

Capsaicin, a spicy component of hot peppers, modulates adipokine gene expression and protein release from obese-mouse adipose tissues and isolated adipocytes, and suppresses the inflammatory responses of adipose tissue macrophages

Ji-Hye Kang^a, Chu-Sook Kim^a, In-Seob Han^b, Teruo Kawada^c, Rina Yu^{a,*}

^a Department of Food Science and Nutrition, University of Ulsan, Ulsan 680-749, South Korea

^b Department of Biological Science, Ulsan 680-749, South Korea

^c Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan

Received 7 July 2007; revised 28 July 2007; accepted 30 July 2007

Available online 14 August 2007

Edited by Robert Barouki

Abstract Adipokines are involved in the obesity-induced chronic inflammatory response that plays a crucial role in the development of obesity-related pathologies such as type II diabetes and atherosclerosis. We here demonstrate that capsaicin, a naturally occurring phytochemical, can suppress obesity-induced inflammation by modulating adipokine release from and macrophage behavior in obese mice adipose tissues. Capsaicin inhibited the expressions of IL-6 and MCP-1 mRNAs and protein release from the adipose tissues and adipocytes of obese mice, whereas it enhanced the expression of the adiponectin gene and protein. The action of capsaicin is associated with NF- κ B inactivation and/or PPAR γ activation. Moreover, capsaicin suppressed not only macrophage migration induced by the adipose tissue-conditioned medium, but also macrophage activation to release proinflammatory mediators. Capsaicin may be a useful phytochemical for attenuating obesity-induced inflammation and obesity-related complications.

© 2007 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Capsaicin; Obesity; Inflammation; Adipokine; MCP-1; IL-6; Adiponectin; Macrophage; Migration; Proinflammatory mediator

1. Introduction

Obesity is characterized by a chronic inflammatory state and obesity-induced inflammation is considered to be closely associated with obesity-related pathologies such as type II diabetes and cardiovascular diseases. Adipokines, which are biologically active molecules secreted from adipose tissues or adipocytes, play major roles in the regulation of food intake, insulin sensitivity, energy metabolism, and the vascular microenvironment [1,2]. Among adipokines, tumor necrosis factor- α (TNF- α) [3], interleukin-6 (IL-6) [4], monocyte chemoattractant protein-1 (MCP-1) [5], and adiponectin [6] are strongly associated with obesity-induced inflammation and obesity-related pathologies. The circulation levels of TNF- α , IL-6, and MCP-1 positively correlate with the levels

of the inflammatory markers such as the C-reactive protein [7,8], whereas adiponectin negatively correlates with it [1].

The dysregulation of adipokine release may result in the chronic inflammatory condition observed in obesity and trigger the development of obesity-related pathologies [1,9,10]. For example, TNF- α and IL-6 impair insulin signaling by suppressing the expression of insulin-sensitive glucose transport 4 and insulin receptor substrate-1 [11–14]. MCP-1 decreases insulin-stimulated glucose uptake rate and the expression levels of adipogenic genes [15]. MCP-1-deficient mice do not develop atherosclerosis [16,17] and exhibit improved insulin sensitivity [5,18]. Recent studies have shown that adipose tissue-derived MCP-1 induces macrophage infiltration into adipose tissues and thus augments the inflammatory response in obesity [15,19,20]. On the other hand, adiponectin is considered to improve insulin sensitivity by upregulating the expression of insulin receptor substrate-1 in skeletal muscles [21], and to attenuate the development of atherosclerosis by suppressing the expression of adhesion molecules in vascular endothelial cells, and inhibiting the accumulation of monocyte/macrophage-derived foam cells in the vascular wall [1,22]. These findings suggest that the dysregulation of the release of these adipokines is critical for the development of obesity-related pathologies; thus, the modulation of adipokines is a useful strategy for preventing not only obesity-induced inflammation, but also the development of obesity-related pathologies.

Studies have shown that anti-inflammatory agents including phytochemicals are effective for the treatment or control of chronic inflammatory conditions such as rheumatism, asthma, atherosclerosis, type II diabetes, and cancer [10,23–25]. However, little is known whether anti-inflammatory phytochemicals can suppress obesity-induced inflammation. Capsaicin, a spicy ingredient of hot peppers, elicits anti-inflammatory properties. Capsaicin inhibits the development of carrageen-induced paw inflammation and adjuvant-induced arthritis [26,27], as well as ethanol-induced inflammation by inhibiting the release of proinflammatory mediators [28]. Moreover, our previous study has also demonstrated that capsaicin inhibits the inflammatory responses of macrophages by inhibiting I κ B- α degradation [29]. In this study, we tested the hypothesis that the anti-inflammatory activity of capsaicin can be used for improving obesity-induced inflammation. We examined whether capsaicin modulates the production of adipokines

*Corresponding author. Fax: +82 52 259 1699.
E-mail address: rinayu@ulsan.ac.kr (R. Yu).

(i.e., IL-6, MCP-1, and adiponectin) from obese adipose tissues and isolated adipocytes, and whether capsaicin alters inflammatory responses of obese adipose tissue macrophages. Our data demonstrate that capsaicin may be a useful phytochemical for attenuating obesity-induced inflammation and obesity-related complications.

2. Materials and methods

2.1. Adipose tissue culture

Adipose tissues were isolated from obese mice fed 45% high-fat diet (Research Diets, Inc., New Brunswick, NJ) for 3 months. The adipose tissues were minced into fragments less than 10 mg in weight and cultured as previously described [20]. All subsequent procedures were performed in a laminar flow hood. In brief, 50 mg of minced adipose tissue fragments was seeded in 2 ml of M199 medium with 1% fatty acid-free albumin in each well of a 6-well plate for 3 h to remove blood cells and soluble factors from the tissue surface. The plate containing the tissue fragments was placed in a humidified incubator at 37 °C in 5% CO₂ atmosphere. After 30 min, the tissue fragments (50 mg/well) were treated with or without capsaicin (Tocris, CA, USA) for 24 h in serum-free M199 medium. Aliquots of the culture medium were stored at –80 °C until use, and the adipose tissue fragments were immediately frozen in liquid nitrogen and stored at –80 °C for RNA extraction.

2.2. Primary adipocyte culture

Adipose tissues were cut with scissors into small pieces and digested with 1 mg/ml collagenase type 2 (Sigma, NY, USA) for 1 h at 37 °C. The digest was filtered through a 100- μ m nylon mesh and adipocytes were isolated by centrifugation at 1500 rpm for 5 min. Fifty microliters of the isolated adipocytes was seeded in 2 ml of M199 medium in each well of a 24-well plate. The plate containing the adipocytes was placed in a humidified incubator at 37 °C in 5% CO₂. The adipocytes were treated with or without capsaicin and/or troglitazone (Cayman, MI, USA) for 24 h in serum-free M199 medium.

