The flavonoid glycoside vaccarin inhibits adipogenesis and stimulates lipolysis via Hedgehog signaling in 3T3-L1 adipocytes

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Abstract

Vaccarin, a flavonoid glycoside isolated from Vaccaria segetalis, is non-toxic to 3T3-L1 cells up to concentrations of 200 μM. Accordingly, we investigated the effects of this natural product on adipogenesis and lipolysis in 3T3-L1 adipocytes. Our results revealed that vaccarin significantly inhibited lipid accumulation by suppressing the adipogenesis-related transcription factors peroxisome proliferator-activated receptor γ (PPARγ) and the CCAAT/enhancer-binding protein α (C/EBPα). Specifically, lipid accumulation decreased by up to 27.7 ± 2.7% when 3T3-L1 adipocytes were treated with a 10 μM concentration of vaccarin. Mechanistic studies showed that the compound inhibited adipogenesis through activation of the Hedgehog (Hh) signaling pathway and so restoring Smo and Gli1 expression at an early stage of differentiation. In mature 3T3-L1 cells, vaccarin significantly increased the secretion of glycerol into the surrounding medium and thus indicating that it accelerated the degradation of triglycerides. In addition, vaccarin, was shown to enhance lipolysis through stimulation of the transcription levels of lipoprotein lipase, monoglycerides lipase, adipose triacylglyceride lipase, hormone-sensitive lipase and adipose differentiated-related protein. All told, vaccarin suppressed lipid accumulation and enhanced lipolysis during adipocyte differentiation by restoring Hh signaling. As such, it is a phytochemical capable of halting adipocyte hyperplasia and, thereby, ameliorating the effects of obesity.

Keywords: Adipogenesis, Lipolysis, Hedgehog signaling, Vaccarin, 3T3-L1 adipocytes
Introduction

Obesity is a worldwide public health problem and closely associated with metabolic complications that can increase the risk of diabetes, cardiovascular diseases, chronic inflammation and cancer [1–3]. It results from in vivo hyperplasia and/or hypertrophy (an increase in the number or volume of adipocytes, respectively) each of which contributes to the accumulation of fat in body tissue [4, 5]. Due to the constancy of adipocyte numbers in adults, hypertrophy is often viewed as the main cause of such accumulations and correlates directly with excessive body mass index (BMI) values and metabolic disease. However, hyperplasia is now also considered to play a pivotal role in the development of obesity as a result of overfeeding in normal-weight adults. Within adult humans, adipocytes encountered within white adipose tissue accumulate at an annual rate of ca. −10% and thus suggesting that hyperplasia contributes to the development of obesity in later life [6]. Preadipocyte hyperplasia acts to counter hypertrophy and so maintain adipose tissue homeostasis [7]. Accordingly, targeting preadipocyte differentiative capacity is considered an important approach to the prevention of obesity and, thence, metabolic disease. During such differentiation, activation of the glucocorticoid receptor, that incorporates the CCAAT-enhancer binding protein (C/EBP) β, C/EBPδ and the cyclic (c)AMP-response element-binding protein, triggers the adipogenesis-related transcription factor C/EBPα and the peroxisome proliferator-activated receptor (PPARγ). As a result, an adipogenic differentiation program is initiated [6]. The resulting (hyper)activation of C/EBPα and PPARγ accelerates hyperplasia. Various studies have demonstrated that multiple pathways, including Wnt, Bone Morphogenetic Protein (Bmp) and Hedgehog (Hh) signaling, are involved in the regulation of adipogenesis [7–10]. Hh signaling is evolutionarily conserved and plays a pivotal role in homeostasis. Further, there are accumulating evidences that activation of the Hh signaling axis can suppress adipogenic differentiation at the preadipocyte stage [11–13].

Hedgehog signaling is notable for its regulation of embryogenesis and tissue regeneration after injury [13–15]. The canonical Hh signaling pathway starts with interaction of the so-called Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh) ligands with the membrane receptor Patched (Ptc). On binding, the G protein-coupled receptor Smoothenened (Smo) is released from Ptc and activates a downstream, glioma-associated oncogene homolog Gli [15]. The activated Gli translocates into the nucleus and then turns on the relevant/target genes. Multiple studies have demonstrated that inhibition of Hh signaling occurs during fat formation in invertebrates and vertebrates [11, 16, 17]. So, for example, in aP2-Sufu mice and fat-specific Hh-activation mutant mice, a total loss of white but not brown fat was observed [18]. Furthermore, it has been shown that constitutive activation of Smo and Gli2 prevented obesity in adult mice with a high-fat diet intervention [19]. Furthermore, activation of the Hh pathway altered adipocyte morphology as well as insulin sensitivity and suppressed adipogenesis by targeting PPARγ and C/EBPα expression that is involved in reducing lipid accumulation and insulin sensitivity [12].

