Peanut arachidin-1 enhances Nrf2-mediated protective mechanisms against TNF-α-induced ICAM-1 expression and NF-κB activation in endothelial cells

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Abstract. Arachidin-1 [*trans*-4-(3-methyl-1-butenyl)-3,5,3',4'-tetrahydroxystilbene] is a polyphenol produced by peanut kernels during germination. The aim of the present study was to investigate the mechanism underlying the anti-inflammatory effect of arachidin-1 in endothelial cells (ECs). The results of cell adhesion and western blotting assays demonstrated that arachidin-1 attenuated tumor necrosis factor (TNF)-α-induced monocyte/EC adhesion and intercellular adhesion molecule-1 (ICAM-1) expression. Arachidin-1 was demonstrated to exert its inhibitory effects by the attenuation of TNF-α-induced nuclear factor-κB (NF-κB) nuclear translocation and inhibitor of κB-α (IκBα) degradation. Furthermore, arachidin-1 upregulated nuclear factor-E2-related factor-2 (Nrf-2), a known mediator of phase II enzyme expression, and increased the transcriptional activity of antioxidant response element. Transfection of ECs with Nrf-2 siRNA blocked the inhibitory effect of arachidin-1 on ICAM-1 expression, NF-κB nuclear translocation and IκBα degradation. In addition, arachidin-1 induced the expression of the phase II enzymes thioredoxin-1, thioredoxin reductase-1, heme oxygenase-1, glutamyl-cysteine synthetase and glutathione S-transferase. Following arachidin-1 pretreatment, the H_2O_2-induced generation of reactive oxygen species was reduced. Therefore, the present results indicate that arachidin-1 suppresses TNF-α-induced inflammation in ECs through the upregulation of Nrf-2-related phase II enzyme expression.

Introduction

Numerous chronic diseases are known to involve the pathogenesis of inflammation, including atherosclerosis, Alzheimer's disease and Parkinson's disease (1). The development of atherosclerosis occurs as a response to injury of the vessel wall (2,3). Endothelial inflammation involves the recruitment of circulating monocytes by cells using a combination of chemokines and cell surface adhesion molecules, such as monocyte chemotactic protein-1 and intercellular cell adhesion molecule-1 (ICAM-1) (3). The inflammatory cytokine tumor necrosis factor (TNF)-α activates the nuclear factor-κB (NF-κB) pathway, which serves a key role in the expression of cell adhesion molecules in endothelial cells (ECs) (2,4). Following stimulation with TNF-α, a kinase cascade induces subsequent inhibitor of κB (IκB) phosphorylation, which rapidly degrades, resulting in the released NF-κB translocating to the nucleus to regulate gene transcription (5).

The prenylated stilbenoids arachidin-1 and arachidin-3 are produced in germinated peanut roots (6). They are prenylated analogs of piceatannol and resveratrol, respectively. Resveratrol and piceatannol are considered to have a number of beneficial effects, including anticancer, anti-atherogenic, anti-oxidative, anti-inflammatory, antimicrobial and estrogenic activity (7). Their prenylated analogs are less studied but display a wide spectrum of biological activity. Arachidin-1 has been reported to possess various pharmacological activities, including antioxidative, anti-inflammatory, anti-virus infection and antitumorigenic effects (7-10). However, to date it is not clear if arachidin-1 also has anti-inflammatory and anti-oxidative effects in ECs. Since arachidin-1 has been demonstrated to be pharmacologically active, the detailed mechanism by which arachidin-1-modulates inflammation in ECs requires further elucidation.

Numerous phytochemicals are electrophilic, a property that has been reported to result in nuclear factor-E2-related factor-2 (Nrf-2) activation and the induction of phase II detoxifying enzyme expression (11,12). Results from *in vitro* and *in vivo* studies have provided evidence that electrophilic stress induces the complexation of Nrf-2 with Kelch-like ECH-associated protein 1 (Keap1), enabling Nrf-2 to escape from Keap1-mediated ubiquitination and degradation (13). As a result, Nrf-2 translocates to the nucleus to form heterodimers with small Maf proteins and binds to the antioxidant response element (ARE) in the promoter region of phase II detoxifying enzymes and antioxidant enzymes, including quinone oxidoreductase and heme oxygenase-1 (HO-1) (11,13). In addition, Nrf-2 has been shown to control the expression of a number of thiol-regulating enzymes, including glutathione S-transferase (GTP), glutamyl-cysteine synthetase, thioredoxin and thioredoxin reductase (14). Increased levels of these intracellular thiol-containing proteins

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may provide cytoprotective effects under conditions of oxidative stress or inflammation. A previous study conducted by the present research team demonstrated that piceatannol, an analog of arachidin-1, induced the expression of the Nrf-2- associated gene HO-1 (15). This phenomenon has broad potential implications regarding the inhibitory effects of arachidin-1 upon inflammation in association with Nrf-2-associated enzymes. For these reasons, the present study investigated Nrf-2 associated pathways in order to elucidate the mechanisms underlying the cytoprotective effects of arachidin-1.

