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Expression of adiponectin receptors in human macrophages and regulation by agonists of the nuclear receptors PPARα, PPARγ, and LXR

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Abstract

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors expressed in macrophages where they control cholesterol homeostasis and inflammation. In an attempt to identify new PPARα and PPARγ target genes in macrophages, a DNA array-based global gene expression profiling experiment was performed on human primary macrophages treated with specific PPARα and PPARγ agonists. Surprisingly, AdipoR2, one of the two recently identified receptors for adiponectin, an adipocyte-specific secreted hormone with anti-diabetic and anti-atherogenic activities, was found to be induced by both PPARα and PPARγ. AdipoR2 induction by PPARα and PPARγ in primary and THP-1 macrophages was confirmed by Q-PCR analysis. Interestingly, treatment with a synthetic LXR agonist induced the expression of both AdipoR1 and AdipoR2. Furthermore, co-incubation with a PPARα ligand and adiponectin resulted in an additive effect on the reduction of macrophage cholesteryl ester content. Finally, AdipoR1 and AdipoR2 are both present in human atherosclerotic lesions. Moreover, AdipoR1 is more abundant than AdipoR2 in monocytes and its expression decreases upon differentiation into macrophages, whereas AdipoR2 remains constant. In conclusion, AdipoR1 and AdipoR2 are expressed in human atherosclerotic lesions and macrophages and can be modulated by PPAR and LXR ligands, thus identifying a mechanism of crosstalk between adiponectin and these nuclear receptor signaling pathways.

Keywords: Gene expression; Vascular wall; Transcription factors; Macrophages; Adipokines

Adipose tissue is considered not only to be a tissue passively storing energy under the form of triglycerides, but also to be a hormonally active system participating in the control of whole body metabolism. Adipokines are a group of adipocyte-derived biologically active molecules which may influence the function as well as the structural integrity of other tissues. Adiponectin is a recently discovered adipokine which is abundantly present in human plasma [1,2]. Low plasma adiponectin levels are found in patients with coronary artery disease, and appears associated with a number of risk factors of cardiovascular disease such as high blood pressure, obesity, and type 2 diabetes [3,4].

PPARs (peroxisome proliferator-activated receptors) and LXRs (liver X receptors) are nuclear receptors activated by fatty acid derivatives and by oxysterols, respectively, as well as by specific synthetic ligands. PPARα and PPARγ as well as LXRs are expressed in cells of the vascular wall, including monocyte-derived macrophages, where they control the inflammatory response and cholesterol homeostasis [5,6]. PPARs and LXRs reduce cytokine secretion and pro-inflammatory gene expression in macrophages [7–9]. PPARα, PPARγ, and LXR activators induce ATP-binding cassette A1 (ABCA1) gene expression and promote apoAI-mediated cholesterol efflux from macrophages [5,10,11]. In macrophages, PPARα activation also reduces the cholesteryl ester content due to reduced cholesterol esterification rates and Acyl-CoA:cholesterol acyltransferase-1 (ACAT1) activity [6]. These effects of PPARα, PPARγ,

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and LXRs result in an anti-atherogenic activity as demonstrated in different studies using preclinical animal models of atherosclerosis [12–15].

In an attempt to identify novel PPAR target genes in human macrophages, we employed whole genome RNA expression profiling experiments using DNA array technology. Interestingly, the recently identified adiponectin receptor AdipoR2 was found to be positively regulated by both PPARα and PPARγ agonists. Furthermore, treatment with T0901317, a ligand for LXRα, a nuclear receptor that is induced by PPARα and γ in macrophages, resulted in the induction of both AdipoR1 and AdipoR2. Interestingly, co-incubation with a PPARα ligand and adiponectin produced an additive effect on the reduction of macrophage cholesteryl ester content, thus suggesting a functional crosstalk between these pathways. Finally, we show that AdipoR1 and AdipoR2 are both detected in human atherosclerotic lesions and vascular cells. These data thus provide mechanistic evidence for crosstalk between these signalling pathways with potential effects on cardiovascular disease.

Materials and methods

Tissue and cell culture. Mononuclear cells isolated from blood of healthy normolipidemic donors by Ficoll gradient centrifugation were suspended in RPMI 1640 medium containing gentamycin (40 mg/ml), glutamine (0.05%), and 10% pooled human serum [16]. Differentiation of monocytes into macrophages occurs spontaneously by adhesion of the cells to the culture dishes. Mature monocyte-derived macrophages were used for experiments after 10 days of culture whereas monocytes were used after 45 min of adherence to the plastic dish.

