ORIGINAL ARTICLE

Peroxisome proliferator-activated receptor gamma expression in peripheral monocytes from rheumatoid arthritis patients

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Abstract  Aim of the work: To study peroxisome proliferator activated receptor gamma (PPARγ) expression levels in the peripheral monocytes from rheumatoid arthritis (RA) patients and to clarify its relation with disease activity, functional disability and drug therapy.

Patients and methods: Thirty RA patients (Group 1) were divided into two subgroups: Group 1A: patients with moderate to high disease activity (n = 15); Group 1B: patients in remission or with low disease activity (n = 15). Thirty healthy volunteers were included as control group. Disease activity score in 28 joints (DAS-28) and Health Assessment Questionnaire (HAQ) were assessed in patients. PPARγ gene expression levels were assessed by real-time PCR in peripheral blood monocytes.

Results: The mean fold increase in monocyte PPARγ expression levels was significantly higher (p < 0.001) in patients (6.87 ± 0.9) compared to control, being significantly higher (p < 0.001) in patients with remission or low activity (7.6 ± 0.63) than patients with active RA (6.13 ± 0.52). In RA patients, monocyte PPARγ expression levels showed significant negative correlations with morning stiffness durations, total joint count, visual analog scale for pain, DAS-28 and HAQ (p > 0.001) and with swelling joint count, erythrocyte sedimentation rate and platelet count (p < 0.05). A significant correlation was present with disease duration (p < 0.05) while there were no statistically significant correlations with any of Larsen score, C-reactive protein, hemoglobin concentrations, white blood cell count, rheumatoid factor or anti-cyclic citrullinated peptide titers (p > 0.05).

Conclusions: Our findings support the role of PPARγ in the pathophysiology of RA and suggest that over-expression of PPARγ protein may have anti-rheumatic effects.

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1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by persistent synovitis, systemic inflammation, production of autoantibodies, and bone destruction preferentially involving the peripheral joints [1]. Macrophages/microphages infiltrate into the joints and produce various bioactive factors, including cytokines and matrix metalloproteinases (MMPs) that cause persistent inflammation and lead to joint destruction [2]. TRIGGERING receptor expressed on myeloid cells-1 (TREM-1) is a recently identified molecule involved in monocyctic activation and inflammatory response. Serum levels of TREM-1 were elevated in Egyptian RA patients and correlated significantly with clinical and laboratory markers of disease activity as well as functional disability [3]. In another study on Egyptian RA patients, proteinase-activated receptor expression in monocytes was consistent with a pathogenic role in the disease and its utility as a marker for disease activity was suggested [4]. It was even reported that RA may be associated with an increased risk of lympho- and myelo-proliferative malignancy [5].

Peroxisome proliferator activated receptors (PPARs) belong to a subfamily of the nuclear receptor superfamily of ligand-inducible transcription factors. Three PPAR isoforms encoded by separate genes have been identified, PPARα, PPARβ/δ, and PPARγ [6]. PPARs can be activated by dietary fatty acids and their metabolites and act as lipid sensors that, upon activation, are able to markedly redirect metabolism that control the expression of gene networks involved in adipogenesis, lipid metabolism, inflammation, and the maintenance of metabolic homeostasis [7]. The PPARγ protein exists in two isoforms that are expressed from the same gene [8]. It was originally described in differentiating adipocytes, the two PPARγ isoforms show a distinct expression pattern: PPARγ1 is abundantly expressed in the adipose tissue, large intestine, and hematopoietic cells, and to a lower degree in the kidney, liver, muscles, pancreas, and small intestine while PPARγ2 is restricted to white and brown adipose tissues under physiological conditions [9]. PPARγ is also expressed in various immune system-related cell types, particularly in antigen-presenting cells such as macrophages and dendritic cells [10]. In these cells, PPARγ does not only regulate genes related to lipid metabolism, but also immunity and inflammation related genes [11]. PPARγ was shown to be highly expressed in mouse thioglycollate-elicited macrophages and its natural and synthetic ligands inhibited the expression of several molecules involved in the inflammatory process like inducible nitric oxide synthase (iNOS), MMP-9 and scavenger receptor A [12].

