β (CCL2) and α (CXCL10) chemokine modulations by cytokines and peroxisome proliferator-activated receptor- α agonists in Graves' ophthalmopathy

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Abstract

No data are present in the literature about the effect of cytokines on the prototype β chemokine (C-C motif) ligand 2 (CCL2) or of peroxisome proliferator-activated receptor α (PPAR α (PPARA)) activation on CCL2 and CXCL10 chemokines secretion in fibroblasts or preadipocytes in Graves' ophthalmopathy (GO). We have tested the effect of interferon γ (IFN γ (IFNG)) and tumor necrosis factor α (TNF α) on CCL2, and for comparison on the prototype α chemokine (C-X-C motif) ligand 10 (CXCL10), and the possible modulatory role of PPAR α activation on secretion of these chemokines in normal and GO fibroblasts or preadipocytes in primary cell cultures. This study shows that IFN γ alone, or in combination with TNF α , stimulates the secretion of CCL2 in primary orbital fibroblasts or preadipocytes from patients with GO at levels similar to those

observed in controls. IFN γ and TNF α also stimulated CXCL10 chemokine secretion as expected. The presence of PPAR α and PPAR γ (PPARG) in primary fibroblasts or preadipocytes of patients with GO has been confirmed. PPAR α activators were able to inhibit the secretion of CXCL10 and CCL2, while PPAR γ activators were confirmed to be able to inhibit CXCL10 but had no effect on CCL2. PPAR α activators were stronger inhibitors of chemokine secretions than PPAR γ agonists. In conclusion, CCL2 and CXCL10 are modulated by IFN γ and TNF α in GO. PPAR α activators inhibit the secretion of the main prototype α (CXCL10) and β (CCL2) chemokines in GO fibroblasts or preadipocytes, suggesting that PPAR α may be involved in the modulation of the immune response in GO.

Journal of Endocrinology (2012) **213**, 183–191

Introduction

Recent evidences have shown that CXC α -chemokines (Th1), especially CXCL9, CXCL10, and CXCL11, play an important role in the initial phases of autoimmune thyroid dysfunction (García-López *et al.* 2001, Antonelli *et al.* 2004, 2006a,b, 2007).

We have recently demonstrated that the secretion of CXCL9, CXCL10, and CXCL11 in primary cultures of Graves' ophthalmopathy (GO) fibroblasts and preadipocytes can be stimulated by interferon γ (IFN γ (IFNG)) and tumor necrosis factor α (TNF α ; Antonelli *et al.* 2006c, 2009a), suggesting that GO retro-orbital cells participate in the self-perpetuation of inflammation by releasing chemokines (under the influence of cytokines) and inducing the recruitment of activated T cells in the orbit.

Furthermore, other studies have demonstrated recently a possible role for chemokine (C-C motif) ligand 2 (CCL2; also known as monocyte chemoattractant protein-1 (MCP1)) in GO. Hwang *et al.* (2009) showed that orbital fibroblasts from

patients with GO overexpress CD40 and that CD154 (the ligand for CD40) upregulated the expression of CCL2. Furthermore, Chen *et al.* (2008) found that the expression of CCL2 was higher in the orbital fat of GO patients than in controls.

The peroxisome proliferator-activated receptors α (PPAR α (PPARA)), PPAR γ (PPARG), and PPAR δ (PPARD) are ligand-activated nuclear receptors with a wide range of effects on metabolism, cell proliferation, and differentiation (Michalik *et al.* 2006). In addition, abundant evidence suggests that the PPARs are also important regulators of the immune system. Of considerable interest, ligands for PPAR γ and PPAR α have therapeutic activity in several rodent models of inflammatory and autoimmune diseases (Spencer *et al.* 1997, Delerive *et al.* 2000, Lovett-Racke *et al.* 2004, Okamoto *et al.* 2005, Lee *et al.* 2007, Oliveira *et al.* 2007), suggesting that they might have similar activity in human disease as well.