Materials and methods

Materials. The p3xARE/Luc vector was constructed as described previously (16). Luciferase assay kits were purchased from Promega Corporation (Madison, WI, USA). Antibodies against HO-1 (SPA-896) and p65 (KAS-TF110) were purchased from Stressgen Biotechnologies (San Diego, CA, USA). ICAM-1 (sc-7891), IκBα (sc-847), Lamin B1 (sc-56143), α-tubulin (sc-53646) and Nrf2 (sc-722) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). TrxR1 antibody (07-613) was purchased from Upstate (Charlottesville, VA, USA). IκBα and Nrf-2 antibodies were obtained from Santa Cruz Biotechnology, Inc. Bacterially derived TNF-α was purchased from Calbiochem (EMD Millipore; Billerica, MA, USA). All other reagents were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

Purification of arachidin-1 and arachidin-3 from peanut. Arachidin-1 and arachidin-3 were isolated using the method originally described by Chang *et al* (17). Briefly, slices of Tainan 14 peanut kernel (1.2 kg; Fengxiang Seed Co., Ltd., Chiayi, Taiwan) were artificially aerated at 25˚C and under 65l/min air flow rate for 16 h. Following pretreatment and freeze drying, the slices of peanut kernel were pulverized into a powder and extracted with methanol. The methanol extract was suspended in water and partitioned with an equal volume of ethyl acetate. The ethyl acetate extract was passed through Sephadex LH-20 and eluted with methanol. The fractions were chromatographed using a LiChroprep RP-18 (2.5x52 cm) column eluted with 0.05% trifluoroacetic acid-CH_3CN (60:40) to obtain arachidin-1 and arachidin-3. The yields of arachidin-1 and arachidin-3 were 0.0045 and 0.0087% and the purities were 96.82 and 98.10%, respectively.

EC cultures. The human umbilical vein cell line EA.hy926 (ATCC CRL-2922; ATCC, Manassas,VA, USA) was cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Gibco-BRL, Carlsbad, CA, USA) at 37˚C under 5% CO_2 in air. When the ECs were grown to confluence, the culture medium was replaced with serum-free DMEM and the cells were incubated for 12 h prior to experimental treatments.

Cell adhesion assay. The THP-1 cells (ATCC) were labeled with 1 μM calcein-AM (Trevigen, Gaithersburg, MD, USA) in PBS for 40 min at 37˚C, and then washed twice with PBS. After treatment, ECs were washed with DMEM and co-cultured with calcein-labeled THP-1 cells for 30 min. After washing twice with RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA), adherent cells were examined using an enzyme-linked immunosorbent assay plate reader (FLx800; Bio-Tek Instruments, Inc., Winooski, VT, USA) at 485 nm excitation and 538 nm emission wavelengths.

Cytotoxicity. The resazurin reduction test, an index of the metabolic activity of living cells, was carried out using an AlamarBlue® assay kit according to the manufacturer's protocol (Serotec; Bio-Rad Laboratories, Inc., Hercules, CA, USA). ECs were plated into 96-well microtiter plates (Falcon; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 20,000 cells/well and incubated with AlamarBlue® reagent for 2 h at 37˚C. Fluorescence was then measured at excitation and emission wavelengths of 570 and 600 nm, respectively.

Western blotting. A total of 10^6 cells were lysed on ice in lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and a protease inhibitor mixture). The total protein concentrations were determined with the use of the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Inc.). Equal amounts (10-20 μg) of whole-cell extracts were boiled for 5 min prior to separation using 10% SDS-PAGE. The proteins were then transferred to a nitrocellulose membrane (EMD Millipore) in Tris-glycine buffer (25 mM Tris-HCl, 192 mM Glycine, pH 8.3) at 10 V for 1.5 h. The membranes were then blocked with PBS containing 5% non-fat milk for 2 h at 4˚C and were subsequently probed with the appropriate primary antibody overnight at 4˚C, with gentle shaking. The next day, following PBS washes, the membranes were incubated with secondary antibodies [horseradish peroxidase-conjugated goat anti-rabbit (34083) or anti-mouse (34081) antibody; Thermo Fisher Scientific, Inc.] for 1 h at room temperature. The results were visualized by chemiluminescence using ECL SuperSignal West Pico kit (Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's protocol.

Preparation of nuclear fractionation for immunoblotting. ECs were collected by scraping and lysed with cell lysis buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride and 0.3% NP-40). The cell lysate was separated into nuclear fractions by centrifugation at 500 x g for 5 min at 4˚C. The nuclei were washed using nuclei washing buffer (320 mM sucrose, 5 mM $MgCl_2$, 10 mM HEPES at pH 7.4) and the nuclear protein was extracted using a buffer containing 25% glycerol, 20 mM HEPES, 0.6 M KCl, 1.5 mM $MgCl_2$ and 0.2 mM EDTA for 15 min at 4˚C.

Luciferase reporter assays. ECs were subcultured at a density of $1x10^6$ cells in 60-mm dishes. ECs were subsequently co-transfected with 100 ng p3xARE/Luc with pSV-β-galactosidase using Lipofectamine-2000 (Invitrogen). Luciferase activity was detected using a luciferase reporter assay system (Promega), and the resulting luciferase activity was measured using a luminometer. For each experiment, luciferase activity was determined in triplicate and normalized with β-galactosidase activity.