The receptor attracted attention as a possible therapeutic target in inflammatory diseases when similar effects were reported for the PPARγ activators in human monocyte-derived macrophages [13]. This study aimed to assess PPARγ expression levels in the peripheral monocytes from RA patients in comparison with healthy volunteers, and to clarify its relation with disease activity, functional disability and drug therapy.

2. Patients and methods

This study was carried out on 30 RA patients attending the outpatient clinic and the inpatient Department of Rheumatology, Rehabilitation and Physical Medicine of Benha University Hospitals. All of them met the American College of Rheumatology/European League against Rheumatism (ACR/EULAR) rheumatoid arthritis classification criteria [14]. They were divided into two subgroups according to the 28-joint disease activity score (DAS-28) [15]: Group 1A included 15 patients with moderate or high disease activity (DAS-28 > 3.2) and Group 1B included 15 patients in remission or with low disease activity (DAS-28 ≤ 3.2). Another 30 apparently healthy volunteers with comparable age, sex and social levels were included as the control group (Group 2).

An informed written consent was taken from patients included in the study and it was approved by the Ethics Committee of Faculty of Medicine of Benha University. Patients and controls were excluded if they were tobacco smokers [16] or had hypertension, diabetes or hypercholesterolemia.

All patients were subjected to full history taking, thorough clinical examination, laboratory investigation, assessment of disease activity by DAS-28 [15] and assessment of functional capacity and disability using Health assessment Questionnaire (HAQ) [17]. Erythrocyte sedimentation rate (ESR) was assessed by the Westergren method, C-reactive protein (CRP) by latex agglutination slide test for qualitative and semiquantitative determination in non-diluted serum, rheumatoid factor (RF) was assessed by latex fixation test and anti-citrullinated peptide antibody (ACPA) detected by ELISA. Lipid profile was performed on blood samples obtained from the patients after overnight fasting including: Total cholesterol (TC) and triglycerides (TG) measured in plasma by the colorimetric method using commercial assays; high density lipoprotein (HDL) using the direct HDL method (BS-300 Chemistry analyzer) and low density lipid lipoprotein (LDL).

2.1. Monocyte PPARγ expression by real time PCR

2.1.1. Sampling

Five ml of antecubital venous blood was collected from patients and controls and immediately put in a heparin containing vacutainer tube. Each sample was mixed and immediately used in monocyte separation.

2.1.2. Isolation of monocytes

The buffy coat containing human monocytes was isolated from heparinized venous blood by standard techniques of Histopaque (density = 1077 g/cm3) gradient centrifugation (400g, 20 min, room temperature) as described [16]. Five ml of blood was mixed with 5 ml of hanks balanced salt solution. After proper mixing, the diluted blood was gently overlaid on the surface of 3 ml of Histopaque. Tubes were centrifuged at 2000 rpm for 20 min. The buffy coat was carefully removed by a pasteur pipette and washed 3 times in hanks balanced salt solution. The cells were re-suspended in hanks balanced salt solution and immediately used in genomic RNA extraction.

2.1.3. Genomic RNA extraction

Total RNA was extracted from the buffy coat using Mammalian Total RNA purification Kit (Thermo scientific), according to the manufacturer’s instructions. The purity of the extracted RNA was detected through measurement by UV spectrophotometer. Readings were taken at wave lengths 260 and 280 nm. Readings at 260 nm were > 0.15. The ratio between the absorbance values at 260 and 280 nm gives an
estimate of RNA purity. A260 and A280 were taken by Nanodrop Spectrophotometer 2000c USA.

2.1.4. cDNA synthesis

Purified RNA samples were immediately reverse transcribed to cDNA using a high-capacity cDNA reverse transcription kit (Intron Biotechnology) according to the manufacturer’s instructions. cDNA was stored at −80.

2.1.5. Relative quantitation of PPARγ mRNA by real time PCR using SYBR green

In this study, the calibrator sample is healthy control. A single-plex reaction is used, where a single primer pair is present in a well. Real-time PCR was carried out in a volume of 25 μL per well in a 96-well optical reaction plate using Super Real Pre Mix Plus (SYBR Green) TIANGEN Biotech (Beijing). According to the manufacturer’s instruction, each reaction contains 12.5 μL of 2 × Super Real PreMix Plus, 0.75 μL of forward primer (10 μM), 0.75 μL of reverse primer (10 μM), 2.5 μL of 50 × ROX Reference Dye, and 4 μL of cDNA template then completed up to 25 μL with RNase-free ddH2O. The plate was run on the ABI 7900HT (Applied Biosystems, USA). The cycling conditions include initial denaturation at 95 °C for 15 min followed by 40 repeated cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 20 s, followed by extension at 72 °C for 30 s. Primer sequence of PPARγ was 5’-CTATG GAGTTCATGCTTGTG-3’ (Forward) and 5’-TGATGTTACCTGTACCATGACATAAT-3’ (Reverse) [18]. Primer sequence of glyceraldehyde phosphate dehydrogenase (GAPDH) as internal control (housekeeping gene) was 5’-TGA TGA CAT CAA GAA GGT GGT GAA G-3’ (Forward) and 5’-TCC TTG GAG GCC ATG TGG GCC AT-3’ (Reverse) [4]. The PCR primers were supplied by Fermentas, Germany. According to the RQ manager program 1.2 ABI SDS software (ABI 7900HT), the relative endogenous quantities of the PPARγ gene normalized against the control (GAPDH) gene fold expression changes are calculated using the equation $2^{-ΔΔc_t}$ [19].

2.2. Radiological assessment

Plain X-rays of both hands and feet were obtained and evaluated according to the Larsen score [20].

2.3. Statistical analysis

The data were recorded on an “Investigation report form”. These data were tabulated, coded and analyzed using the computer program SPSS (Statistical package for social science) version 16. Descriptive statistics were presented in the form of mean and standard deviation. Mann–Whitney test was used for the analysis of 2 non parametric quantitative data. A one way ANOVA was used to compare more than 2 quantitative data. The correlation between variables was performed using Spearman’s rank correlation tests. $p$ value < 0.05 was considered significant.

3. Results

The 30 patients had a mean age of 46.53 ± 11.8 years of whom 23 were females and 7 males (F:M = 23:7). The control group was of matched age and sex with a mean age of 42.3 ± 12.1 years and F:M ratio of 22:8. The clinical and laboratory variables of Group 1A and Group 1B are shown in Table 1. Twenty-six RA patients were on 200 mg/d hydroxychloroquine, 19 patients were on oral prednisolone therapy (7.6 ± 2.6 mg/dl), 17 were on weekly methotrexate (MTX) injection (13.9 ± 2.01/w), 10 patients were on 20 mg leflunomide and 3 were on sulphasalazine 1.8 ± 0.3 mg/d.

PPARγ mRNA expression in the peripheral monocytes showed a highly significant mean fold elevation ($p < 0.001$) in RA patients (6.87 ± 0.9) than in healthy controls (Fig. 1). The peripheral monocyte PPARγ gene expression showed a highly significant mean fold elevation in patients with remission (Group 1B) (7.6 ± 0.63) than in patients with active RA (Group 1A) (6.13 ± 0.52) and controls ($p < 0.001$) (Fig. 1).

The correlation of the monocyte PPARγ gene expression with clinical and laboratory parameters, medications, disease activity (DAS-28), functional disability (HAQ) as well as Larsen score is presented in Table 2.

4. Discussion

The present results showed that PPARγ gene expressions in monocytes from RA patients were significantly higher ($p < 0.001$) than those from the healthy subjects. This is consistent with the previous observations found by Palma et al. [21] who concluded that monocytes obtained from RA patients present a significantly enhanced constitutive expression of PPARγ protein as compared with those from healthy donors. Also, Jiang et al. [22] by evaluating PPARγ gene expression in bone marrow cells obtained from patients with traumatic femoral neck fracture, OA or RA, observed that RA patients had significantly higher PPARγ mRNA expression levels than OA patients and fractured subjects.

PPARγ is a key modulator of macrophage differentiation [16] and the fact that PPARγ mRNA might be increased in inflammatory/immune diseases is not surprising because monocytes/macrophages participate in different inflammatory and autoimmune disorders [23]. PPARγ over-expression in monocytes/macrophages from RA patients not only confers RA as a systemic inflammatory disease, but also suggests that at the joint level, cells can be recruited from a pool of pre-activated peripheral monocytes [21]. Increased PPARγ mRNA levels were also detected in macrophages from patients with active SLE as compared with patients with inactive SLE or infectious diseases and healthy subjects [24]. Koufany et al. [25] demonstrated increased PPARγ mRNA levels in a model of adjuvant-induced arthritis.

Our findings revealed that the PPARγ gene expressions in monocytes were significantly lower in active RA patients than in those with low activity or remission ($p < 0.001$) and PPARγ gene expression was inversely associated with individual markers of disease activity such as morning stiffness durations, TJC, SJC, VAS, ESR/1st h and platelet count. This is in accordance with the findings of Palma et al. [21] who reported a significant inverse correlation between PPARγ protein expression and disease activity evaluated by DAS-28 score ($r = 0.4$, $p < 0.05$). Moreover they found the highest PPARγ protein expression in monocytes from RA patients with a score between 2 and < 2.6 [21], which represents minimal disease
activity rather than remission as documented by Felson et al. [26]. The inverse relationships between the amount of PPAR expression and the degree of disease activity can be explained by the fact that classically activated inflammatory macrophages (M1) that secrete higher levels of pro-inflammatory genes and probably contribute to inflammation and (M2) macrophages, the less inflammatory, alternatively activated macrophages can switch from one phenotype to the other [27]. Therefore the local environment created by the activation of PPAR might induce a switch from M1- toward M2-activated macrophages, thus contributing to the anti-inflammatory effect [28]. Moreover, PPAR agonists exert beneficial effects on inflammatory conditions, including autoimmune diseases, by shifting the pattern of immune response through the modulation of the T-helper 1 (Th1)/T-helper2 (Th2) balance [29]. Guyton et al. [30] found PPAR in the macrophages where its binding to agonists suppresses pro-inflammatory processes. High concentrations

### Table 1  Clinical and laboratory characteristics of rheumatoid arthritis patients with moderate-high disease activity (Group 1A) and with remission/low disease activity (Group 1B).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Rheumatoid arthritis (RA) patients</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td></td>
</tr>
<tr>
<td></td>
<td>All (n = 30)</td>
<td>Group 1A (n = 15)</td>
</tr>
<tr>
<td>Disease duration (y)</td>
<td>7.1 ± 3.9</td>
<td>5.9 ± 2.5</td>
</tr>
<tr>
<td>MS (min)</td>
<td>49.8 ± 46.5</td>
<td>88 ± 36.2</td>
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<tr>
<td>TJC</td>
<td>6.0 ± 5.1</td>
<td>10.4 ± 3.4</td>
</tr>
<tr>
<td>SJC</td>
<td>2.98 ± 2.7</td>
<td>4.8 ± 3</td>
</tr>
<tr>
<td>VAS (cm)</td>
<td>3.8 ± 2.2</td>
<td>5.8 ± 0.9</td>
</tr>
<tr>
<td>DAS-28</td>
<td>3.9 ± 1.5</td>
<td>5.2 ± 0.9</td>
</tr>
<tr>
<td>Larsen score</td>
<td>20.3 ± 14.9</td>
<td>19.9 ± 3.9</td>
</tr>
<tr>
<td>HAQ</td>
<td>1.7 ± 0.7</td>
<td>2.27 ± 0.3</td>
</tr>
<tr>
<td>ESR (mm/1st h)</td>
<td>32 ± 24.9</td>
<td>48 ± 26.2</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>25.5 ± 22.3</td>
<td>10.9 ± 0.7</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>11.1 ± 0.8</td>
<td>7.8 ± 1.6</td>
</tr>
<tr>
<td>WBC (×10³/mm³)</td>
<td>7.3 ± 1.7</td>
<td>7.8 ± 1.6</td>
</tr>
<tr>
<td>Platelets (×10³/mm³)</td>
<td>325.7 ± 91.1</td>
<td>338.9 ± 112.3</td>
</tr>
</tbody>
</table>

Group 1A: moderate-high disease activity, Group 1B: remission/low disease activity, MS: morning stiffness, TJC: tender joint count, SJC: swollen joints count, VAS: visual analog scale, DAS-28: 28-joint disease activity score, HAQ: health assessment questionnaire, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, Hb: hemoglobin, WBC: white blood cells. Bold values are significant between Groups 1A and 1B at p < 0.001.

**Figure 1** Peroxisome proliferator-activated receptor-gamma (PPARγ) mRNA mean fold increase in monocytes from rheumatoid arthritis patients (n = 30); active (n = 15) and inactive (n = 15) as well as in healthy control (n = 30).
of PPARγ ligands were shown to have anti-inflammatory activities by inhibiting the secretion of interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) by stimulated monocytes [31]. Kawahito et al. [32] reported that PPARγ mRNA was expressed in the synovial cells and showed that intraperitoneal injection of PPARγ agonists alleviated adjuvant induced arthritis.

Most of our RA patients were treated with oral prednisone, hydroxychloroquine and methotrexate. Our study revealed no significant correlation between PPARγ expression in monocytes and steroid dose or duration, although there was a significant correlation with the duration of MTX therapy. Palma et al. [21], evaluated the effect of MTX and methylprednisolone on the expression of PPARγ in monocytes and concluded that both enhance expression and act as PPARγ agonists that contribute to overall therapeutic activity, and their clinical efficacy in RA. This was also previously demonstrated in a study showing that indomethacin and other selected non-steroidal anti-inflammatory drugs (NSAIDs), telmisartan and some statins act as PPARγ agonists which contribute to their overall clinical efficacy [33]. In this context, it is worth noting that RA patients with a DAS-28 score < 3.2, which reasonably identifies those patients in whom MTX and corticosteroids are effective in controlling disease severity, express significantly higher PPARγ mRNA levels than patients with a score > 3.2, which could indicate inadequately responding patients.

The findings of Shahin et al. [34] suggest that PPARγ agonist (pioglitazone) could be a potential therapeutic agent for RA. To the best of our knowledge the study by Shahin et al. [34] was the first clinical study evaluating the effects of a PPARγ agonist (pioglitazone) combined with MTX as therapy for RA. In their study, the patients treated with pioglitazone exhibited significant clinical and laboratory improvement. Also, there were significant reductions in the ESR, CRP and DAS28 compared to the placebo treated group of patients. Of note, there was no difference in the blood glucose level between the two groups at the end of the study, indicating that the improvement in disease activity indices and markers was not due to the anti diabetic effect of pioglitazone. These results could be explained by the suggested anti-inflammatory effects of PPARγ ligands. Studies have indicated that many of the cells involved with RA, namely mononuclear leukocytes [35] and chondrocytes [36] express PPARγ receptors. In fact, induction of protective receptors could be a relatively common downstream feature of disease modifying anti-rheumatic drugs (DMARDs).

In conclusion, PPARγ protein expression in RA patients is inversely related to RA disease activity. Our findings support the role of PPARγ in the pathophysiology of RA and suggest that over-expression of PPARγ protein may have anti-rheumatic effects. Further studies on a larger group of RA patients with follow up to explore the anti inflammatory effect of PPARγ and follow the efficiency of therapy are needed. Conduction of more studies on the PPARγ agonist to evaluate its effect on RA disease progression and severity is recommended.

Confict of interest
None.

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