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# Thermo-Sensitive Transient Receptor Potential Vanilloid (TRPV) Channels Regulate IL-6 Expression in Mouse Adipocytes

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## Abstract

**Background:** Hyperthermia may affect adipose cell functions and then adipogenesis. Thermo-sensitive transient receptor potential (TRPs) proteins such as TRPV1-4 play vital roles in cellular functions. We investigated the expression of thermo-sensitive TRPVs and their roles on IL-6 production in mouse 3T3-L1 preadipocytes and differentiated adipocytes.

**Methods:** The conventional and quantitative real-time RT-PCR analysis, western blots, fluorescence activating cell sorting, immunocytochemical staining and [Ca2+]i measurements using fura-2 AM were performed.

**Results:** The conventional and real-time RT-PCR analysis showed the existence of TRPV1, 2 and 4 expressions. Western blotting and immunocytochemical staining also showed the existence of TRPV1, TRPV2 and TRPV4 transcript in 3T3-L1 preadipocytes and differentiated adipocytes. Capsaicin, a TRPV1 agonist, probenecid, a TRPV2 agonist, and GSK1016790A, a TRPV4 agonist, and 2-aminoethoxydiphenyl borate (2-APB), a TRPV1-3 selective agonist, increased [Ca2+]i. These changes were also elicited by a rise in extracellular temperature from 25°C to over 42°C. Treatment with capsaicin, GSK1016790A and probenecid for 24 h increased IL-6 expression, and secretion in differentiated adipocytes. Warm temperature from 25°C to over 42°C also induced IL-6 protein expression.

**Conclusions:** The present study shows that adipose cell possess multiple thermo-sensitive TRPVs (TRPV1, TRPV2, and TRPV4), which may play vital roles in IL-6 production. Thus, thermo-sensitive TRPVs appear to be novel target molecules for promoting IL-6 secretion in adipose tissues.

**Keywords:** 3T3-L1 adipocyte; Transient receptor potential vanilloid (TRPV) channels; Temperature-sensitive channel; IL-6

## Abbreviations

TRP: Transient Receptor Potential; TRPV: Transient Receptor Potential Vanilloid; IL-6: Interleukin-6

## Introduction

Obesity exacts a continuing toll on public health and is a well-known risk factor for diabetes, hypertension, hyperlipidemia, and cardiovascular diseases [1-4]. It is evident that not only hypertrophy of adipocytes and increase of fat deposit but also proliferation of pre-adipocytes and differentiation from pre-adipocytes to adipocytes have important roles for forming obesity. The most intensive and clinical interventions to prevent obesity have been primarily directed to decrease excessive amounts of fat tissue by a change in the balance between intake and expenditure of energy using daily exercise and diet control. On the other hand, the inverse correlation between body mass index (BMI) and the average temperature of January exists, and BMI is low when the temperature is high [5]. In addition, when rats were fed under low or high temperature environment, the amount of subcutaneous fat in rats fed under high temperature was significantly low, compared with that under the low temperature [5]. Thus, hyperthermia may affect adipose cell functions, then adipogenesis, and provides a therapeutic or preventive modality against obesity-related diseases. However, there are few reports concerning the direct effects of hyperthermia stimulation on adipocytes and its underlying mechanism.

The transient receptor potential (TRP) ion channels are important membrane sensors, responding to thermal, chemical, osmotic, or mechanical stimuli by activation of calcium and sodium fluxes. Until now, the mammalian TRP family consists of 28 unique channels, in 6 main subfamilies [6]. Recent studies demonstrated that several members of transient receptor potential vanilloid (TRPV), melastatin (TRPM), and ankyrin (TRPA) subfamilies act as the thermal sensation receptors, responding to moderate or noxious changes in the external temperature. These channels are called heat or cold receptors, depending on the temperature range required for their activation. Heat receptors include TRPV3 and TRPV4, activated by warm temperature (34–38 and 27–35°C, respectively) and noxious heat receptors TRPV1 and TRPV2, with a thermal activation threshold as high as 43 and 52°C, respectively [6-13]. The activation temperatures for two cold receptors, TRPA1 and TRPM8, are 17 and 25–28°C, respectively [6,10,12-19].

It has been reported that heat-sensitive channels are expressed in pre-adipocytes and adipose cells, which regulate adipose function under the physiological and pathophysiological conditions such as obesity. In

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3T3-L1 pre-adipocytes, TRPV1, which is activated by capsaicin, has been reported to inhibit the induction of differentiation into adipocytes from adipose precursor cells [20]. On the other hand, Motter and Ahern [21] reported an opposite result showing that obesity by high fat diet is inhibited in a TRPV1 knockout mouse. Recently, Ye et al. [22] reported that TRPV4 is involved in pre-adipocyte differentiation, inflammation in fat cells and energy metabolism. Furthermore, pre-adipocytes and adipocytes contribute to the obesity-associated increase in circulating interleukin-6 (IL-6) [23]. IL-6, a multifunctional cytokine, is produced by many tissues including adipose tissue. It is now well known that IL-6 production by adipose tissue is enhanced in obesity [24,25], and plays a role in the link between obesity, inflammation and coronary heart diseases [26], and insulin resistance[25,27]. Recently, Ye et al. [22] showed that TRPV4 is involved in adipose function such as IL-6 secretion. And, it has been reported that outcomes of TRPV1 activation in human corneal epithelial cells include enhanced release of IL-6 [28-31], where intracellular $Ca^{2+}$ rises and phosphorylation of kinases belonging to the p38, extracellular regulated kinase (ERK)1/2 and c-jun terminal kinase (JNK)1/2 mitogen-activated protein kinase (MAPK) cascades are involved. Similar results are reported in other cells [32,33]. Thus, there is a possibility that TRPV channel as a temperature sensor is involved in IL-6 secretion in the adipocytes. However, the molecular identities of thermo-sensitive TRPVs and their function such as IL-6 production in adipocytes have not been clarified.

Here, we investigated the molecular identities of thermo-sensitive TRPVs and their roles on IL-6 production in 3T3-L1 preadipocytes and differentiated adipocytes. The present study shows that adipose cell possess multiple thermo-sensitive TRPVs (TRPV1, TRPV2, and TRPV4), which may play vital roles in IL-6 production. Thus, thermo-sensitive TRPVs appear to be novel target molecules for promoting IL-6 secretion in adipose tissues.