Human monocytic THP-1 cells (ATCC, Rockville, Maryland, USA) were maintained in RPMI 1640 medium containing 10% of FCS and differentiated for 72 h with 167 nM PMA. For experiments, medium was changed to medium without serum but supplemented with 1% Nutridoma HU (Boehringer–Mannheim). Ten-day-old primary human macrophages or differentiated THP-1 macrophages were treated or not with the PPARα ligands Wy14643 (25, 50, and 100 μM), bezafibrate (50 μM), fenofibric acid (50 μM), GW647 (600 nM), the PPARγ ligands Rosiglitazone (50, 100 nM and 1μM), GW929 (600 nM) or LXR ligand T0901317 (1 μM).

Human aortic smooth muscle cells (SMC) and human microvascular endothelial cells (HMEC) were cultured as described previously [17,18].

Human atherosclerotic plaques were removed during carotid endarterectomy and collected into RNAlater (Ambion) until RNA extraction.

RNA extraction and analysis. Total cellular RNA was extracted from cells using *Trizol* (Life Technologies, France). For DNA microarray analysis, RNA was extracted from macrophages of five different donors, using the RNeasy kit (Qiagen, France), according to the manufacturer's instructions. For quantitative PCR, total RNA was reverse transcribed using random hexameric primers and Superscript reverse transcriptase (Life Technologies, France). cDNAs were quantified by real-time PCR on a MX 4000 (Stratagene), using specific primers for human AdipoR1: 5′-TTC TTC CTC ATG GCT GTG ATG T-3′ and 5′-AAG AAG CGC TCA GGA ATT CG-3′, human AdipoR2: 5′-ATA GGG CAG ATA GGC TGG TTG A-3′ and GGA TCC GGG CAG CAT ACA-3′ and cyclophilin: 5′-GCA TAC GGG TCC TGG CAT CTT GTC C-3′ and 5′-ATG GTG ATC TTC TTG CTG GTC TTG C-3′ and 28S: 5′-AAA CTC TGG TGG AGG TCC GT-3′ and 5′-CTT ACC AAA AGT GGC CCA CTA-3′. PCR amplification was performed in a volume of 25 μl containing 100 nmol/L of each primer, 4 mmol/L $MgCl_2$, the Brilliant Quantitative PCR Core Reagent Kit mix as recommended by the manufacturer (Stratagene) and SYBR Green 0.33X (Sigma–Aldrich). The conditions were 95 °C for 10 min, followed by 40 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C. AdipoR1 and AdipoR2 mRNA levels were subsequently normalized to cyclophilin or 28S mRNA.

Sample preparation for gene expression analysis. For microarray analysis, total RNA (10 μg) was reverse transcribed using Superscript II (Gibco-BRL) and a oligo(dT) primer containing the T7 RNA polymerase binding site (Genset). Second strand cDNA was then made using DNA polymerase, DNA ligase, and T4 DNA polymerase. Double stranded DNA was purified using phenol:chloroform on Phase Lock gel columns (Eppendorf), ethanol precipitated and resuspended in water. cRNA was synthesised from the cDNA using biotin labeled ribonucleotides and T7 RNA polymerase (Enzo Bioarray) and purified on Qiagen RNeasy columns. cRNA was then fragmented for 35 min at 94 °C in a solution of 40 mM Tris–acetate (pH 8.1), 100 mM potassium acetate, and 30 mM magnesium acetate.

Affymetrix oligonucleotide arrays. For the experiments, fragmented cRNA samples were combined with hybridization controls (Affymetrix), salmon sperm DNA, acetylated BSA and hybridized to microarrays for 16 h at 45 °C. Each sample was hybridized to the Affymetrix Test 3 and the U133A microarray sets. Washing, staining and scanning of the microarrays was performed according to standard protocols (Affymetrix).

Data analysis of Affymetrix microarrays. The raw data from the microarrays was analyzed using the GeneChip Operating Software version 1.0 (GCOS.1, Affymetrix) and Data Mining Tools version 3.1 (DMT 3.1, Affymetrix). Each array was subjected to absolute analysis and all chips showed proper behavior of spiked controls, signal ratio of 5′ to 3′ sequences from housekeeping genes approximately equal to 1 and low background and noise values.