About chemokines, it has been recently shown that fenofibrate represses interleukin 17 (IL17 (IL17a)) and IFN γ expression and improves colitis in IL10-deficient mice by inhibiting the expression of the gene-encoding chemokine

(C-X-C motif) ligand 10 (CXCL10) and by repressing CXCL10 gene promoter activity in TNF α -treated HT-29 cells (Lee *et al.* 2007).

Treatment of fibroblasts and preadipocytes with PPAR γ activators (rosiglitazone and pioglitazone) at near-therapeutic doses significantly inhibited IFN γ -stimulated CXC chemokine secretion, strongly suggesting that PPAR γ might be involved in the regulation of IFN γ -induced chemokine expression in human thyroid autoimmunity and GO (Antonelli *et al.* 2006c, 2009a). More recently, we have shown that CXCL9 and CXCL11 chemokines are modulated by PPAR α agonist secretion in Graves' and normal thyrocytes (Antonelli *et al.* 2010). Furthermore, PPAR α has been found to be expressed in fibroblasts (Pasquali *et al.* 2004) and preadipocytes (Antonelli *et al.* 2012) in GO patients.

However, until now, to our knowledge, no data are present in the literature about the effect of cytokines on CCL2 in GO or of PPAR α activation on CCL2 and CXCL10 chemokine secretions in fibroblasts and preadipocytes in GO. Here, we have tested the effects of IFN γ and TNF α on CCL2 (the prototype Th2 chemokine) and the possible modulatory role of PPAR α activation on CCL2, and for comparison on the prototype Th1 CXCL10 chemokine, secretion in GO fibroblasts or preadipocytes in primary culture.

Materials and Methods

Fibroblast and preadipocyte cell cultures

Orbital adipose/connective tissue samples were obtained from five euthyroid patients (two on L-thyroxine after thyroidectomy) undergoing orbital decompression for severe GO during the inactive phase of the disease (all previously treated with antithyroid medication and systemic corticosteroids; none treated with orbital radiotherapy). All subjects gave their informed consent to the study, which was approved by the local Ethics Committee.

GO tissue explants were minced and placed directly in plastic culture dishes, to let preadipocyte fibroblasts proliferate, as described previously (Bahn *et al.* 1989). Cells were propagated in medium 199 with 20% v/v fetal bovine serum (FBS; Gibco; Invitrogen Ltd.), penicillin (100 IU/ml), and gentamicin (20 μ g/ml) in a humidified 5% CO $_2$ incubator at 37 °C and maintained with medium 199 containing 10% FBS and antibiotics. Orbital cells were grown to confluence in six-well plates in medium 199 with 10% FBS in order to initiate adipocyte differentiation, which was carried out as reported previously (Antonelli *et al.* 2006c, 2010). The differentiation protocol was continued for 10 days, and the medium was replaced every 3–4 days. Separately, fibroblasts obtained from the same patients' orbital tissues were maintained for the same period in the medium lacking various components necessary for complete adipocyte differentiation (i.e. cPGI $_2$, dexamethasone, and IBMX, 3-isobutyl-1-methylxanthine; Antonelli *et al.* 2010). Control fibroblasts and preadipocytes were obtained from dermal tissues (not affected by any pathology) of the same patients.

Orbital preadipocyte fibroblast cultures were plated in medium 199 with 10% FBS, grown to confluence, and subjected to either the differentiation protocol or the nondifferentiation conditions. Cells were washed, stained with filtered 0.21% Oil Red O and subsequently with Mayer's hematoxylin solution (MHS-32; Sigma), and visualized using an Olympus IX50 light microscope and photographed at $\times 20$ as previously reported (Antonelli *et al.* 2009a, 2012).

