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The Inhibitory Effect of Pterostilbene on Inflammatory Responses during the Interaction of 3T3-L1 Adipocytes and RAW 264.7 Macrophages

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ABSTRACT: Chronic inflammation is characterized by the upregulation of proinflammatory cytokines in obese adipose tissue. Accumulations of adipose tissue macrophages enhance a chronic inflammatory state in adipose tissues. Many studies have indicated that the adipocyte-related inflammatory response in obesity is characterized by an enhanced infiltration of macrophages. The aim of this work was to study the inhibitory effects of garcinol and pterostilbene on the change in inflammatory response due to the interaction between 3T3-L1 adipocytes and RAW 264.7 macrophages. In the TNF-α-induced 3T3-L1 adipocyte model, garcinol and pterostilbene significantly decreased the mRNA expression of COX-2, iNOS, IL-6, and IL-1β and IL-6 secretion by suppressing phosphorylation of p-IκBα and p-p65. In a coculture model of 3T3-L1 adipocytes and RAW 264.7 macrophages, pterostilbene suppressed IL-6 and TNF-α secretion and proinflammatory mRNA expression and also reduced the migration of macrophages toward adipocytes. In the RAW 264.7 macrophage-derived conditioned medium (RAW-CM)-induced 3T3-L1 adipocyte and 3T3-CM-induced RAW 264.7 macrophage models, pterostilbene significantly decreased IL-6 and TNF-α secretion and proinflammatory mRNA expression (COX-2, iNOS, IL-6, TNF-α, PAI-1, CRP, MCP-1, resistin, and leptin). Our findings suggest that garcinol and pterostilbene may provide novel and useful applications to reduce the chronic inflammatory properties of adipocytes. We also found that pterostilbene inhibits proinflammatory responses during the interaction between 3T3-L1 adipocytes and RAW 264.7 macrophages.

KEYWORDS: *garcinol, pterostilbene, 3T3-L1 adipocytes, RAW 264.7 macrophages, inflammatory response, coculture*

INTRODUCTION

Obesity is associated with chronic inflammation and is recognized as a risk factor for cardiovascular diseases, type 2 diabetes, hypertension, insulin resistance, and certain cancers.[1,2] Chronic inflammation is characterized by the upregulation of proinflammatory adipocytokines [such as tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein-1 (MCP-1)] and downregulation of adiponectin (an anti-inflammatory adipocytokine) in obese adipose tissue.[3,4] Obesity-related inflammatory responses are partially mediated through multiple cellular stress responses, such as hypoxia, oxidative stress responses, and cellular endoplasmic reticulum (ER) stress responses.[5−7] Many studies have indicated that adipocytes are characterized by an enhanced infiltration of macrophages and that adipocytes are involved in the inflammatory changes that occur during interactions with macrophages.[8,9] Some reports have indicated that the adipocyte-related molecules free fatty acid (FFA) and MCP-1 and the macrophage-related molecule TNF-α establish a vicious cycle that augments inflammatory response changes and insulin resistance in obese adipose tissue.[10,11] Cell models of obesity-related inflammatory responses are generally divided into three types, including (1) inflammatory factor (such as TNF-α)-induced proinflammatory responses in adipocytes, (2) proinflammatory actions due to the interaction between adipocytes and macrophages (coculture model), and (3) inflammatory responses in macrophage-derived conditioned medium (CM)-treated adipocytes or adipocyte-derived CM-treated macrophages.[10−17] Our previous study showed that *p*-coumaric acid, quercetin, and resveratrol inhibit the production of interleukin-6 (IL-6), MCP-1, plasminogen activator inhibitor-1 (PAI-1), and ROS in TNF-α-treated 3T3-L1 adipocytes.[17] These compounds also significantly mitigate the decreases in adiponectin and antioxidant enzyme activities in TNF-α-treated 3T3-L1 adipocytes. The nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways play an important role in obesity-related inflammatory responses.[10,11] Moreover, a decrease in IκB expression is also a key biomarker for the detection of inflammatory responses in adipose tissues. Hirai et al.[15] indicated that diosgenin attenuates inflammatory changes during the interaction of adipocytes and macrophages and in 3T3-L1 adipocyte-derived conditioned medium (3T3-

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CM)-induced RAW 264.7 macrophages. Kang et al.[16] also indicated that resveratrol significantly decreases the inflammatory responses in RAW 264.7 macrophage-derived conditioned medium (RAW-CM)-induced 3T3-L1 adipocytes.

The polyprenylated benzophenone derivative garcinol is obtained from Guttiferae plants in tropical regions, such as *Garcinia indica*, *Garcinia cambogia*, and *Garcinia huillkensis*. *Garcinia* plants used in traditional and modern medicines and foods possess anti-inflammatory, antioxidant, anticarcinogenic, and antiulcer properties.[18−20] Pterostilbene is a stilbenoid chemically similar to resveratrol and is found in grapes, wine, and berries.[21] Some studies have provided pharmacological data on the antioxidant, anti-inflammatory, and anticancer functions of pterostilbene.[22−24] Our previous study showed that garcinol and pterostilbene inhibit cell proliferation and adipogenesis in 3T3-L1 preadipocytes, adipocytes undergoing differentiation, and mature adipocytes.[25] However, the effects of garcinol and pterostilbene on the inflammatory changes that occur during the interaction of macrophages and adipocytes remain unclear.

The objective of this study was to investigate the modulatory actions of garcinol and pterostilbene on TNF-α-induced changes in proinflammatory responses in 3T3-L1 adipocytes. Moreover, the effects of garcinol and pterostilbene on proinflammatory responses in 3T3-L1 adipocytes during coculture with RAW 264.7 macrophages were also investigated. Finally, we investigated the effect of pterostilbene on proinflammatory responses in RAW-CM-treated 3T3-L1 adipocytes and 3T3-L1 adipocytes-derived conditioned medium (3T3-CM)-treated RAW 264.7 cells.

