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Atomic force microscopy, biochemical analysis of 3T3-L1 cells differentiated in the absence and presence of insulin

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ABSTRACT

Background: There are ample evidences to demonstrate that differentiation of preadipocytes is associated with deposition of fat in cells. Still, it is unclear whether the differentiation process also alters membrane topology as well as cholesterol levels and whether insulin contributes to it.
Methods: Membrane scanning of differentiated cells, along with freshly plated and 11 day preadipocytes, was performed using Atomic Force Microscopy (AFM) to gain qualitative information about cell surface properties as well as roughness. Moreover, glucose uptake, lipid analysis, expression profiling of transcription factors and signaling molecules involved in the process of differentiation was also performed.
Results: We report (i) differentiation in the presence of 500 μM isobutylmethylxanthine (IBMX), 0.25 μM dexamethasone (DEX) with or without 0.1 μM (0.57 μg/ml) insulin directly alters membrane topology. (ii) At nano-levels, addition of insulin maintains plasma membrane roughness during differentiation in comparison with IBMX and DEX only. (iii) At macro levels, decreased fat accumulation in preadipocytes exposed to insulin during the initial stages of differentiation is a result of reduced expression and nuclear localization of sterol regulatory element binding protein (SREBP)-1.
General significance: This study reports a significant reduction of membrane cholesterol and total cholesterol ($p<0.01$) in cells differentiated in the presence of insulin.

1. Introduction

Differentiation of preadipocytes to adipocytes is essential for accumulation of fat in adipose tissue. Excess activation of such process results in obesity. Accumulation of fat in vitro in cells using different conditions has been demonstrated in numerous studies and 3T3-L1 preadipocytes are a widely used in vitro model system for studying cellular differentiation. This cell line originally described by Green and Kehinde [1], when maintained at confluence for several weeks differentiates and acquires the phenotype of a mature adipocyte, characterized by intracellular accumulation of triglycerides (TG) in the form of oil droplets. This process can be hastened by a wide variety of agents, including insulin [2]. The most commonly employed procedure to enhance differentiation involves treating postconfluent cells with a cocktail of isobutylmethylxanthine (IBMX) and dexamethasone (DEX) in the absence or presence of insulin. Differentiation markedly alters protein expression pattern [2]. Also, signals that control adipogenesis either endorse or block the cascade of transcription factors that coordinate the differentiation process. CCAAT element binding proteins (C/EBP) and sterol response element binding protein 1 (ADD1/SREBP-1) induce expression of peroxisome proliferator activated receptor-γ (PPAR-γ) during the early stages of the differentiation process [3,4]. Subsequently, activated PPAR-γ co-ordinates with C/EBP-α and stimulates expression of many metabolic genes, ultimately leading to the adipocyte formation [5]. Though, the mechanisms of regulation of fat in cells are known, it is yet unclear to what extent accumulation of fat in cells directly affects cell membrane.

Cellular differentiation, among other complex events that are measured at the macroscale level are in fact regulated by individual molecular events that operate in nanoscale. Considering the fact that, morphologically significant alterations occur upon differentiation and also cell function can be associated with structural changes in the cellular phenotype, we for the first time investigated changes in membrane characteristics before, and after differentiation by using tapping mode atomic force microscopy (AFM). The rationale of this study was to investigate changes in the plasma membrane upon

Abbreviations: AFM, Atomic Force Microscopy; IBMX, Isobutylmethylxanthine; DEX, Dexamethasone; SREBP-1, Sterol regulatory element binding protein-1; TG, Triglycerides; FFA, Free fatty acids; C/EBP, CCAAT element binding proteins; PPAR-γ, Peroxisome proliferators activated receptor-γ; ID, IBMX+DEX; IDI, IBMX+DEX+Insulin; IRS-1, Insulin receptor substrate-1; IR-β, Insulin receptor-β; GLUT-4, Glucose transporter-4; GLUT-1, Glucose transporter-1

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differentiation. In several studies, properties of plasma membrane or alterations in plasma membrane by employing the technique of AFM imaging has been investigated [6–8]. Additionally, we also address the relevance of insulin on differentiation as well on basal glucose transport. Moreover, we investigated sequel of differentiation and expression profile of molecular markers of differentiation, lipid composition and basal glucose transport in adipocytes differentiated in the absence (ID) and presence of insulin (IDI). The present study emphasizes that composition of a differentiation cocktail influences basic properties of resulting adipocytes manifested as striking alterations at the level of plasma membrane, which seems to be a result of increased lipid anabolism within the cell. Our study, for the first time, highlights comparative morphological, biochemical and molecular changes during preadipocyte differentiation in the absence and presence of insulin along with compositional differences in lipids.

