11-2019

Combination of curcumin and luteolin synergistically inhibits TNF-α-induced vascular inflammation in human vascular cells and mice

Lijuan Zhang
Tennessee State University

Xiaoyong Wang
Tennessee State University

Longyun Zhang
Tennessee State University

Carlos Virgous
Meharry Medical College

Hongwei Si
Tennessee State University

Recommended Citation
Lijuan Zhang, Xiaoyong Wang, Longyun Zhang, Carlos Virgous, Hongwei Si, "Combination of curcumin and luteolin synergistically inhibits TNF-α-induced vascular inflammation in human vascular cells and mice", The Journal of Nutritional Biochemistry, Volume 73, 2019, 108222, ISSN 0955-2863, https://doi.org/10.1016/j.jnutbio.2019.108222.

Title: Combination of Curcumin and Luteolin Synergistically Inhibits TNF-α-induced Vascular Inflammation in Human Vascular Cells and Mice

Authors: Lijuan Zhang[1], Xiaoyong Wang[1], Longyun Zhang[1], Carlos Virgous[2], Hongwei Si[1*]

[1] Department of Human Sciences, Tennessee State University, Nashville, TN37209, USA

[2] Animal Care Facility, Meharry Medical College, Nashville, TN 37208, USA

[*] Correspondence: E-mail: hsi@tnstate.edu. Tel.: +1 615 963 5443

Word count for the entire manuscript: 6319

The number of figures: 4

The number of tables: 1

Abbreviations

CI: combination index

CRP: C reactive protein

CVD: cardiovascular diseases

DMSO: Dimethyl sulfoxide

FBS: fetal bovine serum

ICAM-1: intracellular adhesion molecule-1

IL: interleukin

MCP-1: monocyte chemoattractant protein-1

NF-kB: nuclear factor kappa-light-chain-enhancer of activated B

TNF-α: tumor necrosis factor-α

TBST: Tris-buffered saline-Tween

VCAM-1: vascular cell adhesion molecule-1

Abstract: Emerging evidence shows that phytochemicals, the secondary plant metabolites present in a large variety of foods, have the potential ability in reducing the risk of cardiovascular diseases. However, the dosages of phytochemicals in the cellular and animal studies are too high to reach in humans by relevant foods or dietary supplement intake. The aims of this study were to investigate whether and how combined curcumin and luteolin synergistically inhibit tumor necrosis factor-alpha (TNF-α)-induced monocytes adhesion endothelium, a crucial step of the development of endothelial dysfunction, both in human vascular cells and mouse aortic endothelium. Our results show that combined curcumin (1μM) and luteolin (0.5μM) synergistically (combination index is 0.60) inhibited TNF-α-induced monocytes adhesion to human EA.hy926 endothelial cells while the individual chemicals did not have such effect at the selected concentrations. We also found that TNF-α-enhanced protein expressions of vascular cell adhesion molecule 1 (VCAM-1), monocyte chemotactic protein-1 (MCP-1) and nuclear factor (NF)-κB translocation were synergistically reduced by the combined curcumin and luteolin in EA.hy 926 cells while the individual chemical did not have this inhibitory effect. Consistently, two weeks dietary intake of combined curcumin (500 mg/kg) and luteolin (500mg/kg) in C57BL/6 mice synergistically prevented TNF-α-stimulated adhesion of mouse monocytes to aortic endothelium ex vivo as well as the TNF-α-increased aortic protein expression of MCP-1 and VCAM-1. Therefore, combined curcumin and luteolin at physiological concentrations synergistically inhibits TNF-α-induced monocytes adhesion to endothelial cells and expressions of MCP-1 and VCAM-1 via suppressing NF-κB translocation into the nucleus.

Keywords: combination, curcumin, luteolin, synergistic, adhesion, NF-κB pathway

1. Introduction

Elevated chronic inflammation plays a key role in the development and the complications of cardiovascular diseases (CVD), which is characterized by high circulating levels of pro-inflammatory markers such as tumor necrosis factor-α (TNF-α), C reactive protein (CRP) and serum amyloid A [1, 2]. Indeed, increased pro-inflammatory markers trigger the activation and adhesion of monocytes to the endothelium as well as elevation of oxidized LDL uptake, and enhance these monocytes migration into the sub-endothelial space to further stimulate severe inflammation (more macrophages, cytokines and chemokines) and smooth muscle cell proliferation and finally develop foam cells and plaques [3]. This process is primarily mediated by several pro-inflammatory chemokines such as interleukin (IL)-8 and monocyte chemoattractant protein-1 (MCP-1), and several endothelial adhesion molecules including vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1). Increasing studies show that reducing these adhesion molecules and chemokines abolish the adhesion of monocytes to the activated endothelial cells and then inhibits vascular inflammation and the relevant complications [4, 5]. Therefore, attenuating adhesion of monocytes to endothelium and reducing relevant adhesion molecules may be an effective approach to reduce vascular chronic inflammation and prevent CVD prevalence.

While unhealthy lifestyle such as tobacco use, lack of regular physical activity and obesity [6, 7] and aging [8, 9] are the major risk factors of chronic inflammation elevation, increasing evidence supports that selecting healthy foods can reduce chronic inflammation, particularly intake of phytochemicals, secondary plant metabolites present in a large variety of foods including fruit, vegetables, nuts and beverages can significantly attenuate chronic inflammation by reducing pro-inflammatory markers and exerting as antioxidants and thereby and prevent CVD [10, 11]. For instance, curcumin, a polyphenolic compound from

turmeric, shows strong anti-oxidative and anti-inflammatory activities and then widely used to prevent/treat CVD, cancer and other diseases [12, 13]. Similarly, luteolin, a flavone contained in many medicinal plants and vegetables, has the ability to scavenge reactive oxygen species (ROS), suppress pro-inflammatory cytokine expression as well as enhance endothelial nitric oxide (NO) production and therefore protect vasculature [14, 15]. However, the concentrations/dosages of phytochemicals in inhibiting vascular inflammation in the cellular and animal studies are very high and cannot be reached in humans by foods or dietary supplement intake [16]. For example, curcumin significantly inhibits TNF-α induced expression of adhesion molecules VCAM-1, ICAM-1 and E-Selectin in human endothelial cells at 40 μM [17], but the maximum plasma level of curcumin is only 2.30 μg/mL after a high oral dose of 20 g curcumin in healthy humans [18]. One of the approaches of bridging the gap between the required high dosages in cellular/animal studies and very low level of phytochemicals in human blood is to combine two or more phytochemicals. This is supported by increasing studies that combinations of a couple of phytochemicals synergistically improve osteoporosis [19, 20] and suppress obesity [21].

Therefore, the present study investigated whether and how the combination of curcumin and luteolin at physiologically achievable levels synergistically inhibits TNF-α-induced vascular inflammation both in vitro and ex vivo. Our results provide the first scientific evidence that combination of curcumin and luteolin, at low concentrations that individual chemical does not have a significant effect, synergistically inhibited TNF-α-induced monocytes adhesion to human EA.hy926 endothelial cells, and inhibited VCAM-1 and MCP-1 protein expression via regulating nuclear factor kappa-light-chain-enhancer of activated B (NF-κB) nuclear translocation. Moreover, this synergistic inhibitory effect of

combined curcumin and luteolin was also observed in the aortic endothelium isolated from mice after two weeks of dietary intake of curcumin and/or luteolin with TNF-α injection.

