

Lycopene inhibits NF- κ B activation and adhesion molecule expression through Nrf2-mediated heme oxygenase-1 in endothelial cells

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Abstract. The endothelial expression of cell adhesion molecules plays a leading role in atherosclerosis. Lycopene, a carotenoid with 11 conjugated double bonds, has been shown to have anti-inflammatory properties. In the present study, we demonstrate a putative mechanism for the anti-inflammatory effects of lycopene. We demonstrate that lycopene inhibits the adhesion of tumor necrosis factor α (TNF α)-stimulated monocytes to endothelial cells and suppresses the expression of intercellular cell adhesion molecule-1 (ICAM-1) at the transcriptional level. Moreover, lycopene was found to exert its inhibitory effects by blocking the degradation of the inhibitory protein, I κ B α , following 6 h of pre-treatment. In TNF α -stimulated endothelial cells, nuclear factor- κ B (NF- κ B) nuclear translocation and transcriptional activity were abolished by up to 12 h of lycopene pre-treatment. We also found that lycopene increased the intracellular glutathione (GSH) level and glutamate-cysteine ligase expression. Subsequently, lycopene induced nuclear factor-erythroid 2 related factor 2 (Nrf2) activation, leading to the increased expression of downstream of heme oxygenase-1 (HO-1). The use of siRNA targeting HO-1 blocked the inhibitory effects of lycopene on I κ B degradation and ICAM-1 expression. The inhibitory effects of lycopene thus appear to be mediated through its induction of Nrf2-mediated HO-1 expression. Therefore, the findings of the present study indicate that lycopene suppresses the activation of TNF α -induced signaling pathways through the upregulation of Nrf2-mediated HO-1 expression.

Introduction

Atherosclerosis is a chronic inflammatory disease which develops in response to injury in the vessel wall (1,2). A number of chronic diseases are known to be involved in the pathogenesis of inflammation, including atherosclerosis, Alzheimer's disease and Parkinson's disease (3). Endothelial cells (ECs) recruit circulating monocytes via a multi-step process mediated by a combination of cell surface adhesion molecules, such as intercellular cell adhesion molecule-1 (ICAM-1) during inflammation (2). Pro-inflammatory cytokines, such as tumor necrosis factor α (TNF α) commonly found in inflammation, can activate nuclear factor- κ B (NF- κ B) which plays a key role in the expression of cell adhesion molecules (1,4). Following stimulation with TNF α , a kinase cascade is activated that induces subsequent I κ B phosphorylation. Phosphorylated I κ B is rapidly degraded, resulting in the release of NF- κ B, which enters the nucleus and regulates gene transcription (5).

Lycopene is a natural carotenoid that is present in tomatoes and tomato-based products. Other dietary sources of lycopene also include pink grapefruit, papaya, watermelon and dried apricotscarotenoid (6). It has been reported that the dietary intake of tomatoes containing lycopene reduces the risk of chronic diseases and various types of cancer (7). Previous epidemiologic studies have found that the dietary intake of lycopene was significantly associated with plasma lycopene concentrations (8). The mean plasma level of lycopene has been found to be 0.59 μ M, ranging from 0.07 to 1.79 μ M and it contributes to approximately 21-43% of the total carotenoids (8,9). Lycopene exerts several biological functions, such as acting as an antioxidant and reducing low density lipoprotein cholesterol levels (10); it also reduces pro-inflammatory cytokine and chemokine expression in macrophages (11,12). Indeed, lycopene has been found to decrease the expression of several genes by modulating the NF- κ B signaling pathway in ECs (13). These results provide a possible mechanism for the anti-inflammatory effects of lycopene related to its antioxidant activity. However, the detailed mechanisms responsible for the anti-inflammatory effects of lycopene remain to be elucidated.

The transcription factor, nuclear factor-erythroid 2 related factor 2 (Nrf2), which binds to the antioxidant response element (ARE), is essential for the induction of phase II

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detoxification and antioxidant enzymes (14-16). A previous study further demonstrated that the activation of Nrf2 abolishes inflammatory gene expression in ECs (17). Heme oxygenase (HO) is a cytoprotective and a rate-limiting enzyme, and it degrades heme to bilirubin, carbon monoxide (CO) and iron (18). HO-1 is an Nrf2-mediated phase II enzyme upregulated in conditions of oxidative stress, cellular injury and disease (18). In our previous study, we reported that the Nrf2-induced expression of HO-1 and the related signaling pathways exerted anti-inflammatory effects in ECs (19). Recently, we also demonstrated that HO-1 exerts anti-inflammatory effects by its derivative CO production (20,21). However, the detailed mechanisms responsible for the anti-inflammatory effects of lycopene in ECs have not yet been fully elucidated. Thus, in the present study, we investigated the molecular mechanisms underlying the anti-inflammatory properties of lycopene in ECs. We aimed to investigate whether HO-1 expression contributes to lycopene-regulated anti-inflammatory responses. We found that the lycopene-induced accumulation of Nrf2 in the nuclei was closely associated with the upregulation of HO-1, which led to anti-inflammatory effects in ECs.

