SUPPLEMENTARY MATERIAL

Andrographolide and Pterostilbene inhibit adipocyte differentiation by downregulating PPARγ through different regulators.

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Abstract

Adipogenesis involves commitment of stem cells and their differentiation into mature adipocytes. Its, tightly regulated by hormones, nutrients and adipokines. Many natural compounds are being tested for their anti-adipogenic activity which can be attributed to apoptosis induction in adipocytes, blocking adipocyte differentiation, or inhibiting intracellular triglyceride synthesis and accumulation. In this study, we have checked molecular mechanism of two phytocompounds: andrographolide (AN) and pterostilbene (PT) during differentiation of the human MSCs into adipocyte. Interestingly, AN upregulates miR27a, whereas, PT upregulated SIRT1 which inhibits the expression of PPARγ. Thus, our results clearly demonstrate that both AN and PT inhibited adipogenesis by blocking a surge of reactive oxygen species (ROS) during differentiation and inhibiting expression of crucial transcription factors like SREBP1c and PPARγ.

1. Experimental

Chemicals.

AN and PT were purchased from Sigma (USA). HiAdipoXL adipocyte differentiation medium, DMSO and fetal bovine serum (FBS) were purchased from HiMedia laboratories (India). Minimum essential medium (MEM), Phenol free medium, Oil Red O reagent, 2′,7′-dichloroflourescein diacetate (DCFH-DA) and Adipogenesis assay kit were purchased from Sigma (USA). TRIzol reagent was obtained from Invitrogen (USA). Power SYBR Green was purchased from Life Technologies (USA). Components for cDNA synthesis were purchased from New England Biolabs (USA). All the plasticware used for cell culture was purchased from Eppendorf (Germany).

Differentiation and culture of hMSCs.

1×10^4 HiFi human adipose derived mesenchymal stem cells (hMSCs; Himedia laboratories; India) were seeded per well in 96 well plate and grown in MEM supplemented with glutamine at 37°C in 5% CO_2. For inducing differentiation, confluent hMSCs were incubated for 21 days with adipocyte differentiation medium containing 10% FBS. Medium was changed every alternate day.

Cell viability Assay.

Cell viability was measured using MTT (Mossman, 1983). In brief, hMSCs were plated at a density of 1×10^4 cells/well in 96 well plate and cultured for 48 h. They were then exposed to different concentrations of AN (1, 5, 10 and 20 μM) or PT (1, 2, 5 and 10 μM) for 48 h. Following these treatments, MTT solution was added to the culture medium at a final concentration of 0.5 mg/ml and plates were incubated for 4 h at 37 °C. Formazan crystals were solubilized and absorption was measured at 550 nm using multiplate reader (BMG Labtech, Germany).

Later, for checking antiadipogenic activity, hMSCs were treated with AN (10 μM) or PT (5 μM) for 21 days with differentiation medium. Viability of hMSCs exposed to 10 μM AN and 5 μM PT for 21 days was also checked on the last day of differentiation.

Oil red O staining.

Oil red O staining was performed following the protocol of Ramirez-Zacarías et al, (1992). On day 6 and 21 of differentiation, cells were washed (PBS, pH 7.2) and fixed (4% neutral

buffered formaldehyde) for 20 min and stained for ~30 min with oil red O solution (Mittal et al, 2020). Cells were imaged using TE2000U microscope (Nikon, Japan). For oil red O quantification, stained cells were air dried overnight, dissolved in methanol and absorbance was measured at 520 nm on ELISA reader (Multiskan plate reader, Thermoscientific, USA).

Estimation of triglyceride content.

Total intracellular triglycerides were measured using adipogenesis assay kit (Sigma, USA) following manufacturer's instructions. Percent intracellular triglyceride content was calculated considering triglyceride content of control untreated cells as 100%.

Measurement of intracellular reactive oxygen species (ROS).

Total intracellular ROS was measured using DCFHDA dye (Aranda et al, 2013, Gire et al, 2021). Cells were seeded at a density of 10^4 cells/well and incubated in differentiation medium with/without phytocompounds. For estimation of intracellular ROS every day from day 0 to 7 of differentiation, cells were treated with 10 μM DCFHDA in medium for 20 min, washed and fluorescence was measured using spectrofluorimeter (BMG labtech, Germany) at Ex_{485} and Em_{530} wavelengths.

RNA isolation, cDNA synthesis and Quantitative RT PCR.

TRIzol reagent was used for isolation of total RNA. Cells were seeded in a 6 well plate at a density of 2×10^6 cells/well in MEM medium. After 24 h, differentiation medium with/without phytocompounds was added. Cells were harvested on 0, 3rd and 5th days of differentiation in 500 μl TRizol reagent and processed for RNA extraction. RNA was quantitated using nanodrop (Eppendorf, Germany) and cDNA was synthesized using Verso cDNA synthesis kit (Thermoscientific, USA), qRT-PCR was performed on Step-One Plus cycler (ABI, USA) using SYBr Green and gene-specific primers (Supplementary Table 1). Fold change in gene expression was calculated using $2^{-\Delta\Delta Ct}$ method. GAPDH was used as an endogenous reference.

