ORGINAL ARTICLE

Metformin and Pioglitazone Reduce Gene Expression of Inflammatory Factors in Insulin Resistant and Hypertrophied Adipocytes

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Abstract

Objective: In obesity, chronic low grade inflammation, created by induction of pro-inflammatory markers, causes adipocyte dysfunction in adipose tissue. Adipocytes dysfunction is associated with various diseases including insulin resistance and obesity. In obesity, inflammatory factors such as osteopontin (OPN), angiopoietin-like protein 2 (Angptl2) and transforming growth factor-β (TGF-β) are induced in adipose tissue. Metformin and pioglitazone that are used to modulate inflammation, but the relevant mechanism is poorly understood. This study aimed to investigate the effect of metformin and pioglitazone as anti-diabetic drugs, on gene expression of osteopontin, Angptl2 and TGF-β as inflammatory factors in insulin resistance and hypertrophied adipocyte in 3T3-L1 cell line model in vitro.

Materials and Methods: In this experimental research, we differentiated3T3-L1 preadipocytes to adipocytes. The adipocytes treated in insulin resistance and hypertrophied conditions with metformin and pioglitazone and assayed gene expression of OPN, Angptl2 and TGF-β by Real-Time PCR. Data was analyzed by SPSS statistic software.

Results: The results showed that expression of OPN, Angptl2, and TGF-β were increased significantly over 2-fold (P-value< 0.05) in insulin resistance and hypertrophied adipocytes compared to normal adipocytes. Pre- and co-treatment with metformin and pioglitazone led to reduced expression of Angptl2 and TGF-β. Only metformin significantly reduced the expression of Angptl2, TGF-β and OPN in hypertrophied adipocyte.

Conclusion: These results support the proposal that metformin and pioglitazone reduce gene expression of inflammatory factors in insulin resistant and hypertrophied adipocytes.

Keywords: Angptl2, metformin, Osteopontin, Pioglitazone, TGF-β

Introduction

Obesity and insulin resistance are important pathogenic factors for type 2diabetes. Adipose tissue (AT) has been considered not only to be an energy storage but also a secretory organ releasing cytokines and hormones (1). In obesity,

induction of pro-inflammatory factors creates a chronic low-grade inflammatory response which leads to adipocyte dysfunction in adipose tissue. Adipocytes dysfunction associated insulin resistance and type 2 diabetic with obesity (2,3). In particular, adipose tissue pro-inflammatory factors including angiopoietin-like protein2 (Angptl2) (4), transforming growth factor-β (TGF-β) (5), and osteopontin (OPN) (6),which are mostly elevated during obesity, contribute to this inflammation. Angptl2 is a member of Angiopoietin-like protein family that are structurally similar to angiopoietin, but act differently (7). Angptl2 that is mainly produced by adipose tissue, is overexpressed in obesity and contributes to the progression of inflammation (4,8).

Transforming growth factor beta (TGF-β), a multifunctional cytokine, is an important cause of release of inflammatory mediators and collagen deposition in the adipose tissue of obese mice (9). Blockade of TGF-β/Smad3 signaling in mice has been shown to decrease obesity and diabetes, emphasizing the key role of TGF-β in obesity and its related diseases (10). TGF-β induces the expression of the Angptl2 gene via TGF-β/Smad3 pathway in vitro (11). OPN is another inflammatory factor that is synthesized in adipocytes and is elevate in adipose tissues in obesity and insulin-resistance (12). The level of OPN is elevated in adipose tissue inflammation and adipocyte hypertrophy (6,12).

Metformin and pioglitazone are used as anti-diabetic drugs and are valuable in the treatment of type 2 diabetic patients(13,14). Recent studies showed that metformin and pioglitazone could influence inflammation. Metformin prevents inflammation via activation of AMPK–PTEN pathway in vascular smooth muscle cells (15) and also improves low-grade inflammation via AMPK activation in obesity (16). Pioglitazone is also effective in reducing inflammation in patients with Type 2 diabetes (17) and atherosclerotic Inflammation (18), and decreases the risk of cardiovascular disorders (19). Additionally, pioglitazone suppresses palmitate-induced inflammation in pancreatic β-cells (20). However, the mechanism of anti-inflammatory action of metformin and pioglitazone in adipose tissue is not clearly defined yet and requires further study. Therefore the aims of this study were to investigate the effect of metformin and pioglitazone on gene expression of Angptl2, TGF-β and OPN as inflammatory factors, in insulin resistance and hypertrophied adipocyte in vitro.