2. Materials and method

2.1. Material

3T3-L1 preadipocytes (ATCC no CL-173), were obtained from ATCC, VA, USA. DMEM and FBS were purchased from Invitrogen (Carlsbad, CA, USA). ^{14}C 2-DG (15 Ci mmol^{-1}) was obtained from American Radiolabelled Chemicals (St. Louis, USA). PVDF membrane was obtained from Millipore (Billerica, MD, USA). Insulin, DEX, IBMX and cytochalasin were purchased from Sigma (St. Louis, MO, USA). Protease inhibitor cocktail (Tm complete) was purchased from Roche Diagnostics mbH (Mannheim, Germany). Super signal reagent for enhanced chemiluminescence and Coomassie plus protein assay reagent were obtained from Pierce (Rockford, IL, USA). Monoclonal antibody against phosphotyrosine (PY20), polyclonal antibodies for SREBP-1, C/EBP-α, PPAR-γ, IR-β, IRS-1, GLUT-4, GLUT-1, β-actin, β-tubulin and peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cell culture and induction of differentiation

3T3-L1 preadipocytes were maintained in DMEM containing 10% FBS (heat inactivated FBS with no detectable level of insulin). Estimation of insulin levels in FBS was done using ultra sensitive insulin ELISA kit (Mercodia, Sweden) as described previously [9]. 3T3-L1 preadipocytes were induced to differentiate as previously described [10], with minor modifications. Briefly, 3T3-L1 cells were grown to 80–90% confluence in 60 mm coated Petri dishes. Differentiation was induced by incubating cells in DMEM containing 10% FBS, 500 μM IBMX, 0.25 μM DEX with or without 0.1 μM (0.57 μg/ml) insulin on day zero. After 48 h, medium was replaced with DMEM supplemented with 10% FBS and 0.1 μM insulin or DMEM supplemented with 10% FBS only. After additional 48 h, insulin was withdrawn, and medium was changed every second day for 11 days. Differentiation events were photographed immediately before media changes with phase optics at a total magnification of 20× using DP-30 camera, Olympus (Shinjuku-ku, Tokyo, Japan). Considering the fact that when preadipose 3T3-L1 cells in surface culture reach a confluent state, their growth rate decreases greatly and many cells undergo adipose conversion with varied frequency [11,12], freshly plated 3T3-L1 cells of 24 h culture were used as undifferentiated preadipocytes for all experimental purpose.

2.3. Atomic force microscopy

3T3-L1 cells were imaged using Multiview 1000™ AFM (Nanonics Jerusalem, Israel) operating in amplitude modulated tapping mode. Silicon probes (Veeco, CA) with a tip diameter (ϕ) of 10 nm and nominal spring constant (k) 2.8 N/m and resonance frequency (ω_o) 75 kHz (Nanonics, Jerusalem, Israel), were used. Sample surface was scanned by a sharp tip in x, y and z directions and moved by a piezoelectric translator. All images were acquired with a 512 data point resolution and scan delay of 5 ms, which is a delay between each point of AFM imaging and is related to the speed of scan. The AFM was initially performed on fixed cells in air. However, we were unable to locate cells which had differentiated as we could not discriminate between cells in which fat globules were present. Moreover, attempts to do AFM on live cells did not prove to be too fruitful. After innumerable attempts interestingly, we figured out that when fixed cells were moist with water, we could comfortably select cells in which accumulation of oil droplets was clearly visible. After locating the appropriate cell, the position of a probe was fixed and cells were left on a scanning platform for a minute and subjected to scanning. Subsequently, from then onwards, we performed all AFM analyses on fixed cells kept in PBS until scanning, which was done in air. Differentiated cells, 11 day preadipocytes and 3T3-L1 preadipocytes (in culture) were trypsinized and 4×10^4 cells were replated on cover slips. Cells were washed twice with 1× PBS, fixed with 1% formalin for 10 min followed by washing twice with 1× PBS and twice with glass distilled water before AFM measurements.

2.4. Cholesterol, free fatty acids and triglycerides estimation

3T3-L1 preadipocytes differentiated in 24 well plates in the absence and presence of insulin were lysed with 2% Triton X-100 in glass distilled water for 10 min and resulting lysate was used for cholesterol, free fatty acid and TG estimation. Cholesterol estimation kit was obtained from Ensure Biotech., (Hyderabad, India) with a detection range of 3 mg/dl–750 mg/dl. Free Fatty Acid estimation kit was obtained from Randox Laboratories Ltd., with a linear detection in the range 0.0–2.0 mmol/l, (Crumlin, UK), and Triglyceride estimation kit was obtained from Spinreact (Girona, Spain) with a sensitivity of 1 mg/dl=0.0011(A). Plasma membrane fractions were obtained as described previously [13]. Pelleted membrane was resuspended in a detergent-containing lysis buffer (50 mM Tris, pH 8.0, and 0.5 mM DTT, 2 mM EDTA, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM PMSF, 1 mM TPCK, protease inhibitor cocktail and 1% v/v Nonidet P-40,) and assayed for cholesterol using cholesterol estimation kit.