CXCL10 and CCL2 secretion assays

For CXCL10 and CCL2 secretion assays, cells were seeded in 96-well plates at a concentration of 30 000 cells/ml as previously reported (Antonelli *et al.* 2009a) and incubated (24 h) with IFN γ (500, 1000, 5000, and 10 000 IU/ml; R&D Systems, Minneapolis, MN, USA) and 10 ng/ml TNF α (R&D Systems), alone or in combination (Antonelli *et al.* 2009a, 2012). After 24 h, the supernatant was collected and kept frozen at -20 °C until CXCL10 assay.

To establish how the PPAR α or PPAR γ activators affect IFN γ -induced chemokine secretion, cells were treated (24 h) with IFN γ (1000 IU/ml) and TNF α (10 ng/ml) in the presence or absence of increasing concentrations of PPAR α agonists (5, 10, 50, and 100 μ mol/l fenofibrate; 50, 100, 200, and 400 μ mol/l gemfibrozil or ciprofibrate; Sigma–Aldrich Corp.) or PPAR γ agonists (0, 1, 5, 10, and 20 μ mol/l rosiglitazone; Glaxo SmithKline, or pioglitazone; Alexis Biochemicals, Lausen, Switzerland; Antonelli *et al.* 2012).

Supernatants were assayed by ELISA for CXCL10 and CCL2 concentrations. The experiments were repeated three times with the different cell preparations.

Cell cultures and PPAR α or PPAR γ agonist treatment

Cell cultures were treated (24 h) with PPAR γ agonists (0, 1, 5, 10, or 20 μ mol/l rosiglitazone or pioglitazone) or PPAR α agonists (5, 10, 50, and 100 μ mol/l fenofibrate; 50, 100, 200, and 400 μ mol/l gemfibrozil or ciprofibrate). Control cultures were grown (24 h) in the same medium containing vehicle (absolute ethanol, 0.47% v/v) without PPAR α or PPAR γ agonists. Some cultures were examined by phase contrast microscopy using an Olympus IX50 light microscope.

Lysis and homogenization of cell preparations were performed, and the sample was immediately assayed for its protein concentration by conventional methods (Antonelli *et al.* 2006c).

ELISA for CXCL10 and CCL2

Culture supernatants CXCL10 and CCL2 levels were assayed by a quantitative sandwich immunoassay using a commercially available kit (R&D Systems). The mean minimum detectable dose for CXCL10 was 1.35 pg/ml; the intra- and inter-assay coefficients of variation (CV) were 3.1 and 6.8% respectively. The mean minimum detectable dose for CCL2 was 4.6 pg/ml; the intra- and interassay CV were 4.6 and 5.7% respectively.

Table 1 Relative level of peroxisome proliferator-activated receptor α (PPAR α) or PPAR γ mRNA in fibroblasts from Graves' ophthalmopathy (GO) or control fibroblasts by RT-PCR. The amount of target was normalized to the endogenous reference GAPDH mRNA (PPAR α /GAPDH mRNA or PPAR γ /GAPDH mRNA) and expressed as range of values

	PPAR α /GAPDH	PPAR γ /GAPDH
GO fibroblasts	0.41–0.87	0.10–0.21
Control fibroblasts	0.12–0.34	0.04–0.08

RT-PCR for PPAR α and PPAR γ

Total RNA (400 ng) was reverse transcribed using TaqMan RT reagents kit as previously reported (Antonelli *et al.* 2010). Primers and probes for PPAR α and PPAR γ were from Applied Biosystems (TaqMan Gene Expression Assay; Hs00231882_m1 and Hs00234592_m1 respectively).

The amount of target, normalized to the endogenous reference glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Pre-Developed TaqMan Assay Reagents; Applied Biosystems) and relative to a calibrator (quantitative PCR human reference total RNA), was given by $2^{-\Delta\Delta C_t}$ calculation (Antonelli *et al.* 2010).

Data analysis

Values are given as mean $\pm$ S.D. for normally distributed variables (in text), or mean $\pm$ S.E.M. (in figures), otherwise as median and (interquartile range). Mean group values were compared using ANOVA for normally distributed variables, otherwise by the Mann–Whitney *U* (two groups) or Kruskal–Wallis (three groups or more) tests. Proportions were compared by the χ^2 test. *Post hoc* comparisons on normally distributed variables were carried out using the Bonferroni–Dunn test.