MATERIALS AND METHODS

Materials. Garcinol (3-[(3,4-dihydroxyphenyl)-hydroxymethylidene]-6,6-dimethyl-5,7-bis(3-methylbut-2-enyl)-1-[(2*S*)-5-methyl-2-prop-1-en-2-ylhex-4-enyl]bicyclo[3.3.1]nonane-2,4,9-trione) and pterostilbene (*trans*-3,5-dimethoxy-4′-hydroxystilbene) were provided by Professor Chi-Tang Ho (Rutgers University, New Jersey). Pterostilbene was synthesized according to the method reported by Pettit et al.[26] The synthesized pterostilbene was further purified by preparative HPLC, and the purity of pterostilbene was determined by HPLC as higher than 99.7%. Garcinol was prepared from *G. indica* dried fruit rind that was extracted with ethanol. The extract was subjected to silica gel column eluted with hexane-ethyl acetate solvent system. The fractions containing garcinol were concentrated and dried in vacuo. The residue was dissolved in hexane and remained in a freezer for 24 h to get yellow amorphous power from the solution and washed with cold hexane on a glass filter. After recrystallizing the powder in hot acetonitrile at room temperature, pale yellow crystal needles were obtained from the solvent. The compound was identified as garcinol by NMR and MS,[27] and the purity was determined by HPLC as higher than 99.2%. Dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), insulin (INS), and MTT dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were obtained from Sigma-Aldrich Co. (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), bovine calf serum, and antibiotic mixture (penicillin-streptomycin) were purchased from the Invitrogen Co. (Carlsbad, CA). Polyvinyldifluoride (PVDF) membrane for Western blotting was obtained from Millipore (Bedford, MA). Anti-NF-κB p65 antibody and anti-p-NF-κB p65 antibody were obtained from Cell Signaling Technology (Danvers, MA). Anti-COX-2 (cyclooxygenase-2) antibody was obtained from Abcam (San Francisco, CA). Mouse TNF-α and IL-6 enzyme-linked immunosorbent assay (ELISA) Ready-SET-Go! were obtained from eBioscience (San Diego, CA). Chemiluminesence HRP substrate was obtained from Millipore. Tri-Isolation Reagent was obtained from MoBioPlus (Taipei, Taiwan). Smart Quant Green Master Mix with dUTP and ROX kit was obtained from Protech Technology (Taipei, Taiwan). PCR Master Mix 2X Kit and 6X DNA Loading Dye Solution were obtained from Fermentas (Glen Burnie, MD). Transwell 24-well permeable support was obtained from Corning (Lowell, MA). All other chemicals were reagent grade.

Cell Culture. The murine preadipocyte cell line 3T3-L1 was obtained from the Bioresource Collection and Research Center (BCRC 60159, Food Industry Research and Development Institute, Hsin Chu, Taiwan) and cultured in DMEM supplemented with 10% bovine serum, 1.5 g/L sodium bicarbonate, and 1% streptomycin/penicillin (100 units/mL and 100 μg/mL) at 37 °C in a 5% CO_2 incubator. Adipocytic differentiation was induced by adipogenic agents (0.5 mM IBMX, 1 μM DEX, and 1 μM INS) added to the culture medium for 4 days. Afterward, the cells were placed in normal culture medium that was replaced every 48 h. The cells were harvested 8 days after differentiation was initiated. The murine macrophage cell line RAW 264.7 was obtained from the Bioresource Collection and Research Center (BCRC 60001) and cultured in DMEM supplemented with 10% FBS and 1% streptomycin/penicillin (100 units/mL and 100 μg/mL) at 37 °C in a 5% CO_2 incubator.

ELISA Assays. 3T3-L1 adipocytes or RAW 264.7 macrophages were incubated with serum-free medium in a six-well plate for 24 h. The conditioned medium supernatant was then harvested, and TNF-α and IL-6 secretion was measured using a specific ELISA kit according to the manufacturer's instructions (eBioscience).

RNA Extraction, RT-PCR, and Real-Time RT-PCR. After treatment, cells were washed two times with PBS, and Tri-Isolation Reagent (MoBioPlus) was used to extract intracellular RNA. cDNA corresponding to 1 μg of RNA was reverse-transcribed using the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR kit (Invitrogen). PCR analyses were performed on aliquots of the cDNA preparation to detect gene expression using the PCR Master Mix 2X kit (Fermentas). The primers used were the following: COX-2: 5′-GGAGAGACTATCAAGATAGT-3′ (forward); 5′-ATGGTCAGTAGACTTTTACA-3′ (reverse). Inducible nitric oxide synthase (iNOS): 5′-AATGGCAACATCAGGTCGGCCATCACT-3′ (forward); 5′-GCTGTGTGTCACAGAAGTCTCGAACTC-3′ (reverse). IL-6: 5′-GTCGGAGGCTTAATTACACATGTTC-3′ (forward); 5′-ACTCCTTCTGTGACTCCAGCTTATC-3′ (reverse). Interleukin-1β (IL-1β): 5′-TGCAGAGTTCCCCAACTGGTACATC-3′ (forward); 5′-GTGCTGCCTAATGTCCCCTTGAATC-3′ (reverse). β-Actin: 5′-TCATGAAGTGTGACGTTGACATCCGT-3′ (forward); 5′-CCTAGAAGCATTTGCGGTGCACGATG-3′ (reverse). RT-PCR products were analyzed on 1.8% agarose gels. Amplified cDNA bands were detected by ethidium bromide staining. The gel was then photographed under UV transillumination. The relative expression of mRNA was calculated according to the reference bands of β-actin.

Real-time RT-PCR was performed using the SYBR GreenER qPCR SuperMix for smart quant green master mix with dUTP and ROX kit (Protech Technology) using a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) with the following cycling parameters: 95 °C for 15 min for enzyme activation, followed by denaturing at 95 °C for 15 s, and annealing and elongation at 60 °C for 1 min, with a total of 40 cycles. The primers are described as follows: COX-2: 5′-CCTCTGCGATGCTCTTCC-3′ (forward); 5′-TCACACTTATACTGGTCAAATCC-3′ (reverse). iNOS: 5′-TCCTACACCACACCAAAC-3′ (forward); 5′-CTCCAATCTCTGCCTATCC-3′ (reverse). IL-6: 5′-TTCTCTGGGAAATCGTGGAAA-3′ (forward); 5′-TCAGAATTGCCATTGCACAAC-3′ (reverse). TNF-α: 5′-CCCTCACACTCAGATCATCTTCT-3′ (forward); 5′-GCTACGACGTGGGCTACAG-3′ (reverse). Leptin: 5′-CATCTGCTGGCCTTCTCCAA-3′ (forward); 5′-ATCCAGGCTCTCTGGCTTCTG-3′ (reverse). Adiponectin: 5′-GGAGATGCAGGTCTTCTTGG-3′ (forward); 5′-TCCTGATACTGGTCGTAGGTGAA-3′ (reverse). FAS: 5′-TGGGTTCTAGCCAGCAGAGT-3′ (forward); 5′-TACCACCAGAGACCGTTATGC-3′ (reverse). Resistin: 5′-AGACTGCTGTGCCTTCTGGG-3′ (forward); 5′-CCCTCCTTTTCCTTTTCTTCCTTG-3′ (reverse). MCP-1: 5′-GCCCCACTCACCTGCTGCTACT-3′ (forward); 5′-CCTGCTGCTGGTGATCCTCTTGT-3′ (reverse). C-reactive protein (CRP): 5′-GGGTGGTGCTGAAGTACGAT-3′ (forward); 5′-CCAAAGACTGCTTTGCATCA-3′ (reverse). PAI-1: 5′-TCAGCCCTTGCTTGCCTCAT-3′ (forward); 5′-GCATAG-

