Research Article

R-Limonene Enhances Differentiation and 2-Deoxy-D-Glucose Uptake in 3T3-L1 Preadipocytes by Activating the Akt Signaling Pathway

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Adipocyte is an important place for lipid storage. Defects in lipid storage in adipocytes can lead to lipodystrophy and lipid accumulation in muscle, liver, and other organs. It is the condition of mixed dyslipidemia which may favor the development of insulin resistance via lipotoxic mechanisms. Our objective of the study was to investigate the potential role of R-limonene (LM) on differentiation, lipid storage, and 2-deoxy-D-glucose (2DG) uptake in 3T3-L1 preadipocytes. Genes and proteins associated with differentiation, lipid accumulation, 2DG uptake and its signaling pathways in the adipocytes were analyzed using qPCR and western blot methods. LM treatment increased differentiation, lipid accumulation, and the expression of adipogenic and lipogenic markers such as C/EBP-α, C/EBP-β, PPARγ, SREBP-1, RXR, FAS, and adiponectin. However, the LM concentration at 10μM decreased (p < 0.05) adipogenesis and lipogenesis via regulating key transcriptional factors. LM treatment increased activation of Akt by increasing its phosphorylation, but p44/42 activation was not altered. MK-2206, an Akt specific inhibitor, reduced the activation of Akt phosphorylation whereas LM treatment aborted the MK-2206 mediated inhibition of Akt activation. LM enhanced glucose uptake in differentiated adipocytes. Overall data suggested that LM treatment favored lipid storage and glucose uptake in adipocytes via activation of key transcriptional factors through activation of Akt phosphorylation in 3T3-L1 adipocytes.

1. Introduction

Differentiation of adipocytes is an essential process for the lipid storage. Inhibiting adipocyte differentiation alone is not an appropriate way to prevent obesity because adipocytes provide a safe place for lipid accumulation. Defects in adipogenesis can lead to stimulating lipodystrophy. In this condition, lipids are accumulated in muscle, liver, and other organs which develop the insulin resistance [1, 2]. Animal and human experiments have proved that type 2 diabetes mellitus is closely associated with adipogenesis and its factors [3].

Adipogenesis is a complex process that is regulated by a cascade of transcription factor and other regulatory proteins. Numerous positive and negative factors have been involved in adipocyte differentiation [4–7]. Among these, C/EBP-β (CCAAT/enhancer-binding protein-β) and C/EBP-δ act together and induce the expression of PPAR-γ 2 (peroxisome proliferator-activated receptor) and C/EBP-α (CCAAT/enhancer-binding protein-α). These factors are known to regulate genes and proteins associated with adipogenesis and lipogenesis [8].

The Akt kinases play a critical role in adipogenesis and glucose transport [9]. Lack of or inhibition of Akt activation in fibroblast displays an inability to differentiate preadipocyte into adipocytes. In addition, activation of Akt can promote the differentiation of preadipocytes into mature adipocytes.
even in the absence of other factors involved in adipogenesis. Activation of Akt can induce the peroxisome proliferator-activated receptor expression which is the key transcriptional factor in the adipocyte differentiation and lipid accumulation [10–14].

Monoterpenes are the major constituents of the plant kingdom. They are the primary contributor to the organoleptic properties associated with different herbs, spices, citrus fruits, most flowers, spearmint oil, pine oil, and fruits [15]. Monoterpenes are a class of terpenes that consist of two isoprene units and it has many biological functions including the broad spectrum of antimicrobial activities, allelopathic, herbivore deterring, pollinator attracting properties, antioxidant, antiproliferative, antitumor, antiviral, and antinociceptive properties. In addition, they can stimulate glucose uptake in C2C12 cells [16–19]. Among monoterpenes, limonene (Figure 1) and α-pinene exhibit potent antitumor properties [20, 21]; in addition, limonene has been shown to be helpful in relieving heartburn and gastroesophageal reflux disorder; it supports normal peristalsis [22].