RNA interference by small interfering RNA (siRNA) of Nrf-2. The siRNA nucleotide sequence for human Nrf-2 was as follows: 5'-UCCCGUUUGUAGAUGACAA-3' (18). The effects of this siRNA molecule have been demonstrated in a previous study (16). A non-targeting siRNA, 5-GCAAGC

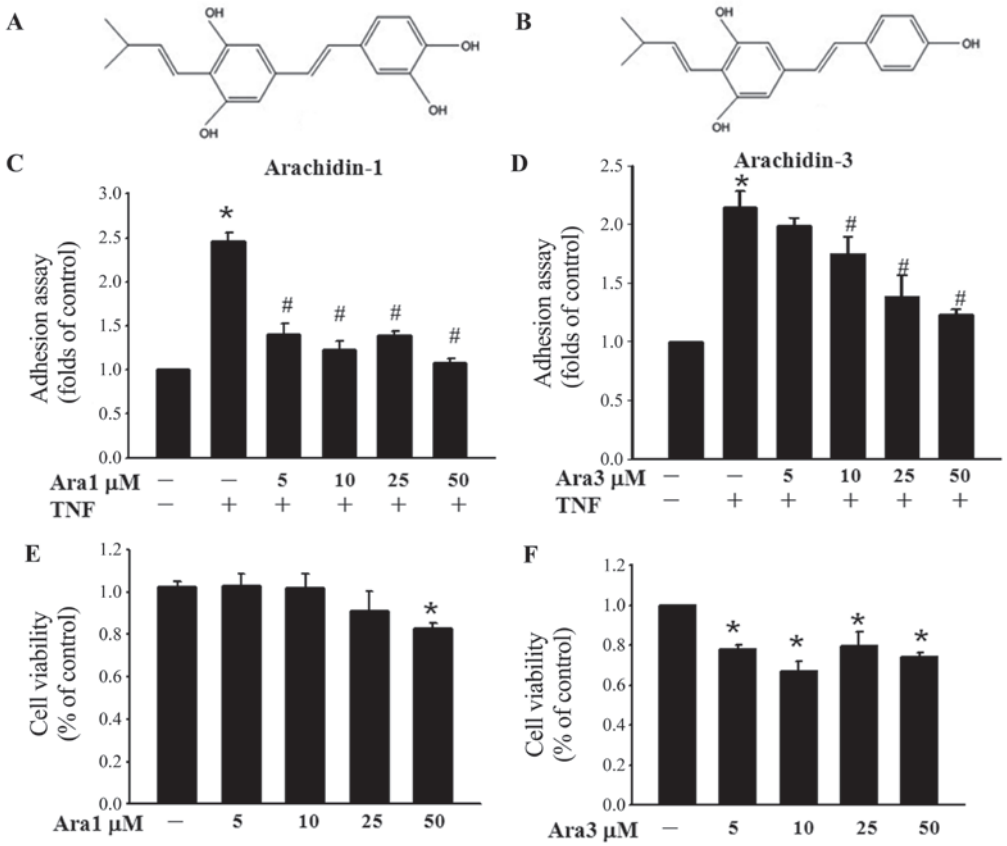

Figure 1. Arachidin-1 inhibits monocyte adhesion to TNF-α-activated ECs, and ICAM-1 expression. (A) Arachidin-1 and (B) arachidin-3 are naturally occurring prenylated analog of piceatannol and resveratrol, respectively. A characteristic of the structure of arachidin-1 is the 3'-hydroxyl group. ECs grown to confluency in 96-well plate were pre-incubated with or without (C) 5-50 μM arachidin-1 or (D) arachidin-3 for 12 h, stimulated with TNF-α (100 U/ml) for 6 h, and then added to $5x10^5$ fluorescence-labeled THP-1 cells and allowed to adhere for 30 min. The data are presented as the relative adherent ratio as compared with untreated ECs. The values represent mean ± standard error of the mean (SEM). *P<0.05 vs. untreated ECs; #P<0.05 vs. TNF-α alone. ECs were incubated with (E) arachidin-1 or (F) arachidin-3 at concentrations of 5, 10, 25 and 50 μM for 24 h and cell viability was measured spectrophotometrically using an Alamar blue assay, according to the manufacturer's protocol. Data are expressed as the mean ± SEN. *P<0.05 vs. control. Ara1, arachidin-1; Ara3, arachidin-3; TNF, tumor necrosis factor; ECs, endothelial cells; ICAM-1, intercellular adhesion molecule-1.

