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Lycopene inhibits TNF-α-induced endothelial ICAM-1 expression and monocyte-endothelial adhesion

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ABSTRACT

Inflammatory mediators such as TNF-α and interleukin (IL)-1β, and IL-8, which can enhance binding of low-density lipoprotein (LDL) to endothelium and upregulate expression of leukocyte adhesion molecules on endothelium during atherogenesis. Lycopene, a natural carotenoid from tomato and other sources, has been shown to prevent cardiovascular diseases in epidemiological studies. However, its anti-inflammatory action mechanism remains unclear. In the present study, we studied the effect of lycopene on TNF-α-induced signaling in human umbilical endothelial cells (HUVECs). We found that TNF-α-induced intercellular adhesion molecule-1 (ICAM-1) expression in HUVECs was inhibited by lycopene, whereas cyclooxygenase-2 (COX-2) and platelet-endothelial cell adhesion molecule (PECAM-1) expression were not affected. A further analysis indicated that lycopene attenuated TNF-α-induced IκB phosphorylation, NF-κB expression, and NF-κB p65 translocation from cytosol to nucleus. In line with this, TNF-α-induced NF-κB-DNA but not AP1-DNA complexes formation was inhibited by lycopene, as determined by the electrophoretic mobility shift assay (EMSA). On the other hand, lycopene did not affect TNF-α-induced p38 and extracellular matrix-regulated kinase1/2 (ERK1/2) phosphorylation and interferon-γ (IFN-γ)-induced signaling, suggesting that lycopene primarily affects TNF-α-induced NF-κB signaling pathway. In a functional study, lycopene dose-dependently attenuated monocyte adhesion to endothelial monolayer but not that adhesion to extracellular matrix. Taken together, we provided here the first evidence showing that lycopene is able to inhibit TNF-α-induced NF-κB activation, ICAM-1 expression, and monocyte-endothelial interaction, suggesting an anti-inflammatory role of lycopene and possibly explaining in part why lycopene can prevent cardiovascular diseases.

1. Introduction

Atherogenic lesions have been observed to arise at regions of the vessel wall exhibiting endothelial activation. Possible causes of such activation comprised elevated and modified low-density lipoprotein (LDL), free radicals arising during oxidative stress and pathological conditions (Osterud and Bjorklid, 2003). Among these, LDL is a major culprit that initiates the signals which promote the recruitment and accumulation of monocytes and T lymphocytes by upregulated expression of specific leukocyte adhesion molecules, notably intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1) (Navab et al., 1996). Another event feeds the process further is the foam cells release inflammatory mediators such as TNF-α, IL-1β, and IL-8, which in turn enhance binding of LDL to endothelium and smooth muscle cells and upregulate expression leukocyte adhesion molecules on endothelium (Hajjar et al., 1989).

Lycopene, a naturally present carotenoid in tomatoes and tomato products, is an open-chain hydrocarbon containing 11 conjugated and two nonconjugated double bonds arranged in a linear array (Britton, 1995). Dietary intakes of tomatoes and tomato products containing lycopene and other carotenoids have been shown to be associated with decreased risk of chronic diseases such as cardiovascular and cancer diseases in numerous studies (Heber and Lu, 2002). Results of epidemiological studies are showing a protective association between plant-derived antioxidants, such as carotenoids, and cardiovascular disease (Kohlmeier and Hastings, 1995). For example, several epidemiological studies have shown that low plasma lycopene concentration is associated with increased intima-media thickness of the carotid artery wall in middle-aged men living in eastern Finland (Rissanen et al., 2000, 2003). The Los Angeles Atherosclerosis Study also suggests that lycopene is protective against intima-media thickness progression among smokers (Dwyer et al., 2004).

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Recently, we have demonstrated that lycopene can directly bind PDGF-BB and inhibit PDGF-BB-induced signaling, proliferation and migration in rat vascular smooth muscle cells (Lo et al., 2007), explaining in part a cardiovascular protective role of lycopene. In parallel, it has been shown that serum lycopene concentration is inversely related to soluble ICAM-1, a marker of endothelial function and inflammation (Herpen-broekmans et al., 2004), suggesting that lycopene may affect endothelial adhesion molecule expression during inflammation. In line with this, Martin et al. (2000) have observed that carotenoids are able to inhibit IL-1β-induced adhesion molecule expression in human endothelial cells. However, how lycopene exhibits its anti-inflammatory effect on endothelial cells is still largely unknown.

The aim of this work was to evaluate lycopene effect on the adhesion of monocyte adhesion to TNF-α-treated endothelial cells, as well as the expression of ICAM-1, which are the critical cytokine and adhesion molecules strongly involved in the atherogenic process. Our results provided the first evidence showing that lycopene is able to inhibit TNF-α-induced ICAM-1 mRNA and protein expression primarily through affecting NF-κB signaling pathway. In striking contrast, lycopene does not affect TNF-α-induced activation of extracellular matrix-regulated kinase1/2 (ERK1/2) and p38 mitogen-activated protein kinase (MAPK) and interferon-γ (IFN-γ)-induced signaling. Finally, a functional study demonstrated that lycopene inhibits TNF-α-induced monocyte-endothelial interaction, suggesting an anti-inflammatory role of lycopene.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA), bovine type I collagen, protease inhibitors for Western blotting, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St Louis, MO, USA). 2′, 7′-*bis*-(2-carboxyethyl)-5-(and-6)-carboxy-fluorescein acetoxymethyl ester (BCECF/AM) was from Molecular Probes (Eugene, OR, USA). Hydrogen peroxide (H_2O_2) was from Merck KGaA Co. (Darmstadt, Germany). The antibody (Ab) raised against phospho-ERK1/2 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The Abs raised against ICAM-1, phospho-STAT1, phospho-p38, total p38, phospho-IκBα, IκBα, NF-κB p65 and p50 were from New England Biolabs, Inc. (Beverly, MA, USA). Human TNF-α, interferon γ (IFN-γ) and the Abs for total ERK1/2 were from R&D systems, Inc. (MN, USA). The Ab for platelet-endothelial cell adhesion molecule (PECAM-1) was from DAKO Cytomation (Glostrup, Denmark) and Ab for COX-2 was from BD Biosciences (San Jose, CA USA). Lycopene was purchased from Extrasynthese (Genay cedex, France) and contained about 93% of *trans*-isomer and 7% of *cis*-isomers by HPLC analysis using the previously described protocol (Rajendran et al., 2005). Tetrahydrofuran (THF) containing 0.025% butylated hydroxytoluene as an antioxidant was purchased from Sigma Chemical Co. and used as a vehicle to dissolve lycopene.

