Activation of Proliferator-activated Receptors α and γ Induces Apoptosis of Human Monocyte-derived Macrophages*

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Peroxisome proliferator-activated receptors (PPARs) have been implicated in metabolic diseases, such as obesity, diabetes, and atherosclerosis, due to their activity in liver and adipose tissue on genes involved in lipid and glucose homeostasis. Here, we show that the PPARα and PPARγ forms are expressed in differentiated human monocyte-derived macrophages, which participate in inflammation control and atherosclerotic plaque formation. Whereas PPARα is already present in undifferentiated monocytes, PPARγ expression is induced upon differentiation into macrophages. Immunocytochemistry analysis demonstrates that PPARα resides constitutively in the cytoplasm, whereas PPARγ is predominantly nuclear localized. Transient transfection experiments indicate that PPARα and PPARγ are transcriptionally active after ligand stimulation. Ligand activation of PPARγ, but not of PPARα, results in apoptosis induction of unactivated differentiated macrophages as measured by the TUNEL assay and the appearance of the active proteolytic subunits of the cell death protease caspase-3. However, both PPARα and PPARγ ligands induce apoptosis of macrophages activated with tumor necrosis factor α/interferon γ. Finally, PPAR inhibits the transcriptional activity of the NFκB p65/RelA subunit, suggesting that PPAR activators induce macrophage apoptosis by negatively interfering with the anti-apoptotic NFκB signaling pathway. These data demonstrate a novel function of PPAR in human macrophages with likely consequences in inflammation and atherosclerosis.

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The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; IFNγ, interferon γ; TNFα, tumor necrosis factor α; RT-PCR, reverse transcriptase-polymerase chain reaction; bp, base pair(s); PBS, phosphate-buffered saline; PG-J2, prostaglandin 15-deoxy-Δ12,14-prostaglandin J2;
in atherosclerotic lesions (26). In addition to apoptosis, in advanced atherosclerotic plaques, macrophages and lipid-loaded foam cells undergo necrosis thereby releasing their intracellular contents due to cytolysis resulting in the formation of the lipid-rich core of the atheromatous plaque (27).

Considering the role of PPARs in lipid metabolism and inflammatory control, we hypothesized a function for PPARs at the level of the vascular wall which, independent of their role in lipoprotein metabolism, could modulate the pathogenesis of atherosclerosis. Therefore, we initiated studies on the expression and functions of PPARs in circulating human monocytes and in macrophages during differentiation, a crucial event in atherosclerosis development and vascular inflammation. Our results show that PPARα is already expressed in undifferentiated human monocytes, whereas PPARγ only becomes expressed in human macrophages upon differentiation. Treatment of differentiated macrophages with PPAR activators induces apoptosis, an effect which is more pronounced in macrophages activated with IFNγ and TNFα. Finally, we show that PPARγ inhibits the transcriptional activity of the NFκB subunit p65/RelA, indicating that PPARγ activators may promote TNFα-induced apoptosis in macrophages by interfering negatively with the anti-apoptotic NFκB pathway (28–30).

EXPERIMENTAL PROCEDURES

Cell Culture—Mononuclear cells were isolated from blood of healthy normolipidemic donors (thrombopheresis residues) (31). After Ficoll gradient centrifugation, the monocytes were suspended in RPMI 1640 medium containing gentamycin (40 μg/ml), glutamine (0.05% (Sigma)), and either 5% pooled human serum or 5% fetal calf serum (apoptosis assays) or 10% pooled human serum (other assays). Cells were cultured at a density of 3 × 10⁵ cells/well in 6-well plastic culture dishes (Primaria, PolyLabo, Strasbourg, France). Differentiation of monocytes into macrophages occurred spontaneously by adhesion of cells to the culture substratum. Polylabo, Strasbourg, France). Differentiation of monocytes into macrophages was induced by addition of human PPARγ1 agonists, such as BRL49653 or PG-J2, or solvent (Me2SO) (32, 33). Monocyte-derived macrophages at day 12 of culture were transfected by the lipofection procedure (DOTAP; Boehringer Mannheim, Mannheim, Germany) with the reporter plasmid (2.5 μg) in 6-well culture dish in RPMI 1640 medium containing 1% Fetal Calf Serum (FCS) and 3% heat-inactivated horse serum. After overnight incubation, cells were washed with PBS and ligands for PPARγ, PPARα, and PPARδ were added (10 μM) into the culture medium. After 24 h at 37°C, cells were washed with PBS and ligands for PPARγ (Wy14,643, Chem-syn, Lenexa, KS) or PPARα (BRL49653 and PG-J2) or solvent dimethyl sulfoxide were added in RPMI 1640 medium containing 5% calf serum delipopolysaccharidic with ultracentrifugation in KBr (1.21 g/ml) and subsequently treated with AG.1.18 resin (Bio-Rad) plus activated charcoal, and cells were incubated further for 36 h.