Limonene can enhance glucose uptake in 3T3-L1 adipocytes via glucose transporters [23]. It possesses antidiabetic effects by preventing dyslipidemia [24]. It also prevents insulin resistance [25], LM enhanced mitochondrial biogenesis, and elevated protein levels of hormonal sensitive lipase (HSL), perilipin (PLIN), AMP-activated protein kinase (pAMPK), Phospho acetyl CoA carboxylase (pACC), Acy coenzyme A oxidase (ACO), cytochrome c oxidase subunit 4 (COX4), carnitine palmitoyltransferase 1 (CPT1), and cytochrome C (CYT-C) at the concentration of 25 and 50μM of limonene, which are involved in lipolysis and lipid catabolism via activation of β-adrenergic receptor-3 (β3-AR) and extracellular signal-regulated kinases (ERK) signaling pathway [26]. Dietary intake of monoterpenes by the human is relatively high. LM with lemons-like odor has been used in differentiation, and glucose uptake in 3T3-L1 adipocytes. In addition, the molecular mechanisms involved in the effect of LM on differentiation and glucose uptake were explored in this study.

2. Materials and Methods

2.1. Cell Culture and Chemicals. The 3T3-L1 preadipocytes cell line was obtained from the American Type Culture Collection [Rockville, MD, USA]. Dulbecco modified Eagle medium [DMEM] and fetal bovine serum (FBS) were procured from Gibco-BRL [Gaithersburg, MD, USA]. Kits for mRNA extraction, cDNA synthesis, and qPCR were purchased from Bio-Rad [Hercules, CA, USA]. R-limonene (Sigma Aldrich, #183164) and Rosiglitazone were obtained from Sigma Aldrich (St. Louis, MO, USA). MK-2206 was obtained from Selleckchem (Houston, TX, USA). Monoclonal antibodies (PPARγ, RXR, C/EBP-α, SREBP-1, aP2, FAS, adiponectin, ACC, TAKT, pAKT (Serine 473), p44/42, pp44/42 (Thr 202/ Tyr204), AMPK-α, pAMPK-α (Thr172), and GAPDH) used in the study were obtained from Cell Signaling Technology (Danvers, MA, USA).

2.2. Cytotoxicity of Limonene (LM). Ez-cytix assay kit (iTS-BiO, Korea) was used to determine the cytotoxic effects of LM. Briefly, 3T3-L1 preadipocytes (ATCC, USA) were seeded into 96-well cell culture plates at the density of 1 × 10^4 cells/well and incubated at 37°C with 5% CO₂ for 24 hours. These cells were then treated with different concentrations of cyclic terpene limonene (LM) and incubated for 1 day, 2 days, and 10 days; every 48 h fresh media was replaced with different concentration of LM until the end of experimental periods. Ten microliters of water-soluble tetrazolium (WST) reagent was added to each well and incubated at 37°C with 5% CO₂ for 1-2 hours. Absorbance (color intensity) of each well was measured at a wavelength of 450 nm using a Spectra Count microplate plate reader [28].

2.3. Differentiation Induction. 3T3-L1 preadipocytes were seeded into 6- and 12-well cell culture plates at a density of 3 × 10^4 and 1.5 × 10^5 cells/well, respectively, and incubated at 37°C with 5% CO₂. Every 48 h, the culture medium was replenished with fresh medium. When cells reached 100%, further incubation was performed for another two days. Growth medium was then replaced with a differentiation induction medium (DMI) (0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 1μg/mL insulin) containing LM, agonist, and antagonist [28].

2.4. Quantification of Lipid Using Oil Red O Staining. Differentiated 3T3-L1 adipocytes in 6-well plates were fixed with 2 mL of 10% formalin for 1 h and then rinsed with 40%
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Figure 2: Cytotoxic effect of LM on 3T3-L1 preadipocytes. (a) LM effect on 3T3-L1 preadipocytes viability after 24 h treatment; (b) LM effect on 3T3-L1 preadipocytes viability after 48 h treatment; (c) LM effect on 3T3-L1 preadipocytes viability after 10 days of treatment.

isopropanol. After rinsing, 3 mL of Oil Red O staining solution was added to each well. Plates were incubated at room temperature for 15 min and washed three times with distilled water. Stained cells were photographed with an inverted microscope [CKX41, Olympus Corporation, Tokyo Japan]. Additionally, Oil Red O stain was eluted with 100% isopropanol and measured at 490 nm [28].

