

Effect of Alpha-Phellandrene on Glucose Uptake and Adipogenesis in Insulin Resistant 3T3-L1 Adipocytes: an *in vitro* and *in silico* Approach

Souprayen Seethalakshmi, Ravishankar Sarumathi, Chandrasekaran Sankaranarayanan*

Department of Biochemistry and Biotechnology, Faculty of Science, Annamalai University, Annamalainagar, Tamil Nadu, INDIA.

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ABSTRACT

Aim: The present study was designated to investigate the effect of alpha-phellandrene, a monoterpene presents in essential oils of mint, turmeric, black pepper, and cinnamon on adipocyte function under insulin resistant condition. **Materials and Methods:** Mature 3T3-L1 adipocytes were exposed to high glucose (25 mM) and 0.6 nM of insulin for 24 hr to convert to IR-3T3-L1 adipocytes. The adipocytes were grouped into group1: normal control, group 2: diabetic control, group 3: diabetic group treated with alpha- phellandrene (65 µM) and group 4: diabetic group treated with rosiglitazone (0.1 µM). Glucose uptake assay, triglyceride accumulation and the activity of glycerol-3-phosphate dehydrogenase were measured. The binding affinity of alpha-phellandrene with PPAR γ and SREBP-1c were analysed by docking studies by using Auto Dock vina (V. 4.0) in Pyrx software. **Results:** Glucose uptake, triglyceride accumulation and activity of glycerol-3-phosphate dehydrogenase were found to be decreased in diabetic control when control to normal control group. Alpha-phellandrene at dose of 65 µM increased glucose uptake, enhanced glycerol-3-phosphate activity and triglyceride accumulation in adipocytes which were found to be comparable with the standard drug rosiglitazone. A high binding affinity of alpha-phellandrene with key transcription factors, PPAR γ and SREBP-1c which are associated with adipogenesis and steroidogenesis were observed in molecular docking studies. **Conclusion:** These results suggest that alpha-phellandrene positively regulates adipocyte function and ameliorate dyslipidaemia and hyperglycaemia in type II diabetes mellitus.

Keywords: Alpha-phellandrene, PPAR γ , SREBP-1c, Glucose uptake, 3T3-L1 adipocytes.

Correspondence:

Chandrasekaran Sankaranarayanan
Department of Biochemistry and Biotechnology, Faculty of Science, Annamalai University, Annamalainagar-608 002, Tamil Nadu, INDIA.

Email: sankarhari05@gmail.com

INTRODUCTION

Diabetes mellitus is a chronic disorder arises from impaired insulin secretion/action and is associated with persistent hyperglycaemia.^[1] Metabolic aberrations causes dysfunction and failure of organ systems predisposing to increased morbidity and mortality.^[2] According to the International Diabetes Federation (IDF) the prevalence of diabetes mellitus may increase from 415 million to 783 million by 2045.^[3]

Adipose tissue is a major insulin dependent tissue that stores excess glucose as triglycerides and is a primary site of insulin resistance in diabetes mellitus. Apart from storage function, adipocytes secrete a variety of adipokines that are involved in insulin sensitivity, food intake and inflammation. Further in response to hormonal and nutritional cues adipocytes maintain metabolic homeostasis. Dysfunction of adipocytes profoundly contributes to metabolic derangements leading to obesity, atherosclerosis, insulin resistance and diabetes mellitus.^[4]

In diabetes mellitus, dysfunctional adipose tissue promotes inflammation, hyperlipidaemia and insulin resistance. These metabolic alterations promote non-alcoholic fatty liver disease, cardiovascular complications with disturbed muscle metabolism.^[5] Currently, a variety of drugs are available to combat insulin resistance in

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adipocytes. However, they are associated with various adverse effects such as hypertension, ischemia, and weight gain. Phytochemical therapy opens new avenues in the pharmacological management of insulin resistance. A variety of phytochemicals are used to overcome insulin resistance in adipose tissue and skeletal muscle.^[6]

α -phellandrene (α -PA) is a major monoterpene present in the essential oil of various dietary species such as cinnamon, black pepper, mint, ginger, and grass.^[7] It possesses diverse pharmacological effects such as anti-inflammatory, antinociceptive, *anti-dermatogenic*, anticancer and antioxidant activities.^[8] The aim of the study was to evaluate the effect of α -PA on insulin sensitivity in insulin resistant 3T3-L1 adipocytes under high glucose conditions.

