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RESEARCH ARTICLE

Sesamin attenuates intercellular cell adhesion molecule-1 expression *in vitro* in TNF-α-treated human aortic endothelial cells and *in vivo* in apolipoprotein-E-deficient mice

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Sesame lignans have antioxidative and anti-inflammatory properties. We focused on the effects of the lignans sesamin and sesamol on the expression of endothelial-leukocyte adhesion molecules in tumor necrosis factor-α (TNF-α)-treated human aortic endothelial cells (HAECs). When HAECs were pretreated with sesamin (10 or 100 μM), the TNF-α-induced expression of intercellular cell adhesion molecule-1 (ICAM-1) was significantly reduced (35 or 70% decrease, respectively) by Western blotting. Sesamol was less effective at inhibiting ICAM-1 expression (30% decrease at 100 μM). Sesamin and sesamol reduced the marked TNF-α-induced increase in human antigen R (HuR) translocation and the interaction between HuR and the 3'UTR of ICAM-1 mRNA. Both significantly reduced the binding of monocytes to TNF-α-stimulated HAECs. Sesamin significantly attenuated TNF-α-induced ICAM-1 expression and cell adhesion by downregulation of extracellular signal-regulated kinase 1/2 and p38. Furthermore, *in vivo*, sesamin attenuated intimal thickening and ICAM-1 expression seen in aortas of apolipoprotein-E-deficient mice. Taken together, these data suggest that sesamin inhibits TNF-α-induced extracellular signal-regulated kinase/p38 phosphorylation, nuclear translocation of NF-κB p65, cytoplasmic translocalization of HuR and thereby suppresses ICAM-1 expression, resulting in reduced adhesion of leukocytes. These results also suggest that sesamin may prevent the development of atherosclerosis and inflammatory responses.

Keywords:
Atherosclerosis / Endothelial cell / Intercellular cell adhesion molecule-1 / NF-κB / Sesamin

1 Introduction

Sesame (*Sesamum indicum* L.) seeds have long been considered as a very popular health food in Asian countries. Sesame oil is highly resistant to oxidative deterioration compared with other edible oils due to its superior anti-oxidative activity [1]. Sesame oil contains a significant amount of sesamin and a small amount of sesamol. Sesamin enhances the hepatic detoxification of chemicals, reduces the incidence of chemically induced tumors, and protects against oxidative stress [2, 3]. Sesamin and sesamol

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Abbreviations: apo-E, apolipoprotein-E; **ARE,** AU-rich element; **EC,** endothelial cell; **ERK,** extracellular signal-regulated kinase; **HAEC,** human aortic endothelial cell; **HuR,** human antigen R; **ICAM-1,** intercellular cell adhesion molecule-1; **MAPK,** mitogen-activated protein kinase; **MTT,** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; **RT-PCR,** real-time PCR; **siRNA,** small interfering RNA; **TNF,** tumor necrosis factor

inhibit Δ-5 desaturase activity, resulting in the accumulation of dihomo-γ-linolenic acid, which can displace arachidonic acid from liver membrane phospholipids and decrease the formation of pro-inflammatory mediators, such as prostaglandin E2 and leukotriene B4 [4]. Sesamin and sesamol have been shown to increase survival after cecal ligation and puncture and increase IL-10 levels in response to a non-lethal dose of endotoxin in mice [5]. Sesamin and sesamolin suppress LPS-induced NO production by microglia and macrophages by inhibiting signal transduction pathways or nuclear transcription factors [6]. Thus, the protective effects of sesamin are related to its anti-inflammatory properties. We were therefore interested in understanding the mechanism of action of sesamin and sesamol on endothelial cells (ECs) stimulated by inflammatory cytokines and whether they affect the expression of adhesion molecules, an important event in atherosclerosis and inflammation.

The expression of cell adhesion molecules, such as intercellular cell adhesion molecule-1 (ICAM-1), on ECs represents one of the earliest pathological changes in immune and inflammatory diseases, such as atherosclerosis [7]. Increased expression of adhesion molecules by ECs in human atherosclerotic lesions may lead to further recruitment of leukocytes to atherosclerotic sites [8]. Regulation of adhesion molecule expression requires a complex array of intracellular signaling pathways involving RNA-binding proteins, mitogen-activated protein kinases (MAPKs), and transcriptional factors [9, 10]. Although these multiple signaling molecules have received considerable attention, little is known about the effects of sesamin and sesamol on adhesion molecule expression and the mechanisms of these effects, and a better understanding of this might provide important insights into the prevention of atherogenesis and inflammation. We therefore tested the ability of sesamin and sesamol to modulate the expression of adhesion molecules, RNA-binding proteins, MAPKs, and transcriptional factors by human aortic endothelial cells (HAECs). In addition, we studied the effects of sesamin on the intimal thickening and adhesion molecule expression seen in apolipoprotein-E (apo-E)-deficient mice. Our results showed that sesamin attenuated ICAM-1 expression both *in vitro* and *in vivo* and that this effect may be mediated by inhibition of human antigen R (HuR) translocation, extracellular signal-regulated kinase (ERK)/p38 phosphorylation, and NF-κB activation. Sesamin also significantly inhibited the adhesion of the human monocytic cell line U937 to HAECs.

2 Materials and methods

2.1 Culture of HAECs

HAECs were obtained as cryopreserved tertiary cultures from Cascade Biologics (Oregon, USA) and were grown in culture flasks in EC growth medium. The cells were cultured at 37°C in a humidified atmosphere of 95% air, 5% CO_2 and used between passages 3 and 8.

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

Cell viability and activity of mitochondrial electron transport chain, as indicator for cytotoxicity, was determined by the capacity of cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan. HAECs were plated at a density of 10^4 cells/well in 96-well plates. After overnight growth, the cells were incubated for 24 h with different concentrations of TNF-α (PeproTech), sesamin (Cayman Chemical), or sesamol (Sigma); then cell viability was measured using the MTT assay.

2.3 Preparation of cell lysates and nuclear and cytosolic fractions

To prepare cell lysates, the cells were lysed for 1 h at 4°C in 20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM PMSF, pH 7.4, then the lysate was centrifuged at 4000 *g* for 30 min at 4°C and the supernatant kept.

To prepare nuclear and cytosolic proteins, the cells were washed with PBS, pelleted, and lysed on ice for 15 min in lysis buffer (0.1 mM EDTA, 10 mM HEPES, 10 mM KCl, 0.1 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 mM Na_3VO_4, and 0.6% NP-40). The lysates were centrifuged at 12 000 *g* at 4°C for 10 min and the resulting nuclear pellet and supernatant (cytosolic fraction) stored at −80°C for Western blot analysis.