Numerous herb-derived phytochemicals have been shown to moderate adipogenesis [20–22]. For example, vaccarin (Figure 1A), a glycosylated flavonoid isolated from Vaccaria segetalis, has been shown to do so amongst its range of notable activities. So, Fei et al have reported that the compound can attenuate vascular endothelial dysfunction associated with diabetic angiopathy by interfering with the ROS/AMPK/miRNA-34a/eNOS signaling cascade [23]. On the other hand, vaccarin administration exerted anti-hypertensive effects in renovascular hypertensive model rats [24]. Furthermore, vaccarin also displayed anti-inflammatory and apoptotic activity [25], induced osteolysis [26], promoted endothelial cell proliferation in vitro and in vivo [27, 28], as well as ameliorating insulin resistance and steatosis through activation of the AMPK signaling pathway [29]. However, the effect of vaccarin on adipogenesis and its possible mode of action in 3T3-L1 cells (ones that can differentiate into an adipocyte-like phenotype) has not been investigated. Accordingly, in this study, the capacity of vaccarin to regulate adipogenic differentiation and lipid accumulation in such cells was pursued and the mechanism by which it activates the Hh signaling pathway has been examined. This research will illustrate the mechanism action of vaccarin for anti-adipogenesis and provide a theoretical guide for vaccarin applied in anti-obesity treatment.

Figure 1 Vaccarin (VAC) suppresses lipid accumulation in MDI-treated 3T3-L1 adipocytes. A. Structure of vaccarin. B. 3T3-L1 cells (5000 cells/well in a 96-well plate) were treated with the indicated concentrations of VAC for 24 h, and cell viability was assessed using the MTT assay. C. D. 3T3-L1 cells (1 × 10^5 cells/well in a 6-well plate) were treated with VAC (1, 5, 10 μM) for 8 days and then stained with Oil Red O and bodipy, respectively; lipid droplets were visualized and quantified. Values represent the mean ± SD (n = 3). Different lowercase letters indicate statistically significant differences at P < 0.05 (one-way analysis of variance followed by Duncan’s test).
Materials and Methods

Reagents and chemicals
Vaccarin (Figure 1A), of 98% purity as determined by HPLC analysis, was purchased from Chengdu Alfa Biotechnology Co. Ltd (Chengdu, China) while Oil Red O, IBMX and DEX and dimethyl sulfoxide (DMSO) were all obtained from Sigma-Aldrich (St. Louis, MO, USA). The Dual-Luciferase Reporter Assay System was obtained from Promega (Madison, WI, USA). The primary antibody against Gli1 was obtained from Cell Signaling Technology (Danvers, MA, USA) while those against PPARγ and C/EBPα were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody against Smo was purchased from Abcam (San Francisco, CA, USA) while β-actin and horse radish peroxidase-conjugated anti-rabbit secondary antibody were secured from Chendu NB Biolab Co., Ltd (Chengdu, China).

Cell culture
The Shh-Light 2 cell line reliably expressing Gli1-dependent firefly luciferase and constitutively expressing Renilla luciferase was cultured with Dulbecco’s modified Eagle’s medium (DMEM, ex. Thermo Fisher Scientific) supplemented with 10% newborn calf serum (NBCS) and 1% penicillin/streptomycin (Life Technologies, Carlsbad, CA, USA). The 3T3-L1 cell line was purchased from Cell Biology Inc (Shanghai, China) and cultured in DMEM supplemented with 10% NBCS (Thermo Fisher Scientific), penicillin (100 U/mL; Thermo Fisher Scientific) and streptomycin (P/S, 100 μg/mL; Thermo Fisher Scientific) at 37 °C and 5% CO₂.