2. Materials and methods

2.1. Cells and reagents

EA.hy926 cells, THP-1 cells, and WEHI 78/24 monocytes were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). RPMI-1640, low glucose Dulbecco's modified eagle's medium (DMEM) and penicillin/streptomycin (P/S) were obtained from Gibco (Carlsbad, CA, USA). Fetal bovine serum (FBS) was from Mediatech (Manassas, VA, USA). Recombinant human TNF-α was purchased from Life Technologies (Grand Island, NY, USA). Recombinant mouse TNF-α was from PeproTech (Rocky Hill, NJ, USA). VCAM-1, MCP-1, β-actin, p65 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (St Louis, MO, USA). Calcein-AM and HBSS buffer were purchased from Corning (NY, USA). Pierce BCA protein assay kit, Supersignal west dura extended duration substrate kit, M-PER mammalian protein extraction buffer, NE-PER nuclear and cytoplasmic extraction reagents kit were purchased from Thermo Fisher Scientific (Rockford, IL, USA). Curcumin, luteolin was purchased from Sigma-Aldrich (St. Louis, MO, USA), dissolved in DMSO at 50 mM, and aliquoted for storage at -20°C freezer.

2.2 Cell culture

EA.hy926 cells, a permanent human endothelial cell line preserving the characteristics of primary human umbilical vein endothelial cells [22], were cultured in low glucose DMEM supplemented with 10% FBS, 1% penicillin/streptomycin（P/S） in 75-cm^2 tissue culture flasks at 37°C in a humidified atmosphere of 5% CO_2/95% air environment. THP-1 cells, a

human leukemia monocytic cell line extensively used to study monocyte/macrophage functions [23], were cultured in RPMI-1640 medium containing 10% FBS and 1% P/S and WEHI 78/24 cells, a mouse monocyte cell line, were maintained in DMEM with 10% FBS and 1% P/S in the CO2 incubator at 37°C in a humidified atmosphere of 5% CO2/95% air environment.

2.3. Monocyte adhesion assay in vitro

The number of monocytes adhering to ECs was conducted as we described [24]. In brief, EA.hy926 cells were grown in 12-well plates to 100% confluent and then treated with/out curcumin and/or luteolin at the relevant concentrations and 10 ng/ml human recombinant TNF-α for 20h. After gently washed with serum-free medium, calcein-AM (10μM) labeled THP-1 cells (1×10^6/ml/well) were added to EA.hy926 cells for 1h incubation. Unbound monocytes were gently washed by HBSS. The adhered monocytes were captured by a fluorescence microscope (fluorescent) and merged with the image of separate endothelial cells (optical), or directly determined by measuring the fluorescence using a Synergy H1 hybrid reader (BioTek Instruments, Inc., Winooski, VT, USA) at excitation and emission wavelengths of 496 nm and 520 nm.

2.4 Optimum combination selection

To select the optimum combination of curcumin and luteolin, different concentrations of curcumin or luteolin were added to monocyte adhesion assay to make the dose-dependent curves of each chemical. The combinations were randomly combined curcumin (0.1μM, 1μM, 5μM, 10μM) and luteolin (0.1μM, 0.5μM, 1μM, 5μM) at the relevant concentrations to determine the inhibitory effect on the TNF-α-induced monocytes adhesion to EA.hy926

cells. The combination index (CI) of each combination was calculated using the classic isobologram equation [25].

$$CI = \frac{D_1}{D_{1x}} + \frac{D_2}{D_{2x}}$$

D1 and D2 in the numerator stand for the concentrations of compounds 1 and 2 in combination to achieve x% inhibition whereas D1x and D2x in the denominator represent concentrations of compounds 1 and 2 to achieve x% inhibition when present alone. CI < 1, CI=1, and CI > 1 indicate synergistic, additive, or antagonistic effects, respectively according to a previous study [25]. The optimum combination was selected based on lower CI and lower concentrations to conduct mechanism experiments.

2.5 Animal study

Male C57BL/6 mice (10 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME and were randomly divided into five groups (8 mice/group, Table 1), which feed either with AIN-93G mineral mix standard food [26], or standard food containing individual or combined curcumin and luteolin for 1 week. The dosages of curcumin (500mg/kg diet, w/w) [27-29] and luteolin (500mg/kg diet, w/w) [30-32] were selected based on previous studies. TNF-α (25 μg/kg daily) was injected intraperitoneally (i.p.) for 7 consecutive days while the treatments were continued. Control mice were i.p. injected HBSS for the same period. All animals were maintained at constant temperature and humidity with a 12:12-hr light-dark cycle permitted the consumption of water and food ad libitum. The body weight and food intake were recorded each day during the entire study period. At the end of the experiment, all mice were euthanized using CO_2 and tissues including the aorta, blood and spleen were collected for immediate assay or frozen at -80°C for further analysis. The aorta was cut into two parts, one section closes the heart was used to conduct *ex vivo* monocyte

adhesion assay immediately, and the second section was frozen at -80 °C for protein extraction. All experimental procedures were approved by the Institutional Animal Care and Use Committee at Tennessee State University in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Table1. Dosages of chemicals in an animal experiment

	Group 1	Group 2	Group 3	Group 4	Group 5
TNF-α (μg/kg)	-	25	25	25	25
Curcumin (mg/kg)	-	-	500	-	500
Luteolin (mg/kg)	-	-	-	500	500

2.6 Ex vivo monocyte adhesion assay

After mice were euthanized, aortas were excised rapidly and washed twice with ice-cold PBS followed by incubation in DMEM for 10 min at 37°C. The aortas were opened longitudinally to expose the endothelium and pinned onto 4% agar in 35-mm plates with 1 ml of DMEM containing 1% heat-inactivated FBS. Fluorescence-labeled WEHI 78/ 24 mouse monocytes (1×10^6) were applied on the endothelium of aorta and incubated for 30 min at 37°C. Unbound monocytes were gently rinsed away by DMEM medium once, firmly bound monocytes to the endothelium of aorta was captured by fluorescence microscope. Three images of each aorta were taken, and all bound monocytes in the entire image were counted using Image-J software. The average number of three images was considered as the number of the mouse, and data are expressed as means ± SEM of 8 mice per group.

