PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA (PPARγ)-INDUCES APOPTOSIS AND INHIBITS AUTOPHAGY OF HUMAN MONOCYTE-DERIVED MACROPHAGES VIA INDUCTION OF CATHEPSIN L: POTENTIAL ROLE IN ATHEROSCLEROSIS.

Dler Faieeq Darweesh Mahmood18, Imene Jguirim-Souissi18, El-Hadji Khadija1, Nicolas Blondeau2, Vimala Diderot1, Souliman Amrani3, Mohamed-Naceur Slimane4, Tatiana Syrovets5, Thomas Simmet5 and Mustapha Rouis1*


2Institut de Pharmacologie Moléculaire et Cellulaire UMR 6097, C.N.R.S/Université de Nice Sophia Antipolis 06560 Valbonne, France

3Laboratoire de biochimie, Faculté des Sciences de Oujda, Maroc.

4Laboratoire de biochimie, Faculté de Médecine de Monastir, Tunisia.

5Institute of Pharmacology of Natural Products and Clinical Pharmacology, Ulm University, D-89081 Ulm, Germany.

§ Equal contributor

Address correspondence to: Mustapha Rouis, UR-04 Vieillissement, Stress et Inflammation, Université Pierre et Marie Curie-Paris 6, Bât. A. 5ème étage/Case courrier 256. 7, Quai St-Bernard. 75252 Paris Cedex 5, France. Tel: 33.1.44.27.20.28. Fax: 33.1.44.27.51.40. Email: mustapha.rouis@snv.jussieu.fr

Macrophages play a pivotal role in the pathophysiology of atherosclerosis. These cells express cathepsin L (CatL), a cysteine protease that has been implicated in atherogenesis and the associated arterial remodeling. In addition, macrophages highly express peroxisome proliferator-activated receptor gamma (PPARγ), a transcription factor that regulates numerous genes important for lipid and lipoprotein metabolism, for glucose homeostasis, and inflammation. Hence, PPARγ might affect macrophage function in the context of chronic inflammation such as atherogenesis.

In the present study, we examined the effect of PPARγ activation on the expression of CatL in human monocyte-derived macrophages (HMDM). Activation of PPARγ by the specific agonist GW929 concentration-dependently increased the levels of CatL mRNA and protein in HMDM. By promoter analysis we identified a functional PPRE-like sequence that positively regulates CatL expression. In addition, we found that PPARγ-induced CatL promotes the degradation of Bcl2 without affecting Bax protein levels. Consistently, degradation of Bcl2 could be prevented by a specific CatL inhibitor confirming the causative role of CatL. PPARγ-induced CatL was found to decrease autophagy through reduction of beclin 1 and LC3 protein levels. The reduction of these proteins involved in autophagic cell death was antagonized either by the CatL inhibitor or by CatL knockdown. In conclusion, our data show that PPARγ can specifically induce CatL, a proatherogenic protease, in HMDM. In turn, CatL inhibits autophagy and induces apoptosis. Thus, the proatherogenic effect of CatL could be neutralized by apoptosis, a beneficial phenomenon, at least in early stages of atherosclerosis.
INTRODUCTION

Atherosclerosis is a complex pathophysiological process that involves interaction of a variety of cells within the vessel wall, including smooth muscle cells, endothelial cells, and monocytes/macrophages. The latter cells play pivotal roles in atherogenesis, mainly through accumulation of oxidatively modified low-density lipoprotein (oxLDL) and production of a variety of inflammatory mediators, cytokines, and extracellular matrix-degrading enzymes (1). Rupture of atherosclerotic plaques is the most common event triggering formation of occlusive thrombi in arteries and subsequent acute ischemic events, such as acute coronary syndromes and stroke (2).

Several cysteine proteases have been implicated in atherogenesis and the associated arterial remodeling. Cysteine cathepsins are members of the papain family of proteases that degrade elastin and collagen (3). Among them, cathepsin L (CatL) is one of the most potent collagenases and elastases cleaving mature insoluble elastin (3). Cat B (4,5) and L (6,7) expression is enhanced in human coronary lesions, but also in human carotid lesions and abdominal aortic aneurysms (8). Cat K, S, and F are also expressed in human atherosclerotic lesions (9,10), and Cat B, D, and L were found in macrophage-derived foam cells in lipid-rich plaque areas (11). The pathophysiological role of Cat is underlined by the findings that deficiency of Cat S and L in LDL receptor knockout-mice reduced atherosclerotic lesions (6,12). Of note, unstable plaque regions contain increased levels of active legumain, a cysteine protease, that promotes intracellular processing of CatL to its mature 25 kDa form (13).