2.3. Preparation of mesenteric adipose tissue-conditioned medium

Male C57BL/6 mice (8 weeks, Hyochang Ltd., Daegu, Korea) were fed a 45% high-fat diet for 3 months, and mesenteric adipose tissue ($n = 6$) was isolated. All subsequent procedures were performed under a laminar flow hood. Adipose tissue was minced into fragments less than 10 mg in weight and cultured as previously described [20]. In brief, 50 mg of minced adipose tissue fragments was seeded in 2 ml of serum medium in each well of a 6-well plate. The plate containing the tissue fragments was placed in a humidified incubator at 37 °C and 5% CO₂. The medium was supplemented with glutamine, 25 mM HEPES, 50 μ g/ml gentamicin and 0.5 μ g/ml amphotericin B. The tissue culture was incubated for 72 h. Aliquots of the culture medium were stored at –80 °C until the use.

2.4. RAW264.7 and 3T3-L1 adipocyte culture

The murine macrophage cell line RAW 264.7 was obtained from a Korean cell Line Bank (KCLB40071, Seoul, Korea). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine serum, 10 mg/l penicillin–streptomycin (Gibco BRL, NY, USA), and 2 mg/l gentamicin (Gibco BRL, NY, USA) at 37 °C in 5% CO₂. 3T3-L1 preadipocytes (ATCC, USA) were cultured in a basal medium consisting of DMEM supplemented with 200 μ M ascorbic acid, 10% fetal bovine serum, 10 mg/l penicillin–streptomycin, and 2 mg/l gentamicin at 37 °C in 5% CO₂ atmosphere. Two days after reaching confluence, the cells were incubated in a differentiation medium containing an inducing mixture (0.25 μ M dexamethasone, 10 μ g/ml insulin, and 0.5 mM 1-methyl-3-isobutylxanthine) in the basal medium. After 40–42 h, the cell culture medium was changed to a maturation medium, which is the basal medium containing 5 μ g/ml insulin. The maturation medium was changed every two days for 6 days.

2.5. Measurement of MCP-1, IL-6, adiponectin, and TNF- α concentrations

The levels of MCP-1, IL-6, adiponectin, and TNF- α in culture supernatants were assessed by enzyme-linked immunosorbent assay

(ELISA). The assay was conducted utilizing an OptEIA™ mouse MCP-1 set, a mouse TNF- α set (BD Biosciences Pharmingen, CA, USA), a mouse IL-6 set (R&D systems, MN, USA), or a mouse adiponectin set (R&D systems, MN, USA). The sample was thawed, appropriately diluted with assay diluent, and assayed. The levels of MCP-1, IL-6, adiponectin, and TNF- α were quantified from a standard curve obtained using the SOFTmax curve-fitting program (Molecular Devices, CA, USA).

2.6. RT-PCR analysis

Total RNA was extracted from 100 mg of a tissue sample using a tri-reagent kit (MCR Inc., USA). Total RNA (0.5 μ g) was used for reverse transcription; it was amplified using a polymerase chain reaction (PCR) technique in a single reaction, using an Access RT-PCR system according to the manufacturer's instructions in a TaKaRa Thermal cycler (TaKaRa Biomedicals, Tokyo, Japan). For semiquantitative analysis, the linearity of the amplifications of MCP-1, IL-6, adiponectin, COX-2, and 36B4 cDNAs was established in preliminary experiments. The following sets of primers were used in the PCR amplification: MCP-1 (GeneBank accession number: NM011333), forward 5'-TCAGCCAGATGCAGTTAACGC-3', reverse 5'-TGGATGCATAGCTTCAGATTCAGATTTACG-3'; IL-6 (NM011333), forward 5'-CATGTTCTCTGGGAAATCGTGG-3', reverse 5'-AACTGATATGCTTAGGCATAACGCAC-3', adiponectin (NM011333), forward 5'-TACAACCAACAGAATCATTATGACGG-3', reverse 5'-GAAAGCCAGTAAATGTAGAGTCGTGTTGA-3', 36B4 (BC011291), forward 5'-TGTGTGTCTGCTGATCGGGTAC-3', reverse 5'-CTTGCGGGATTAGTCAAG-3', COX-2 (M949 67), forward 5'-CAGTTTTTCAAGACAGATCATAAGCG-3', reverse 5'-TGCTCCTGCTTGAGATGTCG-3'. The conditions for PCR for the target genes were as follows: MCP-1 gene: 40 cycles at 85 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min; IL-6 gene: 38 cycle at 85 °C for 30 s, 51 °C for 30 s, and 72 °C for 30 s; adiponectin gene: 40 cycles at 85 °C for 1 min, 64 °C for 1 min, and 72 °C for 1 min; COX-2 gene: 30 cycle at 85 °C for 1 min, 42 °C for 1 min, and 72 °C for 1 min. Amplification products obtained by PCR were electrophoretically separated on a 2% agarose gel. SYBR green-stained bands corresponding to the target genes and 36B4 were photographed with a DS-34 Polaroid camera. The intensity of the bands was densitometrically measured with an NIH Image analyzer. All MCP-1, IL-6, COX-2, and adiponectin signals were normalized to the mRNA level of the housekeeping gene 36B4 and expressed as a ratio.

2.7. Migration assay

The migration of macrophages was assessed in a multiwell microchemotaxis chamber (Neuro Probe Inc., Gaithersburg, MD, USA). Briefly, the above prepared RAW264.7 cells were suspended in M199 medium at 1×10^6 cells/ml and 27 μ l was placed in the upper wells of a 96-well chamber that was separated from lower well by an 8 μ m polycarbonate filter containing adipose tissue-conditioned medium with or without capsaicin and/or MCP-1 (R&D systems, MN, USA). After incubation for 6 h at 37 °C, non-migrated cells were removed by scraping them out and cells that migrated across the filter were fixed and stained with Diff-Quik (Merck Corp, Darmstadt, Germany). Stained cells were counted under a light microscopy in three randomly chosen high-power fields (HPF; 400 \times). Results are expressed as the mean \pm S.E.M. of triplicate samples.

2.8. Measurement of amount of nitric oxide

The amount of nitric oxide in cell-free culture supernatants was measured using Griess reagent [30]. Briefly, 100 μ l of supernatant was mixed with an equal volume of Griess reagent on a 96-well flat-bottom plate. The absorbance at 570 nm was measured after 10 min using a micro-ELISA reader. The amount of nitrite was calculated from the NaNO₂ standard curve.

2.9. Measurement of NF- κ B binding activity

Nuclear extract from treated 3T3-L1 adipocytes was prepared with a Nuclear Extract kit (Active Motif, Rixensart, Belgium) and the protein content in the nuclear lysate was determined using a BCA protein kit (Pierce, Rockford IL, USA). The DNA binding activity of the p65 subunit of NF- κ B in the nuclear protein extract was determined by NF- κ B p65 TransAM assay according to the manufacturer's protocol. Briefly, oligonucleotides containing NF- κ B consensus sequence was immobi-

lized in a 96-well plate. The plate was incubated with the nuclear protein extracts (15 µg/sample) for 1 h at room temperature with gentle agitation, washed and incubated with antibodies against p65 for 1 h without agitation. After successive washing, the plate was incubated with diluted horseradish peroxidase-conjugated secondary antibodies for 1 h. The peroxidase activity was visualized using a developing solution placed in the wells of the plate for 5 min, and the absorbance at 450 nm was measured using a micro-ELISA reader.

