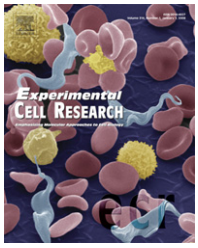
EXPERIMENTAL CELL RESEARCH 314 (2008) 3405–3414

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/yexcr



Research Article

Matrix metalloproteinase-12 gene regulation by a PPAR alpha agonist in human monocyte-derived macrophages

Imen Jguirim Souissi[a,1], *Ludivine Billiet*[b,1], *Clarisse Cuaz-Pérolin*[b], *Mohamed-Naceur Slimane*[a], *Mustapha Rouis*[b,*]

[a]*Research Laboratory on Atherosclerotic Biological and Genetic Factors, Faculty of Medicine, Monastir, TN-5019, Tunisia*
[b]*UMR 7079 Physiologie et Physiopathologie, Université Pierre et Marie Curie-Paris 6, Bât. A. 5ème étage/Case courrier 256, CNRS, 7 Quai Saint-Bernard, 75252 Paris Cedex 5, France*

ARTICLE INFORMATION

Article Chronology:
Received 16 May 2008
Revised version received
4 September 2008
Accepted 4 September 2008
Available online 18 September 2008

Keywords:
Matrix metalloproteinase
Nuclear receptors
Macrophage
Inflammation
Atherosclerosis
Cardiovascular disease

ABSTRACT

MMP-12, a macrophage-specific matrix metalloproteinase with large substrate specificity, has been reported to be highly expressed in mice, rabbits and human atherosclerotic lesions. Increased MMP-12 from inflammatory macrophages is associated with several degenerative diseases such as atherosclerosis. In this manuscript, we show that IL-1β, a proinflammatory cytokine found in atherosclerotic plaques, increases both mRNA and protein levels of MMP-12 in human monocyte-derived macrophages (HMDM). Since peroxisome proliferator-activated receptors (PPARs), such as PPARα and PPARγ, are expressed in macrophages and because PPAR activation exerts an anti-inflammatory effect on vascular cells, we have investigated the effect of PPARα and γ isoforms on MMP-12 regulation in HMDM. Our results show that MMP-12 expression (mRNA and protein) is down regulated in IL-1β-treated macrophages only in the presence of a specific PPARα agonist, GW647, in a dose-dependent manner. In contrast, this inhibitory effect was abolished in IL-1β-stimulated peritoneal macrophages isolated from PPARα$^{-/-}$ mice and treated with the PPARα agonist, GW647. Moreover, reporter gene transfection experiments using different MMP-12 promoter constructs showed a reduction of the promoter activities by ~50% in IL-1β-stimulated PPARα-pre-treated cells. However, MMP-12 promoter analysis did not reveal the presence of a PPRE response element. The IL-1β effect is known to be mediated through the AP-1 binding site. Mutation of the AP-1 site, located at −81 in the MMP-12 promoter region relative to the transcription start site, followed by transfection analysis, gel shift and ChIP experiments revealed that the inhibitory effect was the consequence of the protein–protein interaction between GW 647-activated PPARα and c-Fos or c-Jun transcription factors, leading to inhibition of their binding to the AP-1 motif. These studies suggest that PPARα agonists may be used therapeutically, not only for lipid disorders, but also to prevent inflammation and atheromatous plaque rupture, where their ability to inhibit MMP-12 expression in HMDM may be beneficial.

* *Corresponding author.* Fax: +33 1 44 27 51 40.
E-mail address: mustapha.rouis@snv.jussieu.fr (M. Rouis).
[1] These authors contributed equally.

0014-4827/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexcr.2008.09.002

Introduction

Atherosclerosis is a complex inflammatory process, occurring over several decades and involving a series of events that profoundly affect the structure and composition of the arterial wall. Mature human atherosclerotic plaques are frequently characterized by a lipid-rich core covered by a fibrous cap composed of fibrillar collagens, elastin, proteoglycans and smooth muscle cells. Inflammatory cells, such as macrophages, are abundantly present in vulnerable shoulder regions of atherosclerotic plaques [1]. The accumulation of macrophages correlates with increased local release of matrix-degrading metalloproteinases (MMPs) and weakened fibrous cap tissue [1,2]. These findings suggest a potential role of macrophage-derived MMPs in the weakening and ultimate rupture of plaque structure.

MMPs, a family of more than 25 calcium-activated, zinc-containing, neutral endopeptidases [3–6] are capable of degrading various matrix proteins [7]. Among these MMPs, MMP-12 has been reported to be expressed in atherosclerotic lesions [8–12].

MMP-12 (macrophage elastase) was first identified as an elastolytic metalloproteinase specifically secreted by activated macrophages [13,14]. Besides its elastase activity, MMP-12 displays a wide substrate specificity, being able to act on a variety of extracellular matrix (ECM) proteins such as fibronectin, laminin, vitronectin, heparin sulphate and collagen type IV [15,16]. Thus, MMP-12 not only digests elastin but can also degrade the basement membrane, which enables macrophages to penetrate injured tissues during inflammation [16]. It has been reported that increased activity of MMP-12 from inflammatory macrophages is associated with several diseases involving extracellular matrix destruction, including atherosclerosis [17–19]. Thus, the high expression level of macrophage-derived MMP-12 in animal or human atherosclerotic lesions might be the consequence of macrophage proinflammatory-mediators [18,20,21] and/or modified LDL [18]. Moreover, we have shown in a recent publication that plasma MMP-12 concentration was associated with the presence of coronary artery diseases (CAD) [22]. Interestingly, a common polymorphism in the MMP-12 gene promoter (an A to G substitution at position-82) influences the binding of the transcription factor activator protein-1 (AP-1) [23]. This −82 A/G polymorphism was found to be associated with variations in coronary artery luminal dimensions in diabetic patients with manifest coronary artery disease [23]. These data strongly suggested that excess macrophage-derived MMP-12 production, in cooperation with other MMPs present, might play a pivotal role in both the initiation and progression of atherosclerosis.

A large body of data gathered over these past years has identified the peroxisome proliferator-activated receptors (PPAR) α, γ and β/δ as transcription factors exerting modulatory actions in vascular cells [24]. PPARs regulate transcription of target genes by heterodimerizing with the retinoid X receptor (RXR) and binding to PPAR response elements (PPRE) in transcriptional regulatory regions of target genes [25]. PPARs can also repress gene expression in a DNA-binding-independent manner by interfering with other signalling pathways as well as in a DNA-binding-dependent way through the recruitment of co-repressors [26]. PPARα and PPARγ activators such as fibrates (clofibrate, fenofibrate) and thiazolidinediones (rosiglitazone, pioglitazone) are clinically available and are used to treat lipid disorders and diabetes, respectively [27,28]. The role and development of PPARβ/δ ligands is still under investigation.

Since PPARα [29–31] and PPARγ [29,32,33] are highly expressed in macrophages; they may affect macrophage function and development of atherosclerosis. Indeed, several studies reported that PPARα and PPARγ agonists inhibit the development of atherosclerosis in apolipoprotein E-deficient ($apoE^{-/-}$) mice [34] and in low-density lipoprotein (LDL) receptor-deficient ($LDLr^{-/-}$) mice [35–38]. The inhibition of atherosclerosis occurs through a reduction in inflammatory gene expression as well as through a decrease in chemoattractant and adhesion molecule gene expression [31,39,40].

In this study we investigated the effect of the 3 PPAR isoforms on MMP-12 regulation in HMDM. Our results indicate that neither PPARγ nor PPARβ/δ affected the expression of MMP-12. However, a significant and concentration-dependent down regulation of macrophage MMP-12 expression was observed with a PPARα agonist (GW647) acting in a DNA-binding-independent manner, by interfering with the AP-1 signalling pathway.