Materials and Methods

Cells culture

The 3T3-L1 mouse embryonic fibroblastic cells (ATCC; CL-173) were gotten from the cell bank of Iranian biological resource center (Tehran, Iran). 3T3-L1 pre-adipocytes were cultured in DMEM complemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin and streptomycin mixture and kept at 37 °C in a humidified atmosphere having 5% CO2. In order to induce cellular differentiation, two days after confluence, the cells are incubated in the above medium including 1.5 µg/mL insulin, 1µM dexamethasone (DEX), and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) for 48 hours. The differentiation medium was replaced with medium added with 10% FBS and 1.5 µg/mL insulin for 3 days. The cells were then cultured in medium containing 10% FBS. Seven to nine days after differentiation, adipocyte phenotype was observed in more than 90% cells.

For induction of adipocyte hypertrophy, cells were maintained at 37 °C for 30 day and medium was improved every two days. For creating insulin resistant adipocytes, cells were starved overnight in DMEM with 0.5% BSA followed by treatment with BSA-bound palmitate (200 µm). Control cells were treated with fatty acid-free BSA without palmitate.

MTT assay

The 3T3-L1 adipocytes (2×10^4 cells per well) were seeded in 96-well plates and incubated for 24 h. After differentiation, cells were

treated with different doses of metformin (1-10 mM) and pioglitazone (1-20 μM). After 24 h, the MTT solution (0.5 mg/ml) was added to the cells and incubated for 4 h at 37°C. The formed formazan crystals were solubilized by dimethyl sulfoxide (DMSO) and the absorbance was measured at 570 nm using a multi- well plate reader (Synergy HTX, BioTek Instrument, Inc). The relative cell viability was calculated as the percentage of treated cells relative to that of untreated control cells.

Palmitate/BSA solution preparation

The adipocytes were treated with fatty acid-free bovine serum albumin (BSA)-conjugated palmitate. Sodium palmitate (27.84 mg), after dissolving in 0.1 M NaOH (1 ml), was heated at 70˚C in shaking water bath to create 100 mM of stock solution. Palmitate (0.25 ml of 100mM solution) was added to 4.75 ml of DEMEM medium containing 10% (w/v) FFA-BSA and were mixed for at least 2 h at 37°C while shaking to generate 5 Mm palmitate stock solution. The palmitate/BSA solution was sterile filtered by membrane filter.

Oil red o staining

The oil red O powder (300 mg) was dissolved in 100 ml of 99% isopropanol to make Oil red O stock solution. The stock solution was mixed with deionized water to 3/2 ratio to make oil red working solution. After incubation at room temperature for 10 minutes, the staining solution was ready to use. Cells were washed twice with phosphate-buffered saline (PBS) with pH 7.4 and fixed in 10% formaldehyde prepared in PBS for 1 h at room temperature. Then cells were washed three times for 5 min each in water and stained with oil red O solution for 1h at room temperature. All photographs were taken with ×40 magnification lens on an invert microscope.

Intracellular triglyceride Assay

The cellular triglyceride (TG) contents of were measured by a TG determination kit (Pars Azmoon, Iran, Tehran). In brief, cells were detached with trypsin and washed three times with PBS. Then, the cells were resuspended and homogenized with NP-40 (5%), by heating the samples to 80-100°C water bath until it became cloudy, and then cool down to room temperature. The heating step was repeated once more to completely solubilize the cellular triglyceride. The cell lysate (10 μl) was added to enzyme solution of the kit and incubated for 10 min at 37°C. The absorbance of the samples was measured at 540 nm within 60 min. Total protein concentration of the samples was measured using the BCA Protein colorimetric detection Assay kit that obtained from Takara company (Japan) and used for normalizing the results.

6-Deoxy-D-glucose uptake assay

Differentiated adipocytes were seeded in 24-well plates and serum-starved in 0.5% BSA/DMEM overnight. Subsequently, cells were cultured in DMEM containing palmitate (200 μM) for 24h for creating insulin resistant adipocytes. The adipocytes glucose uptake was assessed following Zhao, Wieman method to confirm insulin resistance in adipocytes (21). After incubating cells with Krebs–Ringer bicarbonate HEPES (KRBH) buffer containing 100 nM insulin for 30 min at 37 °C, cells were washed by KRBH buffer and incubated in the buffer containing 0.1 mM 6-deoxyglucose for 20 min. Then, the cells were washed in ice cold PBS three times and lysed with lysis buffer. Fluorescence intensity was measured by using the microplate reader (λex = 530 to 570 nm, λem = 590 to 620 nm). Total protein content measurement of all samples by the BCA Protein Assay kit was used as normalizer.