The role of cathepsins in apoptosis is now widely recognized in a variety of cell types including macrophages (14,15). Peroxisome proliferator-activated receptor gamma (PPARγ) is a transcription factor that regulates a large number of genes important for lipid and lipoprotein metabolism, for glucose homeostasis, and inflammation (16). PPARγ regulates transcription of target genes by the formation of heterodimers with the retinoid X receptor (RXR) and their subsequent binding to PPAR response elements (PPRE) in the promoter regions of target genes (17). PPARγ can also repress gene expression either in a DNA-binding-independent manner by interfering with other signaling pathways or in a DNA-binding-dependent way through the recruitment of co-repressors (18).

Similar to CatL, PPARγ is highly expressed in macrophages of atherosclerotic lesions and macrophage-derived foam cells (19-21), where it may affect macrophage function and consequently atherogenesis. PPARγ agonists have been shown to inhibit the development of atherosclerosis in apolipoprotein E-deficient (apoE−/−) (22) and in low-density lipoprotein (LDL) receptor-deficient (LDLR−/−) mice (23,24). In addition, mice transplanted with bone marrow from PPARγ−/− chimera mice exhibited a significantly increased atherosclerosis (25,26) suggesting an antiatherogenic role of PPARγ in macrophages.

PPARγ could inhibit atherosclerosis either by direct inhibition of proinflammatory genes (27) or indirectly through liver X receptor alpha (LXRα) (28), further, by negatively interfering with AP-1, NFκB, and STAT signaling pathways (29) or by reducing tumor necrosis factor-α, IL-1, and IL-6 secretion (30). In addition, enhanced PPARγ expression by human and murine monocytes inhibits CC chemokine receptor 2 (CCR2) and suppresses MCP-1-mediated chemotaxis (31). Atheroprotective effects of PPARγ were also implied by data showing that PPARγ agonists induce reduction of endothelial adhesion molecules by reduction of monocyte/macrophage recruitment in a mouse model of atherosclerosis (24,32,33). Moreover, it has been reported that ligand-mediated activation of PPARγ triggers induction of apoptosis of non-activated or activated human macrophages (34). Concomitantly, it was observed that PPARγ inhibits the transcriptional activity of the p65/RelA subunit of NFκB, suggesting that PPAR activators induce macrophage apoptosis by negatively interfering with anti-apoptotic NFκB signaling (34). Upregulation of PTEN by PPARγ may be another mechanism by which PPARγ could induce macrophage apoptosis (35). Only recently, we reported a new mechanistic explanation for the PPARγ-mediated induction of macrophage apoptosis. We demonstrated that PPARγ activation stimulates apoptosis in human macrophages by altering the cellular redox balance via regulation of thioredoxin-1 (Trx-1) and its endogenous
inhibitor, the thioredoxin interaction protein (TXNIP, also called Vitamin D3 Upregulated Protein-1, VDUP-1) (36).

In the present study, we examined the effect of PPARγ activation on the expression of CatL in human monocyte-derived macrophages. Since several studies indicated that PPARγ activation exerts antiatherogenic effects, we hypothesized that PPARγ activation might inhibit the expression of CatL in human monocyte-derived macrophages. Surprisingly, we found that PPARγ concentration-dependently increases the CatL expression.

**EXPERIMENTAL PROCEDURES**

**Isolation and culture of human monocytes**-Mononuclear cells were isolated by Ficoll gradient centrifugation from buffy-coats of healthy normolipidaemic donors. Cells were cultured in RPMI 1640 supplemented with gentamycin (40 µg/ml), glutamine (0.05%) (Sigma, Saint Quentin, France), 10% pooled human serum (Promocell, Heidelberg, Germany) at a density of 6 × 10⁶ cells/well in 60-mm well Primaria-plastic culture dishes (Polylabo, Strasbourg, France). Differentiation of monocytes into human monocyte-derived macrophages (HMDM) was allowed to occur spontaneously by adhesion of cells to the culture dishes and by continued maturation for the subsequent 8 to 12 days.

**RNA extraction and analysis**-Total cellular RNA was extracted using Trizol (Life Technologies, Invitrogen SARL, Cergy-Pontoise, France). For quantitative PCR, reverse transcribed CatL mRNA was quantified by real-time PCR on a MX4000 apparatus (Stratagene, La Jolla, CA), using the forward primer (5’-GCATAATCCATTAGGCCACCATT-3’) and the reverse primer (5’-CAGATCTGTGATTGGAGAGA-3’). PCR amplification was performed in a volume of 25 µl containing 100 nM of each paired primer, 4 mM MgCl₂, and the Brilliant Quantitative PCR Core reagent Kit mix as recommended by the manufacturer (Stratagene). The cycling conditions were 95°C for 10 min, followed by 40 cycles of 30 sec each at 95, 55, and 72°C. Levels of CatL were normalized to the internal control, 36B4 mRNA, using the primer set (forward: 5’-CATGCTCAACATCTCCCCCTCC -3’ and reverse: 5’-GGGAAGGTGAATCCGTCTCCACAG-3’).