2.5. Western blot analysis

Freshly plated cells, cells grown for 11 days, as well as differentiated cells, were washed thrice with ice-cold PBS and lysed in 200 μl of lysis buffer [20 mM HEPES (pH 7.4) containing 1% Triton X-100, 2 mM EDTA, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM PMSF, 1 mM TPCK and protease inhibitor cocktail] per 1×10^6 cells, on ice. Lysates were repeatedly passed through a 261/2 gauge syringe and centrifuged at 15000 rpm for 20 min. An equal amount of protein samples were resolved on 8–10% SDS-polyacrylamide gel and then transferred onto PVDF membrane. The membranes were probed with antibodies against C/EBP-α, PPAR-γ, SREBP-1, IRS-1, IR-β, GLUT-4, GLUT-1, β-actin and β-tubulin. Whenever required, blots were stripped by incubating membrane at 50 °C for 10 min in stripping buffer (62.5 mM Tris–Cl pH 6.7, 100 mM 2-mercaptoethanol, 2% SDS) with intermittent shaking. Membranes were washed thoroughly with TBS and reprobed with required antibodies wherever possible. Otherwise gels run in duplicates were probed for desired proteins by western blotting. Most of the experiments were repeated and representative blots are shown in figures.

2.6. Immunofluorescence confocal microscopy

3T3-L1 cells were plated on 12-well slides (ICN, Ohio, USA) and differentiated for 11 days as described in the previous section. Cells were washed with PBS (1×, pH 7.5) and fixed with 3% paraformaldehyde for 10 min at room temperature. Cells were permeabilized with

