Anti-inflammatory Effects of Extracts of Sweet Basil (Ocimum basilicum L.) on a Co-culture of 3T3-L1 Adipocytes and RAW264.7 Macrophages

Haruka Takeuchi¹, Chie Takahashi-Muto², Mana Nagase³, Masahiro Kassai⁴, Rieko Tanaka-Yachi⁵, and Chikako Kiyose¹,³*

¹ Graduate school of Kanagawa Institute of Technology, Kanagawa 243-0292, JAPAN
² Department of Clinical Nutrition, Kitasato Junior College of Health and Hygienic Sciences, Niigata 949-7241, JAPAN
³ Department of Nutrition and Life Science, Kanagawa Institute of Technology, Kanagawa 243-0292, JAPAN
⁴ S&B food Inc., Tokyo, 174-8651, JAPAN
⁵ Department of Applied Biochemistry, Kanagawa Institute of Technology, Kanagawa 243-0292, JAPAN

Abstract: Obesity, a lifestyle disease resulting from excessive caloric intake and insufficient physical activity, results in a state of chronic inflammation. A food ingredient that suppresses chronic inflammation could help prevent associated diseases. Sweet basil (Ocimum basilicum L.) is a herb from the Lamiaceae family with some reported anti-inflammatory effects. Via this in vitro study, we aimed to investigate whether sweet basil exerts anti-inflammatory effects in obese patients. Fresh sweet basil leaves were freeze-dried and powered. After that, this was extracted with 80% methanol. After 3T3-L1 adipocytes were cultured with sweet basil extracts at final concentrations of either 5 or 25 μg/mL for 24h, RAW264.7 macrophages were seeded onto this adipocytes and co-cultured for 12h. We determined the effects of sweet basil extracts on inflammatory cytokine expression by real-time PCR or western blotting. Sweet basil extracts reduced the expression of inflammatory cytokine mRNA induced by co-culture, including that of IL-6 (Il6), IL-1β (Il1b), TNF-α (Tnf), and CCL2 (Ccl2). In addition, sweet basil extracts suppressed the mRNA expression of NF-κB (Nfκb1), a transcription factor of inflammatory cytokines. In an investigation of costimulatory CD137 (Cd137)/CD137L inflammatory signaling, a member of the TNF super-family, sweet basil extracts inhibited Tnfrsf9 expression induced by the co-culture. Therefore, the results of this study indicated that sweet basil extracts have an anti-inflammatory effect against adipocyte-induced inflammation, possibly through suppression of Tnfrsf9 expression.

Key words: sweet basil, inflammation, obesity, adipocytes, macrophages

1 Introduction

Excessive caloric intake and insufficient physical activity can result in the accumulation of visceral fat, and now, obesity is a worldwide problem. A study of overweight and obesity in 195 countries over 25 years reported that in 2015, there were 107.7 million obese children and 603.7 million obese adults⁴. Visceral fat accumulation is associated with an increased risk of arteriosclerotic diseases through lipid or glucose tolerance, metabolic abnormalities, and hypertension, with cardiovascular diseases accounting for more than two-thirds of deaths related to high body mass index⁴. Obesity is associated with chronic inflammation in the adipose tissue. Inflammation is essentially a defensive physiological response to pathogen invasion, but excessive inflammation can result in the damage or loss of physiological tissue and is a risk factor for various diseases. Macrophages are associated with excess inflammation. Inflammatory factors such as reactive oxygen species and lipopolysaccharides activate macrophages through the phosphorylation of the transcription factor nuclear factor-κB (NF-κB), which upregulates inflammatory cytokines, such as the tumor necrosis factor (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β), and inducible nitric oxide synthase (iNOS). iNOS is accompanied by the increased production of reactive oxygen species and excess...
nitric oxide, eventually resulting in histological damage. Thus, excessive inflammation induces damage or hypoactivity of physiological tissues; therefore, it is a risk factor associated with lifestyle-related diseases.

In obese patients, adipocyte hypertrophy and hyperplasia cause the production of inflammatory cytokines such as IL-6, IL-1β, TNF-α, and C-C motif chemokine ligand-2 (CCL2 or MCP-1), which charge adipose tissue inflammatory response. Obesity also increases macrophage infiltration in adipose tissue, and these may then be involved in the inflammatory response. CCL2 is a chemokine associated with macrophages infiltration in adipose tissue. It is produced by hypertrophy adipocytes, which promote macrophage infiltration via C-C chemokine receptor type 2 (CCR2) signaling. Macrophage infiltration is activated by an interaction with adipocytes and is involved in the inflammatory potentiation of the adipose tissue. Thus, macrophages have important functions in obese adipose tissue, and we considered it important to focus on the interaction between adipocytes and macrophages in seeking to improve inflammation in adipose tissue.

