and anti-phospho-eNOS antibodies were purchased from Cell Signaling Technology (Beverly, MA). All other chemicals used were of analytical grade.

Antioxidative effects of **Curcuma aromatica** *Salisb.* **in vitro**

CAS (500 g, purchased from Dongguk University Gyeongju Oriental Hospital, Gyeongju, Gyeongbuk) was ground and refluxed three times (12 h, 6 h, 3 h) with 70% ethanol (ethanol:water, 70:30, E) solution (20-fold) and then filtered through a glass filter funnel (G4). The extract was then gathered and the ethanol was evaporated under reduced pressure at 45°C in a rotary vacuum evaporator (Buchi, Flawil, Switzerland), followed by lyophilisation. The dried extract was then suspended in 50 mL distilled water and the aqueous suspension was partitioned sequentially with hexane (H), dichloromethane (DCM), ethyl acetate (EA), n-butanol (B) and aqueous (A) in a 1:1 ratio (v/v) at room temperature. The resulting extracts were then evaporated under a rotary vacuum evaporator to dryness to give the **H**, **DCM**, **EA**, and **B** fractions. They were then quantitatively re-dissolved in a 30% ethanol solution. The stock solutions were kept at 4°C in the dark until further analysis. The content of the total phenolic compounds was determined by the Folin-Ciocalteu's reaction [12], using gallic acid (GA) as a standard. The total antioxidant activities of the CSL extracts and CUR were measured by the $ABTS^+$ radical cation ($ABTS^{+}$) decolourisation assay [13]. The DPPH radical scavenging activities of the CAS extract and CUR were determined by the method according to Gyamfi et al. [14] with a slight modification. The conditions of the NBT assay were adapted from Gotoh and Niki [15]. The hydroxyl radical (·OH) scavenging activity of CAS extracts and CUR were assessed using the method described by Halliwell and Gutteridge [16]. A DAF-2 assay [17] was used to measure the nitric oxide radical (NO) scavenging ability. The peroxynitrite ($ONOO^-$) scavenging activities of the CAS extracts and CUR were determined using the method described by Kooy et al. [18], with a slight modification. The relative electrophoretic mobility(REM) of human LDL was determined by agarose gel electrophoresis according to the method described by Yoon et al. [19] with AA used as a positive control.

Anti-atherosclerotic effects of CUR in HUVECs

HUVECs purchased from Lonza Walkersville (Walkersville, MD) were cultured in endothelial cell basal medium-2 (EGM-2) (Clonetics, Walkersville, MD). The cells from passages four to seven were used; cells were maintained in an incubator at 37°C in an atmosphere of 5% CO_2. For all experiments, the HUVECs were grown to 80%–90% confluence and

made quiescent by starvation for at least 18 h. Cell viability was ascertained using a CellTiter96® colorimetric assay (Promega) using 3-(4,5-dimethylthiazol-2-yl]-5-(3-carboxymethoxyphenyl)-2-(4-sulphenyl)-2H-tetrazolium (MTS) according to the manufacturer's instructions. Intracellular ROS determinations were made using the ROS-sensitive fluorescent dye, 2',7'-dichlorofluorescein diacetate (DCF-DA) as previously described [20]. HUVECs (1×10^5) were pre-treated with different concentrations of CUR for 1 h before being incubated with 10 ng/mL TNFα for 12 h. The nitrite concentration in the culture medium was measured as an indicator of NO production, according to the Griess reagent system. A nitrite standard curve was made by making serial dilutions of nitrite solution with media. We added 50 μL of each supernatant to wells and mixed with 50 μL of a sulphanilamide solution, then incubated for 5–10 min at room temperature in the dark. The same volume of naphthyl ethylenediamine dihydrochloride (Merck) solution was added to all the wells, incubated for 5–10 min at room temperature and protected from light. Absorbance of the mixture at 540 nm was measured with a microplate plate reader (Molecular Devices, Sunnyvale, CA). HUVECs (1×10^5) were dispensed into wells of a 24-well plate and pretreated with CUR for 1h, after which they were stimulated with 10 ng/mL TNFα. The culture supernatants were subsequently isolated and the amount of MCP-1 production was measured using an enzyme-linked immunosorbent assay (ELISA, BioRad, MI, USA) kit from according to the manufacturer's instructions. HUVECs (1×10^5) were pre-incubated with various concentrations of CUR for 1 h, before stimulated with 10 ng/mL TNFα. After 12 h of incubation, the cells were washed twice using ice-cold PBS and harvested by scraping in 1 mL of ice-cold PBS. After centrifugation at 13 000 rpm, cell pellets were lysated in a protein extraction solution (Intron Biotechnology, Gyeonggi-do, Korea). Nuclear protein extract obtained from the lysated cells were prepared using the NucBuster™ protein extraction kit (Novagen). Protein concentration was determined with a commercial protein assay kit (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard. Equal protein concentrations were subjected to 7.5%–12.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane (Amersham Pharmacia Biotech). After blocking with PBS containing 5% non-fat dry milk for 1 h at room temperature, each membrane was incubated with the specific primary antibody overnight at 4°C. Anti-β-actin monoclonal antibody (mAb), anti-eNOS polyclonal antibody and anti-phospho-eNOS (Ser1177) polyclonal antibody were purchased from Cell Signaling Technology. Monoclonal anti-human LOX-1 antibody was purchased from R&D Systems. Anti-VCAM-1 polyclonal antibody, anti-ICAM-1 polyclonal antibody, anti-IκBα polyclonal antibody and anti-NFκB p65 mAb were purchased from Santa Cruz Biotechology. After washing twice with PBS containing 0.1% Tween-20 (PBST), each membrane was immunoblotted with horseradish peroxidase-conjugated anti-mouse, anti-rabbit or anti-goat IgG antibodies (Santa Cruz Biotechnology) for 1 h at room temperature, followed by washing three times in PBST and visualisation was by enhanced chemiluminesence (ECL) ,Amersham Pharmacia Biotech. Vision Works Image Software (UVP, Cambridge) was used to measure the band intensities.