Materials and methods

The p3xARE/Luc and NF- κ B/Luc vectors were constructed by introducing the Nrf2 binding site or NF- κ B binding site into the pGL3 promoter plasmid (Promega, Madison, WI, USA), respectively as described in a previous study (22). Luciferase assay kits were purchased from Promega. Antibodies against HO-1 (SPA-896) and p65 (KAS-TF110) were purchased from Stressgen Biotechnologies (SB, San Diego, CA, USA). ICAM-1 (sc-7891), I κ B α (sc-847), lamin B1 (sc-56143), α -tubulin (sc-53646) and Nrf2 (sc-722) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Bacterially-derived TNF α was purchased from Calbiochem (San Diego, CA, USA). All other reagents, including lycopene and tricarbonyl dichlororuthenium (II) dimer (TCDC) were purchased from Sigma (St. Louis, MO, USA). The CO donor, TCDC, was activated by adding the compound to culture medium to liberate CO (20).

Culture of ECs. The human umbilical vein cell line, EA.hy926 (ATCC CRL-2922) was cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum at 37°C under 5% CO₂ in air. When the ECs were grown to confluence, the culture medium was then replaced with serum-free DMEM and the cells were incubated for 12 h prior to the experimental treatments.

Cell adhesion assay. Adhesion assay for monocytes and ECs was performed as previously described (23). Briefly, ECs grown to confluency in a 96-well plate were pre-treated with lycopene for 1-12 h and/or 100 U/ml TNF α for 4 h to allow for the expression of ICAM-1. The cells were washed with control DMEM without supplements and were co-cultured with 5x10⁵ cells of calcein-labeled THP-1 cells (ATCC[®] TIB202[™]) in the control medium for 30 min. After washing twice with RPMI medium, the adherent cells were examined using a Fluoroscan enzyme-linked immunosorbent assay (ELISA) plate reader (FLx800; Bio-Tek Instruments, Inc., Winooski, VT,

USA) at 485 nm excitation and 538 nm emission wavelengths. Fluorescence-labeled adherent cells were photographed using an Axiovert S100 microscope (Zeiss, Jena, Germany).

Western blot analysis. The cells (10⁶) were lysed on ice in lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and a protease inhibitor mixture) and whole-cell extracts were boiled for 5 min prior to separation on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA) in Tris-glycine buffer at 10 volts for 1.5 h. The membranes were then blocked with PBS containing 5% non-fat milk and incubated with primary antibodies for 2 h at 4°C with gentle shaking. After washing with PBS, the membranes were incubated with the secondary antibodies, horseradish peroxidase-conjugated goat anti-rabbit (34083) or anti-mouse (34081) antibody (Thermo Fisher Scientific, Waltham, MA, USA). The antibodies against α -tubulin and lamin were used as internal controls of the total or nuclear protein lysates, respectively. The results were visualized by chemiluminescence using ECL in according with the manufacturer's instructions.

RNA isolation and RT-PCR. Total RNA was isolated using TRIzol reagent and was reverse transcribed using SuperScript II reverse transcriptase (both from Invitrogen, Carlsbad, CA, USA) using an oligo(dT) primer according to the manufacturer's instructions. cDNA was subjected to PCR amplification using the following forward and reverse primer sets: ICAM-1, 5'-AGCAATGTGCAAGAAGATAGCCAA-3' and 5'-GGTCCCCTGCGTGTTCCACC-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5'-TATCGTGGAAGGACTCATGACC-3' and 5'-TACATGGCAACTGTGAGGGG-3'; glutamate-cysteine ligase modifier subunit (GCLM), 5'-CAGCGA GGA GCT TCA TGA TTG-3' and 5'-TGA TCA CAG AAT CCA GCT GTG C-3'; glutamate-cysteine ligase catalytic subunit (GCLC), 5'-GTT CTT GAA ACT CTG CAA GAG AAG-3' and 5'-ATG GAG ATG GTG TAT TCT TGT CC-3' (24). PCR products were separated on 1.5% agarose gels and visualized by ethidium bromide staining.

Preparation of subcellular fractionation for immunoblotting. The ECs were collected by scraping and lysed with cell lysis buffer (10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF and 0.3% nonidet P-40). The cell lysate was separated into cytoplasmic nuclear fractions by centrifugation at 500 x g for 5 min at 4°C, and the supernatant was collected and designated as the cytosolic fraction. The nuclei were washed with nuclei washing buffer and the nuclear protein was extracted using a buffer containing 25% glycerol, 20 mM HEPES, 0.6 M KCl, 1.5 mM MgCl₂ and 0.2 mM EDTA for 15 min at 4°C.

Luciferase reporter assays. The ECs were transfected with NF- κ B/Luc or p3xARE/Luc using Lipofectamine 2000 (Invitrogen), as previously described (22). For the luciferase assays, the cells were lysed and luciferase activity was determined by use luciferase substrate solution (Promega) and the resulting luciferase activity was measured using a luminometer. For each experiment, luciferase activity was determined in triplicate and normalized to β -galactosidase activity.