UGACCCUGAAGUUCAU-3, was purchased from Ambion (Thermo Fisher Scientific, Inc.). Cells were transfected with Nrf-2 siRNA or non-targeting siRNA using Lipofectamine 2000 reagent according to the manufacturer's protocol. Following 24 h of transfection, the ECs were cultured in medium without serum for another 12 h prior to treatment.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA isolated from the ECs using TRIzol was reverse transcribed using SuperScript II reverse transcriptase (both Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The cDNA was subjected to PCR amplification using the following forward and reverse primer sets: GAPDH, 5'-TATCGTGGAAGGACT CATGACC-3' and 5'-TACATGGCAACTGTGAGGGG-3'; GST-Pi, 5'-CCCTCACTGTTTCCCGTTGC-3' and 5'-TGA ATGACGGCGTGGAG-3'; thioredoxin-1 (Trx1), 5'-GGCATG CATTTGACTTCA-3' and 5'-ACGTGATATTCCTTGAAG TAG-3'; glutamate-cysteine synthetase regulatory subunit (GCLM), 5'-CAGCGAGGAGCTTCATGATTG-3' and 5'-TGA TCACAGAATCCAGCTGTGC-3'; glutamate-cysteine synthetase catalytic subunit (GCLC), 5'-GTTCTTGAAACTCTG CAAGAGAAG-3' and 5'-ATGGAGATGGTGTATTCTTGT CC-3'; HO-1, 5'-GGTAAGGAAGCCAGCCAAGAG-3' and 5'-GCCAGCAACAAAGTGCAAGAT-3'. The cDNA samples were amplified using Taq DNA polymerase (Thermo Fisher Scientific, Inc.) for 17-25 cycles of 94˚C (30 sec), 60˚C (30 sec), and 72˚C (30 sec) using Mastercycler personal (Eppendorf, Hamburg, Germany). The PCR products were separated on 1.5% agarose gels and visualized by ethidium bromide staining.

Measurement of intracellular reactive oxygen species (ROS). The fluorescence probe 5-(and-6)-carboxy-2,7,dichlorodihydro fluorescein diacetate (carboxy-H_2DCFDA; Molecular Probes; Thermo Fisher Scientific, Inc.) was used to detect the cellular production of ROS. The final treatment concentration of carboxy-H_2DCFDA was 20 μM, and the cells were incubated with this for 30 min in dark at 37˚C. Following two further washes with PBS, the cells were solubilized with 1% SDS and 5 mM Tris HCl (pH 7.4). H_2DCF reacts with ROS to form the green fluorescent compound DCF, which is detected by spectrofluorophotometry (Rf-5301PC; Shimadzu Corporation, Kyoto, Japan) with excitation and emission wavelengths of 475 and 525 nm, respectively.

Statistical analysis. Results are expressed as the mean ± standard error of the mean of at least three independent experiments. Statistical significance was assessed by one-way analysis of variance followed by Tukey's test using SigmaPlot version 12

(Systat Software Inc., San Jose, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Arachidin-1 inhibits monocyte/EC adhesion and ICAM-1 expression. The anti-inflammatory potential of two peanut-derived prenylated stilbenoids, arachidin-1 and arachidin-3 (Fig. 1A and B), was evaluated via assessment of their effects on the adhesion of monocytes to ECs. The results demonstrated that TNF-α significantly increased monocyte adhesion to the ECs and this adhesion was attenuated by treatment of the ECs with arachidin-1 or arachidin-3 (Fig. 1C and D). In another experiment, the cytotoxic effects of 24 h incubation with 5-50 μM concentrations of arachidin-1 and arachidin-3 in ECs were assessed (Fig. 1E and F). Arachidin-1 exhibited no toxic effects in ECs at dosages ≤25 μM. However, a cytotoxic effect was detectable following treatment with 5 μM arachidin-3. These data suggest that the reduction in monocyte adhesion observed following treatment with arachidin-3 may result from cell damage. These results also indicate that the 3'-hydroxyl group of the treatment agent is critical for determining the levels of cytotoxicity. To further test whether arachidin-1 is a modulator of ICAM-1 induction, ECs were stimulated with TNF-α, with or without arachidin-1 pretreatment. Arachidin-1 appeared to have a dose-dependent inhibitory effect on the TNF-α-induced ICAM-1 expression (Fig. 2A). The pretreatment of ECs with arachidin-1 also appeared to inhibit TNF-α-induced ICAM-1 expression in a time-dependent manner (Fig. 2B). This functional assay implies that arachidin-1 exerts an anti-inflammatory effect via the inhibition of adhesion molecule expression.

Arachidin-1 inhibits TNF-α-induced NF-κB activation and blocks the degradation of IκBα. Since the NF-κB pathway is a well-known inflammatory pathway (5), whether arachidin-1 regulates TNF-α-induced NF-κB activation was examined in the present study. ECs were pretreated with arachidin-1 to examine whether this agent regulates p65 nuclear translocation in TNF-α-treated ECs. Western blotting demonstrated that TNF-α-induced p65 nuclear translocation decreased following pretreatment with 5 μM arachidin-1 from 6 to 12 h (Fig. 3A). Following pretreatment at concentrations of 0.1-10 μM for 12 h, it was observed that TNF-α-induced p65 nuclear translocation was inhibited by ≥1 μM arachidin-1 pretreatment (Fig. 3B). To clarify the inhibitory mechanisms of arachidin-1 in TNF-α-induced NF-κB activation, the degradation of IκBα was determined over time. TNF-α alone induced a marked degradation of IκBα following a 1-h treatment. Pretreatment with 5 μM arachidin-1 for 6 and 12 h inhibited the TNF-α-induced degradation of IκBα in ECs (Fig. 3C). These results indicate that arachidin-1 inhibits NF-κB nuclear translocation and transactivation in a time- and dose-dependent manner.

