Trans, trans-farnesol as a mevalonate-derived inducer of murine 3T3-F442A pre-adipocyte differentiation

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Abstract

Based on our finding that depletion of mevalonate-derived metabolites inhibits adipocyte differentiation, we hypothesize that *trans, trans*-farnesol (farnesol), a mevalonate-derived sesquiterpene, induces adipocyte differentiation. Farnesol dose-dependently (25–75 μ mol/L) increased intracellular triglyceride content of murine 3T3-F442A pre-adipocytes measured by AdipoRedTM Assay and Oil Red-O staining. Concomitantly, farnesol dose-dependently increased glucose uptake and glucose transport protein 4 (GLUT4) expression without affecting cell viability. Furthermore, quantitative real-time polymerase chain reaction and Western blot showed that farnesol increased the mRNA and protein levels of peroxisome proliferator-activated receptor γ (PPAR γ), a key regulator of adipocyte differentiation, and the mRNA levels of PPAR γ -regulated fatty acid-binding protein 4 and adiponectin; in contrast, farnesol downregulated Pref-1 gene, a marker of pre-adipocytes. GW9662 (10 μ mol/L), an antagonist of PPAR γ , reversed the effects of farnesol on cellular lipid content, suggesting that PPAR γ signaling pathway may mediate the farnesol effect. Farnesol (25–75 μ mol/L) did not affect the mRNA level of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate-limiting enzyme in the mevalonate pathway. Farnesol may be the mevalonate-derived inducer of adipocyte differentiation and potentially an insulin sensitizer via activation of PPAR γ and upregulation of glucose uptake.

Keywords: Farnesol, adipocyte, differentiation, HMG CoA reductase, mevalonate, PPAR γ , glucose, GLUT4, adiponectin, FABP4

Experimental Biology and Medicine 2016; 241: 493–500. DOI: 10.1177/1535370215620855

Introduction

Adipocyte differentiation is a well-coordinated multi-step process involving several genes.^{1,2} Two transcriptional factors, peroxisome proliferator-activated receptor γ (PPAR γ) and CCAAT/enhancer-binding protein α (C/EBP α), are known as the major regulators of adipogenesis and are located at the core of the adipogenic cascade. Sterol regulatory element-binding protein 1 (SREBP-1), another key lipogenic transcription factor that produces ligands for PPAR γ , is nutritionally regulated by glucose and insulin.³ In two of the established pre-adipocyte cell lines, insulin or insulin-like growth factor 1 (IGF-1) are the main hormones required for cell differentiation.¹ Insulin induces the translocation of glucose transport protein 4 (GLUT4) vesicles from the cytoplasm to the plasma membrane by activating the phosphatidylinositol-3 kinase-protein kinase B/Akt (PI3K-PKB/Akt) pathway.⁴ Consequently, increased glucose uptake provides the main source of energy for triglyceride synthesis in adipocytes. Dysregulation of adipocyte differentiation and lipogenesis

is associated with pathological conditions such as obesity, type II diabetes, and lipodystrophy.

Our previous studies showed that mevalonate depletion mediated by lovastatin, a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase that is the rate-limiting enzyme in the mevalonate pathway, reduced the intracellular triglyceride content of murine 3T3-F442A adipocytes. Concomitantly, lovastatin downregulated the expression of Ppar γ , Cebp α , leptin, fatty acid-binding protein 4 (Fabp4), adiponectin (AdipoQ), and Srebp-1 genes. Supplemental mevalonate reversed the effects of lovastatin, suggesting that mevalonate or mevalonate-derived metabolites are essential for adipocyte differentiation.

The identity of the mevalonate-derived molecule that promotes adipocyte differentiation remains elusive, though studies have examined a number of mevalonate-derived metabolites, including farnesol and geraniol, for their roles in adipogenesis.^{5,6} The reports that farnesol, a minor constituent of basil oil⁷ shown to have hypoglycemic effect in humans⁸ and rats,⁹ and the farnesol metabolite

farnesyl pyrophosphate (FPP) activate PPAR γ ^{6,10} prompted us to examine the impact of farnesol on the differentiation of 3T3-F442A adipocytes. The effects of farnesol on glucose uptake, PPAR γ , and PPAR γ -regulated adipogenic genes including Fabbp4 and AdipoQ as well as the potential role of PI3K signaling were also examined.