2.4 Western blot analysis

Western blot analyses were performed as described previously [11]. An aliquot of sample (20 μg of total protein) was subjected to 12% SDS-PAGE electrophoresis and transferred to PVDF membranes. To measure ICAM-1 levels, the membranes were incubated with goat anti-human ICAM-1 antibodies, then with HRP-conjugated mouse anti-goat IgG antibodies, bound antibody being detected using Chemiluminescence Reagent Plus (NEN). Anti-β-actin antibodies (1:10 000, Oncogen) and α-tubulin antibodies (1: 3000, Oncogen) were used to quantify β-actin and α-tubulin, used as the internal control. In other studies, the antibodies used were mouse anti-human HuR (Santa Cruz), rabbit anti-human phospho-JNK, mouse anti-human phospho-ERK1/2, rabbit anti-human phospho-p38, rabbit anti-human total JNK, rabbit anti-human total ERK1/2, and goat anti-human total p38 (all 1:1000, Cell Signaling), followed by HRP-conjugated goat anti-rabbit IgG antibodies (1:3000, Sigma) or goat anti-mouse IgG or rabbit anti-goat IgG antibodies (1: 3000, both from Chemicon), as appropriate.

2.5 Quantitative real-time PCR

Total RNA was isolated using a TRIzol reagent kit (Invitrogen, CA, USA). Quantitative real-time PCR (RT-PCR) was performed using a Brilliant II SYBR Green QRT-PCR master mix kit (Stratagene, CA, USA). Primers were as follows: for ICAM-1 forward CTGGACTTCGAGCAAGAGATG and reverse TGATGGAGTTGAAGGTAGTTTCG; and for β-actin, forward CTGGACTTCGAGCAAGAGATG and reverse TGATGGAGTTGAAGGTAGTTTCG. The PCR conditions were 50°C for 60 min, 95°C for 10 min, and 40 cycles of 95°C for 30 s, 60°C for 30 s, and 68°C for 60 s for all genes assayed. Real-time fluorescence monitoring and melting curve analysis were performed using a Stratagene Mx3000P® RT-PCR system and data were analyzed using Stratagene MxPro-Mx3000P QPCR software version 3.00 (Stratagene).

2.6 Cross-linking immunoprecipitation assay for RNA–protein interaction

To determine whether HuR interacted directly with the 3′UTR of ICAM-1 mRNA, immunoprecipitation and RT-PCR were carried out. To induce cross-linking, cells in PBS were irradiated with ultraviolet-B light. The cells were then treated with lysis buffer and the cytoplasmic fraction (500 μg of protein) was incubated with 10 μg of anti-HuR antibody and Protein G-Sepharose (GE Healthcare) for 1 h at 4°C. The RNA in the immunoprecipitated material was extracted using TRIzol reagent and used in a quantitative RT-PCR reaction to detect the presence of the 3′UTR of ICAM-1 mRNA. The mRNA was reverse-transcribed using a Reverse-iT 1st Strand Synthesis Kit (AB gene, USA), followed by quantitative RT-PCR to measure the 3′UTR transcript levels. The PCR primers used for the 3′UTR of ICAM-1 mRNA were CCATCGATGCCTGCTGGATGAGACTCCTGC and CCATCGATAGACTCTCACAGCATCTGCAGC.

2.7 EC–leukocyte adhesion assay

U937 cells, originally derived from a human histiocytic lymphoma and obtained from the American Type Culture Collection (Rockville, MD, USA), grown in RPMI 1640 medium (M.A. Bioproducts, MD, USA), were labeled for 1 h at 37°C with BCECF/AM (10 mM, Boehringer-Mannheim). Labeled U937 cells (10^6) were added to each HAEC-containing well and incubation continued for 1 h. Non-adherent cells were removed by two gentle washes with PBS; then the degree of bound U937 cells was evaluated both by fluorescence microscopy and on a dual scanning spectrofluorimeter (Spectramax Gemini XS, Molecular Devices) after lysing the cells with lysis buffer.

2.8 Knockdown of gene expression using small interfering RNA

Knockdown of ERK, p38, or HuR gene expression was performed by transfection with small interfering RNA (siRNA). Cells (5×10^6) plated on 6-well plates were transfected with siRNA when they reached 30–50% confluence, according to the manufacturer's protocol. Briefly, gene-specific siRNA oligomers (200 μmol/mL) were diluted in 500 μL of Opti-MEM I reduced serum medium (Opti-MEM, Invitrogen) and mixed with 5 μL of transfection reagent (Invitrogen) pre-diluted in 500 μL of Opti-MEM. After 20 min incubation at room temperature, the complexes were added to the cells in a final volume of 1 mL of medium. The ERK siRNAs were AUAUUCUGUCAGGAACCCUGUGUGA and UCACACAGGGUUCCUGACAGAAUAU (catalog♯10620319 124945 F11 & 10620318 124945 F12, Invitrogen), the p38 siRNAs were UUCAUUCACAGCUAGAUUACUAGGU and ACCUAGUAAUCUAGCUGUGAAUGAA (catalog♯10 620319 124945 F09 & 10620318 124945 F10) and HuR siRNAs were GGAUGAGUUACGAAGCCUGTT and CAGGCUUCGUAACUCAUCCTG (Ambion Catalog♯16704). Cells were transfected for 48 h. The Stealth RNAi Negative Control Med GC that has no homology to the vertebrate transcriptome was used as a negative control (siCL). The siRNA results were evaluated by Western blotting.

2.9 Nuclear extract preparation and electrophoretic mobility shift assay

The preparation of nuclear protein extracts and the electrophoretic mobility shift assay conditions have been described previously [11]. The 22-mer synthetic double-stranded oligonucleotides used as NF-κB probes in the gel shift assay were 5′-AGTTGAGGGGACTTTCCCAGGC-3′ and 3′-TCAACTCCCCTGAAAGGGTCC G-5′.

2.10 Immunocytochemical localization of NF-κB p65

To localize NF-κB expression *in situ*, confluent HAECs (controls or cells treated for 24 h with sesamin or sesamol) on slides were incubated with 2 ng/mL of TNF-α for 30 min, fixed in 4% paraformaldehyde in PBS (pH 7.4) for 15 min at 4°C, then reacted for 1 h at room temperature with mouse anti-human NF-κB p65 antibody (1:500 dilution in PBS; Transduction). After washes, the slides were incubated for 1 h at 37°C with FITC-conjugated goat anti-mouse IgG, and then viewed on a fluorescence microscope.