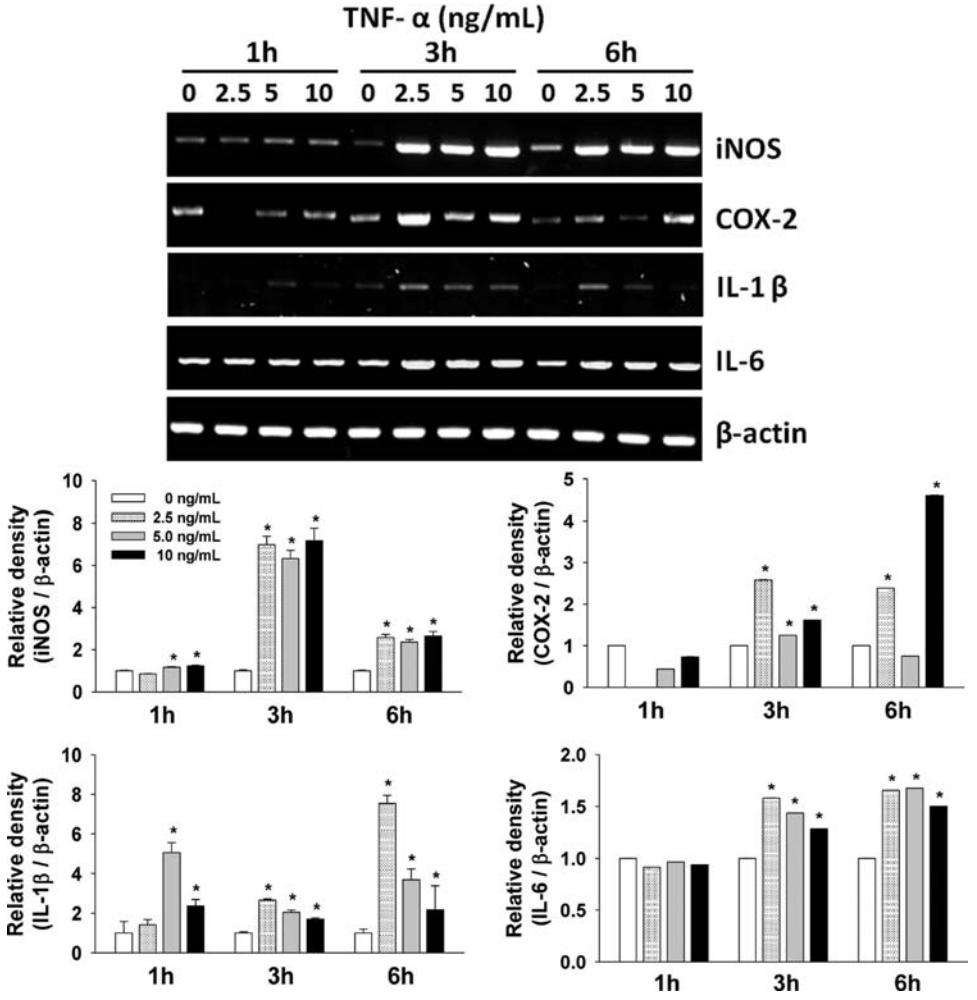

Figure 1. Effect of TNF-α on proinflammatory gene expression of iNOS, COX-2, IL-1β, and IL-6 in 3T3-L1 adipocytes. Cells were treated with 0, 2.5, 5, and 10 ng/mL TNF-α for 1, 3, and 6 h. Values are the mean ± SD for $n = 3$. *$p < 0.05$, and a significant difference from the untreated group was found.

CCAGCACCGAGGA-3′ (reverse). GAPDH: 5′-TCAACGGCACAG-TCAAGG-3′ (forward); 5′-ACTCCACGACATACTCAGC-3′ (reverse). Relative levels of gene expression were quantified using the $\Delta\Delta C_T$ method, which resulted in a ratio of target gene expression relative to an equally expressed housekeeping gene.

Western Blot Analysis. After treatment, 3T3-L1 adipocytes were collected and lysed in ice-cold lysis buffer [20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 500 μM sodium orthovanadate, 1% Triton X-100, 0.1% SDS, 10 mM NaF, 10 μg/mL leupeptin, and 1 mM PMSF]. The protein concentration was estimated with the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as a standard. Total protein was separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% polyacrylamide gel. The proteins in the gel were transferred to a PVDF membrane. The membrane was blocked with blocking buffer for 30 min. Membranes were incubated with primary antibody at 4 °C overnight and then with secondary antibody for 1 h. Membranes were washed in PBST for 10 min three times between each step. The signal was detected using the Amersham ECL system (Amersham-Pharmacia Biotech, Arlington Heights, IL). The relative expression of protein was quantified densitometrically using the software LabWorks 4.5 and calculated according to the reference band of β-actin.

Coculture of 3T3-L1 Adipocytes and RAW 264.7 Macrophages. In the contact system, RAW 264.7 macrophages (3×10^5 cells/well) were plated onto dishes with serum-starved and hypertrophied 3T3-L1 adipocytes overnight. Cocultured cells were incubated with garcinol or pterostilbene (0−5 μM) for 24 h. After treatment, the culture supernatants were collected and stored at −20 °C until use. In the transwell system, cells were cocultured using Transwell Permeable Support (Corning) 6.5 mm inserts with 0.4 μm polycarbonate membranes to separate adipocytes from macrophages. After incubation for 12, 18, and 24 h, the medium in the bottom well was collected for ELISA assay. In conditioned medium experiments, the supernatants of 3T3-L1 adipocytes and RAW 264.7 macrophages were used to replace the medium of each cell type. Briefly, the serum-free medium of hypertrophied 3T3-L1 adipocytes cultured for 12 h was collected as a 3T3-L1-derived conditioned medium (3T3-CM) and stored at −20 °C until use. The serum-free medium of RAW 264.7 macrophages cultured for 24 h was collected as a RAW-CM and stored at −20 °C until use.