## Methods

### Cell culture of 3T3-L1 pre-adipocytes and pre-adipocyte differentiation method

3T3-L1 preadipocytes were obtained from the JCRB Bank (Osaka, Japan) as previously described [34,35]. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) containing 100 μg/ml penicillin and 100 μg/ml streptomycin. Cells were plated and grown until 2 days post-confluence, and then induced differentiation into adipocytes by switching culture medium to DMEM containing 0.5 mM methylisobutylxanthine, 0.25 μM dexamethasone, and 10 μg/ml insulin for 48 h [36]. Using this protocol, >95% of the cells were differentiated into the adipocyte phenotype 3-4 days after initiating differentiation. The differentiated 3T3-L1 adipocytes were switched to fresh DMEM containing 10% FBS.

### Measurement of intracellular $Ca^{2+}$ concentration $[Ca^{2+}]i$ and drugs

Cytosolic free $Ca^{2+}$concentration ($[Ca^{2+}]i$) was determined using the fluorescence method as described previously [37,38]. 3T3-L1 preadipocytes were trypsinized, washed twice in the standard solution, adjusted to a cell density of 106 cells ml−1 and loaded with 2 μM fura-2 AM for 30 min at 37˚C under 5% $CO_2$. After incubation, the medium containing fura-2 AM was removed, and fluorescent cells in suspensions were measured at 37°C while stirred continuously in a cuvette placed by a spectrofluorometer (CAF-100, JASCO Co, Ltd., Tokyo, Japan). The excitation wavelengths were 340 and 380 nm, and emission was 500 nm. In the evaluation of $Ca^{2+}$ responses, the amplitude of $Ca^{2+}$ elevation in response to each stimulant was calculated by the increase of F340/F380 with reference to the value at the resting state.

2-aminoethoxydiphenylborate (2-APB) were obtained from Sigma, and dissolved in DMSO. Fura-2 acetoxymethyl ester (fura-2 AM), capsaicin and probenecid were purchased from SIGMA-ALDRICH (Poole, UK). GSK1016790A (Santa Cruz Biotechnology) was dissolved in DMSO.

### Immunocytochemistry

Immunocytochemistry was performed on 3T3-L1 preadipocytes using the rabbit polyclonal anti-TRPV1, TRPV2, and TRPV4 channel antibodies (Alomone labs, Israel). The cells were cultured on collagen-type 1-coated chamber slide (Becton, Dickinson, NJ), fixed with 4% paraformaldehyde in PBS in 30 min, and then incubated with 1% NP-40 (Nacalai tesque, Kyoto, Japan) in PBS in 15 min at room temperature for membrane permeabilization, then blocked for 30 min with 3% horse serum in PBS. The cells were incubated in a humid chamber overnight at 4˚C with primary antibodies diluted with 1% horse serum in PBS into 1:200. For negative controls, cells were treated with normal rabbit IgG (Vector laboratories, Burlingame, CA) as a substitute for primary antibodies. Alexafluor488 labeled donkey anti-rabbit IgG (H+L) antibody (Life technologies, CA) diluted with 1% horse serum in PBS into 1:1000 was used to visualize the channel expression. A confocal laser scanning microscopy (TCS SL, Leica Microsystems, Wetzlar, Germany) was used for observations.

### RNA extraction, reverse transcriptase/polymerase chain reaction (RT-PCR) and real-time quantitative RT-PCR

Total cellular RNA was extracted from 3T3-L1 pre-adipocytes and differentiated adipocytes, by using the RNeasy mini kit (Qiagen, Cambridge, MA). For RT-PCR, complementary DNA (cDNA) was synthesized from 1 μg of total RNA with reverse transcriptase with random primers (Toyobo, Osaka). The reaction mixture was then subjected to PCR amplification with specific forward and reverse oligonucleotide primers for 35 cycles consisting of heat denaturation, annealing, and extension. PCR products were size-fractionated on 2% agarose gels, and visualized under UV light. Primers were chosen based on the sequence of mouse TRPV1, TRPV2, TRPV3 and TRPV4 as shown in Table 1.

Real-time quantitative RT-PCR was performed with the use of real-time Taq-Man technology and a sequence detector (ABI PRISM® 7000, Applied Biosystems, Foster City, CA) [39]. Gene-specific primers and Taq-Man probes were used to analyze transcript abundance. The 18S ribosomal RNA level was analyzed as an internal control and used to normalize the values for transcript abundance of TRPV family genes. The probes used in this study were purchased as Assay-on-Demand from Applied Biosystems (Foster City, CA) as follows: TRPV1, Mm01246302_m1; TRPV2, Mm00449223_m1; TRPV3, Mm00455003_m1; TRPV4, Mm00499025_m1; 18S rRNA, 4310893E.

| Channel (Gene symbol) | Size (bp) | | Sequence (`5-`3 ) |
|---|---|---|---|
| **TRPV1** | 153 | sense | AGGGTGGATGAGGTGAACTG |
| | | antisense | AACCAGGGCAAAGTTCTTCC |
| **TRPV2** | 253 | sense | GGATGGT ATACCTGATGAGC |
| | | antisense | GCAAAATTCCCTACTCTACCCTGC |
| **TRPV3** | 240 | sense | ACGGTGGAGAACGTCTCC |
| | | antisense | TGTCCGTCTTATGGGTCC |
| **TRPV4** | 478 | sense | GGAGACCTGGAGATGCTGAG |
| | | antisense | CATCTGCGCTTGAGTTCTTG |

