Identification of a peroxisome-proliferator-activated-receptor response element in the apolipoprotein E gene control region

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Apolipoprotein E (apoE) is a protein involved in reverse cholesterol transport. Among other tissues, apoE is expressed in macrophages where its expression increases when macrophages develop into foam cells. It has been recently shown that peroxisome-proliferator-activated receptor gamma (PPAR γ) is involved in this conversion. Northern-blot analysis was carried out in the macrophage cell line THP1 to determine whether apoE mRNA levels were regulated by ciglitazone, a PPAR γ inducer. The results indicated that treatment with ciglitazone doubled the levels of apoE mRNA. To identify a possible PPAR γ response element (PPRE), several portions of apoE gene control region were used to construct luciferase reporter plasmids. In U-87 MG cells, a 185 bp fragment located in the apoE/apoCI intergenic

region was sufficient to induce a 10-fold increase in the luciferase activity of the extract of cells co-transfected with a PPAR γ expression plasmid. Subsequent analysis revealed the presence of a sequence with a high level of sequence similarity to the consensus PPRE. Mutations in this sequence resulted in a lack of functionality both in transient transfection and in electrophoretic-mobility-shift assays. These results demonstrated the presence of a functional PPRE in the apoE/apoCI intergenic region. These results have implications for the regulation of apoE gene expression and could be relevant for understanding the anti-atherogenic effect of thiazolidinediones.

Key words: gene expression, macrophage, thiazolidinedione.

INTRODUCTION

Apolipoprotein E (apoE), a 35000-*M_r* protein, is a member of the apolipoprotein family, which groups several proteins involved in plasma lipid transport. In plasma, apoE is associated with triacylglycerol-rich lipoproteins, their remnants and some high-density lipoprotein particles [1]. Although most plasma apoE is produced in liver [2], apoE is also synthesized in various extrahepatic tissues [3]. It is found abundantly in atherosclerotic vascular lesions [4] in which the foam cells of macrophage origin are its major source [5]. Macrophage-derived foam cells are the hallmark of early atherosclerotic lesions and their death contributes to the formation of the lipid core of advanced atheroma [6]. However, macrophage-specific expression of apoE prevents the development of atherosclerotic lesions in mice in spite of very low plasma apoE concentrations [7,8]. Although the molecular basis for the anti-atherosclerotic properties of macrophage-produced apoE is not well established, it has been shown that apoE expression in macrophages facilitates cholesterol efflux from cholesterol-loaded macrophages to exogenous acceptors (such as high-density lipoproteins) and therefore potentiates reverse cholesterol transport to the liver [9]. Some other local effects of apoE, such as the modulation of platelet capacity to aggregate, the lymphocyte proliferation or the interaction with the extracellular matrix, have also been invoked to explain the anti-atherosclerotic role of apoE (see [10] for a review).

ApoE mRNA levels increase during the differentiation of some macrophage-like cell lines [11], a process in which the peroxisome-proliferator-activated receptor gamma (PPAR γ) is involved [12]. PPAR γ is a ligand-dependent nuclear receptor first identified as an adipocyte-specific transcription factor [13]. There

are several similarities between apoE and PPAR γ expression. Similar to apoE, PPAR γ is overexpressed in the foam cells of atherosclerotic lesions [4,12,14]. PPAR γ activation results in the differentiation of adipocytes [15], a process in which there is also an induction of apoE expression [16]. These findings, together with the fact that PPAR γ regulates the expression of many genes involved in lipid metabolism suggested a possible role for PPAR γ in the regulation of apoE expression.

In this report we present evidence for such a regulation. We also describe a PPAR γ response element (PPRE) within the apoE/apoCI intergenic sequence, a region which contains several elements of control of the extrahepatic expression of both genes [17,18]. These results have implications for the regulation of apoE gene expression and could be relevant for understanding the anti-atherogenic effect of thiazolidinediones.

