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Arginase I Induction by Modified Lipoproteins in Macrophages: A Peroxisome Proliferator-Activated Receptor-γ/δ-Mediated Effect that Links Lipid Metabolism and Immunity

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Macrophages are phagocytic cells that play essential roles in innate immunity and lipid homeostasis. The uptake of modified lipoproteins is an important early event in the development of atherosclerosis. We analyzed the ability of modified low-density lipoprotein (LDL) (oxidized and acetylated) to alter the expression and activity of arginases (ArgI and ArgII) in macrophages. We show that ArgI expression is potently induced by both oxidized and acetylated LDL in macrophages. We further show that this effect is mediated by peroxisome proliferator-activated receptors (PPAR). ArgI expression is highly responsive to agonists for PPARγ and PPARδ but not PPARα. Moreover, the induction of ArgI by both PPAR agonists and IL-4 is blocked in macrophages from PPARγ- and PPARδ-deficient mice. Functionally, PPAR activity induces macrophage activation toward a more Th2 immune phenotype in a model of *Leishmania major* infection. We show that PPARγ and -δ ligands promote intracellular amastigote growth in infected macrophages, and this effect is dependent on both PPAR expression and Arg activity. Collectively, our results strongly suggest that ArgI is a key marker of the alternative program triggered by PPAR in macrophages. (*Molecular Endocrinology* 22: 1394–1402, 2008)

ATHEROSCLEROSIS IS A chronic inflammatory disease triggered by several factors including hypercholesterolemia (1). One of the initial events in the development of atherosclerosis is the uptake and oxidation of low-density lipoproteins (LDL) by resident macrophages in the arterial wall. In the face of hypercholesterolemia, an increased number of monocytes are recruited to the subendothelial space, where they take up LDL and become the so-called foam cells (2).

The uptake and internalization of oxidized LDL (oxLDL) and their lipid content induce important changes in macrophage gene expression, especially those that control cellular lipid homeostasis (3). At the same time, the conversion of these macrophages into foam cells triggers a dramatic change in their activation stage. Therefore, it is reasonable to hypothesize that the cellular response to modified lipids could alter the inflammatory properties of macrophages.

An important family of transcription factors that regulates the expression of genes linked to lipid metabolism is the peroxisome proliferator activated receptor (PPAR) subfamily of nuclear receptors. Peroxisome proliferator-activated receptors (PPAR) are ligand-dependent transcription factors that heterodimerize with the retinoid X receptor (RXR) (4). Early studies showed that PPARγ promotes macrophage gene expression and uptake of oxLDL (5). Several reports have also demonstrated that PPARγ inhibits the expression of proinflammatory genes, including cytokines and inducible nitric oxide synthase (iNOS) (6, 7).

Arginases catalyze the hydrolysis of L-arginine to L-ornithine and urea, and both isoforms are constitutive in resting mouse macrophages (8). Previous studies demonstrated that arginase I is induced by Th2-derived cytokines in macrophages (9) and this enzyme has therefore been considered one of the hallmarks of alternative macrophage activation (10). However, arginases are also coinduced with iNOS and other acute

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Abbreviations: acLDL, Acetylated LDL; BMDM, bone marrow-derived macrophages; 9-cRA, 9-*cis*-retinoic acid; iNOS; inducible nitric oxide synthase; LDL, low-density lipoprotein; LXR, liver X receptor; oxLDL, oxidized LDL; nLDL, native LDL; nor-NOHA, N^{ω}-hydroxy-nor-L-arginine; PPAR, peroxisome proliferator-activated receptors; RXR, retinoid X receptor; WT, wild type.

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immune enzymes under proinflammatory challenge such as bacterial lipopolysaccharide (9).

The potential role of arginases in atherosclerosis is currently a subject of intense investigation. The majority of these studies have analyzed the contribution of arginase in vascular endothelial cells and smooth muscle cells. For example, Ignarro and colleagues (11) demonstrated that arginase I induction in aortic smooth muscle cells by Th2-derived cytokines promoted cell proliferation. Moreover, both arginases are expressed in atherosclerotic lesions as shown in models of hyperlipidemic rabbits (12). Arginase II is expressed predominantly in endothelial cells and reduces nitric oxide release in atherosclerotic mice (13). Remarkably, arginase II expression and activity are up-regulated by oxLDL in human aortic endothelial cells (14). In addition, arginase II was found to be a direct target of liver X receptor (LXR) in macrophages and contributes to some of the antiinflammatory effects observed with LXR agonists on macrophage activation (15).