2.10. Animal treatment

Male C57BL/6 mice (8 weeks) were fed a 45% high-fat diet for 6 weeks. The mice fed a high-fat diet were injected by intraperitoneal administration of capsaicin (2 mg/kg, BW, dissolved in 0.9% saline with 2% ethanol, 10% tween 80, *i.p.* injection) or vehicle (0.9% saline with 2% ethanol, 10% tween 80) only for control [31] five times for 10 days.

2.11. Flow cytometric analysis

Stromal vascular cells were isolated from adipose tissues. Briefly, adipose tissues were cut with scissors into small pieces and digested with 1 mg/ml collagenase type 2 for 1 h at 37 °C. The digest was filtered through a 100 µm-nylon mesh and stromal vascular fraction cells were isolated by centrifugation at 1500 rpm for 5 min. Stromal vascular cells were incubated in the dark at 4 °C on a bidirectional shaker for 30 min in Fc blocking solution (eBioscience, CA, USA), then stained with phycoerythrin-conjugated antimouse F4/80 (eBioscience, CA, USA) and/or fluorescein isothiocyanate-conjugated antimouse CD11b (eBioscience, CA, USA). After incubation with the antibodies, 1 ml of flow cytometric analysis (FACS) buffer was added to the cells. Cells were centrifuged at 1200 rpm for 5 min and resuspended in 1 ml of FACS buffer. The wash was repeated two times. Cells were analyzed on a FACSCalibur (BD Biosciences, CA, USA) with CellQuest software (BD Biosciences, CA, USA).

2.12. Statistical analyses

Results are expressed as means ± S.E.M. Statistical analysis was performed using ANOVA and Duncan's multiple-range test. Differences were considered to be significant when $P < 0.05$.

3. Results

3.1. Effects of capsaicin on expression of adipokine mRNA and protein release from mesenteric adipose tissue of obese mice

Body weight of obese mice was 40.1 ± 0.56 g, and their mesenteric and epididymal fat tissue weights were 1.05 ± 0.10 g

and 2.21 ± 0.06 g, respectively. Visceral fat displays more enhanced lipolytic activity and inflammatory phenotypes than other fat depots, and thus is more implicated in the development of obesity-related complication than other fat depots [19]. Our previous study and others have demonstrated that mesenteric adipose tissue has the greatest potential to release MCP-1 and to induce macrophage infiltration among other adipose tissues (e.g. subcutaneous, epididymal, renal) [20,32]. To examine the effect of capsaicin on the productions of adipokines (MCP-1, IL-6 and adiponectin), mesenteric adipose tissues from obese mice were cultured with or without capsaicin, and the expression levels of the genes encoding these adipokines and protein released were measured by RT-PCR or ELISA. Capsaicin significantly inhibited the expressions of MCP-1 and IL-6 mRNAs and protein release in the mesenteric adipose tissues of obese mice (Figs. 1A, B, 2A, and B), whereas it significantly enhanced the expression of adiponectin mRNA and protein release in the mesenteric adipose tissues (Figs. 1C and 2C).

3.2. Effect of capsaicin on release of adipokine proteins from adipocytes isolated from obese mice

To examine whether capsaicin alters adipokine (IL-6, MCP-1, and adiponectin) releases from adipocytes, we isolated adipocytes from adipose tissues of obese mice and treated the adipocytes with or without capsaicin. Peroxisome proliferator-activated receptor gamma (PPAR γ) agonist, troglitazone, significantly inhibited MCP-1 and IL-6 production, and enhanced adiponectin production from adipocytes (Fig. 3A, B, and C). Capsaicin significantly inhibited the releases of MCP-1 and IL-6 (Fig. 3A and B) in adipocytes isolated from the adipose tissues of obese mice, whereas it significantly enhanced adiponectin (Fig. 3C) release from the adipocytes mimicking troglitazone action. Simultaneous treatment with both capsaicin (50 µM) and troglitazone (10 µM) resulted in greater inhibition (58%, 90%) on MCP-1 and IL-6 production from adipocytes when it was compared with the single treatment of capsaicin (24%, 18%) or troglitazone (27%, 26%) alone at the same concentration.

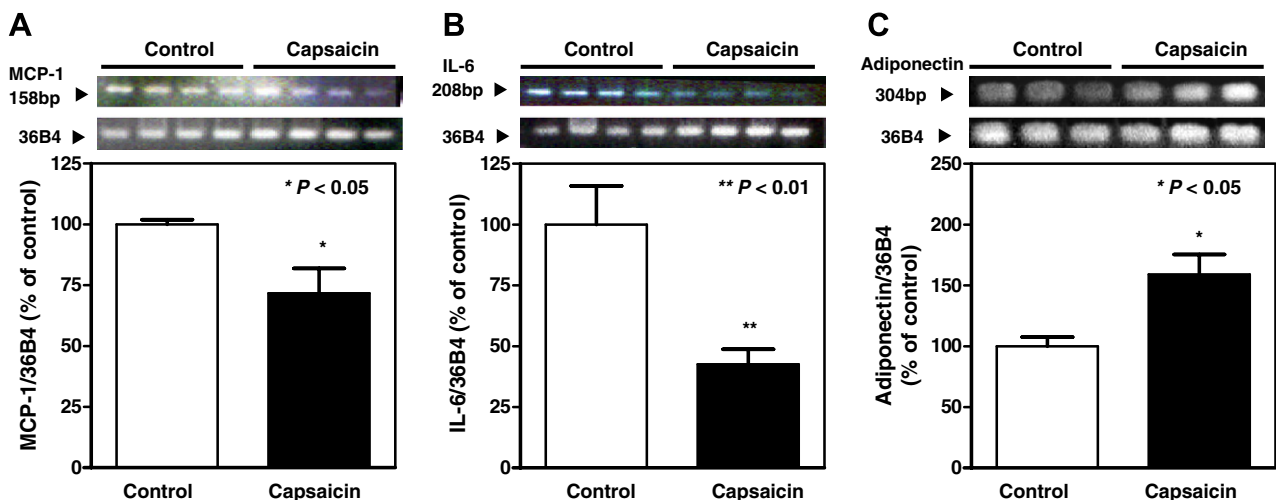


Fig. 1. Effect of capsaicin on expressions of adipokines (i.e., MCP-1, IL-6, and adiponectin) mRNAs from mesenteric adipose tissues of obese mice. Mesenteric adipose tissues (50 mg/well) were treated with or without capsaicin (100 µM) and incubated for 24 h. Adipokines mRNA expression level was measured by RT-PCR. (A) MCP-1, (B) IL-6 and (C) adiponectin mRNA expression levels in mesenteric adipose tissue. The intensity of the bands was densitometrically measured and normalized to the mRNA expression levels of the 36B4 gene. Data represent results of three independent experiments. Values are means ± S.E.M. * $P < 0.05$, ** $P < 0.01$, significantly different from control.

