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Metformin reduces fibrosis factors in insulin resistant and hypertrophied adipocyte via integrin/ERK, collagen VI, apoptosis, and necrosis reduction

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ABSTRACT

Aims: Fibrosis as the hallmark of adipose tissue dysfunction which is associated with insulin resistance and type 2 diabetes, results from deposition of excess extra cellular matrix components like collagen and increased cell death. Here we investigated the effect of antidiabetic drug, Metformin, on the factors that play role in fibrosis such as; integrin/ERK pathway, collagen VI, MMP2, MMP9, apoptosis markers including DAPK1, DAPK3, DAP, SIVA, necrosis markers including RIPK1, RIPK3, and MLKL in insulin resistant and hypertrophied adipocytes.
Methods: 3T3-L1 adipocytes after differentiation to insulin resistant and hypertrophied cells, treated with Metformin, and the gene expression of aforementioned factors assayed by real time PCR. The protein expression of collagen VI and ERK assayed by western blotting.
Key findings: The expression of integrins changed from 0.5 to 6-fold in hypertrophied adipocyte versus adipocyte. Apoptosis and necrosis markers increased >1.5-fold in insulin resistant and hypertrophied adipocytes. Also ECM components and ERK activation increased >2-fold and 1.5-fold, respectively in insulin resistant and hypertrophied adipocytes. Metformin caused reduction of activity of integrin/ERK pathway in Metformin treated insulin resistant and hypertrophied adipocytes compared to untreated group. Metformin also reduced collagen VI in both gene and protein expression level, MMP2 and MMP9 in gene expression, and also the expression of apoptosis and necrosis gene.
Significance: Metformin with reduction of ECM component as collagen VI, MMP2 and MMP9, integrin/ERK pathway, necrosis markers as RIPK1, RIPK3 and MLKL, and apoptosis markers including DAP, DAPK1, DAPK3 and SIVA effects on fibrosis in insulin resistant and hypertrophied adipocytes in vitro.

1. Introduction

Adipose tissue fibrosis is the hallmark of adipose tissue dysfunction that is associated with insulin resistance and type 2 diabetes [1]. In adipose tissue fibrosis extra cellular matrix (ECM) became hard and inflexible because of deposition of excess components like collagen and increased cell death [1,2]. Adipose tissue also controls whole body systemic energy balance, with the secretion of various hormones, cytokines, and metabolites it acts as endocrine and paracrine organs [3]. Adipose tissue is dynamic and modifiable which remodels through changes in the number and size of adipocytes in response to changes in calorie intake [4,5]. Adipogenesis, a necessary process for adipose tissue remodeling, is a bi-phasic process that needs ECM and adipocytes remodeling [5,6]. The constant remodeling of ECM, creates a flexible and stiffness matrix to allow adipose tissue expansion in accordance to nutritional cues via changing cell shape and function of adipocytes [4,6]. ECM remodeling plays a pivotal role in adipogenesis [7]. Mature adipocytes via creating a balance between constructive and destructive enzymes regulate ECM remodeling [6]. Therefore, adipocytes and ECM components have reciprocal effect on each other. Not only ECM remodeling is regulated by adipocytes, and adipocyte dysfunction may lead to instability of ECM, but also the protein composition and dynamics of ECM effect on the function of adipocyte cells. In this way, instability in ECM leads to adipocyte dysfunction [6,8,9]. Integrins as cell receptors communicate between the intracellular and extracellular compartments [10,11]. This interaction is very important for cellular

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function and structure and also for matrix remodeling via gene expression regulation by supporting important biological signaling of mitogen-activated protein kinases (MAPKs), as extracellular-signal-regulated kinases (ERKs) [12–14]. Integrins mediate remodeling of ECM by regulation of enzymes expression such as matrix metalloproteinase that modulate ECM [15,16]. Integrins' gene expression changes in many cellular functions and also in pathological processes [17–19]. Disturbance of ECM remodeling mediates aberrant intracellular signaling networks including ERK 1,2 in pathological processes [20]. The activation of ERKs pathway plays a pivotal role in many essential cellular functions both in normal and pathological processes [21]. Adipocytes dysfunction is the hallmark of insulin resistance and type 2 diabetes, in obesity and non-obesity [22–24]. In adipocyte dysfunction, ECM remodeling disturbed and the components of ECM like collagen VI and ECM modifiers like matrix metalloproteinase (MMPs) including MMP2 and MMP9 increases which makes ECM hard and inelastic [2,25–27]. Also adipocyte death increases via apoptosis and necrotic-like death [28,29]. Increased collagen expression and necrosis are major causes of fibrosis [1,27]. Adipocyte apoptosis is an initial event in obesity -associated metabolic complications [28]. There are many factors that regulate apoptosis, among them is Death-associated protein kinase (DAPK) family like DAPK1, and DAPK3 that regulate cell death via activation of cell death pathways like caspase-linked apoptosis, p53-mediated cell death, and also autophagy [30]. ERK and DAPK interact with each other, DAPK which is activated by ERK, increases conversely, and inhibits ERK signaling in the nucleus via cytoplasmic retention of ERK both in vitro and in vivo. This reciprocal regulation of DAPK and ERK promotes the apoptotic activity of DAPK [31]. Also SIVA and Death-associated protein(DAP), the two proapoptotic protein, have role in apoptosis via association with CD27 and CD60, respectively as tumor necrosis factor receptor superfamily [32,33]. In necrotic-like cell death three proteins including; receptor-interacting protein kinase (RIPK) 1, RIPK3, and mixed lineage kinase domain-like (MLKL) play key role. The expression of necrosis markers increases in many pathological processes such as inflammatory associated diseases and active necrosis death [34]. Also, The increased activity of ERK pathway following aberrant ECM remodeling, dysregulates adipocytokine expression and will enhance inflammatory responses in adipocytes [35]. The activity of ERKs signaling pathway increased in insulin resistance and type 2 diabetes [35]. In hypertrophied adipocytes, b1-integrin/ERK signaling pathway activates which modulates transcription factors and gene expression for adipose function [36]. Metformin as anti-diabetic drugs has a pivotal role in the treatment of type 2 diabetic patients [37]. Therefore the aim of this study, is investigating the effect of Metformin on fibrosis via integrins/ERK pathway, ECM components, apoptosis and necrosis factors in insulin resistance and hypertrophied 3T3-L1 adipocyte in vitro.

2. Materials and method

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum and penicillin–streptomycin were purchased from Gibco BRL (USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), Oil red O powder, fatty acid-free bovine serum albumin, 3-Isobutyl-1-methylxanthine (IBMX), dexamethasone, insulin and palmitate were purchased from Sigma Chemicals (USA). 6-Deoxy-D-glucose was purchased from Cayman Chemicals (USA). Glucose oxidase and triglyceride assay kit were purchased from Pars Azmoon Company (IRAN). RNA extraction kit was prepared from Qiagen (USA). cDNA synthesis kit was purchased from Thermo Fisher Scientific (USA). FITC-Annexin V Apoptosis Detection Kit was obtained from BD Bioscience (Germany). HRP-conjugated anti-mouse IgG and antibodies against β-Actin, phospho-ERK 1/2 (Thr202, Tyr204), ERK 1/2 and collagen VI were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cells culture

Mouse embryo 3T3-L1 cells were obtained from Iranian biological resource center (Tehran, Iran). 3T3-L1 preadipocytes were cultured in high glucose DMEM containing sodium pyruvate (110 mg/L) and 10% fetal bovine serum with 100 U/mL penicillin–streptomycin in a humidified 5% CO2 atmosphere at 37 °C. 3T3-L1 preadipocytes were differentiated to adipocytes from post confluent cells by a regimen of 10% FBS, 1.5 μg/mL insulin, 1 μM dexamethasone (DEX), and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) for 2 days. This medium was removed and replaced with medium supplemented with 10% FBS and 1.5 μg/mL insulin for3 days. Cells were then cultured in DMEM with 10% FBS. Cells were used 9–10 days after initiation of differentiation when >90% cells showed adipocyte phenotype. For adipocyte hypertrophy, adipocytes were maintained at 37 °C for 30 day and medium was changed after every two days. Insulin resistant adipocytes were created by treating with free fatty acid- bovine serum albumin (FFA-BSA)-bound palmitate for 24 h after overnight starvation in DMEM with 0.5% BSA. Control cells were incubated with the same concentration of fatty acid-free BSA without palmitate. The adipocytes treated with 5 mM of Metformin in hypertrophied and insulin resistance conditions for 24 h. Cells untreated with Metformin considered as control in every group.