2.2. Cell culture and lycopene treatment

Human umbilical vein endothelial cells (HUVECs) and THP-1 monocytes were purchased from Food Industry Research and Development Institute (Hsinchu, Taiwan). HUVECs were cultured in M199 medium with heparin (25 U/ml), endothelial cell growth supplement(ECGS, 30 μg/ml), 10% fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 μg/ml) and fungizone (250 ng/ml) (Invitrogen Life Technologies, Carlsbad, CA, USA). HUVECs were identified by their characteristic cobblestone shape, capability of forming tube in Matrigel and the presence of PECAM-1 (CD31) antigen, as previously described by Bachetti and Morbidelli (2000). THP-1 monocytes were cultured in RPMI 1640 medium containing 10% FBS containing 10% FBS, antibiotics and fungizone. For most experiments, HUVECs reaching 80–90% of confluency were starved in 1% FBS-containing M199 medium at 37 °C for 24 h and were preincubated with vehicle or lycopene for 2 h. After preincubation, cells were challenged with TNF-α in the presence or absence of lycopene for the time indicated and then subjected to further analysis.

2.3. Cell lysate preparation and Western blot analysis

For cell lysate preparation, HUVECs were washed with prechilled PBS and lysed in radioimmunoprecipitation assay buffer [20 mM Tris–HCl, pH 7.4, 137 mM NaCl, 2 mM EDTA. 1 mM sodium fluoride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10% glycerol, 1 mM sodium orthovanadate, 1 mM PMSF, and 1 μg/ml aprotinin and leupeptin (freshly prepared)]. After sonication, the lysate was centrifuged (14,000 ×*g* for 10 min at 4 °C), and supernatant was transferred to a tube. The protein content was quantified by Pierce protein assay kit (Pierce, Rockford, IL). Total protein was separated by electrophoresis on SDS-polyacrylamide gels and the proteins were electroblotted onto PVDF membranes and then probed using primary anti-ICAM-1 (1:1000), VCAM-1 (1:1000), COX-2 (1:200), phospho-IκBα (1:2000), total IκB (1:1000), phospho-ERK1/2 (0.2 μg/ml), phospho-p38 (0.2 μg/ml), or phospho-STAT1 (1:1000) mAbs. Immunoblots were detected using Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer, Waltham, MA, USA). For some experiments, PVDF membranes were stripped at 60 °C for 30 min with a striping buffer (62.5 mM Tris–HCl, pH 6.7, 2% SDS and 100 mM β-mercaptoethanol). Membranes were washed with TBS-T (Tris-buffered saline/0.05% tween 20) and probed with anti-total ERK or anti-α-tubulin (0.2 μg/ml) Abs and developed as described above.

2.4. Cell viability assay

Cell viability was determined by MTT assay and luminescence assay of cellular ATP. Briefly, cells were incubated with 0.5 mg/ml MTT for 2 h at 37 °C. Formazan crystals resulting from MTT reduction were dissolved by adding 200 μl DMSO and gently agitated for 20 min. The absorbance of the supernatant was then measured spectrophotometrically in an ELISA reader at 550 nm. The luminescence assay of cellular ATP was performed by using ATPlite 1step kit (PerkinElmer, Waltham, MA, USA) for the quantitative evaluation of cell viability according to the manufacturer's protocol.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of ICAM-1 mRNA expression

Oligonucleotide PCR primers targeting to human ICAM-1 and GAPDH were synthesized. The forward primer for ICAM-1 is 5′-ACCATGGAGCCAATTTCTC-3′ and the reverse primer is 5′-ACAATCCCTCTCGTCCAG-3′. The forward primer for GAPDH is 5′-AACCATGAGAAGTATGACAACAGC-3′ and the reverse primer is 5′-CATGTGGGCCATGAGGTCCACCAC-3′. Total RNA of HUVECs was extracted by Trizol reagents (Invitrogen Technologies) and reverse transcription reaction was performed by using Superscript III First-Strand Synthesis System (Invitrogen Technologies). Briefly, aliquots of 3 μg total RNA were incubated with oligo (dT) primer for 10 min at 65 °C and chilled on ice shortly. After primer annealing, RNA was reverse transcribed by the reverse transcriptase. Reactions were stopped and RNase H was added to remove RNA. Aliquots of transcribed cDNA were subjected to PCR in 25 μl of reaction mixture containing reaction buffer, dNTP, primers, and *Taq* DNA polymerase (Ab peptides, St. Louis, USA). PCR was performed with a hot start at 94 °C for 5 min and then with 30 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, and elongation at 72 °C for 1.5 min on a ABI 7200 Thermal Cycler (Applied Biosystems, Foster city, CA, USA). The amplification products were then analyzed by gel electrophoresis in 2% agarose.