**Western blotting analyses**-HMDM cells were treated with PPARα, γ, or β/δ agonists, or with CatL inhibitor (Merck, ref. 219435) and then scraped into TRIS buffer containing a mixture of protease inhibitors (Sigma). Protein concentrations were measured by Peterson’s method with BSA as standard. Samples were denatured with SDS-loading buffer and subjected to SDS-PAGE (Invitrogen SARL, Cergy-Pontoise, France). The samples were transferred to nitrocellulose membranes blocked with non-fat dried milk. The blots were incubated with monoclonal anti-human CatL antibody (Sigma, 1:500), rabbit anti-human beclin 1 antibody (Cell Signaling, 1:1000), rabbit anti-human LC3 antibody (Cell Signaling, 1:1000), rabbit anti-human Bax antibody (Cell Signaling, 1:1000) or rabbit anti-human Bcl2 antibody (Cell Signaling, 1:1000); after washing, the blots were incubated with peroxidase-conjugated goat anti-mouse IgG (Biorad, Marne la Coquette, France) (1:500 dilution). Human β-actin (Santa Cruz, 1:1000) served as loading control. The blots were visualized with a chemiluminescence kit (Amersham Biosciences, Orsay, France).

**Immunocytochemistry**-HMDM cells were cultured in chamber slides (LabTec, France). After treatment, HMDM were fixed with 4% paraformaldehyde/PBS, permeabilized in 0.3% Tween 20 and blocked with 5% goat serum/PBS at room temperature. HMDM were then incubated with the following rabbit polyclonal antibodies from Cell Signaling: anti-beclin-1 (1:400), anti-cleaved caspase-3 (Asp175, 1:2000) overnight. After washing, the sections were incubated in anti-IgG-Alexa 488-coupled antibodies (1:1000; Molecular Probes, Leiden, Netherlands) in 5% normal goat serum. Hoechst 33342 stain was used to label HMDM nuclei.

To ensure comparable immunostaining, HMDM cultures were processed together under the same conditions. Confocal microscopy was performed with a laser scanning confocal microscope (TCS SP, Leica) equipped with a DMI8IRBE inverted microscope, using a Plan Apo 63x/1.4 NA oil immersion objective. Quantification was done on pictures acquired with the same settings (such as the laser power,
scanning speed and photomultiplier gain). The fluorescence intensity was digitally measured for each cell of five randomly selected fields from each culture condition (ImageJ, NIH). Quantification was carried out by investigators blinded to the experimental condition.

**Plasmids and transient transfection assays**—The human CatL promoter constructs were generated by PCR using *Pfu* polymerase (Stratagene) with human genomic DNA as a template (Genebank AF163338.1). A *Smal* site-linked reverse 5'-primer (5'-CGCACCCCGGATGCCTC-3') was used with *Nhel* site-linked forward 5'-primer (5'-ACCAAAATTgCTAGcACTAAGGAAATG-3') to amplify the -370/+36 fragment, with *Nhel* site-linked forward 5'-primer (5'-ATAATCTGgGCTAAGCAGAG-3') to amplify the -783/+36 fragment or with *KpnI* site-linked forward 5'-primer (5'-TCTTAAAGGTAcCAATGTAC-3') to amplify the -1250/+36 fragment. Inserted nucleotides used to create restriction sites are designated by small characters, restriction sites are underlined. These fragments were transferred into the promoterless firefly luciferase reporter vector pGL3-Basic (Promega, Mannheim, Germany) in the correct orientation. One firefly luciferase reporter vector in pGL3-Basic containing the human CatL promoter (-1250/+36 bp) with mutated PPRE-like (5'-GCAGGCCACGcCcCCTCCCTC-3') was generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). Point mutations were introduced in the human CatL PPRE-like using *Pfu* DNA polymerase, double-stranded plasmid DNA, and complementary oligonucleotide primers containing the desired mutation. The nucleotide sequences of the constructs were verified by automated sequencing. The nucleotide sequences did not show any similarity to coding mRNA sequences devoid of secondary structures, i.e., the loops, were selected using available algorithms (38). The antisense ODN for CatL corresponded to the nucleotides 544-565 of human CatL mRNA (NM_001908.3) 5'-AATACAGGGAGGGAACACAG-3' and for human CatB, they corresponded to the nucleotides 1087-1105 (NM_001980.3) 5'-TTCTTTAAATACTGAG-3'. The control sequences contained the same set of the base pairs in a scrambled order. The sequences were analyzed for lack of secondary structure and oligo pairing. According to blast search, the selected sequences did not show any similarity to coding mRNA. Macrophages were treated for 72 h every 24 h with 5 M of the ODN in RPMI 1640 supplemented with 10% FCS (39). The cells were kept in the presence or absence of the PPARγ agonist for 24 h, lysed, and analyzed for protein expression by Western immunoblotting using antibodies against CatL, CatB, beclin 1, LC3 or β-actin.