Cell Migration Assay. The effects of pterostilbene and garcinol on the transmigration of RAW 264.7 macrophages to 3T3-L1 adipocytes in a transwell coculture system were measured. RAW 264.7 macrophages (3×10^4 cells/well) were placed into the upper transwell inserts of a 24-well culture chamber in serum-free medium. The macrophages were separated from lower wells by 3T3-L1 adipocytes with or without pterostilbene and garcinol in 10% FBS medium and incubated for 6 h. Methanol was used to immobilize RAW 264.7 macrophages on the permeable membrane of the insert for 30 min, and the cells were then stained with Giemsa for 1 h. Cells were counted in 10 randomly chosen fields (200×) using a light microscope (IX71, Olympus, Osaka, Japan), and images were captured by ImagePro (Media Cybernetics, Bethesda, MD). The cell migration induced during coculture of 3T3-L1 adipocytes and RAW 264.7

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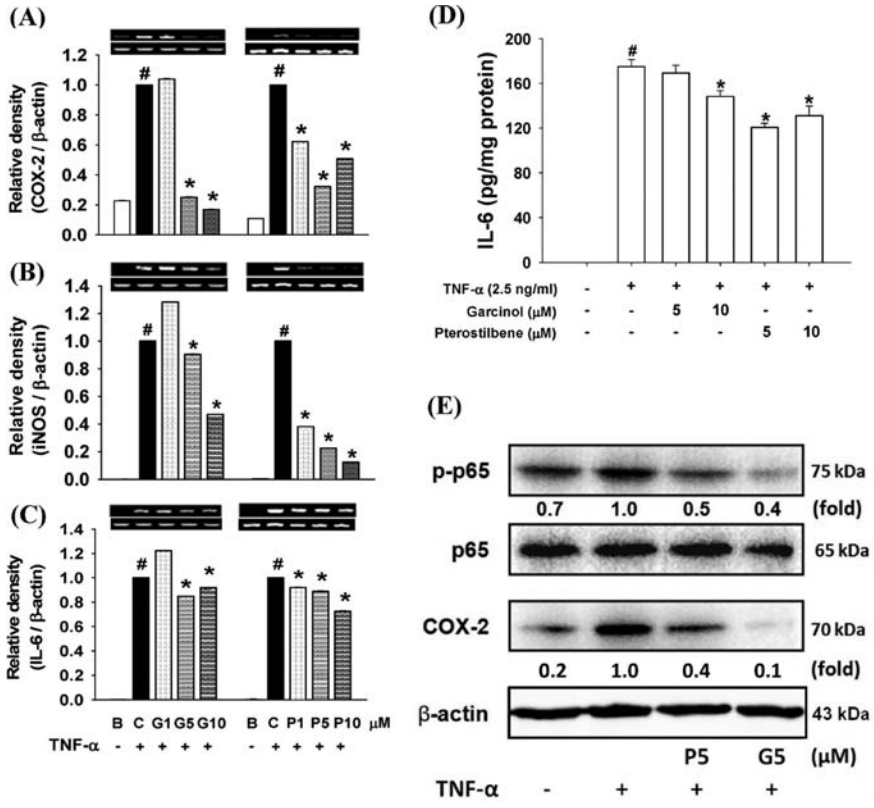

Figure 2. Effects of garcinol and pterostilbene on mRNA expression of COX-2, iNOS, and IL-6 (A–C), IL-6 secretion (D), and protein expression of NF-κB and COX-2 (E) in TNF-α-induced 3T3-L1 adipocytes. Cells were pretreated with pterostilbene for 1 h or garcinol for 3 h and then exposed to TNF-α (2.5 ng/mL) for 3 h. Values are the mean ± SD for $n = 3$. $^{\#}p < 0.05$, and a significant difference from the blank was found. $^{*}p < 0.05$, and a significant difference from TNF-α alone was found. P1, 1 μM pterostilbene; P5, 5 μM pterostilbene; P10, 10 μM pterostilbene; G1, 1 μM garcinol; G5, 5 μM garcinol; and G10, 10 μM garcinol.

macrophages served as the control and was designated as 100% migration for each experiment.

Statistical Analysis. Each experiment was performed in triplicate. The results are expressed as the mean ± SD. Statistical analysis was performed using SAS software. Analysis of variance was performed using ANOVA. Significant differences ($p < 0.05$) between means were determined by Duncan's multiple range test.

RESULTS

Effects of Garcinol and Pterostilbene on Proinflammatory Response Changes in TNF-α-Induced 3T3-L1 Adipocytes. The effect of TNF-α on expression of the proinflammatory genes iNOS, COX-2, IL-1β, and IL-6 in 3T3-L1 adipocytes is shown in Figure 1. These data illustrated that treatment of 3T3-L1 adipocytes with TNF-α for 3 h caused a greater increase in the expression of iNOS, COX-2, and IL-6 than exposure to TNF-α for 1 and 6 h. The results also showed that the expression levels of iNOS, COX-2, IL-1β, and IL-6 were significantly increased by 6.98-, 2.58-, 2.66-, and 1.58-fold, respectively, when 3T3-L1 adipocytes were treated with TNF-α (2.5 ng/mL) for 3 h. To further investigate whether TNF-α treatment affects inflammatory responses, 3T3-L1 adipocytes were treated with 2.5 ng/mL TNF-α for 3 h. Figure 2 shows the effects of garcinol and pterostilbene on expression of the proinflammatory genes COX-2, iNOS, and IL-6, IL-6 secretion and protein expression of NF-κB and COX-2 in TNF-α-induced 3T3-L1 adipocytes. The expression of the proinflammatory genes in 3T3-L1 adipocytes significantly increased when TNF-α (2.5 ng/mL) was added for 3 h. However, garcinol and pterostilbene (1–10 μM, 3 h) significantly decreased the expression of COX-2, iNOS, and IL-6 in TNF-α-treated 3T3-L1 adipocytes (Figure 2A–C). Garcinol (10 μM) and pterostilbene (5 μM) also significantly decreased IL-6 secretion in TNF-α-induced 3T3-L1 adipocytes (Figure 2D). Our previous study[25] indicated that cell numbers in 3T3-L1 adipocytes were not influenced by the treatment with garcinol (0–60 μM, 48–72 h) or pterostilbene (0–200 μM, 48–72 h). In the present study, garcinol (0–10 μM) and pterostilbene (10 μM) inhibited inflammatory responses with no cytotoxicity to the 3T3-L1 adipocytes. The effects of garcinol and pterostilbene on the protein expression of p-p65, p65, and COX-2 in TNF-α-induced 3T3-L1 adipocytes were measured by Western blot analysis (Figure 2E). Our results show that the protein levels of p-p65 and COX-2 were significantly decreased after treatment with 5 μM garcinol or pterostilbene for 3 h. However, pterostilbene caused greater inhibition of TNF-α-induced IL-6 secretion in 3T3-L1 adipocytes. Therefore, pterostilbene was selected for follow up analysis.