2.3. MTT assay

The cytotoxicity of palmitate and Metformin on adipocytes was assayed by measuring viable cells that reduced MTT(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) by considering the concentration of a palmitate and Metformin that kill 50% of cells as LC50 (lethal concentration 50) [38]. 3T3-L1 adipocytes were cultured at a density of 2×10^4 cells per well in 96-well plate and incubated for 24 h. then cells were treated with (0–10 mM) of Metformin, and After serum deprivation in 0.5% BSA/DMEM for one night, treated with various doses (100–400 μM) of palmitate. After 24 h, cells were incubated with MTT solution (0.5 mg/mL) for 4 h at 37 °C. The medium was gently withdrawn and DMSO added to each well. The plate was shaken to dissolve the produced formazan crystal. Finally, absorbance was measured at 570 nm by micro plate reader (Synergy HTX, BioTek Instrument, Inc.). Cytotoxicity was analyzed by measuring cell viability, and the percentage of viable cells was normalized after considering cell viability without treatment as control.

2.4. Palmitate/BSA solution

The palmitate solution used for treatment of the adipocytes was made by conjugating palmitate with bovine serum albumin (BSA). Sodium palmitate (27.84 mg) was dissolved in 0.1 M NaOH (1 mL) and heated at 70–80 °C in a shaking water bath to make 100 mM of stock solution. We prepared a solution of 10% w/v FFA-BSA in DMEM. 0.25 mL of 100 mM palmitate was added to 4.75 mL of 10% FFA-BSA-medium and mixed for a minimum of 2 h to make a 5 mM palmitate stock solution. The palmitate/BSA solution was filtered (0.22 μm, PES Membrane filter, MILLIPORE).

2.5. Oil red O staining

Oil red o stock solution was made by the addition of 300 mg of oil red o powder in 100 mL of 99% isopropanol. 3 parts of oil red o stock solution was combined with 2 parts deionized water to make oil red working solution. This stain is ready after to sit at room temperature for 10 min. Cells before staining with oil red O, were washed two times with phosphate-buffered saline (PBS), pH 7.4 and fixed in 10% formaldehyde in PBS for 1 h at room temperature. Then cells were washed three times each time for 5 min in water and stained with oil red O solution for 1 h at room temperature.

2.6. Triglyceride assay

The cellular content of triglyceride (TG) was determined using a TG determination kit (Pars Azmoon, Iran). Briefly, cells were rinsed three times with PBS and scrapped off the plate. Then, cells were lysed with NP40 (5%) and homogenized by heating the samples to 80–100 °C in a water bath and then cooling down to room temperature. The heating repeated one more time to solubilize all triglyceride. The cell lysate (10 μl) was mixed with 1 mL of the enzyme solution which was included in the kit and incubated for 10 min at 37 °C. Absorbance at 540 nm was measured during 60 min. For internal control, total protein concentration in 3T3-L1 adipocytes was measured by the BCA Protein Assay kit (Takara).

2.7. Glucose metabolic assay

The adipocytes after differentiation were starved overnight in DMEM supplemented with 0.5% BSA in 24-well tissue culture plates. After incubation time, in the cell culture palmitate solution (100–400 μM) was added for 24 h. Then, cells were washed three times, and glucose consumption was performed in Krebs–Ringer bicarbonate HEPES (KRBH) buffer (115 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.25 mM MgSO4, 1.2 mM NaH2PO4, 20 mM NaHCO3, 10 mM HEPES and pH 7.4) containing 5 mM glucose with or without insulin (100 nM) for 3 h. The glucose concentration in medium was measured by the glucose oxidase method. Total protein concentration in 3T3-L1 adipocytes was measured using the BCA Protein Assay kit (Takara) as internal control.

2.8. 6-Deoxy-D-glucose uptake assay

Differentiated adipocytes (7–9 days after initiation of differentiation) in 24-well plates were incubated in 0.5% BSA/DMEM overnight for serum starvation. Cells were treated with palmitate (100–400 μM) for 24 h. Glucose uptake assay was done by Zhao, Wieman et al. 2008 protocol with some modifications [39]. Cells were incubated at 37 °C in 1 mL/well KRBH containing 100 nM insulin for 30 min. Then, cells were washed by KRBH. After washing the cells were incubated in 1 mL KRBH containing 0.1 mM 6-deoxyglucose for 20 min. Then, the cells were washed thrice by ice cold PBS, and lysed by lysis buffer. Fluorescence intensity was measured by micro plate reader (Synergy HTX, BioTek Instrument, Inc.) (λex = 530 to 570 nm, λem = 590 to 620 nm). Non-specific deoxyglucose uptake was measured in the presence of 20 μM cytochalasin B and deducted from the total uptake to get insulin-specific glucose uptake. All samples were normalized by measuring total protein using BCA Protein Assay kit.

2.9. Primer design

Primers were targeted against beta-actin (h-Actin), PPAR-g, col VI, MMP2, MMP9, integrins α and β chains, apoptosis and necrosis factors genes. The primers were specific for mouse genes and chosen based on the below criteria: (i) primer melting temperature of approximately 60 °C, (ii) GC content of approximately 50–55%, (iii) preferably no G at the 5′ end. Specificity and cross-reactivity were checked with the Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST) and the PREMIER Biosoft (http://www.premierbiosoft.com/NetPrimer/AnalyzePrimer.jsp) (Table 1).

2.10. Gene expression

Total cellular RNA was extracted from 3T3-L1 adipocytes using the RNeasy cell mini kit from Qiagen (USA). Quantity and quality of RNA were validated by isolation of RNA to 18s and 28s bands on agarose gel electrophoresis. Total RNA (1 μg) reverse transcribed to cDNA by cDNA synthesis kit from Thermo Fisher Scientific (USA). Quantitative Real-time PCR (qRT-PCR) was performed by primers specific for each cDNA and took place in a 20 μL PCR reaction mixture supplied by Ampliqon SYBR master mix in an ABI-Step One software (version 2.3) equipped Real-Time PCR System (2012 life technologies corporation). All PCRs were normalized to β-actin, and regarding the efficiencies of all primers being ~2, relative expression levels were determined by the $\Delta\Delta Ct$ method [40] to make comparisons between relative abundance from different experiments.

2.11. Western blotting

Cell were lysed with an ice-cold lysis buffer (50 mM Tris-HCl (pH 8.0), 0.4% Nonidet P-40, 120 mM NaCl, 1.5 mM MgCl2, 0.1% SDS, 2 mM phenylmethylsulfonyl fluoride, 80 μg/mL leupeptin, 3 mM NaF and 1 mM DTT). Equal amounts of proteins (40 μg) were mixed with SDS loading buffer. Total protein was came apart by 10% SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane. Membranes were blocked in 5% BSA and hybridized with primary antibodies. After incubation with HRP-conjugated anti-mouse IgG antibody, immunoreactive protein expression was detected by enhanced chemiluminescence ECL assay kit. Anti-β-actin antibody was used as control to normalize the amount of proteins.

2.12. Apoptosis analysis

3T3-L1 adipocytes were seeded in 12-well plates and treated with Metformin (5 mM) for 24 h. Then, cells were rinsed with PBS and harvested by incubation with Accutase solution for 1–6 min. Detachment was controlled via microscopy. The FITC-Annexin V Apoptosis Detection Kit was used to detect Annexin V. The harvested cells, after washing twice with cold PBS, resuspended in binding buffer at a final density of 10^6 cells/mL. FITC-annexin (5 μl) and PI (5 μl) were added to 100 μL of the cell suspension and mixed by gently vortexing, then cells incubated for 15 min at room temperature in dark. 400 μl of binding buffer was added and cells were analyzed by flow cytometry using FACS Calibur (BD Bioscience, Heidelberg, Germany) and the Cellquest Pro software (BD Bioscience).

2.13. Statistical analysis

All experiments were repeated independently for three times in triplicate. Data was analyzed by one-way analysis of variance (ANOVA), using IMB statistic software (version 24) and Prism 5 (Graphpad Software Inc., version 5.0). Data were shown as mean ± SD or SEM. p-Values < 0.05 were considered statistically significant.

3. Result

3.1. Differentiation and hypertrophied adipocytes

3T3-L1 preadipocytes were differentiated to adipocyte by treating with differentiation medium as mentioned. For adipocyte differentiation validation we used PPAR-ɣ gene expression [41] and oil red O staining. Results indicate, differentiated adipocytes had many small fat droplets when stained by oil red O, relative to preadipocytes that hadn't shown any fat droplets (Fig. 1A). While in hypertrophied adipocytes (30 days after differentiation), cells had larger size with one big fat droplet versus freshly differentiated adipocytes (7 days after differentiation) that had smaller cell size with many small fat droplets with various size (Fig. 1A). Also PPAR-ɣ̇ gene expression showed that PPAR-ɣ expressed >900-fold (950 ± 141.1 $p < 0.001$) in differentiated adipocytes versus preadipocytes (Fig. 1B). The above results also validated by triglyceride content assay in preadipocytes, differentiated, insulin resistant and hypertrophied adipocytes. Data analyses shows that while preadipocytes had no recognizable triglyceride content, adipocytes and insulin resistant adipocytes had roughly equal amount of triglyceride

Table 1
Primers sequence.