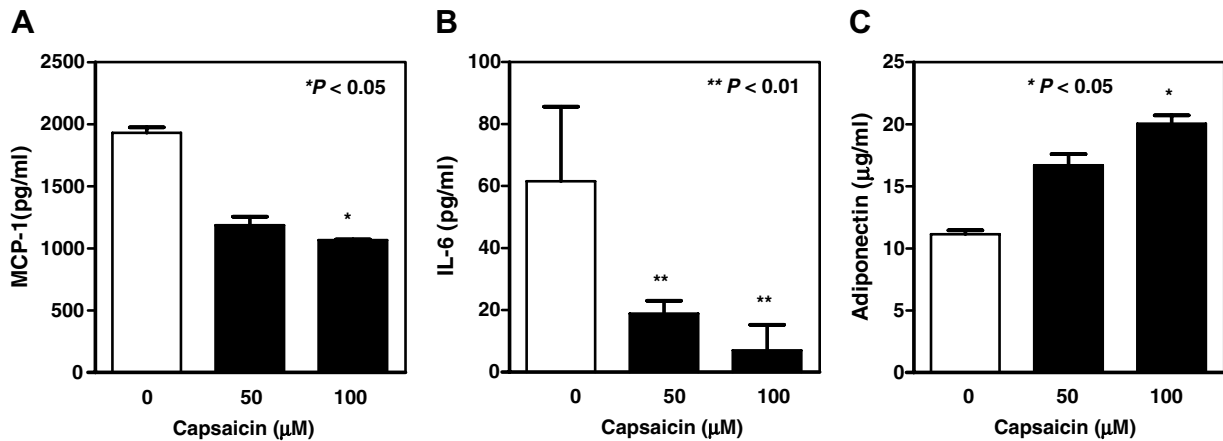


Fig. 2. Effects of capsaicin on expressions of adipokines (i.e., MCP-1, IL-6, and adiponectin) and protein release from mesenteric adipose tissues of obese mice. Mesenteric adipose tissues (50 mg/well) were treated with or without capsaicin (0–100 μM) and incubated for 24 h. Levels of adipokines protein release in the culture medium was measured by ELISA. Levels of (A) MCP-1, (B) IL-6 and (C) adiponectin protein releases in mesenteric adipose tissues. Data represent results of three independent experiments. Values are means ± S.E.M. * $P < 0.05$, ** $P < 0.01$, significantly different from control.

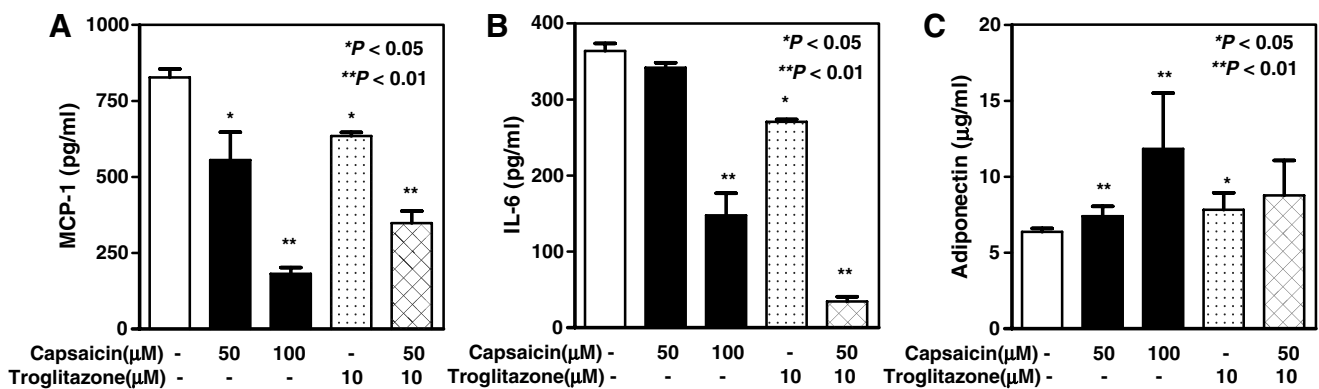


Fig. 3. Effects of capsaicin on releases of adipokines (i.e., MCP-1, IL-6, and adiponectin) from adipocytes isolated from visceral adipose tissues of obese mice. Isolated adipocytes were cultured with or without capsaicin or troglitazone, as described in Section 2. The levels of MCP-1 (A), IL-6 (B), and adiponectin (C) were measured by ELISA. Values are means ± S.E.M. All results shown are representative of results of three independent experiments. * $P < 0.05$, ** $P < 0.01$, significantly different from control.

3.3. Inhibitory effect of capsaicin on macrophage migration induced by obese mice mesenteric adipose tissue-conditioned medium or MCP-1

Our previous study showed that adipose tissue-derived MCP-1 plays a crucial role in macrophage infiltration into the adipose tissues of obese mice; thus, MCP-1 is closely associated with the enhanced inflammatory response in the adipose tissues of obese mice [20]. To test whether capsaicin can alter adipose tissue-derived MCP-1 action, we prepared a mesenteric adipose tissue culture-conditioned medium containing chemotactic factors such as MCP-1 and treated macrophages with it to induce macrophage migration. The mesenteric adipose tissue-derived conditioned medium enhanced RAW264.7 macrophage migration, and capsaicin markedly suppressed RAW264.7 macrophage migration induced by the mesenteric adipose tissue-conditioned medium (Fig. 4A). In addition, capsaicin also inhibited MCP-1-induced macrophage migration in a dose-dependent manner (Fig. 4B).

3.4. Inhibitory effect of capsaicin on macrophage activation stimulated by treatment with mesenteric adipose tissue-conditioned medium

To examine whether capsaicin suppresses adipose tissue macrophage activation, macrophages were treated with the mesenteric adipose tissue-conditioned medium with or without capsaicin, and the productions of proinflammatory mediators (nitric oxide, TNF-α, and MCP-1) were measured by ELISA. The mesenteric adipose tissue-conditioned medium significantly induced RAW264.7 macrophages to release nitric oxide, TNF-α and MCP-1, and capsaicin markedly decreased the proinflammatory mediator productions from the macrophage treated with mesenteric adipose tissue-conditioned medium (Fig. 5). RT-PCR analysis revealed that the expression of hypoxia-inducible factor-1 alpha, a marker of hypoxia, was not altered in the adipose tissue during the preparation of the adipose tissue-conditioned medium for 24 h (data not shown), indicating that the incubation process did not initiate a hypoxic shock.

