

Capsaicin inhibits the production of tumor necrosis factor α by LPS-stimulated murine macrophages, RAW 264.7: a PPAR γ ligand-like action as a novel mechanism

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Received 29 September 2003; revised 28 April 2004; accepted 24 June 2004

Available online 14 July 2004

Edited by Veli-Pekka Lehto

Abstract Capsaicin, a major ingredient of hot pepper, is considered to exhibit anti-inflammatory properties. Our previous study demonstrated that capsaicin inhibited the production of pro-inflammatory mediators through NF- κ B inactivation in LPS-stimulated macrophages. In order to further clarify the mechanism underlying the anti-inflammatory action of capsaicin, we investigated whether capsaicin alters PPAR γ activity, which regulates the production of the pro-inflammatory cytokine TNF α . Capsaicin significantly inhibited the production of TNF α by macrophages in a dose-dependent manner. Simultaneous exposure of the cells to capsaicin and PPAR γ agonist troglitazone or RXR agonist LG100268 resulted in stronger inhibition of TNF α production compared to the cells treated with either capsaicin, troglitazone, or LG100268 alone. Luciferase reporter assay revealed that capsaicin induced GAL4/PPAR γ chimera and full length PPAR γ (PPRE) transactivations in a dose-dependent manner. Furthermore, a specific PPAR γ antagonist T0070907 abrogated the inhibitory action of capsaicin on LPS-induced TNF α production by RAW 264.7 cells, indicating that capsaicin acts like a ligand for PPAR γ . Our data demonstrate for the first time that the anti-inflammatory action of capsaicin may be mediated by PPAR γ activation in LPS-stimulated RAW 264.7 cells.

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Keywords: Capsaicin; Inflammation; Macrophage; RAW 264.7; PPAR γ ; Pro-inflammatory cytokine; TNF α

1. Introduction

Inflammation is a pivotal component of a variety of diseases such as atherosclerosis and tumor progression. Various naturally occurring phytochemicals (e.g., curcumin and flavonoids) exhibit anti-inflammatory activity and are considered to be potential drug candidates against the inflammation-related pathological processes [1]. Capsaicin, the major ingredient of hot pepper, and with a structure similar to that of curcumin, has also been shown to elicit anti-inflammatory properties

[1,2]. Our previous study demonstrated that capsaicin inhibited the production of pro-inflammatory mediators such as PGE₂ and nitric oxide by nuclear transcription factor kappa B (NF- κ B) inactivation in murine peritoneal macrophages [3]. Interestingly, the inhibitory action of capsaicin on the release of pro-inflammatory molecules was not mediated by a vanilloid receptor-1 (VR-1) [3], which is a specific receptor for capsaicin, indicating the involvement of an alternative mechanism.

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily and three subtypes have been identified (PPAR, PPAR δ , and PPAR γ). PPAR γ forms a heterodimer with retinoid X receptor (RXR) and regulates the expression of target genes by binding to the PPAR responsive element (PPRE) [4,5]. It is well known that PPAR γ plays a pivotal role in adipogenesis and fatty acid metabolism [6,7]. Recent studies have shown that PPAR γ modulates the production of certain inflammatory mediators and thus can be implicated in the pathogenesis of inflammatory diseases. Troglitazone or pioglitazone, a synthetic ligand for PPAR γ , suppresses the production of pro-inflammatory cytokines such as TNF α and nitric oxide in LPS-stimulated macrophages [8,9]. Naturally occurring compounds such as fatty acids and the prostaglandin D₂ metabolite 15-deoxy- Δ 12,14-prostaglandin J₂ (15d-PGJ₂), which are major products of arachidonic acid metabolism, are also known to activate PPAR γ and thus regulate inflammatory responses in macrophage [10,11]. The nuclear receptor RXR exerts its gene regulatory activity either as a homodimer (RXR:RXR) or heterodimer with other nuclear receptors such as PPARs [12,13]. The RXR ligands Ro47-5944 or LG100268 inhibited LPS-induced nitric oxide and TNF α production in rat kupffer cells through PPAR γ :RXR activation [8]. These results suggest that ligands for PPAR γ or RXR may exhibit anti-inflammatory properties through the activation of those transcription factors.