Statistical analysis

All experiments were performed at least three times by conducting each assay in triplicate. Data was analysed by SPSS version 17.0 for Windows (SPSS, Chicago, IL) and expressed as the mean ± standard deviation. Statistical analyses were conducted using analysis of variance (ANOVA–Tukey's test) between groups and statistical significance was considered where $p < 0.05$.

Results

Yield of fraction and total phenolics

The total phenolic content of the extracts, as estimated by the Folin-Ciocalteu reagent method, ranged from 5.59 μg GA eq/mg (**A** layer) to 172.72 μg GA eq/mg (**EA** fraction), and increased in the following order: **EA** > **H** > **B** > **DCM** > **H** > **A** layer (Table 1).

Antioxidant activity as determined by the ABTS^{+} and DPPH assays

Table 2 shows the antioxidant capacities of CAS extracts and CUR as determined by the TEAC assay. The extracts showed generally high antioxidant capacities that ranged from 0.548 to 1.112 mmol Trolox equivalents. The antioxidant activities of CUR, which is the major component isolated from EA of CAS, showed a significant difference ($p < 0.05$), their TEAC value was 1.916 mmol Trolox equivalent units. To our knowledge, there have been no prior reports regarding the antioxidant activity of total and organic solvents fractionation from this plant, therefore the data generated by the present study provides valuable preliminary data. However, isolation and characterisation of the individual active components of CAS, as well as evaluation of the *in vivo* relevance of such activity requires further study.

Table 1. Extraction yields and content of total phenolics in the extracts of *Curcuma aromatica* Salisb.

Sample[1]	Yield (%)[2]	Total phenolics (μg GA eq/mg)[3]
E	3.49	88.82 ± 2.99[b]
H	0.15	55.92 ± 0.69[e]
DCM	0.2	65.36 ± 3.22[d]
EA	0.08	172.72 ± 2.1[a]
B	0.15	75.14 ± 1.92[c]
A	0.17	5.59 ± 0.2[f]

[1] **E**, 70% ethanol extract; **H**, hexane fraction; **DCM**, dichloromethane fraction; **EA**, ethyl acetate fraction; **B**, butanol fraction; **A**, aqueous layer.
[2] Extraction yield is expressed as the percentage dry weight of *Curcuma aromatica* Salisb.
[3] Each value represents the mean ± SE of triplicate measurements.
[a–f] Values with different superscripts in the same column are significantly different at a $p < 0.05$ by Tukey's test.

Table 2. Antioxidant activities of the extracts of *Curcuma aromatica* Salisb. and curcumin as determined by the ABTS·+ and DPPH assay.

Sample[1]	TEAC[2](mM Trolox equivalent)	Free radical scavenging activities[3] (IC_{50} = μg/mL)
E	0.9 ± 0.023[d]	1 450.96 ± 124.07[d]
H	0.596 ± 0.028[e]	5 182.66 ± 56.39[a]
DCM	0.548 ± 0.017[e]	3 105.34 ± 157.06[c]
EA	1.112 ± 0.045[c]	902.65 ± 97.52[e]
B	0.582 ± 0.009[e]	4 058.31 ± 108.4[b]
A	0.551 ± 0.012[e]	NA
CUR	1.916 ± 0.162[b]	130.61 ± 11.21[g]
AA	2.349 ± 0.01[a]	72.06 ± 1.72[h]
BHT	1.868 ± 0.03[b]	321.39 ± 8.07[f]

[1]**E**, 70% ethanol extract; **H**, hexane fraction; **DCM**, dichloromethane fraction; **EA**, ethyl acetate fraction; **B**, butanol fraction; **A**, aqueous layer; CUR, curcumin.

[2,3] Each value represents the mean ± SE of triplicate measurements.

[a–h] Values with different superscripts in the same column are significantly different at a $p < 0.05$ by Tukey's test.

The free radical scavenging effects of CAS extract and CUR under investigation on DPPH are shown in Table 2. Among the extracts examined, the **EA** fraction exhibited the strongest efficiency and showed over 50% scavenging effect of the DPPH at a concentration of 902.65 ± 97.52 μg/mL, followed by the **E** extract (IC_{50} = 1 450.96 ± 124.07 μg/mL). These values were higher than that of the positive control, which was 72.06 ± 1.72 (AA).

ROS (superoxide anion and hydroxyl radical) scavenging activity

The half maximal inhibitory concentration (IC_{50}) values of the superoxide anion scavenging activity of all of the test samples from CAS and CUR are shown in Table 3. CAS and CUR had significant scavenging activities on the superoxide anion, and this effect occurred in a dose-dependent manner ($p < 0.05$). The **EA** fraction exerted the strongest scavenging activity (IC_{50} = 2 348.48 ± 90.58 μg/mL) ($p < 0.05$), while the **H** fraction and the **A** layer didn't show any scavenging activities. Taken together, these results suggest that the CAS extracts exhibit scavenging effect on superoxide anion generation that could help prevent or ameliorate oxidative damage. The scavenging activities of CAS and CUR on hydroxyl radicals are shown in Table 3. CAS showed a high enough scavenging activity to be considered a potent hydroxyl radical scavenger. The IC_{50} value of the **EA** fraction of CAS was 275.85 ± 18.12 μg/mL. The ability of the above mentioned extracts to quench hydroxyl radicals seems to be directly related to prevention of propagation of the process of lipid peroxidation and the extract also appears to be a good scavenger of ROS.