Materials and methods

Isolation and culture of human monocytes

Mononuclear cells were isolated from buffy-coats of healthy normolipidaemic donors using Ficoll gradient centrifugation and were subsequently cultured in RPMI 1640 medium containing gentamycin (10 μg/ml) (Sigma, Saint Quentin, France), glutamine (1%) (Invitrogen, France) and 10% pooled human serum (PromoCell, Heidelberg, Germany) at a density of 2×10^6 cells/well for mRNA assays and 1.2×10^6 cells/well for transfection assays in 6-well Primaria-plastic culture dishes (Becton-Dickinson, France). Differentiation of monocytes into macrophages was allowed to occur spontaneously by adhesion of cells to the culture dish and continued maturation during a subsequent 12-day culture period. The cells were washed and either untreated or treated separately with different PPARα, γ and β/δ agonists (GlaxoSmithKline). It is important to note that the different concentrations of the 3 PPAR agonists used in this study were 0.06, 0.6 and 6 μM. Indeed, the general rule, for *in vitro* study, is the use of a concentration of at least $EC_{50}\times100$. The EC_{50} values for GW647 and GW929 were reported to be equivalent to ~0.006 μM [41,42]. However, the EC_{50} for GW516 was reported to be 1.8±0.4 nM [42]. Therefore, we have used GW647 and GW929 at three different concentrations: a concentration equivalent to the $EC_{50}\times100=0.6$ μM, a concentration 10 times lower the $EC_{50}\times100$ (=0.06 μM) and a concentration 10 times higher the $EC_{50}\times100$ (=6 μM). For the agonist, GW516, we have used 0.018 μM, 0.18 μM and 1.8 μM. Nevertheless, since we observed no effect on MMP-12 expression with these latter concentrations of GW516 (data not shown) and for a better comparison between the 3 PPAR agonists, we decided to show here results obtained with the 3 different agonists used at the same concentrations (Figs. 1A and B and 2). It is also important to note that we did not detect any cell toxicity or any nuclear receptor cross activation using these concentrations (not shown).

Isolation of mice peritoneal macrophages

Peritoneal macrophages were isolated from C57BL/6 wild type ($n=4$) or C57BL/6 PPARα knock-out mice ($PPAR\alpha^{-/-}$) ($n=4$) by

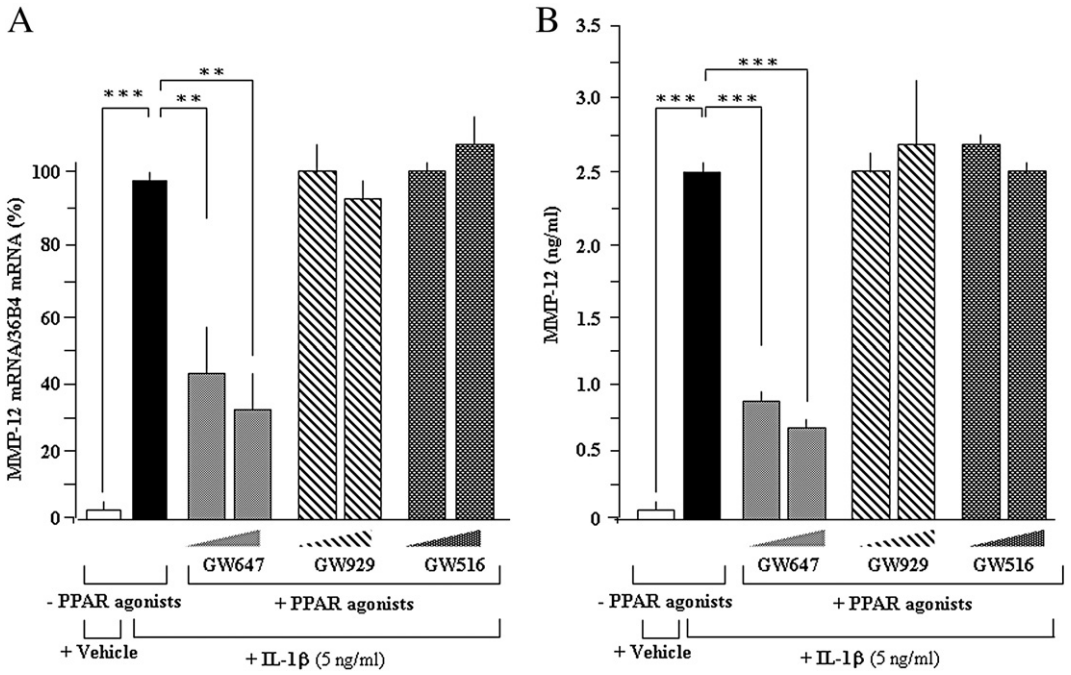


Fig. 1 – Effect of PPARα, γ or β/δ agonists on IL-1β-mediated induction of MMP-12 mRNA and protein in HMDM. Cells were untreated or treated with the PPARα agonist, GW647 (at 0.06 and 0.6 μM), the PPARγ agonist, GW 929 (at 0.06 and 0.6 μM) or the PPAR β/δ agonist, GW 516 (at 0.06 and 0.6 μM) for 1 h. All cells were stimulated for 24 h with vehicle (control) or with Il-1β at 5 ng/ml. (A) Total RNA was extracted and MMP-12 and 36B4 mRNA (for normalization) were evaluated by Q-PCR. (B) Secreted MMP-12 protein levels were determined by ELISA. The cells were washed with PBS and lysed, and cell protein levels were determined with the Lowry procedure. All dishes contained ~ the same level (variation between dishes were <5%). Bars represent means ± S.D. of three separate experiments, each performed in duplicate ($p<0.01$; ***$p<0.001$). 100% (in A) corresponds to the MMP-12/36B4 mRNA ratio of cells which have not been pre-treated with PPAR agonists but stimulated with Il-1β at 5 ng/ml for 24 h.**

intraperitoneal lavage with PBS. These mice were a kind gift of Dr Thierry Pineau from the molecular pharmacology team, INRA, Toulouse, France. Total RNA was extracted by *Trizol* (Life Technologies, France) as described above.

MMP-12 quantification by enzyme-linked immunosorbent assay (ELISA)

MMP-12 concentrations in culture media of HMDM were measured by ELISA. The MMP-12 kit was not commercialised, but was kindly provided by Ingel K Demedts who developed the assay in cooperation with industrial partners (Immunotech, Marseille, France and Schering-Plough LIR, Dardilly, France). Antibodies used for the assay were mAb 701E4.03 for capture and mAb 706F9.01 for detection of MMP-12. Recombinant MMP-12 was immunopurified with a third mAb, 603.E6. Recombinant human MMP-12 with known concentrations was used as a standard. Sensitivity of the assay was <50 pg/ml. It did not detect other related MMPs like MMP-1, MMP-3 and MMP-9. The coefficient of variation was <6%.

MMP-12 activity assay

Following cell treatment, supernatants were collected, centrifuged and kept at −80 °C until use. The cells were washed with serum-free PBS, lysed with buffer (component D of the MMP-12 assay kit) containing 0.1% Triton X-100 (v/v) and MMP-12 activity was measured by the Enzolyte™ 520 MMP-12 Assay Kit (Tebu-Bio, France) which detects MMP-12 activity using a 5-FAM/QXL™ 520 FRET peptide as a substrate with its fluorescence monitored at Ex/Em = 490 nm/520 nm upon proteolytic cleavage. This substrate shows excellent specificity to MMP-12 and minimal cross-reaction with MMP-1, MMP-2, MMP-7, MMP-8, MMP-9, MMP-13 and MMP-14 as stated by the supplier. MMP-12 activity was determined according to the manufacturer's instructions. In addition, we have demonstrated that the RXP407.1 almost completely inhibited the MMP-12 activity indicating that the activity we have measured was specific for MMP-12. Indeed, the RXP407.1 was described by Devel et al. as a highly specific MMP-12 inhibitor [43].