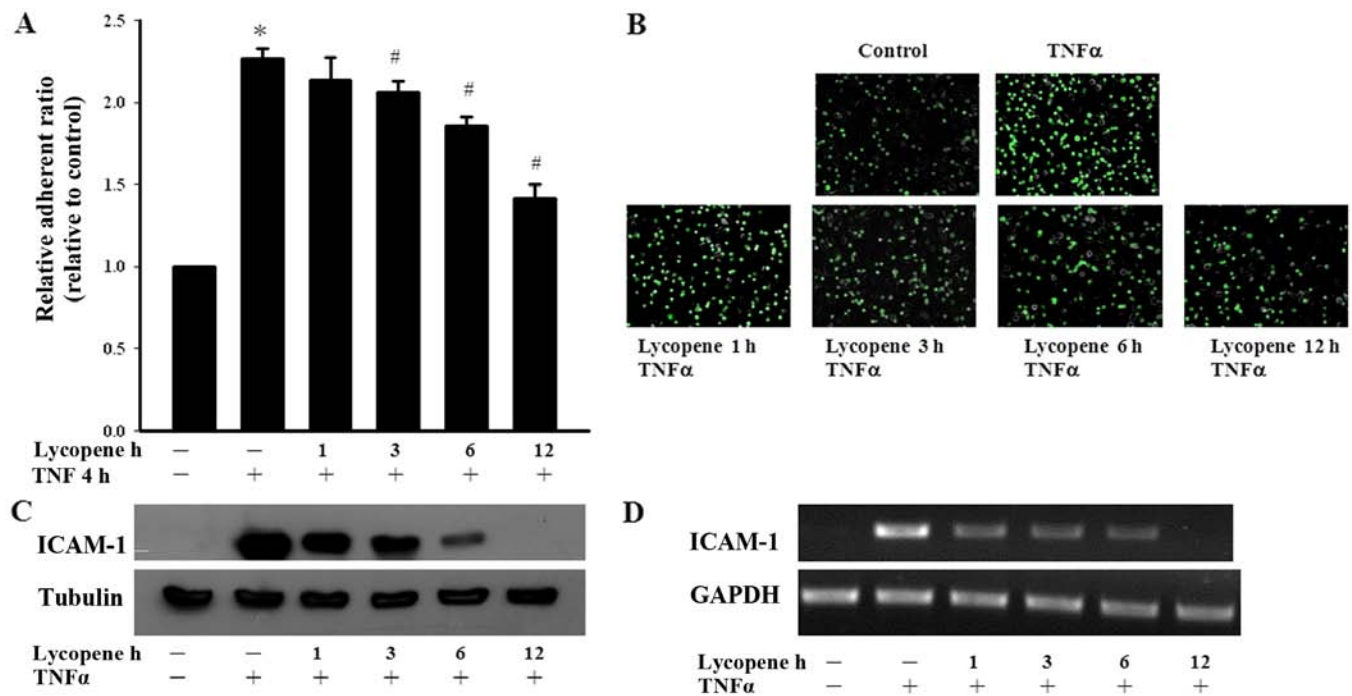


Figure 1. Lycopene inhibits both monocyte adhesion to tumor necrosis factor α (TNF α)-activated endothelial cells (ECs) and intercellular cell adhesion molecule-1 (ICAM-1) expression. (A) ECs were pre-incubated with or without 2 μ M lycopene for 12 h, stimulated without or with TNF α (100 U/ml) for 4 h, and then added to fluorescence-labeled THP-1 cells and allowed to adhere for 30 min. The data are presented by the relative adherent ratio as compared to untreated ECs. The values represent the means \pm SE from 3 independent experiments. * P <0.05 compared with untreated ECs; # P <0.05 compared with ECs exposed to TNF α alone. (B) Fluorescence-labeled adherent cells were photographed using a fluorescence microscope. (C) ECs were pre-incubated with or without 2 μ M lycopene for the indicated time periods and then stimulated with or without TNF α . Cell lysates were prepared and subjected to western blot analysis with antibodies against ICAM-1 or tubulin, as indicated. The tubulin band intensities indicate equal loading of each well. (D) ECs were pretreated with 2 μ M lycopene for the indicated time periods and then incubated with or without TNF α for 2 h and subjected to RT-PCR for ICAM-1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified as an internal control.

Cytotoxicity. To examine cytotoxicity, the resazurin reduction test, which is an index of the metabolic activity of living cells, was carried out using the Alamar blue[®] assay kit according to the instructions provided by the manufacturer (Serotec, Oxford, UK). The ECs were plated into 96-well microtiter plates (Falcon, Pittsburgh, PA, USA) at 20,000 cells/well and incubated in Alamar blue[®] reagent for 2 h at 37°C. Fluorescence was then measured using a Fluorescence Reader (FLx800, Bio-Tek, Winooski, VT, USA) at excitation/emission wavelengths of 570/600 nm.

Determination of GSH levels. The GSH levels were determined using the method originally described by Kamencic *et al* (25). Briefly, the cells cultured in 6-well plates were first washed with PBS, and 40 μ M of the fluorescent probe, monochlorobimane (MCB), with PBS was added followed by incubation for 20 min at 37°C, and then washed again with PBS. Fluorescence was monitored at excitation and emission wavelengths of 405 and 510 nm, respectively, using a spectrofluorophotometer (Shimadzu, Rf-5301PC; Shimadzu, Kyoto, Japan).