EXPERIMENTAL

Materials

Ciglitazone and Wy-14643, respectively the PPAR γ and α ligands, were purchased from Biomol Research Laboratories. 9-*cis* Retinoic acid and bisphenol A diglycidyl ether (BADGE) were purchased from Sigma Chemical Co. (Madrid, Spain) and Fluka (Madrid, Spain) respectively. The anti-PPAR γ antibody, PPAR γ N-20X, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Expression plasmids pCMX-mPPAR γ and pSG5-RXR α were generously given by Dr C. K. Glass (University of California, San Diego, La Jolla, CA, U.S.A.) and Professor P. Chambon (Institut de Genetique et de Biologie Moleculaire et Cellulaire, Strasbourg, France) respectively. The

Abbreviations used: apoE, apolipoprotein E; PPAR, peroxisome-proliferator-activated receptor; PPRE, PPAR γ response element; BADGE, bisphenol A diglycidyl ether; RXR, retinoid X receptor; EMSA, electrophoretic mobility-shift assay; C/EBP, CCAAT-enhancer-binding protein; DR, direct repeat.

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plasmids pSG5-PPAR α and pSG5-PPAR β/δ were obtained from Dr B. Staels (Institut Pasteur de Lille, Lille, France). The plasmid pXP2-TATA in which a TATA box was cloned 5' to the luciferase gene of pXP2 was obtained from Dr A. Corbí (Centro de Investigaciones Biológicas, Madrid, Spain). CCAAT-enhancer-binding protein (C/EBP) expression plasmids were generously given by Dr S. L. McKnight (Department of Biology, John Hopkins University, Baltimore, MD, U.S.A.). The pEpro plasmid was constructed by cloning a *SmaI*–*SacI* fragment from the apoE gene promoter (coordinates –619 to +72 according to [19]) into the *SmaI*–*SacI* sites of the luciferase reporter plasmid pXP2 [20]. Several different fragments from the apoE/apoCI intergenic region produced either by endonuclease digestion or PCR amplification were cloned either into the unique *SalI* site of the pEpro plasmid, or were blunt ended and cloned into the *SmaI* site, 5' with respect to the apoE promoter. The plasmids used in this work were pNde-Hpa (coordinates 1–5500); pSal-Hpa (coordinates 2249–5500); pSal-Sal (coordinates 1–2249); pSal-Hind (coordinates 1–1761); pHind-Hind (coordinates 1761–2754); pHind-Sal (coordinates 1761–2249); pHind-Pvu (coordinates 1761–1891); pPvu-Sal (coordinates 1891–2249); p185 (coordinates 1962–2145); pPME (coordinates 1949–3931) and pPME- Δ Sal (coordinates 2249–3931). The site-specific mutations in the 185 bp fragment shown in Figure 4 were introduced by PCR-mediated mutagenesis.

RNA preparation, Northern blot, labelling of probes and hybridization

All of the procedures, as well as the apoE probe, have already been described [4]. The quantification of band intensity was performed in a Molecular Imager System (Bio-Rad, Hercules, CA, U.S.A.). The levels of rRNA were measured after the transfer of RNA to filters and were used as a control for RNA loading.

Cell culture and transfections

THP-1 cells [21] were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum and an antibiotic/antimycotic mix (Gibco Life Technologies, Rockville, MD, U.S.A.). U-87 MG glioblastoma-astrocytoma cells (ATCC HTB-14) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. For transfection we used 1.5 μ g of plasmid and *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulphate ('DOTAP'; Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.) according to the manufacturer's instructions. For co-transfection with plasmids expressing nuclear receptor genes the transfection mixture contained 20 ng of the corresponding expression plasmid. The plasmid pRL-SV40 (50 ng; Promega, Madison, WI, U.S.A.) was included as a control for transfection efficiency. Luciferase expression was analysed in cell lysates prepared 48 h after transfection. For the lysis and the analysis the Dual Luciferase Reporter assay system (Promega) was used.

Preparation of nuclear extracts for electrophoretic mobility-shift assays (EMSAs)

Extracts were prepared from THP-1 cultures according to the procedure described in [22]. Quantification of the protein concentration in the extracts was carried out according to the method described in [23].

In vitro coupled transcription/translation

cDNAs for mPPAR γ 1 and retinoid X receptor alpha (RXR α) were transcribed and translated *in vitro* using the TnT T7 Quick Coupled Transcription/Translation system (Promega) according to manufacturer's instructions. The presence of translation products of the expected size in the mixtures was confirmed by SDS/PAGE and subsequent Coomassie Blue staining.