Cell viability assay
Cells were incubated in the absence or presence of the test samples at concentrations of 10, 50, 100, 150 or 200 μM for 20 h and treated with 20 μl aliquots of MTT (3-(4,5-dimethylthiazol-2-yl)-5,5′-diphenyltetrazolium bromide (MTT)) (5 mg/mL) then subject to continuous incubation for 4 h at 37 °C. After removal of the culture medium, the formazan crystals were dissolved in DMSO (200 μL) and absorbance measured, quantitatively, at 570 nm using a microplate reader (BioTek, Winooski, VT, USA). The percent cell viability was determined by comparing the absorbance of the test sample with that observed for the control sample treated with the vehicle (DMSO) alone (this being defined as 100%).

Adipocyte differentiation
3T3-L1 cells were seeded in a 6-well plate (1.5 × 10⁵ cell/well) then incubated at 37 °C with 5% CO₂ in DMEM supplemented with 10% NBCS, 1% P/S until they reached confluence (4 days). Adipocyte differentiation was then induced, over a 2-day period using 3-isobutyl-1-methylxanthine (0.5 μM), dexamethasone (1 μM) and insulin (10 μg/mL of a M1D cocktail) in DMEM supplemented with 10% fetal bovine serum (FBS), 1% P/S. Thereafter, the cells were incubated with insulin (10 μg/mL) in DMEM/10% FBS/1% P/S for 4 days with one medium change during this period.

Oil red O staining
Cells were stained with Oil Red O as previously described [30]. Briefly, the cells were washed twice with phosphate-buffered saline (PBS, pH 7.4) and fixed in 10% formalin for at least 1 h at ambient temperatures. After washing with 60% iso-propanol, the cells were stained for 15 min at ambient temperatures with freshly diluted Oil Red O working solution that had been prepared by mixing an Oil Red O stock solution (0.35% Oil Red O in iso-propanol) with H₂O at a 3:2 v/v ratio followed by passage through a 0.22-μm filter. After washing four times with distilled water, stained cells were visualized under a microscope (Olympus, Tokyo, Japan). The lipid droplets were dissolved in 100% iso-propanol and the absorbance of the resulting solution measured at 500 nm.

Bodipy staining
After terminal differentiation of 3T3-L1 cells, the medium was removed and washed with PBS buffer. Boron-dipyrromethene (Bodipy, Sigma-Aldrich) staining solution (500 μM) was added and the resulting mixture allowed to stand in the absence of light at room temperature for 0.5 h. Thereafter, the cells were fixed by treatment with 4% paraformaldehyde for 0.5 h after filtration of the bodipy-staining solution. 4',6-Diamidino-2-phenylindole (DAPI, 1 mg/mL) was then used to stain the cellular nuclei and any fluorescence detected using a fluorescence microscope at 400 × magnification (Leica, DM2500, Berlin, Germany).

Dual-luciferase assay
Shh-Light 2 cells (1 × 10⁵ cell/96-well plate) were treated with varying concentrations of vaccarin in DMEM for 24 h after overnight serum starvation [Shh ligand treatment (100 ng/mL) was employed as the positive control] [30]. The cells were then harvested in 1 × lysis buffer (30 μL, Promega) for 0.5 h and the firefly and Renilla luciferase activities then measured, following the suppliers’ protocols, using a dual luciferase kit and a Veritas luminometer (Turner Biosystems, Sunnyvale, CA, USA).

Quantitative real-time PCR
RNA was extracted from cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed using a PrimerScript RT reagent kit (Thermo Fisher Scientific). Real-time PCR was carried using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with a reaction mixture containing cDNA (200 ng) and appropriate synthetic primers (see Table 1) in iQ5sybrGreen Supermix (20 μL; Bio-Rad, Hercules, CA, USA). Gene expression levels were normalized to that of β-actin and relative quantification was performed using the comparative 2−ΔΔCT method.

Western blotting
The total protein content of each sample was extracted using radioimmunoprecipitation assay buffer (RIPA buffer, Thermo Fisher Scientific) containing a 1% protease/phosphatase inhibitor cocktail (Amresco, Solon, OH, USA). Equivalent amounts of protein samples were isolated by SDS-PAGE as previously described [9] then transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA) that was probed with the appropriate primary and secondary antibodies. Protein expression levels were determined using EZWestLumi plus luminol substrate and the EZ capture MG chemiluminescence imaging system (ATTO, Tokyo, Japan).