Materials and methods

Chemicals

Lovastatin was a gift from Merck Research Laboratories (Rahway, NJ). Farnesol, insulin, rosiglitazone, GW9662, and LY294002 were purchased from Sigma Aldrich (St. Louis, MO). Antibodies against GLUT4 and PPAR γ were purchased from EMD Millipore (Billerica, MA), and those for calnexin and β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Culture and Oil Red-O staining of 3T3-F442A cells

Murine 3T3-F442A cells purchased from Dr. Howard Green (Harvard Medical School) were cultured in six-well (5×10^3 cells/2 mL medium/well) plates in Dulbecco's modified Eagle's medium (DMEM) adjusted by American Type Culture Collection (ATCC, Manassas, VA) to contain 4 mmol/L L-glutamine, 1.5 g/L sodium bicarbonate and 4.5 g/L glucose, supplemented with 10% bovine calf serum (Fisher Scientific Company LLC, Houston, TX), and 1% penicillin/streptomycin (GIBCO, Grand Island, NY) at 37°C in a humidified atmosphere of 10% CO₂. Upon reaching 100% confluency, the 3T3-F442A cells were switched from the growth medium above to DMEM supplemented with 10% fetal bovine serum (FBS, Fisher Scientific Company), 10 μ g/mL insulin, and 1% penicillin/streptomycin (GIBCO) and incubated for 24 h; cells were then changed to differentiation medium (DMEM with 10% FBS) supplemented with test agents. Control cells were incubated in medium containing ethyl alcohol or dimethyl sulfoxide (ATCC) (0.1%, v/v) that was used to dissolve the test agents. Where applicable, 1 μ mol/L rosiglitazone (an agonist of PPAR γ), 10 μ mol/L GW9662 (an antagonist of PPAR γ), and 10 μ mol/L LY294002 (a PI3K inhibitor) were supplemented in the medium. Cells were continued in medium without insulin for additional six to seven days until the approximated 70–80% cells differentiate into adipocytes with lipid droplets. Cells were rinsed with phosphate-buffered saline (PBS) twice and fixed in 1 mL of 10% formalin per well at room temperature for 1 h. Cells were then rinsed with water and stained with 0.5 mL of 0.3% fresh Oil Red-O per well at room temperature for 30 min to visualize cellular neutral lipids. The cells were then washed with 1 mL of water per well for three times before photomicrographs of representative fields of monolayers of 3T3-F442A cells were taken with a Nikon Eclipse TS 100 microscope (Nikon Corporation, Tokyo, Japan) equipped with a Nikon Coolpix 995 digital camera (Nikon Corporation).

AdipoRedTM assay for measuring intracellular triglyceride content

Seven to eight days after the induction of differentiation, lipid content was quantified using an AdipoRedTM assay kit (Lonza, Walkersville, MD) according to manufacturer's instructions. Differentiated cells were rinsed with 2 mL of PBS, and to each well 5 mL of PBS and 140 μ L of AdipoRedTM reagent were added. After 10–15 min of incubation, the plates were positioned in a Tecan Infinite M200 microplate reader (Tecan Systems Inc., Salzburg, Austria), and fluorescence was measured with an excitation wavelength of 485 nm and an emission wavelength of 572 nm.

Cell viability assay

Following seven to eight days of incubation with 0–75 μ mol/L, farnesol in six-well plates containing differentiation medium, detached murine 3T3-F442A adipocytes in culture medium were collected; monolayers of differentiated murine 3T3-F442A adipocytes were trypsinized and combined with floaters. The total number of cells and dead cells with trypan blue staining were counted with a hemocytometer. Viability of cells was expressed as the percentage of total cells that were excluding trypan blue staining.