Statistical Analysis.

All experiments were performed independently for at least three times and data are presented as mean ± SE. All statistical analyses were performed using GraphPad PRISM 7.0d software.

Two-tailed student's *t*-test and ANOVA was performed to determine significant differences between treatment and control. Statistical significance was defined as *$p \leq 0.05$.

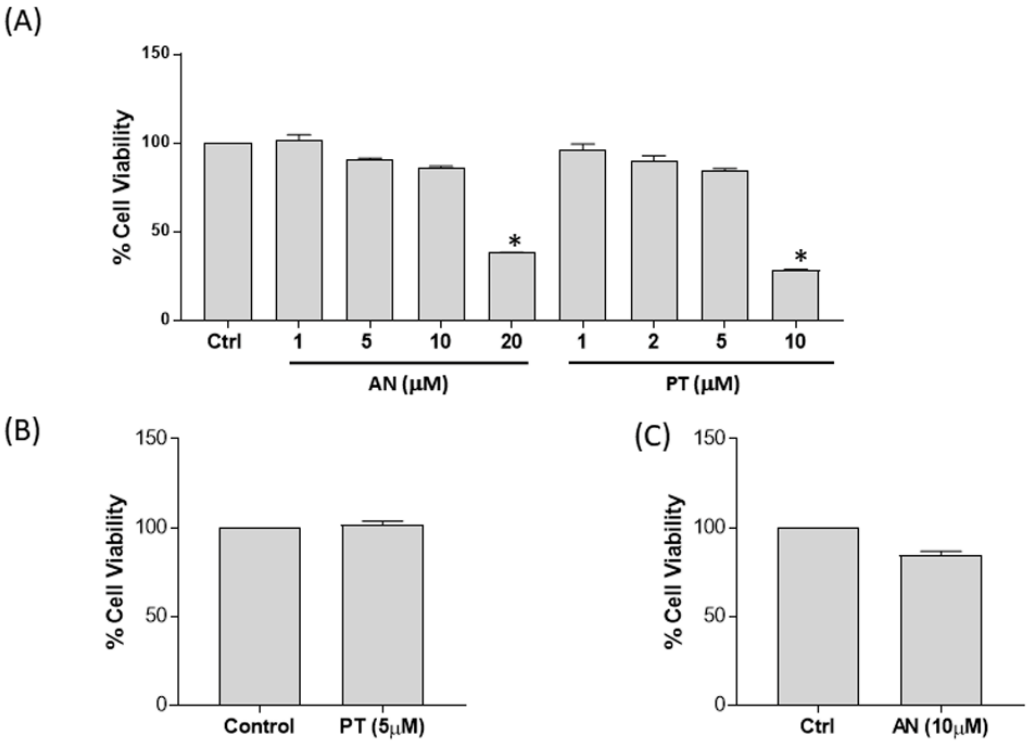

Figure S1: Cell viability of control and treated differentiating hMSCs. (A) Cell viability of differentiating hMSCs treated with different concentrations of AN and PT for 48h. (B) Cell viability of Control and 5μM PT treated cells for 21 days. (C) Cell viability of control and 10 μM AN treated cell for 21 days.