Effect of Pterostilbene on Proinflammatory Response Changes in 3T3-L1 Adipocyte and RAW 264.7 Macrophage Coculture. The effect of pterostilbene on secretion of IL-6 and transmigration of macrophages to adipocytes in 3T3-L1 adipocyte and RAW 264.7 macrophage coculture is shown in Figure 3. When 3T3-L1 adipocytes were cocultured with RAW 264.7 macrophages for 6, 12, 18, and 24 h, IL-6 secretion was significantly increased to 354.9, 967.1, 1998.2, and 2422.1 pg/mL, respectively (Figure 3A). The results also indicated that when pterostilbene was added to 3T3-L1 adipocytes cocultured with RAW 264.7 macrophages for 12, 18, and 24 h, IL-6

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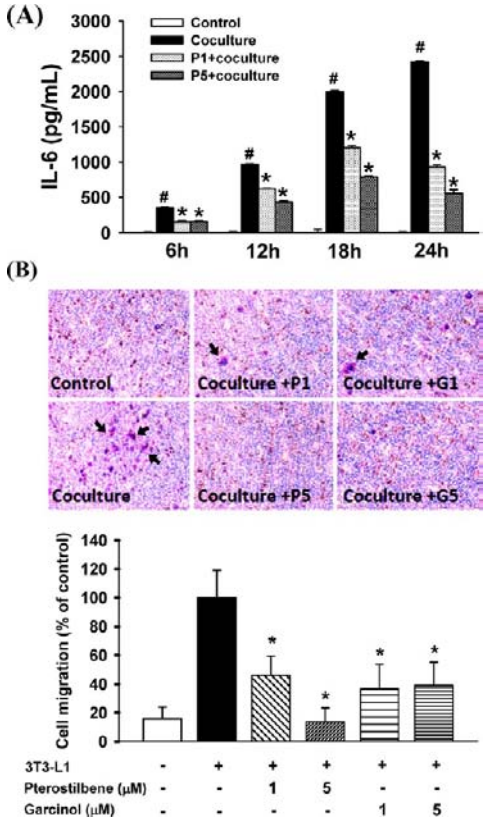

Figure 3. Effect of garcinol and pterostilbene on IL-6 secretion (A) and transmigration of macrophages toward adipocytes (B) in 3T3-L1 adipocyte and RAW 264.7 macrophage coculture. Values are the mean ± SD for $n = 3$. $^{\#}p < 0.05$, and a significant difference from the untreated group was found. $^{*}p < 0.05$, and a significant difference from the transwell coculture alone was found. P1, 1 μM pterostilbene; and P5, 5 μM pterostilbene.

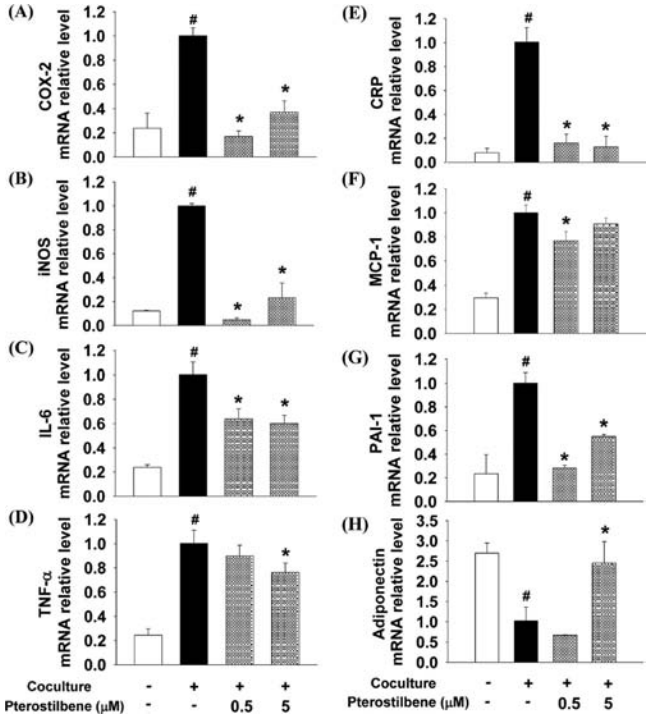

Figure 4. Effect of pterostilbene on proinflammatory gene expression of COX-2 (A), iNOS (B), IL-6 (C), TNF-α (D), CRP (E), MCP-1 (F), PAI-1 (G), and adiponectin (H) in 3T3-L1 adipocyte and RAW 264.7 macrophage coculture. Cocultured cells were treated with pterostilbene for 12 h. Values are the mean ± SD for $n = 3$. $^{\#}p < 0.05$, and a significant difference from the untreated group was found. $^{*}p < 0.05$, and a significant difference from the contact coculture alone was found.

secretion was significantly decreased in a dose-dependent manner. Quantitation of migratory cells by light microscopy demonstrated a marked increase in cell migration in 3T3-L1 adipocytes during coculture with RAW 264.7 macrophages (Figure 3B). The data also showed that pterostilbene (5 μM) and garcinol (5 μM) treatments reduced the migratory ability of 3T3-L1 adipocytes cocultured with RAW 264.7 macrophages by 13.6 and 16.2%, respectively. Figure 4 shows the effect of pterostilbene on the expression of the proinflammatory genes COX-2, iNOS, IL-6, TNF-α, adiponectin, CRP, MCP-1, and PAI-1 in 3T3-L1 adipocytes cocultured with RAW 264.7 macrophages. The protein levels of COX-2, iNOS, IL-6, TNF-α, CRP, MCP-1, and PAI-1 were remarkably decreased when pterostilbene was added to 3T3-L1 adipocytes cocultured with RAW 264.7 macrophages (Figure 4A–G). Moreover, treatment of coculture cells with pterostilbene significantly increased the protein expression of adiponectin (Figure 4H).