RNA extraction and analysis

Total RNA was extracted from cells using *Trizol* (Life Technologies, France). For quantitative PCR (Q-PCR), reverse transcribed MMP-12 mRNA was quantified by real time PCR on a MX4000 apparatus (Stratagene, La Jolla, CA), using specific oligonucleotide primers for human MMP-12 (forward: 5′-GAC AAA TAC TGG TTA ATT AGC AGT TTA AGA CCA GAG CCA AAT TAT C-3′ and reverse: 5′-AAG TAG TGG TAC CCT GAG GAC ATA GCA A-3′), and murine MMP-12 (forward: 5′-CTG GAC AAC TCA ACT CTG GCA A-3′ and reverse: 5′-ATT ATA GAT CCT GTA AGT GAG GTA CCG C-3′). PCR amplification was performed in a volume of 25 μl containing 100 nM of each paired primer, 4 mM $MgCl_2$, and the Brilliant Quantitative PCR Core reagent Kit mix as recommended by the manufacturer (Stratagene).

The thermal cycling conditions were 95 °C for 10 min, followed by 40 cycles of 30 s each at 95, 55, and 72 °C. The levels of MMP-12 mRNAs were normalized to the internal control, 36B4 mRNA, using the human primer set (forward: 5′-CAT GCT CAA CAT CTC CCC CTT CTC C-3′ and reverse: 5′-GGG AAG GTG TAA TCC GTC TCC ACA G-3′) or the murine primer set (forward: 5′-CGT GAT GCC CAG GGA AGA CAG-3′ and reverse: 5′-TTA GTC GAA GAG ACC GAA TC-3′).

Plasmids and transient transfection assays

Plasmids

The human MMP-12 promoter constructs were generated by PCR using *Pfu* polymerase (Stratagene) with human genomic DNA as a template. One XhoI site-linked 5′-primers (5′-GAA TTG CTC GAG TGA TGG ACT AGA TGC-3′) and a Hind III site-linked 3′-primer (5′-CCA AGC TGG TAG AGC TGT TCA GG-3′) specific for the MMP-12 genomic sequence (AY: 856072) were used to amplify and clone a 292 bp genomic fragment. This fragment was transferred into the promoterless firefly luciferase reporter vector pGL3-Basic (Promega) in the correct orientation, using the corresponding digestion enzymes. The firefly luciferase reporter vector in pGL3-Basic containing the human MMP-12 promoter (292 bps) with mutated AP-1 was generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). A point mutation was introduced in the AP-1 site contained within the human MMP-12 promoter using *Pfu* DNA polymerase, double-stranded plasmid DNA and a synthetic complementary oligonucleotide primer containing the desired mutation. The nucleotide sequences of the construct were verified by automated sequencing (Applied Biosystems Inc., Foster City, USA). Plasmid DNA was prepared using the Qiagen Maxi Prep kit (Qiagen). Expression plasmids for human PPARα have been previously described [44]. In transfection assays, HMDM grown in 6-well culture dishes in RPMI 1640 supplemented with 10% pooled human serum, were transiently transfected with the luciferase reporter plasmids (described above) in the presence of c-*fos*, c-Jun and/or pSG5-hPPAR-α expression vectors or corresponding empty vectors, using jetPEI-Man transfection reagent (Ozyme, France). Transfection efficiency, analysed by flow cytometry using a GFP expression control plasmid, was about 30%. The β-galactosidase expression vector (100 ng of a pCH110-β-Gal, Pharmacia, France) was co-transfected as a control for transfection efficiency. Twenty hours post-transfection, the medium was renewed (RPMI 1640 with 1% human serum) and supplemented or not with a PPAR-α agonist. After 24 h, cells were washed with PBS, lysed in 100 µl of Passive Lysis Buffer (Promega) at room temperature for 30 min and subjected to luciferase and β-galactosidase assays as previously described [45]. For each plasmid, the ratio between measured luciferase and β-galactosidase activity in the control cells was set to 1 and subsequently this value was used as a reference for each individual treatment.

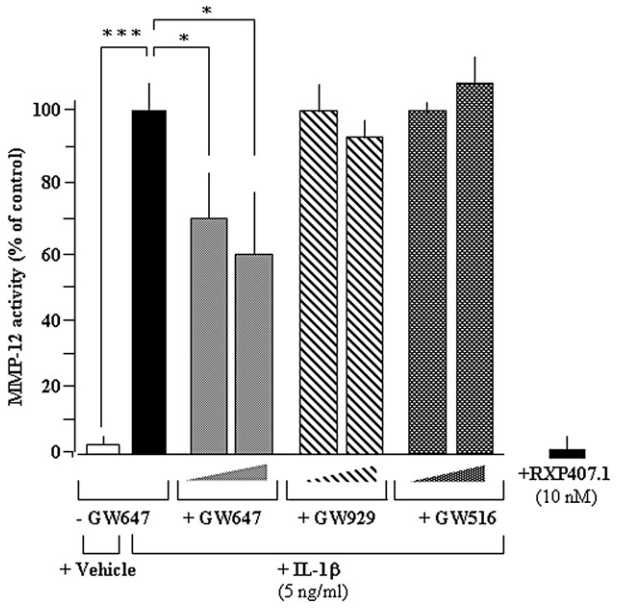


Fig. 2 – Effect of PPARα, γ or β/δ agonists on IL-1β-mediated induction of MMP-12 activity in the supernatant of HMDM. Cells were untreated or treated with the PPARα agonist, GW647 (at 0.06 and 0.6 µM), the PPARγ agonist, GW 929 (at 0.06 and 0.6 µM) or the PPAR β/δ agonist, GW 516 (at 0.06 and 0.6 µM) for 1 h. All cells were stimulated for 24 h with vehicle (control) or with Il-1β at 5 ng/ml. MMP-12 activities were determined in the cell supernatant according to the protocol described in the methods section. Bars represent means ± S.D. of three separate experiments, each performed in duplicate (*$p < 0.05$; ***$p < 0.001$).100% corresponds to the MMP-12 of cells which have not been pre-treated with PPAR agonists but stimulated with Il-1β at 5 ng/ml for 24 h. Similar experiments were performed in the presence of RXP407.1 at 10 nM. In all cases the MMP-12 activity was almost completely inhibited.

Electrophoretic mobility shift assays

Human PPARα, RXRα, c-Fos and c-Jun were synthesized *in vitro* using the TNT Quick Coupled Transcription/Translation System (Promega). A 21-bp (5′-CGC TTG A**TG ACT CA**G CCG GAA-3′) double-stranded oligonucleotide, containing the consensus AP-1 sequence was used as a control AP-1. Double-stranded oligonucleotides, corresponding to a putative AP-1/MMP-12 and its mutated form were end-labeled with [-^{32}P]ATP by using T4-polynucleotide kinase. Either protein (2.5 µl) was incubated for 15 min at room temperature in a total volume of 20 µl with 2.5 µg poly (dI-dC) and 1 µg herring sperm DNA in binding buffer (10 mM Tris, pH 8.0; 40 mM KCl; 0.05% Nonidet P-40; 6% glycerol; and 1 mM dithiothreitol) before the radiolabeled probe was added. Binding reactions were further incubated for 15 min and resolved by 4% non-denaturing polyacrylamide gel electrophoresis in 0.25× TBE buffer. For competition experiments, a 50-fold excess of unlabeled oligonucleotides over the labeled probe were included in the binding reaction.