Glucose uptake assay

Murine 3T3-F442A pre-adipocytes were cultured and induced to differentiate as described above. On day 4 of differentiation, the medium was changed and cells continued incubation until day 8 when an aliquot of medium was sampled along with fresh medium for the measurement of glucose concentration using a Stanbio Glucose LiquiColor kit[®] (Stanbio Laboratory, Boerne, TX). Reduction of medium volume due to evaporation during the incubation period was determined separately and compensated in calculation. The difference in glucose concentration between fresh medium and medium from day 8 was considered as cellular glucose uptake.

Quantitative real-time polymerase chain reaction

Murine 3T3-F442A cells cultured in six-well plates at 5×10^3 cells/2 mL medium/well were incubated for seven to eight days until they fully differentiated. Total cellular RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols. RNA concentration was determined using 25 A_{260 nm}/mg RNA, and purity of the isolated total RNA was determined spectrophotometrically using A_{260 nm}/A_{280 nm}; a minimal ratio of 1.9 was required for further analysis. The integrity of the purified total RNA was verified by analysis of the 28S/18S ribosomal RNA (rRNA) ratio using gel electrophoresis. Samples were run on a 1.5% agarose gel (Tris-acetate (TAE) buffer) at 80 volts for 90 min and visualized by Chemi Doc XRS imaging system (Bio-Rad, Hercules, CA) following the addition of 0.5 μ g/mL ethidium bromide.

The mRNA expression levels of Ppar γ , Fabbp4, AdipoQ, Pref-1, HMG CoA reductase, and GLUT4 were analyzed by reverse transcription followed by quantitative real-time polymerase chain reaction (qRT-PCR). Oligo (dT)₂₀ primer

and SuperScript[®] III first-strand kit (Invitrogen, Grand Island, NY) were used to reverse transcribe 2 µg of total RNA in a 20 µL reaction buffer into cDNA following the manufacturer's instructions. cDNA was diluted by 25-fold with 25 µg/ml acetylated bovine serum albumin solution, and 6 µL of diluted cDNA was amplified in a 25 µL polymerase chain reaction (PCR) solution containing 250 nmol/L of both forward and reverse primers of the gene and iQTM SYBR[®] Green Supermix (Bio-Rad). Primers (Table 1) were designed using Vector NTI Advance version 11 software (Invitrogen). The cDNA was denatured at 95°C for 3 min followed by 40 cycles of PCR (94°C for 30 s, 60°C for 25 s, 72°C for 25 s, and 78°C for 9 s) by means of an iQTM5 multi-color Real-Time PCR Detection System (Bio-Rad) with Bio-Rad iQ5 Optical System Software (version 2.1). The mRNA levels of all the genes were normalized using ribosomal protein L22 (RPL22) as internal control¹¹ and the ΔC_T method. Fold changes of gene expression were calculated by the $2^{-\Delta\Delta C_T}$ method.

Western blot

Following seven to eight days of incubation with the test agents, 3T3-F442A adipocytes were scraped and centrifuged at 1250 r/min for 5 min at 4°C. Cell pellets were washed in ice-cold PBS. Membrane fractions of cell lysates prepared as previously reported¹² were used for GLUT4 blots, and non-membrane fractions were used for PPAR γ blots. Protein concentration of each sample was determined with the BCATM Protein Assay Kit (Pierce, Rockford, IL). Samples were mixed with equal volume of buffer (62.5 mmol/L Tris-HCl, pH 6.8, 15% (w/v) SDS, 8 mol/L urea, 10% (v/v) glycerol and 100 mmol/L dithiothreitol) and 1/6 volume of the 4 × SDS loading buffer (150 mmol/L Tris-HCl, pH 6.8, 12% (w/v) SDS, 30% glycerol, 6% β -mercaptoethanol, and bromophenol blue) and incubated at 37°C for 20 min prior to loading to a 8% SDS polyacrylamide gel.

Electrophoresis with a Mini PROTEAN[®] 3 (Bio-Rad) unit and Western blot was performed as described.¹²

Statistics

One-way analysis of variance (ANOVA) tests were performed to assess the differences between groups using Prism[®] 4.0 software (GraphPad Software Inc., San Diego, CA). Differences in means were analyzed by Dunnett's multiple comparison test unless specified otherwise. Values were mean $\pm$ SD. Levels of significance were designated as $P < 0.05$.