Target gene name	Forward (5′.....3′)	Reverse(5′.....3′)
ITGA1	TCGCCAGCTTTGGAAGTCAT	ATGTACTGGAGTTGGGCAGC
ITGA2	TGTCTGGCGTATAATGTTGGC	TGCTGTACTGAATACCCAAACTG
ITGA3	CTAGCCGAGGTCCAAAGAGC	ACTCTCACGTTGGTTCGGTG
ITGA4	ATGAAGAGCGATAACAAACTCCC	CGGGGCCATCCTTTTACTCA
ITGA5	CTCCCTCTACAACGTCTCAGG	CATCTCCATTGGTATCAGTGGC
ITGA6	GGGATCGTCCGTGTAGAACAA	TCTCTCCACCAACTTCATAGGG
ITGA7	TCGGAAACCAGAAACACCCT	GGAGAAAATCGCACCTCGGA
ITGA8	ATCTCTTGTGCAGTGGGTCG	TTCTTTCTCTTGAGGAACGTGT
ITGA9	TGCTTTCCAGTGTTGACGAGA	TTAAAGGACACGTTGGCATCATA
ITGA10	TCCATGTACCAAAGGCCACC	CCCCCATCAGCATCTGTCTC
ITGA11	GGACCTCCGTTCAACTGTGT	CTGGTCGGTGAAGAAGTCCC
ITGAE	GGCATTCAGTGGTCTGTGCTA	TTCACCAAGGATCGGCAGTT
ITGAL	AAACCTGACCCTGTCATCCC	AATCGCACCCAGTAGGCATC
ITGAM	CATGGTCACCTCCTGCTTGT	CCTCACATACGACTCCTGCC
ITGAV	AAAGACCGTTGAGTATGCTCCA	ATGCTGAATCCTCCTTGACAAAA
ITGAX	TCCAGGTCTGTGTTTGAGTGTC	ATATGAAGGCAGACAGTGGCA
ITGA2B	GCCCTAAACGCAAGTCAAGAG	TATTTCGTGTCTCATCCCTGG
ITGAD	TGCTCAGCTATGGTCCGTG	CCAGGTTAGACCCATGACAGG
ITGB1	ACTGTGATGCCGTATATTAGCAC	GATATGCGTTGCTGACCAACA
ITGB2	AGGAGCATCGCTAATCCTGAG	CCTGGTCGCAAGTAAAGTGTC
ITGB3	GGCGTTGTTGTTGGAGAGTC	CTTCAGGTTACATCGGGGTGA
ITGB4	TCTCTGTGAGGATCTCCGCT	AGCAGTACTCCACCACCTCT
ITGB5	CCTCCTGATTGGGATGGCAC	TTGAGGCTTTGGAACTTGGC
ITGB6	AAGACTTTCACGGCTTCCAGC	AGTAGCAGAAACAGGCAGACC
ITGB7	ATCACACCCGTGCCATCATA	TACTCCCGTCGGTCGTAGAT
ITGB8	GCTTTGCATTATGTGCGGCT	CAAGAACCAAGGCTGGACCT
Col VI	AACCCTCCACATACTGCTAATTC	TCGTTGTCACTGGCTTCATT
DAP	TATCCAACAGCCTCGCAAGT	ACTCTGTGGGAAGTACGGGA
DAPK1	CAGATTCTCAGCGGCGTTTAC	GATCCGAGGTTTGGGCACATT
DAPK3	TAGACGGTGTCCACTACCTGC	CAAAGTCGATGAGCTTAATGCG
SIVA	AGTCTGGTGTGGTAGGAGCC	GCACGATGAACAAGCGATGG
MLKL	TTAGGCCAGCTCATCTATGAACA	TGCACACGGTTTCCTAGACG
RIPK1	AGAAGAAGGGAACTATTCGCTGG	CATCTATCTGGGTCTTTAGCACG
RIPK3	GGCACCCTAGCGTACTTGG	GCTGTAGACATCACTCGCTTT
PPARɣ	GATGCACTGCCTATGAGCACTT	AGAGGTCCACAGAGCTGATTCC
MMP-2	CAGGGAATGAGTACTGGGTCTATT	ACTCCAGTTAAAGGCAGCATCTAC
MMP-9	AATCTCTTCTAGAGACTGGGAAGGAG	AGCTGATTGACTAAAGTAGCTGGA
B-actin	TGTCCACCTTCCAGCAGATGT	AGCTCAGTAACAGTCCGCCTAGA

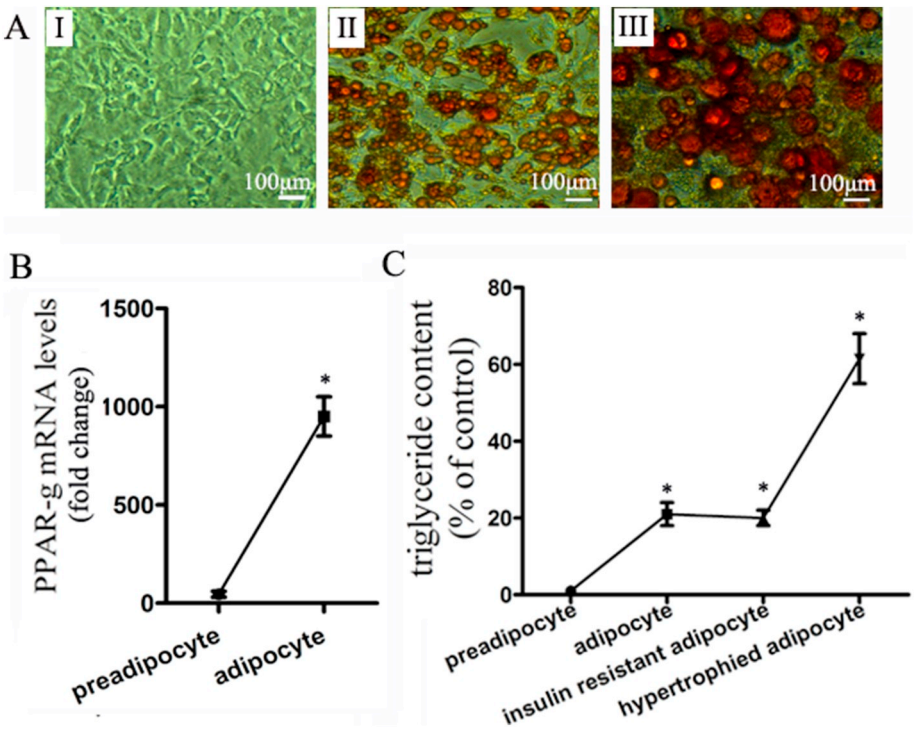

Fig. 1. A: oil red O staining of 3T3-L1 adipocytes. I) 3T3-L1 preadipocytes before differentiation: cells have fibroblastic shape and no fat droplet. II) Freshly differentiated 3T3-L1 adipocyte (7–8 days after differentiation) stained with oil red o; adipocytes have many small fat droplets with spherical shape. III). Oil red o staining of hypertrophied adipocytes (30 days after differentiation); cells are larger than freshly differentiated adipocytes and almost have one large fat droplet that occupies the whole cell.
B: 3T3-L1 preadipocytes were differentiated to adipocyte by treating with differentiation medium. PPAR-ɣ gene expression assayed by real-time PCR. PPAR-ɣ mRNA levels are significantly increased in differentiated adipocytes. The data were displayed with mean ± S.E.M of three separate experiments in triplicate, and ($*p < 0.05$).
C) Triglyceride content assay with calorimetry, in preadipocyte, differentiated, insulin resistant and hypertrophied adipocytes. Data analyses shows that differentiated and insulin resistant adipocytes have about equal amount of triglyceride, which was much more in hypertrophied adipocytes compared to preadipocytes. The data was shown with mean ± S.E.M of three separate experiments in triplicate and analyzed by ANOVA ($*p < 0.05$).