Chromatin immunoprecipitation (ChIP) assays

Experiments were performed with a ChIP assay kit (Upstate), according to the manufacturer's procedures. Briefly, 10×10^6 cells were treated with 1% formaldehyde for 10 min at 37 °C. Subsequent procedures were performed on ice in the presence of protease inhibitors. Cells cross-linked by formaldehyde-treatment were harvested, washed with PBS, and lysed in sodium dodecyl sulphate lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 8.1) for 10 min at 4 °C. Chromatin was sonicated with five 10-s pulses at 30% amplitude (sonifier, Branson Ultrasonic Corp). After centrifugation (10 min, 4 °C, 14,000 *g*), the supernatant was diluted 10-fold with ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl, pH 8.1, 167 mM NaCl). Diluted

extracts were precleared in the presence of salmon sperm DNA–protein A-agarose beads (ChIP assay kit; Upstate). One-tenth of the diluted extract was kept for a direct PCR (Input). The remaining extracts were incubated for 16 h at 4 °C in the presence of 1 μg of specific antibodies per millilitre, followed by 1 h of incubation with salmon sperm DNA–protein A-agarose beads. Anti-c-Fos antibodies (H-125) were purchased from Santa Cruz Biotechnology. Following extensive washing, bound DNA fragments were eluted by a 30-min incubation in elution buffer (1% SDS, 0.1 M NaHCO3). The DNA was recovered for 4 h at 65 °C in elution buffer containing 200 mM NaCl and then incubated in the presence of proteinase K (20 μg/ml) for 1 h at 45 °C. DNA was extracted in the presence of phenol-chloroform and chloroform-isoamyl alcohol and ethanol-precipitated before being subjected to PCR by using specific oligonucleotide primers containing the human MMP-12 AP-1 site (forward: 5′-GCA CCA CTG CTA GCA ATT CTA CCT TTG G-3′ and reverse: 5′-CCA AGC TTG TAG AGC TGT TCA GG-3′).

Statistical analysis

Statistical analyses were performed using the Student's *t*-test and probability values <0.05 were considered significant.

Results and discussion

MMP-12, a macrophage-specific matrix metalloproteinase with large substrate specificity, has been reported to be highly expressed in mice, rabbits and human atherosclerotic lesions [17–19]. In this manuscript, we tested the effect of inflammatory cytokines such as IL-1β and TNF-α, known to be present in atherosclerotic plaques, and lipopolysaccharide (LPS), a component of the gram-negative bacterial wall known to mimic bacterial inflammation. We showed that IL-1β increased both mRNA and protein levels of MMP-12 in HMDM (Figs. 1A and B) and MMP-12 activity in the supernatant of these cells (Fig. 2). The same results were obtained with TNF-α and LPS (not shown). It is interesting to note that increased activity of MMP-12 from inflammatory macrophages is associated with several degenerative diseases such as emphysema [46,47], rheumatoid arthritis [48,49], abdominal aortic aneurysms [50] and atherosclerosis [17–19]. Thus, the high expression level of macrophage-derived MMP-12 in animal or human atherosclerotic lesions might be the consequence of the presence of proinflammatory-mediators such as IL-1β and TNF-α.

Since peroxisome proliferator-activated receptors (PPAR) and particularly PPARα [29–31] and PPARγ [31–33] are highly expressed in macrophages and because PPAR activation has been reported to exert an anti-inflammatory effect on vascular cells, we have conducted studies to investigate the effect of the 3 PPAR isoforms on MMP-12 regulation in HMDM.

Our results showed that MMP-12 expression (mRNA and protein) and MMP-12 activity were down regulated in IL-1β-treated macrophages only in the presence of a specific PPARα agonist GW647 in a concentration-dependent manner: i) ~58±16% and ~69±11% reduction in MMP-12 mRNA levels with 0.06 and 0.6 μM respectively (Fig. 1A); ii) ~65±4% and ~75±4% reduction in MMP-12 protein concentrations in cell culture media with 0.06 and 0.6 μM (Fig. 1B) and iii) ~30±12% and ~42±17% reduction in MMP-12 activity in the supernatants of culture media with 0.06 μM and 0.6 μM respectively (Fig. 2). Similar results were obtained in TNFα or LPS-treated cells (not shown). Therefore, we have focussed our study on MMP-12 gene regulation in HMDM by using only a PPARα agonist.

Next, we reported that the inhibition observed with a PPARα agonist was abolished in IL-1β, TNFα or LPS-stimulated peritoneal macrophages isolated from PPARα$^{-/-}$ mice and treated with the PPARα agonist, GW647, at 0.06 and 0.6 μM (Fig. 3) (not shown for TNFα and LPS). To elucidate the mechanism underlying MMP-12 mRNA inhibition by the PPARα agonist in IL-1β, TNFα or LPS-activated HMDM, we performed reporter gene transfection experiments using −862/+111 bp and −181/+111 bp MMP-12 promoter constructs. Treatment of HMDM with IL-1β (5 ng/ml) increased activity of both fragments by 2.2 to 3.5 fold (Fig. 4). When cells were treated with a PPARα agonist (0.6 μM), promoter activity was reduced by ~50% to 60% (Fig. 4). Similar results were obtained when HMDM were activated by TNFα or LPS then treated with the PPARα agonist, GW647, at 0.6 μM (not shown). PPARα regulates the transcription of target genes by heterodimerizing with the retinoid X receptor (RXR) and binding to PPAR response elements (PPRE) in transcriptional regulatory regions of target genes. Therefore, MMP-12 promoter analysis was performed to identify a PPRE response element. However, no PPRE was found in a ~1 kb fragment of the MMP-12 promoter. This inhibitory effect of the PPARα agonist may be explained by the ability of PPARα to

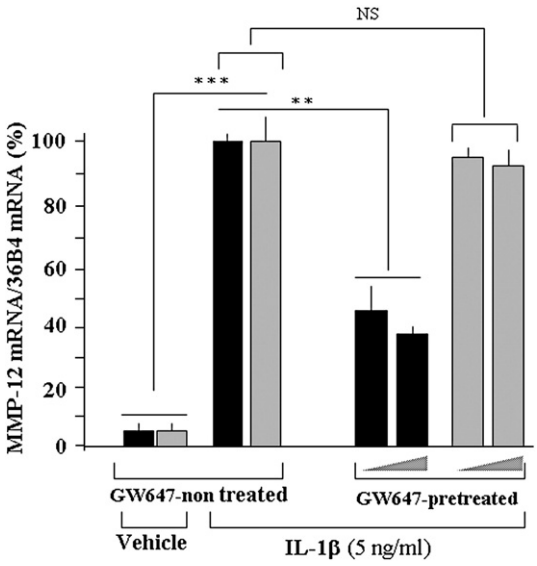


Fig. 3 – Effect of a PPARα agonist on IL-1β-mediated induction of MMP-12 mRNA in peritoneal macrophages isolated from wild type or PPARα-deficient (PPARα$^{-/-}$) mice. Cells were isolated from wild type (*n*=4, black bars) or PPARα$^{-/-}$ (*n*=4, grey bars) mice by peritoneal lavage with PBS. The cells were untreated or treated with the PPARα agonist, GW647 (at 0.06 or 0.6 μM) for 1 h. Cells were stimulated for 24 h with vehicle (control) or with Il-1β at 5 ng/ml. Total RNA was extracted and MMP-12 and 36B4 mRNA levels (for normalization) were evaluated by Q-PCR. Bars represent means±S.D. of 4 separate experiments, each performed in duplicate (*p<0.001; NS=non-significant). 100% corresponds to the MMP-12/36B4 mRNA ratio of cells isolated from wild type mice which have not been pre-treated with a PPARα agonist but stimulated with Il-1β at 5 ng/ml for 24 h.**