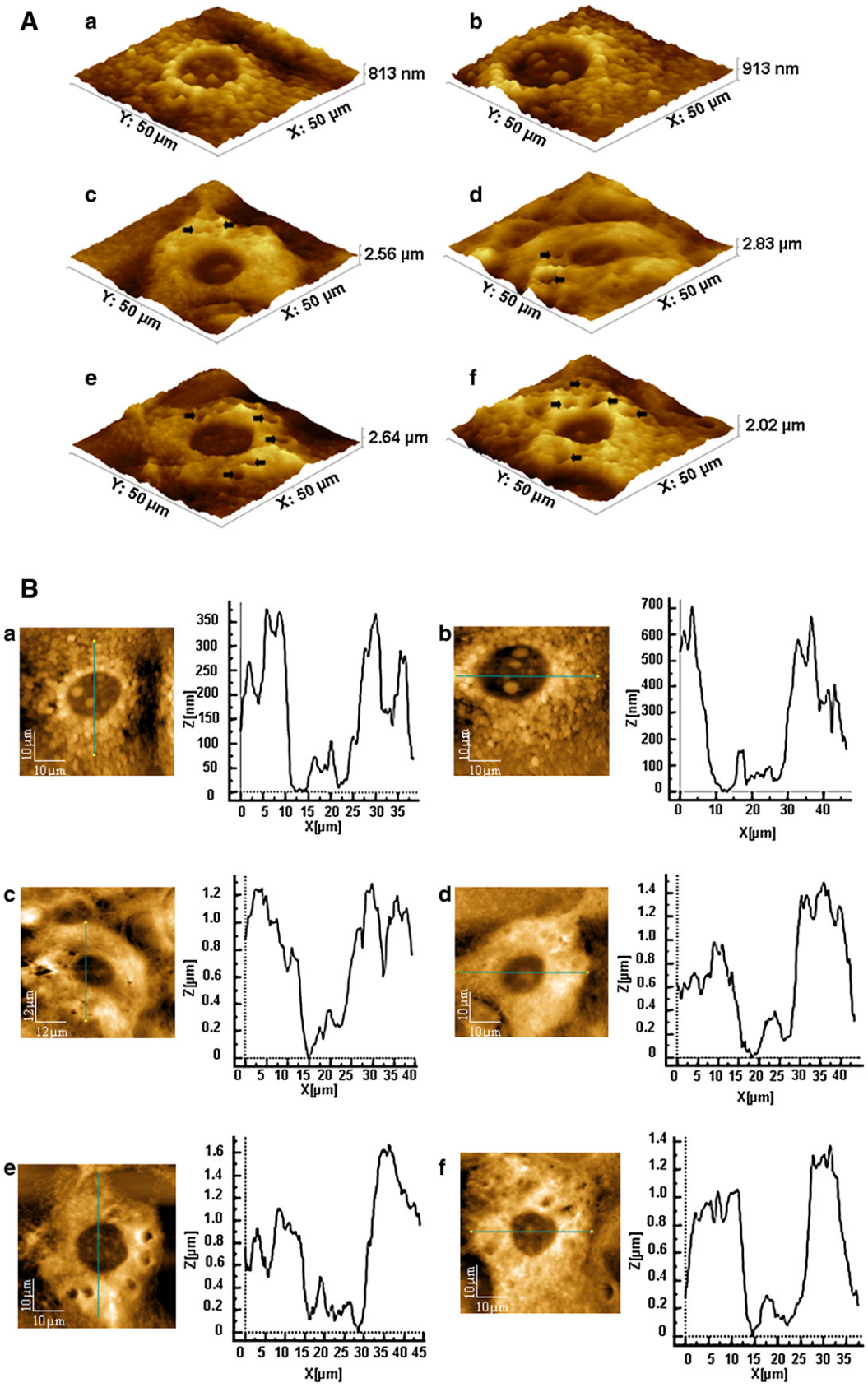


Fig. 1. (A) Three-dimensional AFM profile of 3T3-L1 cells before and after differentiation. 3T3-L1 preadipocytes were differentiated for 11 days in the presence of 500 µM IBMX and 0.25 µM DEX in the absence or presence of 0.1 µM Insulin (insulin treatment was given for 4 days as defined in existing protocol for differentiation). On day 11, cells were washed with 1× PBS (pH 7.4), fixed with 1% formalin for 10 min, again washed with 1× PBS and scanned using tapping mode atomic force microscope with silicon probe. (a) Freshly plated preadipocytes, (b) 11 day preadipocytes, (c) and (d) cells differentiated using ID, (e) and (f) cells differentiated using IDI. (B) Two-dimensional AFM images and respective height profile analysis of 3T3-L1 cells before and after differentiation. (a) Preadipocytes, (b) 11 day preadipocytes, (c) and (d) cells differentiated using ID, (e) and (f) cells differentiated using IDI.

Table 1
Relative levels of cholesterol, free fatty acids and triglycerides in 3T3-L1 preadipocytes and, adipocytes differentiated in the absence (ID) and presence (IDI) of insulin

	Preadipocytes 24 h	Preadipocytes 11 days	ID	IDI
Cholesterol μg/3×10^5 cells	3.37±0.89	8.2±1.3[a]	11.92±3.2	9.71±2.9[**]
Free fatty acids nmol/3×10^5 cells	2.75±1.2	10.0±1.0[a]	15.4±4.0	14.3±1.67[a]
Triglycerides μg/3×10^5 cells	5.5±4.8	26.1±3.5[b]	37.6±4.5[b]	33.8±3.1[b,*]

3T3-L1 cells, freshly plated, cells grown for 11 days and cells differentiated for 11 days in 24 well plates in the absence and presence of insulin were lysed with 2% Triton X-100 for 10 min and resulting lysate was used for cholesterol, free fatty acid and TG estimation using respective kits. ID-IBMX (500 μM)+DEX (0.25 μM), IDI-IBMX (500 μM)+DEX (0.25 μM)+Insulin (0.1 μM).
[a] $p<0.05$ vs. 24 h preadipocytes.
[b] $p<0.01$ vs. 24 h preadipocytes.
[*] $p<0.05$ vs. ID.
[**] $p<0.01$ vs. ID.

0.025% saponin for 10 min and subsequently blocked with 1% BSA at 37 °C for 30 min. Primary antibody against SREBP-1 (1:50) in blocking solution was added and incubated for 1 h at 37 °C. Following incubation cells were washed five times for 2 min each with ice-cold PBS. Fluorescein isothiocyanate conjugated (FITC) secondary antibody (1:100) in blocking agent was added and further incubated for 1 h at 37 °C. Cells were then washed five times for 2 min each with ice-cold PBS. Samples were then examined on confocal microscope (LSM 510, Carl Zeiss, Germany).

2.7. Glucose uptake assay

2-Deoxyglucose (2-DG) uptake measurements were performed as previously described [14], with following modifications. 3T3-L1 cells (1×10^5 per well) were grown in 24 well plates and allowed to differentiate in the absence or presence of insulin for 10 days. Subsequently, freshly plated preadipocytes of 24 h culture, preadipocytes cultured for 10 days and preadipocytes differentiated in the presence of ID or IDI, were serum starved in DMEM containing 1 mg/ml BSA for 3 h. For insulin stimulated uptake, cells were incubated in KRP buffer (10 mM Na_2HPO_4, 137 mM NaCl, 4.7 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$) with 100 nM insulin for 30 min and control cells were incubated with KRP for 30 min. Glucose uptake was initiated by adding 0.2 mM 2-deoxy glucose containing 0.5 μCi ml^{-1} ^{14}C 2-DG for 30 min at 37 °C. Uptake was terminated by washing cells with ice-cold KRP buffer containing 20 mM D-glucose and then solubilized with 0.1% SDS. After protein estimation radioactivity incorporated into the cells was quantified with a top count microplate scintillation counter from Packard (Albertville, MN, USA). Non-specific uptake, measured in the presence of 10 mM cytochalasin B, was subtracted from all values.

2.8. Statistics

Results are expressed as mean±SD. All statistical analyses were performed using students paired *t*-test. A value of $p<0.05$ is considered significant in comparison with the respective control groups.