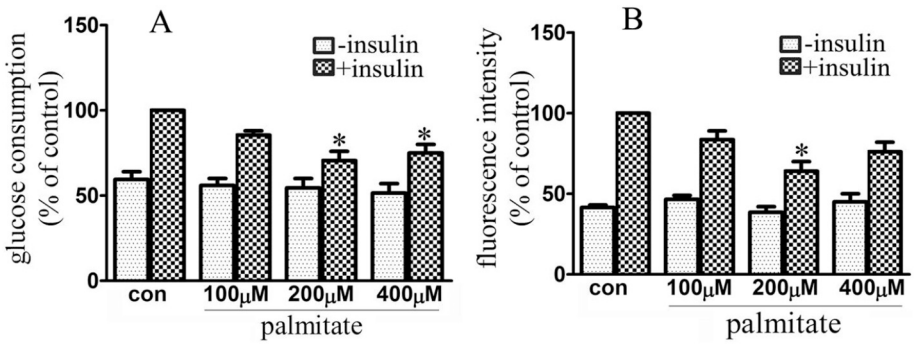

Fig. 2. A: palmitate was used for inducing insulin resistance in 3T3-L1 adipocytes. Glucose consumption of adipocytes treated with palmitate 100–400 μM for 24 h, was performed in KRBH buffer containing 5 mM glucose with or without insulin (100 nM) for 3 h. Cells treated with palmitate (200 μM) for 24 h, induced a significant inhibition of glucose consumption. The data was shown with mean ± S.E.M of three separate experiments in triplicate and analyzed by ANOVA (*$p < 0.05$).
B: Glucose uptake was investigated with 6-deoxy-D-glucose. Glucose uptake of adipocytes treated with palmitate 100–400 μM for 24 h, were measured by using KRBH buffer containing 6-deoxyglucose for 20 min. Fluorescence intensity of uptaken glucose was measured by microplate reader. Cells treated with palmitate (200 μM) for 24 h, induced a significant inhibition of insulin-stimulated 6-deoxy-D-glucose uptake in 3T3-L1 adipocytes versus untreated cells. The data was shown with mean ± S.E.M of three separate experiments in triplicate and analyzed by ANOVA (*$p < 0.05$).

which was 20-fold (21 ± 4.2 $p < 0.05$ and 20 ± 2.8 $p < 0.05$) more than preadipocytes. This value was even larger, 60-fold (61 ± 9.1 $p < 0.001$), in hypertrophied adipocytes compared to preadipocytes (Fig. 1C). Data was normalized by measuring total protein. (Data was shown ±SD and p value)

3.2. Cytotoxicity

The result of the MTT assay shows that there is no meaningful change in cell viability after 24-h treatment with (0–400 μM) palmitate and (0–10 mM) Metformin. (Data not shown).

3.3. Insulin resistance adipocytes

We used palmitate for inducing insulin resistance in 3t3-l1 adipocytes [42]. First, Adipocytes were treated with palmitate (100–400 μM) for 24 h. Glucose consumption and glucose uptake tests were used for demonstration of insulin sensitivity in adipocytes, by evaluating their abilities to consume and uptake glucose. The results show adipocytes treated with palmitate 200 μM for 24 h had about a 30% reduction (71.0 ± 8.0 $p < 0.05$) in glucose consumption compared to untreated cells (Fig. 2A). Also we used glucose uptake test with 6-deoxy-D-glucose for the assessment of insulin sensitivity in adipocytes. The results of glucose uptake after palmitate (100–400 μM) treatment for 24 h showed a significant inhibition, about 36% (64.0 ± 7.2 $p < 0.05$), in insulin-stimulated 6-deoxy-D-glucose uptake in adipocytes by 200 μM palmitate (Fig. 2B). So, we used palmitate 200 μM for inducing insulin resistance in 3T3-L1 adipocytes in other experiments.

3.4. Effect of Metformin on integrins' gene expression:

We evaluated the effect of Metformin on integrins' gene expression in hypertrophied adipocytes versus normal adipocyte from 3T3-L1 cell line by Real-time PCR. The results of integrins gene expression levels in treated and untreated cells versus normal adipocytes as control, were shown in Table 2 and Fig. 3. Collectively, Metformin improved expression of ITGA3 and ITGA8 genes in Metformin treated hypertrophied adipocytes to normal levels while their expressions suppressed in hypertrophied adipocyte (Fig. 3). Metformin caused ITGB6 over expression more than normal level while its expression was suppressed in hypertrophied adipocyte (Fig. 3). Metformin increased expression of ITGA4, ITGA5, ITGB3, ITGB5, and ITGB7 genes in hypertrophied adipocyte while their expressions were in normal range (Fig. 3). Metformin reduced expression of ITGA2, ITGA10, ITGA11, and ITGAL genes that over expressed in hypertrophied adipocyte to normal range. Metformin did not have significant effect on other integrins expression in hypertrophied adipocytes. Data ± SD and significant levels $p < 0.05$ were shown in Table 2, and its chart shown in Fig. 3.

3.5. The effect of Metformin on apoptotic factors:

We evaluated the effect of Metformin on gene expression of apoptotic factors in insulin resistant and hypertrophied adipocytes versus normal adipocyte from 3T3-L1 cell line by Real-time PCR. The expression of DAP increased (1.35 ± 0.07 $p = 0.018$ and 3.0 ± 0.28 $p = 0.005$) in insulin resistance and hypertrophied adipocyte, respectively relative to normal adipocyte as control. Metformin caused reduction of DAP expression in Metformin treated insulin resistant (1.15 ± 0.07 $p = 0.080$) and hypertrophied (1.95 ± 0.21 $p = 0.029$) adipocytes compared to untreated cells (Fig. 4). While DAPK1 over expressed in insulin resistant (5.3 ± 0.42 $p = 0.002$) and hypertrophied (4.5 ± 0.70 $p = 0.014$) adipocytes, Metformin reduced expression of DAPK1 to normal levels (2.3 ± 0.42 $p = 0.007$) in insulin resistant adipocytes and (3.6 ± 0.56 $p = 0.331$) in hypertrophied adipocyte (Fig. 4). The expression of DAPK3 increased in insulin resistant (1.7 ± 0.14 $p = 0.009$) and hypertrophied (2.9 ± 0.14 $p = 0.002$) adipocyte versus normal adipocytes. Metformin increased the expression of DAPK3 in Metformin treated insulin resistant (2.45 ± 0.07 $p = 0.008$) and no significant change was seen in Metformin treated hypertrophied (2.5 ± 0.21 $p = 0.108$) adipocytes versus untreated cells (Fig. 4). The expression of SIVA increased in insulin resistant (1.4 ± 0.14 $p = 0.055$) and hypertrophied (2.2 ± 0.21 $p = 0.006$) adipocytes versus normal adipocytes, while Metformin caused SIVA expression reduction in Metformin treated hypertrophied adipocytes (1.62 ± 0.1 $p = 0.040$) versus untreated cells, it had no significant effect on expression of SIVA in Metformin treated insulin resistant adipocytes (1.33 ± 0.09 $p = 0.779$) versus untreated cells. The data was shown ±SD and p value shows significant levels.

3.6. The effect of Metformin on necrosis factors:

We evaluated the effect of Metformin on gene expression of necrotic factors in insulin resistant and hypertrophied adipocytes by Real-time PCR. Our results shows, while in hypertrophied adipocytes expression of necrotic factors increased; RIPK1 (1.7 ± 0.14 $p = 0.018$), RIPK3 (4.9 ± 0.21 $p = 0.001$), and MLKL (1.8 ± 0.14 $p = 0.013$) versus normal adipocytes, Metformin caused reduction of their expression to normal levels; RIPK1 (1.1 ± 0.14 $p = 0.028$), RIPK3 (2.7 ± 0.42 $p = 0.008$) and MLKL (0.90 ± 0.14 $p = 0.009$) (Fig. 5). Expression of RIPK1 increased almost (1.55 ± 0.07 $p = 0.048$) in insulin resistant adipocytes and no significant change (1.15 ± 0.22 $p = 0.105$) was seen in Metformin treated insulin resistant compared to untreated cells (Fig. 5). The increased expression of RIPK3 (1.5 ± 0.14 $p = 0.084$) in insulin resistant adipocyte versus normal adipocytes was not significant. Metformin reduced RIPK3 expression in Metformin treated insulin resistant adipocyte (0.95 ± 0.21 $p = 0.066$) compared to untreated cells (Fig. 5). Also, the expression of MLKL increased (1.65 ± 0.21 $p = 0.043$) in insulin resistant versus normal adipocytes,

Table 2
Integrins gene expression levels.