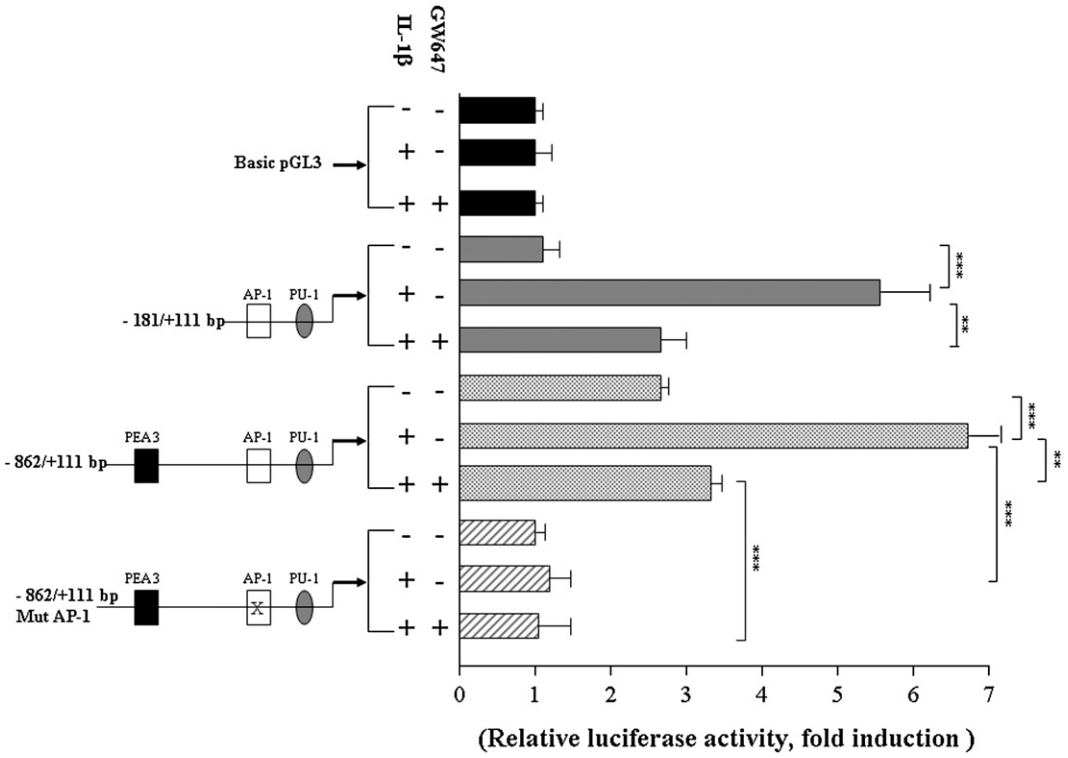


Fig. 4 – Effect of a PPARα activator on IL-1β-induced MMP-12 promoter activity in HMDM. Cells were transiently co-transfected with one of three MMP-12 promoter luciferase reporter constructs (pGL3 −181/+111, pGL3 −862/+111 or pGL3 −862/+111 in which the AP-1 site was mutated), human pSG5 PPARα expression vectors and pCH110, a β-galactosidase vector (for normalization). After 20 h, cells were pre-treated or not for 1 h with GW647 (0.6 μM) and then stimulated with vehicle or with IL-1β at 5 ng/ml for 24 h. The cells were then lysed and both luciferase and β-galactosidase activities were determined as described in Materials and Methods. Bars represent means±SD of three separate experiments, each performed in triplicate (*$p<0.001$).**

repress MMP-12 gene expression in a DNA-binding-independent manner by interfering with other signalling pathways. Indeed, IL-1β, TNFα and LPS are known to activate AP-1 proteins through several cell signalling pathways which often involve members of the mitogen-activated protein kinases including Jun and Fos-regulating kinases [51,52]. Because a putative binding site for AP-1 was identified at position −81 [21,23], a gene transfection experiment was performed using a construct containing the −973 bp promoter fragment in which the AP-1 site (−81) was mutated by site-directed mutagenesis which abolishes its function. The promoter activity was completely abolished after transfection with the mutated construct, even in the presence of IL-1β (Fig. 4). Similar results were observed with TNF-α or LPS (not shown). These data indicate that the AP-1 site is necessary for both basal and inducible MMP-12 promoter activity. Our data are in agreement with data reported earlier by Feinberg et al. [21].

As described above, the PPARα agonist significantly decreased MMP-12 mRNA expression in HMDM that had been stimulated with IL-1β but without reaching the basal level (Fig. 1A). This down regulation could be the consequence of the protein–protein interaction between PPARα and c-Fos or c-Jun, leading to inhibition of their binding to the AP-1 motif, in turn preventing AP-1-mediated gene activation as reported earlier [45]. Indeed, incubation of an oligonucleotide probe containing the putative MMP-12 AP-1 element with an *in vitro* translated c-Fos and c-Jun resulted in the formation of a strong retarded complex between the two transcription factors and the AP-1 motif (Fig. 5A). A similar complex was formed when an oligonucleotide containing the consensus AP-1 site was used (Fig. 5A). However, the specific complex disappeared when it was incubated with a 50-molar excess of unlabeled MMP-12 AP-1 oligonucleotide or oligonucleotide containing the AP-1 consensus element (Fig. 5A). In addition, incubation of c-Fos and c-Jun with a probe containing the MMP-12 AP-1 element that had been mutated did not lead to the formation of a specific complex (Fig. 5A). Moreover, incubation of the oligonucleotide probe containing the putative MMP-12 AP-1 element with the *in vitro* translated c-Fos, c-Jun, PPARα and RXRα did not abolish the specific binding complex within the AP-1 motif. In contrast, the complex completely disappeared when the PPARα agonist, GW647, was added at 0.6 μM. Finally, the result of the ChIP experiment showed a reduced fixation of the complex on the AP-1 site upon activation of PPARα with GW647 (Fig. 5B). Taken together, these results indicate that the PPARα agonist, GW647, inhibited IL-1β-stimulated MMP-12 expression in HMDM, at least in part, through the inhibition of the association of c-Jun and c-Fos on their AP-1 motif located at −81 in the human MMP-12 promoter. Since most MMPs have an AP-1 site located within this region, the observed effect could be general to most MMPs. Indeed, in addition to the MMP-12 expression described in this manuscript, the GW647 agonist reduces, in human macrophages, the IL-1β-stimulated MMP-9, an enzyme known to possess an AP-1 site within its promoter region. In contrast, MMP-2 which does

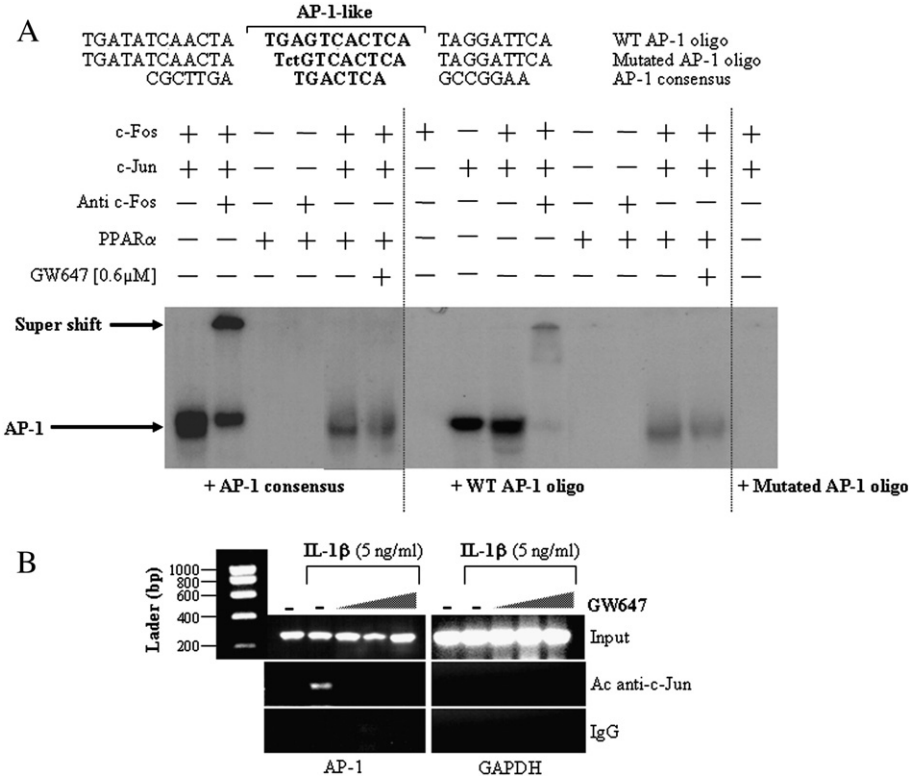


Fig. 5 – PPARα activation with GW 647 inhibited c-Fos and c-Jun binding to the AP-1 site located in the promoter region (−81) of the MMP-12 gene. (A) Gel shift assays with *in vitro* translated human c-Fos, human c-Jun, human PPARα, or GW 647-activated PPARα were performed with end-labeled oligonucleotides representing the consensus AP-1 site, the human putative MMP-12 AP-1 and a mutated version of the human putative MMP-12 AP-1. Oligonucleotides containing the putative AP-1 and the consensus AP-1 sites were used separately for competition experiments. (B) HMDM were treated with or without GW647 (0.06, 0.6 and 6 μM) for 24 h. DNA–protein cross-linking was induced with formaldehyde. After fragmentation, the chromatin was precipitated with rabbit antibody against c-Jun, followed by anti-rabbit IgG. To control non-specific precipitation, the chromatin was incubated with anti-goat IgG alone. Immunoprecipitated chromatin was amplified by PCR using primers (−181/+111) designed to amplify a 292 bp sequence of human MMP-12 promoter containing the AP-1 binding site. As a positive control, lysate chromatin (Input) was also amplified. As a negative control, a fragment of the GAPDH gene was amplified. The ChIP experiment was performed twice with a good reproducibility.