Recombinant human adiponectin purification. The human adiponectin cDNA was obtained by PCR and was inserted into the prokaryotic expressing vector pRSETA. The constructed recombinant plasmid was transferred into *Escherichia coli* for adiponectin expression. Human adiponectin protein was purified by metal chelate affinity chromatography on Ni–NTA agarose and eluted using an imidazole gradient.

Cellular cholesterol measurement. Ten-day-cultured human macrophages were pretreated for 24 h and thereafter every 24 h with the PPARα activator GW647 (600 nM) and cholesterol loaded by incubation with AcLDL (50 μg/ml, containing [^{3}H]-labeled cholesterol) [19] in RPMI 1640 medium supplemented with 1% Nutridoma for 72 h, in the absence or presence of 30 μg/ml adiponectin. Intracellular lipids were extracted by hexane/isopropanol and separated by thin layer chromatography (TLC) in petroleum ether:diethyl ether:acetic acid (180:20:10, vol:vol:vol). Spots corresponding to CE and FC were scraped and radioactivity measured by scintillation counting.

Statistics. Statistically differences between groups were analyzed by ANOVA followed by Student's t test and were considered significant when $p \leqslant 0.05$.

Results

In an attempt to identify new PPARα and PPARγ target genes, DNA microarray experiments were performed on RNA isolated from macrophages treated with GW647 and GW929, specific ligands for PPARα and PPARγ, respectively. Gene expression profiling

analysis revealed AdipoR2, but not AdipoR1, expression to be increased upon PPARα and PPARγ activation. To confirm these results Q-PCR analysis was performed on macrophage RNA isolated from other donors. These results confirmed that AdipoR2 expression is increased by different PPARα (Wy14643) and PPARγ (Rosiglitazone and GW929) ligands (Fig. 1B). This induction by Wy14643 and Rosiglitazone occurred in a dose-dependent manner (Figs. 1C and D). By contrast AdipoR1 mRNA was not affected by the treatment (Fig. 1A). This differential regulation of AdipoR1 and AdipoR2 gene expression by PPARα specific ligands was also observed in THP-1 macrophages (Fig. 2).

Since certain effects of PPARs on macrophage gene expression occurs via LXRα induction, we next investigated the regulation of the adiponectin receptors by LXR agonists in primary differentiated macrophages treated with the LXR ligand, T0901317 [20]. Our results demonstrate that both AdipoR1 and AdipoR2 are induced after LXR agonist treatment in human macrophages (Fig. 3).

In macrophages, adiponectin is able to reduce the intracellular cholesteryl ester (CE) content [21], an effect also observed when macrophages are treated with PPARα ligands [6]. In order to investigate whether the induction of Adipo receptors by PPARα activation could enhance the effect of adiponectin, primary human monocyte-derived differentiated macrophages were loaded with AcLDL (50 μg/ml) for 72 h and treated with the specific synthetic PPARα ligand GW647 (600 nM), added 24 h before cholesterol loading and thereafter every 24 h, alone or in association with adiponectin and the distribution of radiolabeled [^{3}H]-labeled cholesterol between FC and CE was analysed by TLC analysis. Co-treatment with GW647 and adiponectin has an additive effect on the reduction of the amount of [^{3}H]-labeled cholesterol present in the cholesteryl ester fraction, compared to each treatment alone (Fig. 4A). The