Arachidin-1 induces Nrf-2 activation. It has previously been reported that Nrf-2-induced phase II detoxifying and antioxidant enzymes provide cytoprotective effects in ECs (13). In the present study, ECs treated with arachidin-1 exhibited a continuous increase of Nrf-2 nuclear accumulation (Fig. 4A). Nrf-2 regulates the ARE that drives the expression of specific genes (11). The ability of arachidin-1 to increase Nrf-2 transcriptional activity

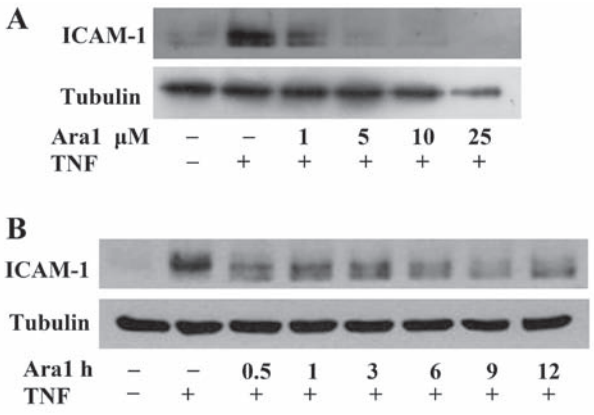

Figure 2. Arachidin-1 inhibits TNF-α-activated ICAM-1 expression. ECs were stimulated with TNF-α for 4 h following (A) pre-incubation with 1, 5, 10 or 25 μM arachidin-1 for 12 h or (B) pretreatment with 5 μM arachidin-1 for the indicated times. Following treatment, cell lysates of the ECs were subjected to western blot analysis with antibodies against ICAM-1 or tubulin. TNF, tumor necrosis factor; ICAM-1, intercellular adhesion molecule-1; ECs, endothelial cells; Ara1, arachidin-1.

was demonstrated by transfecting ECs with an ARE-luciferase reporter construct (Fig. 4B). To investigate whether Nrf-2 contributes to the protective effects of arachidin-1, experiments using Nrf-2 siRNA were performed. Transfection with Nrf-2 siRNA suppressed the inhibitory effects of arachidin-1 on ICAM-1 expression (Fig. 4C), NF-κB nuclear translocation (Fig. 4D) and IκBα degradation (Fig. 4E). These data indicate that arachidin-1 induces Nrf-2 activation and that this mechanism contributes to the anti-inflammatory effects of this compound.

Arachidin-1 induces Nrf-2-associated phase II enzyme expression. The effect of arachidin-1 on phase II enzymes was investigated using ECs in the current study. Cells were treated with arachidin-1 at a concentration of 5 or 10 μM for 12 h. RT-PCR analysis indicated that the mRNA expression levels of GST-Pi, Trx1, HO-1, GCLC and GCLM were increased (Fig. 5A). The protein levels of HO-1 and thioredoxin reductase-1 (TrxR-1) were evaluated by western blot analysis (Fig. 5B). The results suggest that arachidin-1 activates phase II enzymes through increasing their expression at the mRNA and protein levels.

Arachidin-1 reduces H_2O_2-induced ROS levels. To evaluate the antioxidant property of arachidin-1, the effects of arachidin-1 in H_2O_2-treated cells were examined. It was observed that the exposure of cells to 0.5 mM H_2O_2 for 1 h increased the ROS level. Pretreating cells with 5 or 10 μM arachidin-1 for 12 h significantly reduced the intracellular ROS level (Fig. 6A). In addition, the protective effects of arachidin-1 under oxidative stress were examined by subjecting the cells to treatment with 0.5 mM H_2O_2 for 1 h. The results demonstrate that H_2O_2 increases cytotoxicity, and arachidin-1 did not prevent this oxidative stress-induced cell death (Fig. 6B).

Discussion

Arachidin-1 is an electrophilic phytochemical present in germinated peanut roots and the present study provides the first evidence that this compound, at non-cytotoxic