For NFκB and PPARγ co-transfection assays, transient transfections were performed in COS-1 cells at 50–60% confluency by the calcium phosphate co-precipitation procedure using a mixture of plasmids containing the indicated reporter (2 μg) and small interfering RNA (siRNA) plasmid (34) and 0.5 μg of a reference plasmid (pRLTK) into the pG3 luciferase expression vector (34), was used as a reporter vector. Monocyte-derived macrophages at day 12 of culture were transfected by the lipofection procedure (DOTAP; Boehringer Mannheim, Mannheim, Germany) with the reporter plasmid (2.5 μg) in 6-well culture dish in RPMI 1640 medium containing 1% Fetal Calf Serum (FCS) and 3% heat-inactivated horse serum. After overnight incubation, cells were washed with PBS and ligands for PPARγ, PPARα, and PPARδ were added (10 μM) into the culture medium. After 24 h at 37°C, cells were washed with PBS and ligands for PPARγ (Wy14,643, Chem-syn, Lenexa, KS) or PPARα (BRL49653 and PG-J2) or solvent dimethyl sulfoxide were added in RPMI 1640 medium containing 5% calf serum delipopolysaccharidic with ultracentrifugation in KBr (1.21 g/ml) and subsequently treated with AG.1.18 resin (Bio-Rad) plus activated charcoal, and cells were incubated further for 36 h.

Detection Of Apoptotic Cells—Monocyte-derived macrophages (12 days of culture) were incubated for 24 h at 37 °C in RPMI 1640 medium with 1% Fetal Calf Serum, TNFa, and IFNγ and/or Wy14,643, BRL49653, PG-J2, or solvent (Me2SO) and incubated for another 48 h. Luciferase activity was determined on cell extracts using a luciferase buffer (Promega, Madison, WI).

RESULTS

PPARα and PPARγ Are Expressed in Differentiated Human Monocyte-derived Macrophages—To study the expression of PPARα and PPARγ in human monocyte-derived macrophages, a qualitative RT-PCR analysis was performed using specific primers on RNA from freshly isolated human monocytes and macrophages at different stages of differentiation. RT-PCR specificity and functions of PPARα and PPARγ (Fig. 2B) was obtained by centrifugation at 13,000 rpm at 4 °C and protein concentrations were determined using the bicinchoninic acid assay (Pierce Interchim, Montlucon, France). Electrophoresis of the indicated amount of protein lysate was performed through a 10% polyacrylamide gel under reducing conditions (sample buffer containing 10 mDm dithiothreitol). Proteins were transferred onto nitrocellulose membranes and membranes were checked for equal loading by Ponceau red staining. Non-specific binding sites were blocked overnight at 4 °C with 10% skim milk powder in TBST (20 mM Tris, 55 mM NaCl, 0.1% Tween 20). Membranes were subsequently incubated for 4 h at room temperature in 5% skim milk-TBST containing rabbit polyclonal antibodies raised against N-terminal PPARα (amino acids 10–56), PPARγ peptides (33), or a monoclonal antibody against caspase-3 (Transduction Laboratories, Montlucon, France). Specificity of PPAR antibodies was checked by Western blot analysis using in vitro synthesized PPARα and PPARγ protein (Fig. 2B).