Name	Hypertrophied vs control			Hyper. + met(5 mM) vs Hypertrophied		
	Value	*p* value	Sig? P < 0.05?	Value	*p* value	Sig? P < 0.05?
ITGA1	1.05 ± 0.07	0.855	ns	1.25 ± 0.14	0.219	ns
ITGA2	2.6 ± 0.25	0.007	*	1.65 ± 0.21	0.030	*
ITGA3	0.55 ± 0.07	0.032	*	1.05 ± 0.14	0.024	*
ITGA4	0.8 ± 0.14	0.461	ns	2.05 ± 0.21	0.007	*
ITGA5	1.2 ± 0.10	0.216	ns	1.9 ± 0.15	0.014	*
ITGA6	0.92 ± 0.04	0.357	ns	0.95 ± 0.07	0.855	ns
ITGA7	0.46 ± 0.06	0.004	*	0.55 ± 0.07	0.332	ns
ITGA8	0.56 ± 0.06	0.007	*	1.15 ± 0.07	0.003	*
ITGA9	2.8 ± 0.29	0.006	*	2.0 ± 0.21	0.069	ns
ITGA10	1.5 ± 0.14	0.031	*	1.0 ± 0.10	0.046	*
ITGA11	1.5 ± 0.15	0.024	*	0.95 ± 0.07	0.019	*
ITGAV	1.67 ± 0.17	0.047	*	1.45 ± 0.21	0.443	ns
ITGAE	0.75 ± 0.08	0.046	*	0.65 ± 0.07	0.329	ns
ITGAM	3.1 ± 0.29	0.004	*	3.6 ± 0.21	0.143	ns
ITGAX	1.8 ± 0.21	0.029	*	1.5 ± 0.19	0.312	ns
ITGAD	0.87 ± 0.11	0.476	ns	0.82 ± 0.13	0.873	ns
ITGA2B	0.97 ± 0.18	0.980	ns	0.8 ± 0.14	0.469	ns
ITGAL	2.3 ± 0.28	0.010	*	1.4 ± 0.11	0.033	*
ITGB1	1.52 ± 0.10	0.043	*	1.42 ± 0.18	0.708	ns
ITGB2	0.95 ± 0.07	0.685	ns	1.1 ± 0.8	0.080	ns
ITGB3	1.4 ± 0.14	0.304	ns	2.4 ± 0.35	0.046	*
ITGB4	6.5 ± 0.60	0.002	*	6.1 ± 0.51	0.636	ns
ITGB5	1.26 ± 0.1	0.277	ns	1.85 ± 0.22	0.045	*
ITGB6	0.54 ± 0.10	0.225	ns	3.2 ± 0.35	0.002	*
ITGB7	1.06 ± 0.09	0.847	ns	1.62 ± 0.18	0.033	*
ITGB8	1.4 ± 0.21	0.198	ns	1.85 ± 0.25	0.155	ns

Integrins gene expression levels ±SD and *p* value; * significant level: $p < 0.05$, ns: not significant.

while Metformin reduced MLKL expression to normal levels (0.75 ± 0.14 $p = 0.015$). The data was shown ±SD and *p* value shows significant levels (Fig. 5).

3.7. The effect of Metformin on extra cellular matrix components:

We evaluated the effect of Metformin on gene expression of extra cellular factors in insulin resistant and hypertrophied adipocytes by Real-time PCR. Results show, the expression of col VI increased (2.1 ± 0.20 $p = 0.011$ and 2.7 ± 0.14 $p = 0.004$) in insulin resistant and hypertrophied adipocyte respectively compared to normal adipocyte. Metformin decreased expression of col. VI in Metformin treated insulin resistance (1.42 ± 0.17 $p = 0.040$) and hypertrophied (1.6 ± 0.24 $p = 0.015$) adipocytes compared to untreated cells (Fig. 6). The expression of MMP2 increased in insulin resistant (2.7 ± 0.18 $p = 0.005$) and hypertrophied (3.3 ± 0.28 $p = 0.004$) adipocytes versus normal adipocytes, and the expression of MMP2 decreased in Metformin treated insulin resistant (1.8 ± 0.26 $p = 0.031$) and hypertrophied (2.1 ± 0.25 $p = 0.025$) adipocytes compared to untreated cells (Fig. 6). Metformin caused reduced expression of MMP9 in

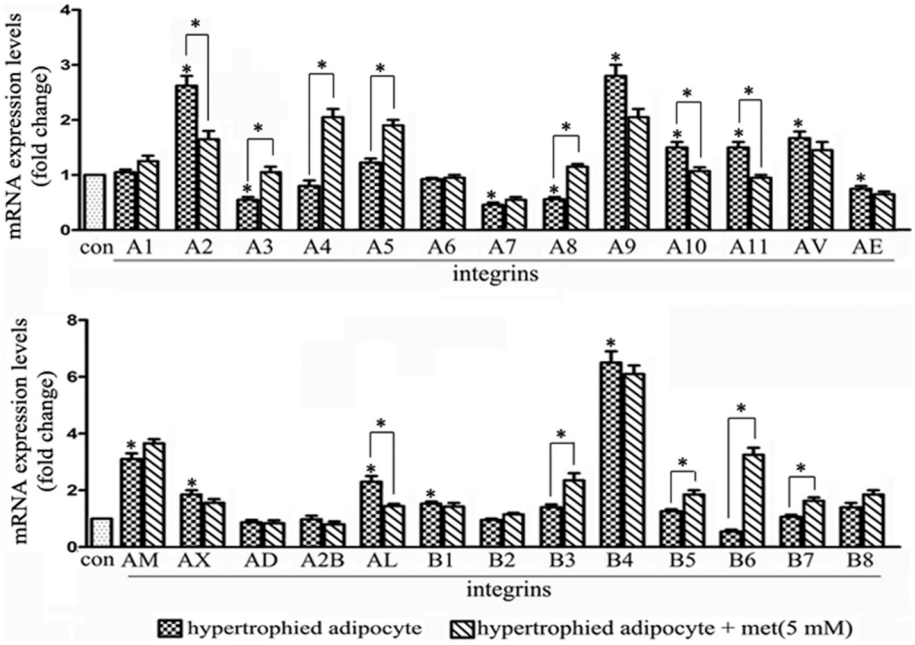

Fig. 3. Real-time PCR quantification of integrins mRNA in metformin treated and untreated 3T3-L1 hypertrophied adipocyte versus adipocytes as control. 1 μg of total RNA from any sample were prepared, and processed for real-time PCR. PCR reactions were performed in triplicates. The relative amount of mRNA was calculated using comparative CT method. The expression of every integrins in metformin treated and untreated hypertrophied adipocytes were shown compared to adipocytes as control. The data was displayed with mean ± S.E.M of three separate experiments in triplicate and analyzed by ANOVA, (*$p < 0.05$).

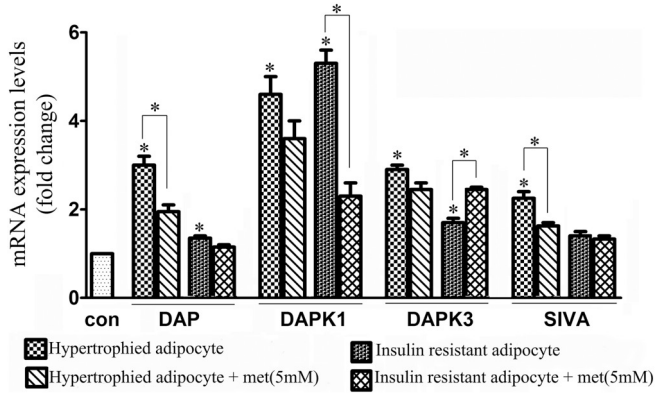

Fig. 4. Real-time PCR quantification of apoptosis markers mRNA in insulin resistant and hypertrophied adipocyte in Metformin treated and untreated cells. mRNA expression levels of DAP, DAPK1, DAPK3, and SIVA increased in insulin resistant and hypertrophied adipocytes versus control (normal adipocyte), Metformin reduced mRNA levels of DAP and SIVA in Metformin treated hypertrophied cell versus untreated cells. Metformin reduced DAPK1 and increased DAPK3 in Metformin treated insulin resistant adipocytes versus untreated cells. The data was displayed with mean ± S.E.M of three separate experiments in triplicate and analyzed by ANOVA (*$p < 0.05$).

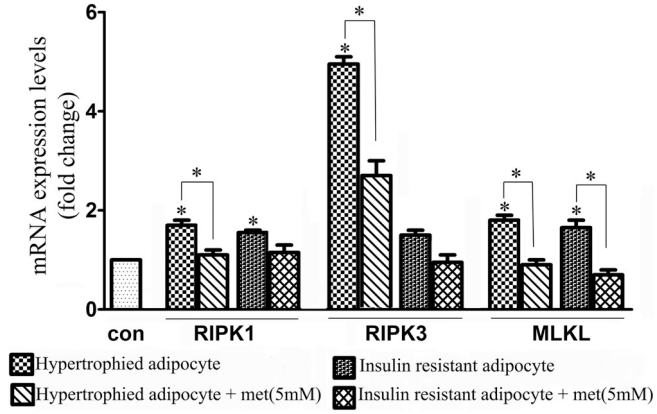

Fig. 5. Real-time PCR quantification of necrosis markers mRNA in insulin resistant and hypertrophied adipocyte in Metformin treated and untreated stats. mRNA expression levels of RIPK1, RIPK3 and MLKL increased in insulin resistant and hypertrophied adipocytes versus control (normal adipocyte), Metformin reduced mRNA levels of RIPK1 and RIPK3 in Metformin treated hypertrophied cell versus untreated cells. Metformin reduced mRNA level of MLKL in Metformin treated insulin resistance and hypertrophied adipocytes versus untreated cells. The data was displayed with mean ± S.E.M of three separate experiments in triplicate, and analyzed by ANOVA (*$p < 0.05$).