Gene expression

Total cellular RNA of 3T3-L1 adipocytes were extracted and purified with the RNeasy cell mini kit from Qiagen (USA). Quantity and quality of RNA was assessed by detection of 18S and 28S bands on agarose gel electrophoresis as well as measurement of the

absorbance at 260 and 280 nm by NanoDrop. Total RNA (1 μg) was reverse transcribed to cDNA by cDNA synthesis kit from Thermo Fisher Scientific (USA). Primers were designed for the amplification of beta-actin (h-Actin), PPAR-γ, Angptl2, TGF-β and OPN. The primers were specific for mouse genes: OPN: Forward: 5′ GACAACAACGGAAAG GGCAG 3′, Reverse: 5′ GATCGGCACTCTC CTGGCT 3′, Angptl2; Forward: AGCCTGAG AATACCAACCGC, Reverse: CCCTTGCT TATAGGTCTCCCAG, TGF-B: Forward: 5′ CCACCTGCAAGACCATCGAC 3′, Reverse: 5′ CTGGCGAGCCTTAGTTTGGAC 3′, PPARɣ: Forward: 5′ GATGCACTGCCTAT GAGCACTT 3′, Reverse: 5′ AGAGGTCCAC AGAGCTGATTCC 3′, B-actin: Forward: 5′ T GTCCACCTTCCAGCAGATGT 3′, Reverse: 5′ AGCTCAGTAACAGTCCGC CTAGA3′ .Quantitative real-time PCR (qRT-PCR) was done by specific primers of cDNAs and PCR reaction mixture supplied from ampliqon SYBR master mix in an ABI- Step One Real-Time PCR System (2012 life technologies corporation). All PCR experiments were normalized to β-actin, and regarding the efficiencies of all primers being ~ 2, the comparison between relative abundance from different experiment were determined by the ΔΔCt method (22).

Statistical analysis

The experiment was repeated three times independently. Data were evaluated with ANOVA by using SPSS statistic software (version 24). Data were displayed as mean ±SD. P-values < 0.05 were considered statistically significant.

Ethical considerations

The protocol of this research was submitted for consideration, approval, and guidance to the Shahid Sadoughi University of Yazd Research Ethics Committee before the study begins. The ethical code of this research is IR.SSU.MEDICINE.REC.1395.26

Results

Differentiation and hypertrophy of adipocytes

3T3-L1 pre-adipocytes were differentiated to adipocytes by treating with differentiation medium for 7 days as mentioned above. The expression of *PPAR-γ* gene was measured to validate the adipocyte differentiation. Results showed that *PPAR-ɣ* gene expression was significantly induced more than 1000-fold (1025(±106); *P*-value< 0.001) in differentiated adipocytes compared to pre-adipocytes. Additionally, in order to confirm lipid accumulation in mature adipocytes, oil red O staining was carried out. In contrast to pre-adipocytes that did not have any fat droplets, many small fat droplets were observed in differentiated adipocytes.

To develop a model for hypertrophied adipocytes, adipocytes were maintained in differentiation medium for 30 days. The results of Oil red O staining in hypertrophied adipocytes showed that the cells had larger size with one big fat droplet compared to differentiated adipocytes which had smaller cells size with many small fat droplets with various sizes.

We also measured triglyceride content in pre-adipocytes, as well as differentiated, insulin resistant, and hypertrophied adipocytes. Data analyses showed that differentiated and insulin resistant adipocytes had significantly higher amount of TG compared to pre-adipocytes 18(±2.8) and 16.5(±2.12) μg/μg protein, respectively). However, TG content was dramatically higher (44(±5.6) μg/μg protein) in hypertrophied adipocytes compared to pre-adipocytes.

The effect of metformin and pioglitazone on cell viability

The results of the MTT assay showed that there was no significant change in cell viability after 24-h treatment with metformin (1-10mM) and pioglitazone (1-20 μM).

Insulin resistance adipocytes

We used palmitate for inducing insulin resistance in 3T3-L1 adipocytes. Glucose uptake test with 6-deoxy-D-glucose was used for assessment of insulin sensitivity in adipocytes. Treatment with 200 μM palmitate for 24 h caused a significant inhibition (1.3-fold; 69 (±6.0), *P*-value< 0.01) of insulin-stimulated 6-deoxy-D-glucose uptake in adipocytes compared to untreated adipocytes.