Using peroxidase-conjugated antibody, signals were visualized by chemiluminescence (Amer sham, Buckinghamshire, United Kingdom). Protein extraction and Western Blot Analysis—Cells were fixed (30 min at room temperature) in 2% paraformaldehyde in PBS (pH 7.4), washed in 0.1% Triton, 0.5% NaCl (pH 7.4) and incubated with 0.1% lysine to avoid nonspecific fluorescence. After permeabilization in cold methanol/aceton (1:1, v/v) for 5 min at room temperature, nonspecific staining was blocked by incubating the cells in TNO (0.01% Triton, 0.5% NaCl, 0.5% ovalbumin) with 1% preimmune goat serum. After incubation with anti-PPARα, anti-PPARγ, mouse monoclonal anti-CD68 antigen (clone KPI), or anti-CD86 antigen (clone T3–4B5) (Dako, Trappes, France) antibodies, proteins were visualized using secondary fluorescein isothiocyanate conjugated anti-rabbit or anti-mouse IgG antibodies using a LEITZ DMR fluorescence microscope.

Protein Extraction and Western Blot Analysis—Cells were washed twice in ice-cold phosphate buffer saline (PBS) and harvested in ice-cold lysis buffer containing PBS, 1% Triton X-100, and a freshly prepared protease inhibitor mixture (ICN, Orsay, France) (10 μg/ml AEBSF, 1 mg/ml leupeptin, 1 mg/ml pepstatin, 5 mg/mll EDTA-Na2) to which 1 μM phenylmethylsulfonyl fluoride was added. Cell homogenates were collected by centrifugation at 13,000 rpm at 4 °C and protein concentrations were determined using the bicinchoninic acid assay (Pierce Interchim, Montlucon, France). Electrophoresis of the indicated amount of protein lysate was performed through a 10% polyacrylamide gel under reducing conditions (sample buffer containing 10 mDm dithiothreitol). Proteins were transferred onto nitrocellulose membranes and membranes were checked for equal loading by Ponceau red staining. Non-specific binding sites were blocked overnight at 4 °C with 10% skim milk powder in TBST (20 mM Tris, 55 mM NaCl, 0.1% Tween 20). Membranes were subsequently incubated for 4 h at room temperature in 5% skim milk-TBST containing rabbit polyclonal antibodies raised against N-terminal PPARα (amino acids 10–56), PPARγ peptides (33), or a monoclonal antibody against caspase-3 (Transduction Laboratories, Montlucon, France). Specificity of PPAR antibodies was checked by Western blot analysis using in vitro synthesized PPARα and PPARγ protein (Fig. 2B). After incubation with a secondary peroxidase-conjugated antibody, signals were visualized by chemiluminescence (Amer sham, Buckinghamshire, United Kingdom). Protein extraction and Western Blot Analysis—Cells were fixed (30 min at room temperature) in 2% paraformaldehyde in PBS (pH 7.4), washed in 0.1% Triton, 0.5% NaCl (pH 7.4) and incubated with 0.1% lysine to avoid nonspecific fluorescence. After permeabilization in cold methanol/aceton (1:1, v/v) for 5 min at room temperature, nonspecific staining was blocked by incubating the cells in TNO (0.01% Triton, 0.5% NaCl, 0.5% ovalbumin) with 1% preimmune goat serum. After incubation with anti-PPARα, anti-PPARγ, mouse monoclonal anti-CD68 antigen (clone KPI), or anti-CD86 antigen (clone T3–4B5) (Dako, Trappes, France) antibodies, proteins were visualized using secondary fluorescein isothiocyanate conjugated anti-rabbit or anti-mouse IgG antibodies using a LEITZ DMR fluorescence microscope.
entiation), staying constant during the following maturation phases (days 6–10) and further increasing in fully differentiated macrophages after 12 days of culture (Fig. 1). In contrast to PPARα, PPARγ mRNA was not detectable by RT-PCR analysis in non-differentiated cells (Fig. 1A). However, PPARγ mRNA was induced during the first stages of macrophage differentiation staying present thereafter (Fig. 1A). Similar results of PPARγ expression were obtained by Northern blot analysis (data not shown).

To determine PPAR protein expression in monocytes and differentiated macrophages, Western blot analysis was performed using PPARα- and PPARγ-specific antibodies, as demonstrated by Western blot analysis using in vitro produced PPARα and PPARγ protein (Fig. 2B). A single band corresponding to PPARα was detected in undifferentiated monocytes (Fig. 2A). Furthermore, the amount of PPARα protein increased upon differentiation (Fig. 2A). By contrast, PPARγ protein was not detectable in monocytes, but its expression appeared in cells undergoing differentiation into macrophages (Fig. 2A). Therefore PPARα and PPARγ protein levels closely follow the changes in mRNA levels (Figs. 1 and 2).