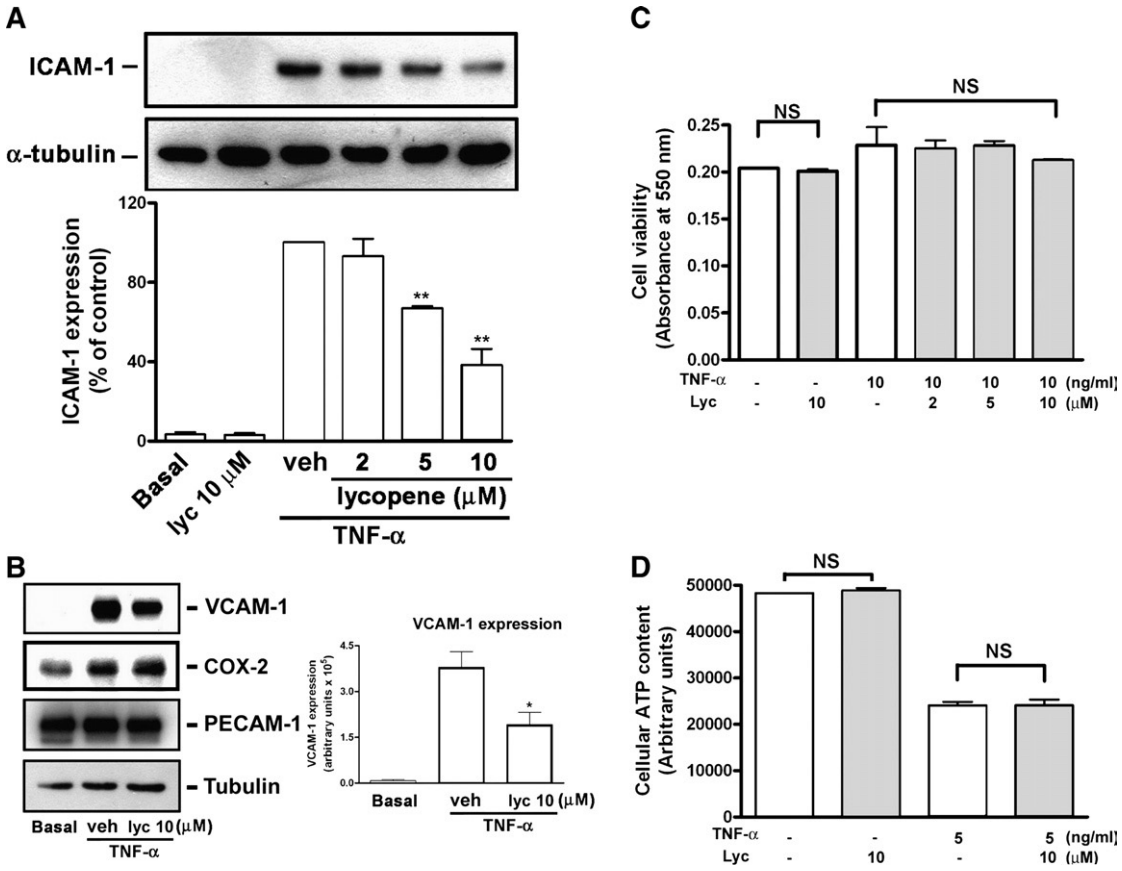

Fig. 1. Effect of lycopene on TNF-α-induced adhesion molecule and COX-2 expression in HUVECs. (A and B) Cells were treated with PBS or TNF-α (5 ng/ml) in the presence of vehicle (veh) or lycopene (lyc). After incubation for 16 h, cells were collected and then analyzed by Western blotting. ICAM-1 and VCAM-1 expression in each independent experiment was quantified by densitometry. Results were expressed as the percentage of control and were mean ± S.E.M. (n = 3). Each blot was the representative from three independent experiments. $^{**}P$ < 0.01 versus TNF-α control. Cell viability at 6 h and 16 were determined by (C) MTT assay and (D) luminescence assay of cellular ATP, respectively. Data were arbitrary units and were mean ± S.E.M. (n = 2–3). NS, non significant.

2.6. Preparation of cytosolic and nuclear fractions

Preparation of cytosolic and nuclear fraction of HUVECs was performed by using Panomics' Nuclear extraction kit (Panomics Inc., Redwood City, CA, USA) according to the manufacturer's protocol. Briefly, HUVECs were washed twice with cold PBS, detached and extracted with the buffer A working reagent on a rocking plateform at 150 rpm for 10 min. After centrifugation, the supernatant (cytosolic fraction) was removed and the remaining cell pellet was resuspended in and extracted with buffer B working reagent on a rocking plateform at 150 rpm for 2 h. The supernatant was the nuclear fraction.

2.7. Electrophoretic mobility shift assay (EMSA)

EMSA was performed by using EMSA 'gel shift' kit (Panomics Inc.) according to the manufacturer's protocol. Oligonucleotides corresponding to canonical NF-κB binding sequence (5′-AGTTGAGGGGACTTTCCCAGGC-3′, from Panomics) and human ICAM-1 promoter NF-κB binding sequence (5′-AGC**TTGGAAATTCC**GGA) or AP1 sequence (5′-GACCG**TGA**TTCAAGC-3′) were used (Roebuck and Finnegan, 1999; Chen et al., 2004). HUVEC nuclear extract (2–4 μg) was incubated with poly d(I-C) at room temperature for 5 min. The nuclear extract was then incubated with biotin-labeled probes in the presence or absence of cold (unlabeled) probe and the incubated at RT for 30 min. In the supershift experiment, 2 μg of p65 or p50 Ab (Santa Cruz Biotechnology, CA, USA) was added before the biotin-labeled probes. After electrophoresis on a 6% polyacrylamide gel, the samples on gel were transferred onto a presoaked immobilon-Ny+ membrane (Millipore, Billerica, MA, USA). The membrane was baked at 80 °C for 1 h, crosslinked in an oven for 3 min, and