2.11 Animal care and experimental procedures

ApoE-deficient mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). After 6 months on a

commercial mouse chow diet, 24 mice were randomly allocated to one of two groups; both of which received a high fat (20% w/w) and high cholesterol (0.15% w/w) semisynthetic modified AIN 76 diet, supplemented, in one group, with 0.5% sesamin. Mice were allowed free access to the diets for the 11 wk of the experiment and were deprived of food overnight at the end of the experiment. The investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). The mice were euthanized by intraperitoneal injection of 35–40 mg/kg of sodium pentobarbital and the thoracic aorta gently dissected, immersion-fixed in 4% buffered paraformaldehyde, paraffin-embedded, then cross-sectioned for morphometry and immunohistochemistry. To examine expression of ICAM-1 protein, immunohistochemistry was performed on sections of the aorta. The sections were then incubated for 1 h at 37°C with goat anti-human ICAM-1 antibody (1:15 dilution in PBS, R&D System) and for 1 h at room temperature with horseradish peroxidase conjugated rabbit anti-goat IgG antibody, bound antibody being detected by 0.5 mg/mL of 3,3′-diaminobenzidine/0.01% hydrogen peroxide in 0.1 M Tris-HCl buffer, pH 7.2, as chromogen (Vector Lab, USA). Negative controls were performed by omitting the primary antibodies.

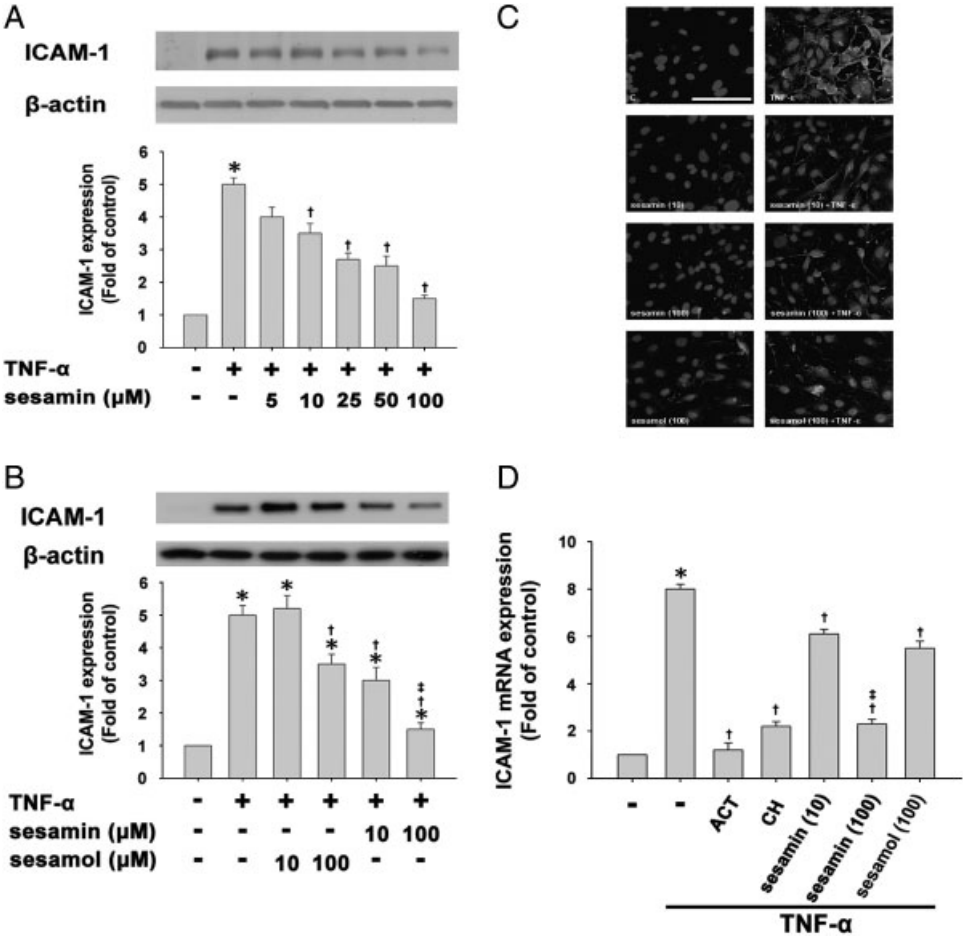

Figure 1. Sesamin and sesamol reduce ICAM-1 mRNA and protein expression in TNF-α-treated HAECs. (A and B) HAECs were incubated for 24 h with various concentration of sesamin or sesamol, then with 2 ng/mL of TNF-α for 12 h in the continued presence of the same concentration of sesamin/sesamol, and ICAM-1 expression was measured in cell lysates by Western blotting. β-actin was used as the loading control. (C) The cells were treated as in (A), then the distribution of ICAM-1 was analyzed by immunofluorescence. ICAM-1 expression is indicated by green (bright) fluorescence (FITC) and nuclei by blue (dark) fluorescence (DAPI). Bar=100 μm. (D) Analysis of ICAM-1 mRNA levels in untreated HAECs or HAECs preincubated with actinomycin D (ACT, 20 μg/mL), cycloheximide (CHX, 20 μg/mL), sesamin (10 or 100 μM), or sesamol (100 μM) for 24 h, then incubated for 4 h with 2 ng/mL of TNF-α in the continued presence of the inhibitor. Total RNA was analyzed by quantitative real-time PCR after normalization to β-actin mRNA levels. In A and C, the data are expressed as a fold value compared with the control value and are the mean ± SEM for three separate experiments. *$p<0.05$ compared with the untreated cells. †$p<0.05$ compared with the TNF-α-treated cells. ‡$p<0.05$ compared with the sesamol (100 μM)+TNF-α-treated cells.