**Table 1:** PCR primers used for amplification of TRPV channel genes.

## Western blotting

Protein lysates were prepared in lysing buffer (1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate in PBS) containing protease inhibitor cocktail (Nacalai tesque). The total protein concentration was determined using BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL). And then, these proteins were separated on 10% (for TRPVs) or 15% (for IL-6) polyacrylamide gel for 50 min at 200 V, and then transferred to Amersham Hybond-P (GE Healthcare UK Ltd., Buckinghamshire, UK) for 60 min at 72 mA with a semi-dry method. After the transfer, the membrane was blocked with Blocking One (Nacalai tesque) at room temperature for 30 min. The membrane was exposed to anti-TRPV1, TRPV2, TRPV4 antibodies (Alomone) into 1:800, and the rabbit polyclonal anti-IL-6 antibody (Abcam, Cambridge, United Kingdom) into 1:1000, and mouse monoclonal anti-beta actin antibody (Sigma-Aldrich, ST. Louis, MO) into 1:10000 in 5% Blocking One in TBS-T (137 mM NaCl, 20 mM Tris, 0.1% Tween20), overnight at 4°C. The probed membrane was washed three times in TBS-T for 15 min each time, and incubated with anti-rabbit IgG or anti-mouse IgG linked to peroxidase (Santa Cruz Biotechnology, Inc., CA) diluted to 1:5000 with 5% Blocking One in TBS-T for 1 h at room temperature. After three additional washes, bound antibodies were detected by Chemi-Lumi One Super (Nacalai tesque) and analyzed with LAS-3000 mini image analyzer (Fuji-Film, Tokyo, Japan).

## Oil red O staining

Differentiated 3T3-L1 adipocytes were rinsed in PBS prior to fixing with 10% formaldehyde. Approximately 15 min after the fixation, Oil-Red O stain was used to stain for lipid accumulation (20 min). After rinsing with the distilled water, photomicrographs were taken with a digital camera to document staining. *Flow cytometry analysis of IL-6.* For flow cytometry analysis using 3T3-L1 differentiated adipocytes, the cells were resuspended in 100 µl of 4% paraformaldehyde in PBS for 30 min. Then, 1 ml of the buffer (3% FBS in PBS) was added and spun for 5 min at 270 g. The cells were resuspended in 100 µl buffer containing 0.1% Tween20 for 15 min for intracellular staining and incubated for 30 min at room temperature with a mixture of mouse monoclonal anti-IL-6 antibody (Abcam). Then, the cells were added with 1 ml buffer, spun at 270 g and incubated with secondary antibody (Alexa Fluor 488-conjugated donkey anti-mouse IgG (H + L) antibody (Molecular Probes Inc., Eugene, OR). Finally, the cells were again resuspended in the buffer. The flow cytometry was performed on a flow cytometer, Easy Cyte plus (Merck Millipore, Darmstadt, Germany).

## IL-6 protein detection using ELISA

To determine the effect of various drugs on the secretion of IL-6, we measured the levels of IL-6 in culture medium collected from differentiated 3T3-L1 adipocytes using ELISA kits (Legend Max Mouse IL-6, Bio Legend, San Diego, CA). The differentiated 3T3-L1 adipocytes were cultured in 24-well plate. After the differentiation, the adipocytes were bathed into DMEM solution without FBS for 24 h. And, then, after 24 h of culture with and without various drugs, the medium was collected and the levels of IL-6 were determined by ELISA assays.

## Data analysis

All values are expressed as means ± S.E.M. Differences between multiple groups were compared by ANOVA. Two-group analysis was performed with a Student *t*-test. Differences were considered significant if $P<0.05$.

# Results

## Expression of TRPV1, TRPV2, and TRPV4 mRNA in mouse 3T3-L1 pre-adipocytes and differentiated adipocytes

First, we investigated the expression of TRPV family member mRNA in mouse 3T3-L1 pre-adipocytes and differentiated adipocytes. The differentiated adipocytes showed lipid deposits stained with oil red (Figure 1A). Figure 1B shows the RT-PCR analysis of the transcripts of TRPV1-4 in mouse 3T3-L1 pre-adipocytes and differentiated adipocytes. Significant expression of TRPV1, TRPV2 and TRPV4 mRNA was detected in 3T3-L1 pre-adipocytes, and the expression of TRPV1, TRPV2, TRPV3 and TRPV4 mRNA was observed in 3T3-L1 differentiated adipocytes. The amplitude of cDNA fragments was of predicted molecular size, identical to cDNA fragments amplified from reversely transcripted mRNA. The expression of TRPV1-V4 mRNA members was also investigated by real-time quantitative RT-PCR analysis (Figure 1C). Transcript levels were normalized to 18S ribosomal housekeeping gene. In Figure 1C, significant expression of TRPV1, TRPV2, and TRPV4 mRNA was detected by real-time quantitative RT-PCR analysis in mouse 3T3-L1 pre-adipocytes and differentiated adipocytes. The expression level of TRPV1 mRNA significantly increased during the differentiation ($P<0.05$). On the other hand, the expression level of TRPV4 tended to decrease during the differentiation.

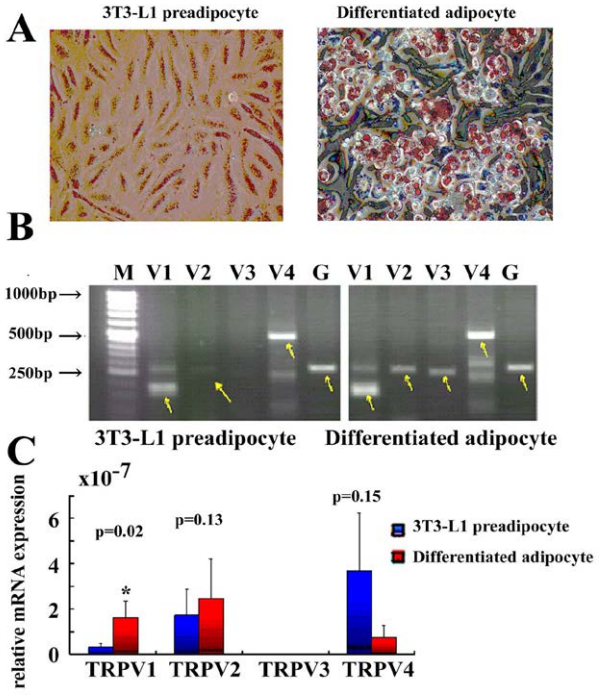


**Figure 1:** A: 3T3-L1 pre-adipocytes and differentiated adipocytes. The differentiated adipocytes were stained with Oil Red O staining. B: Expression of TRPV1-4 channel gene in 3T3-L1 pre-adipocytes and differentiated adipocytes. Marker (M), TRPV1-4 and GAPDH. Note that the expression of TRPV1, TRPV2, and TRPV4 mRNA was detected in 3T3-L1 pre-adipocytes, and the expression of TRPV1, TRPV2, TRPV3 and TRPV4 was observed in 3T3-L1 differentiated adipocytes. B: Quantitative real-time RT-PCR analysis of TRPV1, TRPV2, TRPV3, and TRPV4. The expression levels of TRPV channel genes were normalized to those of the 18S ribosomal RNA levels. Note that the prominent expression of TRPV1, TRPV2, and TRPV4 mRNA, but not TRPV3, was detected in 3T3-L1 pre-adipocytes and differentiated adipocytes. Each data represent the mean ± S.E (n=3).* $P<0.05$ vs. 3T3-L1 pre-adipocytes.