Sweet basil (Ocimum basilicum L.) belongs to the Lamiaceae family and is a native of tropical regions of Asia and Africa. It has been reported to have antipyretic, antiemetic, diuretic, and cardiotoxic properties. In a pharmacological report, the ethanolic extract of leaves of sweet basil showed hepatoprotective effects against hydrogen peroxide and carbon tetrachloride through removing superoxide radicals and nitric oxide. It has also been reported that the ethanolic extract of basil exerted an anti-inflammatory effect on foot edema in rats with carrageenan-induced inflammation.

However, there have been no reports of the anti-inflammatory effects of sweet basil related to obesity. The aim of this in vitro study was to examine the anti-inflammatory effects of sweet basil on inflammation related to obesity by using a co-culture of adipocytes and macrophages.

2 Experimental Procedures
2.1 Materials

Mouse 3T3-L1 preadipocytes were obtained from the American Type Culture Collection CL-173 (Manassas, VA, USA), and RAW264.7 macrophages from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). Sepasol®-RNA II Super and RIPA buffer were purchased from Nacalai Tesque Inc. (Kyoto, Japan). The High Capacity RNA-to-cDNA Kit and Taqman® Gene Expression Assays were purchased from Thermo Fisher Scientific K.K. (Applied Biosystems, Tokyo, Japan). The rabbit anti-IL-6 antibody (DSW4V, #12912) and anti-α-Tubulin antibody (#2144) were purchased from Cell Signaling (Tokyo, Japan). In addition, 10% Mini-PROTEAN TGX Precast Gels, Tris/Glycine/SDS Buffer, Trans-Blot® Turbo™ Transfer Pack, 10× Tris-buffered saline, 0.05% Tween 20 solution Clarity™ Western ECL substrate and goat anti-rabbit horseradish peroxidase conjugate were purchased from BIO-RAD (Tokyo, Japan).

2.2 Preparation of sweet basil extracts

Sweet basil was provided by S&B Food Inc. (Tokyo, Japan). Fresh sweet basil leaves were freeze-dried and powdered, then stored at −80°C. The powder (0.2 g) was extracted three times with 10 mL of 80% methanol. After removing the solvent using an evaporator, the extract was dissolved in dimethyl sulfoxide to produce a final concentration of either 5 or 25 mg/mL and then kept at −80°C.

2.3 Cell culture

Mouse 3T3-L1 adipocytes and RAW264.7 macrophages were maintained in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin and streptomycin, and then incubated at 37°C in humidified 5% CO₂. After passage, the 3T3-L1 adipocytes were cultured in 12-well plates (2.0×10⁵ cells/well). Confluent 3T3-L1 adipocytes were incubated for 10 days with 1 μg/mL insulin, 0.25 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine in high-glucose DMEM containing 10% FBS. During the induction of differentiation, the medium was replaced with fresh medium every 2 days.

2.4 Treatment of the co-culture with sweet basil extract

After the induction of differentiation for 10 days, the 3T3-L1 adipocytes were treated with new serum-free medium for 2 h, followed by sweet basil extract in serum-free medium (at final concentrations of 5 or 25 μg/mL) for 24 h. Control cells were treated with dimethyl sulfoxide only. RAW264.7 macrophages (2.0×10⁵ cells/well) were seeded onto 12-well plates and cultured with the differentiated 3T3-L1 adipocytes in serum-free medium for 12 h. The control cells were mixed with 3T3-L1 adipocytes and RAW264.7 macrophages cultured separately.

2.5 mRNA analysis

Total RNA was extracted using Sepasol®-RNA II Super solution. The amount and purity of the RNA were measured at 260 and 280 nm with a NanoDrop Q5000 spectrophotometer (Tommy Seiko Co., Ltd., Tokyo, Japan). The total RNA was reverse-transcribed into cDNA using a High Capacity RNA-to-cDNA Kit. The mRNA expression of each gene was measured using a 7500 Fast Real Time PCR system (Applied Biosystems, Thermo Fisher Scientific K.K., Tokyo, Japan) and Taqman® Gene Expression Assays. GAPDH was used as the housekeeping gene. The Assay IDs and RefSeqs of the mouse primers used for the quantitative real-time PCR are shown in Table 1. The difference between the Ct values of the sample and GAPDH were cal-
Table 1  Assay ID and RefSeq of mouse primers used for quantitative Real-Time PCR Taqman® Gene Expression Assays.