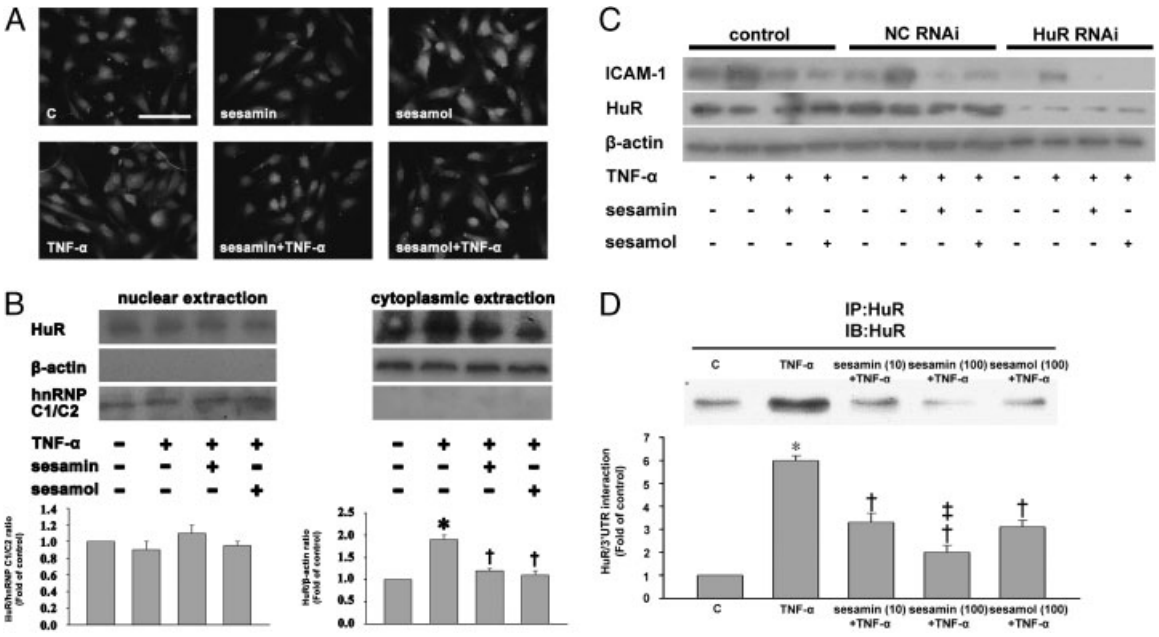

Figure 2. The sesamin- or sesamol-mediated reduction in TNF-α-induced ICAM-1 expression involves inhibition of HuR translocation and the HuR-ICAM-1 mRNA interaction. (A) Subcellular distribution of HuR in HAECs shown by immunofluorescence and confocal microscopy. Bar=120 μm. (B) Western blots showing HuR protein expression in the HAEC cytoplasm and nucleus. β-actin and hnRNP C1/C2 were used as loading controls and were used to ensure lack of cross-contamination of the fractions. (C) After silencing of HuR expression by siRNA, intracellular total HuR levels were measured by Western blot analysis. β-actin was used to monitor the quality of sample loading. (D) The cytoplasmic fractions were analyzed by immunoprecipitation and quantitative real-time PCR using 5 μL of immunoprecipitated material in the quantitative RT-PCR to detect the presence of the 3′UTR of ICAM-1 mRNA. The data are presented as the mean ± SEM and represent the results from three independent experiments.

2.12 Statistical analysis

Values are expressed as the mean ± SEM. Statistical evaluation was performed using one-way ANOVA followed by the Dunnett test, with a p value < 0.05 being considered significant.

3 Results

3.1 Sesamin and sesamol reduce ICAM-1 mRNA and protein expression in TNF-α-treated HAECs

When the cytoxicity of TNF-α or sesamol or sesamin for HAECs was assessed by MTT assay after 24 h of incubation, cell viability was not affected by the presence of 2 ng/mL of TNF-α or 10–100 μM sesamol or sesamin (data not shown). The concentrations of 2 ng/mL of TNF-α and 10–100 μM sesamol or sesamin were therefore used in subsequent experiments.

To determine whether TNF-α alone or together with sesamol or sesamin affected levels of ICAM-1 protein, Western blotting was performed. As shown in Fig. 1A, after treatment of the cells with TNF-α for 12 h, ICAM-1 protein expression was increased by about fivefold and this effect was significantly attenuated by 24 h pretreatment with 10, 25, 50, or 100 μM sesamin (35, 45, 50, or 70% inhibition, respectively). However, sesamol was less effective, causing only 30% inhibition at 100 μM (Fig. 1B). The effect of sesamin and sesamol on ICAM-1 expression was also studied by immunofluorescence staining (Fig. 1C). In untreated cells, ICAM-1 expression was weak. In contrast, cells treated for 12 h with TNF-α showed strong ICAM-1 expression and this effect was inhibited by pretreatment with sesamin (10 or 100 μM) or sesamol (100 μM).

To determine whether TNF-α alone or together with sesamin or sesamol affected ICAM-1 mRNA levels, quantitative RT-PCR was performed. Unstimulated HAECs produced low amounts of ICAM-1 mRNA, and 4 h treatment with TNF-α resulted in a marked increase in levels (Fig. 1D). This increase was markedly inhibited by preincubation with 10 or 100 μM sesamin (25 or 70% inhibition, respectively) or 100 μM sesamol for 24 h (30% inhibition). Furthermore, the addition of 20 μg/mL of actinomycin D (an RNA polymerase inhibitor) or cycloheximide (a protein synthesis inhibitor) for 24 h before incubation for 4 h with TNF-α significantly reduced ICAM-1 mRNA expression in HAECs, showing that TNF-α-induced ICAM-1 expression required *de novo* RNA and protein synthesis.

3.2 Sesamin and sesamol reduce TNF-α-induced ICAM-1 expression by inhibition of HuR translocation and the HuR-ICAM-1 mRNA interaction

HuR is a ubiquitous RNA-binding protein that is located predominantly in the nucleus in the unstimulated cell and is translocated to the cytoplasm in the stimulated cell [12]. We investigated whether sesamin or sesamol pretreatment affected the subcellular distribution of HuR in TNF-α-treated HAECs. As shown in Fig. 2A, in non-treated HAECs, HuR was found predominantly in the nucleus. Treatment with TNF-α caused a marked accumulation of HuR in the cytoplasm and pretreatment with sesamin or sesamol resulted in a significant decrease in cytoplasmic HuR levels in TNF-α-treated HAECs. To confirm the immunocytochemical staining results, Western blotting was performed to measure nuclear and cytoplasmic levels of HuR in HAECs following treatment with TNF-α alone or together with sesamin or with sesamol. As shown in Fig. 2B, TNF-α markedly increased cytoplasmic levels of HuR without a concomitant decrease in nuclear HuR levels. Sesamin and sesamol markedly inhibited the accumulation of cytoplasmic HuR in TNF-α-treated HAECs. Studies were then performed using siRNAs. Western blot analysis revealed the effective reduction of HuR levels in the HuR siRNA-transfected group compared with the negative control siRNA-transfected group and the naive control group (Fig. 2C). TNF-α-induced ICAM-1 mRNA expression was significantly reduced by HuR siRNA, but not by negative