Transfection
3T3-L1 cells (1 × 10⁵ cells/3.5 cm glass bottom dish) were transfected with Gli1-EGFP vector using Transfert®-L-T1 transfection reagent (Mirus Bio, Beijing, China) in serum-free medium for 24 h. The medium was then replaced with culture medium. The levels of Gli1 were determined using a fluorescence microscope with 400 × magnification (Leica, DM2500, Berlin, Germany).

Confocal Microscopy Assays
3T3-L1 cells (1 × 10⁵ cells/3.5 cm glass bottom dish) were transfected with Gli1-EGFP vector for 48 h at the MDI cocktail treatment stage. After removal of the medium by suction, the cells were then fixed with 4% paraformaldehyde for 0.5 h. The fixed cells were then blocked by DAPI mounting solution for nuclear staining (20 min, avoiding light). Finally, pictures were taken with a confocal microscope at 400 × magnification (ZEISS, Oberkochen, Germany).

Lipolysis Assays
Lipolysis was measured by determining the glycerol released from the adipocytes into the surrounding medium. To investigate the lipolytic effects of vaccarin on fully matured 3T3-L1 adipocytes, the 3T3-L1 preadipocytes were subjected to adipocyte differentiation protocols and then challenged with varying concentrations of vaccarin for 48 h. The concentration of glycerol in the surrounding media was then determined as described previously [31]. Briefly, the supernatant medium of each sample (50 μL) was collected into 96-well plate and working solution (150 μL) was added into each well for 15 min

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incubation (37 °C). Then the absorbance of each sample was measured at 550 nm and the quantification of glycerol was calculated based on the standard curve.

Statistical analyses
Data are presented as mean ± standard deviation (SD) and were analyzed using SPSS v.21 software (IBM, New York, NY, USA). All trials repeated triplicated. Differences between groups were evaluated by one-way analysis of variance using Duncan’s post-hoc test, and were considered statistically significant at P < 0.05.

Results
Vaccarin blocks lipid accumulation in 3T3-L1 differentiated adipocytes
As a key prelude to the present study, the cytotoxicity of vaccarin toward 3T3-L1 preadipocytes was determined using an MTT assay and thereby establishing that it had no effect, over a 24 h period, on cell viability until a concentration of 200 μM was reached (Figure 1B). Accordingly, a maximum 10 μM concentration of vaccarin was selected for all subsequent experiments. Lipid accumulation in 3T3-L1 preadipocytes occurring during differentiation induced by MDI treatment was evaluated by Oil Red O staining after vaccarin treatments at concentrations of 1, 5 or 10 μM. The number of lipid droplets decreased notably with the administration of vaccarin at the higher concentrations. (Figure 1C). In particular, at the highest concentration (of 10 μM) lipid droplets numbers were 72.3 ± 2.7% of the MDI control value (100%). Further, body size staining revealed that the fluorescence density of the lipid droplets decreased as the concentration of the administered vaccarin increased (Figure 1D). As such, these results demonstrate that vaccarin inhibits lipid accumulation during differentiation of 3T3-L1 preadipocytes.

Vaccarin suppresses the expression of adipogenesis-related transcription factors in adipocytes
PPARγ and C/EBPα are key adipogenesis-related transcription factors involved in adipocyte differentiation. In the MDI (only) treated positive control group of 3T3-L1 preadipocytes, the mRNA level of PPARγ and C/EBPα increased ca. 1.6 and 1.8-fold, respectively, while vaccarin treatment reverses this (Figure 2A). Vaccarin treatment also suppressed PPARγ and C/EBPα protein expression normally induced by MDI. Specifically, at the highest vaccarin concentration these decreased to 46.9 ± 3.4% and 43.2 ± 5.3%, respectively, relative to the control group (Figure 2B) and thus suggesting that the compound inhibits the key adipogenesis-related transcription factors involved in adipocyte differentiation. As a result, lipid accumulation is diminished.