MATERIALS AND METHODS

Chemicals

α -Phellandrene, Dulbecco's Modified Eagle Medium (DMEM), Foetal Bovine Serum (FBS) and antibiotic-antimycotic solution, insulin and 3-Isobutyl Methylxanthine (IBMX), were purchased from Sigma Aldrich Pvt.Ltd, India. Analytical grade reagents and chemicals were used in this study.

Maturation of adipocytes

The cell line 3T3-L1 preadipocytes were procured from NCCS, Pune, India and was cultured in growth medium containing DMEM with normal glucose enriched with FBS (10%), penicillin (100 units/mL) and streptomycin (100 μ g/mL) in 5% CO₂ at 37°C. After confluence, the cells were placed in differentiation medium containing dexamethasone (1 μ M), IBMX (0.5 mM) and insulin (1 μ g/mL) in DMEM containing 10% FBS. For every 2 days, till maturation of adipocytes the differentiation medium was replaced.^[9]

Development of insulin resistant-3T3-L1 adipocyte

Mature 3T3-L1 cell lines were exposed to high glucose (25 mM) with 0.6 nm/L of insulin for 24 hr to convert to IR-3T3-L1 adipocytes.^[10] The onset of insulin resistance in various groups were evaluated by measuring the residual glucose levels.

Experimental design

Mature adipocytes and IR-adipocytes were grouped as follows:

Group 1: Normal Control: Differentiated 3T3-L1 adipocytes were exposed to normal glucose (5 mM) with 0.6 nm/L of insulin for 24 hr.

Group 2: Diabetic Control (IR-3T3-L1 adipocytes).

Group 3: IR-3T3-L1 cells with α -PA: IR-3T3-L1 adipocytes treated with α -PA (65 μ M) for 24 hr.^[8]

Group 4: IR-3T3-L1 cells with Rosiglitazone: IR-3T3-L1 cells were treated with standard drug rosiglitazone (0.1 μ M) for 24 hr.^[11]

Glucose uptake assay

The glucose uptakes in various experimental groups were assessed by commercial kit. Briefly, mature 3T3-L1 adipocyte was seeded in 96-well plate containing 5x10⁴ cell/well. To that one part of mature 3T3-L1 adipocytes were exposed to high glucose (25 mM) and insulin (0.6 nM) for 24 hr to convert into insulin resistant. After 24 hr, IR-3T3-L1 adipocytes were sub divided into three groups, one served as a diabetic control and the second and third experimental groups were treated with α -PA (65 μ M) and rosiglitazone (0.1 μ M) respectively for 24 hr. Finally, supernatant was collected from each experimental groups and the residual glucose levels were measured and expressed in percentage.^[12]

Measurement of triglyceride content

The intracellular triglyceride content in various experimental cell lines was measured by TG assay kit. The cells from each experimental group were washed using chilled PBS. Then, 1 mL of triglyceride extraction reagent was added and the cells were subjected to disruption using a cell sonicator and were centrifuged at 40°C for 5 min. The supernatant from each group of triglyceride content were measured.^[13]

Oil red O stain

Lipid droplets in experimental cell lines were visualized by Oil red O staining. After washing with PBS cells from each experimental group were fixed in 10% formalin for 15 min. Filtered oil red O solution was added and incubated for 20 min. The cells were incubated in filtered oil red O solution for 20 min at 37°C. The excess stain was removed by rinsing with 50 % ethanol for 3 sec and washing twice with deionised water. Image of stained oil droplets in each experimental group were photographed with the inverted microscope. In each group, the excess dye was removed with 1 mL of isopropanol for 1hr. The dye was dissolved completely, after that the staining was measured at 510 nm using a microporous plate.^[14]