RNS (nitric oxide radical and peroxynitrite) scavenging activity

The CAS extract and CUR inhibited the ·NO induced oxidation of DAF-2 to triazolofluorescein (Table 3), which were indicated by IC_{50} values of 84.07 ± 3.69, 75.46 ± 1.11 and 11.8 ± 0.57 μg/mL for the **E**, **DCM** and **EA** fractions of CAS, respectively. The value of CUR was much lower than that of the positive control, which was 0.92 ± 0.04 μg/mL, implying that CUR could act as a potent scavenger of ·NO. The peroxynitrite scavenging activities of CAS and CUR were investigated and the results were compared with those of reference antioxidants (Table 3). The need for a higher extract concentration to scavenge radicals indicates a lower antioxidant activity. The CAS extract and CUR inhibited the peroxynitrite induced oxidation of the DHR reaction mixture, with the peroxynitrite scavenging activity being the highest in the **DCM** fraction. Taken together, these data imply that CAS extract and CUR are effective scavengers of RNS.

Table 3. ROS (superoxide anion and hydroxyl radical) and RNS (nitric oxide radical and peroxynitrite) scavenging activities of the extracts of *Curcuma aromatica* Salisb.

	ROS (IC_{50} = μg/mL)	
Sample[1]	Superoxide anion[2]	Hydroxyl radical[3]
E	5 682.77 ± 219.49[c]	1 435.73 ± 34.88[c]
H	NA	NA
DCM	7 659.44 ± 223.58[b]	1,880.8 ± 167.94[a]
EA	2 348.48 ± 90.58[e]	275.85 ± 18.12[e]
B	8 601.1 ± 97.1[a]	1 269.48 ± 55.29[c]
A	NA	1 623.19 ± 28.88[b]
CUR	NA	NA
AA	1 829.26 ± 85.58[d]	480.69 ± 26.39[d]
BHT	NA	NA
	RNS (IC_{50} = μg/mL)	
Sample	Nitric oxide radical[4]	Peroxynitrite[5]
E	84.07 ± 3.69[a]	36.33 ± 2.94[d]
H	NA	53.17 ± 2.38[b]
DCM	75.46 ± 1.11[b]	45.16 ± 3.83[c]
EA	11.80 ± 0.57[c]	25.7 ± 1.09[e]
B	NA	53.59 ± 1.96[b]
A	NA	71.27 ± 1.35[a]
CUR	0.92 ± 0.04[e]	29.41 ± 0.82[e]
AA	8.18 ± 0.23[d]	5.14 ± 0.13[f]
BHT	NA	64.96 ± 13.88[a]

[1]**E**, 70% ethanol extract; **H**, hexane fraction; **DCM**, dichloromethane fraction; **EA**, ethyl acetate fraction; **B**, butanol fraction; **A**, aqueous layer; CUR, curcumin.

[2–5] Each value represents the IC_{50}, mean ± SE of triplicate measurements.

[a–f] Values with different superscripts in the same column are significantly different at a $p < 0.05$ by Tukey's test. NA is not active.

Inhibitory effects of CuSO4-induced human LDL oxidation by relative electrophoretic mobility (REM) assay

Figure 1 shows the effect of CAS and CUR on the REM of LDL peroxidation induced by Cu^{2+}. If the REM of native LDL is assumed to be 1, the addition of Cu^{2+} caused the REM to increase to 2.17 in response. In addition, the data showed that LDL peroxidation can be suppressed by the addition of CAS extracts, as indicated by the REM value being reduced to 1.33 and 1 in response to treatment with a concentration of 5 μg/mL of the **EA** fraction and CUR, respectively. In this study, the abilities of CAS and CUR to scavenge free radicals were further confirmed by the inhibition of LDL

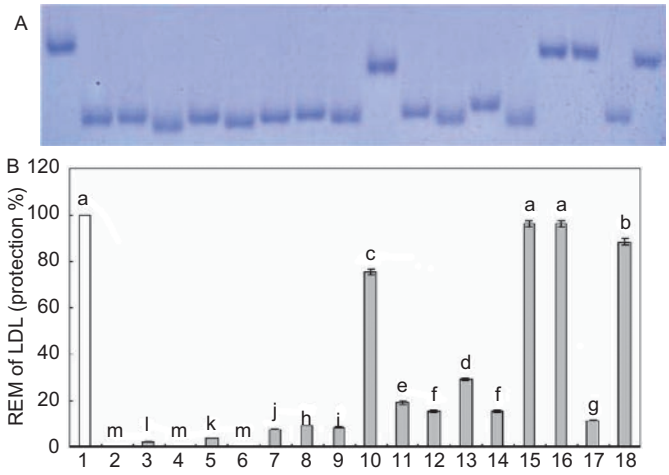

Figure 1. The relative electrophoretic mobility (REM) of human LDL incubated with Cu^{2+} and with or without extracts of *Curcuma aromatica* Salisb. LDL (120 μg/mL) was oxidized with 10 μM $CuSO_4$ at 37°C in the presence of CAS extracts and CUR for 12 h. (A) Lane 1: native LDL; Lane 2: LDL+Cu^{2+}; Lane 3,4: LDL+Cu^{2+}+1, 5 μg of **E**; Lanes 5,6: LDL+Cu^{2+}+1, 5 μg of **H**; Lanes 7,8: LDL+Cu^{2+}+1, 5 μg of DCM; Lanes 9,10: LDLCu^{2+}+1, 5 μg of **EA**; Lanes 11,12: LDL+ Cu^{2+}+ 1, 5 μg of **B**; Lanes13,14: LDL+Cu^{2+}+1, 5 μg of **A**; Lanes 15,16: LDL+Cu^{2+}+1, 5 μg of CUR; Lanes 17, 18: LDL+Cu^{2+}+1, 5 μg of AA (B) Protection rate (%), Each value represents the mean ± SE of triplicate measurements. $^{*a\text{-}l}$ Bars with different letters are significantly different at a $p<0.05$ by Tukey's test.

peroxidation. Based on the data shown in Figure 1, CAS and CUR have the potential to prevent atherosclerosis via suppression of LDL oxidation. Consequently, dietary antioxidants that protect LDL from oxidation may help to reduce atherogenesis and prevent coronary heart disease. These results revealed that CAS extracts could convert free radicals to more stable products and terminate the radical chain reaction, thereby supplying antioxidant action. Collectively, these remarkable properties indicate that CAS has significant antioxidant activity.