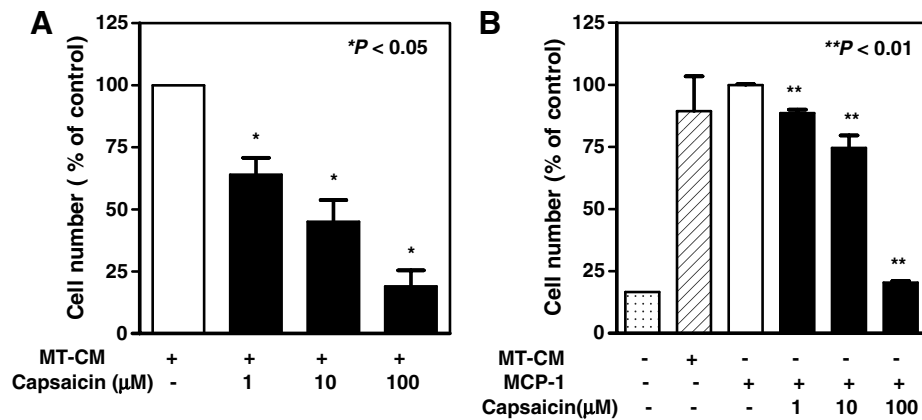


Fig. 4. Inhibitory effect of capsaicin on macrophage migration induced by obese mesenteric adipose tissue-conditioned medium (MT-CM) or MCP-1. RAW 264.7 cells were placed in the upper wells of a 96-well culture chamber that were separated from the lower wells containing different adipose tissue-conditioned media or MCP-1, and incubated for 6 h at 37 °C. The cells that migrated across the filter were fixed and stained with Diff-Quick, and counted under a light microscope in three randomly chosen high-power fields. Values are means ± S.E.M. from three independent experiments. **P* < 0.05, ***P* < 0.01, significantly different from control.

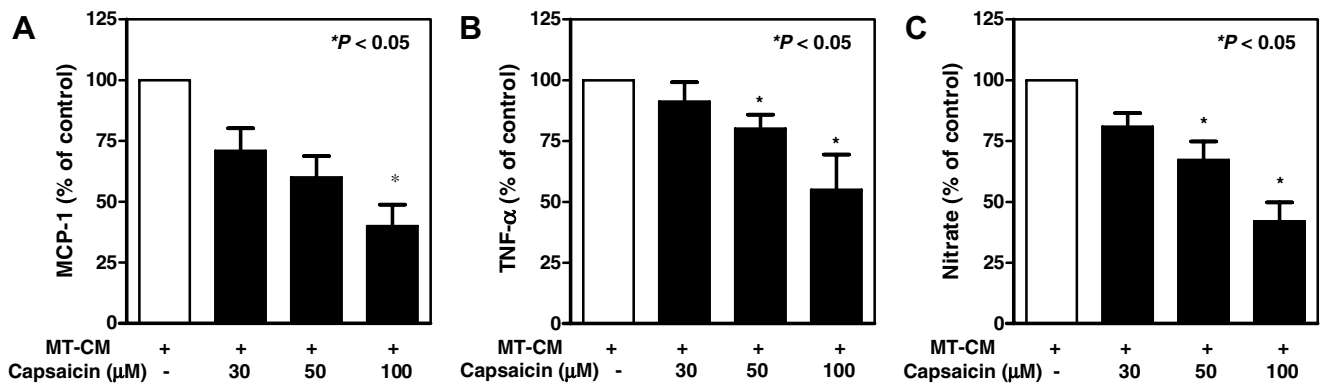


Fig. 5. Inhibitory effect of capsaicin on macrophage activation induced by obese mesenteric adipose tissue-conditioned medium. RAW 264.7 cells were stimulated with the obese mesenteric adipose tissue-conditioned medium and incubated for 24 h with or without capsaicin (30–100 μM). The amount of MCP-1 or TNF-α released in culture medium was measured by ELISA. The amount of nitrite was determined using Griess reagent and the standard curve constructed using NaNO₂ in the culture medium. (A) MCP-1 protein release, (B) TNF-α protein release, and (C) nitric oxide release. Values are means ± S.E.M. Representative results of four independent experiments are shown. **P* < 0.05, significantly different from control.

3.5. Inhibitory effect of capsaicin on NF-κB activation in 3T3-L1 adipocytes induced by adipose tissue-conditioned medium or macrophage-conditioned medium

The inductions of the inflammatory gene, TNF-α, IL-6, and adiponectin are regulated by the transcription factor NF-κB. To examine whether capsaicin action is associated with the NF-κB pathway, we investigated whether capsaicin inhibits the DNA binding activity of NF-κB subunit p65 in adipocytes treated with a macrophage-conditioned medium or an adipose tissue-conditioned medium. As shown in Fig. 6, the DNA binding activity of NF-κB subunit p65 in adipocyte nuclear protein extract, which was markedly increased by the macrophage-conditioned medium and adipose tissue-conditioned medium, significantly decreased in the adipocytes treated with capsaicin, indicating that capsaicin suppresses the DNA binding activity of NF-κB.

3.6. Effects of capsaicin on expression of adipokine genes and macrophage markers from mesenteric adipose tissue of obese mice

We further examined whether capsaicin *in vivo* modulates adipokines production and suppresses macrophage migration

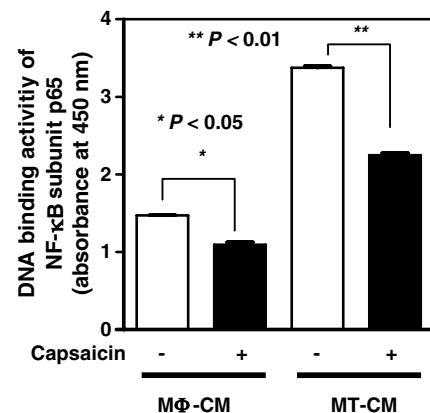


Fig. 6. Inhibitory effect of capsaicin on NF-κB activation induced by adipose tissue-conditioned medium in 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated with and without macrophage-conditioned medium (Mφ-CM), and adipose tissue-conditioned medium and incubated for 18 h with and without capsaicin. Nuclear proteins were extracted from 3T3-L1 adipocytes. NF-κB activation in adipocyte nuclear extract was determined using p65 TransAM assay as described in Section 2. **P* < 0.05, ***P* < 0.01, significantly different from control.

into adipose tissue. C57BL/6 mice (male, 8 weeks) were fed a high-fat diet for 6 weeks, and treated with capsaicin (*i.p.* injection, 2 mg/kg, BW) for 10 days. Our result revealed that the expression levels of MCP-1 and IL-6 decreased in the adipose tissue of the obese mice treated with capsaicin compared with those of the control (Fig. 8A and B). Consistent with this, the expression level of cyclooxygenase-2 (COX-2), which increases under inflammatory conditions, significantly decreased in the adipose tissue of capsaicin-treated obese mice compared with that of the control (Fig. 8C). The expression level of adiponectin, anti-inflammatory adipokine, markedly increased in the adipose tissue of capsaicin-treated obese mice compared with that of the control (Fig. 8D). Moreover, FACS revealed that macrophage population (F4/80+/CD11b+) significantly decreased in the adipose tissue of the obese mice treated with capsaicin compared with that of the control (Fig. 9). These

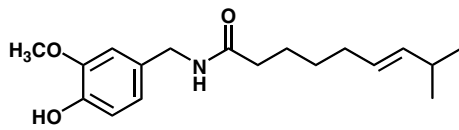


Fig. 7. Chemical structure of capsaicin (8-methyl-*N*-vanillyl-6-nonenamide).