3. Results

3.1. Alterations in membrane properties caused by differentiation

Differentiation of preadipocytes into adipocytes follows an array of morphological, biochemical and molecular changes, which must also involve changes in the characteristics of membrane. In order to study changes in membrane characteristics, we performed tapping mode atomic force microscopy (AFM). AFM 3D images did not show any differences in the appearance of membranes in freshly plated and 11 day preadipocytes, but membranes of adipocytes showed significantly different topography. In freshly plated preadipocytes as well as 11 day preadipocytes, protruding globular structures were observed on the surfaces which were not seen in cells differentiated with ID and appeared significantly diminished in cells differentiated with IDI (Fig. 1A). These structures likely correspond to proteins or protein–lipid complexes [15]. The surface of differentiated cells exhibited decreased roughness in comparison to preadipocytes. Moreover, cells differentiated with ID had a smoother membrane surface than the cells differentiated with IDI. Differentiated cells showed the presence of pit like structures (indicated by black arrows) which were not detected in undifferentiated cells (Fig. 1A). These pit like structures were found to be more in cells differentiated with IDI when compared to cells differentiated with ID. Height profiles of these cell surfaces are also indicative of changes in the roughness status of membranes, before and after differentiation (Fig. 1B). Additionally, three dimensional AFM analyses show changes in cellular dimensions from the order of nanometer in undifferentiated cells to micrometers in cells differentiated with both ID and IDI, along *z*-axis (Fig. 1A). Thus, there is a significant increase in size of the cells during progression from undifferentiated preadipocytes to well differentiated adipocytes. We observed nuclei of these cells as depression (Fig. 1A). Nuclei of other cells processed and scanned in a similar way showed elevated nuclei (data provided as additional figure, not for publication). Central depression in the height profiles corresponds to nuclear region in all images (Fig. 1B).

3.2. Composition of lipids accumulated in cells differentiated in the absence and presence of insulin

We estimated cholesterol, free fatty acids and triglycerides in whole cell lysates prepared from cells which were differentiated for 11 days. Although barely any visual oil droplet accumulation in 11 day preadipocytes was observed (Supplementary Fig. 1), as evident from Table 1, cholesterol, free fatty acids and triglyceride levels in 11 days preadipocytes were elevated when compared to levels in freshly plated preadipocytes. TG level in differentiated cells was elevated (30–45%) in comparison to 11 day preadipocytes and was consistently observed to be higher in cells differentiated in the absence of insulin ($p<0.05$). Moreover, cholesterol level was also significantly elevated by approximately 45% ($p<0.05$) in cells differentiated with ID and by 18% ($p<0.05$) in cells differentiated with IDI, in comparison with

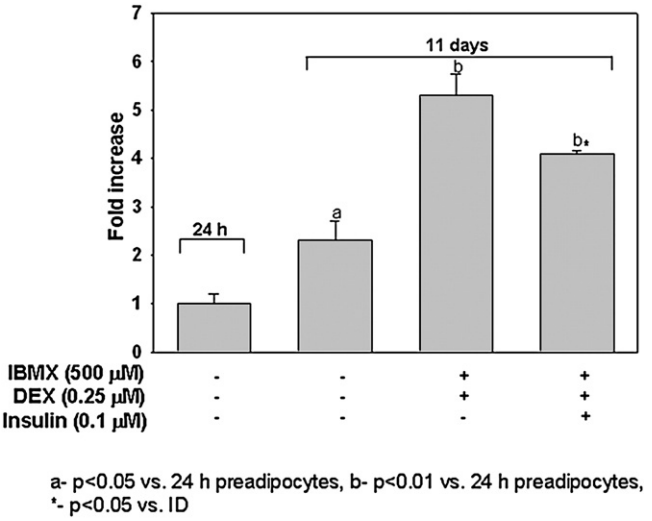


Fig. 2. Membrane cholesterol level in 3T3-L1 cells before and after differentiation. 3T3-L1 preadipocytes were differentiated for 11 days in the presence of 500 μM IBMX and 0.25 μM DEX, in the absence or presence of 0.1 μM Insulin. Membrane fractions of preadipocytes and cells differentiated in the absence and presence of insulin were prepared. Cholesterol estimation was done in these membrane fractions.

11 day preadipocytes. Interestingly, cholesterol level in cells differentiated in the presence of insulin was significantly low ($p<0.01$) in comparison to ID treated cells. A similar trend was observed with free fatty acid level but differences were not significant ($p=0.5$) (Table 1). Free fatty acid levels were higher by approximately 43–55% when compared to 11 day preadipocytes. In addition, membrane cholesterol level increased by 5.2 fold ($p<0.01$) and 4 fold ($p<0.01$), in cells differentiated with ID and IDI respectively. Among differentiated adipocytes, reduced membrane cholesterol by approximately 1.3 fold in cells differentiated with IDI was also significant in comparison to cells differentiated with ID ($p<0.05$) (Fig. 2).

3.3. Alterations in the expression of differentiation markers

In order to find a correlation between decreased fat accumulation in the presence of insulin and alterations in molecular events, we