Effect of CUR on cell viability and ROS generation in TNFα-stimulated HUVECs

To evaluate the effect of CUR on cell growth in HUVECs, viable cell numbers were estimated using an MTS assay. Incubation of CUR alone or TNFα-stimulated HUVECs with 1-25 μg CUR for 12 h did not have a discernable cytotoxic effect (Figures 2A and 2B). To evaluate if the inhibition of oxidative stress by CAS and CUR *in vitro* affect the ROS generation in TNF α-stimulated HUVECs, ROS-specific experiments using the ROS-sensitive fluorescent dye DCF-DA were performed. The level of ROS in TNFα-stimulated HUVECs was markedly increased in comparison with the vehicle. CUR treatment reduced production of ROS in TNFα-stimulated HUVECs to the basal level in a dose-dependent manner; the levels of ROS were suppressed by 4%, 25%, 37% and 50% at concentrations of 1, 5, 10 and 25 μg/mL, respectively (Figure 3).

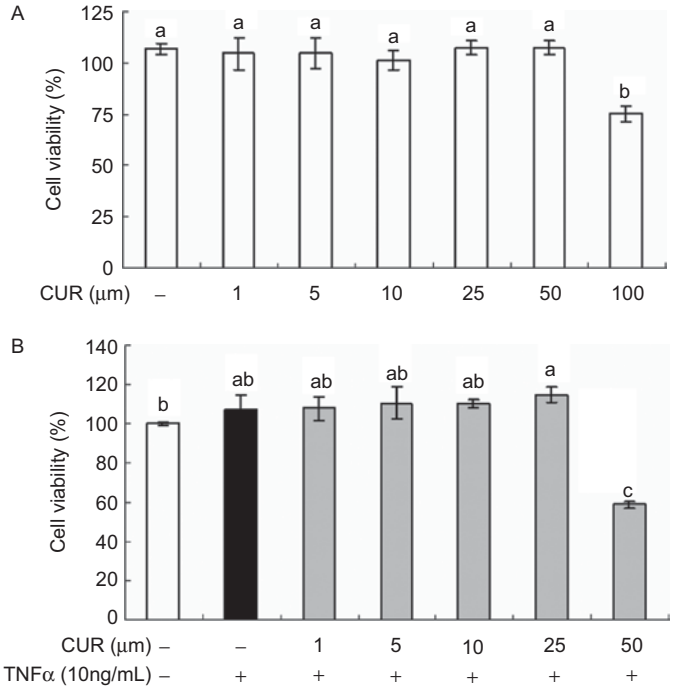

Figure 2. Effect of curcumin on cell viability in HUVECs. Cell viability was evaluated by MTS assay. (A) HUVECs were treated with different concentration of curcumin for 12 h. (B) HUVECs were pretreated with different concentration of curcumin for 1 h before being incubated with 10 ng/mL TNFα for 12 h. Values are mean ± SD (n = 3). *a-cBars with different letters are significantly different at $p<0.05$ by Tukey's test.

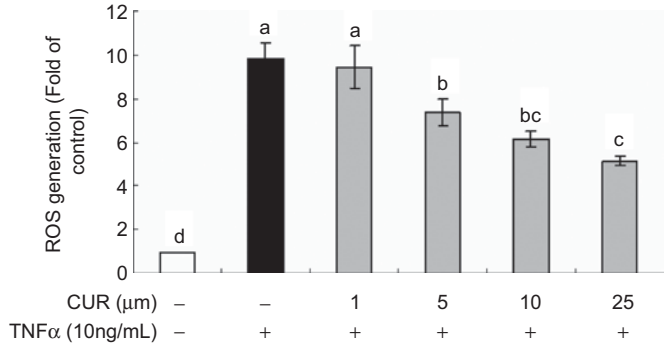

Figure 3. Effect of curcumin on TNFα-induced intracellular ROS generation in HUVECs. HUVECs were pretreated with different concentrations of curcumin for 1 h before being incubated with 10 ng/mL TNFα for 12 h. In the final 30 min of treatment, 10 μM DCF-DA was added. Values are means ± S.D. (n = 3). Values are expressed as compared with TNFα-induced ROS generation without curcumin. *a-dBars with different letters are significantly different at $p<0.05$ by Tukey's test.

Effect of CUR on eNOS phosphorylation and NO production in TNFα-stimulated HUVECs

We tested whether treatment with CUR influences the synthesis of NO and phosphorylation of eNOS in HUVECs. We observed that the increase in NO production secondary to CUR treatment in TNFα-non-stimulated and TNFα-stimulated HUVECs was dose-dependent as expected (Figure 4A and 4B). Phosphorylation of eNOS by CUR

treatment showed a similar tendency with NO production in TNFα-stimulated HUVECs (Figure 4C). NO induction and eNOS phosphorylation reached a maximum of 1.8-fold, with 1.5-fold at a concentration of 25 μg/mL of CUR after 12 h of treatment, respectively. CUR, at low concentrations of 1 μg/mL, didn't induce NO production and eNOS phosphorylation. In conclusion, CUR had an effect on induced NO production by the phosphorylation of eNOS and the suppressed inflammatory events resulted in protection from atherosclerosis.