Results

We first determined the impact of farnesol on the differentiation of murine 3T3-F442A cells. Figure 1(a) (I–IV) shows that farnesol (0–75 µmol/L) induced a concentration-dependent increase in the number of intracellular lipid droplets stained by Oil Red-O and the total amount of visible lipids. As expected, rosiglitazone, an agonist of PPAR γ , at 1 µmol/L augmented intracellular lipid content in 3T3-F442A cells (Figure 1(a)–V), whereas GW9662 (Figure 1(a)–VI), a PPAR γ antagonist, and LY294002 (Figure 1(a)–VII), a PI3K inhibitor, each at 10 µmol/L, reduced lipid content. AdipoRedTM assay confirmed the stimulatory effect of farnesol and rosiglitazone and inhibitory effect of GW9662 and LY294002 on intracellular triglyceride content of 3T3-F442A cells (Figure 1(b)).

To exclude the possible cytotoxicity of farnesol that might have led to reduced number of viable cells and hence reduced cellular triglyceride content, 3T3-F442A adipocytes, following an eight-day differentiation and concomitant incubation with 0, 25, 50, and 75 µmol/L farnesol, were harvested for total cell counts and measure of percentage of viable cells using trypan blue staining. Values for all groups were within 90–110% of the control value (0 µmol/L farnesol) with no statistically significant difference

Table 1 Primer sequences (forward and reverse) and GenBank accession numbers used in the quantitative real-time polymerase chain reaction (qRT-PCR)

Gene	Accession #	PRIMER sequence
Ppar γ	NM_001127330 and NM_011146	5'-AGAGGGCCAAGGATTCATGACCAGG-3' 5'-TTCAGCTTGAGCTGCAGTTCACAGGG-3'
Fabp4	NM_024406	5'-GTGTGATGCCTTTGTGGGAACCTGG-3' 5'-TGCGGTGATTCATCGAATTCACG-3'
AdipoQ	NM_009605	5'-CGGCAGCACTGGCAAGTTCTACTGC-3' 5'-TTGTGGTCCCCATCCCCATACACCT-3'
Pref-1	NM_001190703	5'-CCGTGCCAGAACGGGGGAC-3' 5'-CGGGGGTCAGGCGGTAGGTGA-3'
HMG CoA reductase	NM_008255	5'-GCCAGTGGTGCCTCTCCACG-3' 5'-CATGCCCATGGCGTCCCCCG-3'
GLUT4	NM_009204	5'-GAACCCCTCGGCAGCGAGT-3' 5'-ATCCGGTCCCCAGGACCTTGC-3'
RPL22	NM_009079	5'-GCGACTTTAACTGGGCTGCTGCT-3' 5'-GCCACCAACCCAGCCTCTCG-3'

Ppar γ : peroxisome proliferator-activated receptor γ ; Fabp4: fatty acid-binding protein 4; AdipoQ: adiponectin; Pref-1: pre-adipocyte factor 1; HMG CoA reductase: 3-hydroxy-3-methylglutaryl coenzyme A reductase; GLUT4: glucose transport protein 4; RPL22: ribosomal protein L22.

Figure 1 Farnesol-mediated concentration-dependent induction of 3T3-F442A pre-adipocyte differentiation. (a) Photomicrographs of Oil Red-O-stained 3T3-F442A adipocytes following a eight-day incubation with 0 (I), 25 (II), 50 (III) and 75 (IV) μmol/L farnesol, 1 μmol/L rosiglitazone (V), 10 μmol/L GW9662 (VI) and 10 μmol/L LY294002 (VII). (b) AdipoRed™ assay showing the intracellular triglyceride (TG) content of 3T3-F442A adipocytes as measured by absorbance at 572 nm. Values are mean ± SD, n > 6. Asterisks designate means that are significantly different from control value (0 μmol/L farnesol); **P* < 0.05; ***P* < 0.001. (A color version of this figure is available in the online journal.)

observed between any groups for cell number or viability (data not shown).