Results

PPAR α and PPAR γ expression in GO fibroblasts and preadipocytes

PPAR α and PPAR γ mRNAs were detectable in all primary orbital fibroblast cells. According to RT-PCR results, the relative level of PPAR α expression vs the reference gene

(GAPDH) ranges from 0.4 to 0.9 and the expression of PPAR γ from 0.1 to 0.2. The results obtained in control fibroblasts are summarized in Table 1. PPAR α and PPAR γ were expressed by RT-PCR more in GO fibroblasts than in controls. Similar results were present in preadipocytes (data not shown).

PPAR agonists and chemokines in GO fibroblasts and preadipocytes

CXCL10 was undetectable in the supernatants collected from primary GO fibroblast cultures. IFN γ dose dependently induced the CXCL10 release (ANOVA, $P < 0.001$; Table 2); similar results were observed in preadipocytes (ANOVA, $P < 0.001$). TNF α alone had no effect (chemokines remaining undetectable), while the combination of IFN γ and TNF α had a significant synergistic effect on the CXCL10 secretion both in fibroblasts (ANOVA, $P < 0.0001$) and in preadipocytes (ANOVA, $P < 0.0001$).

Treating GO fibroblasts or preadipocytes with rosiglitazone or pioglitazone, together with IFN γ and TNF α stimulation, CXCL10 release was dose dependently inhibited (ANOVA, $P < 0.0001$) as expected (Antonelli *et al.* 2006c, 2009a).

By treating GO fibroblasts with fenofibrate (Fig. 1A), gemfibrozil (Fig. 1B), or ciprofibrate (Fig. 1C), together with IFN γ and TNF α stimulation, CXCL10 release was dose dependently inhibited. Similar results were observed in preadipocytes, in which fenofibrate, ciprofibrate, or gemfibrozil, dose dependently inhibited the IFN γ - and TNF α -stimulated CXCL10 release (ANOVA, $P < 0.0001$; data not shown).

The inhibition of the CXCL10 chemokine secretion induced by PPAR α agonists, at the highest dosage, was stronger than that induced by PPAR γ activators both in fibroblasts (Fig. 2A) and in preadipocytes (Fig. 2B). However, the relative potency of the PPAR α agonists on the inhibition of the CXCL10 chemokine was different for each compound.

CCL2 was detectable (546 ± 93 pg/ml) in the supernatants collected from primary GO fibroblast or preadipocyte cultures (751 ± 106 pg/ml). IFN γ dose dependently induced CCL2 release in fibroblasts (CCL2: 546 ± 93 , 723 ± 85 , 970 ± 65 , 1076 ± 131 , and 1204 ± 171 pg/ml, respectively, with IFN γ : 0, 500, 1000, 5000, and 10 000 IU/ml; ANOVA,

Table 2 In fibroblasts, from patients with Graves' ophthalmopathy (GO), interferon γ (IFN γ) dose dependently induced the CXCL10 release (ANOVA, $P < 0.001$); similar results were observed in preadipocytes (ANOVA, $P < 0.001$). Tumor necrosis factor α (TNF α) alone had no effect (chemokines remaining undetectable), while the combination of IFN γ and TNF α had a significant synergistic effect on the CXCL10 secretion both in fibroblasts (ANOVA, $P < 0.0001$) and in preadipocytes (ANOVA, $P < 0.001$)

	Control	IFN γ (0 IU/ml)	IFN γ (500 IU/ml)	IFN γ (1000 IU/ml)	IFN γ (5000 IU/ml)	IFN γ (10 000 IU/ml)	TNF α (10 ng/ml)	IFN γ (1000 IU/ml) + TNF α (10 ng/ml)
GO fibroblasts (CXCL10 pg/ml)	0	0	167 ± 62	410 ± 78	563 ± 94	591 ± 107	0	1876 ± 88
Preadipocytes (CXCL10 pg/ml)	0	0	92 ± 32	210 ± 54	361 ± 78	443 ± 81	0	2653 ± 148