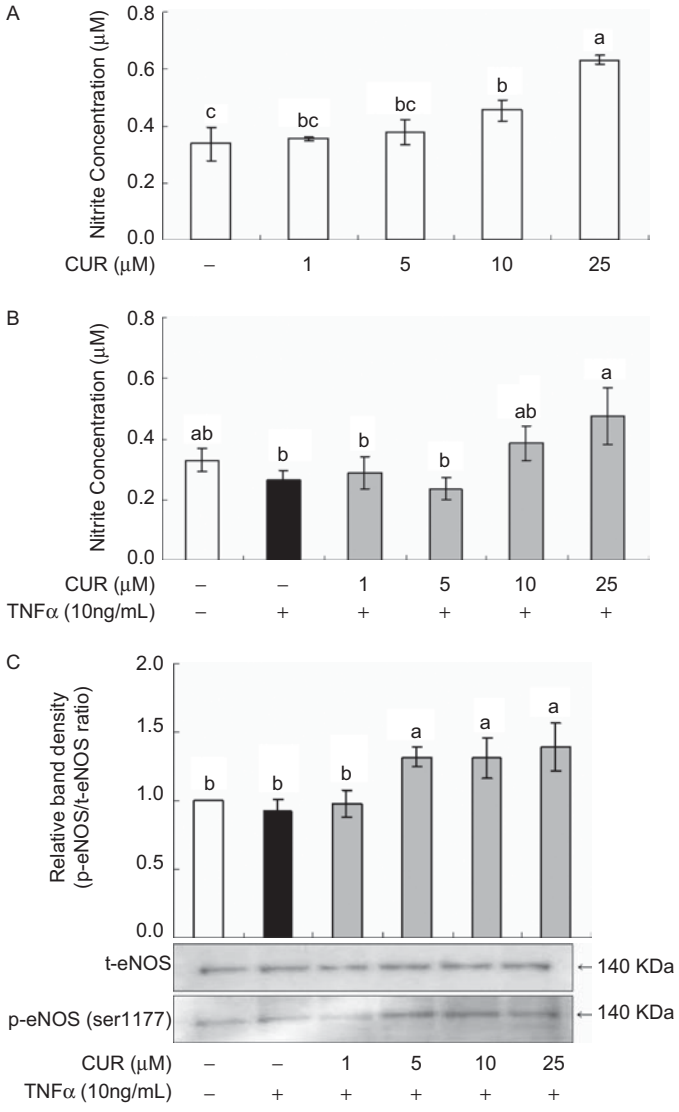

Figure 4. Effect of curcumin on nitric oxide (NO) production and endothelial nitric oxide synthase (eNOS) activation in HUVECs. (A) HUVECs were treated with different concentrations of curcumin for 12 h. (B) HUVECs were pretreated with different concentration of curcumin for 1 h before being incubated with 10 ng/mL TNFα for 12 h. The culture supernatants were subsequently isolated and analysed for nitrite levels. (C) HUVECs were pretreated with different concentrations of curcumin for 1 h before being incubated with 10 ng/mL TNFα for 12 h and subjected to western blotting using an antibody specific for eNOS and phospho-eNOS (Ser1177). The calculated data was compared with the densitometry quantification of bands from the vehicle. Values are mean ± SD (n=3). $^{*a\text{-}c}$Bars with different letters are significantly different at $p < 0.05$ by Tukey's test.

Effect of CUR on LOX-1 expressions in TNFα-stimulated HUVECs

Western blot analysis of cell lysates for LOX-1 expression indicated that the stimulation of cells with TNFα sharply increased LOX-1 expression. The simultaneous incubation of the cells with TNFα and CUR resulted in a significant inhibition of receptor expression in a concentration-dependent manner when >1 μg/mL of CUR was used (Figure 5). In particular, high levels of CUR (25μg/mL) resulted in a significant reduction (80%) in LOX-1 cell expression compared with treatment solely with TNFα.

Effect of CUR on VCAM-1 and ICAM-1 expression in TNFα-stimulated HUVECs

We observed that CUR reduced adhesion molecules in the endothelial cells stimulated with TNFα. To determine if the inhibition of LOX-1 expression on the endothelial cells induced by CUR, was associated with a decrease in the expression of adhesion molecules such as VCAM-1 and ICAM-1, HUVECs were cultured with CUR and TNFα. Protein expression of adhesion molecules in TNFα-stimulated HUVECs were determined by β-actin as an internal standard. TNFα-induced expression of VCAM-1 and ICAM-1 showed a 15- and 36-fold increase, respectively (Figures 6A and 6B), in comparison with control cells. CUR treatment reduced the expression of VCAM-1 and ICAM-1 significantly in the TNFα-stimulated HUVECs. In particular, the expressions of VCAM-1 and ICAM-1 were reduced by 48% and 60% at

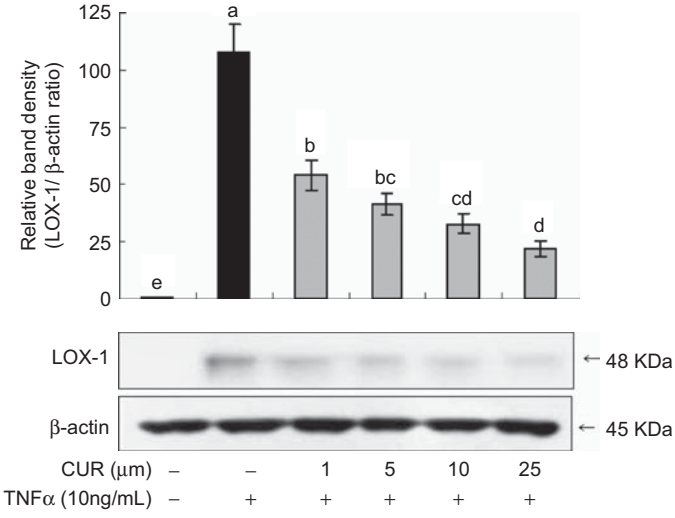

Figure 5. Inhibition of TNFα-induced lectin-like oxidized LDL receptor-1 (LOX-1) expression by curcumin. HUVECs were pretreated with different concentration of curcumin for 1 h before being incubated with 10 ng/mL TNFα for 12 h and subjected to western blotting using antibody specific for human LOX-1. The calculated data was compared with the densitometry quantification of bands from the vehicle. Values are mean ± SD (n = 3). $^{*a\text{-}d}$Bars with different letters are significantly different at $p < 0.05$ by Tukey's test.

concentration of 25 μg/mL, respectively. We reasoned that if TNFα signalled NFκB activation through a pathway that involved ROS, CUR pretreatment might bolster antioxidant defences and thereby suppress activation of the NFκB. We found that with combined CUR and TNFα treatment, the translocation of NFκB to the nucleus was decreased, and the VCAM-1 and ICAM-1 proteins were decreased in a dose-dependent manner when compared with the TNFα-induced control.