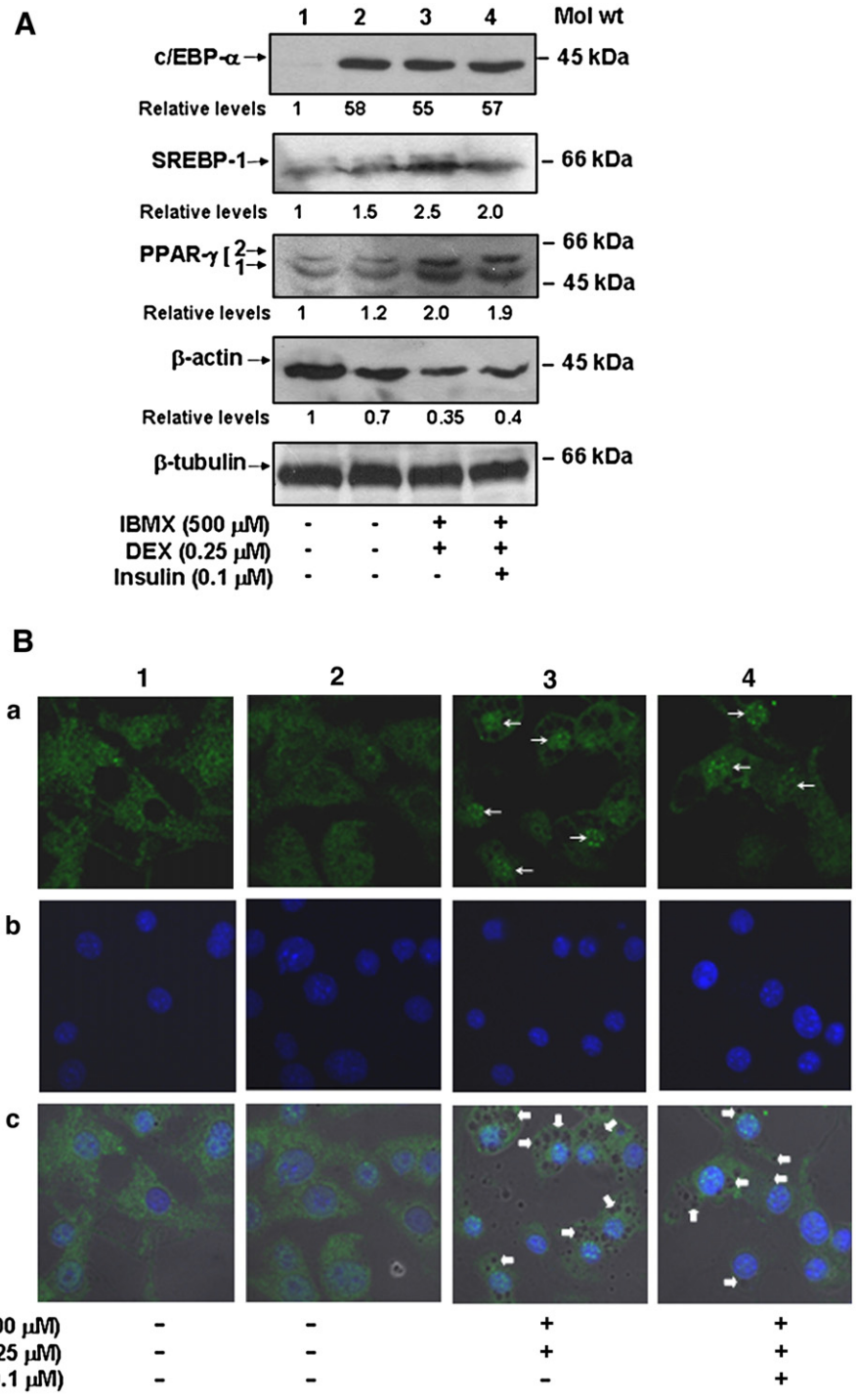
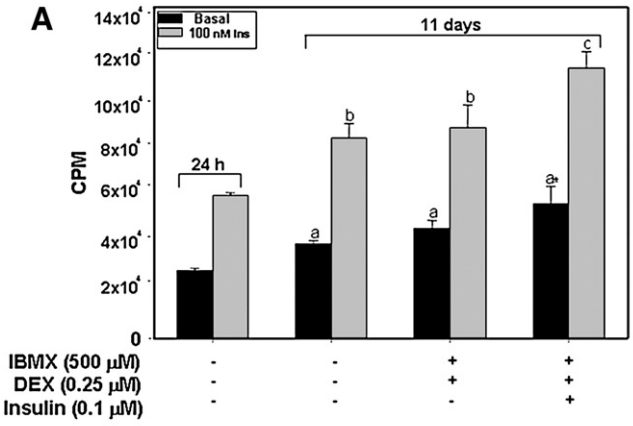


Fig. 3. (A) Expression of molecular markers and transcription factors in 3T3-L1 cells before and after differentiation. (B) Confocal analysis of expression and localization of SREBP-1 in 3T3-L1 cells before and after differentiation. (a) 3T3-L1 cells stained by immunofluorescence for SREBP-1 (green), (b) nuclear staining with DAPI, (c) overlap (a and b) to show nuclear localization of SREBP-1 in differentiated cells. (Lane 1) preadipocytes, (Lane 2) 11 day preadipocytes, (Lane 3) preadipocytes differentiated with ID, (Lane 4) preadipocytes differentiated with IDI. White arrows show the nuclear localization of SREBP-1 in differentiated cells. White block arrows correspond to the lipid droplets accumulated in the adipocytes.