We then examined whether farnesol-induced 3T3-F442A adipocyte differentiation was accompanied by changes in cellular glucose uptake. Farnesol at 50 and 75 μmol/L significantly induced glucose uptake (*P* < 0.001) (Figure 2(a)). Concomitantly, glucose transporter GLUT4 mRNA level was increased by 50 (*P* < 0.05) and 75 (*P* < 0.01) μmol/L farnesol (Figure 2(b)). As a negative control, undifferentiated (UD) 3T3-F442A pre-adipocytes (UD, Figure 2(b)) had undetectable levels of GLUT4 mRNA. Consistent with these observations, Western blot showed farnesol-mediated induction of membrane GLUT4 protein expression. Lovastatin, an inhibitor of 3T3-F442A differentiation,¹² and rosiglitazone down- and up-regulated GLUT4 expression, respectively (Figure 2(c)).

Our previous finding that lovastatin-mediated downregulation of PPARγ expression was reversed by mevalonate

Figure 2 Farnesol-induced cellular glucose uptake and the levels of GLUT4 mRNA and protein in 3T3-F442A adipocytes. 3T3-F442A pre-adipocytes were incubated with 0–75 μmol/L farnesol, 1.25 μmol/L lovastatin and 1 μmol/L rosiglitazone for eight days. (a) Cellular glucose uptake in farnesol-treated groups was measured by the difference in glucose concentration between the medium in day 8 and the fresh medium. (b) At the end of eight-day incubation, differentiated cells treated with farnesol and undifferentiated cells (UD) were lysed and total RNA extracted. Cellular mRNA level of GLUT4 was measured by qRT-PCR. (c) Membrane fractions of cell lysates were subjected to SDS-PAGE and Western blot with antibodies against GLUT4 and calnexin, a marker for membrane protein. Values are mean ± SD, n ≥ 6 (A) or n = 5 (B). **P* < 0.05; ***P* < 0.001 (A) or ***P* < 0.01 (B)

supplementation¹² prompted us to examine next the effect of farnesol, a mevalonate-derived metabolite, on PPARγ expression. Consistent with the aforementioned mevalonate effect, farnesol at 50 and 75 μmol/L upregulated PPARγ mRNA level significantly (Figure 3(a)), parallel to its effect on intracellular lipid content (Figure 1), cellular glucose uptake (Figure 2(a)) and GLUT4 expression (Figure 2(b) and (C)). In contrast, UD 3T3-F442A cells had an extremely low level of PPARγ mRNA (UD, Figure 3(a)). Western blot confirmed farnesol-mediated regulation of PPARγ (Figure 3(b)). Consistent with their effects on GLUT4 expression (Figure 2(c)), lovastatin and rosiglitazone had opposite effects on PPARγ protein expression (Figure 3(b)).

Figure 3 Farnesol-induced PPAR γ mRNA and protein expression, and the PPAR γ antagonist GW9662 attenuated the effect of farnesol on cellular lipid content. 3T3-F442A pre-adipocytes were incubated with 0–75 μ M/L farnesol, 1.25 μ M/L lovastatin and 1 μ M/L rosiglitazone for eight days. (a) At the end of eight-day incubation, differentiated cells treated with farnesol and undifferentiated cells (UD) were lysed and total RNA extracted. Cellular mRNA level of PPAR γ was measured by qRT-PCR. (b) Non-membrane fractions of cell lysates were subjected to SDS-PAGE and Western blot with antibodies against PPAR γ and β -actin, a protein loading control. (c) PPAR γ antagonist GW9662 reversed the effect of farnesol on cellular triglyceride (TG) content in 3T3-F442A adipocytes. 3T3-F442A pre-adipocytes were incubated with 0, 25 and 50 μ M/L farnesol and 10 μ M/L GW9662, individually and in combination, for eight days. At the end of eight-day incubation, the intracellular TG content of 3T3-F442A adipocytes was measured by AdipoRedTM assay using absorbance at 572 nm. Values are mean $\pm$ SD, $n = 5$ (A) or $n \geq 4$ (C). Asterisks designate means that are significantly different from control value (0 μ M/L farnesol); * $P < 0.05$; ** $P < 0.01$ (a) or ** $P < 0.001$ (c)