Metformin treated insulin resistant (1.6 ± 0.12 $p = 0.045$) and hypertrophied (1.8 ± 0.22 $p = 0.012$) adipocytes compared to untreated insulin resistant (2.5 ± 0.30 $p = 0.009$) and hypertrophied (3.0 ± 0.19 $p = 0.003$) adipocytes versus normal adipocytes. The data was shown ±SD and p value shows significant levels (Fig. 6).

3.8. The effect of Metformin on collagen VI and ERK 1,2 protein expressions

The effect of Metformin was evaluated on protein levels of ERK 1,2, phosphorylated ERK1, 2(p-ERK 1,2) and collagen VI in in insulin resistant and hypertrophied adipocytes versus normal adipocyte by western blotting. Results show that protein expression of collagen VI increased (1.48 ± 0.09 $p = 0.010$) in insulin resistant and (1.6 ± 0.15 $p = 0.023$) in hypertrophied adipocytes versus normal adipocytes. Metformin reduced the protein level of col. VI (1.1 ± 0.07 $p = 0.019$) in insulin resistant and (1.15 ± 0.11 $p = 0.049$) in hypertrophied

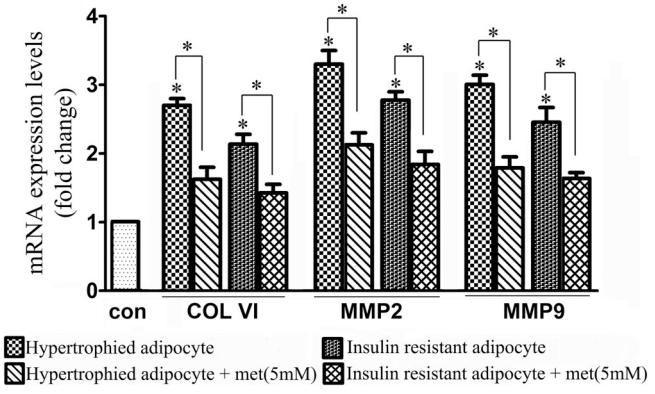

Fig. 6. Real-time PCR quantification of extra cellular matrix components mRNA in insulin resistant and hypertrophied adipocyte in Metformin treated and untreated stats. mRNA expression levels of col. VI, MMP2 and MMP9 increased in insulin resistant and hypertrophied adipocytes versus control (normal adipocyte), Metformin reduced mRNA levels of col. VI, MMP2 and MMP9 in metformin treated in insulin resistant and hypertrophied adipocytes versus untreated cells. The data was displayed with mean ± S.E.M of three separate experiments in triplicate and analyzed by ANOVA (*$p < 0.05$).

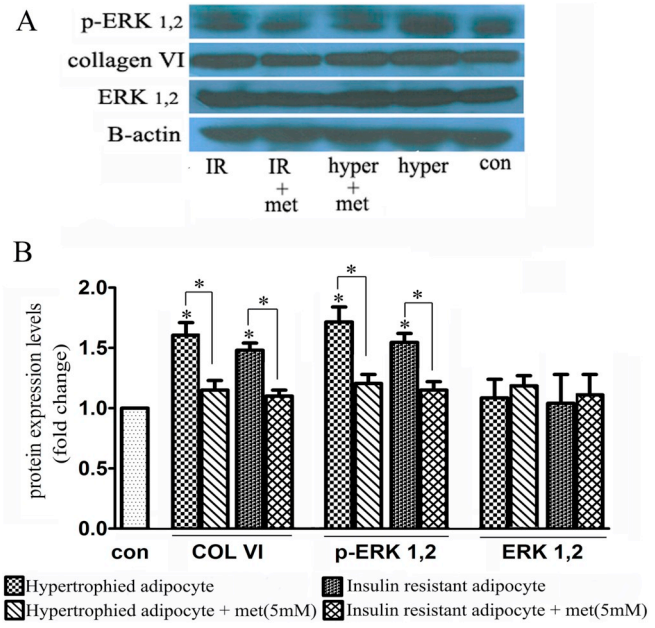

Fig. 7. A: Equal amounts of cell proteins in every group were separated by 10% SDS-PAGE and transferred onto a PVDF membrane. The membranes after blocking and hybridizing with primary antibodies, incubated with HRP-conjugated anti-mouse IgG antibody. Immunoreactive protein expression was detected by enhanced chemiluminescence ECL assay kit. Anti-β-actin antibody was used as control to normalize the levels of proteins.(IR: insulin resistant adipocyte, hyper: hypertrophied adipocyte, met: Metformin (5 mM)).
B: comparison of protein expression of ERK 1,2, p-ERK 1,2 and col VI in insulin resistant and hypertrophied adipocyte in Metformin treated and untreated cells. No significant change of expression for ERK 1,2 protein was seen in cell types. Expression of collagen VI increased in insulin resistant, and hypertrophied adipocyte. Metformin reduced expression of collagen VI in Metformin treated cells versus untreated cells of insulin resistant and hypertrophied adipocytes. Protein p-ERK was activated in insulin resistant and hypertrophied adipocyte. Metformin improved the amount of p-ERK to normal levels. Data was shown with mean ± S.E.M of three separate experiments in triplicate and analyzed by ANOVA (*$p < 0.05$).

adipocytes treated with Metformin compared to untreated groups versus normal adipocytes (Fig. 7). No significant change was seen in the protein expression of ERK 1,2 in insulin resistant and hypertrophied