Figure 1 Increasing doses of fenofibrate (Feno; 5, 10, 50, and 100 μ mol/l; A), gemfibrozil (Gem; 50, 100, 200, and 400 μ mol/l; B), or ciprofibrate (Cipro; 50, 100, 200, and 400 μ mol/l; C) inhibit CXCL10 release from orbital fibroblasts stimulated with interferon (IFN γ) (IFN γ , 1000 IU/ml) and tumor necrosis factor (TNF α) (TNF α , 10 ng/ml), (IFN γ +TNF α ; $P<0.0001$ by ANOVA). Bars are mean $\pm$ S.E.M. * $P<0.05$ or less vs IFN γ +TNF α by Bonferroni–Dunn test.

$P<0.001$) and in preadipocytes (CCL2: 751 ± 106 , 937 ± 96 , 1123 ± 125 , 1411 ± 147 , and 1504 ± 168 pg/ml, respectively, with IFN γ : 0, 500, 1000, 5000, and 10 000 IU/ml; ANOVA, $P<0.001$). TNF α alone had no effect on CCL2 release (with TNF α : 0, 1, and 10 ng/ml). The combination of IFN γ and TNF α had a significant synergistic effect on CCL2 secretion (ANOVA, $P<0.0001$; Fig. 3) in primary GO fibroblasts (Fig. 3A) and in preadipocytes (Fig. 3B).

Treating orbital fibroblasts with fenofibrate (Fig. 4A), gemfibrozil (Fig. 4B), or ciprofibrate (Fig. 4C), together with IFN γ and TNF α stimulation, CCL2 release was dose dependently inhibited. Similar results were observed

in preadipocytes, in which fenofibrate, gemfibrozil, or ciprofibrate dose dependently inhibited the IFN γ - and TNF α -stimulated CXCL10 release (ANOVA, $P<0.0001$; data not shown).

The inhibition of the CCL2 chemokine secretion induced by PPAR α agonists at the highest dosage in GO fibroblasts averaged 65% (Fig. 5A). Treating orbital fibroblasts (Fig. 5A) or preadipocytes (Fig. 5B) with rosiglitazone or pioglitazone,

Figure 2 The inhibition of the CXCL10 chemokine secretion induced by PPAR α agonists at the maximum dosage in GO fibroblasts (A) or preadipocytes (B; fenofibrate (Feno; 100 μ mol/l), gemfibrozil (Gem; 400 μ mol/l), and ciprofibrate (Cipro; 400 μ mol/l)) was stronger than that induced by PPAR γ activators (pioglitazone (Pio 20; 20 μ mol/l) and rosiglitazone (Rosi 20; 20 μ mol/l)). Bars are mean $\pm$ S.E.M. * $P<0.05$ or less vs IFN γ (IFN γ) + TNF α (TNF α) by Bonferroni–Dunn test. * $P<0.05$ or less vs IFN γ +TNF α +Rosi 20 or IFN γ +TNF α +Pio 20 by Bonferroni–Dunn test.

Figure 3 CCL2 was detectable in the supernatants collected from orbital fibroblasts (A). IFN γ (IFN γ , 1000 IU/ml) significantly induced the CCL2 release (ANOVA, $P < 0.001$), while TNF α (TNF α , 10 ng/ml) alone had no effect. The combination of IFN γ (1000 IU/ml) and TNF α (10 ng/ml) had a significant synergistic effect on CCL2 secretion (ANOVA, $P < 0.001$). Similar results were observed in preadipocytes (B; ANOVA, $P < 0.001$). Bars are mean $\pm$ S.E.M. * $P < 0.05$ or less vs control (CT) by Bonferroni–Dunn test; ^o $P < 0.05$ or less vs IFN γ or TNF α by Bonferroni–Dunn test.

together with IFN γ and TNF α stimulation, had no significant effect on the CCL2 release.