Non-steroidal anti-inflammatory drugs such as aspirin, which can suppress the production of pro-inflammatory cytokines (e.g., TNF α and IL-6), are known to activate PPAR γ [10,14,15]. Since target genes for PPAR γ include TNF α as well as iNOS or COX-2, we hypothesized that capsaicin might modulate the PPAR γ pathway. The present study demonstrates for the first time that capsaicin suppresses the

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production of $\text{TNF}\alpha$ by acting as an agonist for $\text{PPAR}\gamma$ in LPS-stimulated murine macrophages RAW 264.7. Capsaicin may be a naturally occurring ligand for $\text{PPAR}\gamma$, which can be useful therapy against inflammation.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle medium (DMEM) and fetal calf serum (FCS) were from Gibco BRL, and lipopolysaccharide (LPS) was from Sigma. Capsaicin was purchased from TOCRIS. Troglitazone and pioglitazone were purchased from Sankyo Co. and Takeda Chemical Industries, respectively. T0070907 was purchased Cayman. LG100268 was synthesized by a method previously described [16]. OptEIATM mouse $\text{TNF}\alpha$ kit was from Pharmingen. Anti-rabbit IgG antibody and Western blotting detection reagent (ECL) were from Amersham Pharmacia Biotech. Anti- $\text{PPAR}\gamma$ antibody was from ABR.

2.2. Cells culture

The murine macrophage cell line, RAW 264.7, was obtained from Korean Cell Line Bank and CV-1 monkey kidney cells were from American Type Culture Collection. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS and 10 mg/l penicillin-streptomycin (Gibco BRL), 2 mg/l gentamicin (Gibco BRL) at 37 °C, 5% CO_2 . The media were changed every 3 days and the cells were separated via trypsinization, using trypsin/EDTA (Gibco BRL).

2.3. Western blot analysis

RAW 264.7 macrophages were cultured in 100-mm dishes and rinsed three times with ice-cold phosphate-buffered saline (PBS), lysed with 100 μl of Tris (25 mM)/EDTA (1 mM) buffer (pH 7.5), and then microcentrifuged at 15000 rpm for 5 min. Protein content of extracts was quantitated by BCA protein kit (Pierce) and stored at -80°C . Equal amounts of protein (30 μg) were loaded and electrophoresed on a 11% sodium dodecyl sulfate-polyacrylamide gels for electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (BIO-RAD). The membrane was blocked in TBS with 5% skimmed milk for 3 h and was treated overnight with a rabbit polyclonal anti- $\text{PPAR}\gamma$ antibody in TBS with 1% skimmed milk (1:1000). After washing, the membrane was incubated for 2 h with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Amersham) in TBS with 5% skimmed milk (1:1000). The immunoreactive protein was detected with a chemiluminescent system (ECL kit, Amersham).

2.4. RT-PCR analysis

Total RNA was extracted from 2×10^7 cells using an RNeasy kit (Qiagen). An aliquot of 0.5 μg of RNA was used for reverse transcription and was amplified using a polymerase chain reaction (PCR) technique in a single reaction, using the Access RT-PCR system according to the manufacturer's instructions in a TaKaRa Thermal cycler (TaKaRa, Biomedicals).

The following sets of primers were used in PCR amplification: β -actin (Gene Bank Accession No. X03672), forward 5'-ATGAAGATCCTG-ACCGAGCGT-3', reverse 5'-AACGCAGCTCAGTAACAGTCCG-3'; VR-1 (AF029310), forward 5'-GTGAGACCCCTAACCGTCATGA-3', reverse 5'-CCTT CCACAGGCCGATAGTA-3'; VRL-1 (BC005415), forward 5'-CAAGTACCTCACTGACTCGGCATAC-3', reverse 5'-TTCTCTACCAAGcagttcagca-3'; and $\text{PPAR}\gamma$ (U01841), forward 5'-GGAGATCTCCAGTGATATCGACCA-3', reverse 5'-ACGGCTTCTACGGATCGA AACT-3'. The conditions of PCR for the target genes were as previously described [3,17].