Effect of CUR on MCP-1 production in TNFα-stimulated HUVECs

To determine if the inhibition of the adhesion molecule expression by CUR was associated with a possible down-regulation in chemoattractant molecules expression, HUVECs were cultured with TNFα and CUR. MCP-1 production was evaluated by ELISA assay. As expected when the cells were TNFα-treated there was an 8% increase in MCP-1 production in comparison with the vehicle (Figure 7). Interestingly, when the cells were cultured with CUR plus TNFα, MCP-1 production was significantly diminished ($p < 0.05$) and the values for TNFα-stimulated HUVECs, TNFα +25 μg/mL CUR-treated HUVECs was similar to that of the vehicle. MCP-1 is critical to the initiation and development of atherosclerotic lesions. In this report, we have examined the role of CUR on the regulation of MCP-1 production in HUVECs. We observed that CUR treatment decreased MCP-1 production in the HUVECs (Figure 7). These findings support the notion that CUR treatment effect on production of MCP-1 via the regulation of NFκB translocation in the TNFα-stimulated HUVECs.

Effect of CUR on IκBα degradation and NFκB nuclear translocation in TNFα-stimulated HUVECs

Since the induction of NFκB activity results in the phosphorylation and rapid loss of IκBα protein through proteolysis, we evaluated if the CUR could decrease both the TNFα-induced IκBα degradation and the nuclear translocation of NFκB (Figures 8A and 8B). The IκB degradation was markedly increased in TNFα-stimulated HUVECs compared with the vehicle, while it significantly decreased the CUR treated HUVECs (Figure 8A). The p65 subunit increased significantly in the nuclear fraction of HUVECs induced by TNFα compared with the non-TNFα treated control cultures ($p < 0.05$), yet these elevations were counteracted by the CUR treatment (Figure 8B), maintaining the nuclear translocation of the vehicle. The CUR concentration used in this test was the same amount as that which reduced protein expression of cell adhesion molecules after TNFα induction. We observed a marked decrease in the NFκB translocation in cells cultured with CUR plus TNFα, these results confirm that CUR has an

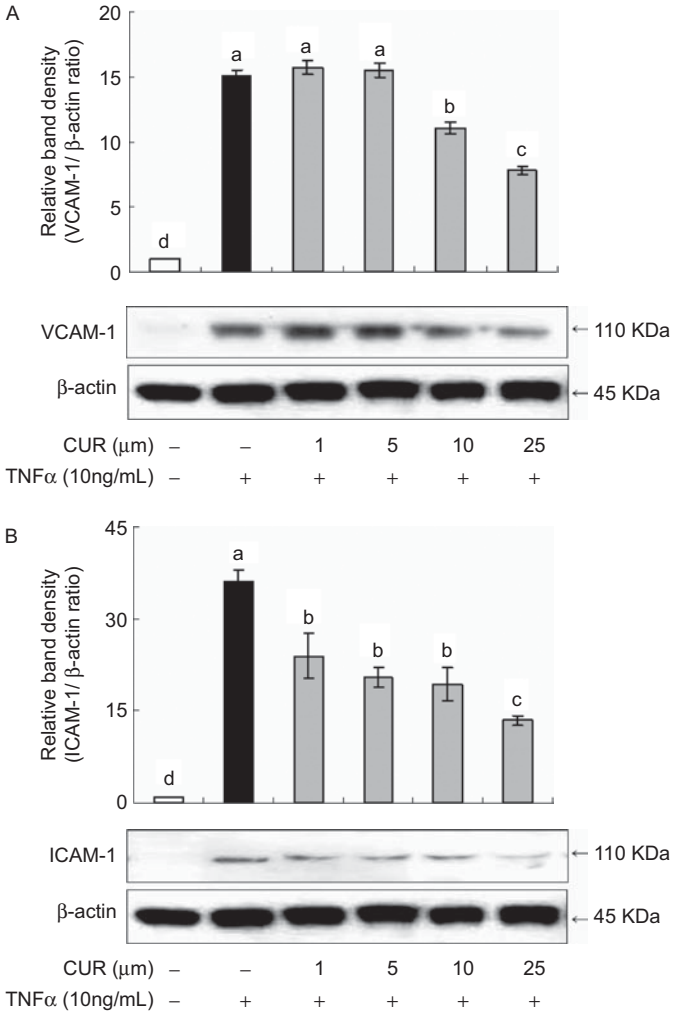

Figure 6. Inhibition of TNFα-induced adhesion molecules vascular cell adhesion molecule-1 (VCAM-1) and intracellular cell adhesion molecule-1 (ICAM-1) expression by curcumin. HUVECs were pretreated with different concentration of curcumin for 1 h before being incubated with 10 ng/mL TNFα for 12 h and subjected to western blotting to analysis expression of VCAM-1 (A) and ICAM-1 (B). A representative figure is calculated and compared with densitometry quantification of bands from the vehicle. Values are mean ± SD (n = 3). [*a-c]Bars with different letters are significantly different at $p < 0.05$ by Tukey's test.