Glycerol-3-phosphate dehydrogenase (GPDH) activity in experimental adipocytes

The activity of Glycerol-3- phosphate dehydrogenase in various experimental cell lines were determined spectrophotometrically. The experimental cells were washed using ice-cold PBS for 2 times and harvested using 300 μ L of ethanolamine/Hcl buffer (pH 7.5)

and EDTA. Then the harvested cells were sonicated for 10 sec. Then centrifuged at 13000 x g for 5 min at 4°C. Finally, supernatant was obtained from each experimental group. The reaction mixture contained triethanolamine/Hcl buffer, EDTA, 2-mercaptoethanol and NADH. Addition of dihydroxyacetone phosphate initiated the reaction and the rate of NADH oxidation was read at 340 nm.^[15]

In silico docking studies

The interactions between α -PA, rosiglitazone, and target proteins PPAR γ (PDB ID: 2PRG) and *SREBP-1c* (PDB ID: 1AM9) were analysed using AutoDock (V. 4.0) in Pyrx software. The docking interactions between the target proteins and α -PA, rosiglitazone were visualized by Biovia discovery studio visualiser 2020. The molecular interaction was calculated based on the binding energy (kcal/mol).^[16]

Statistical Methods

Experimental values expressed as mean \pm standard deviations of three experiments. One-way ANOVA was carried out and values with $p \leq 0.001$ are considered statistically significant. All calculations were done using the SPSS 26.0 version.

RESULTS

Influence of α -PA on glucose uptake in insulin resistant 3T3-L1

The influence of α -PA on glucose uptake in insulin resistant 3T3-L1 adipocytes were measured by GOD-POD method. Mature 3T3-L1 adipocytes were exposed to 25 mM glucose and insulin (0.6 nM) for 24 hr and the changes in the uptake of glucose were determined. In insulin resistant 3T3-L1 cells, the glucose uptake were significantly reduced which indicated the onset of insulin resistance. However, on treatment with

α -PA (65 μ M) for 24 hr a significant improvement in the uptake of glucose was observed in IR-3T3-L1 adipocytes as shown in Table 1. A similar effect was observed in rosiglitazone treated groups.

Role of α -PA on triglyceride content

The effect of α -PA on TG content in 3T3-L1 cells was measured using commercial kit. In IR-3T3-L1 adipocytes, the intracellular triglyceride content was significantly reduced when compared to normal adipocytes. However, on exposure of α -PA (65 μ M) for 24 hr, the triglyceride accumulation was significantly increased when compared to IR-3T3-L1 cells (Figure 1). The effect of α -PA is similar that of rosiglitazone treated cells.

Effect of α -PA on intracellular lipids in IR-3T3-L1

The effect of α -PA on intracellular lipids in IR-3T3-L1 cells were analysed by Oil red 'O' staining method. In IR-3T3-L1 adipocytes, the accumulation of lipids was significantly reduced when compared to normal control. However, on exposure of α -PA (65 μ M) for 24 hr the lipid accumulation was significantly increased (Figure 2). A similar finding was observed in rosiglitazone treated adipocytes.

Influence of α -PA on Glycerol-3-Phosphate Dehydrogenase (G3PD) activity

The activity of glycerol-3-phosphate dehydrogenase was determined in experimental 3T3-L1 cells. In IR-3T3-

Table 1: Effect of α -PA on uptake of glucose in experimental cell lines.