The aim of the present work was to study the regulation of arginase expression by modified lipoproteins and its derivatives in macrophages. We show that modified LDL induce arginase I expression through PPARγ and -δ activation. The induction of arginase I by modified LDL is mimicked by PPARγ and -δ synthetic ligands and blocked by PPAR-specific antagonists. Moreover, response of arginase I expression to synthetic ligands is lost in PPARγ- and PPARδ-deficient cells. Finally, we show that arginase I induction by PPAR favors the growth of *Leishmania major* inside macrophages, suggesting that this enzyme contributes to an immune deactivation program triggered by PPAR.

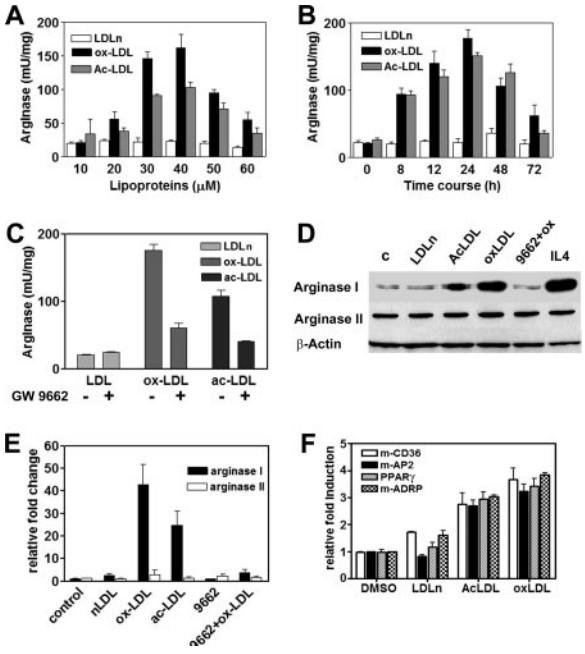

Fig. 1. Modified Lipoproteins Induce Arginase I in BDMD

A, Analysis of arginase activity in cells treated with increasing concentrations of oxLDL, acLDL, and nLDL for 24 h; B, time course of arginase induction in BMDM treated with 30 μM of each type of LDL; C, macrophages were pretreated overnight with 1 μM GW9662 and then stimulated with 30 μM modified LDL for 24 h; D, arginase I and II protein expression. Proteins were analyzed as described in *Materials and Methods*. β-Actin was used as loading control. IL-4, (2.5 ng/ml, 24 h) was used as positive control. c, Control; 9662+ox; GW9662 plus oxLDL. E, Arginase I and arginase II expression levels determined by TaqMan quantitative real-time PCR. The experimental conditions for D and E were identical to those in C. Data are expressed as the mean ± SD of triplicate measurements. F, Macrophages were loaded with LDL for 24 h and then used for RNA extraction. CD36, AP2, PPARγ, and ADRP gene expression were analyzed by real-time PCR SYBR Green assays and normalized to 36B4 gene expression.

RESULTS

OxLDL Induces Arginase I in Bone-Marrow-Derived Macrophages

The metabolic fate of L-arginine in macrophages between NOS and arginase has been demonstrated to be regulated by the activation stage of the cell (16). In the context of atherosclerosis, the internalization of modified lipoproteins inhibits the inducible form of NOS (17), whereas in endothelial cells, arginase is up-regulated in response to oxLDL (14). To analyze the regulation of arginase isoforms in *in vitro* lipid-loaded cells, we measured arginase activity in macrophages treated with increasing concentrations of oxLDL and acetylated LDL (acLDL) (Fig. 1A). Both modified lipoproteins were able to increase arginase activity in a dose-dependent manner, with 30 μM and 24 h treatment being the optimal conditions (Fig. 1B). The response was specific for modified LDL because native LDL (nLDL) did not increase arginase levels significantly. Resting bone marrow-derived macrophages (BMDM) constitutively express arginase I and II (Fig. 1D). Modified lipoproteins selectively induced arginase I mRNA (Fig. 1E) and protein (Fig. 1D).

It is well documented that uptake and internalization of oxidized lipoproteins activate PPARγ expression and activity in macrophages (18). Thus, to better understand the regulation of arginase I by modified lipoproteins, we treated lipid-loaded cells with the PPAR antagonist GW9662 (19). Our results show that pretreatment of macrophages with 1 μM GW9662 potently reduced the induction of arginase I activity by oxLDL and acLDL (Fig. 1C) and also blocked the induction of ArgI protein (Fig. 1D) and mRNA (Fig. 1E). Moreover, the induction of the enzyme by oxLDL was also inhibited by 1 μM of each, GW5393 (20) and T0070907 (21), two structurally different PPAR antagonists (supplemental Fig. 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org).

