Hexane extract of raw ginger enhances adipocyte differentiation through its PPARγ ligand activity on 3T3-L1 preadipocytes

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Abstract

Ginger has been reported to show many health enhancement effects. We prepared a hexane extract of raw ginger (HRG) under low temperature, and examined its anti-obesity effects using 3T3-L1 cells. HRG promoted adipocyte differentiation in a dose-dependent manner and enhanced the expression levels of adipocyte-specific genes, such as adiponectin and Glut4. HRG also exhibited peroxisome proliferator-activated receptor γ ligand activity, similar to antidiabetic drug thiazolidinediones, which promoted adipocyte differentiation and increased small adipocytes. In addition, HRG showed a tendency to suppress the action of tumor necrosis factor-α, which causes insulin resistance by downregulating the expression of adipocyte-specific genes. These findings suggest that HRG would be utilized as a functional food material for the prevention of obesity and related diseases by increasing small adipocytes.

Keywords: ginger, adipocyte, peroxisome proliferator-activated receptor γ, thiazolidinediones

I Introduction

Ginger (Zingiber officinale) is used as a spice and natural medicine, and its extracts are utilized worldwide as nutritional supplements and food additives such as flavorings. Studies on the physiological functions of food materials have been conducted in recent years, and ginger is a good candidate for a functional food material because it is easily harvested and consumed in many countries. It has been reported that ginger components and extracts show antioxidant, anti-inflammatory, antitumor, and anti-obesity effects1–8). Thus, in addition to existing ginger-related food products, the development of various types of novel ginger food materials with functional properties can be expected.

Among the above-described effects of ginger, its effect on obesity appears to be an effective and beneficial way to prevent diabetes. Obesity is a state of excess adipose tissue mass. It is a worldwide problem and represents a major risk factor for type 2 diabetes. Adipocytes were originally thought to be required for lipid storage only, but are now recognized as endocrine cells that control energy homeostasis9, 10). In the non-obese state, small adipocytes secrete adiponectin, a cytokine that enhances insulin sensitivity. However, tumor necrosis factor (TNF)-α and plasminogen activator inhibitor (PAI)-1, which cause insulin resistance and lead to type 2 diabetes, are secreted by large adipocytes in the obese state9–11). Therefore, efforts to increase small adipocytes will form one approach toward the prevention and improvement of diabetes.

Activation of peroxisome proliferator-activated receptor (PPAR) γ can be used as an indicator for differentiation
into small adipocytes\textsuperscript{11, 12}. PPAR\(\gamma\) belongs to a nuclear receptor family and regulates the expression of its target genes\textsuperscript{13}. It plays key roles in adipocyte differentiation, adipogenesis, glucose metabolism, and inflammation\textsuperscript{14-16}. Thiazolidinediones (TZDs) are synthetic PPAR\(\gamma\) ligands that are used as potent antidiabetic drugs\textsuperscript{11, 12, 17-20}. It is proposed that activation of PPAR\(\gamma\) by TZDs promotes adipocyte differentiation, increases small adipocytes, and decreases large adipocytes by apoptosis\textsuperscript{11, 12, 21}. Thus, insulin sensitivity is improved by adiponectin secreted by small adipocytes.

In the present study, as a novel food material from ginger, we prepared a hexane extract of raw ginger (HRG) from the residue obtained after squeezing the juice from raw ginger under low temperature, and examined the effects of HRG on obesity using 3T3-L1 cells. We found that HRG enhanced adipocyte differentiation and promoted the expression of adipocyte-specific genes in a dose-dependent manner. In addition, HRG had PPAR\(\gamma\) ligand activity similar to TZDs and showed a tendency to prevent the downregulation of adipocyte-specific genes by TNF-\(\alpha\). The present results provide evidence that HRG may have potential as a functional food material for the prevention of obesity and obesity-related diseases. We also discuss the components of HRG that may be responsible for its physiological function.