EMSAs

Binding reactions were carried out in 20 μ l of annealing buffer [20 mM Hepes, pH 7.5, 50 mM KCl, 0.175 mM EDTA and 5% (v/v) glycerol] containing 1.5 μ g of poly(dI-dC) (Amersham Pharmacia Biotech, Little Chalfont, Bucks., U.K.) and 0.4 μ g of single-stranded DNA in the presence or absence of either *in vitro* synthesized proteins or 5 μ g of nuclear extracts. The reactions were incubated at 4 °C for 10 min prior to the addition of 0.25 pmol of the ³²P-labelled DNA probe. Then, the reactions were incubated for 15 min at 4 °C. The DNA–protein complexes were resolved from the free probe by electrophoresis at room temperature on a 5% (w/v) polyacrylamide gel in 0.25 $\times$ TBE buffer (TBE buffer is 9 mM Tris, pH 8, 90 mM boric acid, 20 mM EDTA). The sequences of the double-stranded oligonucleotides used were PPREwt 5'GGTGAGGAGGGGAAGGGTCACTGTG3', PPREmut5 5'GGTGAGGtttGGAAGG-GTCAGTGTG3' and PPREmut3 5'GGTGAGGTTTGGAA-GGaaagGTGTG3'. The mutated bases are shown in lowercase in the sequence. The oligonucleotide PU containing the binding site for the transcription factor Spi-1 [24] was used for competition experiments as a control of unspecific binding. In the supershift experiments the antibody (2 μ g) was added to the reaction prior to the addition of the labelled oligonucleotide.

RESULTS

ApoE gene expression is regulated by PPAR γ in THP-1 cells

To study the possible influence of PPAR γ activation on the levels of apoE mRNA, the synthetic PPAR γ ligand ciglitazone was added to THP-1 cultures either in the presence or in the absence of 1 μ M 9-*cis* retinoic acid, a ligand for RXR. The cultures were maintained for 5 days as in [12] and the levels of apoE mRNA were studied by Northern-blot hybridization of total RNAs with an apoE-specific probe. Figure 1 shows a typical Northern-blot analysis in the presence of 12 μ M ciglitazone. The treatment with ciglitazone, retinoic acid or both resulted in increases in apoE mRNA levels of 2.05 ± 0.05 , 2.68 ± 0.07 and 5.44 ± 0.06 respectively (results are means $\pm$ S.D.); a result consistent with the regulation of apoE expression by the activation of the PPAR γ –RXR heterodimer. In another group of experiments the effect of BADGE, a specific PPAR γ antagonist, was tested. In the presence of this compound, at a concentration of 100 μ M [25], the apoE mRNA levels decreased to one third of those of untreated cultures (results not shown). Thus, suggesting that apoE gene mRNA levels are regulated by PPAR γ .

There is a PPRE in the apoE/apoCI intergenic region

The search for a functional PPRE was done in transient transfection assays. Different portions of the apoE gene control region were tested for their ability to modify the luciferase activity of a reporter vector in the presence of a co-transfected

Figure 1 Treatment with ciglitazone and 9-*cis* retinoic acid increased levels of apoE mRNA in THP1 cells

Northern-blot analyses were carried out with total mRNAs from duplicate cultures of untreated cells and cells treated for 5 days with either ciglitazone or 9-*cis* retinoic acid, or both in the concentrations indicated in the text. The upper panel shows the hybridization of the Northern blots with an apoE-specific probe. The lower panel shows the ribosomal RNAs used as control for RNA loading. C, control; RA, 9-*cis* retinoic acid; CG, ciglitazone. The values in the Table, expressed as means $\pm$ S.D., indicate the apoE mRNA/rRNA ratio compared with controls. The number of experiments is shown in parentheses.

plasmid expressing PPAR γ . THP-1 cultures were not well suited for these experiments because (i) they express relatively high basal levels of PPAR γ and (ii) in our hands they transfected very poorly by any of the methods tested. The cell line used in these experiments was U-87 MG, derived from a human astrocytoma. U-87 MG expresses very low levels of PPAR γ mRNA, detectable by reverse-transcriptase PCR but not by Northern-blot analysis (results not shown). Moreover, they have been shown to express the reporter luciferase gene under the control of the apoE promoter [26].

When the construction containing the luciferase gene under the control of the -619 to +72 promoter region of apoE (pEpro) was co-transfected with the PPAR γ expression vector, the reporter gene activity was similar to that observed for the pEpro alone. Therefore, no functional PPRE was present within the apoE promoter (Figure 2).

The human apoE gene is located at the 5' end of a 45 kb cluster together with the genes for apoCI, apoCIV and apoCII [19,27,28] as well as the apoCI pseudogene apoCI' [29]. The intergenic region between apoE and apoCI genes contains several elements for control of the expression of the apoE gene [17].