2.7 Western Blot

At the end of the treatments (20 h), EA. hy926 cells were collected to extract proteins as we described [33]. In brief, after 20 h of incubation with/out treatments, EA. hy926 cells were rinsed once with PBS and added with ice-cold mammalian protein extraction buffer (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM $Na_4P_2O_7$, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4). Cells were then scraped off and collected in 1.5 ml tube and sonicated on ice for 15 seconds 3 times, with 15 seconds interval. After centrifuging 10 min at 12,000g, the supernatant was collected as a protein sample. For the total and nuclear NF-κB (p65), the protein sample was further extracted by a NE-PER nuclear and cytoplasmic extraction reagents kit (Thermo Fisher Scientific, Rockford, IL, USA). Sample protein concentration was measured using a Pierce BCA protein assay kit. An equal amount of protein of samples was mixed with 2x western blot sample buffer and heated at 95˚C for 5 minutes, then subjected to western blot analysis. For animal tissue, mice aorta tissue was weighted (about 20 mg each mouse) and added in mammalian protein extraction buffer with proteinase inhibitor cocktail (Sigma-Aldrich). After cutting 30 times, samples were homogenized by the Precellys 24 tissue homogenizer on ice for 3 times, 15 seconds each time. Then samples were centrifuged 10 min at 12,000g, and supernatants were collected as protein sample to measure total protein level and prepare western blot sample as described above. Samples were separated by 10% SDS-PAGE and the membrane was blocked for 1 hour with Tris-buffered saline-Tween (TBST) containing 5% skim milk at room temperature. After washing 3 times with TBST, the membrane was incubated with the relevant primary antibody at 4°C overnight. On the next day, the membrane was washed 3 times with TBST and then incubated with secondary antibody for one hour at room temperature. Specific bands were detected by SuperSignal West Dura chemiluminescence (ThermoFisher Scientific) and visualization was performed by exposure of the membranes to X-ray films. Band intensities

were quantified by ImageJ software. The value of the specific protein was normalized by the expression of β-actin and expressed as the percentage of the control.

2.8 Statistical analyses

All values of in vitro studies were presented as means ± SEM of at least three independent experiments performed in triplicate. For the animal study, 8 mice/group was determined by conducting a power analysis (using GPOWER software) for a one-way ANOVA at a type error level of 5%. The values of in vivo studies were presented as means ± SEM of 8 mice. All data were analyzed with one-way ANOVA and significant differences between treatment groups were further analyzed using Tukey test. Differences with P value < 0.05 were considered significant.

3. Results

3.1 Combined curcumin and luteolin synergistically inhibited TNF-α-induced monocytes adhesion to EA.hy 926 cells

Since TNF-α-induced monocyte adhesion to endothelial cells is an important step in the development of vascular inflammation, we determined if curcumin or luteolin can block TNF-α-induced adhesion of THP-1 cells to EA.hy 926 cells, a typical model of vascular inflammation research. Our results have shown that exposure of EA.hy 926 cells to TNF-α (10 ng/ml) for 20 h dramatically increased the adhesion of monocytes to EA.hy 926 cells. Curcumin dose-dependently inhibited TNF-α-induced binding of monocytes to ECs at 87% and 73% of TNF-α at 5 μM, 10μM respectively (Fig. 1A). Similarly, luteolin along also dose-dependently inhibited TNF-α-induced binding of monocytes to ECs (Fig. 1B). However, the minimum concentrations of curcumin (5 μM) and luteolin (5 μM) significantly inhibited monocytes to EA.hy 926 cells are still too high to achieve by relevant foods intake. We then combined the two chemicals to test if the combination inhibits monocyte adhesion while the individual chemical does not significantly inhibit at the selected concentrations. To select the optimum combination of luteolin and curcumin, the two chemicals were combined at the relevant concentrations to test in the adhesion assay and CIs were calculated as in Fig. 1 C. The combination of curcumin at 1μM plus luteolin at 0.5 μM has lower CI value (0.60, CI<1 means synergistic) while the individual chemicals at lower concentrations. As shown in Fig. 1D, combined curcumin (1 μM) and luteolin (0.5 μM) synergistically inhibited monocytes adhesion to 84% of TNF-α control, and the inhibitory effect of the combination is significantly different with that of individual curcumin or luteolin, and curcumin and luteolin alone did not significantly inhibit TNF-α-induced monocytes adhesion. Therefore, we selected this combination curcumin (1μM) and luteolin (0.5μM) as an optimum combination

to conduct the following experiments.

3.2 Combined curcumin and luteolin synergistically reduced TNF-α-increased protein expressions of VCAM-1 and MCP-1 in endothelial cells

Elevated VCAM-1, MCP-1, and other adhesion molecules play key roles of adhesion, migration, infiltration of monocytes/macrophages and triggering chronic vascular inflammation. We want to know whether these molecules mediate the inhibitory effect on monocyte adhesion by a combination of curcumin and luteolin. Our western blot results show that the expression of that protein of VCAM-1 was synergistically reduced to 75% of TNF-α control by a combination of curcumin 1 μM plus luteolin 0.5 μM, while the individually treated with curcumin or luteolin at the relevant concentrations did not significantly reduce VCAM-1 expression (Fig. 2A). Similarly, TNF-α-increased MCP-1proteins expression was synergistically reduced to 59% of TNF-α control by combined curcumin and luteolin, and this inhibitory effect is significantly different with the effect by the individual curcumin or luteolin (Fig. 2B). These results are the pattern of monocytes adhesion to EA.hy 926 cells by an individual or combined chemicals, indicating that combined curcumin and luteolin synergistically inhibit TNF-α-induced monocytes adhesion to EA.hy 926 cells by down-regulating proteins expression of VCAM-1 and MCP-1in EA.hy 926 cells.

3.3 Curcumin and luteolin synergistically inhibit TNF-α-induced inflammation via regulating the NF-κB pathway in endothelial cells

To further investigate how combined curcumin and luteolin synergistically inhibits TNF-α-induced monocytes adhesion, we collected treated EA.hy 926 cells to determine the level of NF-κB signaling pathway, a pivotal pathway in the transcriptional regulation of chemotactic cytokines and vascular adhesion molecules [34]. Western blot analysis showed

that combined curcumin and luteolin did not significantly change total P65 protein expression (Fig. 3A), but nuclear P65 protein expression of was significantly decreased to 36% by combined curcumin 1 μM plus luteolin 0.5 μM (Fig. 3B). These data indicate that combined curcumin and luteolin may synergistically inhibit TNF-α-induced monocytes adhesion by attenuating p65 translocation from cytosol to nucleus.

3.4 Dietary supplementation of curcumin and luteolin synergistically reduces TNF-α-induced vascular inflammation in C57BL/6 mice

To further confirm whether combined curcumin and luteolin synergistically prevent TNF-α-induced vascular inflammation in vivo as we recently described [31], individual or combination of curcumin and luteolin were dietarily administrated to mice for 7 consecutive days while TNF-α was injected. The animal condition was observed daily during the experimental period. We did not observe any difference of performance, the reactivity of mice between groups during the entire experimental period, and there was no significant difference of body weight, food intake and drink intake (data not shown). After carefully isolated and opened, the endothelium of isolated mouse aorta was explored to fluorescence-labeled mouse WEHI 78/ 24 monocytes. As shown in Fig. 4A, TNF-α-treatment significantly increased WEHI 78/24 cells binding to the vessel walls of mouse aortas compared with those from control mice, indicating that blood vessels in TNF-α treated mice are activated and inflammatory. While dietary supplementation of individual curcumin or luteolin effectively blocked adhesion of monocytes to the endothelium (Fig. 4A), a combination of curcumin and luteolin synergistically inhibited TNF-α-recruited because of the inhibitory effect of the combination of curcumin and luteolin significantly lower than that of individual curcumin and luteolin. We further measured protein expression of MCP-1 and VCAM-1 in the aorta

and found that TNF-α-increased VCAM-1 (Fig. 4B) and MCP-1(Fig. 4C) were synergistically decreased dietary supplementation of curcumin and luteolin to 52% and 65% of TNF-α control respectively. These results suggest that combined curcumin and luteolin exerts an anti-inflammatory effect in vasculature via inhibiting adhesion molecules production.