Although the PPAR antagonists used here are known to be specific for PPARγ, at the concentrations used in this work (1 or 2 μM), they are inhibiting all PPAR isoforms, according to the references cited above. The mode of action of GW9662 and T0070907 is to covalently modify a cysteine residue in the ligand-binding domain of PPAR (19, 21), whereas GW5393 was designed as a PPAR ligand unable to recruit the coactivator cAMP response element binding protein-binding protein (CBP) to the receptor (20). Because murine macrophages constitutively express more than one PPAR isoform, we used these molecules as pan-antagonists to be sure that the induction of arginase I by modified LDL were dependent on PPAR activation.

Collectively, these results strongly suggest that the induction of arginase I in foam cells is mediated through a PPAR-dependent mechanism. Indeed, under the same experimental conditions, modified lipoproteins triggered the expression of PPARγ and others known PPAR target genes such as CD36, AP2 (22), or ADRP (adipose differentiation-related protein) (Fig. 1F). These genes have been found to be induced by oxLDL and up-regulated in atherosclerotic lesions (23, 24).

It is important to note that in Fig. 1, A and B, the levels of arginase-specific activity were calculated as a function of protein concentration. We paid special attention to this aspect because several reports have demonstrated that oxLDL induces macrophage proliferation (25). In our experimental conditions, we did not observe changes in protein concentration that could significantly affect enzyme-specific activity.

Arginase I Is Selectively Induced by PPARγ and -δ Agonists

To identify the PPAR isoforms responsible for arginase I expression, we next treated primary macrophages with different PPAR agonists. The results are presented in Fig. 2. Interestingly, the natural activators of PPAR, such as the eicosanoid 15dPGJ2 and the oxidized derivative of linoleic acid, (13-HODE), dose-dependently induced arginase I (Fig. 2A). We also used the thiazolidinedione rosiglitazone (Fig. 2B), a potent PPARγ synthetic activator and a widely used insulin sensitizer (26). This drug also induced arginase I and showed maximal effects at concentrations above 1 μM. Finally, GW7845 and GW1929, two non-thiazolidinedione, potent synthetic PPARγ agonists, also significantly increased arginase activity and expression.

We also treated cells with PPARδ and PPARα agonists under the same experimental conditions. Remarkably, the specific PPARδ agonist GW0742 (27) induced arginase activity very efficiently, showing significant induction at 25–100 nM concentrations (Fig. 2C). In contrast, treatment with low micromolar concentrations of two different PPARα ligands (GW7647 and WY14643) did not result in significant changes in arginase activity (data not shown). These results are consistent with the minor expression of PPARα in mouse macrophages, in agreement with previous reports (28). Both RNA expression levels measured by real-time quantitative PCR (Fig. 2D) and protein expression (Fig. 2E) confirmed the induction of arginase I by PPARγ and -δ ligands, whereas the two PPARα activators did not increase protein or mRNA levels. Together, these results demonstrate that arginase I expression and activity are specifically modulated by PPARγ and -δ activators in primary macrophages.

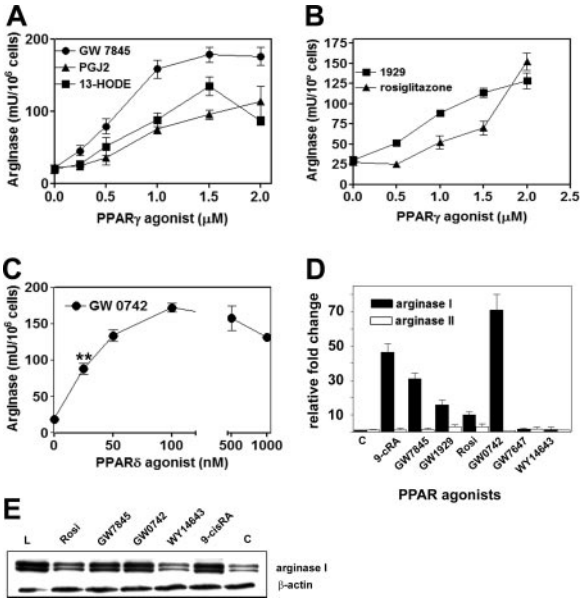

Fig. 2. PPARγ and -δ Agonists Induce Arginase I in BMDM

A and B, Dose response of arginase induction with different PPARγ agonists. Cells were treated with agonists for 24 h and enzyme activity measured as described in *Materials and Methods*; C, arginase activity in cells treated with increasing concentrations of PPARδ agonist GW0742 for 24 h; D, arginase I and arginase II mRNA expression levels from cells treated with the indicated ligands for 24 h. The relative fold induction was calculated by using 18S mRNA as internal control and TaqMan real-time quantitative PCR; E, protein expression by Western blot. The concentrations of agonists used in D or E were 1 μM PPARγ agonists rosiglitazone (Rosi), GW1929, or GW7845; 100 nM PPARδ agonist GW0742; 2 μM of the PPARα agonists GW7647 and WY14643, and 0.5 μM 9-cRA. Lm Liver extract, used as positive control for arginase I. β-Actin was used as loading control. Data represent the mean values and SD of replicate cultures of four independent experiments. **, $P < 0.01$ by the Student's *t* test at 25 nM, compared with the control value.