not possess an AP-1 site does not respond to the PPARα agonist in IL-1β-stimulated macrophages (data not shown).

Moreover, our results showed that the GW647 binding to PPAR is needed to reduce AP-1 binding in the *in vitro* assay (Fig. 5B). Our result is in agreement with data reported in the literature. Indeed, several studies have suggested that nuclear receptors can physically associate with both c-Jun and c-Fos, and thus block their ability to bind to the AP-1 motif. [53–57]. In addition, it has been reported that the human retinoic acid receptor alpha (hRARα) is able to disrupt, in a ligand-dependent manner, the capacity of c-Jun to dimerize either with itself or with c-Fos, which would preclude the formation of DNA-binding-competent AP-1 complexes [58]. These authors also provide evidence that liganded hRARα is able to inhibit dimerization in a cell-specific manner. Thus it is possible that RARs, and other nuclear receptors, can directly associate with c-Jun and/or c-Fos, but the affinity of this direct interaction is not sufficient *in vivo* to modulate transcription. Additional factors, expressed in a tissue-specific manner, may be needed to stabilize the RAR-AP-1 interaction and thus prevent AP-1 dimerization. Therefore, in our study the PPARα ligand, GW647, might either increase the affinity of PPARα-c-Jun and/or c-Fos interactions or it might induce the expression, specifically in macrophages, of factors that are able to reduce the AP-1 binding. Moreover, it is also known that the c-Jun transcriptional activity is enhanced by amino-terminal phosphorylation on Serines 63 and 73 [59,60]. This inducible phosphorylation is mediated by members of the Jun amino-terminal kinase (JNK) subfamily [61–63] and requires recruitment of the transcriptional coactivator CREB-binding protein (CBP) [64,65]. In addition, evidence indicated that hormone-activated nuclear receptors prevent c-Jun phosphorylation on Ser-63/73 and consequently, AP-1 activation, by blocking the induction of the JNK signalling cascade [66]. By comparison, in our study, the PPARα agonist GW647, might inhibit c-Jun phosphorylation leading to the inhibition of the JNK signalling cascade and, ultimately, to the inhibition of the AP-1 complex.

In conclusion, previously reported evidence from intervention studies in animal models has highlighted the role of up regulation of MMP-12 in the progression of atherosclerosis. Indeed, Johnson et al., using the mouse brachiocephalic artery model of plaque instability, were able to show that atherosclerotic lesion area was reduced in brachiocephalic arteries from apoE/MMP-12 double

knock-out mice, supporting the hypothesis that MMP-12 may act as a destructive protease promoting atherosclerotic plaque instability [67]. Moreover, Fan et al. have shown, in MMP-12 transgenic rabbits which specifically overexpress human MMP-12 in tissue macrophages, that MMP-12 accelerated both aortic and coronary atherosclerotic lesion progression [68]. In this context, our study suggests that PPARα agonists may be used therapeutically, not only for lipid disorders, but also to prevent inflammation and atheroma plaque rupture, where their capacity to inhibit MMP-12 expression in HMDM may be beneficial.

Acknowledgments

We thank Dr Mary Osborn-Pellegrin and Dr Athina Kalopisis for critically reading the manuscript, Dr Serge Lebeque from Inserm U-503 Lyon, France and Dr. Ingel Demedts from Ghent University Hospital Department of Respiratory Diseases, Ghent, Belgium for the MMP-12 ELISA kit, Dr Thierry Pineau from the Molecular Pharmacology Team, INRA, Toulouse for the wild type and the PPARα$^{-/-}$ mice and Dr V. Dive from the Commissariat à l'Energie Atomique, Département d' Ingénierie et d'Etudes des Protéines. Saclay, Gif/Yvette, France for the specific MMP-12 inhibitor. The authors would also like to thank C. Copin and C. Lasselin for their expert technical assistance. These studies were supported by an international exchange programme between INSERM (France) and DGRSRT (Tunisia).