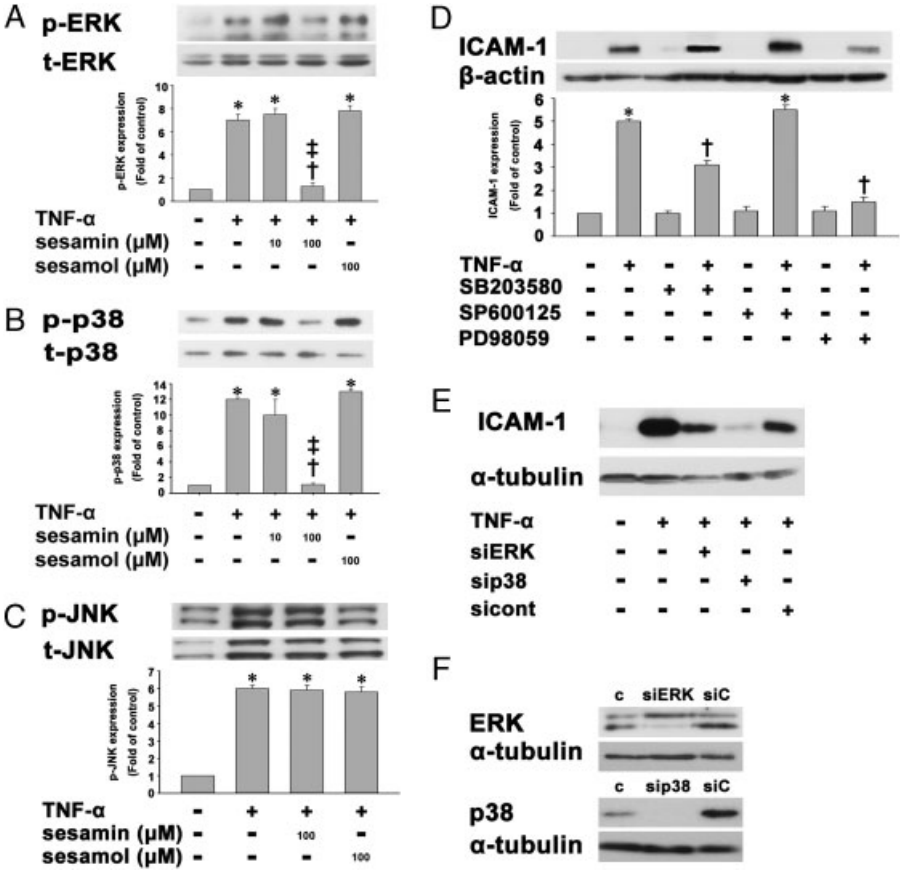

Figure 3. The sesamin-mediated reduction in TNF-α-induced ICAM-1 expression is dependent on inhibition of phosphorylation of ERK and p38. (A–C) Western blot analysis showing the effect of sesamin and sesamol pretreatment on the phosphorylation of (A) ERK1/2, (B) p38, or (C) JNK in TNF-α-treated HAECs. HAECs were incubated for 24 h with or without sesamin (10 or 100 μM) or sesamol (100 μM), then the cells were incubated with 2 ng/mL of TNF-α in the continued presence of the inhibitor for 15 min and aliquots of cell lysate containing equal amounts of protein subjected to immunoblotting with the indicated antibodies. (D) Effect of inhibitors of MAPK phosphorylation on ICAM-1 expression in control and TNF-α-treated HAECs. HAECs were incubated for 1 h with 30 μM PD98059 (ERK1/2 inhibitor), SB203580 (p38 inhibitor), or SP600125 (JNK inhibitor), then for 12 h with or without 2 ng/mL of TNF-α in the continued presence of the inhibitor. ICAM-1 expression was measured by Western blotting. (E) The TNF-α-induced increase in ICAM-1 expression is inhibited by transfection of HAECs with ERK1/2- or p38-specific siRNA (200 μmole/mL), but not control siRNA (sicont). (F) ERK1/2- or p38-specific siRNA (compared with control siRNA; siC) causes a 55- or 90% reduction in ERK1/2 or p38 protein expression. The data are expressed as a fold of the control value and are the mean ± SEM for three separate experiments. Total ERK (t-ERK), total p38 (t-p38), total JNK (t-JNK), β-actin, or α-tubulin was used as the loading control for (B–D), (A–D), or (E and F), respectively.