Cytolocalization of PPARα and PPARγ—To identify the subcellular localization of PPARα and PPARγ in differentiating macrophages, immunofluorescence analysis was performed. Staining with PPARα antibody revealed immunoreactivity in monocytes as well as in macrophages during all phases of the differentiation process (Fig. 3, A, E, I, and O). Interestingly, labeling was detected primarily in the cytoplasmic compartment, indicating that PPARα protein resides predominantly in the cytoplasm of macrophages (Fig. 3, O). Consistent with the results from the Western blot experiments (Fig. 2A), no labeling with anti-PPARγ antibody was observed in monocytes (Fig. 3B). However, in differentiating cells, immunoreactive staining was observed in the nucleus (Fig. 3, L and P), indicating that PPARγ is predominantly nuclear localized in macrophages.

As a control for the monocyte-macrophage maturation process, monocytes and lymphocytes were identified using specific marker antibodies for macrophages (CD68) or lymphocytes (CD3). Along the differentiation process, cells became progressively reactive for the CD68 antibody and after 12 days of culture all adherent cells were CD68 positive (Fig. 3, D, H, N, and R). In contrast, whereas at day 0 a number of cells stained

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**FIG. 1.** PPARα and PPARγ mRNA are expressed upon differentiation of human monocytes into macrophages. RNA was isolated from human monocytes or macrophages after the indicated number of days in culture. A, RT-PCR analysis was performed using PPARα, PPARγ, and glyceraldehyde-3-phosphate dehydrogenase-specific primers (C-RT; C-PCR: negative controls for reverse transcriptase and PCR reaction, respectively). The size in bp of molecular weight markers is indicated. B, top, RNase protection analysis, yielding protected fragments of 188 and 170 bp corresponding to PPARα and internal control 36B4, respectively, was performed as described under “Experimental Procedures.” Middle, prolonged exposure reveals PPARα expression in undifferentiated monocytes. Bottom, PPARα transcripts were quantified as described under “Experimental Procedures” (R.A.U., relative absorbance units).

**FIG. 2.** PPARα and PPARγ proteins are expressed upon differentiation of human monocytes into macrophages. A, Western blot analysis of PPARα and PPARγ protein expression in human monocyte-derived macrophages. Proteins (50 μg) extracted from human monocyte-derived macrophages at the indicated number of days in culture were separated on a 10% SDS-polyacrylamide electrophoresis gel and blotted onto a nitrocellulose membrane. Immunodetection was performed using polyclonal anti-PPARα or anti-PPARγ antibodies and visualized using the chemiluminescence system as described under “Experimental Procedures.” B, Western blot analysis using in vitro produced PPARα and γ protein to determine the specificity of the PPARα and PPARγ antibodies.
positive for CD3, this staining decreased progressively throughout differentiation disappearing completely at day 12, thus indicating the absence of T cells after 12 days of culture (Fig. 3, C, G, M, and Q).

Endogenous PPARs Can be Ligand Activated—Since PPARs are transcription factors, it was determined whether endogenous PPARα and PPARγ are transcriptionally active in differentiated macrophages. Therefore, macrophages were transiently transfected with the PPRE-driven J3TKpGL3 luciferase reporter vector and cells were subsequently treated with PPARα- and PPARγ-specific ligands. Treatment with the PPARα ligand Wy14,643 at a concentration (10 μM) selectively activating PPARα, but not PPARγ, as measured by a co-transfection transactivation assay (data not shown), resulted in a 2-fold induction of luciferase activity (Fig. 4). Treatment with either BRL49653 or PG-J2 resulted in a stronger induction of luciferase activity which was dose-dependent (Fig. 4).