The Induction of Arginase by PPARγ and -δ Ligands Is Increased by RXR Agonists

Because PPAR/RXR heterodimers can be activated through both the PPAR and the RXR arms of the complex, we analyzed arginase activity using the RXR ligand 9-*cis*-retinoic acid (9-cRA), in the presence or absence of different PPAR agonists. The data of Fig. 3

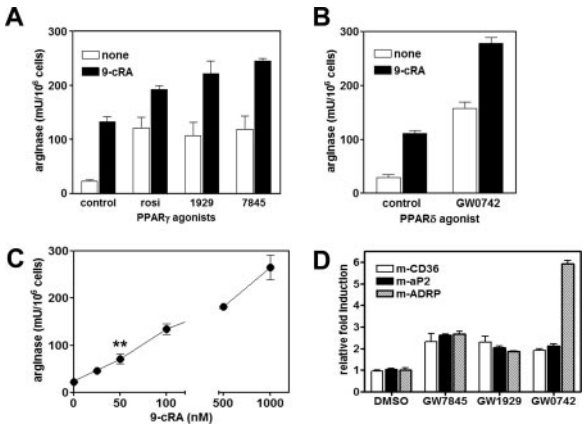

Fig. 3. The Induction of Arginase by PPARγ and -δ Ligands Is Increased in the Presence of the RXR Ligand 9-cRA

A, Arginase activity of cells treated with PPARγ agonists, added at the same dose as in Figs. 1 and 2 (*white bars*) or in combination with 100 nM 9-cRA for 24 h; B, arginase activity in cells treated with 100 nM GW0742 with or without 100 nM 9-cRA for 24 h; C, dose response of arginase induction by 24 h treatment with increasing concentrations of 9-cRA; E, mRNA relative induction of PPAR target genes CD36, AP2, and ADRP. BMDM were treated for 24 h with 1 μM concentration of the indicated PPAR agonists, and relative gene expression was analyzed by real-time PCR SYBR Green assays, normalized to 36B4 expression, as previously described. **, $P < 0.01$ by the Student's *t* test at 50 nM, compared with the control value.

demonstrate an additive effect on arginase activity by cotreatment with PPARγ and -δ activators (Fig. 3, A and B). On the other hand, *cis*-RA alone also induced arginase activity at concentrations from 50 nM (Fig. 3D). As a control, we verified that PPAR ligands increased the expression of the known target genes CD36, AP2, and ADRP (24, 29, 30) by real-time PCR (Fig. 3E).

Arginase I Induction Is Inhibited by PPAR Antagonists and Reduced in Cells from PPARγ and PPARδ Knockout Mice

To confirm that the induction of arginase I expression by PPAR agonists was receptor dependent, we used two different experimental approaches. First, arginase activity and protein levels were significantly inhibited by two structurally unrelated PPAR antagonists, GW9662 and GW5393 (Fig. 4, A and B). Second, we analyzed macrophages with conditional disruption of PPARγ and macrophages from PPARδ-deficient mice. Macrophages from C57BL/6 wild-type (WT) mice showed increased mRNA expression of arginase I when treated for 12 h with 100 nM PPARγ agonist GW7845 or with 100 nM GW0742 (Fig. 4C). However, arginase I induction was completely abrogated in cells from either PPARγ0 or