REFERENCES

[1] R. Ross, Atherosclerosis—an inflammatory disease, N. Engl. J. Med. 340 (1999) 115–126.
[2] C.L. Lendon, M.J. Davies, G.V. Born, P.D. Richardson, Atherosclerotic plaque caps are locally weakened when macrophages density is increased, Atherosclerosis 87 (1991) 87–90.
[3] H. Nagase, Activation mechanisms of matrix metalloproteinases, Biol. Chem. 378 (1997) 151–160.
[4] H. Nagase, J.F. Woessner Jr., Matrix metalloproteinases, J. Biol. Chem. 274 (1999) 21491–21494.
[5] L.M. Matrisian, Metalloproteinases and their inhibitors in matrix remodeling, Trends Genet. 6 (1990) 121–125.
[6] S. Ye, Polymorphism in matrix metalloproteinase gene promoters: implication in regulation of gene expression and susceptibility of various diseases, Matrix Biol. 19 (2000) 623–629.
[7] C.E. Brinckerhoff, L.M. Matrisian, Matrix metalloproteinases: a tail of a frog that became a prince, Nat. Rev. Mol. Cell Biol. 3 (2002) 207–214.
[8] Z.S. Galis, G.K. Sukhova, R. Kranzhofer, S. Clark, P. Libby, Macrophage foam cells from experimental atheroma constitutively produce matrix-degrading proteinases, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 402–406.
[9] Z.S. Galis, G.K. Sukhova, M.W. Lark, P. Libby, Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques, J. Clin. Invest. 94 (1994) 2493–2503.
[10] I. Halpert, U.I. Sires, J.D. Roby, S. Potter-Perigo, T.N. Wight, S.D. Shapiro, H.G. Welgus, S.A. Wickline, W.C. Parks, Matrilysin is expressed by lipid-laden macrophages at sites of potential rupture in atherosclerotic lesions and localizes to areas of versican deposition, a proteoglycan substrate for the enzyme, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 9748–9753.
[11] T.B. Rajavashisth, X.P. Xu, S. Jovinge, S. Meisel, X.O. Xu, N.N. Chai, M.C. Fishbein, S. Kaul, B. Cercek, B. Sharifi, P.K. Shah, Membrane type 1 matrix metalloproteinase expression in human atherosclerotic plaques: evidence for activation by proinflammatory mediators, Circulation 99 (1999) 3103–3109.
[12] R.P. Fabunmi, G.K. Sukhova, S. Sugiyama, P. Libby, Expression of tissue inhibitor of metalloproteinases-3 in human atheroma and regulation in lesion-associated cells: a potential protective mechanism in plaque stability, Circ. Res. 83 (1998) 270–278.
[13] M.J. Banda, Z. Werb, Mouse macrophage elastase. Purification and characterization as a metalloproteinase, Biochem. J. 193 (1981) 589–605.
[14] S.D. Shapiro, D.K. Kobayashi, T.J. Ley, Cloning and characterization of a unique elastolytic metalloproteinase produced by human alveolar macrophages, J. Biol. Chem. 268 (1993) 23824–23829.
[15] S. Chandler, J. Cossins, J. Lury, G. Wells, Macrophage metalloelastase degrades matrix and myelin proteins and processes a tumour necrosis factor-alpha fusion protein, Biochem. Biophys. Res. Commun. 228 (1996) 421–429.
[16] T.J. Gronski Jr., R.L. Martin, D.K. Kobayashi, B.C. Walsh, M.C. Holman, M. Huber, H.E. Van Wart, S.D. Shapiro, Hydrolysis of a broad spectrum of extracellular matrix proteins by human macrophage elastase, J. Biol. Chem. 272 (1997) 12189–12194.
[17] C. Cuaz-Perolin, I. Jguirim, G. Larigauderie, A. Jlassi, C. Furman, M. Moreau, M.J. Chapman, J.C. Fruchart, M.N. Slimane, H. Mezdour, M. Rouis, Apolipoprotein E knockout mice over-expressing human tissue inhibitor of metalloproteinase 1 are protected against aneurysm formation but not against atherosclerotic plaque development, J. Vasc. Res. 43 (2006) 493–501.
[18] S. Matsumoto, T. Kobayashi, M. Katoh, S. Saito, Y. Ikeda, M. Kobori, Y. Masuho, T. Watanabe, Expression and localization of matrix metalloproteinase-12 in the aorta of cholesterol-fed rabbits: relationship to lesion development, Am. J. Pathol. 153 (1998) 109–119.
[19] J. Liang, E. Liu, Y. Yu, S. Kitajima, T. Koike, Y. Jin, M. Morimoto, K. Hatakeyama, Y. Asada, T. Watanabe, Y. Sasaguri, S. Watanabe, J. Fan, Macrophage metalloelastase accelerates the progression of atherosclerosis in transgenic rabbits, Circulation 113 (2006) 1993–2001.
[20] L. Wu, J. Fan, S. Matsumoto, T. Watanabe, Induction and regulation of matrix metalloproteinase-12 by cytokines and CD40 signaling in monocyte/macrophages, Biochem. Biophys. Res. Commun. 269 (2000) 808–815.
[21] M.W. Feinberg, M.K. Jain, F. Werner, N.E. Sibinga, P. Wiesel, H. Wang, J.N. Topper, M.A. Perrella, M.E. Lee, Transforming growth factor-beta 1 inhibits cytokine-mediated induction of human metalloelastase in macrophages, J. Biol. Chem. 275 (2000) 25766–25773.
[22] I. Jguirim-Souissi, A. Jelassi, F. Addad, M. Hassine, M. Najah, K. Ben Hamda, F. Maatouk, M. Ben Farhat, A. Bouslema, M. Rouis, M.N. Slimane, Plasma metalloproteinase-12 and tissue inhibitor of metalloproteinase-1 levels and presence, severity, and outcome of coronary artery disease, Am. J. Cardiol. 100 (2007) 23–27.
[23] S. Jormsjo, S. Ye, J. Moritz, D.H. Walter, S. Dimmeler, A.M. Zeiher, A. Henney, A. Hamsten, P. Eriksson, Allele-specific regulation of matrix metalloproteinase-12 gene activity is associated with coronary artery luminal dimensions in diabetic patients with manifest coronary artery disease, Circ. Res. 86 (2000) 998–1003.
[24] N. Marx, H. Duez, J.C. Fruchart, B. Staels, Peroxisome proliferator-activated receptors and atherogenesis: regulators of gene expression in vascular cells, Circ. Res. 94 (2004) 1168–1178.
[25] K.L. Gearing, M. Gottlicher, M. Teboul, E. Widmark, J.A. Gustafsson, Interaction of the peroxisome-proliferator-activated receptor and retinoid X receptor, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 1440–1444.
[26] C.H. Lee, A. Chawla, N. Urbiztondo, D. Liao, W.A. Boisvert, R.M. Evans, L.K. Curtiss, Transcriptional repression of atherogenic inflammation: modulation by PPARdelta, Science 302 (2003) 453–457.