**Electrophoretic mobility shift assays**—Human PPARα, PPARγ, PPARβ/δ and RXRα were synthesized in vitro using the TNT quick coupled transcription/translation system (Promega). Twenty-bp (5'-GGTTTTGATCCAGTTTCCAGT-3') double-stranded oligonucleotides, containing the consensus PPRE sequence were used as control PPRE (40). The putative CatL PPRE-like (5'-GCAGCCAGTCCCTCCCTCC-3') and its mutated form (5'-GCAGGCCAGC CCTCCCTCC-3') were end-labeled with [γ-
32P]ATP with 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 before the radiolabeled probe was added. Binding reactions were incubated for further 15 min and then resolved by 4% nondenaturing PAGE. 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 x 10⁶ 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 and lysed. Chromatin was sonicated with five 10-s pulses at 30% amplitude (sonifier, Branson Ultrasonic Corp). After centrifugation, the supernatant was diluted 10-fold with ChIP dilution. Diluted extracts were precleared in the presence of salmon sperm DNA-protein A-agarose. One-tenth of the diluted extract was kept for direct PCR (Input). The remaining extracts were incubated for 16 h at 4°C in the presence of 1 µg of specific PPARγ antibody (Santa Cruz Biotech) per ml, followed by 1 h of incubation with salmon sperm DNA-protein A-agarose beads. Following extensive washing, bound DNA fragments were eluted with elution buffer (1% SDS, 0.1 M NaHCO₃). DNA was recovered in elution buffer containing 200 mM NaCl and then incubated in the presence of protease 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 using specific oligonucleotide primers that amplify a fragment containing the human CatL PPRE-like site (forward: 5’-ACGCCCGCGCTCTCTGGAG-3’ and reverse: 5’-TTTAGAAGAATCTGGTAAG-3’).

Statistical analysis-Quantitative results are expressed as mean ± SD. Results were analyzed by multi-group comparison Mann-Whitney U and Newman-Keuls test(s). Differences were considered statistically significant at *p < 0.05.

RESULTS

PPARγ induces expression of human CatL in HMDM-To determine whether CatL is regulated by PPARα, PPARγ or PPARβ/δ, we treated HMDM with 0.06, 0.6, and 6 µM of each agonist. Of the tested agonists, only the selective PPARγ agonist GW929 significantly increased CatL mRNA expression (100 ± 10% at 0.06 µM; 170 ± 50%, p < 0.05 at 0.6 µM and 320 ± 49%, p < 0.01 at 6 µM vs. control cells (100%) (Fig. 1A). In addition, we investigated the effects of the PPARγ agonist GW929 on the expression of the PPARγ target genes CD36 and aP2, and correlated their induction to that of CatL. Indeed, we observed induction of both genes with the agonist GW929, and the expression was 

To determine whether the GW929-induced changes in mRNA expressions would also occur at the protein level, we performed immunoblot experiments. These experiments revealed a significant increase in CatL expression (180 ± 68%, p < 0.05 at 0.6 µM; 285 ± 183%, p < 0.01 at 6 µM vs. control cells, 100%) (Fig. 2). In contrast, treatment of HMDM with the specific PPARα GW647 or the PPARβ/δ agonist GW516 did not increase the CatL expression (Fig. 2). Therefore, we focused our study on the effects of the PPARγ agonist GW929 on CatL expression.

Effects of PPARγ activation on the CatL promoter-Since the expression of CatL was increased upon treatment of HMDM with the specific PPARγ agonist (Fig. 1 and 2), we determined whether this effect was mediated by the promoter region proximal upstream of the CatL gene. First, HMDM cells were transiently transfected with a luciferase reporter vector driven by three different human CatL promoter fragments (-1,250/+36; -783/+36 and -370/+36). Cotransfection with PPARγ and RXRα significantly stimulated the -783/+36 CatL promoter activity (3.45 ± 0.6 fold increase vs.
control with $p < 0.01$ in the presence of 0.6 $\mu$M GW929) (Fig. 3). Transcriptional activation of the CatL gene by PPARγ indicated the presence of a functional PPRE in the CatL promoter located between -783 and -370. A detailed computer analysis was performed in the region of the CatL promoter defined earlier (41). It revealed the presence of a putative PPRE-like motif, located between -652 and -662, with 91% homology with the PPRE consensus defined for PPARs (40). To prove that this putative PPRE-like sequence could be involved in the transcriptional activation of CatL by PPARγ, we next analyzed the activity of a mutated human CatL promoter fragment (-783/+36) using a luciferase reporter assay. Our results showed that cells cotransfected with the mutant CatL promoter did not respond to GW929 (Fig. 3).