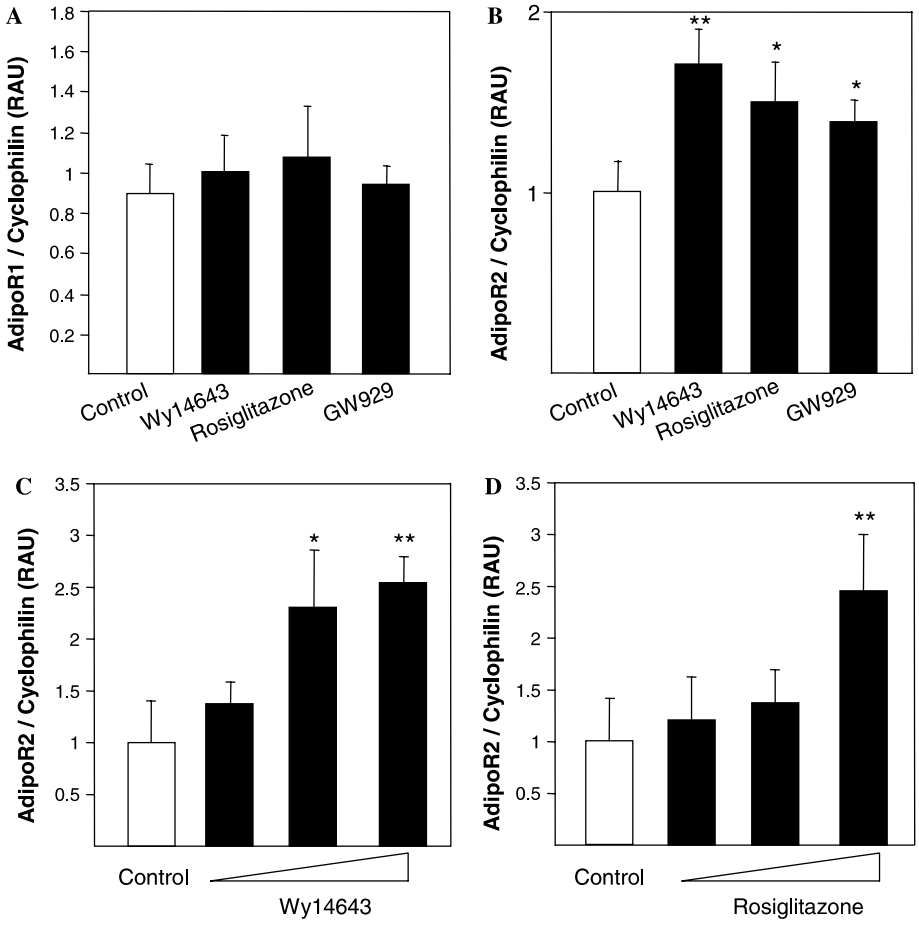

Fig. 1. AdipoR2 but not AdipoR1 gene expression is induced by PPARα and PPARγ ligands in human macrophages. (A,B) Quantitative PCR analysis of AdipoR1 (A) and AdipoR2 (B) and cyclophilin was performed on RNA isolated from human primary macrophages treated or not with Wy14643 (50 μM), Rosiglitazone (100 nM) or GW929 (600 nM) for 24 h. AdipoR mRNA levels were normalized to cyclophilin mRNA and are expressed relative to the levels in untreated cells set as 1. Results are means ± SD of triplicate determinations, representative of three independent experiments. Statistically significant differences between treatments are indicated (t test; $^*p < 0.05$, $^{**}p < 0.01$). (C,D) Induction of AdipoR2 expression in human macrophages treated for 24 h with increasing concentrations of Wy14643 (25, 50, and 100 μmol/L) (C) and Rosiglitazone (50, 100 nM and 1 μM) (D). Statistically significant differences between treatments are indicated (ANOVA followed by t test; $^*p < 0.05$, $^{**}p < 0.01$).

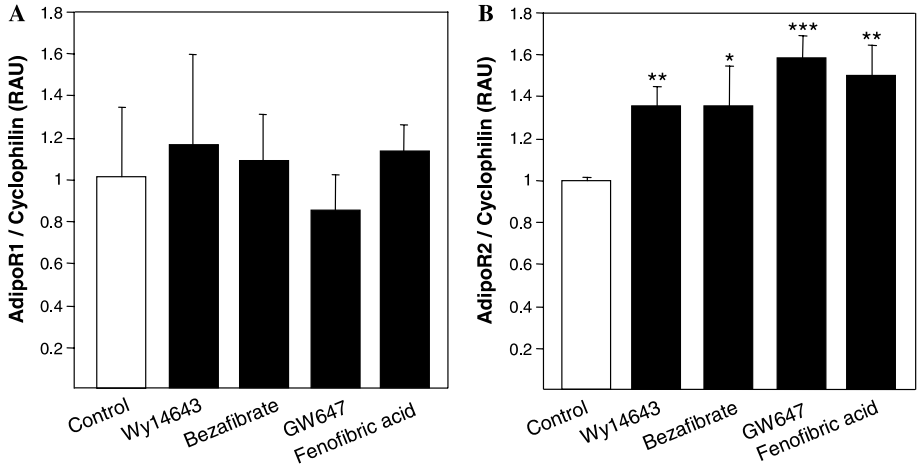

Fig. 2. AdipoR2 is regulated by PPARα in THP-1 macrophages. Quantitative PCR analysis of AdipoR1 (A) and AdipoR2 (B) and cyclophilin was performed on RNA isolated from THP-1 macrophages treated or not with Wy14643 (50 μM), GW647 (600 nM), bezafibrate (50 μM) or fenofibric acid (50 μM) for 24 h. AdipoR mRNA levels were normalized to cyclophilin mRNA and are expressed relative to the levels in untreated cells set as 1. Results are expressed as means ± SD. Statistically significant differences between treatments are indicated (ANOVA followed by t test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