The construction made with a 5.5 kb fragment (pNde-Hpa in Figure 2A), which contains the whole apoE/apoCI intergenic region except the apoCI promoter, responded to the presence of the PPAR γ plasmid with an increase of approximately 10-fold in the luciferase activity (Figure 2A). This result indicated the presence of a functional PPRE within this fragment. To map the PPRE more precisely, several constructions containing different portions of this region in front of the apoE promoter were tested (Figure 2A). The analysis of the luciferase activity in the presence of a co-transfected PPAR γ expression plasmid allowed us to locate the PPAR γ -responsive activity in a 185 bp fragment (plasmid p185 in Figure 2A).

To see whether the putative PPRE described here was functional in another promoter context, two new plasmid constructions were made. In the first one, the 500 bp fragment present in the plasmid pHind-Sal (Figure 2A) was cloned into pGL2pro (Promega) to give the plasmid pGL2proHind-Sal in which the luciferase expression was under the control of the SV40 early promoter. Another plasmid (p185-TATA) was constructed by cloning the 185 bp fragment present in p185 upstream of the TATA box of the plasmid pXP2TATA. Co-transfection of pGL2proHind-Sal or p185-TATA with PPAR γ resulted in increases in the luciferase activity of 10.6 ± 2 or 4.0 ± 0.9

respectively (Figure 2B), which indicate that the induction by PPAR γ was independent of the promoter context.

An enhancer (ME.1), essential for the expression of apoE in both adipocytes and macrophages, has been recently mapped within the apoE/apoCI intergenic region, between the coordinates 3302 and 3922 [18]. This element maps near, but is different to the 185 bp fragment which contains the PPRE (coordinates 1962–2145). This result was surprising since PPAR γ is a transcription factor involved in the processes of both macrophage and adipocyte differentiation. The presence of another PPRE in the ME.1 was tested by making two new constructions. One of them (pPME in Figure 2A) contains 2 kb of the intergenic sequence (coordinates 1949–3931) which include both the PPRE described above and the ME.1 enhancer. This construction showed an induction of the luciferase activity similar to that of p185, which contains only the PPRE (Figure 2A). The deletion of the 300 bp containing the PPRE by *SalI* digestion of pPME produced the pPME Δ Sal plasmid. This deletion completely abolished the ability to respond to PPAR γ . These results suggested the existence of only one PPRE within the apoE/apoCI intergenic region.

The sequence analysis of the region ME.1 revealed the existence of several possible C/EBP response elements [18]. C/EBPs are transcription factors whose involvement in the differentiation of both adipocytes and macrophages has been demonstrated [30]. Thus a series of transient transfection assays were carried out to see whether the presence of the sequence containing the ME.1 enhancer elicited a stronger response to co-transfected C/EBPs. In these experiments three different plasmids were used: pEpro, pPME and pNdeHpa. Both pPME and pNdeHpa contain the ME.1 enhancer and the PPRE (Figure 2A). As shown in Figure 3, the presence of the ME.1 enhancer did not result in an increased response either to C/EBP β or to C/EBP δ . In fact there is a decrease, although not significant, in the luciferase activity encoded by the plasmids PME and pNdeHpa. These results argued against the presence of a functional C/EBP response element within the ME.1 enhancer.

A direct repeat (DR)-1 within the 185 bp fragment responds preferentially to PPAR γ

Within the 185 bp fragment (Figure 4) the sequence 5' AGGGG-AA GGTCA 3' resembled the consensus DR-1 sequence

Figure 2 The intergenic apoE/apoC I region contains a functional PPRE

(A) All the constructs were derivatives of the luciferase plasmid vector pEpro, which contains the -619 to +72 apoE region (shown as a shaded arrow in the Figure) in front of the luciferase gene of the reporter vector pXP2. The different portions of the apoE/apoC I intergenic region (whose map is shown above the constructs) are shown as empty boxes. For the coordinates of the region cloned in each construct see the Experimental section. The presence of a PPRE was tested by co-transfecting U-87 MG cultures with the constructions shown in the Figure and a PPAR γ expression plasmid, and analysing the induction of the luciferase activity of cell extracts. All the results are expressed as the increase of the luciferase (Luc) activity of the plasmids when co-transfected with pCMV-PPAR γ . The results are expressed as means $\pm$ S.D. and the number of experiments is shown in parentheses. (B) The PPRE functions independently of the apoE promoter. The construction of the reporter plasmids pGL2proHind-Sal and p185-TATA is as described in the text. The experiments were carried out in U-87 MG cells as described in (A).