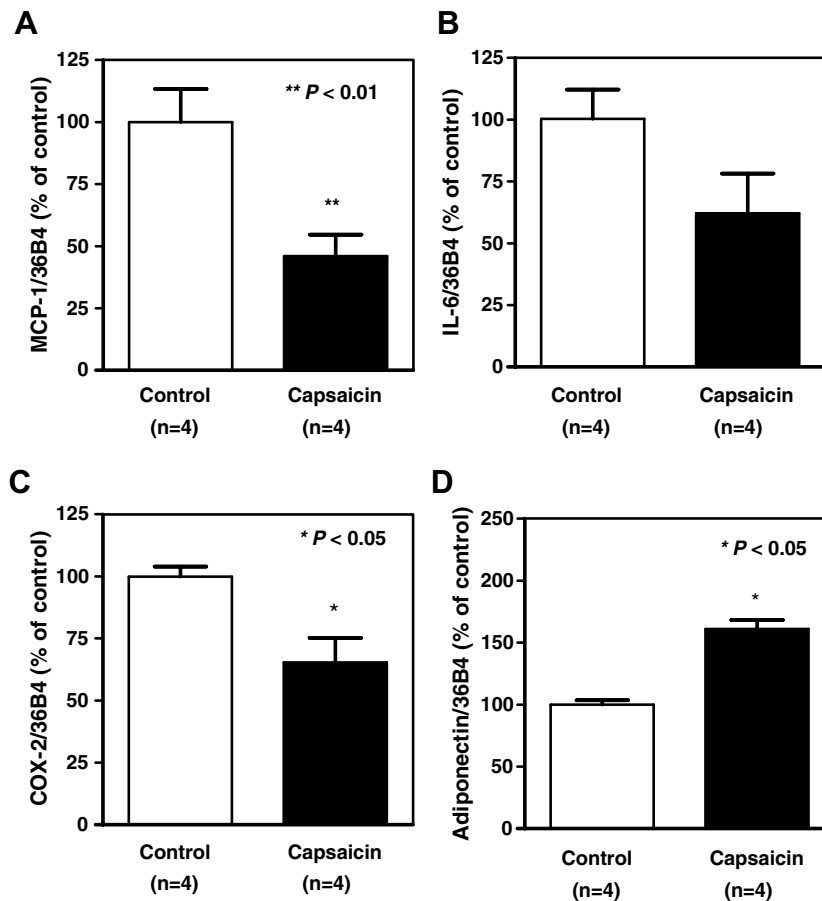


Fig. 8. Effects of capsaicin on expressions of MCP-1, IL-6, COX-2, and adiponectin genes in adipose tissue of obese mice. Adipose tissue was isolated from the obese mice injected with ($n = 4$) or without ($n = 4$) capsaicin solution (2 mg/kg, BW). The expression levels of MCP-1 (A), IL-6 (B), COX-2 (C), and adiponectin (D) genes were measured by RT-PCR. The intensity of the bands was densitometrically measured and normalized to the mRNA expression levels of the 36B4 gene. Values are means \pm S.E.M. * $P < 0.05$, ** $P < 0.01$, significantly different from control.

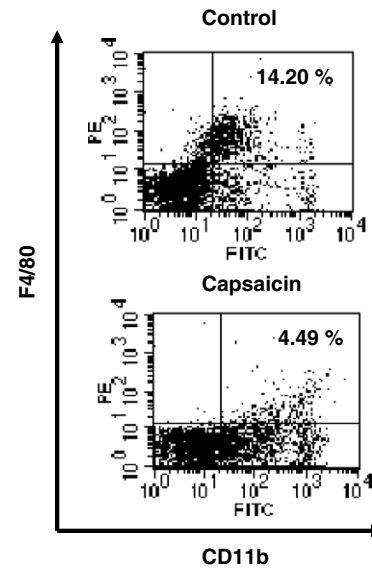


Fig. 9. Decreased macrophage numbers in adipose tissue of obese mice treated with capsaicin. Stromal vascular fraction was isolated from the visceral adipose tissue of the obese mice injected with ($n = 4$) or without ($n = 4$) capsaicin solution (2 mg/kg, BW). The levels of CD11b and F4/80 were measured by FACS as described in Section 2.

results indicate that capsaicin can ameliorate the obesity-induced inflammatory phenotypes by modulating adipokine releases and inhibiting macrophage infiltration into adipose tissue.

4. Discussion

This study demonstrated that capsaicin, a naturally occurring anti-inflammatory phytochemical, can improve the dysregulation of adipokine production from and macrophage behavior in obese adipose tissues.

Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) consists of three main sections, a lipophilic alkyl chain at one end, connected via an acyl-amide linkage to the vanillyl group bearing the polar hydroxyl group at the other end of the molecule (Fig. 7) [33]. Capsaicin has been shown to bind to specific vanilloid receptor-1 (VR-1) on the membrane of sensory neurons, leading to activation of a cation channel and inducing depolarization and Ca^{2+} influx [33]. However, in non-neuronal cells such as macrophages, capsaicin action can occur through NF- κ B pathway not by a VR-1-mediated one [29].

The dysregulation of adipokine release, which causes obesity-induced inflammation, is the common denominator that links obesity to the pathogenesis of insulin resistance and atherosclerosis [10]. Among adipokines, we targeted IL-6, MCP-1, and adiponectin, which are implicated in obesity-related pathologies such as type II diabetes and atherosclerosis [34]. Interestingly, capsaicin decreased the levels of IL-6 and MCP-1 and increased the level of adiponectin released from obese fat tissues and fat cells. This dual action of capsaicin may be favorable for improving the dysregulation of adipokine release through the normalization of the balance between the secretions of proinflammatory and anti-inflammatory adipokines in insulin resistance or atherosclerosis.

The expression levels of macrophage specific markers such as CD68 and F4/80 are upregulated in genetically and diet-induced obese mice [19]. Therefore, adipose tissue macrophages are considered to be crucial for obesity-induced inflammation. Proinflammatory adipokines such as IL-6 and MCP-1 also activate macrophages [13]. We have previously shown that adipose tissue-derived MCP-1 induces macrophage accumulation into the adipose tissues of obese mice and stimulates macrophages to produce proinflammatory mediators [20]. Our current data demonstrated that capsaicin significantly inhibits the macrophage migration induced by MCP-1 and the mesenteric adipose tissue-conditioned medium, and that capsaicin also significantly suppresses macrophage activation to produce inflammatory mediators such as nitric oxide, TNF- α , and MCP-1 induced by the obese-mouse mesenteric adipose tissue-conditioned medium. These findings suggest that capsaicin has potential to suppress the inflammatory response amplified by adipose tissue macrophages in obesity.