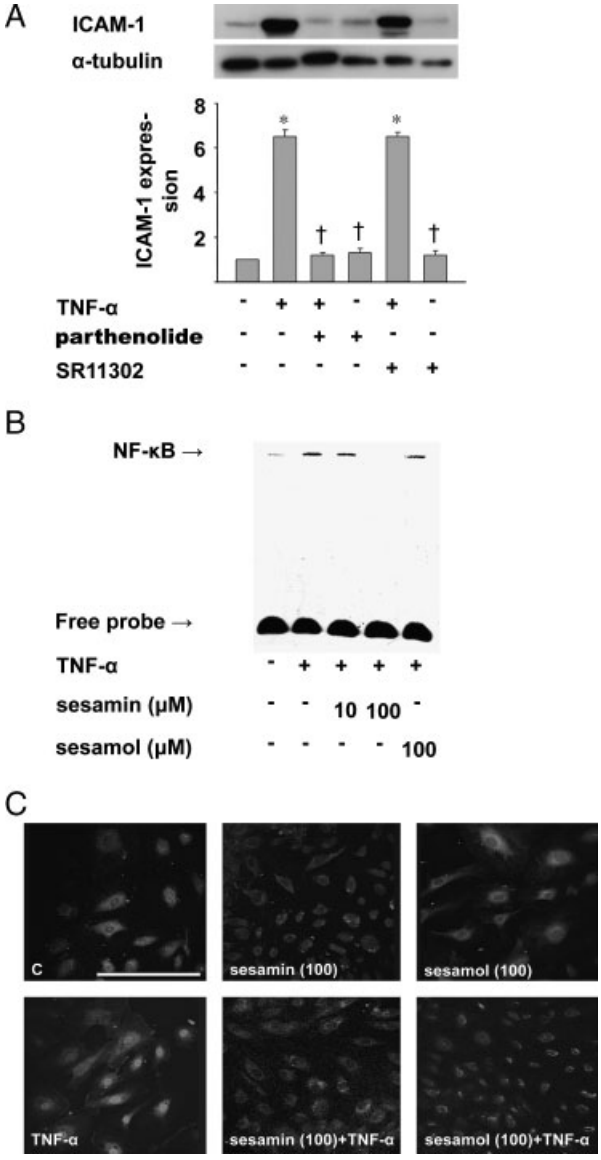

Figure 4. The sesamin- or sesamol-induced upregulation of ICAM-1 expression in TNF-α-stimulated HAECs is mediated by inhibition of both NF-κB activation and NF-κB p65 nuclear translocation. (A) Cells were co-incubated for 24 h with 30 μM parthenolide (NF-κB inhibitor) or 30 μM SR11302 (AP-1 inhibitor) and 2 ng/mL of TNF-α, then cell lysates were prepared and assayed for ICAM-1 on Western blots. (B) Nuclear extracts prepared from untreated cells or from cells with or without sesamin (10 or 100 μM) or sesamol (100 μM) pretreatment for 24 h, then incubated with 2 ng/mL of TNF-α for 30 min in the continued presence of the inhibitor were tested for NF-κB DNA-binding activity by electrophoretic mobility shift assay. (C) Immunofluorescence staining for NF-κB p65. HAECs were incubated for 24 h with 100 μM sesamin or sesamol, then with 2 ng/mL of TNF-α for 30 min. A representative result from three separate experiments is shown. Bar=80 μm.

control siRNA, demonstrating the critical role of HuR in the regulation of ICAM-1 mRNA levels. Sesamin and sesamol significantly reduced TNF-α-induced ICAM-1 expression in the HuR siRNA-treated groups. Based on the cytoplasmic localization of HuR in TNF-α-treated HAECs and the region of the AU-rich element (ARE) recognized by HuR, we postulated that the HuR might interact with the 3′UTR of ICAM-1 mRNA and assessed this possibility using immunoprecipitation with anti-HuR antibody and Protein G- Sepharose, followed by RT-PCR to estimate the amount of 3′UTR of ICAM-1 mRNA in the immunoprecipitate. As shown in Fig. 2D, treatment with TNF-α for 4 h markedly increased the interaction of HuR with the 3′UTR of ICAM-1 mRNA and this effect was markedly reduced by 24 h preincubation with sesamin (10 or 100 μM) or sesamol (100 μM).

3.3 The sesamin-induced reduction of TNF-α-induced ICAM-1 expression is dependent on inhibition of phosphorylation of ERK and p38

A previous study showed that TNF-α can activate MAPKs in the signaling pathway leading to inflammation [10]. In the next set of experiments, the effects of TNF-α on the activation of the MAPK pathway (ERK1/2, JNK, p38), a signaling cascade contributing to ICAM-1 expression, and the effects of sesamin, sesamol, or MAPK inhibitors on TNF-α-stimulated ICAM-1 expression were studied. As shown in Figs. 3A–C, phosphorylation of ERK, p38, and JNK1/2 was increased 7.0-, 12.1-, and 6.0-fold of control levels, respectively, at 15 min after addition of TNF-α. Pretreatment with 100 μM sesamin decreased TNF-α-induced ERK and p38 phosphorylation, but not JNK phosphorylation. Interestingly, pretreatment with 10 μM sesamin or 100 μM sesamol did not reduce TNF-α-induced ERK, p38, or JNK phosphorylation.

As shown in Fig. 3D, the increase in ICAM-1 expression in response to TNF-α treatment was inhibited by 1 h pretreatment with 30 μM PD98059 (an ERK1/2 inhibitor) or SB203580 (a p38 inhibitor), while SP600125 (a JNK inhibitor) had no effect. It was also inhibited by transfection of HAECs with ERK1/2- or p38-specific siRNA (200 μmol/mL) (Fig. 3E). The effectiveness of the siRNA treatment was validated by showing that ERK1/2- or p38-specific siRNA (compared with control siRNA) caused a 55 or 90% reduction in ERK1/2 and p38 protein expression, respectively (Fig. 3F). These results suggest that sesamin inhibits TNF-α-induced ICAM-1 expression by preventing TNF-α-induced phosphorylation of ERK and p38.

3.4 Sesamin and sesamol attenuate activation of NF-κB expression and nuclear translocation of NF-κB p65 in TNF-α-stimulated HAECs

Transcriptional regulation involving NF-κB and AP-1 activation has been implicated in the cytokine-induced