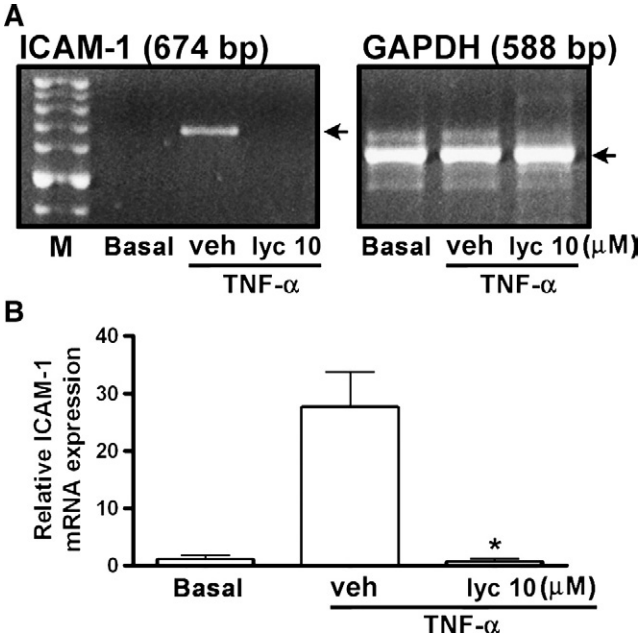

Fig. 2. Effect of lycopene on ICAM-1 mRNA expression in HUVECs. (A) Cells were treated with vehicle (veh) or lycopene (lyc) in the presence or absence of TNF-α (10 ng/ml) for 6 h. At the end of incubation, cells were collected and then analyzed by reverse transcription-polymerase chain reaction. An arrow indicates the PCR product of ICAM-1 and GAPDH. (B) Quantitative analysis of ICAM-1 mRNA expression. Data from (A) and similar experiments were quantified by densitometry. Results were expressed as relative densitometry units and were mean ± S.E.M. (n = 3). $^{*}P$ < 0.05 versus TNF-α control.

then developed by adding the blocking buffer and streptavidin-HRP conjugate. To determine the amount of NF-κB p65 subunit in the cytosol and nucleus, these two fractions were subjected to Western blot analysis.

2.8. Adhesion of monocyte to the endothelial cell monolayer

Adhesion of monocyte to the endothelial cell monolayer was performed using THP-1 monocytes and HUVECs. Confluent HUVECs cultured in 24-well plate were stimulated with TNF-α for 16 h. Serum-starved THP-1 monocytes were loaded with BCECF/AM (10 μg/ml) for 30 min at 37 °C and washed with PBS for 2 times. 5×10^4 of THP-1 monocytes were allowed adhering to TNF-α-treated endothelial monolayer for 1 h. After incubation, nonadherent cells were removed by several washes. The remaining adherent cells were lysed in lysis buffer (20 mM Tris–HCl, pH 7.4, 137 mM NaCl, 2 mM EDTA. 1 mM sodium fluoride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10% glycerol) and quantified by measuring fluorescence intensity on Wallac Victor 3 1420 multilabel counter (Perkin-Elmer, Turku, Finland) using excitation and emission wavelength at 485 and 525 nm, respectively. Alternatively, the adherent THP-1 cells on HUVEC monolayer were analyzed under a Leica DMIL® fluorescence microscope and photographed by a digital camera.

2.9. Monocyte adhesion assay

Ninety-six-well plates (Costar, Cambridge, MA) were coated with 50-μl type I collagen (collagen, 20 μg/ml), fibronectin (20 μg/ml), laminin (20 μg/ml) or 1% BSA in PBS at 4 °C for overnight. After a brief wash with PBS, the plates were blocked with 10% BSA at 37 °C for 1 h. Suspended THP-1 monocytes were labeled with BCECF/AM (10 μg/ml) for 30 min at 37 °C. The labeled cells were washed and resuspended in DMEM to a density of 1.5×10^5 cells/ml. One hundred microliter of the resuspended cells was preincubated with lycopene for 30 min at 37 °C and then allowed adhering to the protein-precoated 96-well plates for 3 h at 37 °C in the absence or presence of lycopene. After washing twice with PBS, the nonadherent cells were removed by aspiration and the 96-well plates were subjected to measurement by Wallac Victor 3 1420 multilabel counter.

2.10. Statistical analysis

Data were expressed as mean ± standard error mean (S.E.M.). Otherwise indicated, comparison of means of two groups of data was made by using the unpaired, two-tailed Student *t* test.

3. Results

3.1. Lycopene inhibits TNF-α-induced ICAM-1 and VCAM-1 expression in HUVECs

To examine whether lycopene affected TNF-α-induced adhesion molecule expression in HUVECs, Western blotting was performed. Since TNF-α has been shown to induce endothelial apoptosis (Robaye