4. Discussion

The critical roles of chemokines and adhesion molecules in the initiation and development of vascular inflammatory process and pathogenesis of CVDs have been well established [35, 36]. For example, chemokine MCP-1 is essential for monocyte rolling, firm adhesion to ECs and the subsequent transmigration into vascular tissue [37][39][46] and adhesion molecules such as ICAM-1, VCAM-1, and E-selectin play a pivotal role in attracting, binding and transmigrating monocytes into sites of inflammation [38][47]. These have been confirmed by that mice lacking receptors for these molecules are less susceptible to vascular disease and have fewer monocytes in vascular lesions [39]. In the present study, we observed that TNF-α-increased protein expression of MCP-1 and VCAM- 1 were synergistically reduced by combined curcumin and luteolin both in human EA.hy 926 endothelial cells (Fig. 2 A&B) and mice aorta (Fig. 4 B&C), the same patterns of the synergistic inhibitory effect on TNF-α-increased monocytes adhesion on ECs by combined curcumin and luteolin both in human EA.hy 926 endothelial cells (Fig. 1D) and mice aorta (Fig. 4 A). In addition to endothelial cells, MCP-1 and other adhesion molecules can be produced by a variety of cell such as fibroblasts, macrophages, monocytes, epithelial, smooth muscle, monocytic and microglial (a specialized population of macrophages that are found in the central nervous system) cells [40], suggesting that combination of curcumin and luteolin may also attenuate the production of these inflammatory molecules from other types of cells. An interesting result from our study is that ICAM-1 protein expression was not significantly reduced by the combination of curcumin and luteolin while this treatment significantly reduced VCAM-1 expression both in human cells and mice aorta as aforementioned, a possible explanation is that VCAM-1 plays a dominant role in the initiation of vascular inflammation [41]. Nevertheless, dietary intake both curcumin and luteolin or

relevant foods may prevent vascular inflammation by inhibiting the release of chemokines and adhesion molecules.

The NF-κB pathway may be the same target both curcumin and luteolin in inhibiting TNF-α-induced vascular inflammation. For instance, curcumin exerts anti-inflammatory through inhibition of the NF-κB pathway [42, 43] and luteolin protects against TNF-α-induced vascular inflammation in human endothelial cells via suppressing IKBα/NF-κB signaling pathway [31]. In fact, NF-κB signaling pathway plays a central role in the regulation of inflammatory responses by governing the expression of chemokines and leukocyte adhesion molecules such as MCP-1, VCAM-1, E-selectin and ICAM-1 [44, 45]. TNF-α activates NF-κB in the cytosol and leads the p50/65 heterodimer of NF-κB p65 translocation into the nucleus, then the p50/p65 dimer binds to the promoter regions of NF-κB-dependent proinflammatory and adhesion genes such as MCP-1 and VCAM-1 to induce the DNA transcription and protein expressions [44, 46]. Therefore, NF-κB is an interesting target for the attenuation of inflammation. Remarkably, we found that TNF-α nuclear p65 protein expression was synergistically reduced by the combined curcumin and luteolin in human EA.hy 926 endothelial cells while the individual chemical did not have this inhibitory effect (Fig. 3B). This result matches the patterns of the synergistic inhibition of combined curcumin and luteolin on the TNF-α-induced monocytes adhesion (Fig. 1D) and expressions of VCAM-1 and MCP-1(Fig. 2), indicating that combined curcumin and luteolin synergistically inhibits TNF-α-induced vascular inflammation via suppressing NF-κB p65 nuclear translocation. Indeed, inhibitors of NF-κB p65 nuclear translocation such as dexamethasone [47] and rolipram [48] have been used to suppress inflammation.

To understand how combination of two or more phytochemicals exerts synergistic anti-inflammatory effects in cells, animals, and humans, we recently summarized literatures and proposed five mechanisms including enhancing the bioavailability of phytochemicals,

increasing antioxidant capacity, interacting with gut microbiome (change microbial profiles, reduce endotoxin and increase gut integrity) and targeting same and/or different signaling pathways by the interactions between phytochemicals [16]. Particularly the bioavailability of curcumin can be massively increased by another chemical. For instance, co-administration of piperine and curcumin to humans and rats enhanced the bioavailability of curcumin by 2000% and 154%, respectively [49]. This may be a result of the inhibition of the glucuronidation of curcumin by piperine because curcumin is heavily metabolized in the form of glucuronide conjugates prior to reaching the plasma and piperine is a well-known inhibitor of hepatic and intestinal glucuronidation [50]. We also observed that adding luteolin with curcumin together significantly increased the cellular uptake of curcumin both in endothelial cells and breast cancer cells (data not shown), indicating the luteolin enhanced curcumin uptake at least partly contributes to the synergistic inhibition on TNF-α-increased vascular inflammation both in human cells and mice.

The complexity of the metabolism of curcumin and luteolin may affect the synergistic anti-inflammatory effect of the combination in vivo. Curcumin is a relatively unstable compound that degrades quickly in neutral and alkaline solutions to ferulic acid, feruloyl methane and vanillin. Compared to curcumin, these degraded products have a much lower anti-cancer effect [51]. Similarly, the major product of in vivo metabolism products curcumin monoglucuronide and curcumin diglucuronide have much lower antioxidant capacity than that of curcumin [52]. However, an increase in blood curcumin glucuronide concentration enhances the concentration of free form curcumin, resulting in the suppression of human colon carcinomas implanted in mice [53]. For luteolin, the two major forms in vegetables are either aglycone or glucosides (e.g., luteolin-7-O-β-D-glucoside, luteolin-7-O-[2-(β-D-apiosyl)-β-D-glucoside]). However, luteolin conjugates, especially luteolin-3′-Oβ-D-

glucuronide, but not luteolin aglycone and luteolin glucosides, are presumably responsible for the physiological effects of luteolin in vivo. Therefore, these luteolin aglycone and luteolin glucosides must be metabolized to luteolin glucuronide (e.g., luteolin-3′-Oβ-D-glucuronide) in rats or luteolin-3′-O-sulfate in humans. Interesting, luteolin glucuronide exerts stronger anti-inflammatory effect than that of luteolin sulfate [54].

In summary, we reported for the first time that combined curcumin and luteolin, at low levels achievable by dietary intake, synergistically inhibited TNF-α-induced monocytes adhesion to human EA.hy 926 endothelial cells, but the individual chemical at the relevant levels did not have a significant inhibitory effect. This synergistic inhibitory effect on monocytes adhesion by combined curcumin and luteolin is in line with the synergistic inhibited TNF-α-increased protein expressions of VCAM-1 and MCP-1 as well as the TNF-α-induced NF-κB p65 nuclear translocation in EA.hy 926 cells. We are also the first reporter that dietary supplementation of the combination of curcumin and luteolin synergistically reduced TNF-α-increased vascular inflammation in C57BL/6 mice. These results suggest that dietary intake of a combination of curcumin and luteolin may be a valuable approach to prevent vascular inflammation and therefore prevent/treat vascular diseases in humans.