(AGGTCA A AGGTCA) [31]. To analyse if this sequence was essential for the response to PPAR γ , two mutants of the plasmid p185 in the DR-1 were constructed by PCR (Figure 5A). As already mentioned, the luciferase activity of plasmid p185 was induced approx. 10-fold by the co-transfection of a plasmid expressing PPAR γ . When the PPAR γ ligand ciglitazone was added to the cultures, the induction was more than 60-fold (Figure 5B). Both mutants lost the ability to be induced by PPAR γ either in the presence or in the absence of ciglitazone

Figure 3 Lack of response of the intergenic region to C/EBPs

U-87 MG cultures were transfected with reporter plasmids pEpro (white bars), pPME (grey bars) or pNdeHpa (black bars) always in the presence of either C/EBP β or C/EBP δ expression plasmids. The luciferase (Luc) activity was measured in cell extracts as indicated and the values are expressed as fractions of the luciferase activity of the extracts of the cells co-transfected with both pEpro and the corresponding C/EBP expression plasmid. A map of the plasmids is shown in Figure 2(A).

ACTGCTCTGA TCACCCACGT GGAGGCAGCA CCCCTCCCCT GGAGATGACT
TGACGAGACT AGTGGGTGCA CCTCCGTCGT GGGGAGGGGA CCTCTACTGA

CACCAGGGCT GAGTGAGCAG **GGGAAGGGTC** AGTGTGCTCA CAGGCAGGGG
GTGGTCCCGA CTCACTCC**TC** **CCCTTCCCAG** TCACACGAGT GTCCGTCGCC

GCCTGGTCTG CTGGGCCTGC TGCTGATTCA CCGTATGTCC AGGAGCTGGA
CGGACCAGAC GACCCGGACG ACGACTAAGT GGCATACAGG TCCTCGACCT

GCAGCACCT CTCAGAGCCT CAGTTTCCCC AGATG
CGTCGTGGGA GAGTCTCGGA GTCAAAGGGG TCTAC

Figure 4 Nucleotide sequence of the 185 bp fragment that contains the functional PPRE

This sequence is part of the apoE/apoC I intergenic region (accession number AJ249921). The putative PPRE is shown in bold-face letters.

(Figure 5B). These results indicated that the ability to respond to PPAR γ lies within the DR-1 element.

PPAR γ belongs to a family that includes the isoforms PPAR α and PPAR β/δ , the response elements being also DR-1. However, there is a certain degree of specificity in the sequences outside the DR-1 which results in the preference of the DR-1 for a given PPAR isoform [32]. To study the isoform specificity of the apoE/apoC I element, the plasmid p185 was co-transfected with PPAR γ , β/δ or α -expression plasmids. As shown above, PPAR γ induced the luciferase activity of the reporter plasmid p185 by a factor of ten which increased to more than 60-fold in the presence of the ligand ciglitazone. The response to PPAR α was significantly lower, 2.6 ± 0.2 and 3.3 ± 0.4 in the absence and in the presence of Wyl4643 (a PPAR α ligand) respectively. No induction was seen when transfecting with PPAR β/δ expression vector (Figure 6). Thus, this PPRE responds preferentially to PPAR γ .

PPRE is able to bind PPAR γ -RXR α in gel-retardation assays

To study whether the putative PPRE was able to bind PPAR γ and/or RXR α , a double-stranded oligonucleotide spanning the

Figure 5 Mutations in PPRE result in the lack of induction of luciferase activity

(A) Alignment of the nucleotide sequence of the wild-type PPRE of the apoE/apoC1 intergenic region and of the mutants constructed for this work. The consensus PPRE sequence is also shown. (B) Induction of the luciferase (Luc) activity of p180 and of the mutants when co-transfected with a PPAR γ -expression vector either in the presence (grey bars) or in the absence (white bars) of 3 μ M ciglitazone. The experiments were carried out in U-87 MG cells at least three times. Black bars indicate luciferase activity of the reporter plasmid in the absence of co-transfected PPAR γ expression plasmid. Error bars represent S.D.