Table 1 The primer sequences used in this study

<table>
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<th>Reverse: 5'-3'</th>
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<td>5'-AAGGCTGTTTACCTGCCAG-3'</td>
</tr>
<tr>
<td>Ptc1</td>
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<td>5'-GGTCTCTTTGCTGCCTGT-3'</td>
</tr>
<tr>
<td>Smo</td>
<td>5'-TCGTTTTTATAGGTTGAAGAGG-3'</td>
<td>5'-CGAAGCTGTCTCAACCGCTG-3'</td>
</tr>
<tr>
<td>PPARγ</td>
<td>5'-CTCCAAAGATACAAAGTGCGA-3'</td>
<td>5'-GCCGATGTCTATCCACACCA-3'</td>
</tr>
<tr>
<td>C/EBPα</td>
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<td>5'-GTCACGTGTCAGCTCAGCAC-3'</td>
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<tr>
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</tr>
<tr>
<td>ADRP</td>
<td>5'-GACCCTTGCTTCGCCTTAT-3'</td>
<td>5'-CAACGGCAATTTGGCTGC-3'</td>
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</table>

Figure 2 Vaccarin suppresses the expression of adipogenesis-related transcription factors in adipocytes. A. B. 3T3-L1 cells (1.5 × 10^5 cells/6-cm dish) were treated with VAC (1, 5, 10 μM) for 8 days and mRNA levels of the adipogenesis-related transcription factors PPARγ and C/EBPα were evaluated by quantitative RT-PCR (A), and protein levels were determined by Western blotting, with β-actin serving as a loading control (B). Values represent mean ± SD (n = 3). Different lowercase letters indicate statistically significant differences at P < 0.05 (one-way analysis of variance followed by Duncan’s test).
Vaccarin reverses the inhibition of Hh signaling during adipogenesis

Previous studies have demonstrated that inhibition of Hh signaling occurs during adipogenic differentiation [32, 33], and so we sought to determine the effect of both the Hh agonist SAG (1 μM) and the antagonist vismodegib (5μM) on lipid accumulation in 3T3-L1 cells (Supplementary Figure 1). In keeping with expectation, we find that lipid production decreased significantly after treatment with SAG. We also examined whether vaccarin can activate Gli1 transcriptional activity in Shh-Light II cells and observed that administration of the compound increased such activity 3-fold (relative to the control) and exerted a similar effect to the Shh ligand that promotes Hh signaling (Figure 3A). The mRNA levels of Smo and Gli1 were reduced, relative to the control (100%), by 42.9 ± 1.2% and 48.7 ± 2.8% in the MDI (only) treated group but these were reversed by vaccarin administration (Figure 3B). Specifically, 10 μM vaccarin treatment resulted in transcriptional levels of Smo and Gli1 being increased 1.8 and 2.5-fold, respectively, relative to those observed in the MDI (only) treated cells. In keeping with such observations, the protein levels of Smo and Gli1 were inhibited by MDI treatment but raised, in a dose-dependent manner, by vaccarin, a trend also observed on administration of SAG (Figure 3C). Furthermore, in cells treated with vaccarin (at 10 μM), the expression of Smo and Gli1 was enhanced by 272.7 ± 5.3% and 256.4 ± 13.9%, respectively, relative to the those simply induced by MDI alone. In addition, for those cells co-treated with vaccarin and vismodegib, lipid accumulation increased and so further demonstrating that the former compound inhibits adipogenesis through activation of Hh signaling (Figure 3D). Notably, the impact of vaccarin on the inhibition of PPARγ and C/EBPα was significantly reduced when the cells were co-treated with vismodegib (Figure 3E).

These results indicate that Hh signaling is suppressed during adipogenesis but that this can be reversed by vaccarin and so decreasing lipid accumulation.

Hh signaling inhibits early-stage lipid accumulation during adipogenic differentiation

To confirm the participation of the Hh signaling pathway in the various stages of adipogenic differentiation, the Gli1-EGFP vector was transfected into 3T3-L1 cells using the protocols outlined in Figure 4A. The oil red O staining results then revealed that the most effective protocol for inhibiting lipid droplet formation. So, for those cells continuously transfected with the Gli1-EGFP vector from day 2 until the end-point (day 10), lipid accumulation was 67.8 ± 12.0% of the MDI-treated control group (representing 100%). Gli1 vector transfection undertaken between days 4 and 6 resulted in superior inhibition of oil droplet accumulation (77.3 ± 5.0%) compared to those observed at later stages – administration between days 6 and 8 or between days 8 and 10 resulted in inhibitions of 88.2 ± 2.0% and 98.1 ± 9.0%, respectively (Figure 4B). These data indicate that Hh signaling inhibits lipid accumulation at the earlier stages of terminal differentiation in 3T3-L1 cells. When 3T3-L1 cells were transfected with the Gli1-EGFP vector at stage 1 and co-treated with vaccarin (10 μM) then confocal microscopy revealed that the fluorescence of Gli1 was significantly decreased in MDI proceeding, while vaccarin increased Gli1 expression in stage II (Figure 4C). These results suggest that vaccarin activates Hh signaling in 3T3-L1 cells at an early stage of differentiation.