**CatL promoter contains a PPRE-like motif that confers responsiveness to PPARγ.** To demonstrate that the putative CatL-PPRE-like sequence could indeed bind the PPARγ/RXRα heterodimer, we performed an electrophoretic mobility shift assay. First, we verified that the *in vitro* translated RXRα and PPARγ proteins produced by the TNT quick coupled transcription/translation system bind the consensus PPRE by heterodimerization. Secondly, in agreement with our previous data (36), incubation of the labeled PPRE-like sequence from the CatL promoter with *in vitro* translated PPARγ and RXRα showed formation of a retarded complex (Fig. 4A). This specific complex was not observed with the mutated PPRE-like oligonucleotide and disappeared when it was incubated with a 50-molar excess of unlabeled CatL-PPRE oligonucleotide as a specific competitor (Fig. 4A). Moreover, incubation of labeled PPRE-like oligonucleotide from CatL promoter with *in vitro* translated PPARγ and RXRα as well as specific antibody against PPARγ resulted in a supershifted band (Fig. 4A). Finally, the result of the ChIP experiment showed an increased fixation of the complex on the PPRE-like site upon activation of PPARγ with GW929 (Fig. 4B). Taken together, these results indicate that the PPARγ agonist GW929 induced CatL expression in HMDM, at least in part, through binding of PPARγ to the PPRE-like motif located between -783 and -1250 of the human CatL promoter.

**CatL increases apoptosis in HMDM.** Next we analyzed the effects of PPARγ activation on the induction of autophagy and apoptosis in HMDM. By quantitative immunocytochemistry we found that the PPARγ agonist GW929 decreased the expression of beclin 1 and LC3, two proteins important for the formation of autophagosomes during autophagy (42). At the same time, treatment with GW929 concentration-dependently increased cleavage of caspase 3, indicating induction of apoptosis in HMDM (Fig. 5).

Next, we examined the role of GW929-induced CatL expression in macrophage apoptosis. HMDM were treated with 0, 0.6, and 6 nM of specific CatL inhibitor in the presence of the PPARγ agonist GW929 (0.6 $\mu$M) and the protein levels of Bax and Bcl-2 were determined using Western immunoblots. The results showed no significant changes in the protein levels of Bax after normalization with β-actin (Fig. 6). As a consequence, the Bax/Bcl-2 ratio was decreased (76 ± 13%, $p < 0.05$ and 51 ± 12%, $p < 0.05$ by 0.6 nM and 6 nM of the CatL inhibitor, respectively) (Fig. 6). A decreased ratio of Bax/Bcl-2 is consistent with a decreased susceptibility to apoptosis, indicating that CatL is involved in the PPARγ-mediated induction of macrophage apoptosis.

**Cathepsin L decreases autophagy in HMDM.** Because macrophages play a central role in atherosclerotic plaque destabilization, selective induction of macrophage death is increasingly gaining attention in cardiovascular medicine as it could stabilize vulnerable, rupture-prone lesions (43). Compared with apoptosis or necrosis, autophagy might to be a favourable type of cell death leading to elimination of macrophages in atherosclerotic plaques, because autophagic macrophages literally digest themselves to death (43). As a consequence of autophagy, the cytoplasmic content progressively decreases so that activation of inflammatory responses, the release of matrix degrading proteases, and the deposition of necrotic debris after postautophagic necrosis is minimal. Therefore, we examined whether autophagy is involved in mechanisms of cell death induced by PPARγ treatment. To do so, we examined the protein levels of beclin 1 (Bcl2 interacting protein) and microtubule-associated protein 1 light chain 3 (LC3), which had previously been shown to promote autophagy (42).
Our results indicate that PPARγ decreases the protein expression of beclin 1 (87 ± 13% at 0.06 µM; 49 ± 21% at 0.6 µM, p < 0.05) (Fig. 7A and Fig. 8). In addition, in the presence of the CatL inhibitor, we observed a concentration-dependent increase of the protein levels of beclin 1 (177 ± 63% and 226 ± 50%, p < 0.05 by 0.6 nM and 6 nM of the CatL inhibitor, respectively) (Fig. 7B). Of note, we did not observe any changes in the mRNA levels of beclin 1 following either PPARγ or CatL inhibitor treatment (not shown).

Similar experiments were conducted to evaluate the level of LC3 protein. Figure 9A shows a concentration-dependent inhibitory effect of PPARγ on LC3 expression (48 ± 15%, p < 0.01 and 39 ± 25% p < 0.01 by 0.06 µM and 0.6 µM GW929, respectively). Moreover, treatment with the CatL inhibitor concentration-dependently prevented the degradation of LC3 (162 ± 14%, p < 0.05 and 220 ± 25%, p < 0.01 by 0.6 nM and 6 nM of the CatL inhibitor, respectively) (Fig. 9B).