Figure 6 DR-1 is specific for PPAR γ

The reporter plasmid p185 was co-transfected into U-87 MG cells with expression plasmids corresponding to the different PPAR isotypes either in the presence or in the absence of specific ligands. The concentrations of the ligands used for these experiments were 3 and 10 μ M for ciglitazone and Wy-14643 respectively. See the text for the numerical values. Error bars represent S.D. Luc, luciferase.

Figure 7 The DR-1 sequence binds PPAR γ -RXR α

The heterodimer PPAR γ -RXR α , but not the individual receptors are able to bind apoE/apoC1 PPRE. Labelled PPREwt oligonucleotide was incubated in the presence of *in vitro* synthesized proteins or in the presence of THP-1 nuclear extracts as indicated. The sequence of PPREwt and of PPREmut5 and PPREmut3 are indicated in the Experimental section. Lane 1, no protein added; lane 2, binding reaction of labelled PPREwt in the presence of *in vitro* synthesized PPAR γ ; lane 3, binding reaction in the presence of *in vitro* synthesized RXR α ; lane 4, binding reaction in the presence of both *in vitro* synthesized PPAR γ and RXR α ; lane 5, as for lane 1; lane 6, binding reaction in the presence of both PPAR γ and RXR α (as for lane 4); lane 7, the same binding reaction as in lane 6 but competed by a 100-fold excess of cold wild-type oligonucleotide; lane 8, the same as in lane 6 but competed by a 100-fold excess of an oligonucleotide mutated in the PPREmut5; lane 9, competition with a 100-fold excess of an oligonucleotide mutated in the PPREmut3; lane 10, competition with a 100-fold excess of an unspecific oligonucleotide corresponding to the binding site of the transcription factor Spi-1; lane 11, binding reaction in the presence of *in vitro* synthesized proteins as in lane 4; lane 12, the same as in lane 11 but in the presence of an anti-PPAR γ antibody (note the decrease in the band for the PPAR-RXR-DNA complex in lane 11 and the appearance of a supershifted band). Lanes 13 and 14 are from the same gel as lanes 11 and 12 but with THP-1 nuclear extracts instead of *in vitro* synthesized proteins. In lane 13 a band in the same position as the PPAR-RXR complex is observed. In lane 14 the PPAR γ -specific antibody was included in the reaction. Note that the band in the position of the PPAR-RXR complex (labelled with an arrow on the right-hand side of the Figure) almost disappeared in the presence of the antibody.

DR-1 (PPREwt) was labelled and used in EMSAs. The first assays were done with *in vitro* translated proteins. Figure 7 shows a composition of typical results of EMSAs. A unique band was retarded in the presence of both PPAR γ and RXR α (lane 4) but not in the presence of either PPAR γ or RXR α alone (lanes 2 and 3 respectively). The band was specific for the oligonucleotide as it disappeared when a 100-fold excess of unlabelled oligonucleotide was added to the mixture (lane 7), but did not with a 100-fold excess of an unlabelled unspecific oligonucleotide containing an Spi-1 binding site [24] (lane 10). Besides, two oligonucleotides with the same mutations as the plasmids used in the previous transfection assays (PPREmut5 and PPREmut3, see Experimental section) at a 100-fold excess were not able to displace the binding of the wild-type oligonucleotide (lanes 8 and 9). Neither labelled mutant oligonucleotide was able to produce any retarded band (results not shown). The addition of a PPAR γ -specific antibody to the mixtures resulted both in a decrease in the retarded band and in the presence of a supershifted band (compare lanes 11 and 12). The presence of PPRE-binding activity was also tested in THP-1 extracts. A band of the same size as the one observed in lane 11 but with less intensity was seen (labelled with an arrow in lane 13). The band almost disappeared in the presence of a PPAR γ -specific antibody (lane 14). These

results confirmed that the DR-1 was able to bind the dimer PPAR γ -RXR α and that this binding activity was present in the extracts of THP-1.