Groups	Glucose uptake (mM/L ⁻¹)
3T3-L1	12.55 \pm 0.08 ^a
IR-3T3-L1	9.15 \pm 0.14 ^b
IR-3T3-L1 + α -PA (65 μ M)	11.31 \pm 0.08 ^c
IR-3T3-L1+Rosi (0.1 μ M)	11.98 \pm 0.09 ^d

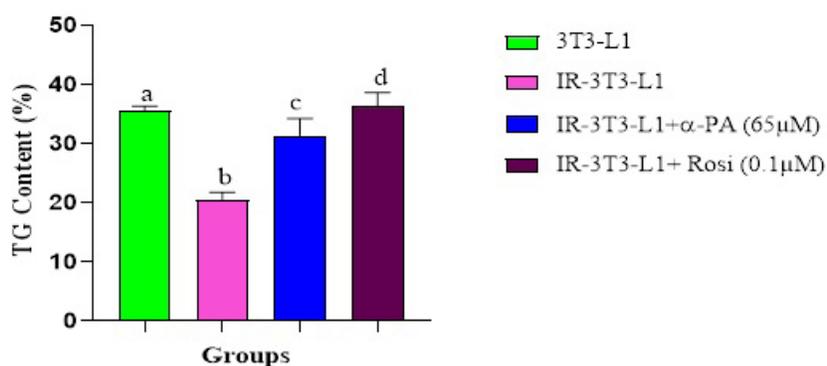


Figure 1: Role of α -PA on triglyceride content in 3T3-L1 cells. Values are represented as the mean \pm SD ($n=3$) and the difference between the groups were evaluated by one-way ANOVA followed by Duncan's Post hoc test $p < 0.001$.

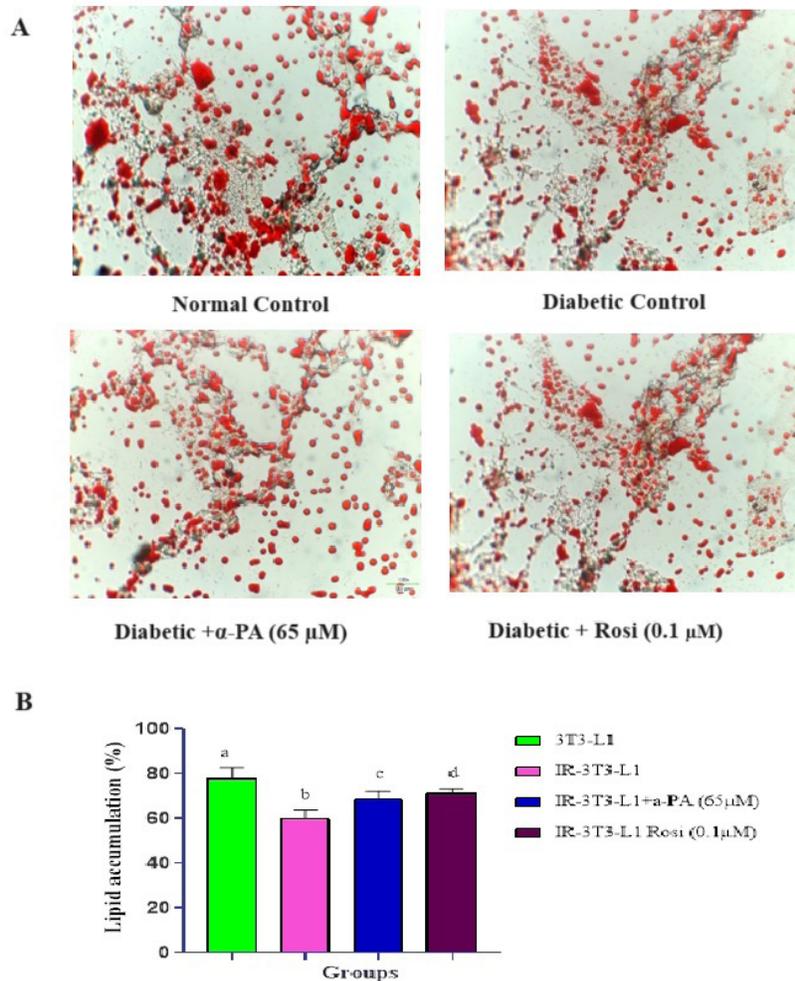


Figure 2: Lipid accumulation in experimental 3T3-L1 cell lines A: Lipid droplets were visualised by Oil red 'O' staining at 40x. B: Lipids were extracted and measured at 510 nm. Values are represented as the mean \pm SD ($n=3$) and the difference between the groups were evaluated by one-way ANOVA and Duncan's Post hoc test $p<0.001$.

