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Short Communication

Tumor necrosis factor-α and interleukin-6 expression in leukocytes and their association with polymorphisms and bone markers in diabetic individuals treated with pioglitazone

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Abstract

Background: Pioglitazone is a peroxisome proliferator-activated receptor gamma (PPARγ) activator used in the treatment of type 2 diabetes (DM2) patients and it has been suggested that can induce bone loss. Tumor necrosis factor-α (TNFα) and interleukin-6 (IL-6) mRNA expression in blood leukocytes and the relationship with polymorphisms and bone markers in DM2 treated with pioglitazone were investigated.

Methods: DM2 (n=53) and normoglycemic (NG, n=52) individuals were included. DM2 patients were treated with pioglitazone (45 mg/day/16 weeks). mRNA expression was evaluated by real-time polymerase chain reaction (PCR). *TNFA* −308G>A and *IL6* −174G>C polymorphisms were detected by PCR-RFLP and high resolution melting polymerase chain reaction (HRM-PCR).

Results: Pioglitazone reduced bone specific alkaline phosphatase (bALP) and increased TNFα in DM2 group ($p<0.001$). DM2 or pioglitazone did not influence TNFα and IL-6 expression ($p>0.05$). *TNFA* −308A allele was associated with reduced basal TNFα mRNA levels in NG and DM2 and reduced alkaline phosphatase (tALP) after treatment ($p<0.05$). *IL6* −174C allele was associated with decreased oral glucose tolerance test (OGTT)−2 h in DM2 individuals ($p<0.05$).

Conclusions: *TNFA −308G>A* polymorphism appear to be involved in regulation of gene expression independently of hyperglycemia and its interaction with pioglitazone may modify tALP, a important bone marker. *IL6* −174G>C variant is related with reduced risk of postprandial hyperglycemia but not with mRNA expression or bone markers.

Keywords: IL-6; pioglitazone; TNFα; type 2 diabetes.

Diabetes has been shown to enhance expression of tumor necrosis factor-α (TNFα) that stimulate formation of osteoclasts and are responsible for resorption of mineralized cartilage and bone (1). Insulin insufficiency, hyperglycemia and oxidative stress, hallmarks type 2 diabetes (DM2), reduce osteoblast differentiation, increase osteoclast activity, alter apoptosis of chondrocytes and osteoblasts leading to impaired fracture healing in diabetic patients (1). In diabetes mice model, it was shown that high level expression of pro-osteoclastogenic factors in chondrocytes is mediated at least in part by TNFα dysregulation in diabetic fracture healing, showing that TNFα is particularly important in enhancing osteoclastogenesis (2). Data indicate that peroxisome proliferator-activated receptor gamma (PPARγ) agonists such as pioglitazone increase insulin sensitivity. Activation of PPARγ has been shown to play an important role in bone morphogenesis favoring osteoclastogenesis. PPARγ promotes osteoclast differentiation by regulating c-fos expression in RANKL signaling pathways that control lineage commitment and osteoclast activation (3). Additionally, there is strong evidence that PPARγ stimulates adipocyte differentiation and suppresses osteoblast differentiation (4). In culture of mononuclear cells, was shown that TNFα could differentiate human peripheral monocytes into activated osteoclasts, and PPARγ agonists may inhibited the TNFα mediated osteoclastogenesis (5). These findings prompted us to examine the effect of PPARγ agonists on TNFα and interleukin-6 (IL-6) mediated osteoclastogenesis in DM2 patients.

The aim of this study was to investigate the mRNA expression of TNFα and IL-6 in peripheral blood leukocytes (PBL) and their association with gene polymorphisms and alterations bone markers levels in DM2 patients treated with pioglitazone.

Fifty-three DM2 and 52 normoglicemic (NG) individuals, aged 30–70 years, were recruited from outpatients at the

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Instituto Dante Pazzanese de Cardiologia (IDPC), Sao Paulo, SP, Brazil. DM2 was diagnosed according to American diabetes association criteria (ADA) (6). Individuals with thyroid, liver or renal disease, pregnant women, or under therapy with β-adrenergic blockers, angiotensin converter enzyme inhibitors, thiazide diuretics, adrenal steroid hormones, fibrates and oral hypoglycemic agents (metformin, repaglinide and acarbose), or insulin were not included in this study. DM2 subjects (never treated) were treated with increasing doses of oral pioglitazone during 16 weeks (15, 30, 45 and 45 mg each 4 weeks once daily). The study protocol was approved by the Ethics Committee of IDPC, Sao Paulo, SP, Brazil. All participants gave written informed consents.