II Materials and Methods

1. Materials

Dulbecco’s modified Eagle’s medium (DMEM) and dimethylsulfoxide (DMSO) were purchased from Nacalai (Kyoto, Japan). Fetal bovine serum (FBS) was from Biowest (Nuaillé, France). Rosiglitazone was obtained from Wako (Tokyo, Japan). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium (MTT) was purchased from DOJINDO (Tokyo, Japan). Insulin, Oil red O, and dexamethasone (DEX) were from Sigma (St. Louis, MO, USA). 3-Isobutyl-1-methylxanthine (IBMX) was purchased from Tocris Bioscience (Ellisville, MO, USA). TNF-\(\alpha\) was obtained from R&D Systems (Minneapolis, MN, USA). TRizol and SuperScript VILO were from Invitrogen (Carlsbad, CA, USA). TURBO DNA-free was purchased from Ambion (Foster City, CA, USA). 2×FAST SYBR Premix was obtained from Applied Biosystems (Foster City, CA, USA). pGL4.35 [luc2P/9xGAL4UAS/Hygro] vector and a Dual-Luciferase Reporter Assay System were from Promega (Madison, WI, USA).

2. HRG samples used for cell assays

We used HRG, “Ginger oil (IDN)”, a hexane extract from frozen raw ginger derived from Indonesia, which was produced by Tsuji Oil Mills Co. Ltd (Matsusaka, Japan). Briefly, \(n\)-hexane was added at double amount to the residue obtained after squeezing the juice from raw ginger and extraction was carried out at room temperature for 15 min. After filtration, HRG was obtained by removing the hexane through distillation in a reduced pressure machine. The yield ratio of HRG was almost 0.5% (w/w). Samples were dissolved in DMSO at 10 mg/mL or 50 mg/mL, and stored at \(-20^\circ\text{C}\) until use. For cell culture, HRG was dissolved in DMSO at an appropriate concentration. The final concentration of DMSO in the cell culture medium was 0.1%.

3. Cytotoxicity assay

Cell proliferation was measured by the MTT assay\textsuperscript{22}. 3T3-L1 cells were seeded in a 24-well plate (1.2×10\(^5\) cells/well). At 2 d after reaching confluence, the cells were exposed to various concentrations of HRG for 8 d. The medium containing HRG was replaced with fresh medium every 2 d until day 8. Subsequently, MTT solution (0.5%) was added to each well, and the plate was incubated for 4 h at 37\(^\circ\text{C}\) to detect viable cells. Cell viability was calculated by measuring the absorbance at 550 nm in a microplate reader (MPR A4i; Tosoh, Tokyo, Japan).

4. Cell culture, adipocyte differentiation, and treatment

3T3-L1 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM supplemented with 100 U/mL penicillin, 100 \(\mu\)g/mL streptomycin, 2 mM l-glutamine, and 10% FBS at 37\(^\circ\text{C}\) under 5% CO\(_2\). To examine the effects of HRG on adipocyte differentiation, 3T3-L1 cells at 2 d after reaching confluence (defined as day 0) were treated with 1 \(\mu\)g/mL insulin with or without HRG or rosiglitazone in DMEM supplemented with 10% FBS, and the medium was replaced with fresh medium every 2 d until day 8. When post-confluent 3T3-L1 cells were treated with 5 ng/mL TNF-\(\alpha\), the cells were induced to undergo adipocyte differentiation with 0.25 \(\mu\)M DEX, 10 \(\mu\)g/mL insulin, and 0.5 \(\mu\)M IBMX in DMEM supplemented with 10% FBS. After 48 h, the cell culture medium was changed to DMEM containing 10% FBS and 5 \(\mu\)g/mL insulin. The medium was replaced with fresh medium every 2 d until day 6. At day 6, the cells were treated with 5 ng/mL TNF-\(\alpha\) for 48 h with HRG.