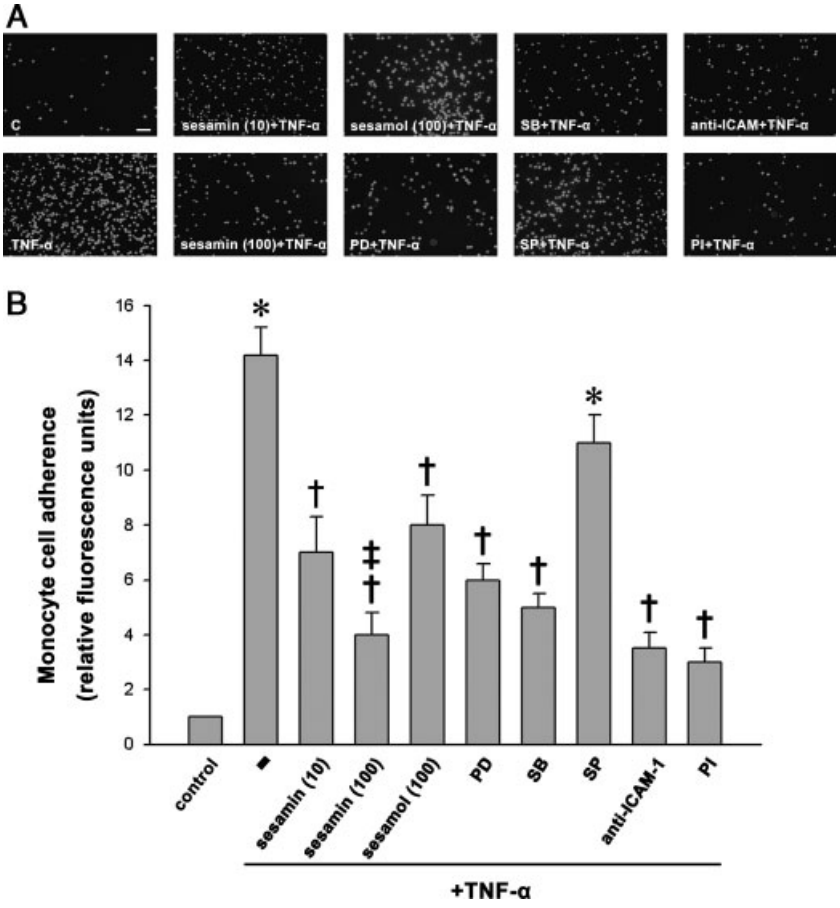

Figure 5. Sesamin or sesamol reduces the adhesion of U937 cells to control and TNF-α-stimulated HAECs. Cells were left untreated or were pretreated for 24 h with sesamin (10 or 100 μM) or sesamol (100 μM) or with 30 μM PD98059 (PD), SB203580 (SB), SP600125 (SP), or parthenolide (PI) or 4 μg/mL of anti-ICAM-1 antibody, then with 2 ng/mL of TNF-α for 12 h in the continued presence of the inhibitor. (A) Representative fluorescent photomicrographs showing the effect on macrophage adhesion to HAECs. C is untreated cells. Bar=100 μm. (B) Fluorescence intensity of the bound monocytes expressed relative to that of the control cells.

expression of adhesion molecules [13]. As shown by Western blots (Fig. 4A), the stimulatory effect of TNF-α on ICAM-1 levels was blocked by co-incubation with parthenolide, an NF-κB inhibitor, but not by co-incubation with SR11302 (an AP-1 inhibitor). Gel-shift assays were performed to determine the effect of sesamin and sesamol on NF-κB activation in TNF-α-treated HAECs. As shown in Fig. 4B, low basal levels of NF-κB-binding activity were detected in control cells and binding was significantly increased by 30 min treatment with TNF-α. The binding activity was blocked by a 100-fold excess of unlabeled NF-κB probe (data not shown). This increase was markedly inhibited by preincubation with 10 or 100 μM sesamin (15 or 95% inhibition, respectively) or 100 μM sesamol (20% inhibition) for 24 h. To determine whether NF-κB activation was involved in the pretranslational effects of sesamin on adhesion molecule expression, we also studied NF-κB p65 protein levels in the nuclei of TNF-α-treated HAECs by immunofluorescence staining. As shown in Fig. 4C, TNF-α-stimulated HAECs showed marked NF-κB p65 staining in the nuclei, while sesamin or sesamol-pretreated cells showed weaker nuclear NF-κB expression, but stronger staining in the cytoplasm.

3.5 Sesamin and sesamol inhibit the adhesion of U937 cells to TNF-α-stimulated HAECs

To explore the effects of sesamin or sesamol on EC–leukocyte interactions, we examined the adhesion of U937 cells to cytokine-activated HAECs. As shown in Fig. 5, control confluent HAECs incubated with U937 cells for 1 h showed minimal binding, but adhesion was substantially increased when the HAECs were pretreated with TNF-α for 12 h. Compared with TNF-α-treated HAECs, pretreatment with 10 or 100 μM sesamin reduced the number of U937 cells adhered to TNF-α-stimulated HAECs by 50 and 70% inhibition, respectively. In total, 100 μM sesamol also significantly reduced the adhesion by 42% inhibition.

The involvement of ICAM-1 in the adhesion of U937 cells to TNF-α-treated HAECs was examined by pretreatment of the cells with anti-ICAM-1 antibody. After HAECs were pretreated with 4 μg/mL of anti-ICAM-1 antibody for 24 h, then incubated with TNF-α, the binding of U937 cells to HAECs was significantly lower than in non-antibody-treated TNF-α-stimulated cells, showing that ICAM-1 plays a major role in the adhesion of U937 cells to TNF-α-treated HAECs. The adherence of TNF-α-treated U937 cells to HAECs was also inhibited by 30 μM PD98059, SB203580, or parthenolide, but not by SP600125.

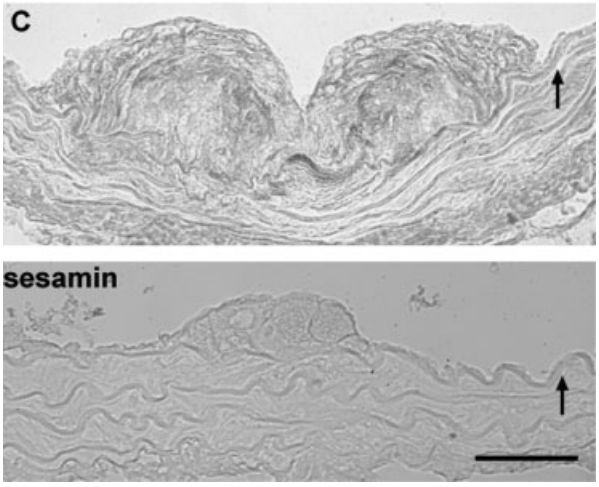

Figure 6. Immunohistochemical staining for ICAM-1 in sections of thoracic aorta from high fat and cholesterol-fed apo-E-deficient mice (C) and sesamin-treated high fat and cholesterol-fed apoE-deficient mice (sesamin). The lumen is uppermost in all sections. The internal elastic membrane is indicated by the arrow. Bar=50 μm.

3.6 Sesamin decreases ICAM-1 protein expression in the thoracic aorta in apo-E-deficient mice

Over the experimental period, there was no difference in weight gain and final weight between high fat and cholesterol-fed apo-E-deficient mice with to without sesamin treatment. Morphometric analysis showed that the intima/media area ratio in the sesamin-treated group ($0.21 \pm 0.03\%$) was significantly lower than that in the control group ($0.52 \pm 0.18\%$). To study the effect of sesamin on ICAM-1 expression in high fat and cholesterol-fed apo-E-deficient mice, immunohistochemical staining with anti-ICAM-1 antibody was carried out on sections of the thoracic aorta. As shown in Fig. 6, in the control group, strong ICAM-1 staining was seen on the thickened intima, while, in sesamin-treated group, the intimal area was reduced and showed weaker ICAM-1 staining.

4 Discussion

In the present study, we found that sesamin treatment effectively blocked ICAM-1 expression both *in vitro* in TNF-α-stimulated HAECs and *in vivo* in the thoracic aorta of apo-E deficient mice. Sesamin inhibited ICAM-1 expression and the binding of the human monocytic cell line U937 to TNF-α-stimulated HAECs, and these effects might be mediated through inhibition of ERK/p38 phosphorylation and NF-κB activation. Sesamol was less effective at inhibiting ICAM-1 expression. Sesamin and sesamol attenuated the TNF-α-induced increase in both the HuR interaction with the 3′UTR of ICAM-1 mRNA and HuR translocation in HAECs.