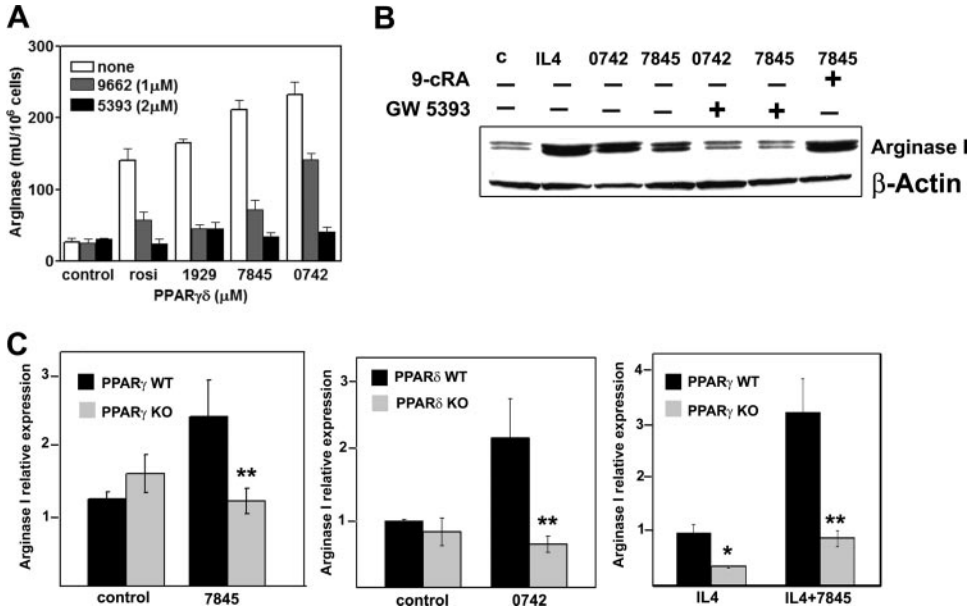

Fig. 4. Arginase I Induction by PPARγ and -δ Is Inhibited by PPAR Antagonists and Reduced in Macrophages from PPARγ- and -δ-Deficient Mice

A, BMDM were pretreated overnight with the PPAR antagonists GW9662 or GW5393 and then triggered with the corresponding agonists for 24 h. After this time, arginase activity was measured in cell lysates. B, Immunoblots show arginase I protein inhibition by PPAR antagonists and increased amount of protein in the presence of PPAR-RXR heterodimer. IL-4 is shown as positive control. C, Arginase I relative expression in PPARγ WT and KO peritoneal macrophages with or without 100 nM GW7845 for 12 h or PPARδ WT and KO cultures treated with 100 nM 0742 for 12 h. Finally, the relative induction of arginase I to IL-4 was analyzed in cells from PPARγ WT and KO pretreated overnight with 2.5ng/ml IL-4 and then with the PPARγ ligand for another 12 h. **, $P < 0.01$ by the Student's *t* test for arginase I expression by PPARγ and -δ agonists in experiments with macrophages from PPARγ- and PPARδ-deficient mice *vs.* the WT mice; *, $P < 0.05$ for arginase I expression by IL-4 in cells from PPARγ WT cells compared with those from the knockout.

PPARδ-deficient mice. Moreover, because arginase I is induced by IL-4 in murine macrophages, we used this Th2-derived cytokine as a positive control in all our experiments.

It has also been documented that IL-4 induces PPARγ expression in macrophages (31). Therefore we checked whether arginase induction by IL-4 could be inhibited by PPAR antagonists. Pretreatment with either GW9662 or GW5393 resulted in more than 50% inhibition of enzyme activity (supplemental Fig. 1). As expected, expression of arginase I mRNA by IL-4 was highly increased by cotreatment with GW7845. Furthermore, the synergistic effect of GW7845 on IL-4-induced arginase I expression was completely suppressed in PPARγ-deficient macrophages.

Together, these results demonstrate that arginase I is specifically induced by activation of PPARγ and -δ isoforms in macrophages.

PPAR Ligands Promote *L. major* Growth in Macrophages in an Arginase-Dependent Manner

The mouse model of *L. major* infection between the susceptible BALB/c and resistant C57BL/6 strains is one of the best characterized examples in which macrophage arginase I, induced in the context of a predominant Th2 response in susceptible mice, is triggering the growth of parasites inside macrophages (32). To determine the functional impact of PPAR-dependent arginase regulation for immune responses, we treated *Leishmania*-infected BMDM with PPAR ligands. The results presented in Fig. 5 clearly demonstrate that both GW7845 and GW0742, when added *in vitro* to infected macrophages, significantly increased the growth of intracellular *Leishmania* amastigotes (Fig. 5, A and B, and supplemental Fig. 2). Moreover, *Leishmania* growth was prevented by pretreatment with 2 μM PPAR antagonist GW5393 or by adding