Figure 3 Vaccarin (VAC) reversed the inhibition of Hedgehog signaling during adipogenesis in adipocytes. A. SHF light-2 cells (10,000 cells/well in a 96-well plate) were treated with VAC (1, 5 and 10 μM) or Shh (100 ng/mL) for 24 h. The transcriptional activity of Gli1 was evaluated with the luciferase assay. B. 3T3-L1 cells (1.5 x 10^4 cells/6 cm dish) were treated with VAC (5 and 10 μM) as well as VIS (5 μM), SAG (1 μM) or VAC/SAG for 8 days, and mRNA levels of Smo and Gli1 were assessed by RT-PCR and normalized using β-actin. C. 3T3-L1 cells (1.5 x 10^5 cells/6 cm dish) were treated with VAC (5 and 10 μM) as well as SAG (1 μM) for 8 days, and the protein levels of Smo and Gli1 were assessed by Western blotting, with β-actin serving as a loading control. D. E. 3T3-L1 cells (1.5 x 10^5 cells/well in a 6-well plate) were co-treated with VAC and vismodegib for 8 days. Cells were then stained with Oil Red O (D) and the protein levels of Gli1 and Smo were analyzed by Western blotting (E). Lipid droplets were visualized and quantified. Shh and SAG were used as positive controls in these experiments. Values represent mean ± SD (n = 3). Different lowercase letters indicate statistically significant differences at P < 0.05 (one-way analysis of variance followed by Duncan’s test). VIS: Vismodegib.
Vaccarin activates Hh signaling at the differentiation stage in 3T3-L1 cells

To investigate the effect of vaccarin on Hh signaling during adipogenic differentiation in 3T3-L1 cells, the compound was administered at varying time points during the differentiation process (Figure 4A). Using a vaccarin concentration of 10 μM, then the levels of lipid accumulation observed under a stage I administration regime (continuous administration between days 2 and 10) were superior to those observed under stage II (days 4-6 only administration), stage III (days 6-8 only administration) and stage IV (day 8-10 only administration) regimes (Figure 5A). However, amongst the two-day treatment regimes, the best one was the stage II (4-6 days) form and so indicating that vaccarin is most effective when administered during the earlier (but not the earliest) stages of differentiation. On administering the compound at stages I and II, lipid accumulation decreased to 64.8 ± 7.0% and 70.1 ± 13.0%, respectively, compared to the MDI-(only)-treated group (100%) (Figure 5B). All told, stage II appears to be the key timeframe within which to inhibit Hh signaling and so diminishing lipid formation most effectively in 3T3-L1 cells. Notably, at stage IV, there was no significant difference in lipid droplet formation compared with the positive control (viz. MDI-(only)-treated group). Given these results, the levels of mRNA associated with Hh signaling and the levels of the adipogenesis-related transcription factors were only determined for stages II (days 4-6) and III (days 6-8) (Figure 5B). Consistent with oil red staining results, this revealed that restoration of Smo and Gli1 started from MDI-stage treatment (stage II), while inhibition of PPARγ and C/EBPα was more pronounced at stage III. These results provide further evidence that vaccarin inhibits adipogenesis via regulation of Hh signaling at the early-stages of terminal differentiation.