5. Oil red O staining

At day 8, the cells were fixed with 10% formalin for 10 min, washed twice with phosphate-buffered saline (PBS), and stained with Oil red O solution (0.2% Oil red O in 60% isopropanol). The stained cells were washed twice with PBS and photographed. The Oil red O was then eluted with 100% isopropanol, and quantified by the absorbance at 550 nm using the MPR A4i microplate reader.
6. RNA preparation and quantitative real-time PCR

Total RNA was isolated from 3T3-L1 cells using TRIzol. After digestion of genomic DNA with TURBO DNA-free, 1 μg of RNA was reverse-transcribed to cDNA using SuperScript VILO with random primers. Quantitative real-time PCR was performed using 2×FAST SYBR Premix in a StepOnePlus System (Applied Biosystems). The primer sequences used were: 18S ribosomal RNA F, 5′-CCTGCGGCTTAATTGGACTC-3'; 18S ribosomal RNA R, 5′-AGACAAATCGCTCCACC-3'; PPARγ2 F, 5′-GGCGTGGAAATCGATCACC-3'; PPARγ2 R, 5′-GTCAAGGGAATGCGAGTGGT-3'; adipocyte fatty acid-binding protein (aP2) F, 5′-GGGGGTGGAATTCGATGAAATCACC-3'; aP2 R, 5′-AACATCCCTCCCCACGCCTGCA-3'; adiponectin F, 5′-CTGGACGACACAAAAGGGGCTC-3'; adiponectin R, 5′-TGCCCCTGCCCATCAACTCGA-3'; Glut4 F, 5′-CGCTACTCGGGCTAATCAGG-3'; Glut4 R, 5′-AGCATAGACTCCAGCCAGCAC-3'.

7. PPARγ ligand assay

The PPARγ ligand activity was measured using a PPAR-GAL4 chimera assay system. CV1 cells, pM-PPARγ vector, and pRL-CMV vector were kindly provided by T. Kawada (Kyoto University, Kyoto, Japan). CV1 cells were cultured in DMEM supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, and 10% FBS at 37°C under 5% CO2. Three plasmids, pM-PPARγ vector, pGL4.35 vector, and pRL-CMV vector, were transfected into CV1 cells cultured on 24-well tissue culture plates. After transfection, the cells were cultured and treated with the ligand assay compounds for 24 h. Subsequently, the ligand activity was measured using the Dual-Luciferase Reporter Assay System.

8. Statistical analysis

All data were represented as mean ± standard deviation. Statistical analyses were performed using R version 3.3.2 (https://www.r-project.org/). To investigate the effect of HRG at each concentration, the data obtained from the experiments were analyzed by one-way analysis of variance (ANOVA) followed by a Tukey multiple test. Values of P < 0.05 were considered statistically significant.

III Results

1. Effect of HRG on cell viability

First, we confirmed that there were no significant differences in cell viability between control cells and cells treated with HRG at final concentrations of 1 to 50 μg/mL using the MTT assay (Fig. 1). Subsequent experiments were performed at a final concentration of 25 μg/mL HRG or less.

Fig. 1. Effect of HRG on cell viability

The cell viabilities after HRG treatment were determined by the MTT assay. Data are presented as mean ± standard deviation (n=3). No significant differences were found between control cells and cells treated with HRG at concentrations of 1, 10, and 25 μg/mL. *P<0.001 vs. control group (0 μg/mL HRG).

2. HRG enhances adipocyte differentiation

As one of the physiological functions of HRG, we investigated its effect on adipocyte differentiation. Post-confluent 3T3-L1 cells were treated with HRG at various concentrations for 8 d in the presence of 1 μg/mL insulin. The accumulated intracellular lipids were then stained with Oil red O. Rosiglitazone, a TZD, was used as a positive control to promote adipocyte differentiation. As shown in Fig. 2, quantification of Oil red O revealed that HRG enhanced adipocyte differentiation in a dose-dependent manner. These results are not consistent with the effects of 6-gingerol, another component of ginger, because 6-gingerol was reported to inhibit adipocyte differentiation23).