Rosiglitazone or pioglitazone, fenofibrate, ciprofibrate, or gemfibrozil alone had no effect and did not affect cell viability or total protein content (data not shown). The data obtained with orbital fibroblasts or preadipocytes were not significantly different from those obtained with control fibroblasts or preadipocytes (data not shown).

Discussion

This study shows that IFN γ alone, or in combination with TNF α , stimulated the secretion of the CC chemokine CCL2 in primary orbital fibroblasts or preadipocytes from patients with GO. IFN γ and TNF α stimulated the CXC chemokine secretion as expected. The presence of PPAR α in primary fibroblasts or preadipocytes from patients with GO has been confirmed (Antonelli *et al.* 2012). PPAR α activators inhibited the secretion of both CXC (CXCL10) and CC chemokines

(CCL2), while PPAR γ activators were confirmed to inhibit CXC chemokines but had no effect on CCL2. PPAR α activators were stronger inhibitors of chemokine secretions than PPAR γ agonists.

Recently, some studies have suggested a possible role for CCL2 in GO. Hwang *et al.* (2009) demonstrated that orbital fibroblasts from patients with GO overexpress CD40 and that CD154 (the ligand for CD40) upregulated the expression of

Figure 4 Increasing doses of fenofibrate (Feno; 5, 10, 50, and 100 μ mol/l) (A), gemfibrozil (Gem; 50, 100, 200, and 400 μ mol/l; B), or ciprofibrate (Cipro; 50, 100, 200, and 400 μ mol/l; C) inhibit CCL2 release from orbital fibroblasts stimulated with IFN γ (IFN γ , 1000 IU/ml) and TNF α (TNF α , 10 ng/ml), (IFN γ +TNF α ; $P < 0.0001$ by ANOVA). Bars are mean $\pm$ S.E.M. * $P < 0.05$ or less vs IFN γ +TNF α by Bonferroni–Dunn test.

Figure 5 The inhibition of the CCL2 chemokine secretion induced by PPAR α agonists at the maximum dosage in GO fibroblasts (A; fenofibrate (Feno; 100 μ mol/l), gemfibrozil (Gem; 400 μ mol/l), ciprofibrate (Cipro; 400 μ mol/l)) ranged from about 60 to 70%. Treating GO fibroblasts with pioglitazone (Pio 20; 20 μ mol/l) or rosiglitazone (Rosi 20; 20 μ mol/l), together with IFN γ (IFN γ ; 1000 IU/ml) and TNF α (TNF α ; 10 ng/ml) stimulation, had no significant effect on the CCL2 release. Similar results were observed in preadipocytes (B; ANOVA, $P < 0.001$). Bars are mean $\pm$ S.E.M. * $P < 0.05$ or less vs IFN γ + TNF α by Bonferroni–Dunn test. ° $P < 0.05$ or less vs IFN γ + TNF α + Rosi 20 or IFN γ + TNF α + Pio 20 by Bonferroni–Dunn test.

CCL2. Furthermore, Chen *et al.* (2008) found that the expression of CCL2 was higher in GO orbital fat than in the orbital fat of controls and suggested that macrophage infiltration may play an important role in the pathogenesis of GO via overexpression of CCL2.

Our results show that CCL2 chemokine was secreted basally by normal fibroblasts or preadipocytes such as those from GO, responded to IFN γ alone, and a synergistic action of IFN γ in combination with TNF α was observed inducing the secretion of the chemokine in primary fibroblasts or preadipocytes from patients with GO. This last result is unexpected because Th2 chemokines are poor responders to IFN γ , but it is in agreement with the results obtained by other studies on murine (Yamana *et al.* 2009) and human fibroblasts (Struyf *et al.* 1998).