To examine whether PPAR γ upregulation mediates the pro-differentiation effect of farnesol, we incubated 3T3-F442A pre-adipocytes with blends of farnesol and GW9662, a PPAR γ antagonist (Figure 3(c)). GW9662 at 10 μ M/L attenuated the upregulation of cellular lipid

Figure 4 Upregulation of AdipoQ and Fabp4 genes and downregulation of Pref-1 gene by farnesol. At the end of eight-day incubation, differentiated 3T3-F442A cells treated with 0–75 μ M/L farnesol and undifferentiated cells (UD) were lysed and total RNA extracted. Cellular mRNA levels of AdipoQ, Fabp4 and Pref-1 were measured by qRT-PCR. Values are mean $\pm$ SD, $n = 4$. Asterisks designate means that are significantly different from control value (0 μ M/L farnesol); * $P < 0.05$; ** $P < 0.01$

content induced by 50 μ M/L farnesol ($P < 0.001$). Though 25 μ M/L farnesol alone did not change lipid content, it attenuated the effect of 10 μ M/L GW9662.

To further confirm the effect of farnesol on PPAR γ and 3T3-F442A differentiation, we measured the mRNA levels of two PPAR γ -regulated genes, AdipoQ and Fabp4, and a pre-adipocyte-specific gene, pre-adipocyte factor 1 (Pref-1) (Figure 4). Farnesol induced the mRNA expression of AdipoQ (Figure 4(a)) and Fabp4 (Figure 4(b)); the mRNA level of both genes stayed low in UD cells. In contrast, farnesol dose-dependently downregulated the mRNA level of Pref-1, a differentiation-inhibitory gene highly expressed in UD cells¹³ (Figure 4(c)).

It has been suggested that lovastatin-mediated mevalonate deprivation interferes with insulin signaling¹⁴ and

Figure 5 PI3K inhibitor LY294002 reduced cellular triglyceride (TG) content in 3T3-F442A adipocytes, an effect not reversed by farnesol. 3T3-F442A pre-adipocytes were incubated with 0, 25 and 50 $\mu\text{mol/L}$ farnesol and 10 $\mu\text{mol/L}$ LY294002, individually and in combination, for 8 days. At the end of eight-day incubation, the intracellular TG content of 3T3-F442A adipocytes was measured by AdipoRedTM assay using absorbance at 572 nm. Values are mean $\pm$ SD, $n \geq 4$. Asterisks designate means that are significantly different from control value (0 $\mu\text{mol/L}$ farnesol); ** $P < 0.001$

reduces adipocyte triglyceride content.¹² In response, we determined the effect of an inhibitor of insulin signaling on farnesol-mediated increase in adipocyte triglyceride content. Consistent with the finding shown in Figure 1 and in contrast to the effect of farnesol (50 $\mu\text{mol/L}$), PI3K inhibitor LY294002 (10 $\mu\text{mol/L}$) significantly reduced the cellular triglyceride content of cells. Farnesol had no effect in rescuing the inhibition mediated by LY294002 (Figure 5).

Discussion

Our previous finding that lovastatin-mediated mevalonate deprivation inhibits adipocyte differentiation suggested the essential role of mevalonate. The observation that $>10 \mu\text{mol/L}$ mevalonate was required to reverse the effect of statins^{12,15} also suggested a mevalonate-derived metabolite, rather than mevalonate per se, plays a more direct role. The additional finding that mevalonate-derived non-sterols, including geraniol and with a greater potency, farnesol, attenuated statin-induced inhibition of pre-adipocyte differentiation⁵ prompted us to evaluate the role of farnesol in 3T3-F442A adipocytes, a proven model¹² to study the role of mevalonate-deprived agents in adipogenesis.

Our present study shows that farnesol concentration-dependently induces the differentiation of 3T3-F442A adipocytes. The induction of differentiation is accompanied by upregulated uptake of glucose and expression of GLUT4, a PPAR γ -regulated gene.¹⁶ Concomitantly, PPAR γ expression and other PPAR γ -regulated adipogenic genes including AdipoQ, a biomarker for insulin sensitivity,¹⁷ and Fabp4

are also upregulated. Consistent with farnesol-mediated induction of differentiation, the Pref-1 gene, a negative regulator of adipocyte differentiation,¹³ was downregulated by farnesol.

Our finding of farnesol as an adipocyte differentiation-promoting agent via PPAR γ upregulation, further confirmed by the attenuation of farnesol effect by the PPAR γ antagonist GW9662, is consistent with the observation that FPP, a downstream metabolite of mevalonate, is an endogenous PPAR γ agonist;¹⁰ in that study, the effect of FPP on 3T3-L1 differentiation was also counteracted by a PPAR γ antagonist. The concentration of FPP (1 $\mu\text{mol/L}$)—much lower than that reported herein for farnesol—required for PPAR γ activation and adipocyte differentiation suggests phosphorylation of farnesol and conversion to FPP¹⁸ as a possible mode of action for farnesol. A separate study with 3T3-L1 cells also showed that 50 $\mu\text{mol/L}$ farnesol, a level comparable to those used in the present study, activated PPAR γ in a reporter assay; farnesol had the highest potency among several isoprenoids evaluated therein in activating PPAR γ -regulated FABP4 expression.⁶ Farnesyl phosphate and FPP competed with rosiglitazone for PPAR γ binding with IC₅₀ values for ligand displacement approximating 19 $\mu\text{mol/L}$, a value much lower than that of farnesol; the latter was nevertheless more efficacious in activating PPAR γ in CV-1 cells transfected with a reporter gene,¹⁹ suggesting a possible farnesol metabolite with higher potency than those of the phosphorylated products of farnesol. In human HCT, 116 colorectal cancer cells, farnesol at 50 and 100 $\mu\text{mol/L}$ increased PPAR γ promoter activity, an effect that mediates the growth-inhibitory activity of farnesol.²⁰

Literature also suggests that farnesol and FPP may promote adipocyte differentiation through protein prenylation. The expression of geranylgeranyl pyrophosphate (GGPP) synthase, an enzyme that catalyzes the conversion of FPP to GGPP, another substrate for protein prenylation, is upregulated by 20-fold in differentiating 3T3-L1 adipocytes and by 5–20-fold in adipose tissue from ob/ob mice.²¹ Insulin, a strong promoter of adipocyte differentiation, stimulates the activities of farnesyl transferase²² and geranylgeranyl transferase II²³ and the prenylation of small G proteins including Ras,²² Rho,²⁴ Rab-3, and Rab-4²³ in 3T3-L1 adipocytes, whereas inhibitors of farnesyl- and geranylgeranyl-transferases blunt 3T3-L1 differentiation. Insulin-mediated upregulation of the mevalonate pathway is not restricted to adipocytes because both HMG CoA synthase and HMG CoA reductase were shown to be upregulated by insulin in keratinocytes.²⁵

Coincidentally, the mevalonate-, FPP- and GGPP-producing HMG CoA reductase expression is upregulated in differentiating²⁶ and differentiated²⁷ 3T3-L1 cells and adipose tissues from obese rodents²⁸ and humans.²⁹ One of the potential mediators of the mevalonate-dependent and insulin-stimulated adipocyte differentiation is the Ras protein, which requires farnesylation for its membrane attachment and biological activity. Whether other prenylated proteins mediate PPAR γ upregulation or stimulate differentiation independent of PPAR γ signaling remains elusive.