For semiquantitative analysis, the linearity of amplification of VR-1 and $\text{PPAR}\gamma$ and β -actin cDNAs was established in preliminary experiments. Amplification products obtained by PCR were electrophoretically separated on a 2% agarose gel. SYBR Green-stained bands corresponding to the target genes and β -actin were photographed with a DS-34 Polaroid camera.

2.5. Measurement of $\text{TNF}\alpha$

The concentration of $\text{TNF}\alpha$ in culture supernatants was assessed by enzyme-linked immunosorbent assay (ELISA). The assays were con-

ducted utilizing the OptEIATM Mouse $\text{TNF}\alpha$ kit (Pharmingen). The sample (triplicate) was thawed, diluted properly in assay diluent, and assayed. The absorbance of each well was read at 450 nm on a Vmax Kinetic Microplate Reader (Molecular Devices). Cytokine was quantitated from standard curve using the SOFTmax curve-fitting program (Molecular Devices).

2.6. Luciferase reporter assays

$\text{PPAR}\gamma$ luciferase assay was performed by the method previously described [7,17]. Briefly, using GAL4/ PPAR chimera protein, we transfected p4xUASg-tk-luc (a reporter plasmid) and pM- $\text{PPAR}\gamma$ (an expression plasmid for GAL4/ PPAR -ligand binding domain chimera protein) into CV1 cells cultured on 24-well tissue culture plates. In the case of a PPARE system, p4xPPRE-tk-luc (0.2 μg /well) and pDEST-h $\text{PPAR}\gamma$ (full-length PPAR expression vectors) (0.2 μg /well) were also transfected into CV1 cells cultured on 24-well tissue culture plates. The transfection was performed by LipofectAMINE (Invitrogen, USA) using the manufacturer's protocol. Twenty four hours after transfection, the transfected cells were cultured in medium containing each compound for an additional 24 h. Luciferase assays were performed using the dual luciferase system according to the manufacturer's protocol.

2.7. Cell viability

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay was used to assess the viability of RAW 264.7 cell after treatment [3]. Cell viabilities were determined colorimetrically by using an automated 96-well plate reader (Molecular Devices) and SOFTmax software to measure absorbance at 570 nm.

2.8. Statistical analysis

The data were presented as means \pm S.E.M. and were statistically analyzed using ANOVA and the unpaired *t*-test. Differences were considered significant when *P* was <0.05 .

3. Results

3.1. Expression of VR-1, VRL-1, or $\text{PPAR}\gamma$ in murine macrophages, RAW 264.7

We examined whether VR-1 or VR-1-like protein (VRL-1) mRNA was expressed in RAW 264.7 by RT-PCR analysis. The specific expression of RT-PCR products of VR-1 mRNA in RAW 264.7 was not detected, although the products of VR-1 were clearly expressed in rat and mouse brain (positive controls) (Fig. 1A). VRL-1 (non-sensitive to capsaicin) mRNA was expressed (Fig. 1B). It has been shown that VR-1 is mainly localized at the plasma membrane in VR-1 transfected cells [2]. We confirmed that VR-1 protein was also not expressed in RAW 264.7 cells (Fig. 1C), while it was detected in human keratinocyte (positive control). The expression pattern was similar to peritoneal macrophage observed in our previous study [3]. We also examined the expression of $\text{PPAR}\gamma$ mRNA and the protein in RAW 264.7 and 3T3-L1 (positive control) by RT-PCR and Western blot analysis. We found that RAW 264.7 macrophage highly expressed $\text{PPAR}\gamma$ mRNA and protein (Fig. 1D and E).