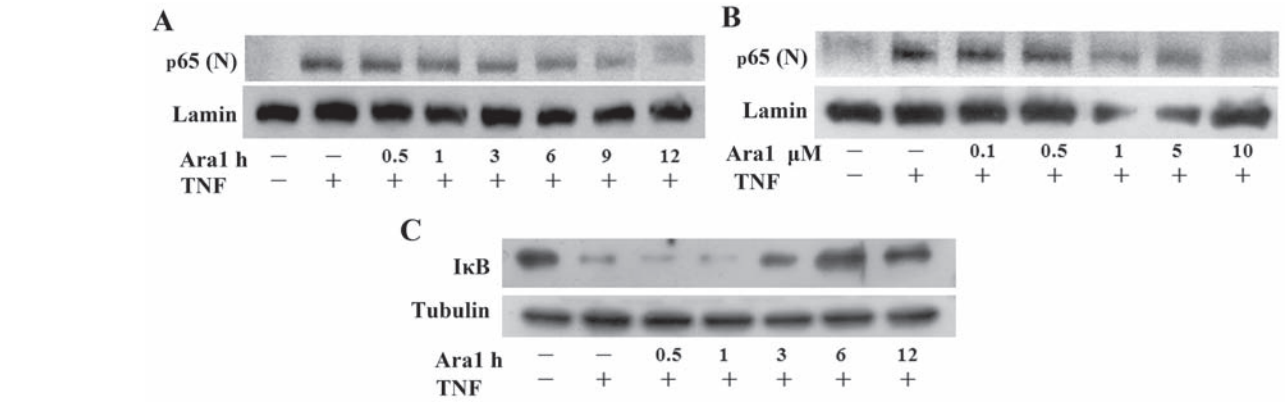

Figure 3. Arachidin-1 inhibits TNF-α-induced NF-κB activation and blocks the degradation of IκBα. (A) ECs were pretreated with 5 μM arachidin-1 for the indicated times and then stimulated with TNF-α. Nuclear extracts (N) were then prepared and subjected to western blot analysis using p65 antibodies. The lamin band intensity serves as a loading control. (B) Western blot analysis of ECs pretreated with the indicated concentration of arachidin-1 for 12 h and then stimulated with TNF-α. (C) ECs were pretreated with 5 μM arachidin-1 for the indicated times and then incubated with TNF-α for 1 h and subjected to western blotting. Cell lysates were then prepared and subjected to western blot analysis with antibodies against IκBα and tubulin. TNF, tumor necrosis factor; NF-κB, nuclear factor κB; IκBα, inhibitor of κB-α; ECs, endothelial cells; N, nuclear; Ara1, arachidin-1.

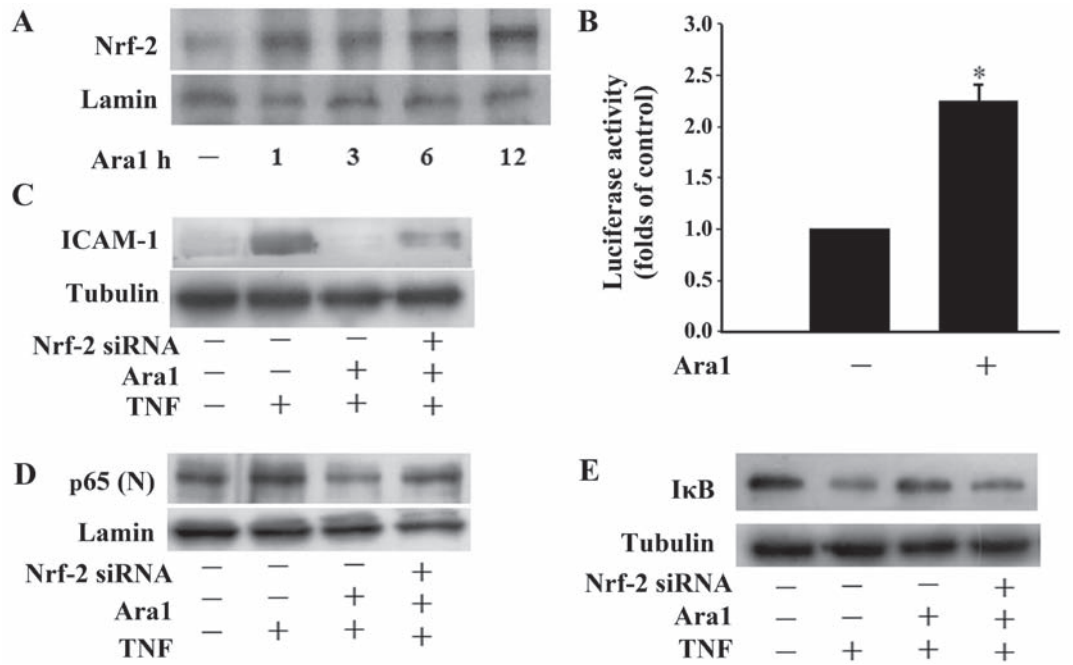

Figure 4. Arachidin-1 induces Nrf-2 activation. (A) Nuclear extracts from ECs were prepared following treatment with 5 μM arachidin-1 for the indicated time periods. Immunoblots of the nuclear lysates were probed with Nrf-2-specific antibodies. (B) The effects of 5 μM arachidin-1 on the transcriptional induction of the ARE in ECs. Cells were transfected with an ARE-luciferase construct. After 12 h, the cells were treated with 5 μM arachidin-1. The cell lysates were analyzed for luciferase activity as described in 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 mean ± SEM. *P<0.05. (C-E) Cells were transfected with control or Nrf-2 siRNA for 36 h and then exposed to 5 μM arachidin-1 for 12 h. Cells were treated with TNF-α for 4 h, and cell lysates or nuclear extracts were prepared and subjected to western blotting with antibodies against (C) ICAM-1, (D) p65 (D) or (E) IκBα. Nrf-2, nuclear factor-E2-related factor-2; ECs, endothelial cells; ARE, antioxidant response element; siRNA, small interfering RNA; TNF, tumor necrosis factor; ICAM-1, intercellular adhesion molecule-1; IκB, inhibitor of κB; Ara1, arachidin-1. .