[27] J.M. Lehmann, L.B. Moore, T.A. Smith-Oliver, W.O. Wilkison, T.M. Willson, S.A. Kliewer, An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma), J. Biol. Chem. 270 (1995) 12953–12956.
[28] B. Staels, J. Dallongeville, J. Auwerx, K. Schoonjans, E. Leitersdorf, J.C. Fruchart, Mechanism of action of fibrates on lipid and lipoprotein metabolism, Circulation 98 (1998) 2088–2093.
[29] M. Ricote, J. Huang, L. Fajas, A. Li, J. Welch, J. Najib, J.L. Witztum, J. Auwerx, W. Palinski, C.K. Glass, Expression of the peroxisome proliferator-activated receptor gamma (PPARgamma) in human atherosclerosis and regulation in macrophages by colony stimulating factors and oxidized low density lipoprotein, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 7614–7619.
[30] G. Chinetti, S. Griglio, M. Antonucci, I.P. Torra, P. Delerive, Z. Majd, J.C. Fruchart, J. Chapman, J. Najib, B. Staels, Activation of proliferator-activated receptors alpha and gamma induces apoptosis of human monocyte-derived macrophages, J. Biol. Chem. 273 (1998) 25573–25580.
[31] M. Ricote, A.C. Li, T.M. Willson, C.J. Kelly, C.K. Glass, The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation, Nature 391 (1998) 79–82.
[32] N. Marx, U. Schonbeck, M.A. Lazar, P. Libby, J. Plutzky, Peroxisome proliferator-activated receptor gamma activators inhibit gene expression and migration in human vascular smooth muscle cells, Circ. Res. 83 (1998) 1097–1103.
[33] P. Tontonoz, L. Nagy, J.G. Alvarez, V.A. Thomazy, R.M. Evans, PPARgamma promotes monocyte/macrophage differentiation and uptake of oxidized LDL, Cell 93 (1998) 241–252.
[34] Z. Chen, S. Ishibashi, S. Perrey, J. Osuga, T. Gotoda, T. Kitamine, Y. Tamura, H. Okazaki, N. Yahagi, Y. Iizuka, F. Shionoiri, K. Ohashi, K. Harada, H. Shimano, R. Nagai, N. Yamada, Troglitazone inhibits atherosclerosis in apolipoprotein E-knockout mice: pleiotropic effects on CD36 expression and HDL, Arterioscler. Thromb. Vasc. Biol. 21 (2001) 372–377.
[35] A.C. Li, C.J. Binder, A. Gutierrez, K.K. Brown, C.R. Plotkin, J.W. Pattison, A.F. Valledor, R.A. Davis, T.M. Willson, J.L. Witztum, W. Palinski, C.K. Glass, Differential inhibition of macrophage foam-cell formation and atherosclerosis in mice by PPARalpha, beta/delta, and gamma, J. Clin. Invest. 114 (2004) 1564–1576.
[36] V.R. Babaev, H. Ishiguro, L. Ding, P.G. Yancey, D.E. Dove, W.J. Kovacs, C.F. Semenkovich, S. Fazio, M.F. Linton, Macrophage expression of peroxisome proliferator-activated receptor-alpha reduces atherosclerosis in low-density lipoprotein receptor-deficient mice, Circulation 116 (2007) 1404–1412.
[37] A.C. Li, K.K. Brown, M.J. Silvestre, T.M. Willson, W. Palinski, C.K. Glass, Peroxisome proliferator-activated receptor gamma ligands inhibit development of atherosclerosis in LDL receptor-deficient mice, J. Clin. Invest. 106 (2000) 523–531.
[38] A.R. Collins, W.P. Meehan, U. Kintscher, S. Jackson, S. Wakino, G. Noh, W. Palinski, W.A. Hsueh, R.E. Law, Troglitazone inhibits formation of early atherosclerotic lesions in diabetic and nondiabetic low density lipoprotein receptor-deficient mice, Arterioscler. Thromb. Vasc. Biol. 21 (2001) 365–371.
[39] S.B. Joseph, A. Castrillo, B.A. Laffitte, D.J. Mangelsdorf, P. Tontonoz, Reciprocal regulation of inflammation and lipid metabolism by liver X receptors, Nat. Med. 9 (2003) 213–219.
[40] G. Chinetti, J.C. Fruchart, B. Staels, Peroxisome proliferator-activated receptors (PPARs): nuclear receptors at the crossroads between lipid metabolism and inflammation, Inflamm. Res. 49 (2000) 497–505.
[41] T.M. Willson, P.J. Brown, D.D. Sternbach, B.R. Henke, The PPARs: from orphan receptors to drug discovery, J. Med. Chem. 43 (2000) 527–550.
[42] M. Seimandi, G. Lemaire, A. Pillon, A. Perrin, I. Carlavan, J.J. Voegel, F. Vignon, J.C. Nicolas, P. Balaguer, Differential responses of PPARalpha, PPARdelta, and PPARgamma reporter cell lines to selective PPAR synthetic ligands, Anal. Biochem. 344 (2005) 8–15.
[43] L. Devel, V. Rogakos, A. David, A. Makaritis, F. Beau, P. Cuniasse, A. Yiotakis, V. Dive, Development of selective inhibitors and substrate of matrix metalloproteinase-12, J. Biol. Chem. 281 (2006) 11152–11160.
[44] O. Barbier, D. Duran-Sandoval, I. Pineda-Torra, V. Kosykh, J.C. Fruchart, B. Staels, Peroxisome proliferator-activated receptor alpha induces hepatic expression of the human bile acid glucuronidating UDP-glucuronosyltransferase 2B4 enzyme, J. Biol. Chem. 278 (2003) 32852–32860.
[45] P. Delerive, K. De Bosscher, S. Besnard, W. Vanden Berghe, J.M. Peters, F.J. Gonzalez, J.C. Fruchart, A. Tedgui, G. Haegeman, B. Staels, Peroxisome proliferator-activated receptor alpha negatively regulates the vascular inflammatory gene response by negative cross-talk with transcription factors NF-kappaB and AP-1, J. Biol. Chem. 274 (1999) 32048–32054.
[46] R.D. Hautamaki, D.K. Kobayashi, R.M. Senior, S.D. Shapiro, Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice, Science 277 (1997) 2002–2004.
[47] S.D. Shapiro, Diverse roles of macrophage matrix metalloproteinases in tissue destruction and tumor growth, Thromb. Haemost. 82 (1999) 846–849.
[48] X. Wang, J. Liang, T. Koike, H. Sun, T. Ichikawa, S. Kitajima, M. Morimoto, H. Shikama, T. Watanabe, Y. Sasaguri, J. Fan, Overexpression of human matrix metalloproteinase-12 enhances the development of inflammatory arthritis in transgenic rabbits, Am. J. Pathol. 165 (2004) 1375–1383.
[49] M. Liu, H. Sun, X. Wang, T. Koike, H. Mishima, K. Ikeda, T. Watanabe, N. Ochiai, J. Fan, Association of increased expression of macrophage elastase (matrix metalloproteinase 12) with rheumatoid arthritis, Arthritis Rheum. 50 (2004) 3112–3117.
[50] J.A. Curci, S. Liao, M.D. Huffman, S.D. Shapiro, R.W. Thompson, Expression and localization of macrophage elastase (matrix metalloproteinase-12) in abdominal aortic aneurysms, J. Clin. Invest. 102 (1998) 1900–1910.
[51] M. Karin, The regulation of AP-1 activity by mitogen-activated protein kinases, J. Biol. Chem. 270 (1995) 16483–16486.
[52] P. Angel, M. Karin, The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation, Biochim. Biophys. Acta 1072 (1991) 129–157.
[53] C. Jonat, H.J. Rahmsdorf, K.K. Park, A.C. Cato, S. Gebel, H. Ponta, P. Herrlich, Antitumor promotion and antiinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone, Cell 62 (1990) 1189–1204.
[54] H.F. Yang-Yen, J.C. Chambard, Y.L. Sun, T. Smeal, T.J. Schmidt, J. Drouin, M. Karin, Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein–protein interaction, Cell 62 (1990) 1205–1215.
[55] H.F. Yang-Yen, X.K. Zhang, G. Graupner, M. Tzukerman, B. Sakamoto, M. Karin, M. Pfahl, Antagonism between retinoic acid receptors and AP-1: implications for tumor promotion and inflammation, New Biol. 3 (1991) 1206–1219.
[56] M.I. Diamond, J.N. Miner, S.K. Yoshinaga, K.R. Yamamoto, Transcription factor interactions: selectors of positive or negative regulation from a single DNA element, Science 249 (1990) 1266–1272.
[57] M. Touray, F. Ryan, R. Jaggi, F. Martin, Characterisation of functional inhibition of the glucocorticoid receptor by Fos/Jun, Oncogene 6 (1991) 1227–1234.
[58] X.F. Zhou, X.Q. Shen, L. Shemshedini, Ligand-activated retinoic acid receptor inhibits AP-1 transactivation by disrupting c-Jun/c-Fos dimerization, Mol. Endocrinol. 13 (1999) 276–285.
[59] B.J. Pulverer, J.M. Kyriakis, J. Avruch, E. Nikolakaki, J.R. Woodgett, Phosphorylation of c-*jun* mediated by MAP kinases, Nature 353 (1991) 670–674.
[60] T. Smeal, B. Binetruy, D.A. Mercola, M. Birrer, M. Karin, Oncogenic and transcriptional cooperation with Ha-Ras requires phosphorylation of c-Jun on serines 63 and 73, Nature 354 (1991) 494–496.
[61] M. Hibi, A. Lin, T. Smeal, A. Minden, M. Karin, Identification of an oncoprotein- and UV-responsive protein kinase that binds and

potentiates the c-Jun activation domain, Genes Dev. 7 (1993) 2135–2148.
[62] B. Derijard, M. Hibi, I.H. Wu, T. Barrett, B. Su, T. Deng, M. Karin, R.J. Davis, JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain, Cell 76 (1994) 1025–1037.
[63] A. Minden, A. Lin, T. Smeal, B. Derijard, M. Cobb, R. Davis, M. Karin, c-Jun N-terminal phosphorylation correlates with activation of the JNK subgroup but not the ERK subgroup of mitogen-activated protein kinases, Mol. Cell. Biol. 14 (1994) 6683–6688.
[64] J. Arias, A.S. Alberts, P. Brindle, F.X. Claret, T. Smeal, M. Karin, J. Feramisco, M. Montminy, Activation of cAMP and mitogen responsive genes relies on a common nuclear factor, Nature 370 (1994) 226–229.
[65] A.J. Bannister, T. Oehler, D. Wilhelm, P. Angel, T. Kouzarides, Stimulation of c-Jun activity by CBP: c-Jun residues Ser63/73 are required for CBP induced stimulation in vivo and CBP binding in vitro, Oncogene 11 (1995) 2509–2514.
[66] C. Caelles, J.M. Gonzalez-Sancho, A. Munoz, Nuclear hormone receptor antagonism with AP-1 by inhibition of the JNK pathway, Genes Dev. 11 (1997) 3351–3364.
[67] J.L. Johnson, S.J. George, A.C. Newby, C.L. Jackson, Divergent effects of matrix metalloproteinases 3, 7, 9, and 12 on atherosclerotic plaque stability in mouse brachiocephalic arteries, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 15575–15580.
[68] J. Fan, X. Wang, L. Wu, S.I. Matsumoto, J. Liang, T. Koike, T. Ichikawa, H. Sun, H. Shikama, Y. Sasaguri, T. Watanabe, Macrophage-specific overexpression of human matrix metalloproteinase-12 in transgenic rabbits, Transgenic. Res. 13 (2004) 261–269.