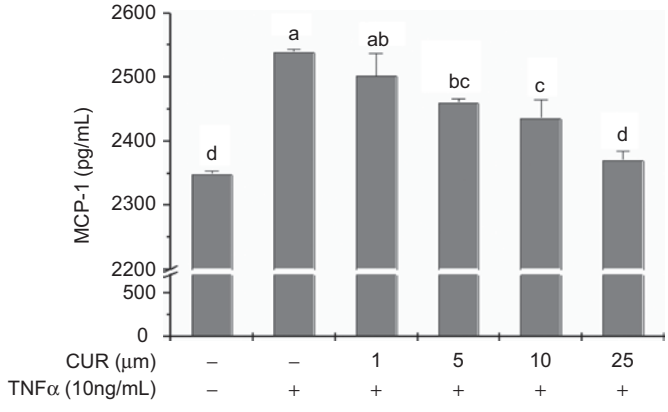

Figure 7. Inhibition of TNFα-induced monocyte chemotactic protein-1 (MCP-1) production by curcumin. HUVECs were pretreated with different concentration of curcumin for 1 h before being incubated with 10 ng/mL TNFα for 12 h. The culture supernatants were subsequently isolated and the MCP-1 production was measured using an ELISA kit according to the manufacturer's instructions. A representative figure is calculated and compared with densitometry quantification of bands from the vehicle. Values are mean ± SD (n = 3). [*a-d]Bars with different letters are significantly different at $p < 0.05$ by Tukey's test.

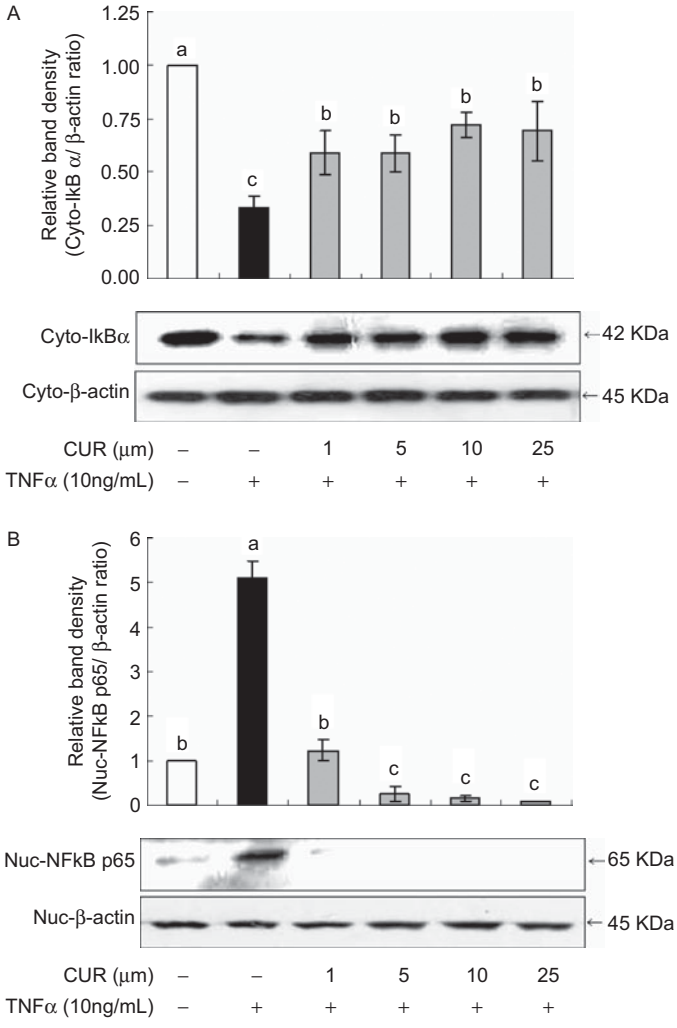

Figure 8. Effect of curcumin on TNFα -induced nuclear factor-κB (NFκB) activation in HUVECs. HUVECs were pretreated with different concentrations of curcumin for 1h before being incubated with 10 ng/mL TNFα for 12h and subjected to western blot to analyse the expression of cytosolic IκBα (A) and nuclear NFκB p65 (B). A representative figure was calculated and compared with quantification of bands from the vehicle by densitometry. Values are mean ± SD (n = 3). *[a-c]Bars with different letters are significantly different at $p < 0.05$ by Tukey's test.

anti-inflammatory effect through the partial interference of NFκB activation. We further showed that CUR abolished partially the TNFα-induced degradation of IκBα, which was associated with the effect observed on the translocation of NFκB.

Discussion

The CAS extracts under investigation were revealed to be very effective against the evaluated pro-oxidant species, including ROS and RNS. The scavenging effects and inhibitory effects on LDL oxidation of CAS were found to be potent in each of the evaluated reactive species. These data imply that at least part of the observed antioxidant activity may be a result of the phenolic compounds of CAS and showed that CAS can be used as an easily accessible source of natural antioxidants. Furthermore, these extracts might be helpful for preventing lipid peroxidation and protecting excipient bases and medicines from oxidative damage. Moreover, the observed antioxidant activity can justify the therapeutic use of CAS for the treatment of inflammatory diseases. Our results suggest that pharmacological concentrations of CUR, the major compound of CAS, interfere with the atherosclerosis process by inhibiting the secretion of adhesion molecules, chemoattractant molecules by an NFκB dependent pathway thus diminishing the inflammatory milieu.

There is considerable evidence that ROS molecules such as superoxide anion ($O_2^{\cdot-}$) contribute to endothelial barrier dysfunction in response to TNFα and ROS can directly activate mitogen activated protein kinases (MAPKs) and the transcription factor NFκB, which regulate the expression of many genes [21,22]. Recent evidence suggests that ROS up-regulates NFκB, which plays a pivotal role in the release of proinflammatory cytokines, such as IL-1, IL-6, and TNFα, leading to an increased expression of endothelial adhesion molecules [23,24]. The findings that IL-1β- and TNFα-induced VCAM-1 expression are inhibited by antioxidants also suggest that expression of adhesion molecules on vascular endothelium is ROS dependent [25]. The TNFα-induced increase of ROS was suppressed by CUR (Figure 3). The inhibition of ROS production by CUR may have contributed to the protection against oxidative damage by preventing the decrease of antioxidant enzyme activity [26,27].