Effect of Pterostilbene on Proinflammatory Response Changes in RAW-CM-Induced 3T3-L1 Adipocytes. Figure 5 shows the effect of pterostilbene on IL-6 and TNF-α secretion and COX-2, iNOS, IL-6, TNF-α, resistin, CRP, PAI-1, leptin, and adiponectin expression in RAW-CM-induced 3T3-L1 adipocytes. RAW-CM significantly increased IL-6 and TNF-α secretion in 3T3-L1 adipocytes (Figure 5A). The data also indicated that pterostilbene (5 and 10 μM) significantly decreased IL-6 and TNF-α secretion in RAW-CM-induced 3T3-L1 adipocytes. Pterostilbene inhibited the expression of COX-2, iNOS, IL-6, TNF-α, resistin, CRP, PAI-1, and leptin and upregulated the expression of adiponectin at the mRNA level in RAW-CM-induced 3T3-L1 adipocytes (Figure 5B-J).

Effect of Pterostilbene on Proinflammatory Response Changes in 3T3-CM-Induced RAW 264.7 Macrophages. The effect of pterostilbene on IL-6 secretion and expression of the proinflammatory genes COX-2, iNOS, IL-6, MCP-1, PAI-1, and TNF-α in 3T3-CM-induced RAW 264.7 macrophages is shown in Figure 6. 3T3-CM significantly increased IL-6 secretion in RAW 264.7 macrophages (Figure 6A). The data also indicated that pterostilbene (5 μM) significantly decreased IL-6 secretion in 3T3-CM-induced RAW 264.7 macrophages. In addition, pterostilbene significantly decreased the expression of COX-2, iNOS, IL-6, MCP-1, PAI-1, and TNF-α mRNA in 3T3-CM-induced RAW 264.7 macrophages (Figure 6B–G).

DISCUSSION

Proinflammatory cytokines such as TNF-α and IL-6 appear to participate in the induction and maintenance of the chronic inflammatory response and are observed in obesity-related diseases.[28,29] In the present study, the effects of garcinol and pterostilbene on inflammatory changes during the interaction of adipocytes and macrophages were investigated. We investigated the effects of garcinol and pterostilbene on proinflammatory response changes in TNF-α-induced 3T3-L1 adipocytes, during coculture of 3T3-L1 adipocytes and RAW 264.7 macrophages, in RAW-CM-induced 3T3-L1 adipocytes and in 3T3-CM-induced RAW 264.7 macrophages. TNF-α plays a critical role in inflammatory response, cell proliferation,

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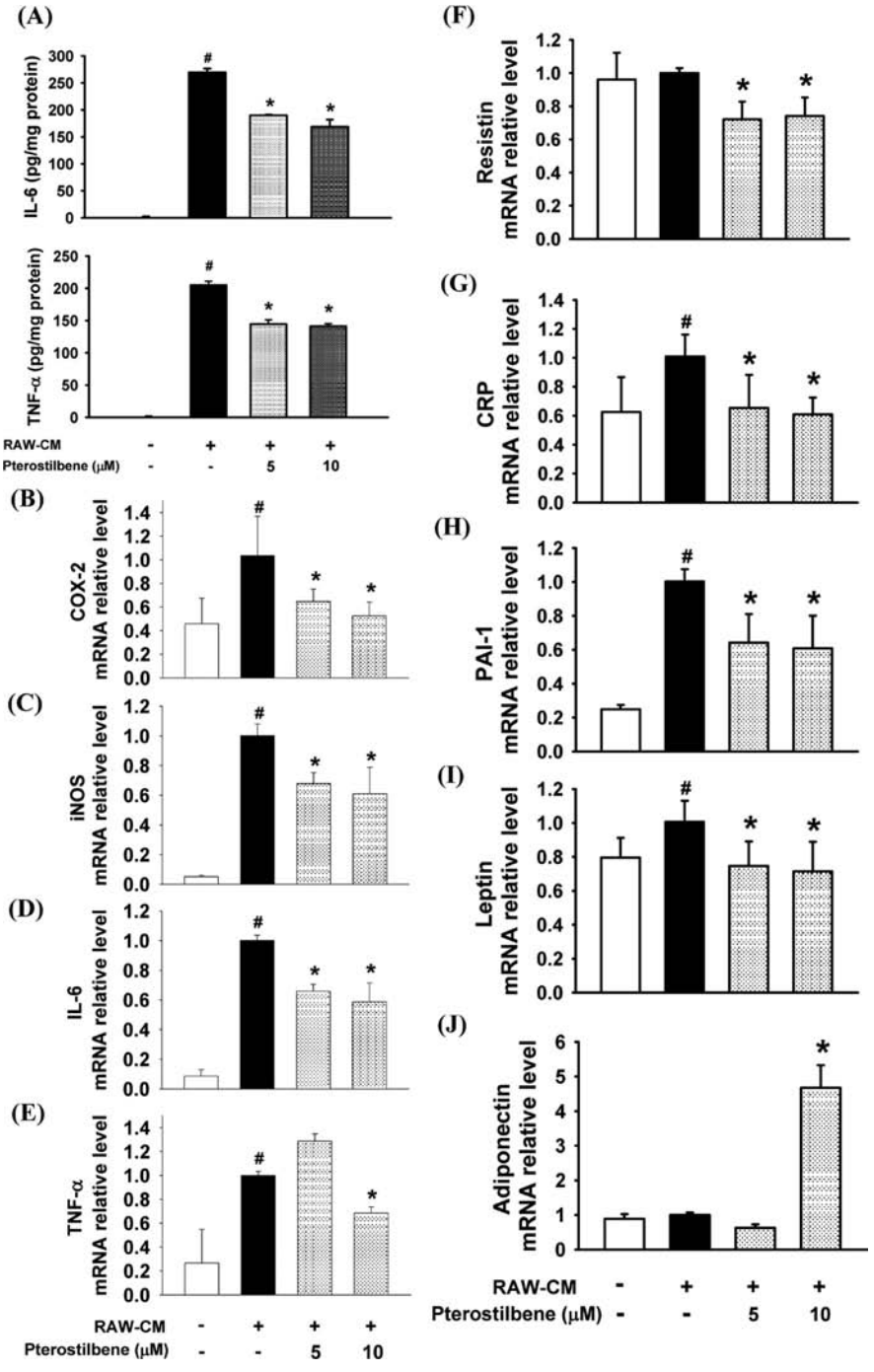