Several lines of evidence suggest that ligands for PPAR γ such as thiazolidinediones (TZDs), which are used as antidiabetic drugs, suppress TNF- α , IL-6, and MCP-1 gene expressions and protein release, and enhance adiponectin release [35]. Previously, we showed that capsaicin can act as a PPAR γ ligand, resulting in the inhibition of TNF- α production by LPS-stimulated macrophages [36]. We observed that PPAR γ ligand, troglitazone, significantly inhibited MCP-1 and IL-6 production, and enhanced adiponectin production from adipocytes. Therefore, the dual action of suppressing IL-6 and

MCP-1 releases and enhancing adiponectin release from obese adipose tissues and adipocytes may be due to the ligand action of capsaicin for PPAR γ . It has also been shown that PPAR γ ligands inhibit monocyte/macrophage chemotaxis and activation [36,37], indicating that the inhibitory action of capsaicin on macrophage behavior in our observation is also associated with its ligand action for PPAR γ .

The molecular events that involve inflammation include the activation of proinflammatory transcription factor NF- κ B, besides PPAR γ . Troglitazone, a PPAR γ ligand, has been shown to suppress intranuclear NF- κ B activation and increase cellular I κ B level in leukocytes [38]. We previously showed that capsaicin inhibits the NF- κ B pathway by inhibiting I κ B degradation in LPS-stimulated macrophages, leading to the suppression of the release of proinflammatory mediators [29]. In this study, we confirmed that NF- κ B in 3T3-L1 adipocytes could be activated by a mesenteric adipose tissue-derived conditioned medium or a macrophage-derived conditioned medium, and that NF- κ B activation in these adipocytes is significantly inhibited by capsaicin. These findings taken together strongly suggest that PPAR γ and NF- κ B are involved in the inhibitory action of capsaicin on the release of inflammatory adipokines such as IL-6 and MCP-1.

We further investigated whether capsaicin can be applied to the suppression of obesity-induced inflammatory responses in vivo. It has been shown that intraperitoneal injection of capsaicin (6 mg/kg, BW) in vivo alters lipid energy metabolism [31] and dietary capsaicin supplementation (0.014%) lowers the perirenal adipose tissue weight and levels of serum triglyceride in obese rats [39]. Our data demonstrated that intraperitoneal injection of capsaicin (2 mg/kg, BW) significantly decreased the expression levels of MCP-1, IL-6, and COX-2 genes in the adipose tissue of obese mice, but increased the expression level of adiponectin gene. Moreover, there was a significant decrease in macrophage infiltration into the adipose tissue of the obese mice. These results indicate that the beneficial dual action of capsaicin in vitro can be relevant in vivo.

In summary, capsaicin suppressed the expressions of the IL-6 and MCP-1 genes and protein release from the obese adipose tissues and isolated adipocytes, whereas it enhanced the expressions of the adiponectin gene and the protein release. Moreover, capsaicin inhibited macrophage migration induced by the obese mesenteric adipose tissue-conditioned medium or MCP-1, and inhibits macrophage activation to release proinflammatory mediators. Our findings suggest that capsaicin suppresses obesity-induced inflammation by modulating adipokines release. Thus, capsaicin may be a useful phytochemical for attenuating obesity-induced inflammation and obesity-related pathologies.

Acknowledgements: This work was supported by a grant from Korean Science and Engineering Foundation (KOSEF R01-2005-000-10408-0). TK was supported by the Research and Development Program for New Bio-industry Initiatives and Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sport, Science and Technology of Japan (15081205 and 19380074).

References

- [1] Matsuzawa, Y., Funahashi, T., Kihara, S. and Shimomura, I. (2004) Adiponectin and metabolic syndrome. *Arterioscler. Thromb. Vasc. Biol.* 24, 29–33.