Induction of Macrophage Apoptosis by PPAR Activators—Interestingly, treatment of differentiated macrophages with BRL49653 or PG-J2 at concentrations within the range of their Kᵦ for PPARγ binding consistently induced pronounced changes of cellular morphology resulting eventually in cell death. Since these effects were very marked, were reproduced in several experiments using different macrophage preparations, and occurred primarily with the PPARγ ligands BRL49653 and PG-J2 at concentrations below those classically used in transactivation and adipocyte differentiation assays (and much less with the PPARα ligand Wy14,643 at 100-fold higher concentrations), it was analyzed whether PPAR activation of macrophages induced cellular apoptosis, a form of programmed cell death. Therefore, the induction of apoptosis was measured by the TUNEL assay in differentiated human monocyte-derived macrophages treated with either Wy14,643, BRL49653, or PG-J2 at a concentration and time point before changes in cell morphology became microscopically apparent. Both BRL49653 and PG-J2 induced an intense nuclear staining (Fig. 5, C and D), indicating the appearance of apoptotic cells. The number of cells staining positively after PG-J2 treatment increased in a dose-dependent fashion (data not shown),
similar as the activation of PPARγ transcriptional activity (Fig. 4). In contrast, when cells were treated with Wy14,643 at concentrations specifically activating PPARα, staining was comparable to solvent background (Fig. 5, A and B), indicating absence of apoptosis induction by Wy14,643. Next, it was analyzed whether PPAR activators could promote apoptosis in the presence of TNFα, a known inducer of macrophage apoptosis. Activation of differentiated macrophages by TNFα and IFNγ resulted in a significant number of cells staining positive by TUNEL labeling (Fig. 5E). When macrophages were simultaneously treated with TNFα/IFNγ and BRL49653 or PG-J2, nuclear labeling was observed in a high number of cells which stained much more intensely than in the presence of each compound alone (Fig. 5, G and H). Interestingly, in the presence of TNFα/IFNγ, treatment with Wy14,643 resulted also in a significant number of cells stained positive by TUNEL labeling (Fig. 5F). Quantitative analysis of apoptosis showed that treatment with BRL49653 resulted in an approximate 2-fold induction of cellular DNA fragmentation compared with solvent, whereas Wy14,643 was without effect (Fig. 6). Activation of macrophages with TNFα/IFNγ resulted in a 2-fold induction of cellular DNA fragmentation, an effect which was enhanced in cells simultaneously treated with TNFα/IFNγ and either BRL49653 or Wy14,643 (Fig. 6).

Since cells undergoing apoptosis execute the death program by activating cysteine proteases of the caspase family (36), it was analyzed whether treatment of differentiated macrophages with PPAR activators resulted in the activation of caspase-3/cystein protease protein 32, a key executioner of apoptosis (37). Western blot analysis showed the appearance of the enzymatically active 17- and 12-kDa proteolytic cleavage products of the inactive 32-kDa caspase-3 precursor in differentiated macrophages treated for 24 h with either BRL49653, PG-J2, or TNFα/IFNγ alone (Fig. 7). Interestingly, compared with each compound alone, simultaneous activation of cells with TNFα/IFNγ and BRL49653 or PG-J2 significantly increased the amount of 17- and 12-kDa cleavage products (Fig. 7). In the presence of both TNFα/IFNγ and Wy14,643 a similar but less pronounced increase was observed (Fig. 7). Altogether these data indicate that PPAR activators potentiate TNFα-induced apoptosis.

Since during the preparation of this article it was shown that PPARγ activators can negatively regulate transcription from a NFκB-response element driven promoter (38) and since NFκB via its p65/RelA subunit has been shown to protect macrophages from TNFα-induced cell death (28–30), the effects of
PPARs are lipid-activated transcription factors that function as important regulators of lipid and glucose metabolism, adipocyte differentiation, and energy homeostasis. PPARα and PPARγ mediate, respectively, the action of the hypolipidemic fibrates and the anti-diabetic thiazolidinediones. PPARs therefore play a role in metabolic conditions, such as dyslipidemia and type II diabetes, leading to atherosclerosis development. Several indirect observations suggest that PPAR activators not only regulate plasma cholesterol and triglyceride concentrations, major risk factors for atherosclerosis, but may also exert an activity at the level of the vascular wall. First, in cholesterolfed rabbits, treatment with the PPAR activator fenofibrate decreases atherosclerotic plaque formation in thoracic aorta, in the absence of any effect on lipoprotein levels (39), suggesting a direct anti-atherosclerotic action of fibrates, most likely by negatively interfering with the antiapoptotic NFκB signaling pathway.