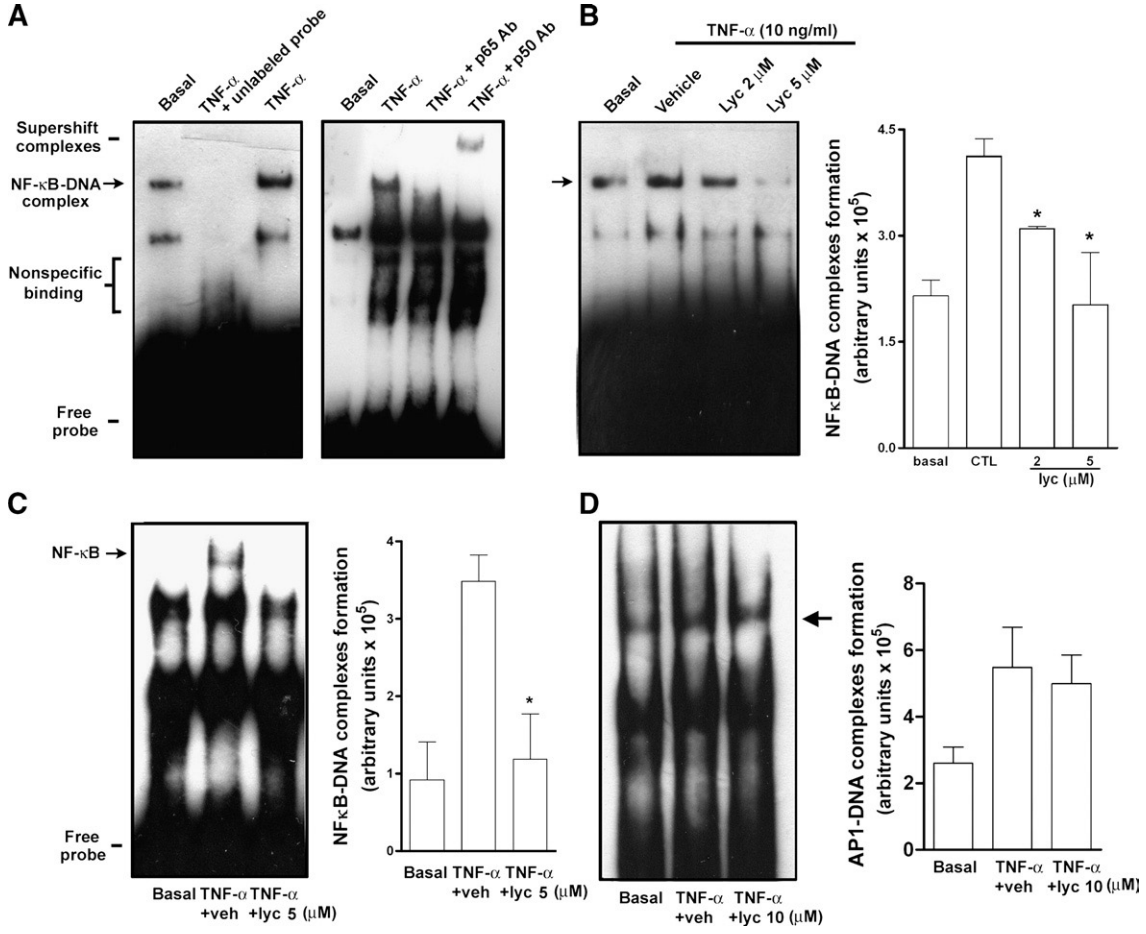

Fig. 3. Effect of lycopene on TNF-α-induced transcription factor-DNA complexes formation in HUVECs. Cells were treated with PBS or TNF-α (10 ng/ml) in the presence of vehicle (veh) or lycopene (lyc) for 6 h, the nuclear fraction was isolated and EMSA was performed using (A and B) canonical NF-κB binding sequence or (C) human ICAM-1 promoter NF-κB and (D) AP1 binding sequences. Each blot was representative from three similar experiments. Effect of lycopene on TNF-α-induced NF-κB-DNA and AP1-DNA complexes formation was quantified by densitometry. Arrows indicate transcription factor-DNA complexes formation. Data were expressed as the arbitrary units and were mean ± S.E.M. ($n=3$). $^*P<0.05$ versus TNF-α control.

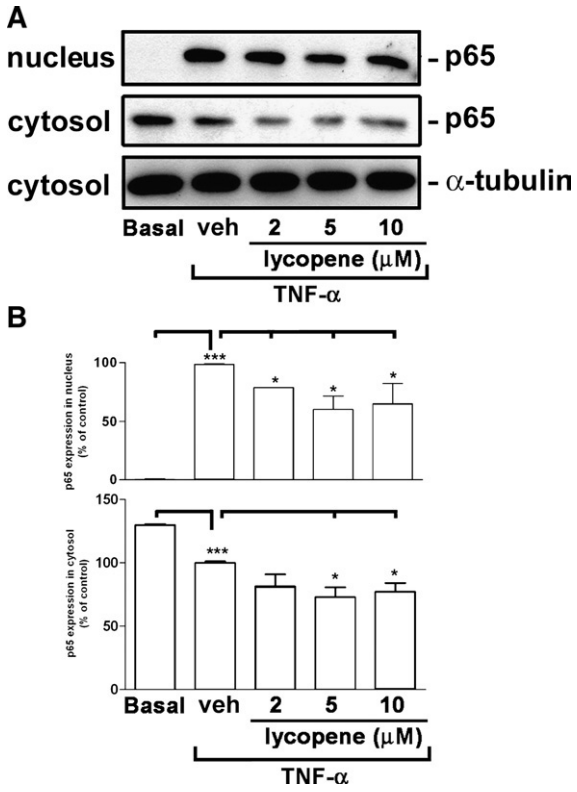

Fig. 4. Effect of lycopene on TNF-α-induced NF-κB translocation in HUVECs. (A) Cells were treated with PBS or TNF-α (10 ng/ml) in the presence of vehicle (veh) or lycopene for an additional 6 h. The separated nuclear and cytosolic fractions were analyzed by Western blotting. (B) Data from (A) and other similar experiments were quantified by densitometry. Data were expressed as the percentage of control and were mean ± S.E.M. (n = 3–5). Each blot was the representative from three to five independent experiments. $^{*}P<0.05$, $^{***}P<0.001$.

et al., 1991), 5 ng/ml of TNF-α was used in the long-term (16-h) incubation with HUVECs to prevent excessive cell death. It was shown that TNF-α markedly induced ICAM-1 expression in HUVECs but the expression was significantly inhibited by lycopene (Fig. 1A). In parallel, lycopene also inhibited TNF-α-induced VCAM-1 expression but did not affect TNF-α-induced cyclooxygenase-2 (COX-2) and basal PECAM-1 and tubulin expression in HUVECs (Fig. 1B). The inhibition was not due to lycopene's cytotoxicity because that lycopene alone and in combination with TNF-α did not increase cell death in HUVECs, as determined by MTT assay and luminescence assay of cellular ATP (Fig. 1C and D). Moreover, lactate dehydrogenase (LDH) assay demonstrated that lycopene treatment for 16 h did not induce HUVEC necrosis (data not shown).

3.2. Lycopene inhibits ICAM-1 mRNA expression

To further examine if lycopene's inhibitory effect on TNF-α-induced ICAM-1 expression was associated with possible down-regulation in ICAM-1 mRNA expression, HUVECs were treated with lycopene, TNF-α or both. ICAM-1 mRNA expression at 6 h was evaluated by reverse transcription-polymerase chain reaction. As shown in Fig. 2, ICAM-1 mRNA was upregulated by TNF-α but was inhibited in the presence of lycopene, whereas expression of GAPDH mRNA was not affected. This suggests that lycopene interferes with TNF-α-induced ICAM-1 expression at a transcriptional level.