- [2] Hausman, D.B., DiGirolamo, M., Bartness, T.J., Hausman, G.J. and Martin, R.J. (2001) The biology of white adipocyte proliferation. *Obes. Rev.* 2, 239–254.
- [3] Aldhahi, W. and Hamdy, O. (2003) Adipokines, inflammation, and the endothelium in diabetes. *Curr. Diab. Rep.* 3, 293–298.
- [4] Fontana, L., Eagon, J.C., Trujillo, M.E., Scherer, P.E. and Klein, S. (2007) Visceral fat adipokine secretion is associated with systemic inflammation in obese humans. *Diabetes* 56, 1010–1013.
- [5] Weisberg, S.P., Weisberg, S.P., Hunter, D., Huber, R., Lemieux, J., Slaymaker, S., Vaddi, K., Charo, I., Leibel, R.L. and Ferrante, A.W. (2006) CCR2 modulates inflammatory and metabolic effects of high-fat feeding. *J. Clin. Invest.* 116, 115–124.
- [6] Ahima, R.S. (2006) Metabolic actions of adipocyte hormones: focus on adiponectin. *Obesity (Silver Spring)* 14 (Suppl. 1), 9S–15S.
- [7] Kern, P.A. (1997) Potential role of TNF α and lipoprotein lipase as candidate genes for obesity. *J. Nutr.* 127, 1917S–1922S.
- [8] Vozarova, B., Weyer, C., Hanson, K., Tataranni, P.A., Bogardus, C. and Pratley, R.E. (2001) Circulating interleukin-6 in relation to adiposity, insulin action, and insulin secretion. *Obes. Res.* 9, 414–417.
- [9] Shimomura, I., Funahashi, T., Takahashi, M., Maeda, K., Kotani, K., Nakamura, T., Yamashita, S., Miura, M., Fukuda, Y., Takemura, K., Tokunaga, K. and Matsuzawa, Y. (1996) Enhanced expression of PAI-1 in visceral fat: possible contributor to vascular disease in obesity. *Nat. Med.* 2, 800–803.
- [10] Shoelson, S.E., Lee, J. and Goldfine, A.B. (2006) Inflammation and insulin resistance. *J. Clin. Invest.* 116, 1793–1801.
- [11] Hotamisligil, G.S., Shargill, N.S. and Spiegelman, B.M. (1993) Adipose expression of tumor necrosis factor- α : direct role in obesity-linked insulin resistance. *Science* 259, 87–91.
- [12] Feinstein, R., Kanety, H., Papa, M.Z., Lunenfeld, B. and Karasik, A. (1993) Tumor necrosis factor- α suppresses insulin-induced tyrosine phosphorylation of insulin receptor and its substrates. *J. Biol. Chem.* 268, 26055–26058.
- [13] Hotamisligil, G.S. (2003) Inflammatory pathways and insulin action. *Int. J. Obes. Relat. Metab. Disord.* 27 (Suppl. 3), S53–S55.
- [14] Lagathu, C., Bastard, J.P., Auclair, M., Maachi, M., Capeau, J. and Caron, M. (2003) Chronic interleukin-6 (IL-6) treatment increased IL-6 secretion and induced insulin resistance in adipocyte: prevention by rosiglitazone. *Biochem. Biophys. Res. Commun.* 311, 372–379.
- [15] Sartipy, P. and Loskutoff, D.J. (2003) Monocyte chemoattractant protein 1 in obesity and insulin resistance. *Proc. Natl. Acad. Sci. USA* 100, 7265–7270.
- [16] Gu, L., Okada, Y., Clinton, S.K., Gerard, C., Sukhova, G.K., Libby, P. and Rollins, B.J. (1998) Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. *Mol. Cell* 2, 275–281.
- [17] Gosling, J., Slaymaker, S., Gu, L., Tseng, S., Zlot, C.H., Young, S.G., Rollins, B.J. and Charo, I.F. (1999) MCP-1 deficiency reduces susceptibility to atherosclerosis in mice that overexpress human apolipoprotein B. *J. Clin. Invest.* 103, 773–778.
- [18] Kanda, H., Tateya, S., Tamori, Y., Kotani, K., Hiasa, K., Kitazawa, R., Kitazawa, S., Miyachi, H., Maeda, S., Egashira, K. and Kasuga, M. (2006) MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J. Clin. Invest.* 116, 1494–1505.
- [19] Weisberg, S.P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R.L. and Ferrante Jr., A.W. (2003) Obesity is associated with macrophage accumulation in adipose tissue. *J. Clin. Invest.* 112, 1796–1808.
- [20] Yu, R., Kim, C.S., Kwon, B.S. and Kawada, T. (2006) Mesenteric adipose tissue-derived monocyte chemoattractant protein-1 plays a crucial role in adipose tissue macrophage migration and activation in obese mice. *Obesity (Silver Spring)* 14, 1353–1362.
- [21] Kadowaki, T., Yamauchi, T., Kubota, N., Hara, K., Ueki, K. and Tobe, K. (2006) Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. *J. Clin. Invest.* 116, 1784–1792.
- [22] Ouchi, N., Kihara, S., Funahashi, T., Matsuzawa, Y. and Walsh, K. (2003) Obesity, adiponectin and vascular inflammatory disease. *Curr. Opin. Lipidol.* 14, 561–566.
- [23] Treves, R. (1995) Low-dose glucocorticoid therapy in rheumatoid arthritis. *Rev. Rhum. Engl. Ed.* 62, 611–617.
- [24] Habtemariam, S. (2001) Antiinflammatory activity of the anti-rheumatic herbal drug, gravel root (*Eupatorium purpureum*): further biological activities and constituents. *Phytother. Res.* 15, 687–690.
- [25] Gescher, A. (2004) Polyphenolic phytochemicals versus non-steroidal anti-inflammatory drugs: which are better cancer chemopreventive agents? *J. Chemother.* 16 (Suppl. 4), 3–6.
- [26] Ahmed, M., Bjurholm, A., Srinivasan, G.R., Lundeberg, T., Theodorsson, E., Schultzberg, M. and Kreicbergs, A. (1995) Capsaicin effects on substance P and CGRP in rat adjuvant arthritis. *Regul. Pept.* 55, 85–102.
- [27] Marsh, D., Dickenson, A., Hatch, D. and Fitzgerald, M. (1999) Epidural opioid analgesia in infant rats II: responses to carrageenan and capsaicin. *Pain* 82, 33–38.
- [28] Park, J.S., Choi, M.A., Kim, B.S., Han, I.S., Kurata, T. and Yu, R. (2000) Capsaicin protects against ethanol-induced oxidative injury in the gastric mucosa of rats. *Life Sci.* 67, 3087–3093.
- [29] Kim, C.S., Kawada, T., Kim, B.S., Han, I.S., Choe, S.Y., Kurata, T. and Yu, R. (2003) Capsaicin exhibits anti-inflammatory property by inhibiting I κ B- α degradation in LPS-stimulated peritoneal macrophages. *Cell. Signal.* 15, 299–306.
- [30] Granger, D.L., Taintor, R.R., Boockvar, K.S. and Hibbs Jr., J.B. (1996) Measurement of nitrate and nitrite in biological samples using nitrate reductase and Griess reaction. *Methods Enzymol.* 268, 142–151.
- [31] Kawada, T., Watanabe, T., Takaishi, T., Tanaka, T. and Iwai, K. (1986) Capsaicin-induced beta-adrenergic action on energy metabolism in rats: influence of capsaicin on oxygen consumption, the respiratory quotient, and substrate utilization. *Proc. Soc. Exp. Biol. Med.* 183, 250–256.
- [32] Bruun, J.M., Lihn, A.S., Pedersen, S.B. and Richelsen, B. (2005) Monocyte chemoattractant protein-1 release is higher in visceral than subcutaneous human adipose tissue (AT): implication of macrophages resident in the AT. *J. Clin. Endocrinol. Metab.* 90, 2282–2289.
- [33] Szallasi, A. and Blumberg, P.M. (1999) Vanilloid (Capsaicin) receptors and mechanisms. *Pharmacol. Rev.* 51, 159–212.
- [34] Fantuzzi, G. (2005) Adipose tissue, adipokines, and inflammation. *J. Allergy Clin. Immunol.* 115, 911–919, (quiz 920).
- [35] Ghanim, H., Dhindsa, S., Aljada, A., Chaudhuri, A., Viswanathan, P. and Dandona, P. (2006) Low-dose rosiglitazone exerts an antiinflammatory effect with an increase in adiponectin independently of free fatty acid fall and insulin sensitization in obese type 2 diabetics. *J. Clin. Endocrinol. Metab.* 91, 3553–3558.
- [36] Park, J.Y., Kawada, T., Han, I.S., Kim, B.S., Goto, T., Takahashi, N., Fushiki, T., Kurata, T. and Yu, R. (2004) Capsaicin inhibits the production of tumor necrosis factor alpha by LPS-stimulated murine macrophages, RAW 264.7: a PPAR-gamma ligand-like action as a novel mechanism. *FEBS Lett.* 572, 266–270.
- [37] Tanaka, T., Masuzaki, H., Ebihara, K., Ogawa, Y., Yasue, S., Yukioka, H., Chusho, H., Miyanaga, F., Miyazawa, T., Fujimoto, M., Kusakabe, T., Kobayashi, N., Hayashi, T., Hosoda, K. and Nakao, K. (2005) Transgenic expression of mutant peroxisome proliferator-activated receptor gamma in liver precipitates fasting-induced steatosis but protects against high-fat diet-induced steatosis in mice. *Metabolism* 54, 1490–1498.
- [38] Ghanim, H., Garg, R., Aljada, A., Mohanty, P., Kumbkarni, Y., Assian, E., Hamouda, W. and Dandona, P. (2001) Suppression of nuclear factor-kappaB and stimulation of inhibitor kappaB by troglitazone: evidence for an anti-inflammatory effect and a potential antiatherosclerotic effect in the obese. *J. Clin. Endocrinol. Metab.* 86, 1306–1312.
- [39] Kawada, T., Hagihara, K. and Iwai, K. (1986) Effects of capsaicin on lipid metabolism in rats fed a high fat diet. *J. Nutr.* 116, 1272–1278.