Figure Legends

Fig. 1. Combined curcumin and luteolin synergistically inhibited TNF-α-induced monocytes adhesion to EA.hy 926 cells. After 100% confluent, EA.hy 926 cells were treated TNF-α (10 ng/ml) with/out individual or combination of curcumin (Cur) or/and luteolin (Lut) for 20 h. Cells were gently washed with serum-free medium and incubated with Calcein-AM labeled THP-1 cells for 1h. Unbound monocytes were gently rinsed away by HBSS. The adhered

monocytes were captured by a fluorescence microscope or directly determined by measuring the fluorescence using a Synergy H1 hybrid reader at excitation and emission wavelengths of 496 nm and 520 nm. Individual curcumin (**A**) and luteolin (**B**) dose-dependently inhibited TNF-α-induced monocytes adhesion to EA.hy 926 cells. Combination index (CI, **C**) were calculated from various combinations to select the optimum combination. **D**, combined curcumin (1 μM) and luteolin (0.5 μM) synergistically inhibited TNF-α-induced monocytes adhesion to EA.hy 926 cells as shown in representative images (merged from two separate images of endothelial cells (optical) and monocytes (fluorescent)) at the magnification of 40× and bar graphs. Data are reported as the means ± SEM *, $P < 0.05$; **, $P < 0.01$ compared to the TNF-α control. #, $p < 0.05$ compared to the combined curcumin and luteolin treatment.

Fig. 2. Combined curcumin and luteolin synergistically reduced TNF-α-increased proteins expression of VCAM-1(**A**) or MCP-1(**B**) in EA.hy926 cells. 20 h treated TNF-α (10 ng/ml) with/out individual or combination of curcumin (Cur) or/and luteolin (Lut) EA.hy926 cells were collected to measure proteins levels by Western blot. Target protein expression was normalized to β-actin. Data are expressed as the means ± SEM from three independent experiments. *, $P < 0.05$; **, $P < 0.01$ compared to the TNF-α control. #, $p < 0.05$ compared to the combined curcumin and luteolin treatment.

Fig. 3. Combined curcumin and luteolin synergistically reduced TNF-α-increased NF-κB p65 nuclear translocation. 20 h treated TNF-α (10 ng/ml) with/out individual or combination of curcumin (Cur) or/and luteolin (Lut) EA.hy926 cells were collected to measure proteins levels by Western blot. Total NF-κB (p65) protein (**A**) and nuclear p65 protein (**B**) protein expression were normalized to β-actin. Data are expressed as the means ± SEM from three independent experiments. *, $P < 0.05$; **, $P < 0.01$ compared to the TNF-α control. #, $p < 0.05$ compared to the combined curcumin and luteolin treatment.

Fig. 4. Dietary supplementation of a combination of curcumin and luteolin synergistically reduced TNF-α-induced aortic inflammation in C57BL/6 mice. Individual or combination of curcumin and luteolin were dietarily administrated to mice for 7 consecutive days while TNF-α was injected. Ex vivo adhesion assay was conducted between the endothelium isolated from mouse aorta and fluorescence-labeled mouse WEHI 78/ 24 monocytes. Combination of curcumin and luteolin synergistically inhibited TNF-α-recruited monocytes adhesion (**A**), and TNF-α-increased aortic VCAM-1 (**B**) and MCP-1(**C**) protein expression. Representative images of monocytes adhesion on ECs are shown at the magnification of 40×. Target protein expression was normalized to β-actin. Data are expressed as means ± SEM (n=8). *, $P < 0.05$; **, $P < 0.01$ compared to the TNF-α control. #, $p < 0.05$ compared to the combined curcumin and luteolin treatment.

Disclosure statements

The authors have nothing to disclose.

Acknowledgments

This work was supported by grants from the National Institute of Food and Agriculture in the United States Department of Agriculture (TENX-2011-0255 and TENX-1814-FS to Hongwei Si).

References

[1] Ruparelia N, Chai JT, Fisher EA, Choudhury RP. Inflammatory processes in cardiovascular disease: a route to targeted therapies. Nature Rev Cardiol 2017; 14. (3): 133-144

[2] Danesh J, Wheeler JG, Hirschfield GM, Eda S, Eiriksdottir G, Rumley A, et al. C-reactive protein and other circulating markers of inflammation in the prediction of coronary heart disease. N Engl J Med. 2004;350:1387-97.

[3] Zhu M, Lei L, Zhu Z, Li Q, Guo D, Xu J, et al. Excess TNF-alpha in the blood activates monocytes with the potential to directly form cholesteryl ester-laden cells. Acta Biochim Biophys Sin (Shanghai). 2015;47:899-907.

[4] Carluccio MA, Siculella L, Ancora MA, Massaro M, Scoditti E, Storelli C, et al. Olive oil and red wine antioxidant polyphenols inhibit endothelial activation: antiatherogenic properties of Mediterranean diet phytochemicals. Arterioscler Thromb Vasc Biol. 2003;23:622-9.

[5] Mayer K, Merfels M, Muhly-Reinholz M, Gokorsch S, Rosseau S, Lohmeyer J, et al. Omega-3 fatty acids suppress monocyte adhesion to human endothelial cells: role of endothelial PAF generation. Am J Physiol Heart Circ Physiol. 2002;283: H811-8.

[6] Ford ES, Bergmann MM, Kroger J, Schienkiewitz A, Weikert C, Boeing H. Healthy Living Is the Best Revenge Findings From the European Prospective Investigation Into Cancer and Nutrition-Potsdam Study. Arch Intern Med. 2009;169:1355-62.

[7] Kuroda M, Sakaue H. Adipocyte Death and Chronic Inflammation in Obesity. J Med Invest. 2017;64:193-6.

[8] Prasad S, Sung B, Aggarwal BB. Age-associated chronic diseases require age-old medicine: role of chronic inflammation. Prev Med. 2012;54 Suppl: S29-37.

[9] Franceschi C, Garagnani P, Vitale G, Capri M, Salvioli S. Inflammaging and 'Garb-aging'. Trends Endocrinol Metab. 2017;28:199-212.

[10] Pop RM, Popolo A, Trifa AP, Stanciu LA. Phytochemicals in Cardiovascular and Respiratory Diseases: Evidence in Oxidative Stress and Inflammation. Oxid Med Cell Longev. 2018;2018:1603872.

[11] Bellik Y, Hammoudi SM, Abdellah F, Iguer-Ouada M, Boukraa L. Phytochemicals to prevent inflammation and allergy. Recent Pat Inflamm Allergy Drug Discov. 2012;6:147-58.

[12] Gupta SC, Patchva S, Koh W, Aggarwal BB. Discovery of curcumin, a component of golden spice, and its miraculous biological activities. Clin Exp Pharmacol Physiol. 2012;39:283-99.