<table>
<thead>
<tr>
<th>Gene</th>
<th>AssayID</th>
<th>RefSeq</th>
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<tbody>
<tr>
<td>Il1b</td>
<td>Mm00434228_m1</td>
<td>NM_008361.3</td>
</tr>
<tr>
<td>Ccl2</td>
<td>Mm00441242_m1</td>
<td>NM_011333.3</td>
</tr>
<tr>
<td>Tnf</td>
<td>Mm99999068_m1</td>
<td>NM_013693.2</td>
</tr>
<tr>
<td>Il6</td>
<td>Mm00446190_m1</td>
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<tr>
<td>Nfkb1</td>
<td>Mm00476361_m1</td>
<td>NM_008689.2</td>
</tr>
<tr>
<td>Tnfrsf9</td>
<td>Mm00441899_m1</td>
<td>NM_001077508.1</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Mm99999915_g1</td>
<td>NM_008084.2</td>
</tr>
</tbody>
</table>

culated and the logarithm of this difference was taken as the measured value for each sample.

2.6 Western blot analysis

Cells were recovered with phosphate buffered saline and lysed with RIPA buffer. The cellular lysate was incubated on ice for 15 min and then centrifuged for 10 min at 10,000 × g at 4°C. Sample solutions were applied to 10% MINI-PROTEAN TGX Precast Gels and separated on a Tris/glycine/SDS buffer. After electrophoresis, the proteins were transferred onto polyvinylidene difluoride membrane of a Trans-Blot® Turbo™ Transfer Pack Transfer System. The membranes were blocked with 5% skimmed milk in Tris-buffered saline with 0.05% Tween 20 solution (1 × TTBS) at room temperature for 40 min, and then incubated overnight at 4°C with the primary antibodies (IL-6 at a concentration of 1:500 and α-tubulin at a concentration of 1:1000). After washing the membranes three times with 1 × TTBS, they were incubated with the secondary antibody at a concentration of 1:2000 for 1h at room temperature. They were then washed three times with 1 × TTBS, treated with reagents in Clarity™ Western ECL substrate, and detected with the Universal Hood II system (BIO-RAD, Tokyo, Japan).

2.7 Statistical analysis

The data are presented as mean ± SD. Differences were evaluated using one-way ANOVA followed by Bonferroni post hoc testing, and were considered statistically significant at \( p < 0.05 \). The analysis was performed using IBS SPSS Statistics 21 (IBM Corp., Armonk, NY, USA).

3 Results

The expression levels of Il1b and Ccl2 genes were significantly increased in the co-culture group compared with the control group, but these increases were significantly attenuated by treatment with the sweet basil extracts (Fig. 1(a), (b)). The expression of the Tnf gene was decreased in the co-culture group compared with the control group, and further decreased by treatment with the sweet basil extracts (Fig. 1(c)). The expression of the Il6 gene was significantly increased in the co-culture group compared with the control group, but this increase was significantly attenuated by treatment with the sweet basil extracts (Fig. 2(a)). In addition, the expression of IL-6 protein tended to be lower with the sweet basil extracts than in the co-culture group (Fig. 2(b)). There was no significant difference in Nfkb1 gene expression between co-culture group and control group, but there was a significantly decrease in expression in the sweet basil extracts group compared with
measurement of the expression of the \textit{Tnfrsf9} gene located upstream of NF-κB showed that this was significantly increased in the co-culture group compared with the control group, with significant attenuation of this increase following treatment with the sweet basil extracts (Fig. 4). These findings showed that treatment with sweet basil extracts exerted an anti-inflammatory effect on inflammation induced by 3T3-L1 adipocytes through the suppression of \textit{Tnfrsf9} expression.

**Discussion**

This study investigated the anti-inflammatory effects of sweet basil extracts on a co-culture of 3T3-L1 adipocytes and RAW264.7 macrophages. Treatment with the sweet basil extracts suppressed the expression levels of \textit{Il6} and \textit{Il1b} genes and protein that had been increased by the co-culture, demonstrating that the extracts exerted anti-inflammatory effects against the inflammation induced by the co-culture. In addition, treatment with sweet basil extracts inhibited the expression of the \textit{Ccl2} gene, which is promoted by adipocyte hypertrophy. This suggested that sweet basil extracts suppress the inflammatory potentiation resulting from the interaction of adipocytes and macrophages by blocking macrophage infiltration into the adipose tissue.

NF-κB is a transcription factor that directly controls the
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expression of inflammatory cytokine genes by binding to the promoter element after entering the nucleus. Several in vitro herb studies have suggested that the pathway influenced by anti-inflammatory effects is NF-κB signaling\textsuperscript{14,16}. To clarify the mechanism underlying the anti-inflammatory effects of sweet basil extracts, we examined whether treatment with the extracts influenced the expression of Nfkbi. The co-culture did not increase the expression of Nfkbi, but the expression was suppressed by treatment with the sweet basil extracts. Dai et al. reported that iNOS and NF-κB gene expression induced using Quinocetone decreased by treatment of curcumin in human hepatocytes. They assumed that curcumin inhibited inflammatory response of hepatocyte and may involve in inhibition of NF-κB pathway\textsuperscript{16}. According to this result, it can guess that the decrease in the gene expression of NF-κB may lead to the inhibition of inflammation response. Therefore, this suggested that treatment with sweet basil extracts reduced NF-κB transferring into nuclear by inhibiting the gene expression of Nfkbi itself, which in turn suggested that the suppression of the inflammatory cytokine genes by the sweet basil extracts was through inhibition of the function of NF-κB as their transcription factor.