RNA interference using siRNA against HO-1. The siRNA nucleotide sequence for human HO-1 was as follows: 5'-CUG UGUCCUCUCUCUGGA-3' and was obtained from Sigma (NM_002133). A non-targeting siRNA, 5-GCAAGCUGA CCCUGAAGUU CAU-3, was purchased from Ambion (Austin, TX, USA). The EA.hy926 cells were transfected with siRNA against HO-1 (HO-1 siRNA) or non-targeting siRNA using

Lipofectamine 2000 reagent according to the manufacturer's instructions. After 24 h of transfection, the ECs were then cultured in medium without serum for another 12 h prior to the treatments.

Statistical analysis. The values are expressed as the means \pm standard error of the mean (SEM) of at least 3 independent experiments. Statistical significance was assessed by one-way analysis of variance (ANOVA) followed by Tukey's test. A confidence limit of P <0.05 was considered to indicate a statistically significant difference.

Results

Lycopene inhibits both monocyte adhesion and ICAM-1 expression in ECs. We first examined the effects of lycopene on monocyte adhesion to ECs. Monocyte adhesion was quantified by measuring the fluorescence intensity using fluorescent-labeled monocytes adhering to lycopene and/or TNF α pre-treated ECs. We found that TNF α significantly increased monocyte adhesion to the ECs and this adhesion process was abolished by treatment with lycopene (Fig. 1A and B). We then investigated the inhibitory effects of lycopene upon the TNF α -induced expression of adhesion molecules in ECs. We found that pre-treatment of the ECs with 2 μ M lycopene after 12 h significantly inhibited the TNF α -induced ICAM-1 expression at the protein and mRNA level (Fig. 1C and D). In the present study, we examined the cytotoxicity of lycopene in ECs using

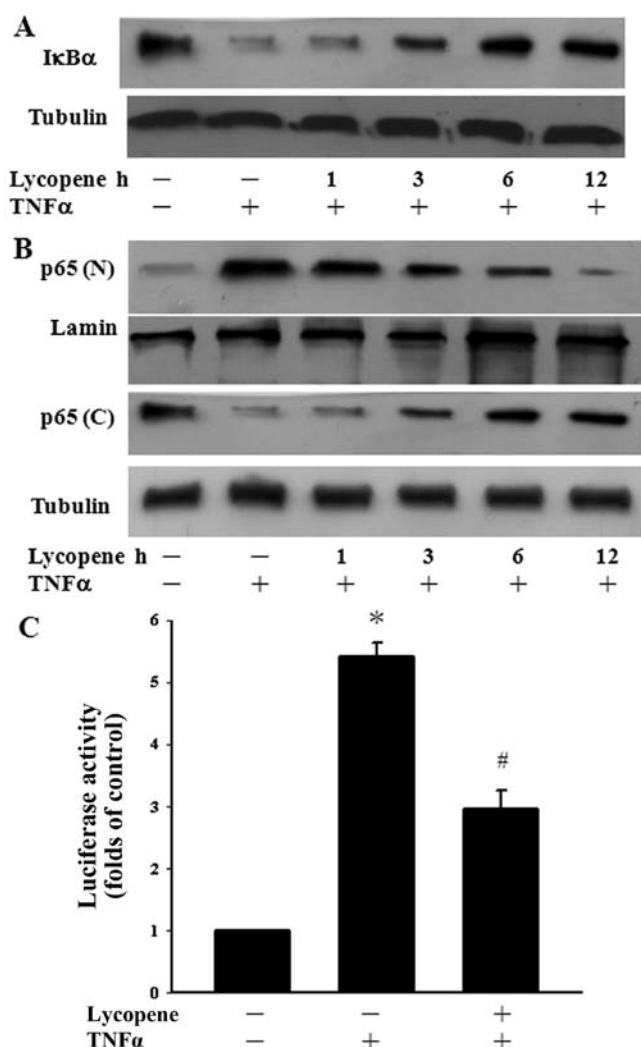


Figure 2. Influence of lycopene on tumor necrosis factor α (TNF α)-induced I κ B α degradation and nuclear factor- κ B (NF- κ B) nuclear translocation. (A) Endothelial cells (ECs) were pre-incubated with or without 2 μ M lycopene for the indicated time periods, stimulated without or with TNF α (100 U/ml). Nuclear (p65 N) and cytosolic (p65 C) extracts were subjected to western blot analysis. The tubulin and lamin band intensities indicate equal loading, respectively. (B) ECs were stimulated with TNF α for 30 min and a portion of these cells was then pre-treated with 2 μ M lycopene for the indicated time periods. Cell lysates were then prepared and subjected to western blot analysis with antibodies against I κ B α and tubulin, as indicated. (C) ECs were co-transfected with the NF- κ B luciferase reporter construct and β -galactosidase for 16 h. Cells were then exposed to 2 μ M lycopene for 12 h and to 100 U/ml TNF α for a further 6 h. Luciferase activity was normalized to co-transfected β -galactosidase activities. Induction is indicated relative to the control. All values are presented as the means \pm SEM with $P < 0.05$. * $P < 0.05$ compared with untreated ECs; # $P < 0.05$ compared to cells exposed to TNF α alone (means \pm SEM).

an Alamar blue assay, which indicated no adverse effects (data not shown).