[13] Naksuriya O, Okonogi S, Schiffelers RM, Hennink WE. Curcumin nanoformulations: a review of pharmaceutical properties and preclinical studies and clinical data related to cancer treatment. Biomaterials. 2014;35:3365-83.

[14] Ruiz PA, Haller D. Functional diversity of flavonoids in the inhibition of the proinflammatory NF-κB, IRF, and Akt signaling pathways in murine intestinal epithelial cells. Nutritional Immunology. 2006:664-71.

[15] Si H, Wyeth RP, Liu D. The flavonoid luteolin induces nitric oxide production and arterial relaxation. Eur J Nutr. 2014;53:269-75.

[16] Lijuan Zhang, Carlos Virgous, Si H. Synergistic anti-inflammatory effects ,and mechanisms of combined phytochemicals. JNutri Biochem. 2019;69:19-30.

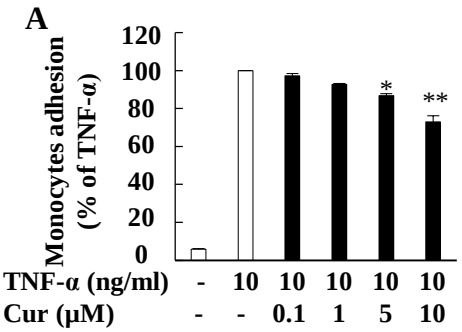
[17] Babita Gupta, Ghosh B. Curcuma longa inhibits TNF-α induced expression of adhesion molecules on human umbilical vein endothelial cells. Int J Immunopharmacol. 1999;21:745-57.

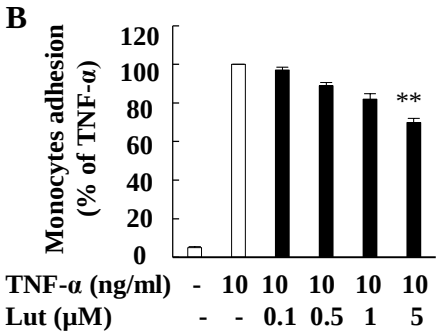
[18] Vareed SK, Kakarala M, Ruffin MT, Crowell JA, Normolle DP, Djuric Z, et al. Pharmacokinetics of curcumin conjugate metabolites in healthy human subjects. Cancer Epidemiol Biomarkers Prev. 2008;17:1411-7.
[19] Rayalam S, Della-Fera MA, Baile CA. Synergism between resveratrol and other phytochemicals: Implications for obesity and osteoporosis. Mol Nutr Food Res. 2011;55:1177-85.
[20] Rayalam S, Della-Fera MA, Yang JY, Park HJ, Ambati S, Baile CA. Resveratrol potentiates genistein's antiadipogenic and proapoptotic effects in 3T3-L1 adipocytes. J Nutr. 2007;137:2668-73.
[21] Murakami A, Takahashi D, Koshimizu K, Ohigashi H. Synergistic suppression of superoxide and nitric oxide generation from inflammatory cells by combined food factors. Mutat Res Fund Mol Mech Mut. 2003;523:151-61.
[22] Ahn K, Pan S, Beningo K, Hupe D. A permanent human cell line (EA.hy926) preserves the characteristics of endothelin converting enzyme from primary human umbilical vein endothelial cells. Life Sci. 1995;56:2331-41.
[23] Chanput W, Mes JJ, Wichers HJ. THP-1 cell line: an in vitro cell model for immune modulation approach. Int Immunopharmacol. 2014;23:37-45.
[24] Babu PV, Si H, Fu Z, Zhen W, Liu D. Genistein prevents hyperglycemia-induced monocyte adhesion to human aortic endothelial cells through preservation of the cAMP signaling pathway and ameliorates vascular inflammation in obese diabetic mice. J Nutr. 2012;142:724-30.
[25] Yang J, Liu RH. Synergistic effect of apple extracts and quercetin 3-beta-d-glucoside combination on antiproliferative activity in MCF-7 human breast cancer cells in vitro. J Agric Food Chem. 2009;57:8581-6.
[26] Reeves PG, Nielsen FH, Fahey GC, Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr. 1993;123:1939-51.
[27] Ejaz A, Wu D, Kwan P, Meydani M. Curcumin inhibits adipogenesis in 3T3-L1 adipocytes and angiogenesis and obesity in C57/BL mice. J Nutr. 2009;139:919-25.
[28] Kim SJ, Son TG, Park HR, Park M, Kim MS, Kim HS, et al. Curcumin stimulates proliferation of embryonic neural progenitor cells and neurogenesis in the adult hippocampus. J Biol Chem. 2008;283:14497-505.
[29] Jiang G, Zhang L, Wang J, Zhou H. Baicalein induces the apoptosis of U251 glioblastoma cell lines via the NF-kB-p65-mediated mechanism. Anim Cells Sys. 2016;20:296-302.
[30] Jang S, Kelley KW, Johnson RW. Luteolin reduces IL-6 production in microglia by inhibiting JNK phosphorylation and activation of AP-1. Proc Natl Acad Sci U S A. 2008;105:7534-9.
[31] Jia Z, Nallasamy P, Liu D, Shah H, Li JZ, Chitrakar R, et al. Luteolin protects against vascular inflammation in mice and TNF-alpha-induced monocyte adhesion to endothelial cells via suppressing IKappaBalpha/NF-kappaB signaling pathway. J Nutr Biochem. 2015;26:293-302.
[32] Kang KP, Park SK, Kim DH, Sung MJ, Jung YJ, Lee AS, et al. Luteolin ameliorates cisplatin-induced acute kidney injury in mice by regulation of p53-dependent renal tubular apoptosis. Nephrol Dial Transplant. 2011;26:814-22.
[33] Zhang L, Zhang L, Wang X, Si H. Anti-adipogenic effects and mechanisms of ginsenoside Rg3 in pre-adipocytes and obese mice. Front Pharmacol. 2017;8:113.