3.3. Lycopene inhibits TNF-α-induced NF-κB signaling pathway

A previous study has shown that TNF-α induces ICAM-1 and VCAM-1 expression through NF-κB signaling pathway in human endothelial cells (Zhou et al., 2007). The ability of lycopene to perturb TNF-α-stimulated NF-κB nuclear translocation was studied by the electrophoretic mobility shift assay (EMSA) using the canonical and human ICAM-1 NF-κB oligonucleotide-binding sequences. As shown in Fig. 3A, evaluation of NF-κB in nuclear extracts by EMSA revealed two slight bands in unstimulated cells (basal) but a marked increase in the two bands in TNF-α-treated cells, which can be abolished by adding an excessive unlabeled cold probe (left panel). The identity of the upper band was demonstrated to be a NF-κB p65-p50-DNA complex by a supershift assay using NF-κB p65 or p50 Abs (right panel). It was found that lycopene exhibited an inhibitory effect on TNF-α-induced NF-κB-DNA complexes formation in HUVECs (Fig. 3B, left panel). Quantitative analysis by densitometry revealed that lycopene's inhibitory effect on NF-κB-DNA complexes formation was concentration-dependent (right panel). The results were confirmed by EMSA using human ICAM-1 promoter NF-κB binding sequence. A supershift assay also showed that TNF-α induced NF-κB p65-p50-DNA complex formation (data not shown). Again, lycopene inhibited TNF-α-induced NF-κB-DNA complexes formation (Fig. 3C). However, it did not inhibit TNF-α-induced AP1-DNA complexes formation under this condition (Fig. 3D).

NF-κB proteins are predominantly localized in the cytoplasm in unstimulated cells and are translocated into nucleus in response to stimulation (Yamamoto and Gaynor, 2004). Therefore, NF-κB expression in both cytosolic and nucleic fractions was determined. As shown in Fig. 4A, NF-κB p65 subunit expressed abundant in cytosol but less in

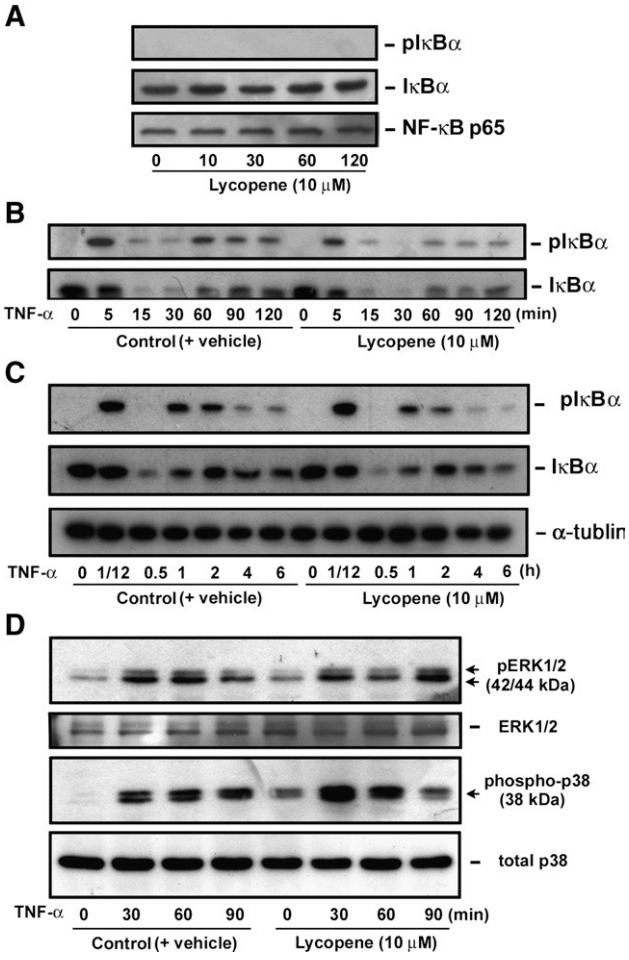

Fig. 5. Effect of lycopene on IκB phosphorylation, expression, and MAPKs phosphorylation in HUVECs. (A) Cells were treated with lycopene for the indicated time points. (B–D) Cells were treated with PBS or TNF-α (10 ng/ml) in the presence of vehicle or lycopene. After incubation, cells were collected and subjected to be analyzed by Western blotting. Each blot was the representative from four independent experiments.

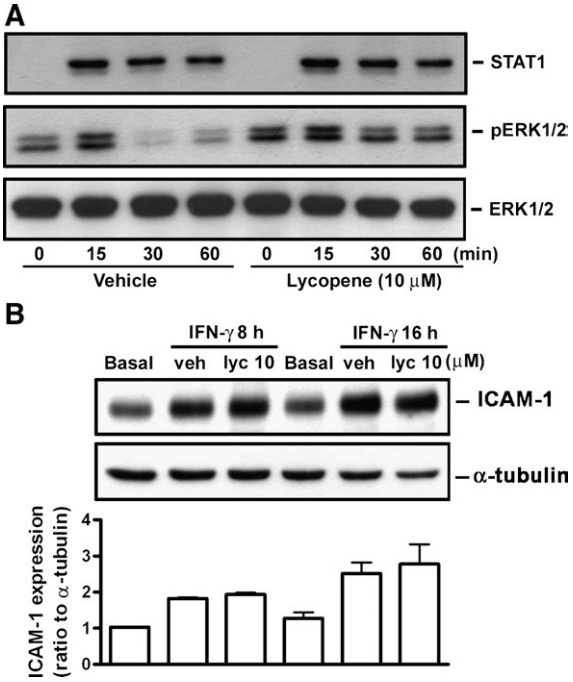

Fig. 6. Effect of lycopene on IFN-γ-induced signaling. HUVECs were treated with (A) 20 or (B) 50 ng/ml of IFN-γ in the presence of vehicle (veh) or lycopene (lyc) for the indicated times. Cell lysates were analyzed by Western blotting. Each blot was representative from two similar experiments. ICAM-1 protein levels in (B) and a similar experiment were determined by densitometry. The data were expressed as mean±S.E.M. and were ratio of arbitrary units of ICAM-1 to α-tubulin.

nucleus in unstimulated cells; however marked translocation of p65 NF-κB from the cytosol to the nucleus was observed in TNF-α-stimulated cells (lanes 1 and 2). Lycopene dose-dependently decreased NF-κB p65 subunit translocation to nucleus but surprisingly also decreased NF-κB p65 expression in cytosol, while it did not affect α-tubulin expression in cytosol (Fig. 4A, lanes 3–5). A quantitative analysis showed that NF-κB p65 expression in cytosol and nucleus was significantly decreased in the presence of lycopene (Fig. 4B), suggesting that lycopene affects NF-κB-DNA complexes formation through inhibition of NF-κB expression and translocation.