Influence of lycopene on TNF α -induced I κ B α degradation and NF- κ B nuclear translocation. Since the NF- κ B pathway is a well-known inflammatory pathway (2), we examined whether lycopene regulates TNF α -induced NF- κ B activation. To clarify the inhibitory mechanisms of lycopene in TNF α -induced NF- κ B activation, the degradation of I κ B α was determined over a time course manner. TNF α alone induced the significant

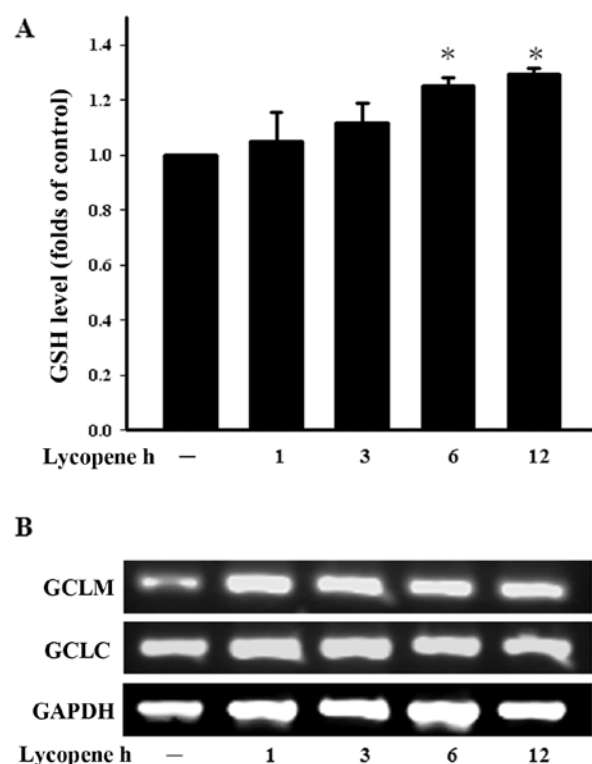


Figure 3. Lycopene increases the intracellular GSH level in endothelial cells (ECs). (A) ECs were exposed to 2 μ M lycopene for the indicated time periods and the intracellular GSH levels were then measured. All values shown as the means \pm SEM. * $P < 0.05$ compared with untreated ECs. (B) ECs were treated with 2 μ M lycopene for the indicated time periods and then subjected to RT-PCR analysis for GCLM and GCLC.

degradation of I κ B α after 1 h of exposure. However, pre-treatment with lycopene for 6 and 12 h inhibited the TNF α -induced degradation of I κ B α in ECs (Fig. 2A). The cells were also pre-treated with lycopene over a 12-h time period to examine whether this agent regulates p65 translocation to the nucleus in TNF α -stimulated ECs. We found the TNF α -induced p65 nuclear translocation decreased following pre-treatment with lycopene for 6 to 12 h (Fig. 2B). In addition, we examined the inhibition of TNF α -induced p65 activation by lycopene at the transcriptional level. Following pre-treatment with lycopene for 12 h, we found that the TNF α -induced NF- κ B activation was indeed inhibited using a luciferase assay (Fig. 2C). Our results thus indicate that lycopene inhibits NF- κ B nuclear translocation and transactivation following pre-treatment with lycopene for 12 h.

Lycopene increases the GSH level in ECs. GSH, a well-studied tri-peptide, plays numerous protective roles in cells, protecting them against oxidative stress and maintaining the cellular thiol redox status. We therefore examined the GSH levels in ECs treated with lycopene in a time course manner. The intracellular GSH levels were found to be increased following treatment with lycopene for 6 h and this increased persisted until 12 h of treatment (Fig. 3A). The enzyme in the *de novo* synthesis of GSH is GCL and consists of GCLC and GCLM. Treatment with lycopene increased the GCLM expression levels over the indicated incubation period (Fig. 3B). These findings demon-

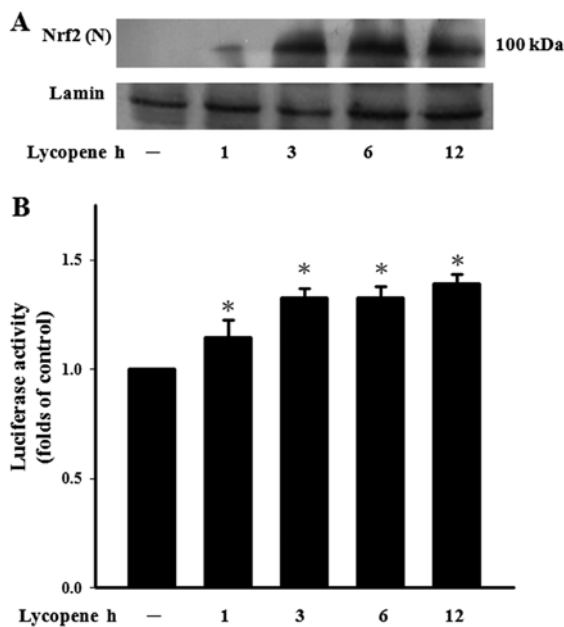


Figure 4. Lycopene induces nuclear factor-erythroid 2 related factor 2 (Nrf2) activation. (A) Endothelial cells (ECs) were treated with 2 μ M lycopene for the indicated time periods. Nuclear lysates were prepared and subjected to western blot analysis to quantify the nuclear translocation of Nrf2. The bottom panel shows the internal control (lamin). (B) The cells were transfected with the ARE-luciferase construct (ARE) and after 12 h were maintained in low serum medium for a further 6 h, and then stimulated with 2 μ M lycopene for the indicated time periods. The cell lysates were analyzed for luciferase activity as described in the Materials and methods. Luciferase activity was normalized to co-transfected β -galactosidase activities. Induction is indicated relative to the control. All values are presented as the means \pm SEM with * P <0.05.

strate that lycopene increases the GSH levels and modulates the cellular redox status.