Figure 5. Effect of pterostilbene on IL-6 and TNF-α secretion (A) and proinflammatory gene expression of COX-2 (B), iNOS (C), IL-6 (D), TNF-α (E), resistin (F), CRP (G), PAI-1 (H), leptin (I), and adiponectin (J) in RAW-CM-induced 3T3-L1 adipocytes. The cells were treated with 0−10 μM pterostilbene in the presence or absence of RAW-CM for 24 h. Values are the mean ± SD for $n = 3$. $^{\#}p < 0.05$, and a significant difference from the untreated group was found. $^{*}p < 0.05$, and a significant difference from the RAW-CM treatment alone was found.

apoptosis, and lipid metabolism.[30] Hotamisligil et al.[3] and Sartipy and Loskutoff[4] indicated that chronic inflammatory response is characterized by the upregulation of TNF-α and MCP-1 and downregulation of adiponectin in adipocytes. Salerno et al.[31] indicated that increased expression of COX-2 and iNOS at both the protein and the mRNA levels is associated with an inflammatory reaction. In this study, our data indicated that TNF-α caused a greater increase in the expression of the proinflammatory genes iNOS, COX-2, and IL-6 in 3T3-L1 adipocytes (Figure 1). Garcinol and pterostilbene significantly decreased COX-2, iNOS, and IL-6 expression as well as IL-6 secretion in TNF-α-induced 3T3-L1 adipocytes (Figure 2). At the transcriptional level, COX-2 and iNOS expression is mainly through the activation of a key transcription factor, NF-κB, which plays an important role in inflammatory conditions.[32] By Western blot analysis, garcinol and pterostilbene significantly decreased the protein expressions of p-p65 and COX-2 in TNF-α-induced 3T3-L1 adipocytes (Figure 2E). Moreover, our previous data indicated that *p*-coumaric acid, quercetin, and resveratrol can attenuate

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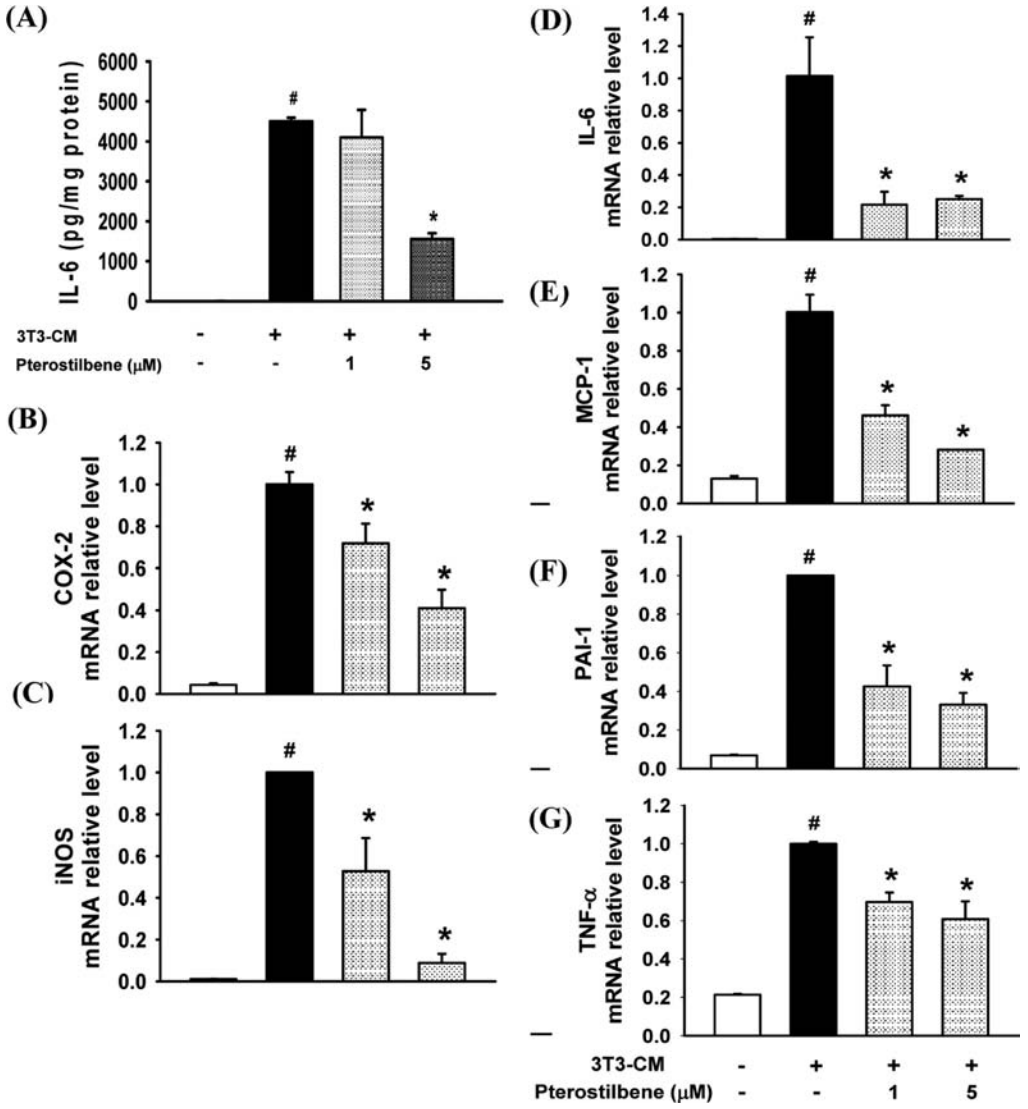

Figure 6. Effect of pterostilbene on IL-6 and TNF-α secretion (A) and proinflammatory gene expression of COX-2 (B), iNOS (C), IL-6 (D), MCP-1 (E), PAI-1 (F), and TNF-α (G) in 3T3-CM-induced RAW 264.7 macrophages. The cells were treated with 0−5 μM pterostilbene in the presence or absence of 3T3-CM for 12 h. Values are the mean ± SD for $n = 3$. $^{\#}p < 0.05$, and a significant difference from the untreated group was found. $^{*}p < 0.05$, and a significant difference from the 3T3-CM treatment alone was found.