The fact that CCL2 can be produced by GO fibroblasts or preadipocytes, under the influence of cytokines such as IFN γ or TNF α (released by activated Th1 lymphocytes), suggests a possible role of these cells on recruitment of monocytes and T lymphocytes to the tissue from the blood in GO.

IFN γ and TNF α also stimulated CXC chemokine secretion as expected (Antonelli *et al.* 2006c, 2009a). These results, on the whole, are in agreement with the view that autoimmune disorders evolve from an initial Th1 phase to a later Th2 prevalent immune response with progression toward fibrosis (Romagnani 1997, Antonelli *et al.* 2008). For example, the early expression of CXCL10 and CCL2 suggests their involvement in the initiation and perpetuation of disease in severe granulomatous experimental autoimmune thyroiditis, which progresses to fibrosis (Chen *et al.* 2005).

PPAR α has been confirmed to be expressed more in GO fibroblasts or preadipocytes than in controls. These results are in agreement with our data (Antonelli *et al.* 2012) and the ones obtained by Pasquali *et al.* (2004). In our studies, no significant adipocyte change was observed in fibroblasts after a 24 h incubation period with PPAR α , according to the results obtained by Pasquali *et al.* (2004) that showed that few subpopulations (1–2%) of fibroblasts from Graves' ophthalmopathy (FGOs) exposed to fenofibrate changed their typical fibroblastic appearance after 24 h, such as in controls. Our results suggest that PPAR α agonists are able to modulate both the Th1 and Th2 immune responses as suggested by their powerful action on the inhibition of chemokines. In fact, PPAR α can modulate both CXC (CXCL10) and CC (CCL2) chemokines.

The anti-inflammatory action of PPAR α has been widely investigated by many studies, and some studies advocate PPAR α agonists as therapy for autoimmune disease (Cuzzocrea *et al.* 2004, Gervois *et al.* 2004, Lovett-Racke *et al.* 2004, Lo Verme *et al.* 2005, Okamoto *et al.* 2005, Lee *et al.* 2007, Oliveira *et al.* 2007). The effect of PPAR α on CXC chemokines has been less deeply investigated. In the study by Lee *et al.* (2007), fenofibrate repressed the expression of the gene encoding CXCL10 and repressed CXCL10 gene promoter activity in TNF α -treated HT-29 cells. Our results are in agreement with a previous study that has demonstrated that CXCL9 and CXCL11 chemokines are modulated by PPAR α agonists in Graves' and normal thyrocytes (Antonelli *et al.* 2010).

The results of this study shed new light on the effect of PPAR α on CXC chemokines, demonstrating a powerful effect on the secretion of CXCL10 both in normal fibroblasts

and in preadipocytes, such as in cells obtained by GO, inhibiting the stimulatory effect of IFN γ and TNF α on the secretion of these chemokines. Interestingly, the effect of PPAR α has been exerted at near-therapeutic doses as also suggested by the study of Lovett-Racke *et al.* (2004). The potency of the PPAR α agonist used is maximum on the secretion of CXCL10 reaching about a 60% of inhibition by fenofibrate, while about a 40–50% of inhibition was observed with the others. This may suggest that the different PPAR α agonist compounds may act through different pathways that remain to be explored.

In the study by Lee *et al.* (2007), fenofibrate repressed the expression of a CXCL10 promoter–luciferase construct, indicating that the repression of CXCL10 mRNA expression occurs at least partly by a transcriptional mechanism. TNF α -stimulated transcription of the CXCL10 gene is largely mediated by nuclear factor κ B (NF- κ B) binding to 2 κ B binding sites located in the CXCL10 proximal promoter (Majumder *et al.* 1998).

PPAR ligands repress transcriptional activation by NF- κ B via a mechanism known as ligand-dependent transrepression (Delerive *et al.* 1999, Pascual *et al.* 2005); thus, NF- κ B is a likely target for repression of CXCL10 transcription by fenofibrate.