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## Expression of TRPV1, TRPV2, and TRPV4 protein in mouse 3T3-L1 pre-adipocytes

**(Immunocytochemistry and Western Blotting)**

To confirm TRPV1, TRPV2 and TRPV4 protein expression, western blot analysis was performed in 3T3-L1 pre-adipocytes as shown in Figure 2A. A specific antibody for TRPV1, TRPV2, and TRPV4 protein revealed a strong band. In addition, expression of TRPV1, TRPV2 and TRPV4 was confirmed by immunocytochemistry in mouse 3T3-L1 preadipocytes as shown in Figure 2B. No expression was detected in negative controls with normal rabbit IgG instead of a primary antibody (Figure 2B). Thus, these western blotting and immunocytochemical analysis showed the expression of TRPV1, TRPV2, and TRPV4 channel protein in mouse 3T3-L1 pre-adipocytes.

## Effects of warm temperature on $[Ca^{2+}]i$

To examine whether 3T3-L1 pre-adipocytes express functional thermo-sensitive (heat)-ion channels, the response of the cells to rapid changes in bath solution temperature was examined by continuous measurement of $[Ca^{2+}]i$. The cells were maintained in a bath solution chamber at various temperatures. The cells were exposed to rapid changes in bath solution temperatures, in ranges of heating to 20-50°C. As shown in Figure 3A, the bath temperature was increased from 37°C to 50°C, resulting in increasing $[Ca^{2+}]i$. And, after decreasing the bath temperature from 50°C to 16°C, the increased $[Ca^{2+}]i$ returned to the control level. In addition, temperature-dependent effects of heat on $[Ca^{2+}]i$ were investigated as shown in Figure 3B. The bath temperature was increased from 37°C to 50°C in stages, and returned back to 20°C. An increase of $[Ca^{2+}]i$ was caused by a stepwise elevation in bath temperature. The $[Ca^{2+}]i$ responses to various degrees of temperature were also compared as shown in Figure 4. The basal $Ca^{2+}$ level in the absence of extracellular $Ca^{2+}$ was similar, irrespective of temperature (20-50°C). On the other hand, when the bath temperature was increased from 37°C to 50°C in stages, $[Ca^{2+}]i$ increased in a temperature-dependent manner, proposing that heat stimulation induced $Ca^{2+}$ entry, but not $Ca^{2+}$ release in 3T3-L1 pre-adipocytes. In addition, as shown in Figures 4B and 4C, $[Ca^{2+}]i$ was increased at temperatures higher than 25°C and, it more increased above 40°C, compared with the temperature below 40°C. Thus, it is likely that different heat sensitive channels (25~40°C and >40°C) exist in 3T3-L1 pre-adipocytes.

## Effects of TRPV specific chemical activators and blockers on $[Ca^{2+}]$

Specific chemical activators and blockers were used to identify which specific thermo-sensitive TRP channels are functionally expressed on 3T3-L1 preadipocyte. Capsaicin (20 μM, Figure 5A), an agonist of TRPV1 noxious heat receptor [6,7,16], increased $[Ca^{2+}]$ i at concentrations (up to 10 μM). The activating effects of capsaicin were inhibited by the application of capsazepine (data not shown), an antagonist of TRPV1. Probenecid (1 μM, Figure 5B), a TRPV2 agonist [40-42], and GSK1016790A (200 nM), a TRPV4 agonist [43,44], also increased [Ca2+]i. Capsaicin (20 μM, A) and probenecid (1 μM, B and C) increased $[Ca^{2+}]i$, and the additional application of GSK1016790A (200 nM, A and C) further increased $[Ca^{2+}]i$ in 3T3-L1 preadipocytes. 2-APB (100-300 μM, Figure 5C), a TRPV1-3 selective agonist [45], significantly increased $[Ca^{2+}]i$. (3C). On the other hand, capsaicin, GSK1016790A, 2-APB (Figure 5D) and probenecid (data not shown) did not significantly increased $[Ca^{2+}]i$ in the absence of extracellular $Ca^{2+}$, suggesting that these agents mainly increased $[Ca^{2+}]i$ due to $Ca^{2+}$ entry, but not $Ca^{2+}$ release from intracellular store sites.

## Effects of heat on IL-6 production in 3T3-L1 differentiated adipocytes

As shown in Figure 6A, the fluorescence activating cell sorting (FACS) analysis showed the existence of IL-6 protein in 3T3-L1 differentiated adipocytes. The effects of heat stimulation on IL-6 protein expression were examined (Figure 6B). The cells were stimulated with heat (42°C) or control temperature (37°C) for 1 h, and the expression level of IL-6 protein was investigated. In addition, the

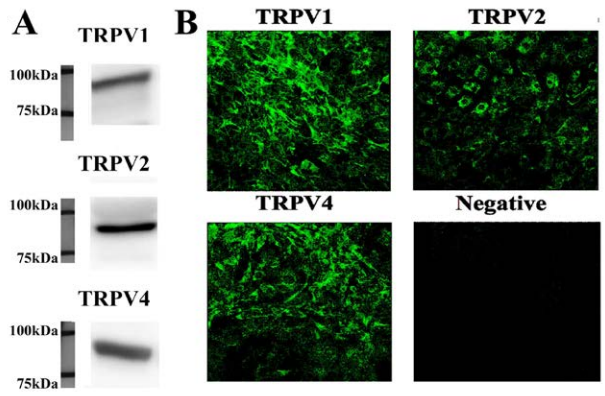


**Figure 2**: TRPV expression in 3T3-L1 pre-adipocytes. A: Western blotting for TRPV1, TRPV2, and TRPV4 in 3T3-L1 pre-adipocytes. B: Immunostaining for TRPV1, TRPV2, TRPV4, and negative control without the antibody. Note that 3T3-L1 pre-adipocytes have significant expression for TRPV1, TRPV2, and TRPV4 channel protein.