Sesamin and sesamol were chosen for testing as they have long been known as health foods and used as traditional Chinese medicines. Antioxidative and anti-inflammatory actions are two of the pharmacological properties proposed to underlie their beneficial effects [14–16]. Sesamin can inhibit cholesterol absorption [17] and can regulate the transcription of hepatic enzymes for metabolizing alcohol and lipids [18]. Sesamin has a protective effect on hypoxia-induced apoptosis-like cell death in neuronal cells [19]. Our previous report demonstrated that sesame ingestion effectively decreases plasma triglycerides levels by 5% and LDL-C levels by 10% in postmenopausal women, and, in addition, also effectively improves γ-tocopherol bioavailability and decreases levels of LDL-thiobarbituric acid reactive substances [20]. The present study is the first to report that sesamin strongly reduces the expression of ICAM-1 mRNA and protein in TNF-α-treated HAECs. The present results also showed that, at the same concentration, sesamin had a much greater inhibitory effect than sesamol on ICAM-1 mRNA and protein expression.

ICAM-1 mRNA seems to be regulated at the post-transcriptional level by TNF-α treatment [21]. Post-transcriptional regulation of ICAM-1 mRNAs (stability, localization, and translation) is now recognized as an important control point in mRNA metabolism during inflammatory processes. AREs, located in the 3′UTR of the mRNAs coding for certain cytokines, including ICAM-1 mRNA, are strong determinants of cytoplasmic mRNA turnover. HuR, a ubiquitous protein belonging to the embryonic lethal abnormal vision family of RNA-binding proteins, selectively binds to AREs and stabilizes ARE-containing mRNA [12]. TNF-α upregulates angiogenic factor expression in malignant glioma cells, and RNA stabilization *via* the AREs in the 3′UTR contributes to this upregulation [22]. Our data provide evidence that HuR interacts directly with the 3′UTR of ICAM-1 mRNA and that HuR is an essential regulator of ICAM-1 expression in TNF-α-stimulated HAECs. In addition, sesamin and sesamol reduced TNF-α-induced ICAM-1 expression through posttranscriptional regulation and this effect was mediated by inhibition of cytoplasmic shuttling of HuR and cytoplasmic HuR expression.

The phosphorylation status of MAPKs plays an important role in the signal transduction of extracellular stimuli to cellular responses and is associated with vascular inflammation and disease [10]. Our study showed that TNF-α caused strong activation of three MAPK subtypes in HAECs, as reported in the previous study [11]. However, the involvement of their activation in the protective mechanism of sesamin or sesamol remains unclear. In the present study, the increase in ICAM-1 expression induced by TNF-α was markedly suppressed in the presence of an ERK inhibitor or a p38 inhibitor, but not a JNK inhibitor. ICAM-1 expression was also inhibited by ERK or p38-specific siRNA. Sesamin decreased TNF-α-induced ERK and p38 phosphorylation. Thus, one of the mechanisms by which sesamin reduces TNF-α-induced ICAM-1 expression involves a reduction in

ERK1/2 and p38 activation. Consistent with our results, sesamin has been shown to reduce lipopolysaccharide-induced NO and IL-6 production in BV2 microglia by suppression of the p38 MAPK signaling pathway [6, 23]. Another study showed that sesamin and sesamolin inhibit the phosphorylation of JNK, p38, and caspase, but not of ERK, in BV-2 cells under conditions of hypoxia [24]. The sesamin- and sesamolin-induced inhibition of ERK, p38, and JNK phosphorylation correlate well with the reduction in lactate dehydrogenase release from hypoxic neuronal and PC12 cells [19]. The differences between the above results in terms of the pathways involved may be related to differences in cell type and the cytokines and inducers. Interestingly, the sesamol-induced decrease in TNF-α-induced ICAM-1 expression was not mediated by MAPKs and further experiments are needed to determine the pathway involved.

The binding of TNF-α to its receptors causes activation of two major transcriptional factors, NF-κB and AP-1, which, in turn, induce the expression of genes involved in chronic and acute inflammatory responses [13]. NF-κB and AP-1 transcriptional activity can be modulated through phosphorylation by MAPKs. In the present study, pretreatment with an NF-κB inhibitor suppressed the TNF-α-induced increase in ICAM-1 expression, whereas an AP-1 inhibitor had no effect. These findings raise the possibility that sesamin and sesamol reduce ICAM-1 expression through a reduction in NF-κB activity. Our study demonstrated that the sesamin- or sesamol-induced decrease in ICAM-1 expression was mediated through inactivation of NF-κB binding activity. This is consistent with a previous report that sesamin inhibits LPS-induced IL-6 production by reducing the activation of NF-κB [23]. NF-κB is activated by signals possibly involving phosphorylation of the IκB subunit and its dissociation from the inactive cytoplasmic complex, followed by translocation of the active p50/p65 dimer to the nucleus [25]. We demonstrated that the sesamin- or sesamol-induced decrease in ICAM-1 expression was mediated through inhibition of p65 translocation.

It has been reported that a sesame oil-containing diet significantly reduces atherosclerotic lesion formation in LDL receptor-negative mice [26]. Rats fed a sesamin-supplemented diet show a significant reduction in the production of proinflammatory mediators, such as TNF-α and prostaglandin E2 [27]. Atherosclerotic lesions, the result of a chronic inflammation, result from the adherence of monocytes/macrophages to ECs and their accumulation within the arterial intima [28]. In the present study, sesamin and sesamol reduced monocyte-EC adhesion and this effect was mediated through the decrease in ICAM-1 expression. In addition, sesamin was shown to significantly reduce the area of atheroma and ICAM-1 expression in the aorta of apoE-deficient mice. On the basis of the probable involvement of ICAM-1 in monocyte recruitment to early atherosclerotic lesions, our findings suggest an additional mechanism by which sesamin treatment may be important in preventing the progress of atherosclerosis.

In conclusion, this study provides the first evidence that sesamin reduces the expression of ICAM-1 both *in vitro* and *in vivo* and also decreases leukocyte adhesion to HAECs. The present data suggest that these effects might be mediated through inhibition of ERK/p38 phosphorylation, NF-κB activation and HuR translocation. Since monocyte recruitment into the vascular wall after their adhesion to ECs is a crucial step in the pathogenesis of atherosclerosis, our study implies that sesamin may have an, as yet, unexplored therapeutic potential in the prevention of atherosclerosis and inflammation.

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