Vaccarin stimulates lipolysis in mature 3T3-L1 adipocyte

We have also investigated the effect of vaccarin on lipolysis in mature 3T3-L1 cells. As revealed in Figure 6A, vaccarin enhanced glycerol eflux in 3T3-L1 adipocytes in a dose-dependent manner. To begin, compared to the control group, the eflux of glycerol in the MDI-(only)-treated group increased from 1.52 ± 0.17 to 2.54 ± 0.19 μmol/μg of protein in the culture medium (an increase of 67.1%). In contrast, treatment of the mature cells with vaccarin (10 μM) resulted in glycerol release levels of 3.48 μmol/μg of protein, clearly indicating that the compound accelerates lipolysis in mature adipocytes. In keeping with expectations for the phenotype, the levels of lipoprotein lipase (LPL), monoglycerides lipase (MGL), adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL), and adipose differentiated-related protein (ADRP)-associated mRNA in the cells were raised by 103.6% on being treated with 10 μM vaccarin (Figure 6B). Consistent with these results, at 1 μM concentrations SAG also enhanced glycerol eflux in 3T3-L1 adipocytes as well as the transcription of the lipolysis-related genes (Figure 6A and B). The effect of vaccarin on Hh signaling in mature 3T3-L1 adipocytes was also determined (Figure 6C) and shown to dramatically enhance lipolysis (via hedgehog signaling in such cells).
Figure 5 Vaccarin-activated Hedgehog signaling at the early stage of differentiation in 3T3-L1 cells. A. 3T3-L1 cells (1.5 × 10⁵ cells/well in a 6-well plate) were treated with VAC (10 μM) at different stages and stained with Oil Red O; lipid droplets were visualized and quantified. B. the levels of mRNA associated with Hh signaling (Gli1, Smo and Pich) and adipogenesis-related transcription factors (PPARγ and C/EBPα) were determined after treatment with VAC (5, 10 and 15 μM) at different stages by quantitative RT-PCR. Values represent mean ± SD (n = 3). Different lowercase letters indicate statistically significant differences at P < 0.05.

Figure 6 Vaccarin-stimulated lipolysis through Hedgehog signaling in 3T3-L1 adipocytes. 3T3-L1 cells (1.5 × 10⁵ cells/well in a 6-well plate) were cultured for 8 days and then treated with VAC (5, 10 μM) for 48 h. A. the glycerol levels within the medium were determined as described in the materials and methods section. B. the mRNA levels associated with LPL, MGL, ATGL, HSL and ADRP were measured by quantitative RT-PCR. C. the mRNA levels associated with Gli1 and Smo were determined using quantitative RT-PCR. Values represent mean ± SD (n = 3). Different lowercase letters indicate statistically significant differences at P < 0.05.
Discussion

Vaccarin, a flavonoid mainly found in Vaccaria segalensis, has been shown to prevent diabetic angiopathy, hypertension, insulin resistance and steatosis both in vitro and in vivo [23, 24, 29]. Given that such diseases are linked to obesity, in which there is an imbalance of energy intake and expenditure, then this compound might be expected to suppress adipogenesis and/or stimulate lipolysis and so exerting anti-obesity effects. In keeping with such expectations, the present study reveals that vaccarin reverses adipogenic differentiation in 3T3-L1 preadipocytes and without toxic effects at concentrations up to 200 μM (Figure 1). When administered during the earlier stages of the differentiation of 3T3-L1 preadipocytes, vaccarin both restores Hh signaling and limits lipid accumulation (Figure 3 and 5). Post-differentiation, vaccarin stimulates lipolysis in the mature 3T3-L1 adipocytes (Figure 6). As such, vaccarin is a phytochemical with the potential to alleviate obesity and treat obesity-associated metabolic disease.

Extracellular signaling pathways known to affect adipogenesis in vitro include Wnt, Hh and bone morphogenetic protein (BMP) [33, 34]. In a recent porcine cohort study, the concentration of Hh-interacting protein (Hhip), a negative regulator in tissue remodeling, was determined to be 6.5± 4.66, 5.79 ± 4.33, and 3.97 ± 3.4 ng/ml in normal weight, overweight, and obese groups, respectively. This suggests that Hh signaling exerts anti-obesity effects [32]. Other studies suggest that Hh signaling responds, in a dynamic manner, to the adipogenesis and osteoblastogenesis of mouse mesenchymal cells by suppressing C/EBPα and PPARγ [33, 35, 36]. Further, the activation of Hh signaling is involved in bone formation (osteogenesis) and the inhibition of adipogenic differentiation [37]. Indeed, during adipogenesis, Hh signaling is suppressed and Hh antagonists such as cyclopamine and vismodegib exert no effect on the formation of lipid droplets in 3T3-L1 cells [9, 38]. Conversely, the Hh agonists SAG and purmorphamine as well as Hh ligands attenuate adipogenesis and so resulting in a decrease in the accumulation of lipid droplets [39] (Supplementary Figure 1A). Multiple natural phytochemicals have been shown to inhibit adipogenesis by regulating Hh signaling [9, 22]. In present study, vaccarin has been shown to significantly promote Hh signaling through the increased expression of the Smo and Gli1 components (Figure 3) and thus showing a similar behavior to SAG.