2.5. Glucose Uptake Assay. Eight-day differentiated cells were incubated with serum-free media for 12 h. Then, serum-starved cells were treated with LM (5 μM), insulin (10 μM), and MK-2206, an antagonist for Akt (8 nM) individually for 2 h at 37°C with 5% CO2 followed by addition of 10 mM 2DG and incubated for another 20 min. Glucose uptake was then performed using glucose uptake assay kit according to the manufacturer’s protocol (glucose uptake colorimetric assay kit, Abcam, UK).

2.6. Quantification of Gene Expression Using Quantitative RT-PCR. Total RNA was extracted from experimental cells using RNeasy lipid mini kit (Qiagen, MD, USA) and quantified by Spectramax i3 (Molecular devices, CA, USA). Total RNA (500 ng) was then reverse transcribed using iScript cDNA synthesis kit (Hercules, CA, USA). Gene transcripts in the experimental samples were quantified with SYBR Green-based qPCR using specific primers for C/EBP-α (F-gcaggaggaatcaggaag, R-acagacctcaatcctccaaca); C/EBP-β (F-gtctggtgccttgtgctctac, R-acccaccacccgagaa); PPAR γ2 (F-ctgcccaactgtccttccttg, R-gtgctgcctagctgtctctg); Adiponectin (F-gctgcttcaacactgac, R-tccctcctccatcactac); FAS (F-cccacccataagttaca, R-atcgggaatcagcaaca); SREBP-1 (F-ggaatgggagagcgcct, R-tatctcctaaagctcgac); β-actin (F-ctcctcctcagctc, R-agtccacagcagttc) on a CFX 96 Real Time PCR detection system (Hercules, CA, USA). All gene expressions were normalized against housekeeping gene β-actin [28].

2.7. Protein Extraction and Immunoblotting. Proteins were extracted from experimental adipocytes with RIPA lysis buffer containing protease and phosphatase inhibitors cocktail (Roche, Switzerland and Sigma Aldrich, USA). Protein quantification was performed with the Pierce BCA protein assay kit (Thermo Fisher Scientific, MA, USA). Immunoblotting was performed with Cell Signaling Technology monoclonal antibodies [29]. The intensity of immunoreacted bands was quantified with ImageJ software version 1.49 (32 bit), (Wayne Rasband, National Institute of Health, USA).

2.8. Statistical Analysis. Experimental data were subjected to one-way ANOVA with post hoc test and multivariate comparisons with Duncan test using the statistical package of social science [SPSS-Version 16.0, SPSS, Inc., USA]. Statistical significance was considered when p value was less than 0.05.

3. Results

3.1. Cytotoxic Effects of LM on 3T3-L1 Preadipocytes. Preadipocytes were treated with different concentrations (10 μM to 300 μM) of LM and incubated for 1 day, 2 days, and 10 days. LM treatment failed to affect the cell viability significantly at a concentration of 10 μM or less. Furthermore, the increase of LM concentration slightly reduced cell viability compared to the control. However, there was no significant difference in cell viability between control and LM treatment at any concentration tested (Figure 2).

3.2. Effects of LM on 3T3-L1 Preadipocyte Differentiation. Based on microscopic observation, adipocytes treated with
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3.3. Effect of LM on mRNA Expression in 3T3-L1 Adipocytes. We next investigated how LM regulated the expression of PPARγ and C/EBPs in adipocytes. Expression levels of C/EBP-α, C/EBP-β, and PPARγ were significantly (p < 0.05) upregulated in adipocytes treated with LM at 5 μM compared to those in control adipocytes (Figure 4(a)). LM treatment at a concentration of 10 μM significantly (p < 0.05) downregulated PPARγ, C/EBP-α, C/EBP-β, SREBP-1, FAS, and adiponectin mRNA expression levels in differentiated adipocytes compared to control (data not shown).