3.2. Effects of capsaicin and $\text{PPAR}\gamma$ agonist on LPS-induced $\text{TNF}\alpha$ production by murine macrophages, RAW 264.7

To examine whether capsaicin inhibits $\text{TNF}\alpha$ production, murine macrophages (RAW 264.7) were stimulated with LPS in the presence or absence of capsaicin. Either capsaicin or $\text{PPAR}\gamma$ agonists at the concentrations used in this study did not affect cell viability (data not shown). Capsaicin significantly inhibited LPS-induced $\text{TNF}\alpha$ production by RAW 264.7 cells in a dose-dependent manner (Fig. 2). $\text{PPAR}\gamma$

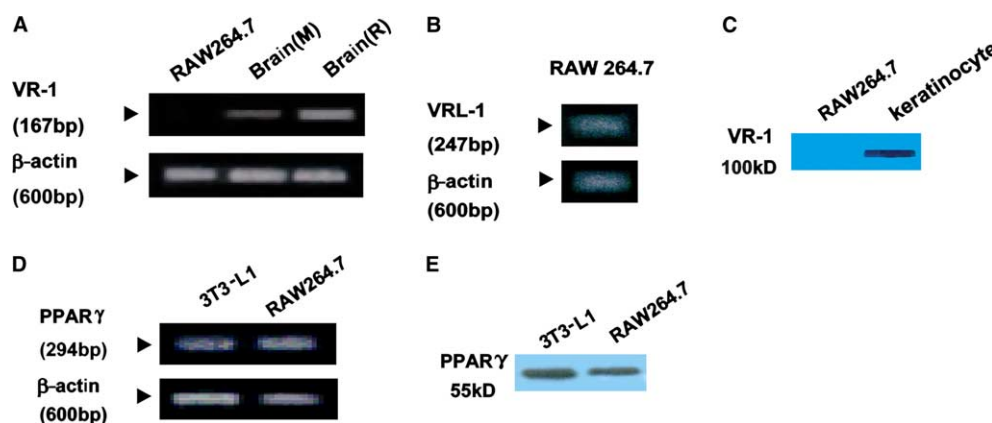


Fig. 1. Expression of VR-1 or PPAR γ in murine macrophages, RAW 264.7. The expression of VR-1 (A), VRL-1 (B), and PPAR γ (D) mRNA was determined by RT-PCR. Total RNA was extracted from murine macrophages RAW 264.7 used for RT-PCR analysis as described in Section 2. R, rat; M, mouse. The expression of VR-1 (C) and PPAR γ (E) protein in RAW 264.7 was determined by Western blot analysis. Total protein extracts were prepared from cultured RAW 264.7 or 3T3-L1 cells. Protein extracts (30 μ g) were analyzed by 11% SDS-PAGE and Western blotting with antibody against PPAR γ protein. Results are representatives of two separate experiments.

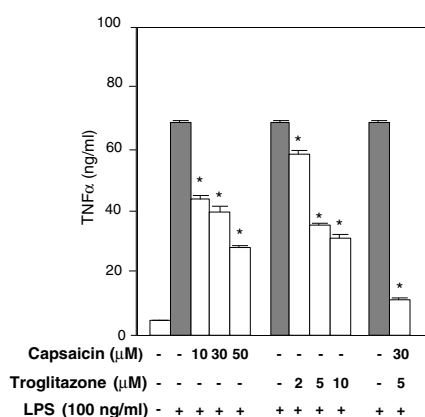


Fig. 2. Effect of capsaicin and PPAR γ specific agonist on LPS-stimulated TNF α production by RAW 264.7 cells. Murine macrophages, RAW 264.7 (1×10^6 /well), were pretreated with various concentrations of capsaicin or PPAR γ agonist (troglitazone) for 4 h at 37 $^{\circ}$ C. The cells were stimulated with LPS (100 ng/ml) and incubated for 16 h. The levels of TNF α in the culture medium were measured by ELISA. The values are means \pm S.E.M. of three separate experiments. (A) Capsaicin and/or troglitazone treatment. *Significantly different from the levels of TNF α production in RAW 264.7 cells treated with LPS alone, $P < 0.01$.