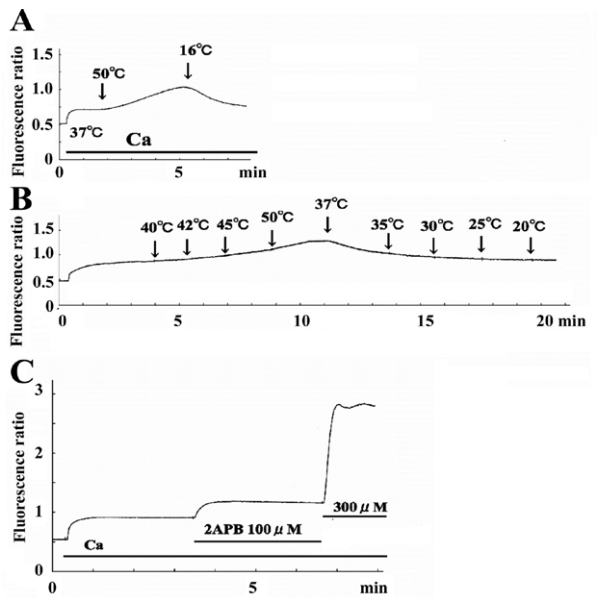


**Figure 3:** Effects of heat on $[Ca^{2+}]i$ in mouse 3T3-L1 pre-adipocytes. A: Effects of heat from 37°C to 50°C on $[Ca^{2+}]i$. The $Ca^{2+}$-free bathing solution contained 0.9 mM EGTA in the absence of $Ca^{2+}$ at 37°C, and 0.9 mM $Ca^{2+}$was added to the bath solution. The $[Ca^{2+}]i$ responses to various degree of temperature were compared, and the typical data are shown. In A, the bath temperature was increased from 37°C to 50°C, resulting in increasing $[Ca^{2+}]i$. And, after decreasing the bath temperature from 50°C to 16°C, the increased $[Ca^{2+}]i$ returned to the control level. B: Temperature-dependent effects of heat on $[Ca^{2+}]i$. The bath temperature was increased from 37°C to 50°C in stages, and returned back to 20°C. Note that an increase of $[Ca^{2+}]i$ is caused by a stepwise elevation in bath temperature. C: Effects of 2-aminoethoxydiphenyl borate (2-APB) on $[Ca^{2+}]i$ in 3T3-L1 pre-adipocytes. The typical data obtained from 4 different experiments were shown in this figure.

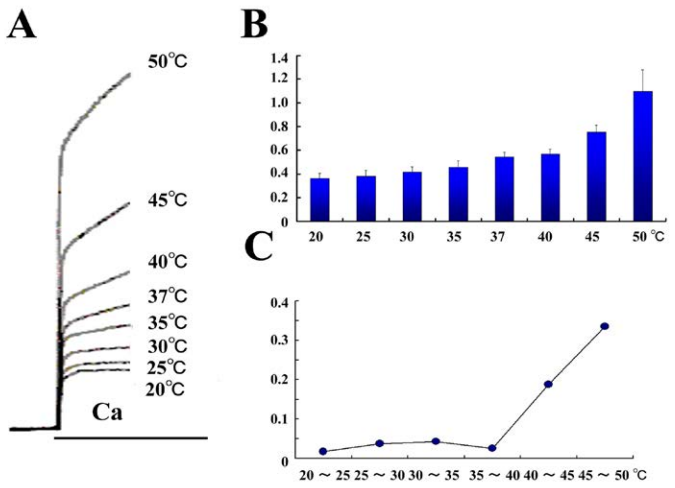


**Figure 4:** Effects of heat on $[Ca^{2+}]i$ in mouse 3T3-L1 pre-adipocytes. A: Temperature-dependent effects on $[Ca^{2+}]i$. The $Ca^{2+}$-free bathing solution contained 0.9 mM EGTA in the absence of $Ca^{2+}$ at various temperature (20-50°C). The $[Ca^{2+}]i$ responses to various degree of temperature were compared, and the typical data are shown in A. The basal $Ca^{2+}$ level in the absence of extracellular $Ca^{2+}$ was similar, irrespective of temperature (20-50°C). In B, the increased value in F340/F380 induced by the addition of $Ca^{2+}$ into the bath solution was plotted against each temperature. In C, the increased value in F340/F380 induced by the addition of $Ca^{2+}$ into the bath solution was plotted each time 5 degrees of temperature are raised.
Note that $[Ca^{2+}]i$ was increased at temperatures higher than 25°C and, it more increased above 40°C, compared with the temperature below 40°C. Each data represent the mean ± S.E of paired four different experiments.

cells were stimulated with heat (42°C) for 1 h, and then returned to a control temperature (37°C) for 6 h. As shown in Figure 6B, the rise in temperature to 42°C for 1 h increased IL-6 expression in 3T3-L1 differentiated adipocytes. The increased IL-6 protein was still observed after returning to a control temperature (37°C) for 6 h.

### Effects of capsaicin, GSK1016790A (GSK) and probenecid on IL-6 expression and secretion in mouse 3T3-L1 differentiated adipocytes

Figure 7 shows the effects of treatment with capsaicin, GSK1016790A and probenecid on IL-6 expression in differentiated adipocytes. The differentiated adipocytes were treated with various conditions (control, DMSO (0.1%), capsaicin (Cap, 200 μM), GSK1016790A (GSK, 2 μM), and probenecid (Prob, 1 μM) for 24 h, and the expression level of IL-6 was examined by western blotting analysis (Figure 7A). DMSO (0.1%) did not significantly affect the expression of IL-6. On the other hand, capsaicin (200 μM), GSK1016790A (2 μM) and probenecid (1 μM) significantly increased IL-6 expression. Therefore, we also investigated the effects of these agents on IL-6 secretion in 3T3-L1 differentiated adipocytes (Figure 7B). The cells were treated with capsaicin (Figure 7Ba) and probenecid (Figure 7Bb) in mouse 3T3-L1 differentiated adipocytes, and IL-6 secretion into the supernatant solution was measured by ELISA as shown in Figure 7B. Treatment with capsaicin (20 μM), and probenecid (1 μM) for 24 h increased IL-6 secretion into the supernatant solution in 3T3-L1 differentiated adipocytes.