Finally, knockdown of CatL in PPARγ agonist-treated HMDM (Fig. 10A) protects beclin 1 and LC3 from degradation (Fig. 10B). In contrast, knockdown of CatB in identically treated cells did not affect the expression of both proteins (Fig. 10B) confirming the specific effect of CatL on beclin 1 and LC3 degradation.

**DISCUSSION**

Our study shows that PPARγ activation increases the levels of CatL mRNA and protein in HMDM. Using promoter analysis, we identified a functional PPRE-like sequence in the CatL promoter region that confers an increased CatL regulation by PPARγ. In addition, we show that PPARγ-induced CatL decreases the levels of Bcl2 and increases those of BAX indicating involvement of CatL in the PPARγ-mediated induction of macrophage apoptosis. We also found that PPARγ-induced CatL decreases autophagy through the reduction of beclin 1 and LC3. The reduction of proteins involved in autophagic cell death was antagonized by a CatL inhibitor and following CatL knockdown in PPARγ agonist-treated HMDM. To the best of our knowledge, this is the first study showing that PPARγ inhibits autophagy and stimulates apoptosis through induction of CatL in human macrophages. Previous studies have shown that PPARγ can induce apoptosis through other pathways. Thus, Trx-1, a 12 kDa protein, which plays a major role in the regulation of the cellular redox balance, can interact with ASK-1 and inhibits apoptosis (44). In addition, we have previously shown that the PPARγ agonist GW929 concentration-dependently increased HMDM expression of VDUP-1, a specific endogenous inhibitor for Trx-1, suggesting that PPARγ activation stimulates apoptosis in human macrophages by altering the cellular redox balance via upregulation of VDUP-1 (36). Trx-1 may also antagonize apoptosis by binding to PTEN and the subsequent inhibition of its phosphatase activity (45). The reduced expression/activity of Trx-1 in cells treated with PPARγ agonists would increase PTEN activity and contribute to decreased cell survival by inhibition of Akt signaling. Furthermore, it has been demonstrated that activation of PPARγ by its selective ligand rosiglitazone directly upregulates PTEN expression in several cell types including macrophages, and reduces the rate of macrophage proliferation (35). Taken together, PPARγ may increase macrophage apoptosis in several ways, such as direct upregulation of PTEN gene expression, through reduction of Trx-1 activity or, as we have demonstrated in the present study, by increasing the CatL expression, or by a combination of these mechanisms.

It is important to note that death of lesional macrophages by apoptosis was observed throughout all stages of atherosclerosis. However, the consequences of this event were considered to be very different in early versus late atherosclerotic lesions (46). One explanation based on in vivo investigations is that phagocytic clearance of apoptotic macrophages in early atherosclerotic lesions is highly efficient (47-50), whereas, phagocytic clearance in advanced lesions is less effective due to the high number of apoptotic macrophages in advanced atherosclerotic lesions (47,51-53).

Recently, Liu et al. (54) reported that macrophage apoptosis suppresses the development of atherosclerosis in LDLR−/− mice. On this background, our results would imply that the PPARγ-mediated increase in CatL expression in macrophages may represent a new approach for the inhibition of atherosclerosis progression. However, CatL was reported rather to induce than to reduce the progression of atherosclerosis (6,12).

On the other hand, PPARγ activation was found to exert both atherogenic and anti-
atherogenic effects. In general, the beneficial effects of PPARγ activation are considered to be greater than its atherogenic effects (55). We therefore hypothesized that the induction of CatL by PPARγ activation could eliminate macrophages by apoptosis or/and autophagy, and thus counteract atherogenesis. Autophagy is a catabolic pathway for bulk turnover of long-lived proteins and organelles via lysosomal degradation. A growing body of evidence suggests that autophagy is playing a role in many diseases by promoting or preventing their progression (56). In atherosclerotic plaques, basal autophagy is a survival mechanism safeguarding plaque cells against oxidative injury, metabolic stress and inflammation, by removing harmful oxidatively modified proteins and damaged components. Hence, autophagy is anti-apoptotic and contributes to cellular recovery. Basal autophagy can be intensified by appropriate drugs. Hence, pharmacological approaches have been developed to stabilize rupture-prone plaques through selective induction of macrophage autophagic death, without affecting the plaque stabilizing smooth muscle cells (for review see (56)).

Because macrophages play a central role in atherosclerotic plaque destabilization, selective induction of macrophage death aiming at stabilization of vulnerable, rupture-prone lesions is gaining increased attention (43). Compared with apoptosis or necrosis, autophagy seems to be a favorable type of cell death to eliminate atherogenic macrophages within atherosclerotic plaques. As a consequence, the release of matrix degrading proteases, the deposition of necrotic debris, and the activation of inflammatory responses would be minimal.