DM2 patients had higher baseline concentrations of fasting and oral glucose tolerance test (OGTT)–2 h glucose, glycohemoglobin (HbA1c), insulin and HOMA-IR [homeostasis model assessment-(insulin resistance)], and lower ionized calcium compared with NG individuals ($p<0.05$) (Table 1). Lower levels of ionized calcium found may result from increasing bone resorption in DM2 individuals. Activation of osteoclasts removes the mineralized matrix of bone, releasing ionized calcium into extracellular fluid and altering calcium homeostasis and signaling (7).

Reduction of fasting and OGTT–2 h glucose, HbA1c, fasting insulin and HOMA-IR, after treatment of DM2 patients (Table 1) demonstrates the hypoglycemic and insulin sensitizer effects of pioglitazone (8). Pioglitazone also showed an inhibitory effect on bone-specific alkaline phosphatase (bALP) levels in DM2 patients. A randomized controlled trial has shown that rosiglitazone (4 mg) reduces plasma bALP in diabetic postmenopausal women, indicating an inhibitory effect on activity of osteoblasts cells (9). DM2 did not influence TNFα and IL-6 serum levels, while serum TNFα was increased by pioglitazone ($p=0.001$). Treatment of DM2 patients with pioglitazone (30 mg) plus sitagliptin (100 mg) for 12 months was shown to decrease TNFα in serum (10). These differential

Table 1 Clinical and laboratory data of DM2 and normoglycemic individuals.

Variables	NG (52)	DM2 (53)		DM2 (53)	
		Basal	p^1	Pioglitazone	p^2
Age, years	55.6±10.2	60.2±8.9	0.015[b]		
Women, %	69.8 (37)	34.0 (18)	0.001[a]		
Arterial hypertension, %	45.3 (24)	35.8 (19)	0.429[a]		
Obesity, %	37.7 (20)	45.3 (24)	0.554[a]		
BMI, kg/m^2	28.9±7.0	30.7±4.8	0.129[b]		
Hypercholesterolemia, %	5.8 (3)	15.1 (8)	0.214[a]		
Menopause, %	67.6 (25)	66.7 (12)	0.811[a]		
Tobacco consumption, %	7.7 (4)	9.4 (5)	0.927[a]		
Alcohol consumption, %	9.6 (5)	5.7 (3)	0.650[a]		
Physical activity, %	34.6 (18)	34.0 (18)	0.894[a]		
Family history of CAD, %	48.1 (25)	32.1 (17)	0.099[a]		
Fasting glucose, mmol/L	5.1±0.7	6.7±1.3	0.001[b]	5.9±1.1	0.001[d]
OGTT–2 h glucose, mmol/L	5.8±1.9	10.3±3.2	0.001[b]	8.4±2.5	0.001[d]
Hb1Ac, %	5.8±0.4	6.5±1.0	0.001[c]	6.3±1.4	0.019[e]
Fasting insulin, mol/L	8.7±5.8	13.9±9.2	0.001[c]	10.4±6.5	0.002[d]
HOMA-IR, %	2.1±1.5	4.1±2.6	0.001[b]	2.7±1.7	0.001[d]
HOMA-β, %	119.8±83.1	100.6±80.8	0.151[c]	104.4±104.8	0.959[e]
Total calcium, mmol/L	2.3±0.3	2.3±0.1	0.578[c]	2.3±0.1	0.541[e]
Ionized calcium, mmol/L	1.2±0.1	1.1±0.1	0.002[b]	1.16±0.11	0.319[d]
Phosphate, mmol/L	1.11±0.2	0.17±0.2	0.426[c]	1.1±0.2	0.897[d]
tACP, U/L	3.5±1.9	3.3±1.7	0.561[b]	3.2±0.8	0.643[d]
tALP, U/L	74.9±27.1	70.9±22.6	0.415[b]	67.2±21	0.224[d]
bALP, U/L	30.4±15.4	29.0±17.3	0.660[b]	13.6±6.6	0.001[d]
TNFα, pg/mL	6.5±2.2	6.4±3.0	0.902[c]	13.0±6.0	0.001[d]
IL-6, pg/mL	4.8±3.7	6.8±11.6	0.826[c]	6.8±9.5	0.500[e]
Minor allele frequency, %					
TNFA –308G>A, %	11.3 (11)	16.4 (17)	0.570[a]		
IL6 –174G/C, %	26.9 (28)	34.9 (37)	0.006[a]		