3. HRG promotes the expression of adipocyte-specific genes

During adipocyte differentiation, various transcription factors and adipocyte-specific genes are transcribed24). PPARγ is a master regulator of adipogenesis and induces the expression of adipocyte-specific genes such as PPARγ2, aP2, adiponectin, and Glut4.

Therefore, we performed quantitative RT-PCR analyses to assess the effects of HRG on the expression of these genes. As shown in Fig. 3, HRG significantly upregulated the expression levels of these adipocyte-specific genes in a dose-dependent manner. These findings suggest that HRG upregulates the expression of adipocyte-specific genes and enhances adipocyte differentiation.
4. HRG has PPARγ ligand activity

Increasing the number of small adipocytes is important for the prevention of diabetes. Therefore, we examined whether HRG has PPARγ ligand activity, similar to the case for TZD antidiabetic drugs that enhance adipocyte differentiation and increase the expression levels of adipocyte-specific genes through PPARγ ligand activity\(^1\), \(^12\), \(^24\). As shown in Fig. 4, HRG showed significant PPARγ ligand activity in a dose-

**Fig. 2. Effect of HRG on adipocyte differentiation**

Post-confluent 3T3-L1 cells were incubated in medium containing 1 \(\mu\)g/mL insulin with or without HRG for 8 d. (A) The cells were stained with Oil red O and photographed. Bars, 100 \(\mu\)m. 0: 0 \(\mu\)g/mL HRG; 10: 10 \(\mu\)g/mL HRG; 25: 25 \(\mu\)g/mL HRG; Rosi: 10 \(\mu\)M rosiglitazone. (B) Oil red O was eluted and quantified by the absorbance at 550 nm. Data are presented as mean ± standard deviation (n=3). *\(P<0.05\), **\(P<0.001\) vs. control group (0 \(\mu\)g/mL HRG).

**Fig. 3. Effects of HRG on adipocyte-specific genes**

Post-confluent 3T3-L1 cells were incubated in medium containing 1 \(\mu\)g/mL insulin with or without HRG for 8 d. Total RNA was extracted and analyzed by real-time PCR. 0: 0 \(\mu\)g/mL HRG; 10: 10 \(\mu\)g/mL HRG; 25: 25 \(\mu\)g/mL HRG. Data are presented as mean ± standard deviation (n=3). *\(P<0.01\), **\(P<0.001\) vs. control group (0 \(\mu\)g/mL HRG).

**Fig. 4. HRG has PPARγ ligand activity**

CV1 cells were transfected with vectors expressing a PPARγ/GAL4 reporter assay system. 0: 0 \(\mu\)g/mL HRG; 10: 10 \(\mu\)g/mL HRG; 25: 25 \(\mu\)g/mL HRG; Rosi: 10 \(\mu\)M rosiglitazone. Data are presented as mean ± standard deviation (n=3). *\(P<0.05\), **\(P<0.001\) vs. control group (0 \(\mu\)g/mL HRG).
dependent manner. These results suggest that HRG promotes the differentiation of preadipocytes into small adipocytes through PPARγ ligand activity. It should be noted that HRG showed PPARγ ligand activity although 6-shogaol with the activity was little detected in the HRG (see Discussion).

5. HRG prevents the action of TNF-α

We further examined whether HRG can suppress the action of TNF-α which causes insulin resistance\(^{16}\). TNF-α is a cytokine secreted by adipose tissue in the obese state\(^8, 10\), and is known to downregulate the expression of adipocyte-specific genes, such as adiponectin and Glut4\(^{25}\). Meanwhile, TZDs are known to suppress the action of TNF-α through PPARγ activation\(^{11, 12, 16}\). As shown in Fig. 5, HRG showed a tendency to prevent the decreases in the expression levels of adipocyte-specific genes by TNF-α, similar to the case for TZDs. These findings suggest that HRG suppresses the action of TNF-α and enhances the expression of adipocyte-specific genes.