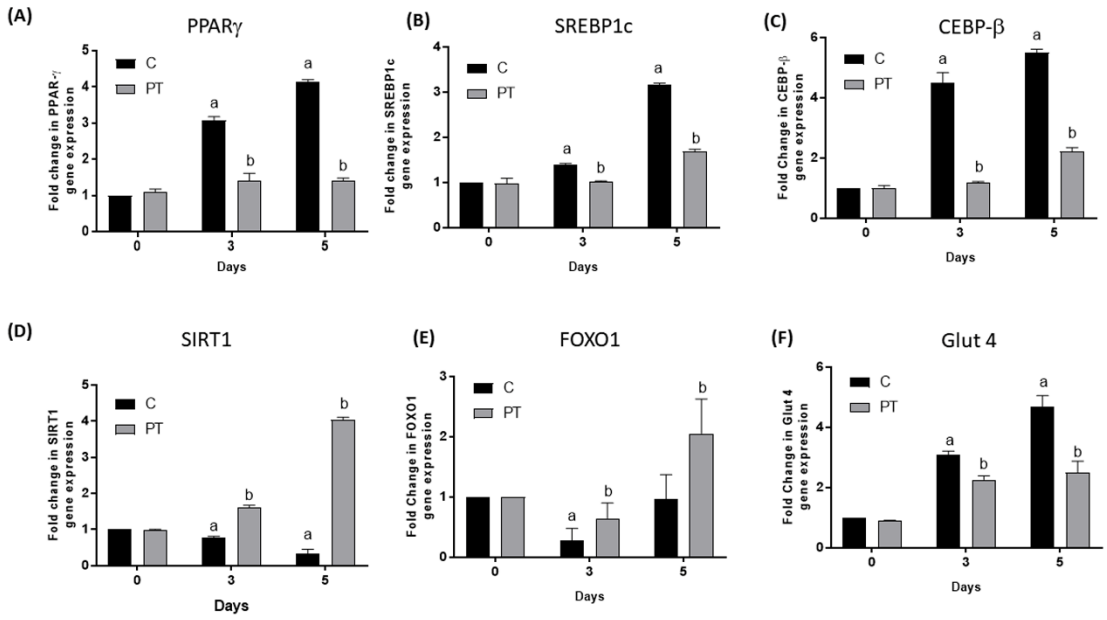

Figure S2. Expression of (A) PPARγ (B) SREBP1c (C) C/EBPβ (D) SATB2 (E) miR-27a and (F) Glut4 in untreated control and cells treated with 10 μM AN. Data are presented as mean ± SE of three independent experiments. Data was analysed using ANOVA. In figure alphabet 'a' indicates significant difference ($p \leq 0.05$) compared to day 0 control and 'b' indicates significant difference ($p \leq 0.05$) compared to respective day control (C: Control, AN: Andrographolide).

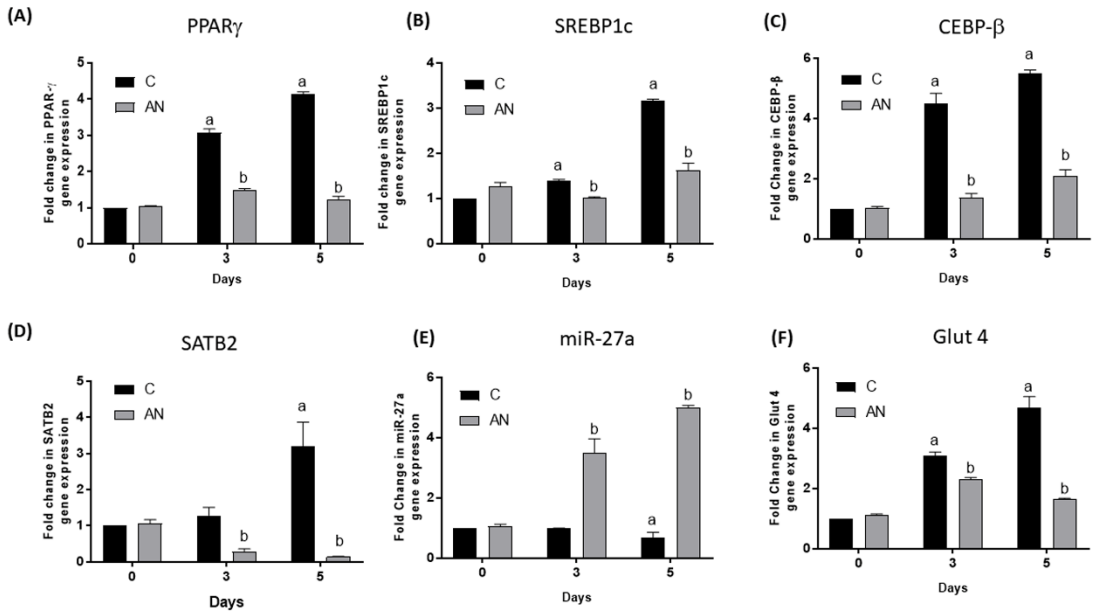

Figure S3. Expression of (A) PPARγ (B) SREBP1c (C) C/EBPβ (D) SIRT1 (E) FOXO1 and (F) Glut4 in untreated control and cells treated with 5 µM PT. Data are presented as mean ± SE of three independent experiments. Data was analysed using ANOVA. In figure alphabet 'a' indicates significant difference ($p \leq 0.05$) compared to day 0 control and 'b' indicates significant difference ($p \leq 0.05$) compared to respective day control (C: Control, PT: Pterostilbene).

Supplementary Table 1: Oligos used for qRT-PCR

Sr.no.	Primer Name	Primer Sequence 5' to 3'
1	CEBPβ F	GCAAGAGCCGCGACAAG
2	CEBPβ R	GGCTCGGGCAGCTGCTT
3	PPARγ F	GATACACTGTCTGCAAACATATCACAA
4	PPARγ R	CCACGGAGCTGATCCCAA
5	Glut4 F	GCCGGACGTTTGACCAGAT
6	Glut4 R	TGGGTTTCACCTCCTGCTCTA
7	GAPDH F	TGCACCACCAACTGCTTAGC
8	GAPDH R	GGCATGGACTGTGGTCATG
9	SIRT1 F	TGCCGGAAACAATACCTCCA
10	SIRT1 R	TGAAACAGACACCCCAGCTC
11	SATB2 F	CCGCACCAGAAGAAGACACC
12	SATB2 R	AGGGACTGCTCACGGTCT
13	SREBP1c F	GCAAGGCCATCGACTACATT
14	SREBP1c R	GGTCAGTGTGTCCTCCACCT
15	FOXO1 F	ACGAGTGGATGGTCAAGAGC
16	FOXO1 R	AATTGAATTCTTCCAGCCCGC
17	miR-27a SL	GTCGTATCCAGTGCAGGGTCCGAGGTAT TCGCACTGGATACGACTGCTCA
18	miR-27a F	TATAGGGCTTAGCTGCTTG
19	miR-27a R	GTGCAGGGTCCGAGGT