Interestingly, however, in the study by Lee *et al.* (2007), all five chemokine genes tested are known to be activated by NF- κ B, but two of these genes, CXCL8 (IL8) and CXCL1 (GRO1), were not repressed significantly by fenofibrate. This suggests that the promoter context and perhaps the presence or absence of corepressor complexes associated with promoters in the inactive state may determine which NF- κ B-activated promoters are repressed by fenofibrate (Pascual *et al.* 2005).

In addition to repressing TNF α -mediated activation of the CXCL10 promoter, fenofibrate also repressed activation of the promoter by IFN γ . This repression very likely acts via IFN response factor-containing complexes that bind to the IFN-stimulated response element of the CXCL10 promoter after stimulation with IFN γ (Majumder *et al.* 1998).

PPAR γ ligands show an inhibitory activity on CXC chemokines, confirming the results of our previous studies (Antonelli *et al.* 2006c, 2009a). However, the relative potency of PPAR α agonists on the inhibition of the secretion of CXC chemokine is higher than that of PPAR γ ligands. This could suggest that PPAR α agonists may act, at least in part, through different pathways than PPAR γ agonists (Marx *et al.* 2000, Schaefer *et al.* 2005, Lombardi *et al.* 2008, Antonelli *et al.* 2009b).

The effect of PPAR α agonists on CCL2 has been investigated but not in GO cells. In fact, PPAR α activators suppress lipopolysaccharide-stimulated Ccl2 transcription and release in activated rat glia (Lee *et al.* 2005). Furthermore, PPAR α activators inhibit CCL2 expression induced by high glucose concentration in human endothelial cells (Dragomir *et al.* 2006).

The results of this study show a potent inhibition (more than 60%) of the cytokine-stimulated CCL2 secretion by PPAR α agonists both in normal and in GO cells. Interestingly, PPAR γ agonists show no effect on the secretion of CCL2 in GO cells. This discrepancy is in agreement with

the results of other studies on different cells (Nie *et al.* 2005). The different effects of PPAR α and PPAR γ agonists on the secretion of chemokines in GO cells may be, at least in part, explained by the different pathways involved.

Recently, it has been demonstrated that rosiglitazone was associated with an increased risk of stroke, heart failure, and all-cause mortality in elderly patients (Graham *et al.* 2010), and the European Medicines Agency (EMA) recommended on September 2010 that rosiglitazone be suspended from the European market. More recently, the EMA extended the review of safety to pioglitazone (Moynihan 2011). Even if these arguments cannot be automatically translated in GO field, they do not actually advise PPAR γ agonists for the therapy of GO.

The presence of PPAR α in GO fibroblasts or preadipocytes and the immunomodulatory effect of PPAR α agonists on the production of Th1 and Th2 chemokines suggest that PPAR α may be involved in the modulation of the immune response both in normal and GO cells, probably by interaction with endogenous ligands. A therapeutic use of PPAR α ligands in GO remains to be explored, but it is suggested by the fact that their effect on chemokines has been exerted at near-therapeutic doses. Despite anti-inflammatory actions of PPAR α , its proadipogenic functions in the orbit might worsen the disease, contraindicating the use of these agents in GO (Pasquali *et al.* 2004). Thus, further studies are necessary to establish whether in active GO the anti-inflammatory effects of PPAR α activation can be exploited without the risk of expanding retrobulbar fat mass, for example, combining PPAR α agonists and non-steroidal anti-inflammatory drugs or glucocorticoids that may synergize to exert their anti-inflammatory action.

In conclusion, this study shows that CCL2 is modulated by IFN γ and TNF α in GO. PPAR α activators are able to inhibit the secretion of the main prototype α (CXCL10) and β (CCL2) chemokines in GO fibroblasts or preadipocytes, suggesting that PPAR α may be involved in the modulation of the immune response in GO.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

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Received in final form 23 February 2012

Accepted 28 February 2012

Made available online as an Accepted Preprint

29 February 2012