Number of individuals is in parenthesis. Obesity (BMI>30 kg/m^2), hypertension (blood pressure >140/90 mm Hg or use of anti-hypertensives), hypercholesterolemia (LDL cholesterol >4.14 mmol/L), current cigarette smoking (daily intake ≥1 cigarettes), alcohol consumption (intake of beer, wine, and distilled spirits of ≥1 g/day), physical activity (practice of sports, for at least 2 h/week). [a]Categorical variables were compared by χ^2-test; [b]continuous variables are presented as mean±SD and compared by t-test; [c]Mann-Whitney; [d]paired t-test or [e]Wilcoxon signed rank test. p^1, DM2 compared with NG; p^2, pioglitazone compared with basal; BMI, body mass index; CAD, coronary artery disease; OGTT, oral glucose tolerance test; Hb1Ac, glycohemoglobin; HOMA-beta, homeostasis model assessment for beta cell function; HOMA-IR, homeostasis model assessment-(insulin resistance); tACP, total acid phosphatase; tALP, alkaline phosphatase; bALP, bone specific alkaline phosphatase; IL6, interleukin-6; TNFα, tumor necrosis factor-α.

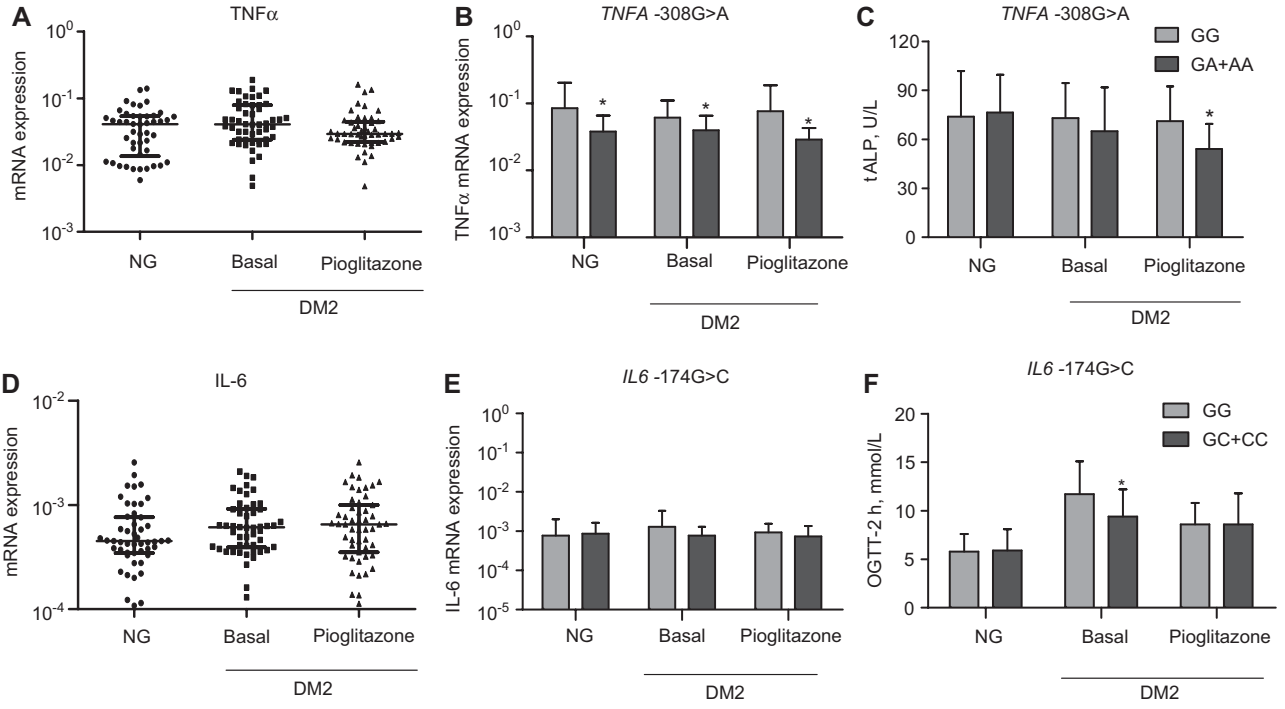

Figure 1 TNFα mRNA expression (A), influence of *TNFA* −308G>A variant on TNFα mRNA expression (B) and tALP (C) and IL-6 mRNA expression (D), influence of *IL6* −174G>C variant on IL-6 mRNA expression (E) and OGTT−2 h (F) of NG and DM2 before and after pioglitazone therapy.