DISCUSSION

PPARs are lipid-activated transcription factors that function as important regulators of lipid and glucose metabolism, adipocyte differentiation, and energy homeostasis. PPARα and PPARγ mediate, respectively, the action of the hypolipidemic fibrates and the anti-diabetic thiazolidinediones. PPARs therefore play a role in metabolic conditions, such as dyslipidemia and type II diabetes, leading to atherosclerosis development. Several indirect observations suggest that PPAR activators not only regulate plasma cholesterol and triglyceride concentrations, major risk factors for atherosclerosis, but may also exert an activity at the level of the vascular wall. First, in cholesterolfed rabbits, treatment with the PPARα activator fenofibrate decreases atherosclerotic plaque formation in thoracic aorta, in the absence of any effect on lipoprotein levels (39), suggesting a direct anti-atherosclerotic action of fibrates. Second, in the BCAIT (Bezafibrate Coronary Atherosclerosis Intervention Trial) (40) and LOCAT (Lopid Coronary Angiography Trial) (41) intervention trials, fibrate treatment prevented the progression of coronary atherosclerosis, in the absence of a marked lowering of plasma atherogenic lipoprotein concentrations. Third, treatment with the PPARγ activator troglitazone inhibits vascular smooth muscle cell proliferation (42) and decreases the intima and media thickness of carotid arteries in man (43). Finally, although no in vivo data are available for PPARγ, PPARα has been shown to play a role in anti-inflammatory control, since PPARα knockout mice exhibit a prolonged inflammatory response (10). Therefore, PPAR activators may not only interfere with atherogenesis by decreasing plasma lipid concentrations, but also by exerting anti-inflammatory activity at the vascular wall.

Macrophages are key players in vascular wall inflammation and atherogenesis. In this report, we demonstrate the expression of both PPARα and PPARγ in differentiated human macrophages. Furthermore, we show that PPARα expression is already detectable in monocytes and increases along the differentiation process into macrophages. By contrast, PPARγ expression is not detectable in monocytes, but is strongly induced upon differentiation into macrophages. Recently it was reported that PPARγ may promote monocyte/macrophage differentiation (44). Furthermore, PPARγ is activated by oxidized lipid components present in atherogenic oxidized low density lipoprotein and PPARγ activation results in the induction of macrophage scavenger receptor CD36 expression and enhanced uptake of oxidized low density lipoprotein (45). These observations may point to a role for PPARγ in foam cell formation (45). Since PPARγ expression is undetectable in circulating human monocytes and appears only several hours after induction of differentiation, it is unlikely that PPARγ is involved in the initial differentiation process. Furthermore, in these studies maximal activity on monocyte/macrophages was observed with the less specific PPARγ agonist PG-J2 in the presence of 9-cis-retinoic acid receptor agonists (44), suggesting that activation of additional signaling pathways is required to stimulate these differentiation processes. Nevertheless, PPARγ appears to be an important component of further downstream processes in macrophage differentiation and function.

Our transient transfection experiments demonstrate that endogenous PPARs are transcriptionally activated by their ligands at K<sub>g</sub> concentrations confirming a functional role for PPARs in transcription control of human macrophages. Whereas BRL49653 and PG-J2 stimulate gene transcription severalfold, Wy14,643 appears less active. This low activation of PPARα by Wy14,643 might be due to either the small amount of PPARα in the nucleus compared with PPARγ, or to the relative weaker affinity of Wy14,643 to human PPARα than BRL49653 to PPARγ (9, 10). The observation that PPARs are transcriptionally active raises the question on which are the target genes for PPARs in human macrophages. Since PPARs regulate lipid and lipoprotein metabolism, genes involved in macrophage lipid metabolism, such as lipoprotein lipase, which is expressed in macrophages and foam cells (44, 45), are likely candidates. Interestingly, a PPRE was identified in the human lipoprotein lipase gene promoter, via which fibrates and thiazolidinediones regulate lipoprotein lipase expression in liver and adipose tissue, respectively (46). Furthermore, the macrophage scavenger receptor CD36, which also functions as a fatty acid transporter in adipose tissue (47), has been identified as a PPARγ target gene in macrophages (44). In addition, PPARs also regulate genes involved in inflammatory control, since PPAR activators have been shown to inhibit the activation of macrophages by interfering with the transcriptional induction of genes such as TNFα, iNOS, and gelatinase B by inflammatory agents (38, 48).