## Discussion

The major findings of the present study are as follows. 1) In mouse 3T3-L1 pre-adipocytes and differentiated adipocytes, TRPV1, 2 and 4 expressions were confirmed by real-time RT-PCR. 2) The western blotting and immunocytochemical staining also showed the existence of TRPV1, TRPV2 and TRPV4 transcripts. 3) In fura 2-loaded cells, capsaicin, a TRPV1 agonist, probenecid, a TRPV2 agonist, and GSK1016790A, a TRPV4 agonist, and 2-aminoethoxydiphenyl borate (2-APB, 100-300 μM), a TRPV1-3 selective agonist, increased $[Ca^{2+}]i$. These changes were also elicited by rises in extracellular temperature from 25°C to over 42°C. 4) The rise in temperature to 42°C for 1 h increased IL-6 expression in 3T3-L1 differentiated adipocytes. 5) Treatment with capsaicin, GSK1016790A, and probenecid for 24 h increased IL-6 expression and secretion into the supernatant solution in 3T3-L1 differentiated adipocytes. These results suggest that adipose cell possess multiple thermo-sensitive TRPVs (TRPV1, TRPV2, and TRPV4), which may play vital roles in IL-6 production. Thus, thermo-sensitive TRPVs appear to be novel target molecules for promoting IL-6 secretion in adipose tissues. Several members of TRPV, TRPM, and TRPA subfamilies act as the thermal sensation receptors, responding to moderate or noxious changes in the external temperature. Heat receptors include TRPV3 and TRPV4, activated by warm temperature (34–38 and 27–35°C, respectively) and noxious heat receptors TRPV1 and TRPV2, with a thermal activation threshold as high as 43 and 52°C, respectively [6-13]. In the present study, we provided the evidence showing that the rises in extracellular temperature from 25°C to over 42°C increased $[Ca^{2+}]i$ in 3T3-L1 pre-adipocytes. The basal $Ca^{2+}$ level in the absence of extracellular $Ca^{2+}$ was similar, irrespective of temperature (20-50°C). On the other hand, when the bath temperature was increased from 37°C to 50°C in stages, $[Ca^{2+}]i$ increased in a temperature-dependent manner, proposing that heat stimulation induced $Ca^{2+}$ entry, but not $Ca^{2+}$ release in 3T3-L1 pre-adipocytes. And, $[Ca^{2+}]i$ was increased at temperatures higher than 25°C and, it more increased above 40°C, compared with the temperature below 40°C. Thus, it is likely that different heat sensitive channels (warm temperature 25~40C and heat temperature >40°C) exist in 3T3-L1 pre-adipocytes. The RT-PCR and immunoblot analyses revealed that TRPV1, TRPV2, and TRPV4 mRNA and protein are definitely expressed in mouse 3T3-L1 pre-adipocytes and differentiated adipocytes, which is compatible with the previous paper [46]. From these observations, it is likely that adipose cell possess multiple functional thermo-sensitive TRPVs, where TRPV4 and TRPV1/TRPV2 are involved.

Many of the thermoreceptor channels display significant ligand promiscuity and can be activated by additional modalities, such as hypotonicity and mechanical stretch (TRPV2 [47], TRPV4 [48,49]), extracellular acidification (TRPV1, TRPV4 [49]), chemical ligands (TRPV1, vanilloids and cannabinoids [50,51]; TRPV2, probenecid [40,41]; TRPV4, arachidonic acid metabolites [52] and GSK1016790A [44]). In the present study, we used fura-2 cytosolic free calcium concentration ($[Ca^{2+}]i$) measurement to demonstrate the direct involvement of TRPV channels in mouse 3T3-L1 pre-adipocyte calcium response elicited by the agonist stimulation. In fura-2-loaded cells, capsaicin, a TRPV1 agonist, probenecid, a TRPV2 agonist, and GSK1016790A, a TRPV4 agonist, increased $[Ca^{2+}]i$ in 3T3-L1 pre-adipocytes, suggesting that 3T3-L1 pre-adipcytes have functional TRPV1, TRPV2, and TRPV4. These changes were also elicited by rises in extracellular temperature from 25°C to over 42~50°C. Recently, Che et al. [53] have reported that TRPV2 and TRPV4, but not TRPV1, and TRPV3, were abundantly expressed in human pre-adipocytes, and a TRPV2 activator, probenecid, and a TRPV4 channel activator, 4α-phorbol 12-13-dicaprinate, enhanced the intracellular $Ca^{2+}$ signals, which suggest the functional expression of TRPV2 and TRPV4 in human pre-adipocytes. The functional existence of TRPV1 has also been described in 3T3-L1 pre-adipocytes [20]. Zhang et al. [20] reported that the expression of TRPV1 mRNA and protein in 3T3-L1 pre-adipocytes was observed, and capsaicin (1 μM) increased $[Ca^{2+}]i$.