probed for expression levels of molecular markers in cellular lysates by western blotting. C/EBP-α expression was not altered in differentiated cells in comparison to 11 day preadipocytes. This observation is in accordance with earlier reports demonstrating that 3T3-L1 preadipocytes when left confluent slowly undergo differentiation and thus express molecular markers involved in differentiation, which precedes the attainment of morphological and biochemical characteristics of adipocytes [1,16]. Levels of a cleaved form of SREBP-1 corresponding to a molecular weight of 68 kDa were approximately 2 fold higher in cells differentiated in the absence of insulin in comparison with cells differentiated in the presence of insulin (Fig. 3A). More importantly, as evident from immunofluorescence studies, differentiated cells (Fig. 3B, lanes 3 and 4) exhibited an increased nuclear localization of SREBP-1 in comparison to preadipocytes (Fig. 3B, lanes 1 and 2). Relative nuclear localization of SREBP-1 was high in cells differentiated with ID when compared to cells differentiated with IDI (Fig. 3B, lane 3 vs lane 4). Western blot analysis exhibits the presence of two isoforms of PPAR-γ which is in accordance with earlier reports [17]. However, expression level of both these isoforms of PPAR-γ was similar in cells differentiated in ID or IDI media (Fig. 3A). Thus, our results indicate that except for alterations in the expression and nuclear localization of SREBP-1, no change in levels of c/EPB-α or PPAR-γ was detected in cells differentiated in the absence or presence of insulin. Concomitant with an earlier report, marked decrease in β-actin content was observed in differentiated cells when compared to preadipocytes [18].



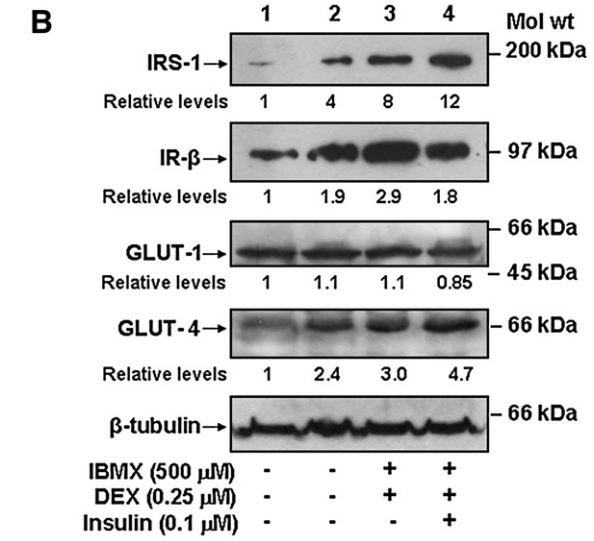


Fig. 4. (A) Basal glucose uptake in 3T3-L1 cells before and after differentiation. 3T3-L1 preadipocytes were differentiated for 11 days in the presence of 500 μM IBMX and 0.25 μM DEX in the absence or presence of 0.1 μM insulin. On day 11 glucose uptake assay was done. Glucose uptake assay was initiated by adding 0.2 mM 2-deoxy glucose containing 0.5 μCi ml^{-1} ^{14}C 2-DG for 30 min at 37 °C. Uptake was terminated by washing the cells with ice-cold KRP buffer containing 25 mM D-glucose and then solubilized with 0.1% SDS. After protein estimation radioactivity incorporated into the cells was quantified. Non-specific uptake, measured in the presence of 10 mM cytochalasin B, was subtracted from all the values. (B) Expression profile of various signaling molecules involved in glucose uptake pathway in 3T3-L1 cells before and after differentiation. Comparative profiles of molecules were either compiled from the same gel or from various duplicate gels.

3.4. Glucose uptake and expression of molecules involved in glucose uptake pathway

Basal as well as insulin stimulated glucose uptake was significantly higher in 11 day preadipocytes and differentiated cells, in comparison to 24 h preadipocytes (Fig. 4A). Surprisingly, basal glucose uptake increased by 20% in cells differentiated with IDI when compared to cells differentiated with ID, and this was significant ($p<0.05$). The extent of differentiation under both conditions was similar (Fig. 4A). Glucose uptake in cells differentiated with ID was only marginally higher than preadipocytes cultured for 11 days. Glucose uptake was further enhanced by treatment of cells with 0.1 μM insulin and the maximum increase in stimulation by 50% was detected in adipocytes differentiated in the presence of insulin (Fig. 4A). Stimulation of glucose uptake by insulin was not significantly different between ID and IDI treated cells ($p=0.07$). Differentiation remarkably increased basal expression of IRS-1 and IR-β in comparison to undifferentiated preadipocytes (Fig. 4B). Also, an increase in phosphorylation status of IR-β and IRS-1 was observed (Supplementary Fig. 3). Expression levels of GLUT-4 (65 kDa) [19], increased in adipocytes and 11 day preadipocytes, in comparison with freshly plated preadipocytes (Fig. 4B). GLUT-1(54 kDa) [20] levels were not altered significantly in differentiated cells in comparison to preadipocytes (Fig. 4B). Many molecules in the molecular ranges of 45–200 kDa show apparent changes in tyrosine phosphorylation status upon differentiation (Supplementary Fig. 3).