3.4. Effect of LM on Adipogenic and Lipogenic Proteins Expression in 3T3-L1 Adipocytes. Expression levels of protein expressions related to adipocyte differentiation in adipocytes on the 10th day after treatment with LM were analyzed. Treatment with LM at 5 μM significantly (p < 0.05) increased protein levels of PPARγ, RXR, C/EBP-α, SREBP-1, and their downstream targets such as aP2, FAS, ACC, and adiponectin, compared to control adipocytes (Figure 4(b)).

3.5. Comparative Effects of LM and Rosiglitazone (RGZ) on Differentiation and PPARγ Expression. We also compared the effects of LM with those of PPAR-γ agonist (RGZ) in adipocytes. Confluent preadipocytes were treated with RGZ or LM (0.1 μM or 5 μM, respectively) for 48 h. Results showed that RGZ treatment significantly increased lipid accumulation and PPAR-γ protein expression. Similarly, LM treatment significantly increased lipid accumulation and PPAR-γ protein expression compared to control adipocytes (Figures 5(a)–5(c)).
Figure 4: (a) Impact of LM on adipogenic and lipogenic mRNA expression in 3T3-L1 adipocytes. (b) Effects of LM on adipogenic and lipogenic proteins on day 10. Confluent 3T3-L1 adipocytes were differentiated with DMI in the presence/absence of LM for 10 days. Experiments were performed in triplicate and repeated three times with the same results. Bars display mean ± SEM and statistical analysis was performed by one-way ANOVA. *p < 0.05 indicates a statistically significant difference between the control and LM treated adipocytes.

3.6. Effects of LM on Signaling Pathways. Next, signaling pathways involved in the regulation of adipocyte differentiation and lipid accumulation in control and LM treated adipocytes were investigated. LM treatment induced activation of Akt by increasing its phosphorylation at serine 473 but decreased AMPK-α phosphorylation at Thr172 in adipocytes on the 10th day compared to the control adipocytes. However, p44/42 pathway remained the same in the control and experimental adipocytes (Figure 5(d)).

3.7. A Competitive Study between LM and MK-2206 on Akt Phosphorylation. MK-2206, an antagonist of Akt, was used in this study to further evaluate the role of Akt involved in the effect of LM on adipocyte differentiation. Adipocytes treated with MK-2206 for 48 h inhibited lipid accumulation and downregulated PPAR-γ expression compared to the control adipocytes. Cotreatment with both LM and MK-2206 (Akt inhibitor) significantly (p < 0.05) increased lipid accumulation and upregulated PPAR-γ expression in
differentiated adipocytes, indicating that LM treatment could abolish MK-2206 mediated downregulation of PPAR-γ and optical density of Oil Red O stain. These data confirmed that LM treatment could increase differentiation and lipid accumulation via Akt activation by increasing its phosphorylation at serine 473 (Figures 6(a) and 6(b)).