Lycopene induces Nrf2 activation. Lycopene increases the intracellular GSH levels which are synthesised by Nrf2-related glutamylcysteine synthetase (26). It has previously been reported that Nrf2 encoding phase II detoxifying and antioxidant enzymes provide cytoprotective effects in ECs (27). In this study, the ECs treated with lycopene exhibited a continuous increase in Nrf2 nuclear accumulation for up to 3 h (Fig. 4A). Nrf2 regulates the ARE that drives the expression of specific genes (27). We found lycopene that indeed led to an increase in Nrf2 transcriptional activity by transfecting ECs with ARE-luciferase reporter construct (Fig. 4B).

Lycopene induces HO-1 expression. In our previous study, we reported that the Nrf2-induced expression of HO-1 exerted anti-inflammatory effects in ECs (19). Hence, in this study, we examined the HO-1 levels over the time course of lycopene pre-treatment and found that the HO-1 protein levels increased after 6 h of lycopene pre-treatment and this increased persisted for up to 12 h (Fig. 5A). In the ECs treated with various concentrations of lycopene, HO-1 protein expression was increased (at the concentrations of 1-5 μ M) (Fig. 5B).

The inhibitory effects of lycopene are dependent on the induction of HO-1 expression. To investigate whether HO-1 induction contributes to the protective effects of lycopene, HO-1

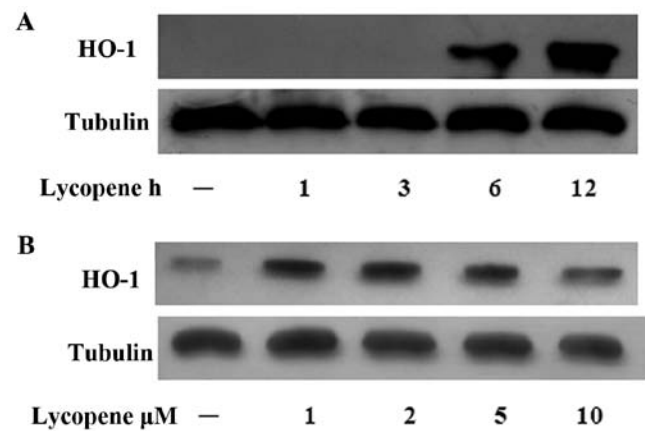


Figure 5. Lycopene induces heme oxygenase-1 (HO-1) expression. (A) EC cultures were incubated with 2 μ M lycopene for the indicated time periods. Western blot analysis was then performed with antibodies against HO-1 or tubulin, as indicated. (B) Western blot analysis of HO-1 expression was carried out in cells treated with 1, 2, 5 or 10 μ M lycopene for 12 h.

siRNA experiments were performed. Indeed, transfection with HO-1 siRNA suppressed the inhibitory effects of lycopene on ICAM-1 expression (Fig. 6A). Consistently, transfection of the ECs with HO-1 siRNA also reversed the inhibitory effects of lycopene on I κ B degradation following treatment with lycopene for 12 h (Fig. 6B). Our previous data found that CO seems to be the major mediator of the anti-inflammatory effects of HO-1 (20,21). In this study, we found that pre-treatment of the ECs with 25 μ M CO donor [tricarbonyldichlororuthenium (II) dimer] (TCDC) for 3 h inhibited TNF α -induced ICAM-1 expression (Fig. 6C). Therefore, our data indicate that the lycopene-induced expression of HO-1 is involved in the anti-inflammatory effects in ECs.

Discussion

Lycopene is a natural carotenoid that is present in tomatoes and tomato-based products and our present findings indicated that lycopene suppressed TNF α -induced monocyte adhesion to ECs by downregulating the expression of ICAM-1. Our data also indicated that the inhibitory effects of lycopene upon the activation of inflammatory transcriptional factor, NF- κ B, were mediated through the blocking of the degradation of I κ B α . However, with this chemical, this effect appears to be exerted through the induction of Nrf2-related genes, particularly HO-1. HO-1 was suggested as the major effector of lycopene for its anti-inflammatory effects and the increase in the levels of GSH and HO-1 expression were mediated by promoting Nrf2 nuclear translocation (Fig. 7).