In the present study we showed that treatment with CUR influences the synthesis of NO and phosphorylation of eNOS in HUVECs (Figures 4 and 5). There are three isoforms of this enzyme, type I (neuronal nitric oxide synthase, nNOS), type II (inducible nitric oxide synthase, iNOS), and type III (endothelium nitric oxide synthase, eNOS) [28]. High levels of NO have been described in a variety of pathophysiological processes, including various forms of circulatory shock, inflammation and carcinogenesis [29]. A recent study has reported that antioxidants can up-regulate eNOS activity in vascular endothelial cells, suggesting that this direct effect, leading to NO production, may contribute to the improvement of endothelial function [30]. NO produced by eNOS appears to be a key mediator in vascular homeostasis and reduced activity of eNOS is responsible for vasoconstriction, decreased endothelial cell viability, impaired angiogenesis and the initiation of inflammatory events leading to the formation of atherosclerotic plaques [31,32]. In this study, the effect of CUR on induced NO production by phosphorylation of eNOS and suppressed inflammatory events resulted in protection from atherosclerosis.

Furthermore, we observed that the expression of LOX-1 and adhesion molecules such as VCAM-1 and ICAM-1 were significantly inhibited in TNFα-stimulated HUVECs by CUR (Figures 6 and 7). LOX-1 was originally identified from cultured aortic endothelial cells as a receptor for oxidised LDL. As ox-LDL plays a role in the initiation of pathologic processes such as atherosclerosis [33], it was chosen as a possible marker of endothelial dysfunction. Furthermore, both *in vitro* and *in vivo* experiments have shown the receptor to be up-regulated by pro-atherogenic factors including

shear stress, TNFα, angiotensin II (Ang II), and ox-LDL itself [34]. Adhesion molecules such as VCAM-1 and ICAM-1 play a significant role in the process of atherosclerosis as they ensure the recruitment of inflammatory cells. While VCAM-1 is primarily an inducible molecule, ICAM-1 is also constitutively expressed on resting endothelial cells and both molecules are up-regulated by pro-atherogenic factors. The attenuated LOX-1 expression observed presently might be due to the decrease of oxidative stress by CUR. Furthermore, expression of TNFα-stimulated VCAM-1 in HUVECs is also reduced by antioxidants such as probucol [35], apigenin [36] and hematein [37]. These observations suggest that CUR may also suppress the expression of VCAM-1 and ICAM-1 in HUVECs through the same mechanism.

MCP-1 is expressed by a variety of cell types including monocytes, smooth muscle cells, and vascular endothelial cells in response to several different stimuli such as IL-1β and TNFα [38,39]. The expression of MCP-1 is strongly dependent on activation of the transcriptional factor NFκB [40]. In bone marrow transplantation studies, over-expression of MCP-1 in vessel wall macrophages led to increased foam cell formation and increased atherosclerosis [41]. Therefore, MCP-1 is critical to initiate and develop atherosclerotic lesions. In this reports, we have examined the role of CUR on the regulation of MCP-1 expression in HUVECs. We observed that CUR treatment decreased MCP-1 expressions in HUVECs (Figure 7). These findings support the notion that CUR treatment has an effect on the expressions of MCP-1 via regulation of NFκB translocation in TNFα-stimulated HUVECs.

The early phases of the atherosclerosis process are characterised by an increased adhesion of leukocytes and other inflammatory cells to the vascular endothelium. Leukocyte adhesion requires the expression of specific adhesion molecules on endothelial cells [42]. The expression of these adhesion molecules on the endothelial cell surface during the primary inflammatory response is dependent on NFκB activation [43]. Here, we determined if the protection of CUR against atherosclerosis is associated with the regulation in the expression of some NFκB-related inflammatory adhesion molecules in endothelial cells. It is well established that NFκB is a major transcription factor in the development of atherosclerotic injury, mediates cell migration, endothelial cell activation and controls the balance between cell proliferation and apoptosis [44]. The activation of NFκB in endothelial cells is associated with the activation of genes responsible for an increased transcription of adhesion molecules, cytokines and chemokines [45,46]. We observed a marked decrease in the NFκB translocation in cells cultured with CUR plus TNFα and these results confirm that CUR has an anti-inflammatory effect through the partial interference of NFκB activation. We further showed that CUR partially abolished the TNFα-induced degradation of IκBα, which was associated with the effect observed on the translocation of NFκB. Since other anti-inflammatory molecules such as interleukin-10 inhibit NFκB by blocking IκB kinase activity, it would be interesting to evaluate if CUR also affects IκB kinase activity.

Since atherosclerosis is a chronic inflammatory disease associated with increased oxidative stress on the vascular endothelial cell, it is reasonable to conclude that the anti-atherosclerotic effects of CUR are due to its ability to support anti-oxidative defence mechanisms. Our results suggest that pharmacological concentrations of CUR interfere with the atherosclerosis process by inhibiting the secretion of adhesion molecules and chemoattractant molecules through an NFκB-dependent pathway by an anti-oxidative mechanism thus diminishing the inflammatory milieu.

This is the first evidence that pharmacological concentrations of CUR inhibits production of ROS, expression of LOX-1, adhesion molecules, chemoattractant molecules, activation of NFκB and attenuates production of NO by p-eNOS in HUVECs. The anti-inflammatory and anti-atherosclerotic activities of CUR in HUVECs suggest that it could be useful in the prevention of atherosclerosis.

Acknowledgements

Hye-Sook Lee and Min-Ja Lee contributed equally to this work as first author.

Declaration of interest

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