3.4. Lycopene reduces TNF-α-induced IκB phosphorylation

The increase of NF-κB activity results from the phosphorylation and rapid loss of IκB protein through proteolysis (Brown et al., 1993). We next determined if lycopene affected IκB phosphorylation. We showed that lycopene alone did not affect basal IκB and NF-κB activity, including IκB phosphorylation and expression and NF-κB p65 expression (Fig. 5A). The addition of TNF-α to HUVECs caused rapid IκBα phosphorylation with a concomitant decrease of IκBα protein levels. A two-phase activation of IκBα phosphorylation was observed when cells were stimulated with TNF-α more than 1 h. Treatment of cells with lycopene resulted in a slight inhibition on TNF-α-induced IκBα phosphorylation and expression but not on α-tubulin expression (Fig. 5B and C). In contrast, TNF-α-induced ERK1/2 and p38 activation was not obviously affected by lycopene in HUVECs (Fig. 5D).

3.5. Effect of lycopene on IFN-γ-induced signaling

It has been reported that IFN-γ can induce ICAM-1 expression but acts via a signaling pathway different from TNF-α in endothelial cells (Roebuck et al., 1995; Look et al., 1995). To determine whether lycopene primarily affected TNF-α-induced signaling pathway, IFN-γ-induced cellular signaling, including the activation of ERK1/2 and STAT1 transcription factor, were examined by Western blotting. As shown in Fig. 6A, IFN-γ induced STAT1 and ERK1/2 phosphorylation at the tested time points in HUVECs and were not affected by lycopene. Moreover, IFN-γ-induced ICAM-1 expression at 8 and 16 h were not affected by lycopene (Fig. 6B), suggesting that lycopene primarily affects TNF-α-induced signaling pathway.

3.6. Lycopene inhibits monocyte adhesion to TNF-α-stimulated endothelial cells

It has been suggested that inhibition of adhesion molecule expression reduces the adhesive properties of the endothelial cell monolayer, leading to a reduction of leukocyte recruitment (Zhang et al., 2006). To test the functional consequences of TNF-α-induced NF-κB signaling inhibition by lycopene, we performed cell adhesion assay using THP-1 monocytes and HUVECs. As shown in Fig. 7A and B, some fluorescent-labeled adherent THP-1 cells on HUVEC monolayer were detected in the absence of TNF-α, whereas the number of adherent THP-1 cells dramatically increased about 2.5 folds in the presence of TNF-α. Treatment of HUVECs with lycopene resulted in the reduction of TNF-α-mediated monocyte adhesion to HUVEC monolayer in a concentration-dependent manner, as quantified by fluorescence

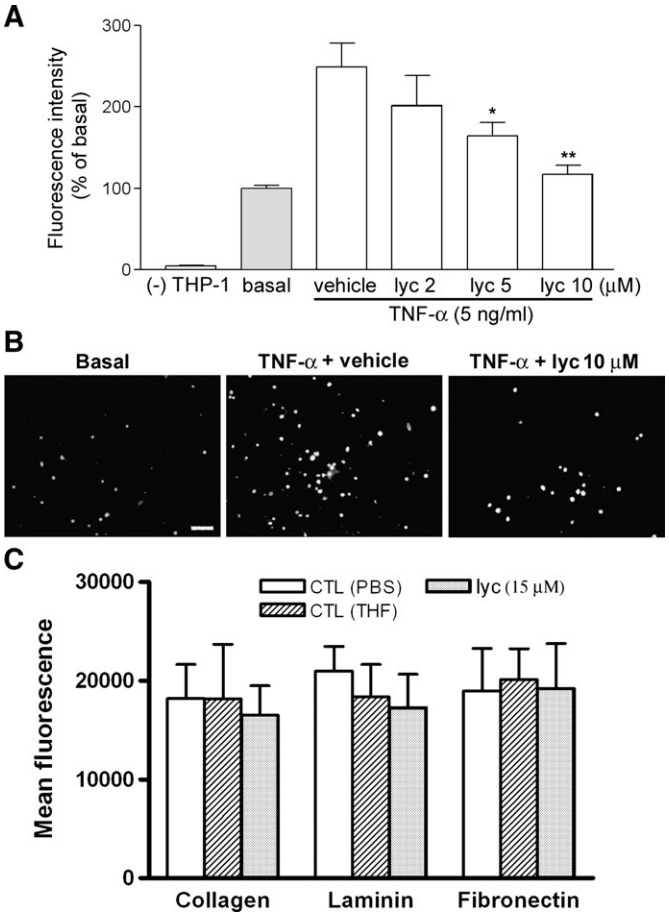

Fig. 7. Effect of lycopene on monocyte adhesion to endothelial monolayer and matrix proteins. (A) HUVEC monolayers were stimulated with PBS or TNF-α (5 ng/ml) in the presence of vehicle or lycopene for 16 h. THP-1 monocytes were allowed to adhesion to HUVEC monolayer in the presence of vehicle or lycopene for 1 h. The number of adherent cells was (A) measured by the fluorescence plate reader or (B) photographed under the fluorescence microcope. Each experiment was performed in duplicate. Data were expressed as the percentage of basal (without TNF-α) and were mean±S.E.M. (n=3). (C) Suspended THP-1 monocytes were loaded with BCECF/AM and pretreated with PBS, vehicle or lycopene (lyc). Cell adhesion was performed by adding of cells to 96-well plates precoated with collagen, laminin, or fibronectin for 3 h at 37 °C and measured by a fluorescence plate reader. $^{*}P<0.05$ and $^{**}P<0.01$ versus TNF-α control.

measurement of adherent cells and analyzed by fluorescence microscopy. It has been reported that endothelial cells secret stromal and ECM components, including collagen I, II, fibronectin, elastin, and other matrix proteins (Bachetti and Morbidelli, 2000). To exclude the possibility that lycopene's effect was due to its inhibition on monocyte-ECM interactions, monocyte adhesion assay was performed. Fig. 7C showed that lycopene did not significantly influence monocyte adhesion to type I collagen, fibronectin, and laminin even at a higher concentration (15 μM). Taken together, our results suggest that downregulation of ICAM-1 expression in HUVECs by lycopene is involved in the reduction of monocyte adhesion to endothelial cells.