3.8. LM on Glucose Uptake in Differentiated 3T3-L1 Adipocytes. Differentiated adipocytes were then treated with LM, insulin, MK-2206, and MK-2206+LM separately and incubated for 2 h in a CO₂ incubator after serum starvation. Insulin treatment increased 2DG uptake in 3T3-L1. Treatment with MK-2206, an antagonist for Akt, reduced glucose uptakes. LM treatment significantly enhanced 2DG uptake in 3T3-L1 adipocytes. MK-2206 + LM treatment also increased glucose uptakes as compared to control adipocytes. Overall data suggest that the LM could stimulate 2DG uptake in differentiated adipocytes through the Akt signaling pathway in differentiated adipocytes (Figure 6(c)).
Figure 6: (a)-(b) A competitive study between LM and MK-2206 on Akt phosphorylation at serine 473 during the differentiation of 3T3-L1 preadipocytes. Adipocytes were differentiated in DMI with LM (5μM), or MK-2206 (8nM), or LM + MK-2206 individually. LM alone or cotreatment of LM with MK-2206 increased PPAR-γ and Akt activation. MK-2206 alone treated adipocytes exhibited downregulation of PPAR-γ and Akt activation. (a) Western blot analysis of experimental proteins. (b) An optical density of extracted Oil Red O stains from experimental adipocytes. Experiments were performed in triplicate and repeated three times with the same results. Bars display mean ± SEM and statistical analysis was performed by one-way ANOVA. Different letters, A, B, and C, within a column indicate a statistically significant difference (p < 0.05). (c). Effects of LM on 2DG glucose uptake in 3T3-L1 adipocytes. Differentiated adipocytes in differentiation induction medium (DMI) were exposed to LM (5μM), Insulin (10μM), MK-2206 (8nM), and MK+LM separately for 2h and glucose uptake was then analyzed in 3T3-L1 adipocytes using a glucose uptake assay kit with kinetic mode (Abcam, USA). Experiments were performed in six replicates and repeated three times with the same results. Bars display mean ± SEM and statistical analysis was performed by one-way ANOVA. Significance was at p < 0.05 level between the groups.

4. Discussion

Adipogenesis is a sequential process accompanied by the dramatic increase in the expression of adipocyte genes [7]. The dysfunctional adipose tissue is characterized by reduced adipogenesis, increased cellular senescence, and inflammation. Such dysfunction impairs lipid storage and dysregulates production of adipokines and cytokines, leading to metabolic disorder, adipose tissue inflammation, insulin resistance, and type-2 diabetes [30]. An adequate adipogenesis is essential
to sequester lipids in adipose tissues to prevent ectopic fat deposition and insulin resistance development. Increases in lipid storage capacity are believed to play a vital role in metabolic regulation [31]. In the present study, LM treatment induced differentiation and lipid accumulation in adipocytes at 5th and 10th day after treatment. However, cells treated with LM at the concentration higher than 10 μM inhibited lipid accumulation with slight morphology changes compared to control.

Furthermore, we analyzed effects of LM on expression levels of C/EBP-β, PPARγ, and C/EBP-α known to be essential factors that work sequentially and cooperatively to induce differentiation and lipid accumulation in adipocytes [7]. In the present study, LM induced C/EBP-β, C/EBP-α, PPAR-γ2, and RXR expression. Crosstalks among PPAR-γ, RXR, and C/EBPα at common regulatory sites are important in the regulation of genes involved in adipocyte differentiation, for lipogenic function requires PPAR-γ/RXR heterodimers while the vital function is mediated by PPAR-γ/RXR α or PPAR-γ/RXR α [32]. C/EBP-β is mainly involved in adipocyte differentiation. It activates key transcriptional factors such as PPAR γ and C/EBP-α [33]. Upregulation of these genes was consistent with lipid accumulation results based on Oil Red O staining.

Adiponectin is exclusively secreted in fully differentiated adipocytes. It regulates many metabolic processes via enhancing insulin sensitivity in muscle and liver or by activating fatty acid oxidation in different tissues [34]. In the present study, LM treatment increased mRNA of adiponectin and their protein expression levels in differentiated adipocytes compared to control, suggesting that LM might promote the glucose uptake by cells.