L1cells, the glycerol-3-phosphatase dehydrogenase activity was found to be significantly decreased when compared to normal control. On exposure with α -PA (65 μ M) for 24 hr, the G3PD activity was increased significantly when compared to IR-3T3-L1 adipocytes (Table 2). Similar effects were observed in rosiglitazone treated groups.

Table 2: Effect of α -PA glycerol-3-phosphate dehydrogenase activity in experimental cell lines.

Groups	Glycerol-3-Phosphate Dehydrogenase (μ mol/g Protein)
3T3-L1	5.73 \pm 0.14 ^a
IR-3T3-L1	1.95 \pm 0.07 ^b
IR-3T3-L1 + α -PA (65 μ M)	4.11 \pm 0.17 ^c
IR-3T3-L1+Rosi (0.1 μ M)	5.04 \pm 0.05 ^d

Binding affinities of α -PA and Rosiglitazone with PPAR γ and SREBP-1c transcription factors

A strong binding affinity was observed between the transcription factors PPAR γ and SREBP-1c with α -PA and rosiglitazone. The results were summarised in Figure 3 and Table 3 as follows:

DISCUSSION

Adipose tissue an energy balance "hub" regulates energy homeostasis by integrating the requirements of diverse organ systems.^[17] It has been found that inadequate adipogenesis and ineffective hyperplasia causes inflammation and insulin resistance in adipocytes. Alteration in adipocyte function leads to insufficient energy storage and perturbation of glucose and lipid metabolism resulting in insulin resistance,

Table 3: Binding energy and aminoacids interactions of α -PA and rosiglitazone with PPAR γ and SREBP-1.

Target	Binding energy with α -PA (kcal/mol)	Binding energy with rosiglitazone (kcal/mol)	Amino acids interacting with α -PA	Aminoacids interacting with rosiglitazone
PPAR γ	-5.9	-8.1	PHE 282, GLN 286, LEU 453, MET 463, LEU 465, SER 464, LYS 457.	PHE 282, GLN 286, LEU 453, VAL 450, MET 463, SER 464, ILE 456, ASP 475, LYS 474, LEU 465, TYR 473, LYS 457.
SREBP-1c	-5.1	-5.9	LEU 349, VAL 350, TYR 369, LYS 365, ASP 368, PHE 372.	LEU 349, VAL 350, GLN 374, ASP 368, LYS 365, PHE 372, HIS 375, ARG 371.

abnormal insulin secretion, inflammation, dyslipidemia and hyperglycaemia.^[18] The dysfunctional adipocytes resulting from insulin resistance elevates plasma Free Fatty Acids (FFA) and brings metabolic alterations leading to lipotoxicity. Further, free fatty acids drained via portal vein gets deposited in the hepatic tissue.^[19] Thus surplus energy levels and loss of healthy adipose tissue expandability are found to be detrimental for the whole organism. As Adipose tissue exhibits plasticity, agents that promote adipogenesis improve insulin sensitivity and mitigates inflammation thereby protecting tissues from metabolic consequences.^[20] Currently, thiazolidinediones group of drugs are available to improve adipogenesis and normalize adipocyte function. However, these are associated with adverse effects. In this context, there is a search for compounds with glitazone like activity exerting minimal side effects.^[21]