The effect of metformin and pioglitazone on gene expression of inflammatory factors

We first evaluated the effects of insulin resistance and hypertrophy in adipocytes on gene expression of major inflammatory factors including Angptl2, TGF-β and OPN by real-time PCR. Results showed that induction of insulin resistance in adipocytes caused significant elevation in the gene expression of Angptl2 (3.3 (±0.28), *P*-value= 0.001), TGF-β (2.4 (±0.21), *P*-value= 0.001) and OPN (2.7 (±0.21), *P*-value= 0.001) compared to the control group (untreated adipocyte) (Table 1).

Next we investigated the changes in the gene expression in response to metformin and pioglitazone together with the induction of insulin resistance by palmitate at three models of pre-, co- and post- treatment. In the pre-treatment model of metformin, there was a significant reduction in Angptl2 (1.5 (±0.21), *P*-value= 0.001), TGF-β (1.7 (±0.15), *P*-value= 0.021) and OPN (1.5 (±0.14), *P*-value=0.007) compared to palmitate group. Co-treatment of adipocytes with both palmitate and metformin also significantly reduced the gene expression of Angptl2 (1.7 (±0.28), *P*-value= 0.001), TGF-β (1.4 (±0.17), *P*-value= 0.007) and OPN (1.35 (±0.22), *P*-value=0.003) compared to palmitate group. On the contrary, post-treatment with metformin only reduced gene expression of OPN (1.5 (±0.14), *P*-value= 0.007) while no significant change was detected in gene expression ofAngptl2 (2.7 (±0.35), *P*-value= 0.597) and TGF-β (1.95 (±0.07), *P*-value= 0.254) compared to palmitate group (Table 1).

The effect of pioglitazone was also assessed in the same models as mentioned above. Pre-treatment with pioglitazone significantly attenuated the gene expression of Angptl2 (1.7 (±0.35), *P*-value= 0.007), TGF-β (1.7 (±0.16), *P*-value= 0.047) and OPN (1.75 (±0.4), *P*-value= 0.026) compared to palmitate group. Co-treatment with pioglitazone also significantly reduced the gene expression of TGF-β (1.55 (±0.21), *P*-value= 0.017) and OPN (1.7 (±0.14), *P*-value= 0.02), but no significant change in the expression of Angptl2 expression was observed (2.6 (±0.21), *P*-value= 0.380). Post-treatment of adipocytes with pioglitazone only reduced the gene expression of OPN (1.65 (±0.35), *P*-value= 0.015) and did not cause any significant change in the gene expression of Angptl2 (3.0 (±0.35), *P*-value= 0.978) and TGF-β (2.25 (±0.35), *P*-value= 0.947) compared to palmitate group (Table 2).

Induction of hypertrophy in adipocytes was accompanied by elevated gene expression of Angptl2 (4.05 (±0.35), *P*-value=0.002), TGF-β (3.9 (±0.14), *P*-value= 0.001) and OPN (3.25 (±0.35), *P*-value= 0.007) compared to control group (non-hypertrophied adipocytes). Hypertrophied adipocytes were treated with metformin and the results showed reduced expression of Angptl2 (2.6 (±0.28), *P*-value= 0.034), OPN (1.8 (±0.42), *P*-value= 0.041) and TGF-β (1.9 (±0.14), *P*-value=0.001) compared to untreated hypertrophied adipocyte. Treatment of hypertrophied adipocytes with pioglitazone did not exert any significant change in the expression of Angptl2 (3.5 (±0.42), *P*-value= 0.4), TGF-β (3.2 (±0.28), *P*-value= 0.055) and OPN (3.0 (±0.28), *P*-value= 0.849) compared to untreated hypertrophied adipocyte (Tables 1- 2).

Discussion

The aim of this study was to identify the effect of metformin and pioglitazone, two anti-diabetic drugs on gene expression of Angptl2, TGF-β and OPN as inflammatory factors in insulin resistant and hypertrophied adipocytes generated from 3T3-L1 pre-adipocyte cell line.