PPARγ and C/EBP-α can regulate their downstream targets such as aP2, FAS, LPL, leptin, and adiponectin. These downstream targets can trigger the synthesis of fatty acids and triglycerides synthesis [5]. Adipocytes can dramatically induce lipogenesis after differentiation induction, leading to insulin sensitivity. FAS, aP2, and ACC are known to be increased 10- to 100-fold during differentiation [35]. In the present study, LM treatment increased FAS, ACC, and adiponectin levels in differentiated adipocytes as compared to the control, indicating that LM may trigger insulin sensitivity by activating adipocyte differentiation and fatty acid metabolism by regulating key transcriptional factors and their downstream targets. Adipocyte binding protein (aP2) is a key mediator involved in the transport of fatty acids and their metabolism regulation. It is regulated by PPARγ during differentiation and lipid accumulation [36]. LM significantly upregulated aP2 expression in differentiated adipocytes as compared to the control. SREBPs can enhance genes associated with cholesterol and fatty acid biosynthesis and uptake [37]. Furthermore, SREBP-1c induces PPARγ expression in adipocytes. In collaboration with C/EBP-α, SREBP-1c can activate adipocyte markers [38]. Furthermore, activation of AMPK directly phosphorylates precursor of SREBP-1c at ser 372 residue which controls proteolytic maturation and translocation of mature SREBP-1c into the nucleus which leads to reducing the expression of ACC and FAS [39–41]. The present study is indicating that LM treatment could increase mature SREBP-1c expression via AMPK-α downregulation and increased ACC and FAS expression levels which are the key enzymes involved in the lipogenesis.

The insulin signaling pathway plays a crucial role in preadipocyte differentiation [29]. Akt is particularly important in adipocyte differentiation and insulin metabolic functions [42]. Many researchers have reported that Akt regulates PPARγ and adipocyte differentiation [12, 43]. In the present study, insulin, DEX, and IBMX mixture increased Akt phosphorylation at ser 473. Furthermore, activation of Akt by increasing its phosphorylation was accelerated by LM treatment. These data suggest that activation of Akt can activate genes associated with adipocyte differentiation. It is known that Akt phosphorylation can promote adipocyte differentiation via upregulating PPAR γ [44]. In addition, cotreatment with Akt inhibitor and LM significantly increased lipid accumulation and differentiation, indicating that LM treatment could abrog inhibit of Akt phosphorylation induced by Akt inhibitor and increase phosphorylation of Akt. Taken together, these results suggest that LM might accelerate adipogenesis and lipogenesis via Akt signaling pathway in adipocytes. In the meantime, the Erk1/2 pathway remained the same in control and LM treated cells.

Finally, we examined the effect of LM on glucose uptake properties of cells because LM upregulated Akt phosphorylation at serine 473 and adiponectin level in 3T3-L1 adipocytes. It is known that insulin signaling pathway is activated under nutrient availability. GLUT-4 translocation is required for insulin-dependent PI3K/AKT activation [45]. Akt kinase, a Ser/Thr kinase, is activated by insulin and certain growth factors. Several reports have shown that Akt function is downstream of the PI3 kinase. Akt kinase critically contributes to glucose uptake and metabolism through PI3K kinase in the insulin signaling pathway [14]. In this study, adipocyte treated with LM or insulin increased glucose uptake in 3T3-L1 adipocytes. However, MK-2206, a specific inhibitor for Akt activation, caused a reduction in glucose uptake. These results confirmed that LM could enhance glucose uptake in adipocytes via activating Akt signaling pathway.

5. Conclusions

In summary, the present study showed that limonene (LM) could induce differentiation and glucose uptake in 3T3-L1 preadipocytes. LM regulated adipogenesis and lipogenesis via induction of key transcriptional factors such as PPARγ, C/EBP-α, and C/EBP-β as well as their downstream targets by activation of Akt signaling pathway. Our overall data suggest that limonene is a promising compound found in the natural products that can favor lipid storage in adipocytes and glucose uptake via the Akt signaling pathway.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.
Conflicts of Interest

The authors have no conflicts of interest to disclose.

Authors’ Contributions

Ilavenil Soundharrajan and Ki Choon Choi conceived and designed the experiments; Ilavenil Soundharrajan, Da Hye Kim, Srigopalram Srisesharam, and Palaniselvem Kuppusamy performed the experiments; Srigopalram Srisesharam and Palaniselvem Kuppusamy helped in writing the paper; Ki Choon Choi contributed reagents/materials/analysis tools. Ilavenil Soundharrajan and Da Hye Kim contributed equally to this work.

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