Adipogenesis, a physiological process that prevent lipotoxicity in peripheral organs by safely sequestering lipids. It is associated with development and accumulation of adipocytes in various parts of the body. Adipocytes stores energy as fat and mobilises free fatty acids when energy expenditure exceeds intake.^[4] Defective adipocyte metabolism causes ectopic deposits of lipids promoting atherosclerosis, insulin resistance and metabolic syndrome. A strong correlation between metabolic health and adipogenesis are documented in several studies on animal models and humans. As adipose depot health rather than size is important, adipogenesis emerges as a viable therapeutic target. Under insulin resistant conditions, decreased glucose uptake is associated with defective adipogenesis and dyslipidaemia.^[22] In the present study, treatment with α -PA significantly increased adipogenesis in 3T3-L1 cells as it is evidenced by increased glucose uptake and activation of transcription factor Peroxisome Proliferator-Activated Receptor gamma (PPAR γ) a master regulator of adipogenesis and lipid storage.^[23] Several studies reported that PPAR γ plays a vital role in

lipid metabolism. It exists in three isoforms viz., PPAR α , PPAR γ , and PPAR β/δ which show variation in tissue distribution. Among the three, PPAR γ , activates and upregulates the key genes associated with lipogenesis and triglyceride storage. PPAR γ is a therapeutic target for various chronic metabolic diseases such as atherosclerosis, type 2 diabetes, insulin resistance and obesity.^[24] Docking studies revealed a high binding affinity of α -PA with PPAR γ . The binding data a comparable with standard drug (Table 3). Our results are in line with Anushree *et al.* who reported that puniceic acid increased glucose uptake and PPAR γ activation in IR 3T3-L1.^[25]

Liver and adipose tissue are the principal sites of lipogenesis that include fatty acid and triglyceride synthesis. Apart from metabolic tissues, lipogenesis in immune and nervous system cells are crucial for the proliferation and differentiation. Aberration in lipid homeostasis arising from excessive, or insufficient lipogenesis are related to dyslipidaemia, fatty liver and diabetes.^[26] Evidence from literature reveals that insulin and various nutrients governs the expression of lipogenic genes through transcription factor SREBP1-c. Insulin-mediated SREBP-1c expression increases glycolysis by the activation of glucokinase and lipogenesis by Glycerol-3-Phosphate Dehydrogenase (GPDH) activity. Under insulin resistant condition, decreased gene expression of SREBP-1c results in decreased activity of glycerol-3-phosphate dehydrogenase, a crucial enzyme associated in triglyceride production in adipocytes.^[27] In this study, treatment with α -PA improved the activity of glycerol-3-phosphate and triglyceride accumulation in insulin resistant adipocytes. A strong binding affinity between SREBP-1c and α -PA was observed suggesting it as a potent SREBP-1c agonist. A recent studies shows that caloric restriction enhances fatty acid synthesis and metabolic remodelling in adipose tissue via SREBP-1c dependent processes. The findings of the present study clearly demonstrates that α -PA significantly restored insulin sensitivity as

evidenced by improved adipo/lipogenesis in IR-3T3L1 adipocytes exposed to hyperglycemic conditions. The plausible mechanism of α -PA may be attributed to its efficient interaction and modulation of activities of key transcription factors PPAR γ and SREBP-1c in insulin resistant adipocytes. Recently Maliheh *et al.* reported that leaf and bark extracts of *Psidium guajava* improved insulin sensitivity in high glucose induced adipocyte cell lines.^[28]

SUMMARY AND CONCLUSION

From this we conclude that α -PA potentiated glucose uptake and intracellular storage of triglycerides in IR-3T3-L1 adipocytes by interacting with the key transcription factors PPAR γ and SREBP-1c. Thus, α -PA contributes to the improvement in adipocyte function under insulin resistance condition. The strength of the present study is the availability of α -PA in common dietary sources which may be a cost-effective therapeutic agent for restoring adipocyte function in insulin resistant conditions. However, the limitation lies in elucidating similar beneficial role in *in vivo* experimental models for the potential application α -PA in the management of adipocyte physiology in diabetes mellitus.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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