agonists (troglitazone) also inhibited TNF α production by the macrophage, as previously reported [8]. Capsaicin at a concentration of 10, 30, and 50 μ M inhibited TNF α production by 36%, 42%, and 59% in LPS-stimulated RAW 264.7 cells, while troglitazone at a concentration of 2, 5, and 10 μ M inhibited LPS-stimulated TNF α production by 15%, 48%, and 54%, respectively. Simultaneous treatment with both capsaicin (30 μ M) and troglitazone (5 μ M) resulted in greater inhibition (83%) on TNF α production when it is compared to the single treatment of capsaicin (42%) or troglitazone (48%) alone at the same concentration (Fig. 2). Combined treatment with both capsaicin and pioglitazone also resulted in greater inhibition on LPS-induced TNF α production when it was compared to either capsaicin or pioglitazone alone (data not shown).

3.3. Effects of capsaicin and RXR agonist on LPS-induced TNF α production by murine macrophages, RAW 264.7

The nuclear receptor PPAR γ can exert its transcriptional activity as a heterodimer with RXR or heterodimer with other nuclear receptors [11]. To examine whether capsaicin can act as a ligand for PPAR γ , we investigated the effect of capsaicin on LPS-stimulated TNF α by RAW 264.7 in the presence of the synthetic RXR agonist. RXR agonist (LG100268) at a concentration of 2 nM did not affect LPS-induced TNF α and LG100268 at a concentration of 5 nM resulted in an inhibition of TNF α production by 12% in RAW 264.7 cells (Fig. 3). However, simultaneous treatment with both 30 μ M capsaicin and 2 nM LG100268 markedly inhibited the LPS-induced TNF α production by 94%.

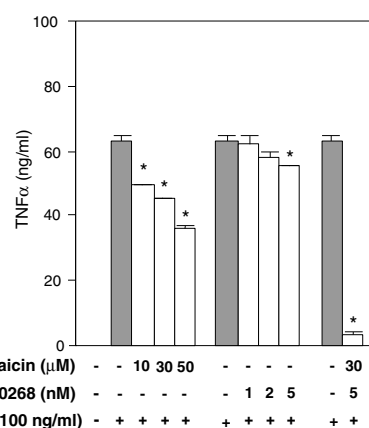


Fig. 3. Effect of capsaicin and RXR specific agonist on LPS-stimulated TNF α production by murine macrophages, RAW 264.7. Murine macrophages, RAW 264.7 (1×10^6 /well), were pretreated with various concentrations of capsaicin or RXR agonist (LG100268), for 4 h at 37 $^{\circ}$ C. The cells were stimulated with LPS (100 ng/ml) and incubated for 16 h. The levels of TNF α in the culture medium were measured by ELISA. The values are means \pm S.E.M. of three separate experiments. *Significantly different from the levels of TNF α production in RAW 264.7 cells treated with LPS alone, $P < 0.01$.

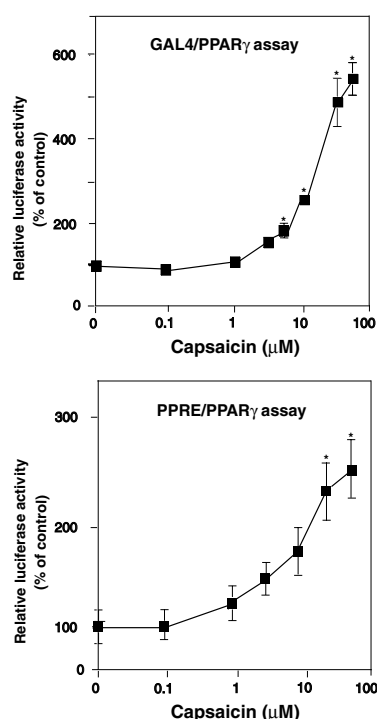


Fig. 4. Effect of capsaicin on the activation of PPAR γ or PPRE binding in luciferase assays. CV-1 cells are transfected with (A) p4xUASg-tk-luc (a reporter plasmid) and pM-PPAR γ (an expression plasmid for GAL4/PPAR-ligand binding domain chimera protein), (B) p4xPPRE-tk-luc and pDEST-hPPAR γ (full-length PPAR expression vectors) in the presence of LipofectAMINE. Twenty four hours after transfection, the transfected cells were cultured in medium containing capsaicin for additional 24 h. The activity of a vehicle control was set at 100% and the relative luciferase activities were presented as fold induction to that of the vehicle control. The values are means \pm S.E.M. of four separate experiments. * $P < 0.05$ compared with vehicle controls.