Data are shown as median and interquartile ranges (A, D) and as mean ± SE (B, C, E, F) of values compared by Mann-Whitney test and Wilcoxon signed rank test. Chi-square analysis was used to test the Hardy-Weinberg equilibrium (HWE). For *TNFA* −308G>A polymorphism, individuals carrying GG genotype were compared with the GA+AA genotypes carriers. For *IL6* −174G>C variant, GG genotype carriers were compared with those carrying CG+CC genotypes. *TNFA* −308G>A (rs 11800629) and *IL6* −174G>C (rs 1800795) polymorphisms were determined by HRM-PCR and PCR-RFLP respectively, using the sequences of primers previously described (13, 14). *$p<0.05$. TNFα, tumor necrosis factor-α; IL-6, interleukin-6; OGTT, oral glucose tolerance test.

results may be influenced by time, dosage and combination of the pioglitazone treatment that remains to be investigated.

The distribution of *TNFA* −308A and *IL6* −174C genotypes was as expected from Hardy-Weinberg equilibrium (HWE) in both groups (data not shown). *TNFA* −308G>A variant had similar genotype and allele frequencies between DM2 (A allele: 16.4%) and NG (A allele: 11.3%) groups ($p>0.05$), suggesting lack of relationship with DM2, as previously reported (11). *IL6* −174C allele was more frequent in DM2 (34.9%) than in NG (26.9%, $p<0.05$). Moreover, individuals carrying C allele (GC+CC genotypes) had two times higher risk for DM2 (OR: 2.43, 95% CI: 1.11–5.34, $p=0.032$) than non-carriers. In a previous study, the frequency of the C allele was significantly higher in DM2 individuals with metabolic syndrome compared to those without this metabolic disease (12) suggesting a relationship of this allele with worst metabolic control in DM2 patients.

Global TNFα and IL-6 mRNA levels in PBL were not influenced neither by hyperglycemic status (DM2) nor by treatment ($p>0.05$) (Figure 1A and D, respectively). *TNFA* −308A allele carriers had decreased TNFα mRNA expression than those carrying GG genotypes, in both NG and DM2 before and after treatment ($p<0.05$, Figure 1B). *IL6* −174G>C polymorphism did not influence gene expression in NG and DM2 (Figure 1E). This is the first evidence that there is a relationship between *TNFA −308G>A* polymorphism and TNFα mRNA expression in leukocytes in both DM2 and NG individuals. It has been suggested that the *TNFA −308G>A* region may play a role in TNFα transcription in human T cell model (15).

Reduction of alkaline phosphatase (tALP) in response to pioglitazone was found in *TNFA* −308A allele carriers ($p=0.017$) (Figure 1C). This result suggests that the interaction of *TNFA* −308A allele and pioglitazone treatment may influence the osteoblastic activity, that is reflected by the reduction of the bone marker tALP (9). A multivariate analysis model was performed in order to identify significant differences in sex and other variables, with mRNA expression and genotypes. However, there were no statistically significant differences between groups (data not shown).

IL6 −174C allele was associated with decreased OGTT−2 h in DM2 individuals ($p<0.05$) (Figure 1F) but not to other metabolic markers (data not shown). It was previously shown that *IL6* −174C allele is associated with higher plasma levels of IL-6, particularly in inflammatory situations, showing that IL-6 might have an aetiological role in insulin signaling (16). These findings may explain the results obtained in this study,

demonstrating the insulin influence, decreasing OGTT–2 h in *IL6* –174C allele carriers.

In conclusion, the *TNFA –308G>A* polymorphism appear to be involved in regulation of gene expression in PBL independently of the hyperglycemic status and its interaction with pioglitazone may modulate bone markers such as tALP. *IL6* –174G>C variant is related with reduced risk of postprandial hyperglycemia but not with mRNA expression nor bone markers.

Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.
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