It has been reported that the regulation of ICAM expression involves the NF- κ B pathway. The activation of the transcription factor, NF- κ B, by TNF α is required for the transcriptional activation of EC adhesion molecules, and NF- κ B is believed to be a pivotal transcription factor in chronic inflammatory diseases (5). Overall, this suggests that lycopene inhibits adhesion molecule expression, possibly by blocking NF- κ B activation. Our data revealed that lycopene inhibited the TNF α -induced degradation of I κ B α , following pre-treatment with lycopene for more

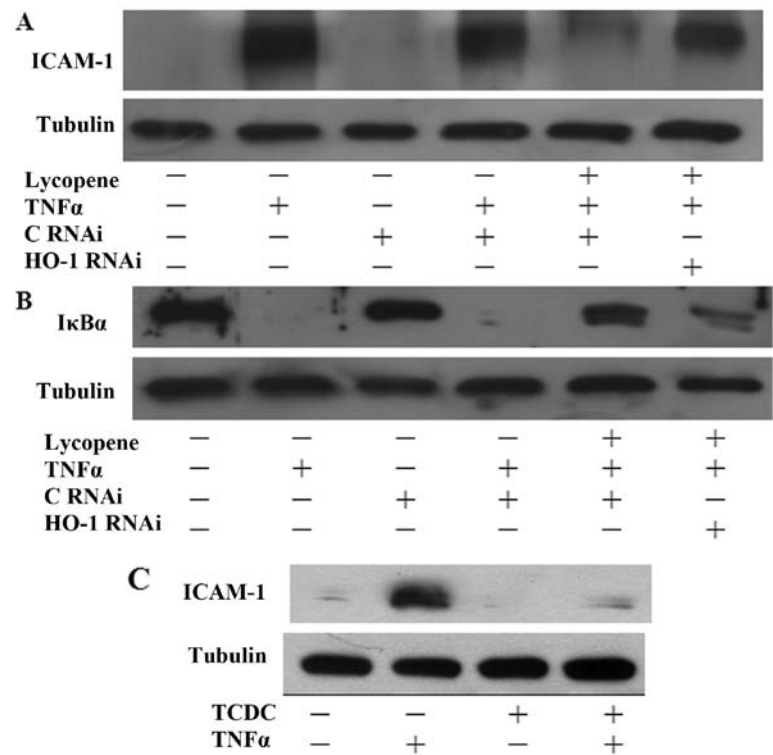


Figure 6. Inhibitory effects of lycopene are dependent on the induction of heme oxygenase-1 (HO-1) expression Cells were transfected with siRNA or si-HO-1 and transfection efficiency was examined. (A) Cells were transfected with non-targeting siRNA (C RNAi) or HO-1 siRNA (HO-1 RNAi) for 36 h and then treated with 2 μM lycopene for 12 h. Endothelial cells (ECs) were treated with or without 100 U/ml tumor necrosis factor α (TNFα) for 4 h, and cell lysates were prepared and subjected to western blot analysis with antibodies against intercellular cell adhesion molecule-1 (ICAM-1). (B) Cells were transfected with non-targeting siRNA or HO-1 siRNA and then treated with lycopene as described above. ECs were treated with or without 100 U/ml TNFα for 0.5 h, and cell lysates were subjected to western blot analysis with antibodies against IkBα. (C) ECs were pre-treated with 25 μM TCDC for 3 h without or with TNFα (100 U/ml) for 4 h. Cell lysates were prepared and subjected to western blot analysis with antibodies against ICAM-1.