concentrations, enhances the suppression of TNF-α-induced monocyte adhesion to ECs by downregulating the expression of ICAM-1. The present data also demonstrate that the inhibitory effects of arachidin-1 on the activation of the inflammatory transcriptional factor, NF-κB, are mediated via blocking the degradation of IκBα, and exerted through the induction of Nrf-2-associated phase II detoxifying and antioxidant enzymes. The Nrf-2-associated protective mechanisms may be the major effecter of the anti-inflammatory and anti-oxidative effects arachidin-1.

Resveratrol and piceatannol are dietary polyphenols that are recognized to have numerous health-promoting effects, including anti-oxidative, anti-inflammatory and anticancer properties (19). Although arachidin-1 and arachidin-3 are naturally occurring prenylated analogs of piceatannol and resveratrol, respectively, the biological activity of these prenylated stilbenoids is less studied. The addition of a prenyl group to these compounds increases their hydrophobicity, which may facilitate their attachment to cell membranes or translocation into cells. Theoretically, prenylated stilbenoids should have much more potent biological activity

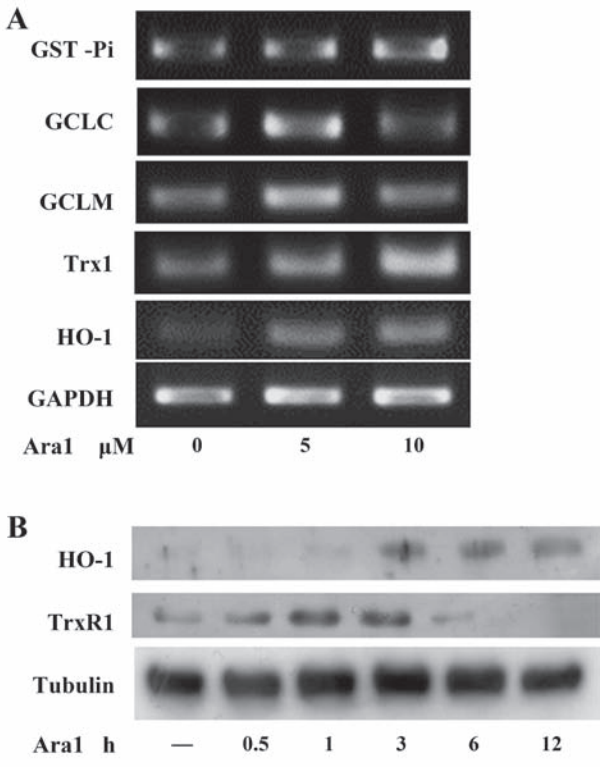

Figure 5. Arachidin-1 induces Nrf-2-associated phase II enzyme expression. (A) ECs were treated with 5 or 10 μM arachidin-1 at 12 h and then subjected to reverse transcription-polymerase chain reaction analysis of GST-Pi, GCLM, GCLC, Trx-1, HO-1 and GAPDH. (B) EC cultures were incubated with 5 μM arachidin-1 for the indicated time periods. Western blot analysis was then performed with antibodies against HO-1, TrxR-1 or tubulin as indicated. Nrf-2, nuclear factor-E2-related factor-2; ECs, endothelial cells; GST, glutathione S-transferase; GCLM, glutamate-cysteine synthetase regulatory subunit; GCLC, glutamate-cysteine synthetase catalytic subunit; Trx-1, thioredoxin-1; HO-1, heme oxygenase-1; TrxR-1, thioredoxin reductase-1; Ara1, arachidin-1.