3.4. Capsaicin activates PPAR γ in luciferase assays

The effect of capsaicin on TNF α release by RAW 264.7 indicated that capsaicin might have potential acting as a PPAR γ ligand. To support the evidence that capsaicin can alter activity of PPAR γ , we performed luciferase ligand assays using the PPAR γ /GAL4 chimera system for capsaicin. Interestingly, we found that capsaicin activated GAL4/PPAR γ chimera transactivations in a dose-dependent manner (Fig. 4A). Moreover, we examined whether capsaicin activated full length PPAR γ (PPRE) using p4xPPRE-tk-luciferase reporter plasmid. As shown in Fig. 4B, capsaicin activated PPRE-dependent luciferase activities in a dose-dependent manner in human PPAR γ -transfected CV1 cells. Our data clearly indicate that capsaicin can act as a PPAR γ ligand.

3.5. Capsaicin action is abrogated by a specific PPAR γ antagonist

To obtain direct evidence on the PPAR γ ligand action of capsaicin, we tested whether a PPAR γ antagonist blocked capsaicin action on TNF α release by LPS-stimulated macrophage. A specific PPAR γ antagonist, T0070907, abrogated the inhibitory action of capsaicin on LPS-stimulated TNF α production in Raw 264.7 cells. The result indicates that capsaicin acts as PPAR γ ligand (Fig. 5).

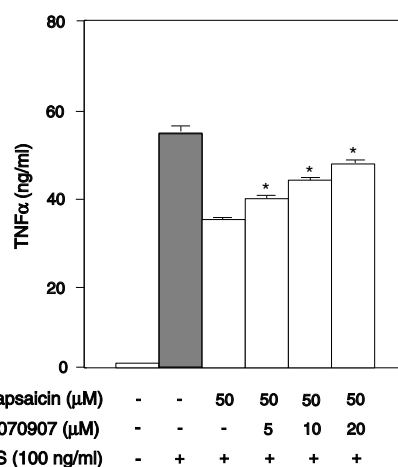


Fig. 5. Effect of PPAR γ antagonist on the inhibitory action of capsaicin on LPS-stimulated TNF α production by murine macrophages, RAW 264.7. Murine macrophages, RAW 264.7 (1×10^6 /well) were pretreated with various concentrations of capsaicin or PPAR γ antagonist (T0070907), for 4 h at 37 °C. The cells were stimulated with LPS (100 ng/ml) and incubated for 16 h. The levels of TNF α in the culture medium were measured by ELISA. Each bar represents the mean \pm S.E.M. of triplicate determinations. *Significantly different from the levels of TNF α production in RAW 264.7 cells treated with capsaicin alone, $P < 0.01$.

4. Discussion

This study demonstrated that capsaicin suppresses pro-inflammatory cytokine TNF α production in LPS-stimulated RAW 264.7 cells and that the inhibitory action of capsaicin was associated with PPAR γ activation. Capsaicin may be a naturally occurring ligand for PPAR γ , which could be useful for ameliorating inflammatory diseases.

Anti-inflammatory activity of capsaicin in non-neuronal cells is known to occur via vanilloid receptors-independent manner [3,18], indicating that capsaicin action is associated with an alternative signaling pathway. Studies have shown that PPAR γ ligand exhibits anti-inflammatory properties [8,9]. Troglitazone or pioglitazone, a synthetic ligand for PPAR γ , is known to inhibit LPS-induced TNF α production in rat kupffer cells [8] and PMA-induced TNF α production in monocytes [9]. In the present study, we found that RAW 264.7 did not express VR-1, but expressed PPAR γ . Indeed, the production of TNF α by LPS-stimulated RAW 264.7 macrophages was inhibited by capsaicin treatment. The results suggest that the inhibitory action of capsaicin on the TNF α release may be associated with PPAR γ , but not VR-1 pathway in RAW 264.7 cells. We also found that simultaneous treatment of capsaicin and the PPAR γ agonist, troglitazone or pioglitazone, resulted in greater inhibitory effect on TNF α production compared to their single treatment. The result indicates that capsaicin has a potential to exhibit an agonistic activity for PPAR γ activation in a similar manner to troglitazone or pioglitazone.