[34] Kumar M, Sharma G, Misra C, Kumar R, Singh B, Katare OP, et al. N-desmethyl tamoxifen and quercetin-loaded multiwalled CNTs: A synergistic approach to overcome MDR in cancer cells. Mater Sci Eng C Mater Biol Appl. 2018;89:274-82.
[35] Norata GD, Cattaneo P, Poletti A, Catapano AL. The androgen derivative 5alpha-androstane-3beta,17beta-diol inhibits tumor necrosis factor alpha and lipopolysaccharide- induced inflammatory response in human endothelial cells and in mice aorta. Atherosclerosis. 2010;212:100-6.
[36] Galkina E, Ley K. Vascular adhesion molecules in atherosclerosis. Arterioscler Thromb Vasc Biol. 2007;27:2292-301.
[37] Gerszten RE, Garcia-Zepeda EA, Lim Y-C, Masayuki Yoshidak, Ding HA, J MAG, et al. MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. mnature. 1999;398:718-23.
[38] Martin J, Collot-Teixeira S, McGregor L, McGregor JL. The Dialogue Between Endothelial Cells and Monocytes: Macrophages in Vascular Syndromes Current Pharmaceutic Design. 2007;13:1751-9.
[39] Hong JJ, Jeong TS, Choi JH, Park JH, Lee KY, Seo YJ, et al. Hematein inhibits tumor necrotic factor-alpha-induced vascular cell adhesion molecule-1 and NF-kappaB-dependent gene expression in human vascular endothelial cells. Biochem Biophys Res Commun. 2001;281:1127-33.
[40] Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): an overview. J Interferon Cytokine Res. 2009;29:313-26.
[41] Cybulsky MI, Iiyama K, Li H, Zhu S, Chen M, Iiyama M, et al. A major role for VCAM-1, but not ICAM-1, in early atherosclerosis. J Clin Invest. 2001;107:1255-62.
[42] SK B, D M, LA J, IL M, I R. Curcumin induces glutathione biosynthesis and inhibits NF-kappaB activation and interleukin-8 release in alveolar epithelial cells: mechanism of free radical scavenging activity. Antioxid Redox Signal. 2005;7:32-41.
[43] JW C, KS L, CW K. Curcumin attenuates the expression of IL-1ß, IL-6, and TNF-α as well as cyclin E in TNF-··-treated HaCaT cells; NF-κB and MAPKs as potential upstream targets. Int J Mol Med. 2007;19:469-74.
[44] Chen JW, Chen YH, Lin FY, Chen YL, Lin SJ. Ginkgo biloba extract inhibits tumor necrosis factor-alpha-induced reactive oxygen species generation, transcription factor activation, and cell adhesion molecule expression in human aortic endothelial cells. Arterioscler Thromb Vasc Biol. 2003;23:1559-66.
[45] Mukherjee TK, Nathan L, Dinh H, Reddy ST, Chaudhuri G. 17-epiestriol, an estrogen metabolite, is more potent than estradiol in inhibiting vascular cell adhesion molecule 1 (VCAM-1) mRNA expression. J Biol Chem. 2003;278:11746-52.
[46] Li Y, Reddy MA, Miao F, Shanmugam N, Yee JK, Hawkins D, et al. Role of the histone H3 lysine 4 methyltransferase, SET7/9, in the regulation of NF-kappaB-dependent inflammatory genes. Relevance to diabetes and inflammation. J Biol Chem. 2008;283:26771-81.
[47] Auphan N, DiDonato JA, Rosette C, Helmberg A, Karin M. Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. Science. 1995;270:286-90.
[48] Thomas AJ, DaSilva JN, Lortie M, Renaud JM, Kenk M, Beanlands RS, et al. PET of (R)-11C-rolipram binding to phosphodiesterase-4 is reproducible and sensitive to increased norepinephrine in the rat heart. J Nucl Med. 2011;52:263-9.

[49] Shoba G, Joy D, Joseph T, Majeed M, Rajendran R, Srinivas PS. Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers. Planta Med. 1998;64:353-6.
[50] Reddy L, Odhav B, Bhoola KD. Natural products for cancer prevention: a global perspective. Pharmacol Ther. 2003;99:1-13.
[51] Zhu J, Sanidad KZ, Sukamtoh E, Zhang G. Potential roles of chemical degradation in the biological activities of curcumin. Food Funct. 2017;8:907-14.
[52] Tsuda T. Curcumin as a functional food-derived factor: degradation products, metabolites, bioactivity, and future perspectives. Food Funct. 2018;9:705-14.
[53] Ozawa H, Imaizumi A, Sumi Y, Hashimoto T, Kanai M, Kakeya H. Curcumin β-D-glucuronide plays an important role to keep high levels of free-form curcumin in the blood. Biol Pharm Bull. 2017;40:1515-24.
[54] Hayasaka N, Shimizu N, Komoda T, Mohri S, Tsushida T, Eitsuka T, et al. Absorption and Metabolism of Luteolin in Rats and Humans in Relation to in Vitro Anti-inflammatory Effects. J Agric Food Chem. 2018;66:11320-9.

Figure 1.





C

	Cur 0.1	Cur 1	Cur 5	Cur 10
Lut 0.1	1.20	0.91	0.88	0.87
Lut 0.5	0.86	**0.60**	0.58	0.52
Lut 1	0.85	0.59	0.56	0.50
Lut 5	0.77	0.53	0.49	0.47

D

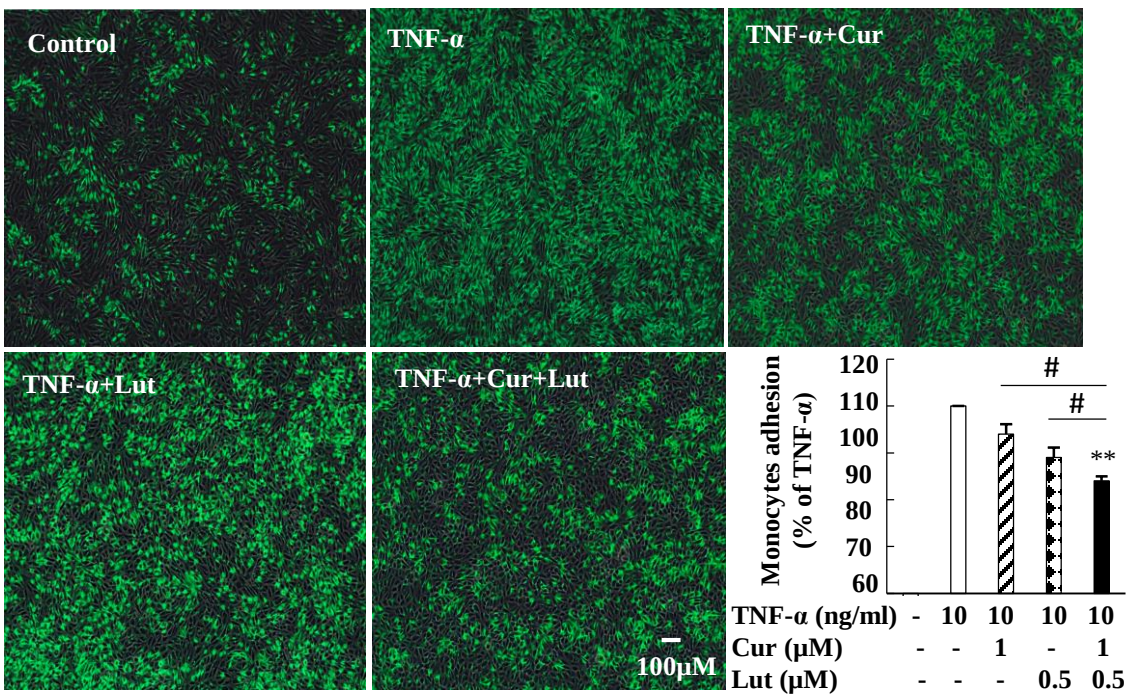


Figure 2.