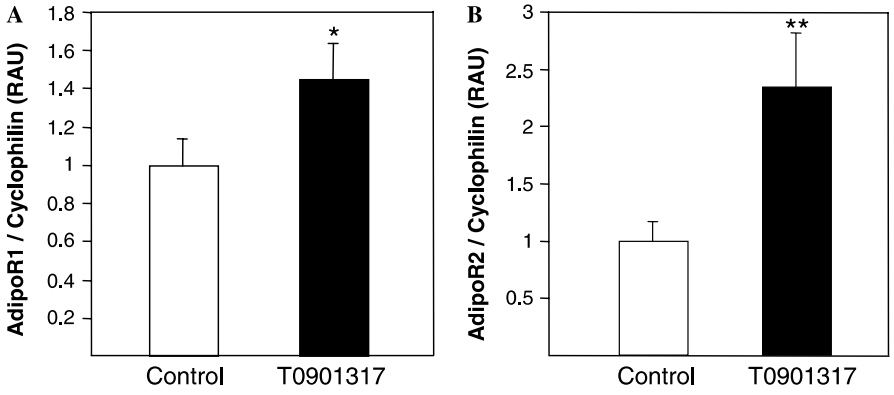

Fig. 3. AdipoR1 and AdipoR2 are induced by LXR agonist treatment in human macrophages. Quantitative PCR analysis of AdipoR1 and AdipoR2 mRNA in primary macrophages treated or not with the LXR ligand T0901317 (1 μM). AdipoR1 and AdipoR2 mRNA levels were normalized to cyclophilin mRNA and are expressed relative to the levels in untreated cells set as 1. Results are means ± SD of triplicate determinations, representative of three independent experiments. Statistically significant differences between treatments are indicated (t test; *$p < 0.05$, **$p < 0.01$).

total amount of intracellular [^{3}H]-labeled cholesterol was not influenced by the treatment (Fig. 4B). These results suggest that through the induction of AdipoR2 expression, PPARα might potentiate the effect of adiponectin in macrophages.

Since it has been reported that adiponectin has anti-atherogenic properties in apoE deficient mice [22], we determined whether AdipoR1 and AdipoR2 are present in human atherosclerotic lesions. Both AdipoR1 and AdipoR2 are present in lipid-rich atherosclerotic plaques isolated by carotid endarterectomy and their expression was slightly higher in lesion areas compared to the healthy carotid zone (Figs. 5A and B). Lipid-rich lesions contain macrophages which were found to express both receptors in vitro. The presence of AdipoRs in the non-diseased carotid zone suggests that cells of the vascular wall other than macrophages, such as SMC and endothelial cells could also express these two receptors. Analysis of AdipoRs expression levels showed that both AdipoR1 and AdipoR2 are expressed in primary human aortic SMC as well as endothelial HMEC cells at a level comparable with the primary differentiated macrophages (Figs. 5C and D). Finally, we studied whether AdipoR1 and AdipoR2 are regulated during differentiation of primary monocytes into macrophages. AdipoR1 is more abundant than AdipoR2 in monocytes and its expression decreases upon differentiation into macrophages, dropping already after 3 days and remaining constant during further macrophage differentiation (Fig. 6A). By contrast, AdipoR2 expression is not affected during the differentiation process (Fig. 6C). Interestingly, both freshly isolated monocytes (day 0) as well as fully differentiated macrophages (day 12) express more AdipoR1 than AdipoR2 mRNA (Figs. 6B and D).