Recent studies have shown that inflammatory cytokine (e.g., TNF α and IL-1) treatment suppresses the expression of target genes for PPAR γ through NFB-mediated inhibition of the binding of PPRE by PPAR γ /RXR α heterodimers [19]. Ruan et al. [20] have demonstrated that troglitazone inhibits transcriptional regulatory function of NF- κ B, leading to

antagonize TNF α -induced adipocyte gene expression, suggesting that PPAR activation may modulate NF- κ B pathway. Previously, we revealed that capsaicin inhibited NF- κ B activation in LPS-stimulated murine peritoneal macrophages, leading to the reduction of pro-inflammatory mediators (e.g., COX-2, PGE₂, iNOS, and NO) [3]. Combining this and our findings in the present study, the inhibition of NF- κ B activation by capsaicin may be associated with PPAR γ activation. When we examined whether capsaicin could be a ligand for PPAR γ using luciferase report assay, we found that capsaicin induced GAL4/PPAR γ chimera and full length PPAR γ (PPRE) transactivations in a dose-dependent manner. Furthermore, using a PPAR γ antagonistic ligand T0070907, we demonstrated that PPAR γ antagonist abrogated the inhibitory action of capsaicin on TNF α production in RAW 264.7 cells. These results clearly reveal that capsaicin can act as a ligand for PPAR γ , indicating that the anti-inflammatory action of capsaicin is mediated by PPAR γ pathway.

Interestingly, the RXR agonist LG100268 inhibited LPS-induced TNF α production, and combined treatment with capsaicin markedly enhanced the inhibitory effect on TNF α production. RXR forms heterodimers with PPAR γ , and this type of heterodimer can be activated by the binding of the respective ligand to either RXR or PPAR γ . Studies have shown the enhanced or synergistic effects of PPAR γ ligands in combination with RXR ligands, resulting in either activation or suppression of a specific gene [21–24]. Recently, Berger et al. [8,25] have revealed that PPAR γ forms a functional heterodimer with RXR and occupancy of both ligand binding domains is required for maximal receptor activity. Therefore, the enhanced inhibitory effect of capsaicin with LG100268 observed in our study supports the idea that capsaicin acts as a PPAR γ ligand in LPS-stimulated RAW 264.7 macrophages.

In conclusion, capsaicin inhibited the production of pro-inflammatory cytokine TNF α by LPS-stimulated RAW 264.7 in a dose-dependent manner. Simultaneous exposure of the cells to capsaicin and PPAR γ agonist troglitazone or RXR agonist LG100268 resulted in stronger inhibition compared to the cells treated with either capsaicin, troglitazone, or LG100268 alone. Luciferase assay revealed that capsaicin induced GAL4/PPAR γ chimera and full length PPAR γ (PPRE) transactivations in a dose-dependent manner. Moreover, PPAR γ antagonist T0070907 abrogated the inhibitory action of capsaicin on LPS-induced TNF α production by RAW 264.7 cells. Taken together, our data indicate that the inhibitory action of capsaicin against TNF α production in LPS-stimulated RAW 264.7 cells is mediated by PPAR γ activation. The PPAR γ ligand-like action of capsaicin may be important to understand a novel mechanism in the anti-inflammatory action of capsaicin.

Acknowledgements: This work was supported by grants of KOSEF 98-0402-04-01-3 and the Ministry of Health & Welfare and partially by University of Ulsan, Korea. T. Kawada was supported by a grant-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (16380087, 15081205).

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