Recently, it has been proposed that CD137 (TNFRSF9) and CD137L (TNFSF9) costimulatory signaling enhances inflammation in obese adipose tissue. Tu et al. reported that CD137/CD137L costimulatory signaling, which belongs to the TNF super-family, is involved in the obesity inflammatory response\textsuperscript{25}. They claimed that interactive signaling by CD137/CD137L is induced by co-culturing adipocytes and macrophages, and that the production of inflammatory cytokines such as IL-6, CCL2, and TNF-α was promoted via the signaling pathway of adipocytes and macrophages, respectively. Furthermore, they found that CD137 was expressed on adipocytes and CD137L was expressed on macrophages, and that stimulation of CD137 in adipocytes activated three pathways, e-Jun N-terminal kinase, p38 MAP kinases (p38MAPK) and IκB kinase (IKK)\textsuperscript{17}. As yet, there has been little investigation on the effect of food ingredients on CD137/CD137L costimulatory signaling. In this study, therefore, we examined whether the sweet basil extracts influenced the expression of the Tnfrsf9 gene. We found that the expression of Tnfrsf9 was indeed suppressed by treatment with the sweet basil extracts. This suggested that the underlying mechanism by which sweet basil extracts exerted an anti-inflammatory effect in adipocytes was by suppressing the inflammatory signaling pathway through a reduction in Tnfrsf9 gene expression. Treatment with sweet basil extracts also resulted in a decrease in Nfkbi gene expression, suggesting that NF-κB signaling was inhibited via suppression of the expression of upstream regulator CD137. Little has been reported on the mechanisms that control the receptor, so the regulation of receptor expression by sweet basil extracts remains unclear.

In an in vivo study of obese mice fed a high-fat diet for 9 weeks, Kim et al. investigated the expression and role of CD137/CD137L in adipose tissue\textsuperscript{18}. They found that Tnfrsf9 gene expression was significantly increased in the epididymal adipose tissue, and that body weight and the levels of cytokines and infiltrating macrophages were reduced in the CD137-deficient mice. A future study should investigate whether sweet basil powder demonstrates anti-inflammatory effects when added to the high-fat diet of obese mice.

We expected the gene expression of Tnf and Nfkbi increased by the co-culture of 3T3-L1 adipocytes and RAW264.7 cells. However, there were not increased. We guessed that co-culture time and RAW264.7 cell numbers might influence for this phenomenon\textsuperscript{9,17}, and we will perform the examination more about this point in future. Treatment with sweet basil extracts further reduced the expression of Tnf significantly decreased compared with the level in the co-culture, indicating that the sweet basil extracts exerted an effect on Tnf gene in co-culture. Future studies are needed to investigate this further with various culture conditions.

In addition to CD137/CD137L costimulatory signaling, the interaction of adipocytes and macrophages induces inflammation through the participation of various cell surface receptors. Suganami et al. reported that adipose tissue induced inflammation by the activation of macrophages, with the free fatty acids of the adipocytes binding to the TLR4 of the macrophages\textsuperscript{19}. The present study showed that the Nfkbi gene expression was reduced by treatment with sweet basil extracts, suggesting that the extracts influenced TLR4-NF-κB signaling.

Sweet basil shows strong antioxidant activity, thought to be due to its phenolic content\textsuperscript{20}. Reported phenolic compounds in sweet basil include rosmarinic, caffeic, and chieoric acids\textsuperscript{11–23}. It has been suggested that multiple phenolic components participate in the anti-inflammatory effects of sweet basil extracts. However, components of sweet basil extracts are unclear in this experiment. We suggest that some phenolic compounds or other active components in sweet basil extracts are involved in the anti-inflammatory effects.

In summary, this study investigated the anti-inflammatory effects of sweet basil extracts on inflammation induced by adipocytes in a co-culture with macrophages. As the results, we found that Il6, Il1b and Ccl2 gene expressions significantly decreased by treatment with sweet basil extracts in co-culture of 3T3-L1 adipocytes and RAW264.7 cells. In future, it is necessary to examine the phosphorylation of NF-κB and IkB to apparent the influence on the NF-κB signaling pathway by sweet basil extracts.
References


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