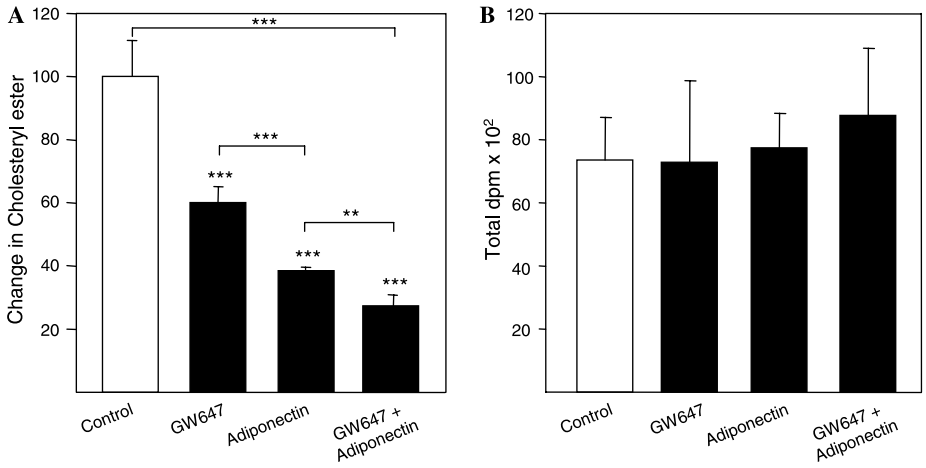

Fig. 4. Adiponectin and PPARα ligand treatment additively decreases cholesteryl ester content in human macrophage-foam cells. Human macrophages were cholesterol-loaded with [^{3}H]-labeled cholesterol-containing AcLDL (50 μg/ml) for 72 h in the absence or presence of 30 μg/ml adiponectin. GW647 (600 nM) was added 24 h before cholesterol-loading and thereafter every 24 h. Lipids were extracted and separated by TLC. (A) Spots corresponding to CE and FC were scraped and radioactivity measured by scintillation counting. Results are expressed relative to untreated cells set as 100%. Statistically significant differences between treatments are indicated (ANOVA followed by *t* test; $^{**}p < 0.01$, $^{***}p < 0.001$). (B) Total cellular cholesterol-loading was calculated as the sum of radioactivity of the CE and FC spots. Results are expressed as dpm × 10^2.

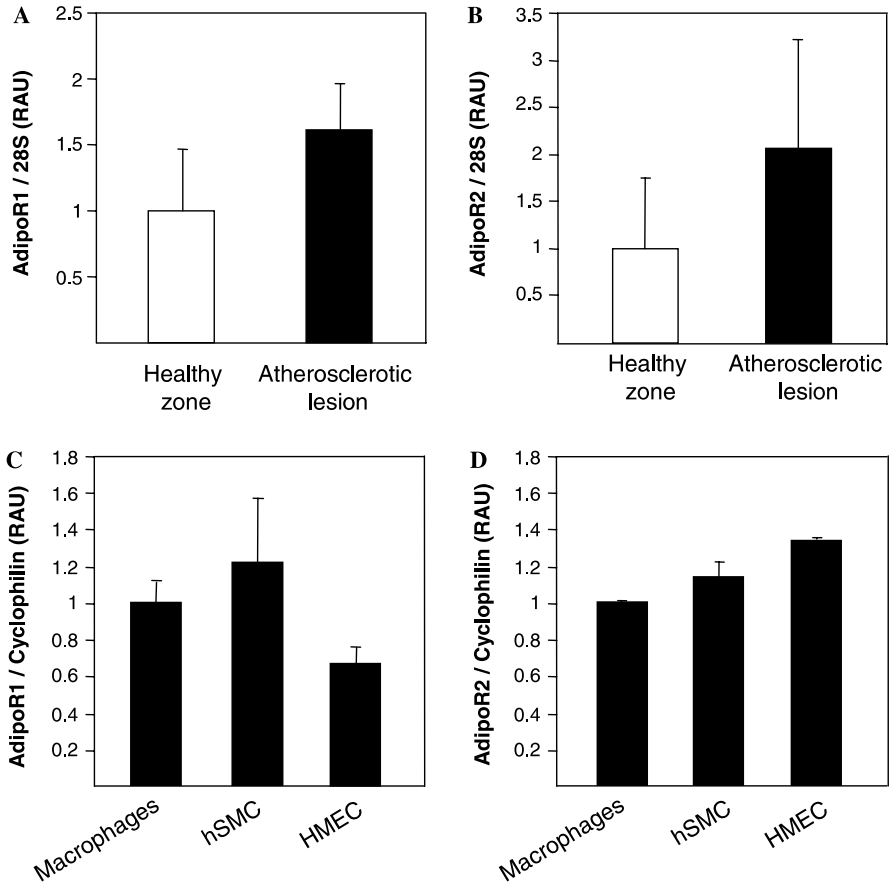

Fig. 5. AdipoR1 and AdipoR2 are present in human atherosclerotic plaques in vivo and in cells of the vascular wall in vitro. (A,B) Quantitative PCR analysis of AdipoR1 (A) and AdipoR2 (B) on RNA from lipid-rich atherosclerotic lesions obtained by endarterectomy from four patients. Results are expressed relative to the level of expression in the healthy carotid zone. (C,D) Quantitative PCR analysis of AdipoR1 (C) and AdipoR2 (D) mRNA levels in primary human aortic SMC, HMEC, and differentiated macrophages normalized to cyclophilin mRNA. Values are expressed relative to the levels in human differentiated macrophages set as 1.