4. Discussion

Recruitment of leukocytes from the peripheral blood to the intima of the vessel wall is a primordial event in atherogenesis (Osterud and Bjorklid, 2003). The events during leukocyte adhesion and transmigration are complex, including upregulation of adhesion molecules on endothelial cells for mediating of leukocyte rolling and firm adhesion. It is now well accepted that ICAM-1 and VCAM-1 are two important adhesion molecules expressed on endothelial cells during this process. Lycopene, a carotenoid from tomato, has been shown to inhibit IL-1β-induced adhesion molecule expression in human endothelial cells (Martin et al., 2000). However its action mechanism on TNF-α-primed endothelial cells has not been elucidated. In the present study, we therefore determined if lycopene affected TNF-α-induced adhesion molecule expression in human endothelial cells. Our results indicated that lycopene inhibited TNF-α-induced ICAM-1 and VCAM-1 expression. However, lycopene did not affect basal PECAM-1 expression and TNF-α-induced COX-2 expression, suggesting its specificity for ICAM-1 and VCAM-1 in HUVECs. A further analysis demonstrated that lycopene inhibited ICAM-1 mRNA expression (Fig. 2), indicating lycopene affects TNF-α-induced ICAM-1 expression at a transcriptional level.

It has been reported that transcriptional regulation of ICAM-1 and VCAM-1 gene by TNF-α in human endothelial cells critically depends on NF-κB binding to their promoter (Ledebur and Parks, 1995; Zhou et al., 2007). In this study, we found that TNF-α-induced NF-κB-DNA but not AP1-DNA complexes formation was attenuated in the presence of lycopene (Fig. 3), suggesting lycopene may affect NF-κB binding to ICAM-1 promoter region and/or NF-κB translocation. Indeed, our analysis indicated that lycopene decreased NF-κB p65 translocation from cytosol to nucleus (Fig. 4). However, it was surprising that cytosolic NF-κB p65 expression was also decreased in the presence of lycopene (Fig. 4). As described earlier, the dissociation of NF-κB from IκB requires phosphorylation of IκB, which results in rapid and ubiquitous degradation of IκB. In our study, TNF-α-induced IκB phosphorylation was attenuated by lycopene. However, TNF-α-induced decrease in IκB expression was not concomitantly increased upon lycopene treatment (Fig. 5). Therefore, it is possible that lycopene interferes with NF-κB and IκB protein expression via affecting their synthesis and/or increasing their degradation. The inhibitory effect on NF-κB expression and translocation appears to be primarily responsible for its action in HUVECs. Lycopene's inhibitory effect resulting from its cytotoxicity was excluded due to the observations that lycopene did not affect TNF-α-induced COX-2 and PECAM-1 expression and endothelial viability (Fig. 1B–D). Moreover, lycopene alone did not affect basal NF-κB p65 and p50 expression (Fig. 5A and data not shown).

It has been documented that all carotenoids possess certain common chemical features: a polyisoprenoid structure, a long conjugated chain of double bonds in the central portion of the molecule, and near symmetry around the central double bond (Britton, 1995). Therefore lycopene is structurally similar to vitamin A (retinol) and retinoic acid (El Agamey et al., 2004). In this regard, all-*trans*-retinoic acid (ATRA) has been shown to induce NF-κB activation and affect IL-8 production and matrix metalloproteinase-9 expression in normal human keratinocyts and human SK-N-BE 9N neuroblastoma cells, respectively (Farina et al., 2002; Dai et al., 2004). Moreover, ATRA acts synergistically with TNF-α on IL-8 gene activation in human melanoma cell line G-361 (Harant et al., 1996). However, in contrast to ATRA's inducing effect on NF-κB, lycopene alone did not interfere with basal IκB and NF-κB activity, including IκB expression, phosphorylation, and NF-κB p65 expression (Fig. 5A). According to our observations we suggested that lycopene primarily affects TNF-α-induced NF-κB signaling. Several lines of evidence supported our hypothesis. Firstly, lycopene did not affect TNF-α-induced COX-2 expression and p38 and ERK1/2 phosphorylation (Figs. 1 and 5). Secondly, lycopene inhibited TNF-α-induced NF-κB-DNA but not AP1-DNA complexes formation (Fig. 3). Thirdly, lycopene did not affect intracellular H_2O_2 level and ERK1/2 phosphorylation induced by exogenous H_2O_2 (data not shown), a reactive oxygen that can activate NF-κB transcription factor in human cells (Schreck et al., 1991; Roebuck et al., 1995). Fourthly, lycopene did not inhibit IFN-γ-induced ERK1/2 and STAT1 phosphorylation (Fig. 6A), which have been suggested to play a key role in activating ICAM-1 transcription via a signaling pathway different from TNF-α (Look et al., 1995; Roebuck and Finnegan, 1999). This was confirmed by the observation that lycopene did not affect IFN-γ-induced ICAM-1 expression at a short- and long-term incubation (Fig. 6B).

In summary, in the present study we demonstrated that lycopene inhibits TNF-α-induced ICAM-1 protein and gene expression in human endothelial cells through affecting NF-κB signaling pathway. More importantly, the inhibition functionally leads to attenuation in monocyte adhesion to TNF-α-primed endothelial monolayer. Since monocyte-endothelial interaction plays a critical role during atherogenesis, the data presented here not only suggest that lycopene possesses anti-inflammatory activity but also explain in part why lycopene has a protective effect in cardiovascular diseases.

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