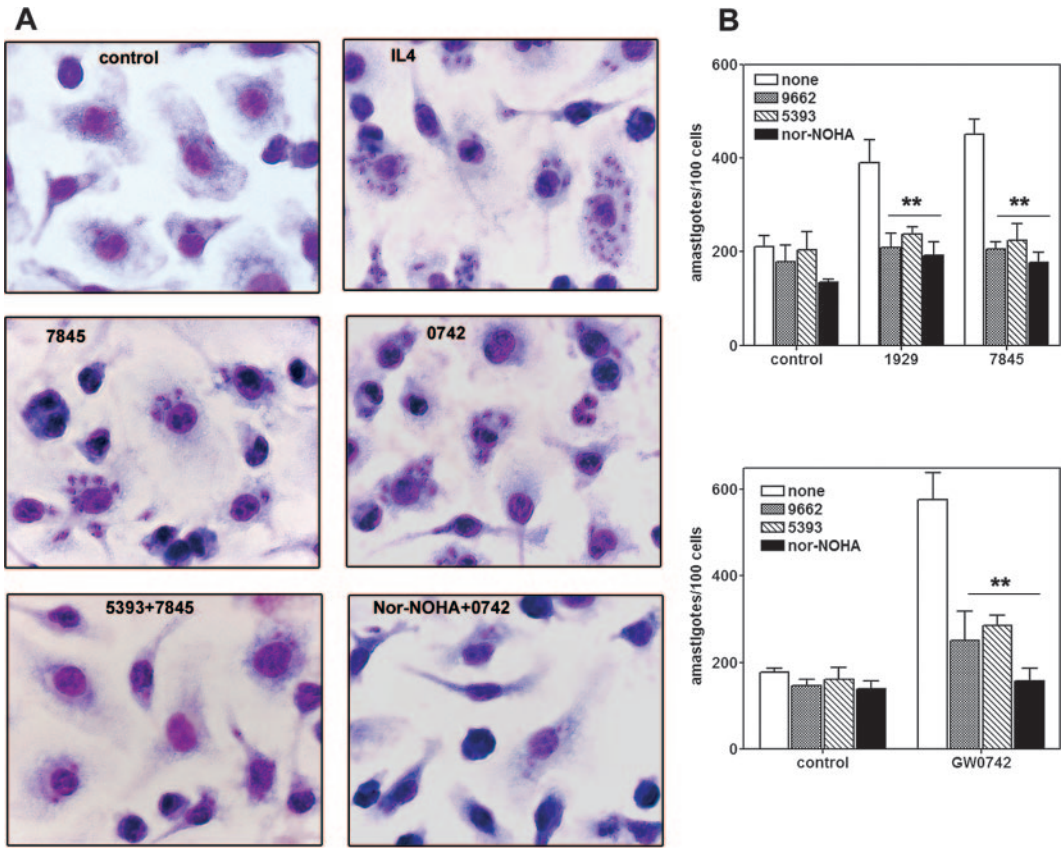

Fig. 5. PPARγ/δ-Dependent Arginase I Induction Promotes the Growth of *L. major* Inside Macrophages

A, Micrographs of *L. major*-infected BMDM. Cells were treated with PPAR agonists (1 μM 7845 or 100 nM 0742) for 2 h or alternatively pretreated with 1 μM concentration of each antagonist for 24 h and PPAR agonist during 2 h and finally infected with stationary phase *L. major* promastigotes for 24 h. After this time, cultures were stained as described in *Materials and Methods*. Original magnification, ×100. B, Enumeration of intracellular amastigotes in infected BMDM. Data are the results of counting the parasites from micrographs of A, where the *errors bars* represent results obtained from three independent macrophage infections performed in triplicate. *White bars* are control infected cultures or cells pretreated 2 h with PPAR agonists, infected, and counted 24 h after infection. PPAR antagonists were added 24 h before the agonists, and 100 μM Nor-NOHA (*black bars*) was added at the same time as the agonists. **, $P < 0.01$ by the Student's *t* test of intracellular parasite counts of infected cells treated with PPAR ligands alone compared with those pretreated with PPAR antagonists of nor-NOHA.

N^{ω}-hydroxy-nor-L-arginine (nor-NOHA), the specific arginase inhibitor, together with either the PPARδ agonist GW0742 (Fig. 5, A and B) or PPARγ ligand GW7845 (supplemental Fig. 2). We also show the potent induction of parasitic growth achieved in the presence of IL-4 as a positive control for arginase I induction. Intracellular amastigote growth was strictly correlated with the levels of macrophage arginase I activity. In cells cotreated with PPAR/RXR ligands (supplemental Fig. 2), the amount of intracellular parasites was similar to those infected in the presence of IL-4, and both effects were reverted by Nor-NOHA.

Together, these results establish a role for PPARγ and PPARδ in arginase I expression and alternative macrophage activation.

DISCUSSION

The role of arginase in the context of vascular pathology has recently become an area of intense investigation (33). Both arginase isoforms are present in human and mouse atherosclerotic lesions (34), and recent studies have established a direct correlation between arginase I polymorphisms and the risk of myocardial infarction (35). However, the mechanisms that regulate arginase expression in macrophages are not completely understood.

Here we have analyzed the expression of arginase isoforms in lipid-loaded macrophages. We showed that low doses of modified LDL (oxLDL and acLDL) induce arginase I expression and that this effect is mediated by activation of PPAR signaling. Finally, we showed that induction of arginase expression by the PPAR pathway renders macrophages more susceptible to *L. major* infection. These studies demonstrate that regulation of cellular metabolism by PPAR have a direct consequence for immune responses.