REFERENCES

ACKNOWLEDGEMENTS

This work was supported by Fondation Coeur et Artères (FCA), Fondation de France and the CMCU exchange program between Tunisia and France.

FIGURE LEGENDS

Fig. 1. PPARγ activation induces CatL, aP2 and CD36 mRNA expression. HMDM cells were treated with either vehicle (control), GW647, GW929, or GW516 for 24 h. A, effects of PPAR activation on CatL mRNA and 36B4 mRNA (for normalization). B, effects of GW929 on human aP2, CD36 and CatL mRNA. Compounds were used at 0.06, 0.6, and 6 µM and their effects on mRNA expression were analyzed by real-time Q-PCR. Results are the mean ± SD of 4 separate experiments, each was performed in triplicates, (*p < 0.05, **p < 0.01, NS, not significant, compared to control).

Fig. 2. PPARγ activation induces protein expression of CatL. HMDM cells were treated either with vehicle (control), GW647, GW929, or GW516 (each at 0.06, 0.6 and 6 µM) for 24 h. CatL protein levels were evaluated by three independent Western immunoblots. Results are the mean ± SD; one representative Western immunoblot was inserted for each agonist, (*p < 0.05, **p < 0.01, NS, not significant, compared to control).

Fig. 3. PPARγ activation elicits CatL promoter activity. HMDM were co-transfected with one of three constructs of the CatL promoter (pGL3-1250/+36, pGL3-783/+36, pGL3-370/+36, or PPRE-like mutated CatL-luciferase expression vectors) together with human pSG5-PPARγ and/or RXRα expression vectors and pCH110, a β-galactosidase vector for 20 h. Cells were then incubated in the presence or absence of GW929 (0.6 µM) for 36 h. Results are the mean ± SD of three separate experiments, each performed in triplicates (***p < 0.01 compared to non-treated cotransfected cells).

Fig. 4. PPARγ and RXRα bind as heterodimer to a PPRE-like site located in the 5′-flanking region of the CatL gene. A, gel retardation assays with in vitro-translated hPPARγ and hRXRα were performed with end-labeled oligonucleotides representing the consensus DR1 PPRE, the human putative CatL PPRE-like,
and a mutated version of the human putative PPRE-like. The consensus DR1 PPRE was used as unlabeled competitor. Supershift analysis was performed using a specific antibody against human PPARγ. B, HMDM were treated with or without GW929 (0.06, 0.6 and 6 µM) for 24 h. DNA-bound proteins were cross-linked to DNA, and after fragmentation, chromatin was precipitated with rabbit polyclonal antibody against human PPARγ, or anti-rabbit IgG. As a positive control, input chromatin of the cell lysate was also amplified.

**Fig. 5.** PPARγ activation induces caspase 3 activation. HMDM cultured in chamber slides for 7 days were treated with the PPARγ agonist GW929 for 24 h. The cells were then washed, fixed, permeabilized and blocked. HMDM were then incubated overnight with anti-cleaved caspase-3 (Asp175) antibody, followed by anti-IgG Alexa 488-coupled antibodies. Hoechst 33342 staining was used to label HMDM nuclei. Confocal microscopy was performed with a DMIRBE inverted microscope, using a Plan Apo 63x/1.4 NA oil immersion objective. Quantification was done on pictures acquired with the same settings. The fluorescence intensity was digitally measured for each cell of five randomly selected fields from each culture condition, and presented as mean ± SD (**p < 0.01 compared to control cells).

**Fig. 6.** CatL inhibitor reduces the Bax/Bcl2 ratio in PPARγ-activated macrophages. HMDM were treated with PPARγ agonist GW929 (0.6 µM) for 24 h, followed by treatment with CatL inhibitor at 0.6 or 6 nM for additional 24 h. The cells were washed, lysed and analyzed by Western immunoblotting. Bax, Bcl2 and β-actin protein levels were analyzed and the results are presented as mean ± SD of four independent Western immunoblots. One representative Western blot for each protein was inserted, (*p < 0.05 compared to control).

**Fig. 7.** PPARγ-induced decrease in beclin 1 levels is reversed by CatL inhibitor. A, HMDM were treated with the PPARγ agonist GW929 (0.06 and 0.6 µM) for 24 h. The cells were washed, lysed and protein levels of beclin 1 and β-actin (for normalization) were analyzed by Western immunoblotting. Results are the mean ± SD of four independent experiments. One representative Western blot for each protein was inserted, (*p < 0.05 compared to control). B, HMDM were treated with the PPARγ agonist GW929 (0.6 µM) for 24 h. The cells were incubated for additional 24 h in the presence or absence of the CatL inhibitor at 0.6 or 6 nM. The cells were washed, lysed and beclin 1 and β-actin (for normalization) protein levels were analyzed. Results are presented as mean ± SD of four independent Western immunoblots. One representative Western blot for each protein was inserted, (*p < 0.05 compared to control).