DISCUSSION

The results presented here clearly show the induction of apoE gene expression in cell cultures by a combination of ciglitazone, a PPAR γ ligand, and 9-*cis* retinoic acid, a ligand of RXR, the obligatory heterodimer partner of PPAR γ . These results suggested that apoE gene expression is regulated by the induction of PPAR γ . Furthermore, the treatment of the cultures with BADGE, a specific PPAR γ inhibitor, resulted in a reduction of apoE mRNA levels. BADGE acts by binding to PPAR γ and antagonizing the ability of agonist ligands to activate the transcriptional activity of the receptor [25]. The decrease of apoE mRNA upon BADGE treatment might indicate that PPAR γ is also contributing to the maintenance of apoE gene expression basal levels. Although this is the first report of an induction of apoE gene expression by PPAR γ , this regulation was not unexpected as both apoE and PPAR γ mRNAs are induced during the processes of macrophage [11,12] and adipocyte differentiation [16,33]. The regulation of the apoE gene by PPAR γ was also supported by the presence of the sequence 5'-AGGGGAAGGGTCA3', which resembles the DR-1 consensus sequence recognized by PPAR, in the apoE/apoCI intergenic region. Functionally, this sequence behaved as a PPRE since several DNA fragments containing it conferred PPAR γ responsiveness to the apoE promoter in transient expression assays. The PPAR γ responsiveness was abolished by mutation of either halves of the DR-1. This functional characteristic was confirmed also by gel-retardation assays in which this sequence was able to specifically interact with the PPAR γ -RXR α dimer. A PPRE-binding activity was present in THP-1 cells which agrees with previous results from other groups [12].

The PPRE described here is specific for PPAR γ and no significant induction was seen by co-transfection with plasmids expressing either the PPAR α or the PPAR β/δ subtypes. In the CYP4A6 element, with a rather weak DR-1 element, the sequence flanking the DR-1 in the 5' region could help to discriminate between PPAR α -RXR α and other DR-1 binding proteins. The 5' flanking region of the element described here is different from the consensus described for the 5'-flanking sequences of known PPRES [31]. This could explain why this element responds to PPAR γ and not to PPAR α , as PPAR α binding is more dependent on the 5'-flanking region [32].

By studying the influence of different parts of the apoE/apoCI intergenic region in the expression of human apoE in transgenic mice, Simonet et al. [17] identified a region specific for expression in spleen between coordinates 1700 and 4000 3' to the apoE gene. The expression of apoE in spleen is fundamentally due to the presence of macrophages [3,5]. The fact that the PPRE described here (coordinates 2029–2049) mapped within this region, made us initially think that this element might be essential for the macrophage-specific expression of apoE. However, very recent experiments have shown that a 620 bp region denominated ME.1, which maps between coordinates 3302 and 3922, is sufficient for the expression of apoE in the macrophages of transgenic mice. This region was not only different to the one described here but also it did not contain any sequence with an obvious similarity to a PPRES [18]. Our gene reporter assays also indicated that this region does not have the ability to respond to PPAR γ . The ME.1 enhancer contains several sequences similar to the consensus binding site of C/EBP, a family of transcription factors whose expression is also induced during the process of

macrophage and adipocyte differentiation [30]. However, our transient transfection data (Figure 3) argue against a direct interaction of C/EBPs with this region of the apoE gene during the differentiation process. Thus, the PPRES described here is the only sequence of the apoE/apoCI intergenic region with a demonstrated ability to respond to the inducers of macrophage/adipocyte differentiation. Why this region is not essential for apoE expression in the macrophages of transgenic mice is an issue that needs further research, but it is possible that in the absence of PPAR γ there is still a considerable level of apoE expression as it happens in THP-1 cultures when treated with the PPAR γ inhibitor BADGE. It would be interesting to test the influence of this PPRES on the levels of apoE mRNA in the transgenic system used in [18].

In summary, the results presented here indicate that apoE expression is regulated by PPAR γ ligands and demonstrate the existence of a PPRES located in the apoE/apoCI intergenic region. These results could be of importance as it has been recently demonstrated that PPAR γ inducers could favour foam cells formation by inducing the expression of CD36, the scavenger receptor gene [12]. These results questioned the convenience of treating people with high risk of atherosclerosis with PPAR γ inducers. On the other hand thiazolidinediones, the most used PPAR γ inducers, are known to be anti-atherogenic [34]. It has been pointed out that the sole formation of foam cells is not necessarily a pro-atherogenic process [35] if the cell is provided with a mechanism that allows it to get rid of the excess of cholesterol. Such a mechanism might well be a direct induction of apoE expression. This hypothesis has received further support by the recent finding of a liver-X-receptor response element within the ME.1 enhancer which provides a mechanism for the induction of apoE gene expression as a consequence of the loading of cells with lipids [36]. A further induction of apoE gene expression by treatment with thiazolidinediones could avoid the saturation of the defence mechanism provided by liver X receptor. This hypothesis needs further testing but if confirmed would help to explain the anti-atherogenic properties of thiazolidinediones.

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