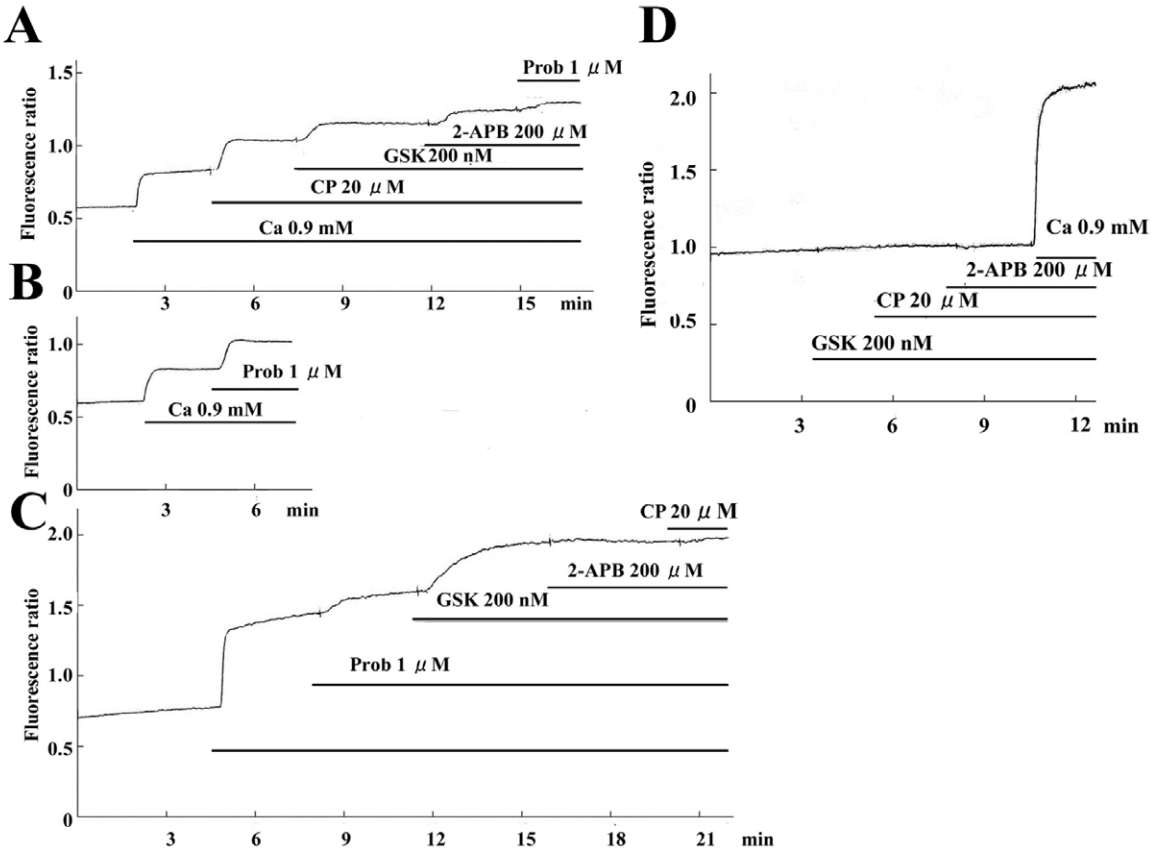


**Figure 5:** Effects of capsaicin, GSK1016790A and probenecid on $[Ca^{2+}]i$ in mouse 3T3-L1 preadipocytes. The $Ca^{2+}$-free bathing solution contained 0.9 mM EGTA in the absence of $Ca^{2+}$ at 37°C. In A-C, the $[Ca^{2+}]i$ responses to capsaicin (20 μM), GSK1016790A (200 nM), probenecid (1μM) and 2-APB (200 μM) were examined, and the typical data obtained from three different experiments are shown.
Note that capsaicin (20 μM, A) and probenecid (1 μM, B and C) increased $[Ca^{2+}]i$ (A), and the additional application of GSK1016790A (200 nM) further increased $[Ca^{2+}]i$ in 3T3-L1 pre-adipocytes. D: Effects of GSK1016790A, capsaicin and 2-APB on $[Ca^{2+}]i$ in the absence of $Ca^{2+}$ at 37°C.

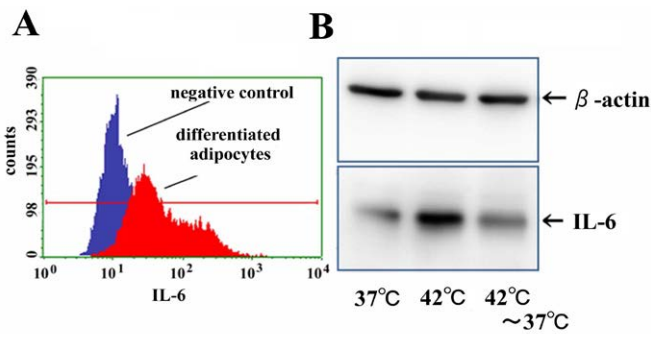


**Figure 6:** Effects of heat stimulation on IL-6 expression in 3T3-L1 differentiated adipocytes. A: FACS analysis of IL-6 in 3T3-L1 differentiated adipocytes. The staining of IL-6 in 3T3-L1 differentiated adipocytes is shown by red areas, and the negative control is indicated by blue areas. B: Effects of heat stimulation on IL-6 expression. The differentiated adipocytes were bathed into three different conditions, and the expression level of IL-6 was examined by western blotting analysis. The cells were stimulated with heat (42ºC) or control temperature (37ºC) for 1 h, and the expression level of IL-6 was compared with the internal control β-actin. In addition, the cells were stimulated with heat (42ºC), and then returned to a control temperature (37ºC) for 6 h. The similar results were obtained from three different experiments.

On the other hand, Motter and Ahern [21] have reported that capsaicin (10 μM) failed to increase $[Ca^{2+}]i$ in 3T3-L1 preadipocytes. The reason of these discrepancies remains unknown, but it may depend on the different cell conditions and different drug concentrations used in these studies. In the present study, capsaicin at concentrations higher than 10 μM increase $[Ca^{2+}]i$ in 3T3-L1 pre-adipocytes. The expression of TRPV1, TRPV2, and TRPV4 were also confirmed in native adipose tissues obtained from C57BL/6J male mice (unpublished results). Thus, it is likely that adipose cell possess multiple functional thermo-sensitive TRPVs (TRPV1, TRPV2, and TRPV4).