IL-6, MCP-1, and PAI-1 secretion in TNF-α-induced 3T3-L1 adipocytes.[17]

IL-6 is one inflammatory cytokine that plays a key role in linking adipocytes and macrophages.[33] Weisberg et al.[8] indicated that adipocyte-related inflammatory responses in obesity are characterized by an enhanced infiltration of macrophages. In the present study, the effects of pterostilbene on proinflammatory responses of 3T3-L1 adipocytes cocultured with RAW 264.7 macrophages were also investigated. Our data indicated that IL-6 secretion was significantly increased in 3T3-L1 adipocytes cocultured with RAW 264.7 macrophages (Figure 3A). Moreover, pterostilbene (5 μM) and garcinol (5 μM) treatments resulted in a significant reduction of migratory abilities of cocultured 3T3-L1 adipocytes and RAW 264.7 macrophages (Figure 3B). Hirai et al.[15] indicated that diosgenin attenuates inflammatory changes in cocultured adipocytes and macrophages and 3T3-CM-induced RAW 264.7 macrophages. By Western blot analysis, pterostilbene significantly decreased the expression of the proinflammatory proteins COX-2, iNOS, IL-6, TNF-α, CRP, MCP-1, and PAI-1 and increased the protein expression of adiponectin in 3T3-L1 adipocyte and RAW 264.7 macrophage coculture (Figure 4). Kanda et al.[34] indicated that adipocyte-derived MCP-1 enhances the infiltration of macrophages into adipose tissue and increases TNF-α proinflammatory cytokines, which is followed by adipocyte dysfunction. Hirai et al.[12] indicated that naringenin chalcone inhibits MCP-1, TNF-α, and nitrite production 3T3-L1 adipocyte and RAW 264.7 macrophage coculture.

Finally, we investigated the inhibitory effect of pterostilbene on proinflammatory response in RAW-CM-induced 3T3-L1 adipocytes and in 3T3-CM-induced RAW 264.7 macrophages. Previous studies have indicated that RAW-CM significantly increases IL-6, TNF-α, and resistin gene expression and IL-6 and TNF-α secretion in 3T3-L1 adipocytes.[13,16] Our data indicated that RAW-CM-induced 3T3-L1 adipocytes and 3T3-CM-induced RAW 264.7 macrophages secrete significantly increased amounts of IL-6 and TNF-α (Figures 5A and 6A). Kang et al.[16] indicated that resveratrol significantly decreases inflammatory responses in RAW-CM-induced 3T3-L1 adipocytes through ERK 1/2- and NF-κB-dependent pathways. Our

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data indicated that pterostilbene significantly decreased the secretion of IL-6 and TNF-α and the gene expression of COX-2, iNOS, IL-6, TNF-α, resistin, CRP, PAI-1, and leptin but upregulated expression of adiponectin in RAW-CM-induced 3T3-L1 adipocytes (Figure 5B–J). Ando et al.[14] indicated that 3T3-CM significantly increases inflammatory cytokine secretion (TNF-α, MCP-1, and nitrite) and proinflammatory protein expression (TNF-α, MCP-1, and iNOS) in 3T3-L1 adipocytes. They also indicated that luteolin can attenuate inflammatory cytokine secretion and protein expression in 3T3-CM-induced RAW 264.7 macrophages. Moreover, our data indicated that pterostilbene significantly decreased IL-6 secretion and proinflammatory gene expression (COX-2, iNOS, IL-6, MCP-1, PAI-1, and TNF-α) in 3T3-CM-induced RAW 264.7 macrophages (Figure 6B–G).

In conclusion, garcinol and pterostilbene are able to inhibit proinflammatory mRNA and protein expression and cytokine secretion in TNF-α-induced 3T3-L1 adipocytes. Pterostilbene also inhibits proinflammatory responses in cocultured 3T3-L1 adipocytes and RAW 264.7 macrophages, in RAW-CM-induced 3T3-L1 adipocytes, and in 3T3-CM-induced RAW 264.7 macrophages. Our results demonstrate that pterostilbene downregulates inflammatory TNF-α, IL-6, COX-2, iNOS, IL-1β, MCP-1, CRP, PAI-1, resistin, and leptin expression by inhibiting the activation of NF-κB (Figure 7).

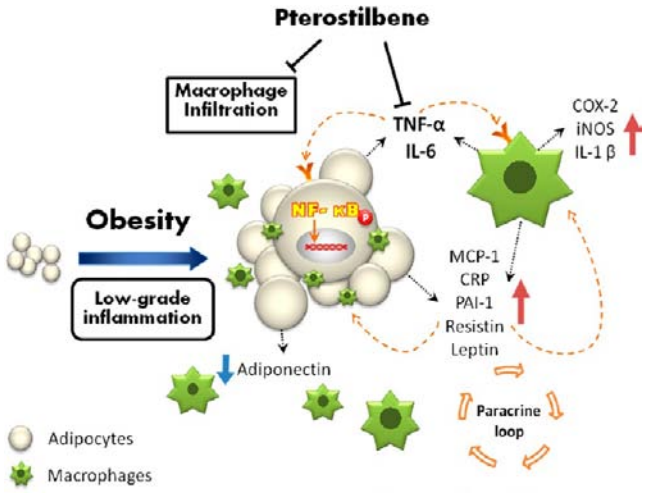

Figure 7. Modulatory action of pterostilbene on inflammation during the interaction between adipocytes and macrophages.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

COX-2, cyclooxygenase-2; CRP, C-reactive protein; ELISA, enzyme-linked immunosorbent assay; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; IL-1β, interleukin-1β; MCP-1, monocyte chemoattractant protein-1; NF-κB, nuclear factor-kappa B; PAI-1, plasminogen activator inhibitor-1; RAW-CM, RAW 264.7 macrophage-derived conditioned medium; TNF-α, tumor necrosis factor-α; 3T3-CM, 3T3-L1 adipocyte-derived conditioned medium

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