Previous studies have shown that PPARγ as well as arginase I expression is induced by the Th2-derived cytokine IL-4 (31, 9), providing another link between the two genes. Furthermore, PPARγ and arginase I may also be connected in the context of inflammation.

Activation of PPARγ in macrophages leads to transcriptional repression of a cohort of inflammatory genes induced by external insults, including iNOS, the inducible form of NOS (36). Arginase and iNOS share L-arginine as a substrate, and both enzymes are believed to play reciprocal roles when both are present in immune cells (37). Finally, arginase II has been shown to be a target of LXR (15), another nuclear receptor previously shown to be up-regulated by PPAR ligands in human macrophages (38).

Previous studies have also demonstrated that arginase II is regulated by oxLDL in human aortic endothelial cells (14). Arginase II induction in the Ryoo *et al.* (14) model contributed to endothelial dysfunction by down-regulation of NO production. In contrast, in our experimental conditions, the addition of oxLDL to resting macrophages did not change either iNOS arginase II expression or nitric oxide release (17), and thus, arginase I is the only arginine-metabolizing enzyme induced in our system.

The demonstration that arginase I expression is regulated by PPARγ/δ in lipid-loaded cells reinforces previous studies showing that PPAR contributed to the modification of macrophage functions and the reprogramming of their activation status. Interestingly, arginase I induction has been proven to be a major component of this alternative status, triggered by a Th2-predominant immune response (16). Our results indicate that macrophage PPAR activity impacts the balance of Th1/Th2 responses through specific induction of arginase I expression and activity. During the preparation of the present manuscript, Chawla and colleagues (39) reported the identification of a peroxisome proliferator response element in the promoter region of arginase I. Their results also suggest that PPAR activity participates in transcriptional programs that switch the macrophage toward an alternative activation state.

We showed here using an *L. major* infection model that arginase induction by PPAR changes macrophage functions to a more permissive stage that is suitable for parasite growth. This activation status closely resembles the one triggered by a Th2 response, because the magnitude of parasite proliferation in the presence of PPAR ligands is comparable to that achieved in the presence of IL-4. Inhibition of parasite growth by both PPAR antagonists and arginase inhibition clearly demonstrates that these nuclear receptors are key factors in macrophage alternative activation. Our results are consistent with previous studies by Kopf and colleagues (40) in which dyslipidemia inhibited protective Th1-type immunity and increased host susceptibility to *L. major* infection.

Recently, macrophage arginase I has been identified as candidate gene for atherosclerosis resistance (41). Arginase I is highly expressed in rabbits genetically resistant to atherosclerosis. PPAR agonists exert anti-atherogenic roles in murine models of atherosclerosis. We hypothesize that arginase I induction in foam cells may inhibit atherogenic inflammation. Arginases compete for arginine with NOSs. Therefore, the induction of arginase in foam cells may counter the accumulation of reactive nitrogen intermediates that contribute to the inflammatory process during plaque development. Future studies will be required to test the potential roles of macrophage arginase I activity in atherosclerosis.

It is important to note that arginase I expression is triggered efficiently by PPARγ and PPARδ activity. PPAR have been recently associated with protective pathways that control cellular repair (reviewed in Ref. 42). PPARγ activation potentiates antiinflammatory and antifibrotic actions, whereas PPARδ activation induces cellular proliferation and enhances the expression of fibrotic markers. Therefore, we speculate that induction of arginase I by PPAR could have different

consequences depending on the stage of atherosclerosis development. At early stages, arginase I and II isoforms, triggered by PPARγ and LXR receptors, respectively, could counteract inflammation by competing with iNOS. However, as macrophages change their activation programs and as atherosclerosis progresses, the ornithine generated by arginase could be used for cell proliferation and collagen synthesis, both processes essential for tissue remodeling but also profibrotic in pathogenic conditions.

MATERIALS AND METHODS

Reagents and Chemicals

Lipoproteins, oxLDL, acLDL, and nLDL, were from Biomedical Technologies, Inc. (Stoughton, MA) and used according to the manufacturer's instructions. The RXR agonist 9-cRA; PPARα agonists WY14643 and GW7647, PPARγ agonist GW1929, the PPAR antagonist GW9662 (2-chloro-5-nitrobenzanilide), and the natural PPAR ligands 15dPGJ2 and 13-HODE were purchased from Sigma-Aldrich (Madrid, Spain), whereas the PPARδ agonist GW0742, PPARγ agonist GW7845, and the PPAR antagonist GW5393 (1,3,4-oxadiazole) were provided by Tim Willson and Jon Collins (GlaxoSmithKline, Research Triangle Park, NC). The PPAR antagonist T0070907 (2-chloro-5-nitro-*N*-4-pyridinyl-benzamide) and rosiglitazone were purchased from Cayman Chemical (Ann Arbor, MI). PPAR and RXR ligands were dissolved in dimethylsulfoxide before use. Recombinant murine IL-4 was obtained from PeproTech, EC Ltd. (London, UK) and used in complete culture media at a concentration of 2.5 ng/ml.