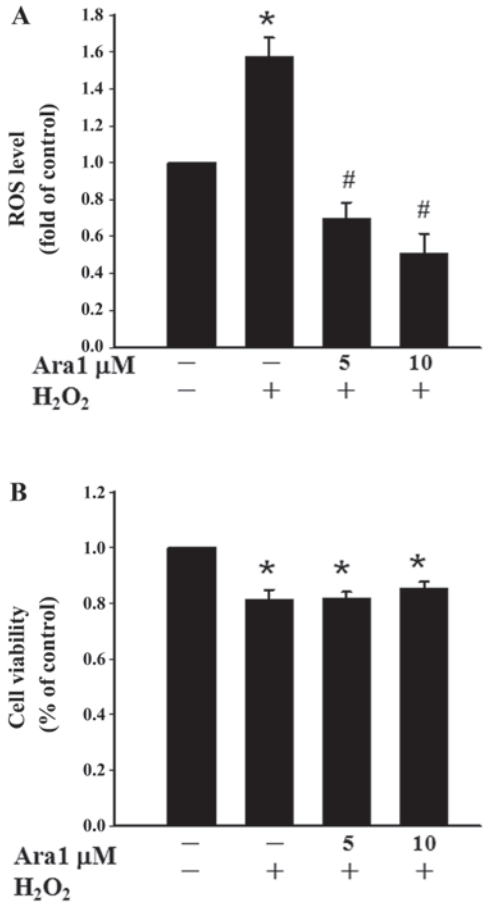

Figure 6. Arachidin-1 reduces H_2O_2-increased ROS level rather than cytotoxicity. Cells were pre-incubated without or with 5 or 10 μM arachidin-1 for 12 h, stimulated with H_2O_2 (0.5 mM) for 1 h and the (A) intracellular ROS levels or (B) cell viability were then measured. The results are shown as the mean ± standard error of the mean. *P<0.05 vs. untreated cells; $^\#$P<0.05 vs. H_2O_2 alone. ROS, reactive oxygen species; Ara1, arachidin-1.

and bioavailability than stilbenoids. Dietary resveratrol has been shown to have a low bioavailability; a previous study revealed that for a dietary 25-mg oral dose of resveratrol, only <5 ng/ml amounts of unchanged resveratrol were detected in plasma (20). Therefore, arachidin-1 may have greater potential applicability than resveratrol. A previous study by the present research team demonstrated that piceatannol induced Nrf-2-related HO-1 expression in ECs, which peaked at 6 h with 25 μM piceatannol treatment (15). Other researchers demonstrated that 30 μM piceatannol treatment significantly upregulated the expression of HO-1 at 6 h in human breast epithelial cells (21). In the present study, it was demonstrated that prenylated piceatannol, arachidin-1, induced HO-1 expression when applied to ECs at a concentration of 5 μM for 3-12 h. A comparison of these findings indicates that the prenylated stilbenoid has a greater efficiency in cell protection than the unprenylated molecule.

The present study provides the first evidence in ECs that arachidin-1 is anti-inflammatory at non-cytotoxic concentrations. The results show that the inhibitory effect differed between arachidin-1 and arachidin-3, indicating that even subtle changes of the chemical structure significantly affect the potency. Notably, the only structural difference between these two prenylated stilbenoids is the 3'-hydroxyl group present in arachidin-1 (Fig. 1A and B). It is notable that arachidin-3 was cytotoxic. Thus, the reduction of cytotoxicity is a crucial factor in the modification of the structure of prenylated stilbenoids.

The redox-sensitive transcription factor Nrf-2 serves a pivotal role in the induced expression of a number of cytoprotective enzymes (13). The present study findings demonstrate that arachidin-1 induces Nrf-2 translocation and activates ARE-luciferase promoter activity, indicating that it directly induces Nrf-2 via its ARE (Fig. 4A). They also indicate that the anti-inflammatory effects of arachidin-1 exerted through protection against ICAM-1 expression and IκBα degradation were reduced by the transfection of Nrf-2 siRNA (Fig. 4C and D). Furthermore, arachidin-1 was found to induce Nrf-2-associated phase II enzyme expression. These data suggest that arachidin-1 induces Nrf-2-activated phase II enzymes involved in the anti-inflammatory effect in ECs. Although the exact mechanisms by which arachidin-1 activates the induction of Nrf-2-associated genes remain unknown, arachidin-1 may contribute to the activation of Nrf-2 through its catechol moiety (22). Although arachidin-1 possesses reducing properties, it may also act as an electrophile. The catechol moiety of arachidin-1 can be oxidized to the electrophilic *o*-quinone form (23). The electrophilicity of the quinone may lead to the activation of the Nrf-2/Keap1 pathway due to the modification of specific cysteine residues of Keap1, which allows Nrf-2 to translocate to the nucleus and bind

to ARE, leading to an increased expression of Nrf-2-associated genes. The characteristic catechol moiety of arachidin-1 appears to be critical for the induction of Nrf-2 activation and subsequent upregulation of phase II enzymes.

The induction of Nrf-2-associated enzymes has been recognized as a mechanism underlying the antioxidant properties of phytochemicals (24). A previous study demonstrated that piceatannol protects cells from glutamate-mediated oxidative injury by upregulating cellular Nrf-2-associated HO-1 expression (25). In the present study, arachidin-1 was revealed to induce the expression of HO-1, GST and other thiol-containing enzymes in ECs, indicating a protective effect of arachidin-1 against oxidative stress. Although arachidin-1 did not prevent H_2O_2-induced cell damage, it significantly reduced the H_2O_2-induced increase in ROS levels (Fig. 6). These findings suggest that the anti-inflammatory effect of arachidin-1 was in accordance with its Nrf-2-associated anti-oxidative property. However, whether the reduction in oxidative stress was dependent on phase II enzyme induction requires further investigation.

In conclusion, the present study demonstrates that an Nrf-2-regulated antioxidant response serves a pivotal role in the anti-inflammatory mechanism underlying the inhibition of NF-κB activation in arachidin-1-treated ECs. A clearer understanding of the mechanisms of action of arachidin-1 in the future may contribute to a potential therapeutic application for the treatment of pathological inflammation.

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