4. Discussion

Adipose cells differentiate in culture, and in vitro differentiation leads to activation of most of the same set of genes characteristic of adipose tissue in vivo [21]. In addition, an important role of adipocytes and excessive adiposity in syndromes of obesity and diabetes provides a separate rationale for understanding differentiation and genetic control of these cells, in the absence or presence of insulin. Arousal of interest in studying development of adipocytes and their exact role in human physiology is a result of a gradual establishment of adipose tissue as an important endocrine organ. Moreover, obesity has not only been proposed as a major risk factor for Type II Diabetes and hypertension but also suspected to be a molecular link to various cancers [22,23]. Thus, it is apparent that a thorough understanding of the differentiation process of adipocytes certainly would help in the elucidation of the process and progression of a number of diseases associated with obesity.

AFM, which allows various approaches to analyze a cell surface, can be exploited as an excellent tool to investigate membrane alterations. On one hand, three dimensional cell surfaces can be analyzed microscopically at a single cell level and on the other hand, the surface can be analyzed at nanoscale. It also offers an outstanding resolution in the vertical *z*-axis [24]. We have performed membrane analysis of fixed preadipocytes and adipocytes for the first time, and scanning was done in air. The AFM studies have been carried out in live cells [6,7] as well as in fixed cells [6,25], based on the purpose of

the study. Either of the approaches is informative about membrane characteristics. As reported in a study wherein comparative analysis of AFM images of fixed epithelial and fibroblast cells in balanced salt solution and air was done, images of fixed cells show no discernible differences in surface features when visualized in air or under physiologic solution. Also, as stated by Braet et. al., "Membrane structures, such as ruffles, lamellipodia, microspikes and microvilli, could only clearly be observed after fixing the cells with 0.1% glutaraldehyde" and thus, fixing of cells appears to be more advantageous in certain cases [7]. The scanning microscopy revealed striking differences in the appearance of cell membranes mainly manifested as a change in roughness status, before and after differentiation. The membrane of undifferentiated preadipocytes appears to be constituted of protruding globular structures. The presence of similar structures has also been reported on the membrane of live CV-1 cells by Christian et. al. Notably, a similar organization was observed for supported isolated plasma membranes examined under liquid [26,27]. The increase in membrane cholesterol levels by 4 to 5 folds may contribute towards these changes or appears to influence membrane appearance. Differentiation is followed by an increase in various proteins and transporters, which aid in maintaining high metabolic rates in these cells, and thus, an increase in roughness in the membranes can be expected upon differentiation [24]. However, here we surprisingly observe decreased membrane roughness upon differentiation. Undifferentiated cells show maximum roughness in the membrane and cells differentiated with ID show smooth membrane topography as compared to cells differentiated with IDI. Increase in membrane cholesterol in these cells upon differentiation implies that there are alterations in lipid compositions of the membrane itself, which may have serious implications in physiology and metabolism of these adipocytes as well as in its appearance.

In accordance with the results of Rubin et. al., [28], in present studies, we have demonstrated that differentiation of preadipocytes also occurs in the presence of an adipogenic regimen consisting of IBMX and DEX only. Accumulation of lipids is hallmark of the differentiation process [23] and our results indicate that the amount of lipids accumulated depends on the composition of differentiation media. Notably, reduction in cholesterol, FFA and TG levels were observed in the presence of insulin. Moreover, in the absence of insulin, the expression of SREBP-1 was higher, which parallels with increased cholesterol, FFA and TG levels in cells. Recently, insulin has been implicated for improvement in lipid profile, as well as a decrease in circulating cholesterol [29]. Moreover, earlier it has been reported that insulin sensitizers, which increase responsiveness of various cells and tissues to insulin lead to reduced cholesterol synthesis in various cell lines [30]. These observations thus strengthen our findings that insulin in the differentiating media decreases cellular cholesterol, FFA and TG levels in 3T3-L1 differentiated preadipocytes. AFM data shows an increase in membrane nano-enfoldings in the presence of insulin, when compared to adipocytes differentiated with ID only and insulin has been implicated in the increased level of receptors on the plasma membrane [31].

The process of differentiation follows an increase in expression of differentiation markers [32]. Data presented in this manuscript suggests that these effects cannot be attributed to the contribution of insulin in differentiating media because in cells differentiated without insulin these markers are also elevated. Also, increased glucose uptake in cells differentiated in the presence of insulin is due to increased levels of GLUT-4. Although insulin is known to increase glucose uptake in adipocytes [33], increased basal glucose uptake due to the presence of insulin in initial stages of differentiation has not been documented. Moreover, glucose uptake is high in cells differentiated with IDI and in response to insulin, glucose uptake is further enhanced. Collectively, data presented here indicates that differentiation of 3T3-L1 cells leads to alterations in the plasma membrane at nano-levels which is possibly caused due to altered lipid composition. Lipid profile is altered with significant reduction in the membrane cholesterol and total cholesterol coupled with noticeable differences seen on AFM images of differentiated adipocytes. This suggests possible implications of insulin in modulating lipid composition and maintaining membrane nano-structures during differentiation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbagen.2008.08.008.

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