Cell Culture

BMDM were obtained by flushing the femurs from 6-wk-old BALB/c mice (Harlam Iberica, Barcelona, Spain) and cultured in hydrophobic Teflon bags (Cell Genix, Freiburg, Germany) in DMEM containing 10% inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Sigma, Spain), and 15% of L929 supernatant as a source of macrophage colony-stimulating factor.

Measurement of Arginase Activity

Arginase activity was measured in macrophages lysates as previously described (43). One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol urea/min.

RNA and Protein Analysis

RNA was isolated using TRIzol reagent (Sigma-Aldrich, Spain). Reverse transcription was made by using the high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. TaqMan or SYBR Green real-time quantitative assays were performed using an Applied Biosystems 7500 sequence detector system. The relative expression of arginase I (Arg1) and arginase II (Arg2) genes were analyzing by using TaqMan Gene Expression Assays, and the relative expression in each experiment was compared by the $2^{-\Delta\Delta Ct}$ calculation method as described (44). The culture conditions used in this study did not alter the expression of 18S rRNA, thus validating its use as endogenous control. Primers and probes for arginase I and II and 18S rRNA were obtained from Applied Biosystems.

The rest of the genes were analyzed using SYBR Green gene expression assays. In these experiments, each sample was run in duplicate and was normalized to 36B4, as previously described (45). Primers and probes are published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org (supplemental Table 1).

Protein expression was assayed by Western blot using macrophage lysates with the same protein content (assayed by the Bradford method; Bio-Rad, Spain). Briefly, Proteins were separated by 10% SDS-PAGE, transferred to a polyvinylidene difluoride membranes, and then incubated with anti-arginase I (1:1000; BD Transduction Laboratories, Lexington, KY), anti-arginase II (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA), or anti-β-Actin (1:3000; Sigma) which served as loading control. Goat antirabbit IgG-horseradish peroxidase secondary antibody (1:10000; Santa Cruz Biotechnology) was used against arginase II and β-actin, and goat antimouse IgG (1:3000; Pierce, Rockford, IL) were used against arginase I. Detection was made with and ECL-Plus western blotting detection system (Amersham Biosciences, Uppsala, Sweden).

Leishmania Infection

L. major promastigotes (MHOM/IL/80/Friedlin) were cultured as previously described (31). Cells were cultured onto round slides inside 24-well plates. PPAR antagonists were added to macrophage cultures 24 h before infection, and PPAR agonists were added 2 h before the infection. Then, stationary-phase promastigotes were added to BMDM at a parasite to cell ratio of 3:1. After 24 h infection, slides were mounted and stained with Diff-Quik (Panreac, Barcelona, Spain). Results are presented as the number of intracellular amastigotes per 100 cells.

Arginase I Expression in Macrophages from PPARγ- and PPARδ-Deficient Mice

Primary peritoneal macrophages were elicited from thioglycollate-injected mice. Cells were derived from floxed PPARγ-MxCre$^{-/+}$ mice and PPARδ$^{-/-}$ mice (C57BL/6 background). The PPARγ fl/fl mice were provided by Ron Evans, and the PPARδ$^{-/-}$ mice were provided by Frank Gonzalez. Mice were maintained on standard chow diet in pathogen-free conditions. The cells were flushed and cultured in DMEM with 10% fetal bovine serum (Omega Scientific, Tarzana, CA) and 1% penicillin/streptomycin (Cellgro, Lawrence, KS). BMDM were derived by flushing cells from the femur and tibia of floxed PPARγ-MxCre$^{-/+}$ and PPARδ$^{-/-}$ mice. Cells were then differentiated using DMEM, 20% fetal bovine serum, 1% penicillin/streptomycin, and 30% L929-conditioned media. Cells were activated by ligand for 12 h before harvesting total RNA using TRIzol reagent (Invitrogen, Carlsbad, CA). Alternatively, cells were pretreated with 2.5 ng/ml IL-4 for 12 h before activating with ligand for 24 h. Total RNA was harvested after 24 h using TRIzol reagent (Invitrogen). Expression of arginase I was analyzed by quantitative real-time PCR (SYBR Green) using an Applied Biosystems 7900 sequence detector.

Statistical Analysis

All data were processed by using GraphPad Prism 4.0 software and are the result of at least three independent experiments performed in triplicate. Errors bars were calculated as SD of the data, and when necessary, we have analyzed significance by the Student's *t* test (paired *t* test).

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