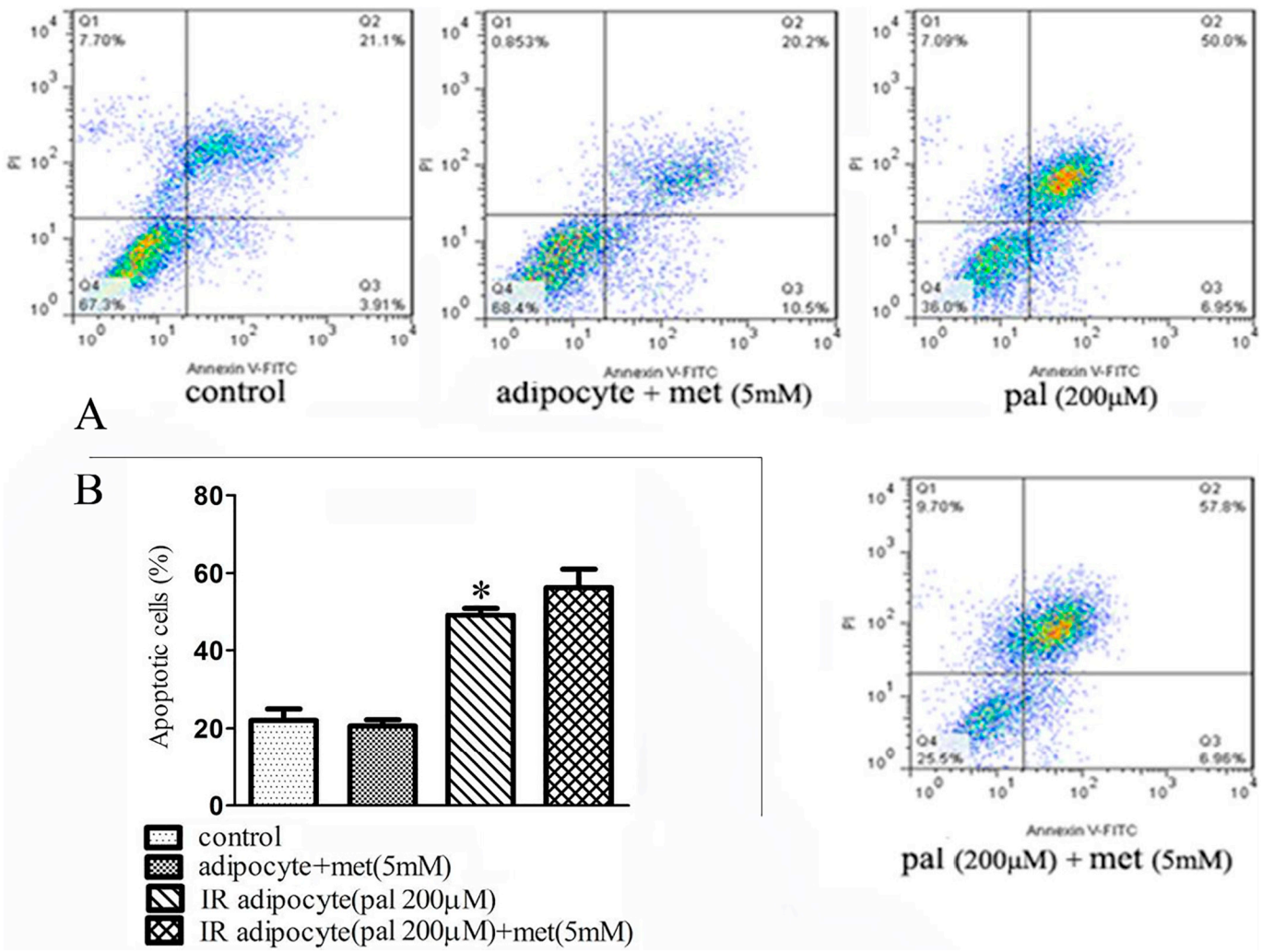

Fig. 8. A: Annexin V in 3T3-L1 adipocytes and insulin resistant adipocytes treated with Metformin assayed by flow cytometry. Cells were rinsed with PBS and harvested. The suspension of floating and harvested cells 10^6 cells/ml was prepared in binding buffer. FITC-annexin and PI were added to 100 μL of the cell suspension, after 15 min at room temperature in the dark, 400 μl of binding buffer was added and cells were analyzed by flow cytometry.(pal: palmitate, met: metformin).

B: comparison of apoptosis in normal and insulin resistant adipocytes in Metformin treated and untreated cell. Metformin in normal adipocyte had no significant effect on apoptosis. While apoptosis increased in insulin resistant adipocyte, Metformin had no significant effect on apoptosis in metformin treated insulin resistant versus untreated cells. (IR: insulin resistant, pal: palmitate, met: metformin). The data was displayed with mean ± S.E.M of three separate experiments and analyzed by ANOVA (*$p < 0.05$).

adipocytes compared to normal adipocytes (Fig. 7). While protein expression of p-ERK 1,2 was increased (1.54 ± 0.11 $p = 0.015$) in insulin resistant and (1.72 ± 0.18 $p = 0.019$) in hypertrophied adipocytes versus normal adipocytes. Metformin improved p-ERK 1,2 to normal range (1.15 ± 0.10 $p = 0.036$) in insulin resistant and (1.2 ± 0.11 $p = 0.047$) hypertrophied adipocytes treated with Metformin compared to untreated groups (Fig. 7). The data was shown ±SD and p value shows significant levels.

3.9. Effect of Metformin on apoptosis by FITC-annexin V flow cytometry

We evaluated the effect of Metformin on apoptosis in normal adipocytes and insulin resistant adipocytes from 3T3-L1 cell line by FITC-annexin V flow cytometry. Results show that in insulin resistant apoptosis significantly increased about 30% (49% ± 2.8 $p = 0.010$) compared to control (normal adipocytes) (22% ± 4.2) (Fig. 8). After treatment, Metformin had no effect on apoptosis of normal adipocytes (20.6% ± 2.3 $p = 0.987$) in Metformin treated adipocytes. In insulin resistant adipocytes, Metformin increased apoptosis about 7% (56.2% ± 6.7 $p = 444$) in Metformin treated insulin resistant adipocytes compared to untreated cells that was not significant (Fig. 8). The data was shown ±SD and p value shows significant levels.

4. Discussion

The aims of this study was investigating the effect of Metformin on fibrosis factors including integrins/ERK pathway, ECM components, apoptosis, and necrosis in insulin resistant and hypertrophied 3T3-L1 adipocyte in vitro by real-time PCR, western blotting, and flow cytometry. Results show that, Metformin has an improving effect on fibrosis markers in insulin resistant and hypertrophied adipocytes. Adipose tissue fibrosis is the hallmark of adipose tissue dysfunction that is associated with insulin resistance and type 2 diabetes [1,22]. Although adipose tissue fibrosis is the result of aberrant extracellular matrix (ECM) remodeling and increased adipocyte apoptosis and necrosis following adipocyte dysfunction, it has key role in the impairment of adipocyte function [1,27, 43]. Adipose tissue constantly remodeled through adipocyte and ECM remodeling [4–6]. Adipocytes and ECM component are interacting. In fibrosis and adipocyte dysfunction, the component of ECM as collagen VI and also ECM modifiers as matrix

metalloproteinase (MMPs) including MMP2 and MMP9 increases [2,25–27]. In our study it is shown that collagen VI expression both in gene and protein levels increased as well as the gene expression of MMP2 and MMP9 in adipocytes for insulin resistant and hypertrophied conditions. That confirms the adipocytes dysfunction in these conditions. Metformin effects on adipocytes dysfunction and improves the function of adipocytes by reduction of expression of collagen VI, MMP2 and MMP9. Thus has a role in improving fibrosis markers in adipocytes. The relationship between cells with their extra cellular matrix is regulated by integrins via activation of extracellular-signal-regulated kinases (ERKs) [12]. The activity of ERKs signaling pathway increased in insulin resistant and type 2 diabetes [35]. In our study, the gene expression of integrins varied in hypertrophied versus normal adipocytes. Metformin with improving some integrins gene expression to normal adipocytes levels acts as a modifier on integrins expression.

Even though the activity of ERK 1,2 signaling pathway increased in insulin resistant and hypertrophied adipocytes, Metformin reduced the activity of this pathway. According to the previous studies, integrins collaborate with ECM in the regulation of gene expression by activation of signaling pathways such as ERK [12–14]. In our study, the expression of integrins varied in hypertrophied adipocytes. The ECM components as collagen VI and MMP2, 9 and also ERK pathway activation increased in insulin resistant and hypertrophied adipocytes.

Metformin effects on fibrosis markers in insulin resistant and hypertrophied adipocytes via effecting on integrins/ERK pathway and improving expression of extra cellular components such as collagen VI and modifiers such as MMP2 and MM9 to normal adipocytes levels.

Fibrosis is a multifactorial process that various factors play role in it. In adipose tissue fibrosis, cell death increased via apoptosis and necrosis [28,29, 43]. Adipocyte apoptosis, as an initial event in adipocyte dysfunction [28], is regulated by many markers including Death-associated protein kinase (DAPK) family [30]. In our study the expression of DAPK family such as DAPK1, DAPK3, and also proapoptotic markers, DAP, and SIVA increased in insulin resistant and hypertrophied adipocytes. By examining apoptosis via annexin V assay, Metformin showed no effect on apoptosis, while in gene expression assay, Metformin showed different effects. It seems, the result of Metformin on apoptosis is an ensemble made of effect outcomes of Metformin on all components of apoptosis. However Metformin increased apoptosis in insulin resistant adipocytes, in small amount and significantly reduced apoptosis gene expression in hypertrophied adipocytes. On the other hand, ERK and DAPKs are interacting. DAPK activated by increased ERK [31]. Therefore, Metformin effect on apoptosis in insulin resistant and hypertrophied adipocytes is not only via affecting gene expression of apoptotic components but also via improving effect on ERK pathway.

One of the important hallmarks of fibrosis is cell death via necrosis [1, 43]. In our study, three important factors that have role in necrosis such as RIPK1, RIPK3, and MLKL significantly increased in insulin resistant and hypertrophied adipocytes which confirms the activation of cell death via necrotic-liked death in adipocyte dysfunction associated complications. Metformin by the reduction of expression in necrotic factors such as RIPK1, RIPK3, and MLKL has a role in decreasing necrosis cell death in insulin resistant and hypertrophied adipocytes. RIPK3 is the main factor of necrosis that in hypertrophied adipocyte strongly increased versus insulin resistant adipocytes. This shows when adipocytes size increases, necrosis is the dominant cell death. Metformin decreased RIPK3 in hypertrophied and insulin resistant adipocytes. So, Metformin as an anti-diabetic drug that has pivotal role in the treatment of type 2 diabetic patients 37 has extensive effects on adipocytes dysfunction complications such as fibrosis.

5. Conclusion

Metformin, as an anti-diabetic drug which ameliorates fibrosis via effecting and modifying integrins expression, also, improving expression of extracellular matrix components and ERK pathway efficiency. On the other hand Metformin by effecting on markers of cellular death via apoptosis and necrosis prevents cell death and its following fibrosis.

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Declaration of Competing Interest

There is no conflict of interest.