**Fig. 8.** PPARγ activation reduces beclin 1 expression in HMDM. HMDM, cultured in chamber slides for 7 days, were rinsed and treated with the PPARγ agonist GW929 for 24 h. HMDM were incubated overnight with anti-beclin-1 antibody followed by anti-IgG Alexa 488-coupled antibodies. Hoechst 33342 staining was used to label HMDM nuclei and all chamber slides were rinsed and mounted with Fluoprep mounting medium. Confocal microscopy was performed with a DMIRBE inverted microscope using a Plan Apo 63x/1.4 NA oil immersion objective. Quantification was done on pictures acquired with the same settings. The fluorescence intensity was digitally measured for each cell of five randomly selected fields from each culture condition and presented as mean ± SD (***p < 0.01 compared to control cells).

**Fig. 9.** PPARγ activation induces a CatL-dependent decrease of autophagy markers. A, HMDM were treated with the PPARγ agonist GW929 (0.06 and 0.6 µM) for 24 h. The cells were analyzed for LC3 and β-actin (for normalization) expression using Western immunoblotting. Results are presented as mean ± SD of four independent Western blots. One representative Western blot for each protein was inserted, (***p < 0.01 compared to control). B, HMDM were treated with the PPARγ agonist GW929 (0.6 µM) for 24 h followed by incubation in the presence or absence of CatL inhibitor at 0.6 or 6 nM for additional 24 h. The cells were washed, lysed and LC3 and β-actin (for normalization) protein levels were evaluated. Results are the mean ± SD of four independent Western immunoblots. One representative Western blot for each protein was inserted, (*p < 0.05, **p < 0.01 compared to control).
Fig. 10. In vitro knockdown of CatL reverses the PPARγ-mediated decrease of autophagy markers. A, downregulation of CatL by phosphothioate ODN. HMDM were treated with 5 µM ODN complementary to the corresponding mRNA sequence devoid of secondary structures for 72 h. Downregulation of CatL was analyzed by Western blotting. A representative blot is shown. The control ODN contained the same set of the bases in a scrambled order. B, HMDM were treated for 72 h with 5 µM of either ODN followed by treatment with the PPARγ agonist GW929 (0.6 µM) for additional 24 h. Expression of CatL, beclin 1, LC3 or β-actin was analyzed by Western immunoblotting. Representative blots are shown.
Figure 2

CatL

β-Actin

vehicle GW647 GW929 GW516

CATL / β-Actin (% of control)

+ Vehicle GW647 GW929 GW516

NS

NS

* NS

**
### Figure 4

#### A

<table>
<thead>
<tr>
<th></th>
<th>$^{32}$P-labeled</th>
<th>Wild type PPRE-like oligo</th>
<th>Mutated PPRE-like oligo</th>
<th>PPRE consensus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-PPAR$\gamma$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PPAR$\alpha$</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PPAR$\gamma$</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PPAR$\delta$</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RXR$\alpha$</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Competitor</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

#### B

- **Input**
- **GW929**
- **PPRE-like**
- **IgG**
Figure 5
Figure 6

Bax

Bcl2

β-Actin

GW929 + + +
CatL inhibitor - + +

GW929 + + +
CatL inhibitor - - -

Bax/Bcl2 (% of control)

0 25 50 75 100

*
Figure 7

A

Beclin 1

β-Actin

Vehicle

GW929

B

Beclin 1

β-Actin

GW929

CatL inhibitor

Beclin 1/β-Actin (% of control)

0

25

50

75

100

Vehicle

GW929

Beclin 1/β-Actin (% of control)

0

100

200

300

GW929

CatL inhibitor

*
Figure 8
Figure 9

A

LC3-I
LC3-II
β-Actin

Vehicle
GW929

B

LC3-I
LC3-II
β-Actin

GW929
CatL inhibitor

+ + +

GW929
CatL inhibitor

+ + +

Vehicle
GW929

GW929
CatL inhibitor

+ + +

**

*
Figure 10

A

B

<table>
<thead>
<tr>
<th></th>
<th>Control ODN</th>
<th>CatL ODN</th>
<th>Control ODN</th>
<th>CatB ODN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beclin 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LC3-II</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GW929</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
Dler Faieeq Darweesh Mahmoud, Imene Jguirim-Souissi, Khadija El-Hadri, Nicolas Blondeau, Vimala Diderot, Souliman Amrani, Mohamed-Naceur Slimane, Tatiana Syrovets, Thomas Simmet and Mustapha Rouis

J. Biol. Chem. published online June 23, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M111.273292

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts