Effect of PPAR-γ agonists on macrophage activation in type2 diabetes mellitus

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Abstract
Aim: Recently, it has been proposed that inflammation triggered by macrophages as the pathogenic mechanism linked to the development of obesity-related insulin resistance. Peroxisome proliferator-activated receptor-γ (PPAR-γ) agonists which increase the insulin receptor sensitivity, might improve glycemic control by enhancing insulin sensitivity and ameliorate the impaired lipid profile. In this study, we assessed the effect of rosiglitazone (PPAR-γ agonist) on the insulin resistance and inflammatory markers. Material and Method: Thirty patients (11 men, 19 women) with type II diabetes mellitus (DM) were taken into the study. They were treated by rosiglitazone in a dose of 4mg/day as well as a diet for 12 weeks. Results: Rosiglitazone treatment significantly decreased HbA1c (p<0,01), IR-HOMA, hsCRP (p<0,01), triglyceride levels (p<0,05), CETP activity (p<0,01) and basal serum oxidation (TBARS levels, p<0,05). However, Chitotriosidase activity significantly increased after Rosiglitazone treatment (p<0,01). TNF, IL-6, sTNFR1, and sTNFR2 levels showed no statistically significant difference compared to their basal levels. Discussion: Rosiglitazone might treat diabetes by mediating glucose/lipid metabolism and preventing lipid oxidation in patients with DM. On the other hand, it has a dual role on inflammation; while it might induce macrophage activation suggesting its pro-inflammatory effect, it might also reduce the CRP levels by lowering gene expression suggesting its anti-inflammatory effect.

Keywords
Insulin Resistance; Chitotriosidase; PPAR-Gamma; TNF
Introduction
Many patients with type 2 diabetes (DM) have hyperglycemia as a result of β-cell dysfunction and/or insulin resistance. Insulin resistance is a state of reduced insulin sensitivity, that is, the inability of insulin to lower plasma glucose levels through suppression of hepatic glucose production. Recently, it has been proposed that oxidative stress is the pathogenic mechanism linking insulin resistance with the dysfunction of both beta cells and endothelium, eventually leading to DM. Treatment strategies have focused on increasing the insulin receptor sensitivity. It has been clearly shown that Peroxisome proliferator-activated receptor-γ (PPAR-γ) agonists such as Rosiglitazone might improve glycemic control by enhancing insulin sensitivity [1] and ameliorate the impaired coronary arteriolar dilation by reducing oxidative stress. In spite of this positive effect on insulin sensitivity of Rosiglitazone, some authors indicated its adverse effects including fluid retention, weight gain, hepatotoxicity, plasma-volume expansion, hemodilution, edema, bone fractures [1]. From 2007 in which was noticed the adverse effect of Rosiglitazone in development of atherosclerosis [2] till today, many conflicting meta-analyses have reported on the cardiovascular effect of thiazolidinediones [3-5]. Atherosclerosis is initiated by inflammatory activation induced by many different factors such as LDL oxidation, hyperglycemia which increase the expression of pro-inflammatory cytokines and chemokine genes [3]. It has been shown that the levels of inflammatory cytokines in insulin-treated diabetic patients were significantly higher than in OHA-treated patients. This information speculated that inflammation plays a significant role in the pathogenesis and severity of the disease. In addition, markers of inflammation seem to be associated with obesity and glucose homeostasis for obese patients, in the presence or absence of DM [6]. Pro-inflammatory cytokines (especially TNF α, IL-1, IL-6, IL-18) are responsible for vascular endothelial dysfunction and activation during the atherosclerotic process. Up-regulation of TNF α expression in the heart is found to be related to myocardial infarction and ischemia. TNF-α is a proinflammatory cytokine, which mediates its effect by two distinct receptors, 55-kDa TNF-R1 and 75-kDa TNF-R2 [7]. TNF-R1 is the main receptor subtype in most cell types, especially in the heart, and it is responsible for the negative ionotropic activity of TNF-α. TNF-R2 expressed in hematopoietic cells is responsible for TNF-α-induced apoptosis. Chitotriosidase is a 50-kDa active enzyme, firstly recognized by its secretion by lipid-laden macrophages in Gaucher disease, and has been proposed as a biochemical marker of macrophage activation in several lysosomal diseases [8]. In this study, we assessed the effect of Rosiglitazone as a PPARγ agonist on insulin resistance, inflammatory markers, and antioxidant enzymes, in order to clarify its effect on the atherosclerotic process.

Material and Method
Thirty patients (11 men and 19 women) with type II DM who have admitted to the Hospital between the years of 2009-2010, were included in the study. The mean age was 48.3 ± 8.8 between 29-79. Their waist measurements were between 86-132 cm (104±12). Seventeen patients (56%) were hypertensive, and they were being treated with oral antidiabetic drugs. Exclusion criteria included the following: None of the above subjects had any clinically-detectable inflammatory or liver disease, cancer, or had taken any regular medications or antioxidant supplements, and had no history of coronary artery disease. None were smokers, and regular alcohol consumption was acceptable.

They were treated by Rosiglitazone in a dose of 4mg/day as well as were following a regular diet for 12 weeks. Blood samples were collected at the beginning of treatment and after 12 weeks treatment.

Serum Antioxidant activity: TEAC (Trolox equivalent antioxidant capacity): ABTS (2,2′-azinobis-3-ethylbenz thiazoline sulfonate) and potassium persulphate (1/1:v/v) solution were mixed with serum, and the absorbance was read in 734nm in a spectrophotometer. Phosphate buffer and Trolox were used as controls and standards, respectively.

TAO (Total antioxidant activity): The solution of 0.1mM DPPH (1,1-diphenyl-2-picrylhydrazyl) was rapidly mixed well with a serum sample. The decline in absorbance was recorded at 550nm against an ethanol blank.

FRAP (Ferric Reducing Antioxidant Power): The mixing solution (10:1:1, v/v/v) of acetate buffer (pH=3.6), TPTZ (2,4,6 tripyridyl-s-triazine) and FeCl3 were added to the serum sample and stored at room temperature for 30 minutes. Readings were done in 620nm in a microplate reader.

In vitro serum oxidation was determined by using TBARS directly in plasma samples. TBARS measurements were performed by TBARS solution as described previously [9]. Susceptibility to oxidation of LDL was determined by inducing LDL oxidation via using 5mM CuSO4. Briefly, plasma samples were incubated with 5mM CuSO4, in vitro TBARS levels were also determined at the 2nd hour of initiation. Serum TNFα, IL-6, sTNFR1, sTNFR2 and hsCRP levels were determined using commercially available ELISA kits. Chitotriosidase activity was measured fluorometrically [10].

Results
Rosiglitazone treatment resulted in significant decreases in HbA1c (p<0.01), IR-HOMA, and triglyceride levels (p<0.05). It had no effect on total, LDL, and HDL cholesterol levels during the 12-week treatment period (Table-1).

Basal and stimulated serum oxidation (TBARS levels, p<0.01) decreased following Rosiglitazone treatment (Figure-1). While Chitotriosidase activity significantly increased after Rosiglitazone treatment (p<0.05), hsCRP (p<0.01) levels decreased (Figure-1). TNF, IL-6, sTNFR1 and sTNFR2 levels showed no statistically significant difference compared to their basal levels (Figure 1 and Table-1).

There was a negative correlation between hsCRP level and chitotriosidase activity in patients before (p=0.019, r=-0.432) and after treatment (p=0.019, r=-0.432) with Rosiglitazone. Since there was no correlation between IL6 and hsCRP levels before treatment, we found a positive correlation between IL6 and hsCRP levels after the treatment (p=0.025, r=0.423).

There was a positive correlation between TNF and TNF-R1 in patients before (p=0.005, r=0.511) and after treatment (p=0.005, r=0.529) with Rosiglitazone.
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Discussion

We determined the effect of Rosiglitazone on LDL-oxidation, inflammatory markers and macrophage activation in type II DM patients. While Rosiglitazone decreased the glucose levels, insulin resistance, serum oxidation and hsCRP levels following the treatment period, it had no effect on TNF alpha, TNF receptors, IL-6 except for an increase in chitotriosidase activity. Depletion in lipoprotein oxidation levels by the treatment of PPAR-γ activation might be related to upregulation of CD36 gene by PPAR γ. It has been proposed that the induction of PPAR-γ, leading to increased expression of CD36, which in turn would increase the uptake of oxLDL [11]. CRP is an acute phase reactant induced by cytokines like TNF-α and IL-6 and is a factor responsible for the subclinical inflammatory state in arteriosclerosis [12]. In our 12-week prospective study, Rosiglitazone showed an anti-diabetic and antioxidant effect and led to a decrease in hsCRP. In accordance with our data, many authors showed that Rosiglitazone treatment decreased CRP levels in 3-26 weeks treatment periods independently from the Rosiglitazone dose in patients with insulin resistance [3]. Since there was no statistically significant difference in other inflammatory cytokine levels (TNF, IL-6, ICAM-1, VCAM-1), this depletion in CRP levels by Rosiglitazone might be explained by the direct inhibitory effect on the liver gene expression of these mediators through the induction of the PPAR-γ receptor gene transcription [5,12]. Previously it has been shown that Thiazolidinediones reduced CRP levels in DM and non-DM subjects by a significant acute effect and prolonged inhibition of the liver gene expression of these mediators [3]. CRP is produced by different cells, including smooth muscle cells, macrophages, lymphocytes and adipocytes, and activates the classical complement pathway [8].Thiazolidinediones insignificantly decreased TNF alfa levels in Type 2 DM patients or had no effect on TNF-α and sCD40L; our data are consistent with the Manning et al.’s study [12, 13]. Recently, Cao X et al. suggested that Toll-like receptor 4 (TLR4) played a critical role in vascular inflammation, lipid accumulation, and atherosclerosis development [14]. Activation of TLR4 signaling via oxidized low-density lipoprotein (oxLDL) inhibited expression of peroxisome proliferator-activated receptor gamma (PPARγ), liver X receptor alpha (LXRα) and ABCG1. PPARγ activation by Rosiglitazone induced LXRα and ABCG1 expression and reduced TLR4 induced inflammation. Cao et al. showed that Rosiglitazone treatment inhibited IL-6 and MCP-1 mRNA expression but not that of TNF-α [14]. It has been suggested that the anti-inflammatory effect of PPARγ activators was related to PPARγ-dependent or PPARγ-independent mechanisms [14, 15].

Dzienis-Straczkowska et al. (2003) indicated that soluble-TNFα receptors, especially sTNFR2, might be a better marker of TNFα action in insulin-resistant states than the plasma TNFα level itself [16]. While Bullo et al. [7] reported a relationship of both receptors with HOMA-IR, Fernandez-Real et al. [17] observed a correlation between sTNFR2, but not sTNFR1, and insulin resistance. All of these studies did not provide any data on the source of elevated TNF receptor levels in patients with insulin resistance [7,16,17]. It might be speculated as a result of increasing release of TNF cytokines from adipocytes due to high mass of adipocytes or induction by hyperglycemia in obese patients. On the other hand, the TNFα system might contribute to the deterioration of insulin sensitivity in these patients. Our data indicated a decrease in HOMA-IR levels as a marker of insulin resistance, but no significant change in TNFα and both receptors following Rosiglitazone treatment. Since there was no change in the BMI and waist measurements of patients following the treatment, we suggested that elevated TNFα and receptor levels were related to adipocyte mass rather than an inductive effect of hyperglycemia. Interestingly, Rosiglitazone
stimulated the macrophage activation determined by an increase in chitotriosidase activity in spite of no change in TNF levels in this study. ChT is synthesized by neutrophilic granulocyte progenitors, and it has been proposed as a biochemical marker of macrophage activation in several lysosomal diseases as well as atherosclerosis [9]. It has been suggested a clear connection between ChT expression and lipid-laden macrophages inside the atherosclerotic vessel wall. Chitotriosidase is an inflammatory protein, but not an acute-phase response protein and is selectively expressed in chronically activated tissue macrophages [18]. Our previous study showed no correlation between CRP and chitotriosidase activity in coronary patients [9]. Malaguerena et al. showed that Chitotriosidase activity and the levels of CHIT mRNA increased by stimulation of macrophages with IFN-γ, TNF-α, and LPS [19]. Although an increase in chitotriosidase activity, as well as inflammatory molecules in patients with DM, has been shown, so far there is no data on the effect of PPAR-γ activation on chitotriosidase activity in patients with insulin resistance [20]. Rosiglitazone treatment increased chitotriosidase activity which is released from activated macrophages probably due to PPAR-γ activation-induced expression of scavenger receptors (CD36) and increased uptake of oxLDL in macrophages [11]. In accordance with this data, Desmet C et al. noticed an increase in TNFα induced proinflammatory cytokine expression by Rosiglitazone, and this effect was independent on PPARγ activation [21]. It has been shown that CHIT1 levels increased significantly during the process of macrophage maturation towards the phenotypes of M1/M2 macrophages. While stimulation by TLR ligand/IFN-γ leads to classical M1 activation, stimulation by IL-4/IL-1 leads to M2 activation [22]. It has been reported that IL-4 treatment induced the expression of mRNA CHIT1 and activated PPAR-γ [23, 24]. IL-4 is also an antagonist of the M1 response. Therefore, macrophage pro-inflammatory properties showed that CHIT1 levels increased in M2 macrophages which had a role in both generation of fibrosis and resolution of inflammation, CHIT might have a role in the modulation of the extracellular matrix during the tissue remodelling processes [25]. It might be concluded that increase in CHIT activity with Rosiglitazone treatment might be related to M2 macrophage activation via IL-4 pathway rather than PPAR gamma activation.

Since Rosiglitazone administration led to a significant decrease in HbA1c and insulin resistance, serum oxidation as well as hsCRP, we suggested that Rosiglitazone has anti-diabetic, antioxidant effects in patients with DM. In conclusion, Rosiglitazone might treat DM by mediating glucose/lipid metabolism and preventing lipid oxidation in patients with DM. On the other hand, RSG has a dual role in inflammatory markers, while it might induce macrophage activation suggesting its proinflammatory effect, it might also reduce the CRP levels by lowering gene expression suggesting its anti-inflammatory effect.

**Animal and Human rights**

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Scientific Responsibility Statement**

The authors declare that they are responsible for the article’s scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

**Animal and human rights statement**

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

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**Conflict of interest**

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