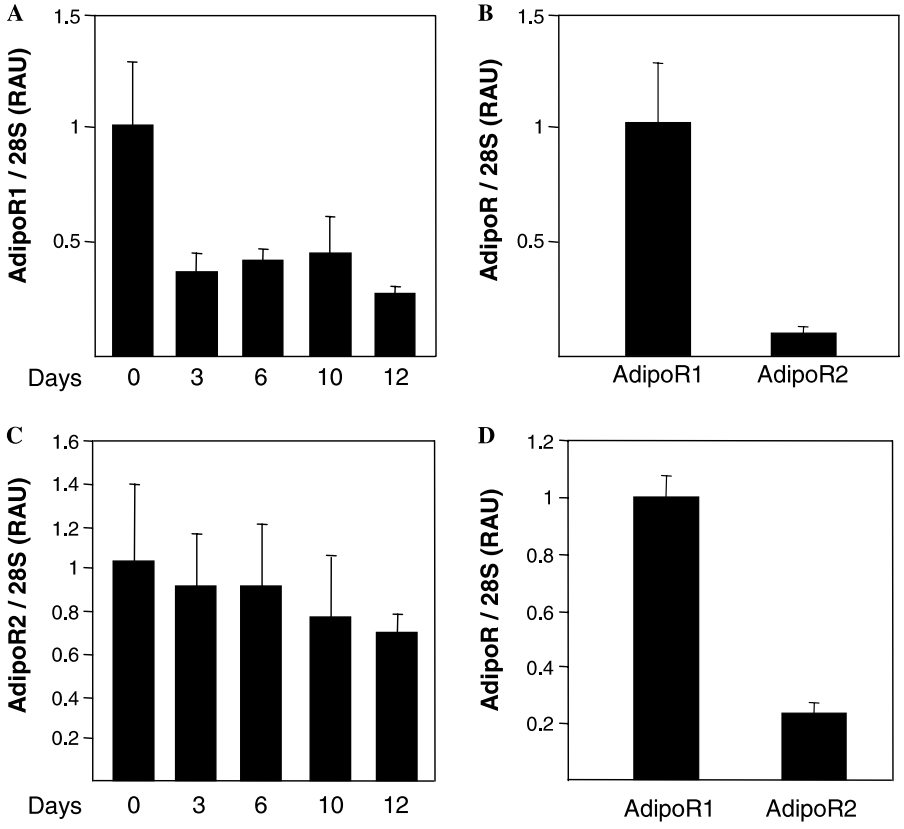

Fig. 6. AdipoR1 and AdipoR2 are differently expressed and regulated during monocyte–macrophage differentiation. Quantitative PCR analysis of AdipoR1, AdipoR2, and 28S was performed on RNA isolated from human primary monocytes and differentiating macrophages at the indicated number of days of culture. AdipoR1 (A) and AdipoR2 (C) mRNA levels were normalized to 28S mRNA and are expressed relative to the levels in freshly isolated monocytes (day 0). (B,D) Relative abundance of AdipoR1 and AdipoR2 in freshly isolated monocytes (day 0, B) and in fully differentiated macrophages (day 12, D). AdipoR1 level was set as 1. Results are means ± SD of triplicate determinations, representative of two independent experiments.

Discussion

Adiponectin, a recently discovered adipokine [1,2], is secreted by fat cells and circulates in the blood. Plasma adiponectin concentration is reduced in patients with coronary artery disease and inversely correlated with certain risk factors of cardiovascular disease [3,4]. Adiponectin stimulates fatty acid oxidation, decreases plasma triglyceride levels, and improves glucose metabolism by increasing insulin sensitivity [23,24].