It has been well known that intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]i$) plays an essential role in cellular processes such as proliferation, differentiation and secretion in various cells. Oguri et al. [35] reported that the $Ca_V3.1$ T-type $Ca^{2+}$ channel encoded by α1G subtype is the dominant $Ca_V$ in mouse pre-adipocytes and may play a role in regulating pre-adipocyte proliferation, a key step in adipose tissue development. Involvement of TRPV2 and TRPM7 on pre-adipocyte proliferation has also been described in human pre-adipocytes and 3T3-L1 pre-adipocytes [53,54]. On the other hand, IL-6, a multifunctional cytokine, is produced by many tissues including adipose tissue. There are several papers showing the relations between TRPV and IL-6 secretion. Outcomes of TRPV1 activation in human corneal epithelial cells include enhanced release of IL-6 [28-31], where intracellular $Ca^{2+}$ rises and phosphorylation of MAPK cascades are involved. Similar

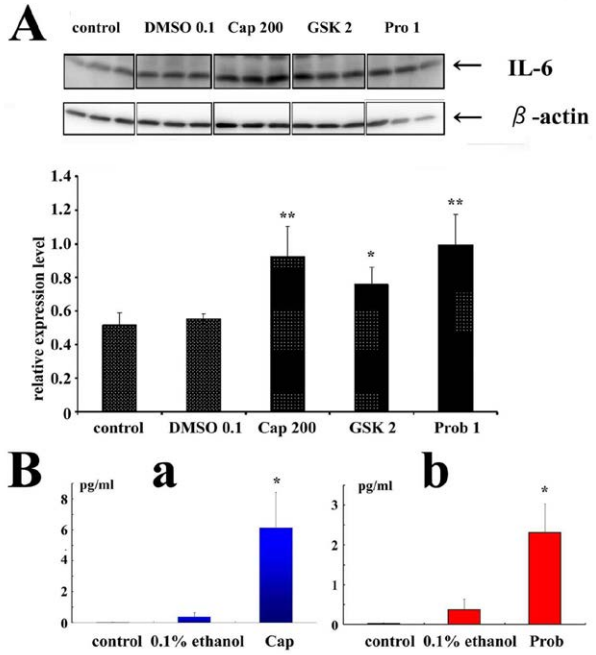


**Figure 7:** Effects of treatment with capsaicin, GSK1016790A, and probenecid on IL-6 expression in differentiated adipocytes. The differentiated adipocytes were treated with various conditions (control, DMSO (0.1%), 2ABP (20 μM), capsaicin (Cap, 200 μM), GSK1016790A (GSK, 2 μM), and probenecid (Prob, 1 μM) for 24h, and the expression level of IL-6 was examined by western blotting analysis. Note that capsaicin, GSK1016790A, and probenecid increased IL-6 expression in 3T3-L1 differentiated adipocytes. B: Effects of capsaicin and probenecid on IL-6 secretion in mouse 3T3-L1 differentiated adipocytes. The cells were treated with 0.1% ethanol, capsaicin (Cap, 20μM, a), and probenecid (Prob, 1 μM, b) in mouse 3T3-L1 differentiated adipocytes, and IL-6 secretion into the supernatant solution was measured by ELISA. Data were obtained from three different experiments. *p<0.05 vs. control

results are reported in other cells [33]. Also, the lipopolysaccharide (LPS)-induced transient increase in $[Ca^{2+}]i$ is reported to be required for cytokine increase in macrophages [55]. In the present study, the FACS analysis showed the existence of IL-6 protein in 3T3-L1 differentiated adipocytes. The application of heat stimuli to 42°C for 1 h increased IL-6 expression. The increased IL-6 protein was still observed after returning to a control temperature (37°C) for 6 h. These results suggest that the heat stimulation is a unique method to promote IL-6 production in adipocytes. The similar results have been reported in mouse C2C12 myoblasts and skeletal muscle [56]. In humans, it has been also reported that therapeutic hyperthermia, which is used for cancer treatment (41.8°C core temperature for 30 min), induces a striking elevation of IL-6 [57]. Recently, Ye et al. [22] showed that TRPV4 is involved directly in adipocyte functions such as IL-6 secretion. The present study also showed that TRPV1 (capsaicin) and TRPV2 (probenecid) as well as TRPV4 activators (GSK1016790A) elicited an increase of IL-6 expression and secretion in 3T3-L1 differentiated adipocytes. Thus, TRPVs activators appear to be novel stimuli for IL-6 production in adipocytes.

IL-6 production by adipose tissue is known to be enhanced in obesity [24,25], and plays a role in the link between obesity, inflammation, coronary heart diseases [26], and insulin resistance [25,27]. TRPV2 and TRPV4 have been known to be activated by additional modalities, such as hypotonicity and mechanical stretch (TRPV2 [47], TRPV4 [48,49]. Because adipocytes become very large in obesity, it is possible that this cellular distention activates TRPV2 and TRPV4, subsequently leading to the changes in gene programs, i.e., IL-6 production. In fact, it has been reported that TRPV4 deficiency protected mice from diet-induced obesity and insulin resistance [22]. Also, a very recent study of Trpv4−/− mice has shown a resistance to diet-induced obesity [58]. The present study also showed the functional expression of TRPV2 in 3T3-L1 pre-adipocytes and differentiated adipocytes. Thus, it is likely that TRPV2 as well as TRPV4 involves IL-6 production from adipocytes under the pathophysiological conditions such as obesity, and the target of TRPVs in particular may be a very promising target for treating obesity and type 2 diabetes.

Alternatively, IL-6 is well known to be transiently secreted from skeletal muscles during exercise, called as a myokine [59]. Such transient IL-6 secretion may rather improve the pathological conditions such as obesity, by inducing lipolysis, and improving insulin resistance [60,61]. Wallenius et al. [62] have demonstrated that IL-6-deficient mice develop mature-onset obesity. Thus, myokines such as IL-6 may be involved in mediating the beneficial health effects against chronic diseases associated with low-grade inflammation such as diabetes and cardiovascular diseases. The present study showed that hyperthermia increased IL-6 production from adipocytes, which may be a novel transient stimulation for IL-6 production from adipocytes. Therefore, it is likely that hyperthermia may affect adipose cell functions including IL-6 production, and provide a therapeutic or preventive modality against obesity-related diseases. Further studies are needed to clarify this possibility.

## Conclusion

Adipose cell possess multiple thermo-sensitive TRPVs (TRPV1, TRPV2, and TRPV4), which may play vital roles in IL-6 production. Thus, thermo-sensitive TRPVs appear to be novel target molecules for promoting IL-6 secretion in adipose tissues.

### Conflict of Interests

No conflicts of interest, financial or otherwise, are declared by the author(s).

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