In the present report, we demonstrate a role for PPARs in the control of macrophage apoptosis. PPARγ activation results in a pronounced induction of apoptosis, whereas PPARα activators are much less pro-apoptotic in unstimulated differentiated human macrophages. This induction of apoptosis by PPARγ activators occurs at concentrations well within the range of their K<sub>g</sub> values. By contrast, the recently reported anti-inflammatory activity of synthetic PPARγ activators are observed only at much higher concentrations (10<sup>2</sup>-10<sup>3</sup>-fold
above the $K_D$ (38). Similarly, induction of scavenger receptor CD36 and foam cell formation occurs at severalfold higher concentrations (44). Therefore, the induction of apoptosis might be the physiologically primary event, resulting in a secondary general inhibition of macrophage activation. Alternatively, PPARγ activators may have a dual activity: at low concentrations they may be proapoptotic and, as a result, prevent foam cell formation, whereas at high concentrations they may favor foam cell formation through induction of scavenger receptors in those macrophages which rescue from apoptosis.

Apoptosis induction by PPARγ ligands was more pronounced in macrophages activated with TNFα and IFNγ. Furthermore, PPARα ligands induced apoptosis only in activated macrophages. In macrophages, TNFα affects cell survival via two distinct signaling pathways with opposing functions: a proapoptotic NFκB-independent and an anti-apoptotic NFκB-dependent pathway (49). Indeed, in macrophages NFκB activation by TNFα induces a negative feedback mechanism protecting the cells from TNFα-induced apoptotic cell death (30). PPARγ-mediated apoptosis induction could therefore occur either via stimulation of the proapoptotic effects of TNFα, or alternatively by interfering negatively with the protective NFκB signaling pathway. Interestingly, recently it was suggested that, in murine macrophages, PPARγ may inhibit the inflammatory response in part by antagonizing the activities of transcription factors of the AP-1, STAT, and NFκB families (38). Therefore it is likely that PPARγ induces cellular apoptotic death by interfering negatively with the antiapoptotic NFκB signaling pathway. Our results from co-transfection experiments show that PPARγ inhibits the transcriptional activity of p65/RelA, a key component of the anti-apoptotic NFκB signaling pathway (28). Thus, in addition to the previous described inhibition of transcriptional activity of members of the E2F/DP family (50), interfering with the NFκB signaling function constitutes a novel mechanism through which PPARγ may interfere with cell cycle control and survival.

The observation that PPARRα activators only induce apoptosis of activated macrophages is intriguing and may be of importance for the treatment of atherosclerosis. Although the reasons for this selective effect of PPARRα, as opposed to PPARγ activators are presently unclear, this is likely to be due to a lower level of PPARRα expression, since its expression is higher than PPARγ in macrophages in all steps of the differentiation process. Furthermore, recent work from our laboratory in human smooth muscle cells indicates that PPARRα can also interfere negatively with the p65/RelA subunit of the NFκB signaling pathway (51). The major difference between PPARγ and PPARRα in macrophages is that PPARγ protein is predominantly localized in the nucleus of differentiated macrophages, whereas PPARRα resides primarily in the cytoplasmic compartment. Therefore the different subcellular localization of PPARRα and PPARγ in macrophages may be involved in the differential effects of PPARRα and PPARγ on macrophage apoptosis. TNFα and IFNγ may modify PPAR activity and cytolocalization through phosphorylation leading to a modified activity on p65/RelA. Indeed, PPARs are phosphoproteins whose activity has been shown to be modulated through phosphorylation induced by factors as insulin and TNFα (52, 53).

Both unspecific cell necrosis as well as programmed cell death by apoptosis occur in advanced atherosclerotic lesions (54). Macrophages and smooth muscle cells make up the bulk of the activated cells in the atherosclerotic plaque (27, 54, 55) and proapoptotic proteins, such as caspase-3, are expressed in and colocalize with the apoptotic macrophages, T-lymphocytes, and smooth muscle cells in human atheroma (56). It remains to be determined whether induction of macrophage apoptosis by PPAR activators also occurs in vivo in the atherosclerotic plaque and what the (patho)physiological consequences might be.

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