Our previous study established that the inhibition of Hh signaling occurs mainly at early-stage differentiation in 3T3-L1 preadipocytes while in mature 3T3-L1 cells the activation of Hh signaling only slightly reduced the formation of lipid droplets during adipogenesis [9]. In the present study it has been shown that Gli1 expression and nuclear translocation are significantly reduced at the differentiation but not the mature stage (Figure 4C). In investigating the effects of vaccarin on adipogenesis, we have shown that the activation of Hh signaling starts upon MDI treatment (Figure 5B) and, through a morphological study, that lipid droplet formation significantly decreased upon early-stage administration of the compound (Figure 5A). As such, Hh signaling plays a pivotal role in mediating adipogenesis, most notably during the differentiation of preadipocytes. However, the adipogenic translated genes associated with PPARγ and C/EBPα changed significantly after stage II (Figure 5B) and thus indicating Hh signaling was affected before alteration of these adipogenic genes.

Hh signaling also been shown to play a vital role in the lipid metabolism of body fat in the common fruit fly Drosophila [40]. However, the relationships between Hh signaling and lipid metabolism are multifaceted with the appearance of the Hh ligands such as Shh, Ihh and Dhh transporting lipids moieties in ways that alter during the course of cellular maturation [41]. Fatty acids and cholesterol can modify Hh ligands such that these display a higher affinity for the cellular membrane and for localized lipid-raft micro-domains and triggering Hh signaling transduction [41]. The activation of Smo is dependent on the levels of phospholipid phosphatidylinositol-4 phosphate (P4P), a polar lipid largely synthesized and localized to the Golgi membrane [42]. Conversely, the activation of Hh signaling can increase the levels of P4P controlled by Pch1 [43]. Indeed, the genetic or pharmacological modulation of Hh signaling can have impacts on Ca2+ signaling, on cytoskeletal rearrangement, on axon guidance and in adipose tissue as well as on skeletal muscle metabolism. This can occur through either canonical or non-canonical Hh signaling [41]. Worthy of mention, aP2-Sufu−/− mice showed a unique lipidoprotein phenotype without any signs of metabolic dysfunction and retaining white adipose tissue [18]. Aggressive metabolism within mature adipocytes is observed in mice fed three times more glucose than a control group comprised of their wild-type counterparts [18].

In this research, the administration of vaccarin accelerated the metabolism of lipids in mature 3T3-L1 adipocytes. During lipolysis, lipases act on TAG and release glycerol. Hh signaling has also been shown to regulate the levels of ATGL, a key lipase involved in the conversion of triglycerides into diglycerides and so promoting lipolysis in adipose tissue [44]. Vaccarin also can enhance the transcription of ATGL in mature 3T3-L1 adipocytes, HSL and MGL are lipases that act on diglycerides and monoglycerides [45]. As with SAG, the administration of vaccarin influenced, by activating Hh signaling, the transcriptional levels of HSL and MGL as well as those of LPL and ADRP (Figure 6B and C). Compared with Gli1, Smo was more strongly affected by vaccarin treatment, an observation consistent with previous reports that Smo is intimately involved in lipid metabolism in adipose tissue [46]. Thus, all available evidence indicates that vaccarin promotes lipolysis via activation of Hh signaling in adipocytes.

Overall, then, vaccarin notably inhibits adipogenesis in preadipocytes while activating lipolysis in mature adipocytes. The transcription and expression levels of the adipogenic-related markers PPARγ and C/EBPα were significantly suppressed by vaccarin. Further, the cellular levels of the hedgehog signaling components Smo and Gli1 in 3T3-L1 preadipocytes were suppressed after MDI-induced differentiation began but reversed by vaccarin treatment. In addition, glycerol efflux significantly increased when the cells were treated with either vaccarin and SAG. Its seems that these effects result from the activation of Hh signaling and the attendant expression of those genes, viz. LPL, MGL, ATGL, HSL, associated with lipid metabolism. Given its defined mechanisms of action and levels of efficacy in vitro, vaccarin would appear to be a useful agent for regulating adipogenesis and modulating lipid metabolism.

References


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