Fig. 5. Effect of HRG on TNF-α inhibition
Post-confluent 3T3-L1 cells were induced to undergo adipocyte differentiation. At day 6, the cells were treated with 5 ng/mL TNF-α for 48 h with or without HRG. 0: 0 μg/mL HRG; 10: 10 μg/mL HRG; 25: 25 μg/mL HRG. Data are presented as mean ± standard deviation (n=3). *P<0.05, **P<0.01, ***P<0.001 vs. control group (0 μg/mL HRG).

### IV Discussion

In this study, we examined the anti-obesity effects of HRG using 3T3-L1 cells. HRG promoted adipocyte differentiation and enhanced the expression levels of adipocyte-specific genes. We further showed that these effects could be attributed to PPARγ activation by HRG. Moreover, HRG showed a tendency to prevent the downregulation of the expression of adipocyte-specific genes induced by TNF-α.

Ginger is used as a herbal medicine, and has recently been shown to have effective physiological functions such as anti-inflammatory, antitumor, and anti-obesity effects\(^1, 4-8\). However, these effects were mainly shown for extracts prepared with ethanol, a solvent with low extraction efficiency. Therefore, ginger extracts prepared from raw ginger using high extraction efficiency solvents, such as hexane, without a heating process may have currently unidentified useful properties and retain volatile flavor ingredients. We prepared...
a raw ginger extract under low temperature with n-hexane, an organic solvent used as a processing aid in the manufacture of food flavorings. According to the previous HPLC analysis, 6-gingerol was detected more than 10% in the HRG, while 6-shogaol, the dehydrated form of 6-gingerol, was hardly detected\(^{(29)}\). Volatile components were also detected in HRG. Meanwhile, the HPLC analysis of an n-hexane extract from dried ginger revealed that the level of 6-shogaol was higher and the levels of volatile components were lower than those in HRG (data not shown). Thus, we concluded that HRG can be prepared without causing chemical changes or losing volatile components during heating and extraction processes.

Since little is known about the physiological functions of raw ginger, we evaluated the anti-obesity effects of HRG extracted from raw ginger, in addition to the properties of HRG which retains native organic components as described above. We found that HRG enhanced adipocyte differentiation in a dose-dependent manner. Since 6-gingerol, the major component of HRG, was reported to inhibit adipocyte differentiation\(^{(23)}\), the observed promotion of adipocyte differentiation by HRG is considered to be caused by substances in HRG other than 6-gingerol.

**Activation of PPAR\(\gamma\) is essential for adipocyte differentiation and expression of adipocyte-specific genes.** The TZD antidiabetic drugs have potent PPAR\(\gamma\) ligand activity and induce marked adipocyte differentiation\(^{(11)}\). Some plant compounds, such as aurapten and flavanone, were reported to serve as PPAR\(\gamma\) ligands\(^{(27-30)}\). While a previous study showed that 6-shogaol acts as a PPAR\(\gamma\) ligand\(^{(11)}\), HRG was found to contain little 6-shogaol, suggesting that HRG contains other compounds that can overcome the inhibition of adipocyte differentiation by 6-gingerol.

Adipogenesis is accompanied by the expression of various adipocyte-specific genes\(^{(11, 24)}\). We confirmed that HRG enhanced the expression levels of these adipocyte-specific genes in a dose-dependent manner. PPAR\(\gamma\) is a master regulator of adipogenesis. It has two splice isoforms, PPAR\(\gamma1\) and PPAR\(\gamma2\), with both abundantly expressed in adipose tissue\(^{(32)}\). PPAR\(\gamma\) regulates adipocyte-specific genes such as aP2, adiponectin, and Glut4\(^{(25)}\). aP2 is expressed in fully differentiated adipocytes and is involved in intracellular transport and fatty acid metabolism\(^{(33)}\). Adiponectin is a cytokine that increases insulin sensitivity and is secreted by adipocytes\(^{(34)}\). Glut4 is expressed in adipose and muscle tissue, and plays a critical role in whole-body glucose homeostasis\(^{(35)}\).