References

[1] K. Sun, J. Tordjman, K. Clément, P.E. Scherer, Fibrosis and adipose tissue dysfunction, Cell Metab. 18 (4) (2013) 470–477.
[2] D. Lin, T.-H. Chun, L. Kang, Adipose extracellular matrix remodelling in obesity and insulin resistance, Biochem. Pharmacol. 119 (2016) 8–16.
[3] L. Luo, M. Liu, Adipose tissue in control of metabolism, J. Endocrinol. 231 (3) (2016) R77–R99.
[4] S.S. Choe, J.Y. Huh, I.J. Hwang, J.I. Kim, J.B. Kim, Adipose tissue remodeling: its role in energy metabolism and metabolic disorders, Front. Endocrinol. 7 (2016) 30.
[5] A.T. Ali, W.E. Hochfeld, R. Myburgh, M.S. Pepper, Adipocyte and adipogenesis, Eur. J. Cell Biol. 92 (6–7) (2013) 229–236.
[6] E.C. Mariman, P. Wang, Adipocyte extracellular matrix composition, dynamics and role in obesity, Cell. Mol. Life Sci. 67 (8) (2010) 1277–1292.
[7] T.-H. Chun, Peri-adipocyte ECM remodeling in obesity and adipose tissue fibrosis, Adipocyte 1 (2) (2012) 89–95.
[8] V. Catalan, J. Gomez-Ambrosi, A. Rodriguez, G. Fruehbeck, Role of extracellular matrix remodelling in adipose tissue pathophysiology: relevance in the development of obesity, Histol. Histopathol. 27 (12) (2012) 1515–1528.
[9] B.D. Pope, C.R. Warren, K.K. Parker, C.A. Cowan, Microenvironmental control of adipocyte fate and function, Trends Cell Biol. 26 (10) (2016) 745–755.
[10] R.O. Hynes, Integrins: bidirectional, allosteric signaling machines, cell 110 (6) (2002) 673–687.
[11] E.F. Plow, T.A. Haas, L. Zhang, J. Loftus, J.W. Smith, Ligand binding to integrins, J. Biol. Chem. 275 (29) (2000) 21785–21788.
[12] N.J. Boudreau, P.L. Jones, Extracellular matrix and integrin signalling: the shape of things to come, Biochem. J. 339 (3) (1999) 481–488.
[13] L.A. Cary, J.-L. Guan, Focal adhesion kinase in integrin-mediated signaling, Front. Biosci. 4 (15) (1999) D102–D113.
[14] S.-H. Kim, J. Turnbull, S. Guimond, Extracellular matrix and cell signalling: the dynamic cooperation of integrin, proteoglycan and growth factor receptor, J. Endocrinol. 209 (2) (2011) 139–151.
[15] M. Larsen, V.V. Artym, J.A. Green, K.M. Yamada, The matrix reorganized: extracellular matrix remodeling and integrin signaling, Curr. Opin. Cell Biol. 18 (5) (2006) 463–471.
[16] J. Ashkenas, C.H. Damsky, M.J. Bissell, Z. Werb, Integrins, signaling, and the remodeling of the extracellular matrix, Integrins, Elsevier, 1994, pp. 79–109.
[17] J. Liu, S.M. DeYoung, M. Zhang, M. Zhang, A. Cheng, A.R. Saltiel, Changes in integrin expression during adipocyte differentiation, Cell Metab. 2 (3) (2005) 165–177.
[18] C.H. Streuli, Integrins as architects of cell behavior, Mol. Biol. Cell 27 (19) (2016) 2885–2888.
[19] A. Taherian, X. Li, Y. Liu, T.A. Haas, Differences in integrin expression and signaling within human breast cancer cells, BMC Cancer 11 (1) (2011) 293.
[20] J.F. Hastings, J.N. Skhinas, D. Fey, D.R. Croucher, T.R. Cox, The extracellular matrix as a key regulator of intracellular signalling networks, Br. J. Pharmacol. 176 (1) (2019) 82–92.
[21] F. Bost, M. Aouadi, L. Caron, B. Binétruy, The role of MAPKs in adipocyte differentiation and obesity, Biochimie 87 (1) (2005) 51–56.
[22] A. Guilherme, J.V. Virbasius, V. Puri, M.P. Czech, Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes, Nat. Rev. Mol. Cell Biol. 9 (5) (2008) 367.
[23] G.R. Hajer, T.W. van Haeften, F.L. Visseren, Adipose tissue dysfunction in obesity, diabetes, and vascular diseases, Eur. Heart J. 29 (24) (2008) 2959–2971.
[24] A.J. Henninger, B. Eliasson, L.E. Jenndahl, A. Hammarstedt, Adipocyte hypertrophy, inflammation and fibrosis characterize subcutaneous adipose tissue of healthy, non-obese subjects predisposed to type 2 diabetes, PLoS One 9 (8) (2014) e105262.
[25] C. Buechler, S. Krautbauer, K. Eisinger, Adipose tissue fibrosis, World J. Diabetes 6 (4) (2015) 548.
[26] G. Derosa, I. Ferrari, A. D'Angelo, C. Tinelli, S. Salvadeo, L. Ciccarelli, et al., Matrix metalloproteinase-2 and -9 levels in obese patients, Endothelium 15 (4) (2008) 219–224.
[27] T. Khan, E.S. Muise, P. Iyengar, Z.V. Wang, M. Chandalia, N. Abate, et al., Metabolic dysregulation and adipose tissue fibrosis: role of collagen VI, Mol. Cell. Biol. 29 (6) (2009) 1575–1591.
[28] N. Alkhouri, A. Gornicka, M.P. Berk, S. Thapaliya, L.J. Dixon, S. Kashyap, et al., Adipocyte apoptosis, a link between obesity, insulin resistance, and hepatic

steatosis, J. Biol. Chem. 285 (5) (2010) 3428–3438.
[29] S. Cinti, G. Mitchell, G. Barbatelli, I. Murano, E. Ceresi, E. Faloia, et al., Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans, J. Lipid Res. 46 (11) (2005) 2347–2355.
[30] Y. Lin, T.R. Hupp, C. Stevens, Death-associated protein kinase (DAPK) and signal transduction: additional roles beyond cell death, FEBS J. 277 (1) (2010) 48–57.
[31] C.H. Chen, W.J. Wang, J.C. Kuo, H.C. Tsai, J.R. Lin, Z.F. Chang, et al., Bidirectional signals transduced by DAPK–ERK interaction promote the apoptotic effect of DAPK, EMBO J. 24 (2) (2005) 294–304.
[32] K. Prasad, Z. Ao, Y. Yoon, M.X. Wu, M. Rizk, S. Jacquot, et al., CD27, a member of the tumor necrosis factor receptor family, induces apoptosis and binds to Siva, a proapoptotic protein, Proc. Natl. Acad. Sci. 94 (12) (1997) 6346–6351.
[33] M.-L. Liou, H.-C. Liou, The ubiquitin-homology protein, DAP-1, associates with tumor necrosis factor receptor (p60) death domain and induces apoptosis, J. Biol. Chem. 274 (15) (1999) 10145–10153.
[34] R. Weinlich, A. Oberst, H.M. Beere, D.R. Green, Necroptosis in development, inflammation and disease, Nat. Rev. Mol. Cell Biol. 18 (2) (2017) 127.
[35] K.-i. Ozaki, M. Awazu, M. Tamiya, Y. Iwasaki, A. Harada, S. Kugisaki, et al., Targeting the ERK signaling pathway as a potential treatment for insulin resistance and type 2 diabetes, Am. J. Phys. Heart Circ. Phys. (2016).
[36] C. Farnier, S. Krief, M. Blache, F. Diot-Dupuy, G. Mory, P. Ferre, et al., Adipocyte functions are modulated by cell size change: potential involvement of an integrin/ERK signalling pathway, Int. J. Obes. 27 (10) (2003) 1178.
[37] Y.-W. Wang, S.-J. He, X. Feng, J. Cheng, Y.-T. Luo, L. Tian, et al., Metformin: a review of its potential indications, Drug design, development and therapy 11 (2017) 2421.
[38] R. Hussain, A. Nouri, R. Oliver, A new approach for measurement of cytotoxicity using colorimetric assay, J. Immunol. Methods 160 (1) (1993) 89–96.
[39] Y. Zhao, H.L. Wieman, S.R. Jacobs, J.C. Rathmell, Chapter twenty-two mechanisms and methods in glucose metabolism and cell death, Methods Enzymol. 442 (2008) 439–457.
[40] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method, methods 25 (4) (2001) 402–408.
[41] F.M. Gregoire, C.M. Smas, H.S. Sul, Understanding adipocyte differentiation, Physiol. Rev. 78 (3) (1998) 783–809.
[42] M. Van Epps-Fung, J. Williford, A. Wells, R.W. Hardy, Fatty acid-induced insulin resistance in adipocytes, Endocrinology 138 (10) (1997) 4338–4345.
[43] M. Lafontan, Adipose tissue and adipocyte dysregulation, Diabetes Metab. 40 (1) (2014) 16–28.