Adiponectin is present in the subendothelial space of the injured aorta of humans in which macrophages accumulate during atherogenesis [21]. Adiponectin inhibits the expression of adhesion molecules and prevents the attachment of monocytes in TNFα-stimulated human endothelial cells [3]. In smooth muscle cells, adiponectin controls DNA synthesis and diminishes cell migration and proliferation [25]. Disruption of the adiponectin gene in mice results in enhanced neointimal formation in response to external cuff injury [26]. Adiponectin also has an inhibitory effect on the proliferation of myelomonocytic progenitors and decreases phagocytic activity and cytokine production in macrophages [27]. In macrophages, adiponectin also decreases cholesteryl ester accumulation and inhibits the uptake of acetylated LDL, thus acting as a modulator of macrophage-to-foam cell transformation [21]. Finally, recombinant adiponectin reduces atherosclerotic lesions in apoE deficient mice, thus providing evidence for a potential anti-atherogenic effect of this adipokine [22]. Taken together these actions suggest that this adipocyte-derived cytokine may exert anti-inflammatory and anti-atherogenic effects, especially in macrophages and therefore may play a protective role in experimental models of vascular injury as well as in early atherogenesis.

The molecular basis for the cellular action of adiponectin became more evident with the recent identification of 2 trans-membrane proteins, AdipoR1 and AdipoR2, which serve as receptors for adiponectin [28]. AdipoR1 is abundantly expressed in skeletal muscle, whereas AdipoR2 is predominantly expressed in the liver. AdipoR1 and AdipoR2 serve as receptors for globular and full length adiponectin, and mediate adiponectin stimulation of AMP kinase, PPARα ligand

activities, fatty acid oxidation and glucose uptake [28]. Here we report that AdipoR1 and AdipoR2 are present in human non-diseased carotid artery areas and that their expression is slightly induced in lipid-rich atherosclerotic lesions (Fig. 5). This observation could be explained by the presence of lipid-enriched macrophages into the lipid core of the plaques. The presence of AdipoR1 and AdipoR2 in human plaques could mediate the effects of adiponectin in the subendothelial space of the injured aorta which contains macrophages [21]. Furthermore, the presence of AdipoRs in the non-diseased carotid zone suggests that cells of the vascular wall other than macrophages, such as SMC and endothelial cells, also express these receptors. Our findings that human SMC and endothelial cells express AdipoR1 and AdipoR2 are consistent with this (Fig. 5). Both AdipoR1 and AdipoR2 are expressed in monocytes whereas AdipoR1 expression decreases during differentiation of monocytes into macrophages, with an expression level of AdipoR1 higher than AdipoR2 during all steps of the differentiation process (Fig. 6). The regulation of Adipo receptors during monocyte differentiation suggests that adiponectin could modulate monocyte differentiation to macrophages. It has already been reported that markers for macrophage differentiation, such as lipoprotein lipase and Scavenger Receptor-A, as well as macrophage phagocytic activity are reduced upon adiponectin treatment [21,27].

Macrophage functions and gene expression can be modulated by specific nuclear receptor pathways. Among those nuclear receptors, PPARα and PPARγ as well as LXRs control the inflammatory response and cholesterol homeostasis in macrophages. Our findings indicate that both AdipoR1 and AdipoR2 are targets for LXR, as shown by the induction of their expression upon treatment with the specific LXR ligand T0901317 (Fig. 3). By contrast, AdipoR1 and AdipoR2 are differently regulated when cells are stimulated with specific ligands for PPARα and PPARγ. Only AdipoR2 is positively regulated, in a dose dependent manner, by PPARα and PPARγ activators both in primary and THP-1 differentiated macrophages (Figs. 1 and 2). To our knowledge this is the first report showing the regulation of adiponectin receptors by nuclear receptors. It has been already described that thiazolidinediones, a class of antidiabetic drugs acting through PPARγ activation, increase plasma adiponectin concentrations in insulin-resistant humans and rodents [23,24,29]. Our results on the regulation of AdipoR2 could thus provide a novel additional mechanism by which PPARγ influences the adiponectin pathway.

One of the key features of atherosclerosis is the presence of foam cells in the lesions, which are essentially lipid-loaded macrophages. It has been reported that due to its action on SRA expression, adiponectin suppresses the macrophage uptake of AcLDL and drastically reduces cholesteryl ester content [21]. Similarly, a reduction in the cholesteryl ester is also observed in macrophages upon PPARα activation [6]. Co-activation of macrophages with adiponectin and a PPARα ligand additively reduces the cholesteryl ester content, without affecting total cholesterol loading (Fig. 4). Thus combination of PPARα and adiponectin action could have a beneficial role in the modulation of macrophage foam cell formation, without affecting cholesterol accumulation.

It is tempting to speculate that pharmacological modulation of adiponectin signalling could provide beneficial effects in the treatment of cardiovascular disease in selected patients. Our findings open novel therapeutic applications for drugs activating nuclear receptors acting by enhancing adiponectin signalling via their effects on its receptors.

Acknowledgments

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