Table 1. The effect of metformin on inflammatory factors in insulin resistant and hypertrophied adipocytes

Adipocytes / Genes	Untreated (control)	Insulin resistant	Hypertrophied	Insulin resistant- treated			Hypertrophied -treated
				pre-treat	co-treat	post-treat	
ANGPTL2	1	3.3±0.28 *p=0.001	4.05±0.35 p=0.002	1.5±0.21 p=0.001	1.7±0.28 p=0.001	2.7±0.35 p=0.597	2.6±0.28 p=0.034
OPN	1	2.7±0.21 p=0.001	3.25±0.35 p=0.007	1.5±0.14 p=0.007	1.35±0.22 p=0.003	1.5±0.14 p=0.007	1.8±0.42 p=0.041
TGF-B	1	2.4±0.21 p=0.001	3.9±0.14 p=0.001	1.7±0.15 p=0.021	1.4±0.17 p=0.007	1.95±0.07 p=0.254	1.9±0.14 P=0.001

* *P*-value

Table 2. The effect of pioglitazone on inflammatory factors in insulin resistant and hypertrophied adipocytes

Adipocytes / Genes	Untreated (control)	Insulin resistant	Hypertrophied	Insulin resistant- treated			Hypertrophied-treated
				pre-treat	co-treat	post-treat	
ANGPTL2	1	3.3±0.28 *p=0.001	4.05±0.35 p=0.002	1.7±0.35 p=0.007	2.6±0.21 p=0.380	3.0±0.35 p=0.978	3.5±0.42 p=0.400
OPN	1	2.7±0.21 p=0.001	3.25±0.35 p=0.007	1.75±0.4 p=0.026	1.7±0.14 p=0.02	1.65±0.35 p=0.015	3.0±0.28 p=0.849
TGF-B	1	2.4±0.21 p=0.001	3.9±0.14 p=0.001	1.7±0.16 p=0.047	1.55±0.21 p=0.017	2.25±0.35 p=0.947	3.2±0.28 p=0.055

* *P*-value

Many studies have shown that inflammatory factors are increased in obesity and obesity-related insulin resistance (2,3).

In obesity, the expression of pro-inflammatory factors such as Angptl2, OPN and TGF-β are augmented concomitant with increasing the volume of adipocytes and expansion of adipose tissue and results in chronic inflammation(4-6). This inflammation is associated with insulin resistance which may lead to type 2diabetes (3). The expression levels of Angptl2 and OPN have been reported to be increased in adipose tissues in obesity and insulin-resistance (8,12). TGF-β has also been introduced as an important cause of releasing inflammation mediators in the adipose tissue of obese mice (9).

In the current study we developed insulin resistant and hypertrophied adipocytes as an in vitro model of obesity and insulin resistance and showed that the expression of inflammatory factors including Angptl2, OPN and TGF-β was induced in response to insulin resistance and adipocyte hypertrophy.

One approach to prevent the occurrence of obesity-related diseases and improve insulin sensitivity is to attenuate the expression of inflammatory factors. Recent studies have shown that metformin via the activation of AMPK as well as pioglitazone are able to influence inflammatory processes in obesity and Type 2 diabetes (16,17). Pioglitazone reduces palmitate-induced inflammation in pancreatic β-cells (20). Here we showed that metformin and pioglitazone prevent excessive gene expression of inflammatory factors such as AGNPTL2, OPN and TGF-β prior to and during the occurrence of insulin resistance. These two anti-diabetic drugs also reduced OPN expression after the insulin resistance was established in adipocytes.

Metformin had improving effect on increased AGPTL2, OPN and TGF-β expression in treatment of hypertrophied adipocyte. Pioglitazone had no improving effect on increased AGPTL2, OPN and TGF-β expression in hypertrophied adipocyte, Of course may need to more time for effective impact. These results are indicative of the preventive effect of both metformin and pioglitazone on the inflammatory processes associated with the progression of insulin resistance. However, the effect of these drugs would be limited after the insulin resistance is established and some inflammatory factors would become irresponsive to the ameliorative effect of both metformin and pioglitazone. So, metformin and pioglitazone as anti-diabetic

drugs that have pivotal role in the treatment of type 2 diabetic patients have extensive effects on inflammation in insulin resistant and hypertrophied adipocytes. Identification of signaling pathways that are responsible for the effect of these drugs on the inflammation in adipose tissue will be beneficial in the development of novel strategies for the management of insulin resistance. For example, metformin has been shown to act through activation of AMPK/PTEN pathway in vascular smooth muscle cells (15). Also this study assayed the effect of metformin and pioglitazone on three inflammatory markers in cell culture. There are many inflammatory factors, that more study is needed.

Conclusions

Metformin and pioglitazone not only prevent but also ameliorate inflammation in insulin resistant adipocytes through modulating the gene expression of inflammatory factors. However the efficacy of these drugs is reduced after the insulin resistance is established. Metformin is more effective on inflammation in hypertrophied adipocytes compared with pioglitazone. Clarification of these mechanisms might be beneficial in designing more effective treatment strategies with metformin and pioglitazone in obesity and insulin resistance.

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Conflict of Interest

There is no conflict of interest.

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