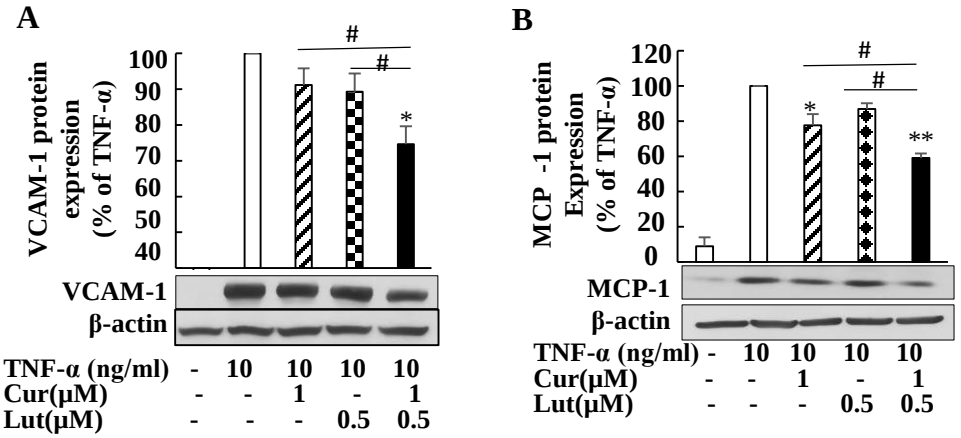

A
VCAM-1 protein expression (% of TNF-α)
100
90
80
70
60
50
40
#
#
*
VCAM-1
β-actin
TNF-α (ng/ml) - 10 10 10 10
Cur(μM) - - 1 - 1
Lut(μM) - - - 0.5 0.5
B
MCP -1 protein Expression (% of TNF-α)
120
100
80
60
40
20
0
#
#
*
**
MCP-1
β-actin
TNF-α (ng/ml) - 10 10 10 10
Cur(μM) - - 1 - 1
Lut(μM) - - - 0.5 0.5

Figure 3.

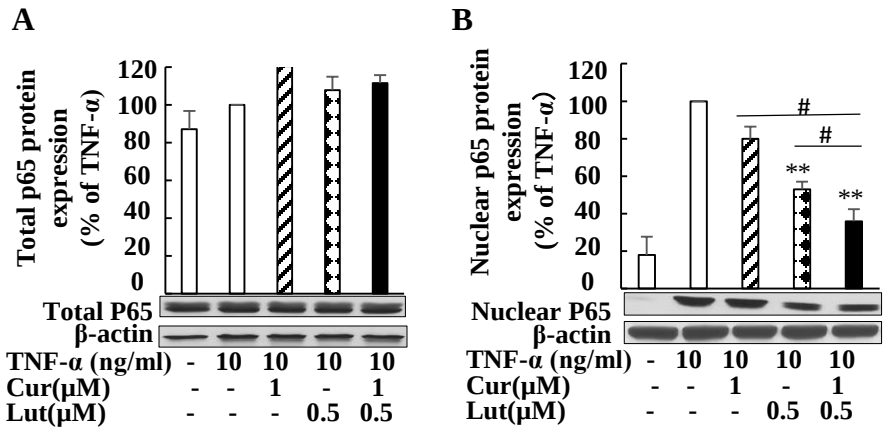
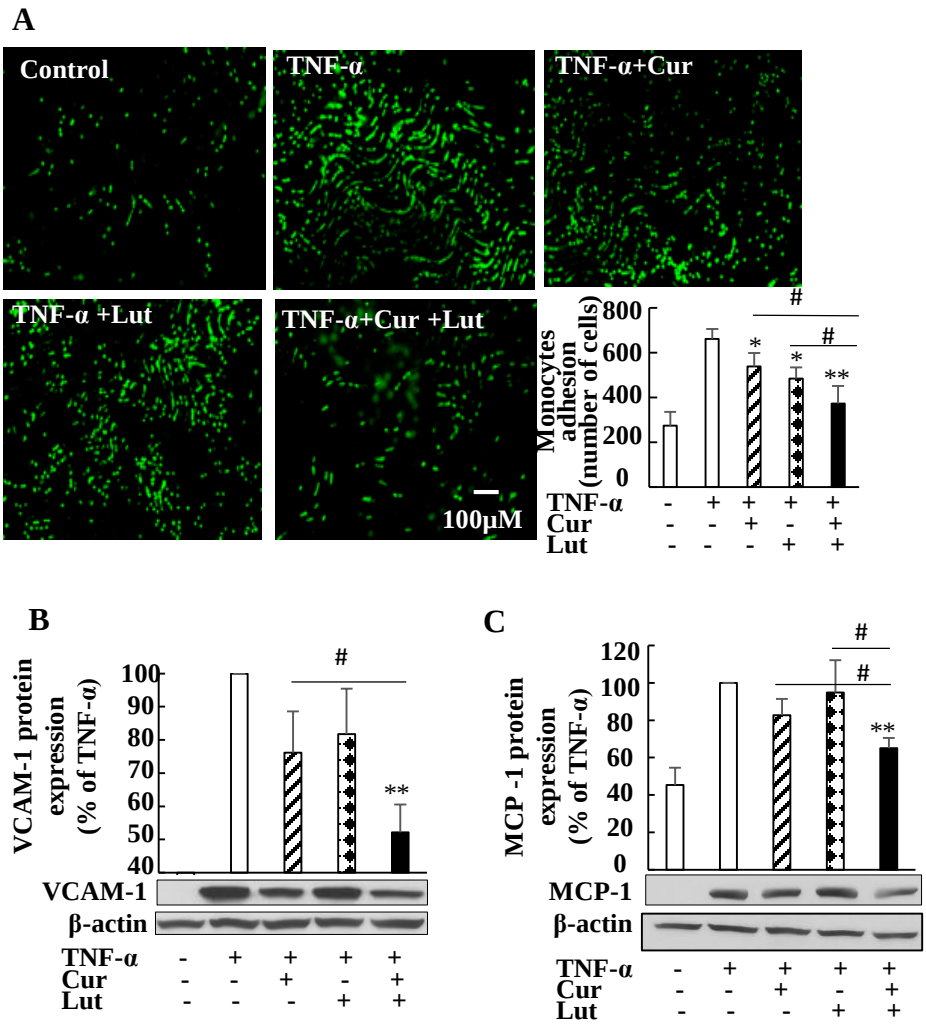

A
Total p65 protein expression (% of TNF-α)
120
100
80
60
40
20
0
Total P65
β-actin
TNF-α (ng/ml) - 10 10 10 10
Cur(μM) - - 1 - 1
Lut(μM) - - - 0.5 0.5
B
Nuclear p65 protein expression (% of TNF-α)
120
100
80
60
40
20
0
#
#
**
**
Nuclear P65
β-actin
TNF-α (ng/ml) - 10 10 10 10
Cur(μM) - - 1 - 1
Lut(μM) - - - 0.5 0.5

Figure 4.


A
Control
TNF-α
TNF-α+Cur
TNF-α +Lut
TNF-α+Cur +Lut
100μM
Monocytes adhesion (number of cells)
800
600
400
200
0
TNF-α - + + + +
Cur - - + - +
Lut - - - + +
B
VCAM-1 protein expression (% of TNF-α)
100
90
80
70
60
50
40
VCAM-1
β-actin
TNF-α - + + + +
Cur - - + - +
Lut - - - + +
C
MCP -1 protein expression (% of TNF-α)
120
100
80
60
40
20
0
MCP-1
β-actin
TNF-α - + + + +
Cur - - + - +
Lut - - - + +