Figure 6 Farnesol did not affect the level of HMG CoA reductase mRNA. At the end of eight-day incubation, differentiated 3T3-F442A cells treated with 0–75 μmol/L farnesol were lysed and total RNA extracted. Cellular mRNA levels of HMG CoA reductase were measured by qRT-PCR. Values are mean ± SD, n = 3

Binding of insulin with its receptor activates PI3K and PKB/Akt, which have a pro-adipogenic role in adipocytes.³⁰ In contrast, inhibition of PI3K with wortmannin and LY294002 blocks the adipocyte differentiation in 3T3-L1 cells.^{30,31} Additionally, PKB/Akt-knockout mice have impaired development of adipose tissue.³² PI3K-PKB/Akt pathway also mediates insulin-induced glucose uptake by adipocytes.³³ In the basal state, only 10% of GLUT4 is present at the plasma membrane,³⁴ whereas more than 90% of GLUT4 is sequestered in the intracellular compartments and is located in “GLUT4 storage vesicle”, which are the main target of insulin-induced GLUT4 translocation.³⁵ The activation of PI3K is essential for the activation of Rac1, a Rho family of small G proteins involved in the remodeling of actin that is required for GLUT4 translocation.³⁶ We stipulated that a blend of farnesol and the PI3K inhibitor LY294002 would illustrate the role of insulin signaling in farnesol-mediated differentiation. On the contrary, the inability of farnesol to reverse the inhibitory effect of LY294002 (Figure 5) indicated that the farnesol effect was either independent of PI3K pathway or insufficient to attenuate the strong effect of LY294002. Whether farnesol acts through the insulin signaling pathway warrants further investigation.

It is interesting to note that geranylgeraniol, also a mevalonate-derived isoprenoid, activates PPARγ in 3T3-L1 cells⁶ and HCT 116 cells,²⁰ albeit with a slightly lower potency than that of farnesol. Geranylgeraniol can be phosphorylated and converted to GGPP, much like the way farnesol is converted to FPP.¹⁸ A separate study confirmed activation of PPARγ by GGPP,³⁷ again with slightly lower potency than that of FPP. However, geranylgeraniol, but not farnesol, has been shown to enhance the degradation of HMG CoA reductase³⁸ and hence suppress the production of mevalonate-derived metabolites, an action that might counteract the geranylgeraniol- or GGPP-mediated PPARγ activation. Consistent with the observation by Sever *et al.*³⁸ in fibroblasts, in our hands farnesol at concentrations up to 75 μmol/L did not affect the expression of HMG CoA reductase mRNA (Figure 6) following a seven-day incubation with 3T3-F442A adipocytes. The lack of negative

impact on HMG CoA reductase may have rendered farnesol a stronger inducer of adipocyte differentiation than geranylgeraniol. In human HepG2 hepatoma cells and Caco-2 colon carcinoma cells, troglitazone-induced PPARγ activation led to downregulation of SREBP-2 and HMG CoA reductase mRNA and SREBP-2 protein levels.³⁹ What contributes to the lack of impact of farnesol on HMG CoA reductase despite its PPARγ activation, or whether the PPARγ-HMG CoA reductase relationship is cell type-specific remains obscure.

It should be noted that the regulation of adipogenesis in established cell lines such as the 3T3-F442A cells used in the present study may be different from those in primary stromal-vascular cells due to diverse exposures to hormone and growth factors.⁴⁰ The murine origin of 3T3-F442A cells may also limit the applicability of current findings in humans.

In summary, we propose that farnesol and its metabolite, FPP, serve as mevalonate-derived adipogenic mediators in activating—directly or indirectly via farnesylated Ras—PPARγ signaling to induce glucose uptake and adipocyte differentiation. By enhancing the role of PPARγ in adipocyte differentiation, farnesol and other PPARγ-activating sesquiterpenes⁴¹ found in plant-based diets may prove to be promising insulin sensitizers. The mevalonate pathway may turn out to be a valid target in interventions for obesity, diabetes, and glucose homeostasis.

Authors' contributions: Both authors participated in the design, execution, and interpretation of the studies, analysis of the data, and writing and review of the manuscript.

ACKNOWLEDGEMENTS

This work was supported by Texas Department of Agriculture Food and Fiber Research Program and Texas Woman's University Research Enhancement Program. The authors thank Dr. Nathaniel Mills and Ms. Hoda Yeganehjoo for technical assistance, and Dr. Brian W. Beck for BLAST search.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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(Received August 18, 2015, Accepted November 9, 2015)