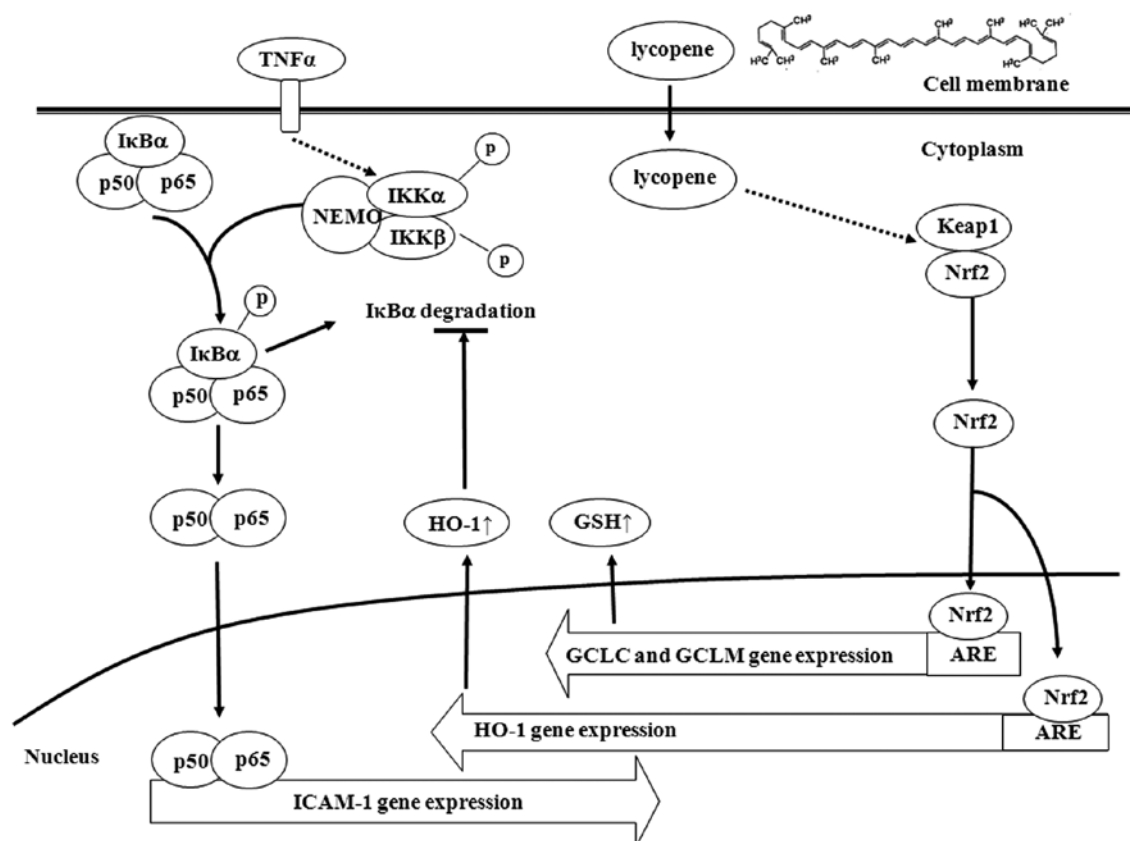


Figure 7. Possible mechanisms responsible for the protective effects of lycopene against tumor necrosis factor α (TNFα)-induced nuclear factor-κB (NF-κB) activation. The blocking of IκBα degradation may lead to the prevention of NF-κB activation; a relevant mechanism is also by the lycopene-induced expression of heme oxygenase-1 (HO-1) through the nuclear factor-erythroid 2 related factor 2 (Nrf2) pathway. Please see the 'Discussion' for details.

than 3 h (Fig. 2A). Hence, the inhibition of NF- κ B activity by lycopene was mediated through the modulation of upstream targets in the NF- κ B pathway. However, lycopene exerted an inflammatory effect via the NF- κ B pathway and it modulated EC activation according to different incubation times. A short incubation (3 h) with lycopene only had a partial inhibitory effect on the nuclear appearance of NF- κ B in ECs (Fig. 2B). Otherwise, the significant inhibitory effects of lycopene on p65 translocation were observed at 12 h. These findings further indicate that the cytoprotective effects of lycopene are more prominent with prolonged treatment.

GSH is a well-studied tri-peptide and has numerous roles in the protection of cells from oxidants and maintaining the cellular thiol redox status. Our previous studies have demonstrated that the intracellular GSH levels are a major modulator of the effects of EGCG and piceatannol (19,28). In this study, we therefore investigated the intracellular level of GSH following lycopene treatment. We observed that the GSH levels increased after 6 h of lycopene treatment. Additional experiments demonstrated that lycopene increased the gene expression of GCL, the key rate-limiting enzyme in GSH synthesis (Fig. 3B). The reversible redox reactions of GSH regulate diverse biological processes, including enzyme catalysis, gene expression, cell proliferation and atherosclerosis (29). In a previous study of ours, we found that GSH played a critical role in protein glutathionylation during mild oxidative stress (30). Thus, the regulation of GSH by Nrf2 plays an important role in the glutathionylation of NF- κ B and this may be one of the potential mechanisms underlying the anti-inflammatory effects. Thus, we cannot exclude the mechanisms involved in the elevated intracellular GSH levels. It has been reported that GCLC and GCLM expression are mediated by Nrf2 activation (26). Subsequently, additional experiments are required to evaluate the role of lycopene in regulating Nrf2 activity, since GCLC was found to be regulated by Nrf2 activation.

Nrf2 has previously been reported to be a key factor inducing phase II detoxifying and antioxidant enzymes (27). A recent study also demonstrated that lycopene enhanced the activation of the phosphoinositide 3-kinase/Akt pathway, followed by the induction of Nrf2 nuclear translocation in ECs (31). Our present findings indicated that lycopene induced phase II enzyme GCL and HO-1 expression and further demonstrate that Nrf2 translocates into the nucleus and binds to ARE to activate transcription.

It has been previously determined that HO-1 functions as part of a cytoprotective mechanism derived from its antioxidant and anti-inflammatory properties, and thus has potential as a therapeutic target for cardiovascular diseases (20). In atherosclerotic plaques, the increased expression of HO-1 attenuates atherosclerosis and this further establishes the protective role of HO-1 against this disease (32). The anti-inflammatory effects of lycopene-induced HO-1 have been investigated in previous studies (33,34). In this study, we found that the anti-inflammatory effects of lycopene were mediated through the inhibition of I κ B α degradation and ICAM-1 expression was reduced by transfection with HO-1 siRNA (Fig. 6A and B). Previous studies have shown that CO donors exert their effects via HO-1 induction in various systems (20,21). In the present study, as shown in Fig. 6C, CO donor mediated the inhibition of ICAM-1 expression.

In conclusion, the findings of the present study indicated that there is a pivotal role of Nrf2-regulated pathways in the mechanisms underlying the inhibition of NF- κ B in lycopene-treated ECs. The cytoprotective effects of lycopene require the upregulation of HO-1 expression in ECs. A clearer understanding of the working mechanisms of lycopene in the future may contribute to the development of a therapeutic application which may be used in inflammation-associated diseases.

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