It is known that PPAR\(\gamma\) activation leads to enhanced adipocyte differentiation and increased expression of adipocyte-specific genes. The present study showed that HRG promoted both adipocyte differentiation and expression of adipocyte-specific genes. Therefore, HRG was predicted to serve as a PPAR\(\gamma\) ligand. In PPAR\(\gamma\) ligand assays, HRG showed PPAR\(\gamma\) ligand activity in a dose-dependent manner. Thus, HRG can be expected to enhance differentiation into small adipocytes and improve glucose and lipid metabolism.

Obesity involves chronic inflammation\(^{(37)}\). In the obese state, adipocytes and macrophages secrete inflammatory cytokines such as TNF-\(\alpha\), TNF-\(\alpha\) is a major cytokine for insulin resistance. TNF-\(\alpha\) downregulates adipocyte-specific genes such as adiponectin and aP2, which are related to insulin responsiveness\(^{(38)}\). Activation of PPAR\(\gamma\) by TZDs prevents the action of TNF-\(\alpha\)\(^{(11, 12, 16)}\). Since HRG exhibited PPAR\(\gamma\) ligand activity, we evaluated its effect on TNF-\(\alpha\) in this study. We found that HRG showed an inhibitory effect against TNF-\(\alpha\). 6-Gingerol is also known to prevent the downregulation of adipocyte-specific genes by TNF-\(\alpha\), independently of PPAR\(\gamma\) activation\(^{(31)}\), and HRG contains a large amount of 6-gingerol. Therefore, HRG may have an inhibitory effect on the action of TNF-\(\alpha\) in both PPAR\(\gamma\) activation-dependent and -independent manners.

In this study, we found that HRG had an anti-obesity effect, at least through its PPAR\(\gamma\) ligand activity. Therefore, we performed an LC-MS analysis to reveal unknown components of HRG. However, we were unable to identify any compounds because many peaks were detected in the MS spectrum (data not shown). Although the components responsible for this physiological function remain unclear, HRG, a highly concentrated extract of raw ginger, has potential as a functional food material for the prevention of obesity and related diseases such as diabetes. In addition to other physiological functions of HRG, future studies to identify one or more currently unknown components of HRG with a similar function to TZDs are expected.

**V Acknowledgement**

We wish to thank T. Kawada for providing CV1 cells, pM-PPAR\(\gamma\) vector and pRL-CMV vector.

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論文

生鮮ショウガのヘキサン抽出物はPPARγリガンド活性を通して3T3-L1細胞の脂肪細胞への分化を促進する
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概要

ショウガは多くの健康増進効果を有することが報告されてきた。我々は生鮮ショウガのヘキサン抽出物（HRG）を揮発性香気成分や有機成分を保持するように低温で調製し、3T3-L1細胞を用いて肥満についてのHRGの効果を検討した。HRGは量依存的に脂肪細胞の分化を促進し、adiponectinやGlut4といった脂肪細胞特異的遺伝子の発現を高めた。HRGはまた、抗糖尿病薬で脂肪細胞の分化を促進し、小型脂肪細胞を増加させるthiazolinedioneと同様、peroxisome proliferator-activated receptor γリガンド活性も有していた。加えて、HRGは脂肪細胞特異的遺伝子の発現を下方向けすることでインスリン抵抗性を引き起こすtumor necrosis factor-αの作用を抑制する傾向を示した。これらの結果は、HRGが小型脂肪細胞を増加させることで肥満や肥満関連疾患を予防するための機能性食品素材として利用できる可能性を示唆している。