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Peroxisome Proliferator-Activated Receptor β regulates acyl-CoA synthetase 2 in reaggregated rat brain cell cultures

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Running title: PPARβ and ACSs in brain cell aggregates

SUMMARY

Peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors that regulate the expression of many genes involved in lipid metabolism. The biological roles of PPARα and γ are relatively well understood, but little is known about the function of PPARβ. To address this question and since PPARβ is expressed to a high level in the developing brain, we used reaggregated brain cell cultures prepared from dissociated fetal rat telencephalon as experimental model. In these primary cultures, the fetal cells form random aggregates initially, which progressively acquire a tissue specific pattern resembling that of the brain. PPARs are differentially expressed in these aggregates with PPARβ being the prevalent isotype. PPARα is present at a very low level and PPARγ is absent. Cell type-specific expression analyses revealed that PPARβ is ubiquitous and most abundant in some neurons, while PPARα is predominantly astrocytic. We chose acyl-CoA synthetases (ACS) 1, 2 and 3 as potential target genes of PPARβ and first analyzed their temporal and cell type-specific pattern. This analysis indicated that ACS2 and PPARβ mRNAs have overlapping expression patterns, thus designating the ACS2 gene as a putative target of PPARβ. Using a selective PPARβ activator, we found that the ACS2 gene is transcriptionally regulated by PPARβ, demonstrating a role for PPARβ in brain lipid metabolism.

INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily. Three PPAR isotypes, α, β (also called δ, FAAR and NUC1) and γ, have been cloned from *Xenopus*, rodents and human. PPARs regulate gene expression by binding as heterodimers with retinoid X receptors to peroxisome proliferator response elements in the promoter of genes involved in lipid metabolism (1-3). The biological roles of PPARα and γ are relatively well understood, not least because specific ligands for these isotypes have been identified (4). PPARα regulates genes involved in peroxisomal and mitochondrial β-oxidation as well as lipoprotein metabolism (2;5). PPARα also suppresses apoptosis in cultured rat hepatocytes (6) and reduces inflammatory responses (7;8). PPARγ stimulates adipogenesis, enhances insulin sensitivity, and is involved in cell cycle control and regulation of tumor growth (9-13).

In contrast, the functions of PPARβ are poorly understood, due partly to its ubiquitous expression and the lack of a selective ligand. The objective of this study was to start unraveling PPARβ functions using an experimental model easy to manipulate and expressing high levels of PPARβ compared to PPARα and γ. The nervous system seemed an appropriate target for this, as PPARβ is abundantly expressed in brain from embryogenesis to adulthood, while PPARα and γ are barely detectable (14-17). The brain is the organ with the highest lipid concentration in the body, second only to adipose tissue. Brain lipids serve primarily in modifying the fluidity, structure and functions of the membranes, and both anabolic and catabolic pathways of lipid metabolism are important in brain development (18;19). Fatty acids need to be activated to their acyl-CoA by acyl-CoA synthetases (ACS), the activities of which have been found in the brain (20;21). Moreover, ACS1, 2 and 3 mRNAs have been analyzed in the postnatal rat brain, and the levels of ACS2 and 3 vary during its development (22). Given the key role of ACSs in fatty acid utilization, we speculated that they could be potential targets of PPARβ in the brain.

We chose cultures of reaggregated neural cells prepared from the telencephalon of rat embryos which provide a three-dimensional network of different neural cell types that progressively acquire

a tissue specific pattern resembling that in the brain (23;24). We first determined the expression pattern of PPARs during maturation of brain aggregates, providing evidence for PPARβ being the prevalent isotype. Then, using a selective activator for PPARβ, we demonstrate that the ACS2 gene is regulated by PPARβ at the transcriptional level.

EXPERIMENTAL PROCEDURES

Cell culture: Reaggregated brain cell primary cultures were prepared from mechanically dissociated telencephalon of 16-day rat embryos as previously described (24). Cultures were initiated and grown in serum-free, chemically defined medium consisting of Dulbecco's modified Eagle's medium (DMEM) with high glucose (25 mM), and supplemented with insulin (0.8 μM), triiodothyronine (30 nM), hydrocortisone-21-phosphate (20 nM), transferrin (1 μg/ml), biotin (4 μM), vitamin B12 (1 μM), linoleate (10 μM), lipoic acid (1 μM), L-carnitine (10 μM), and trace elements (listed in ref.24). Gentamicin sulfate (25 μg/ml) was used as antibiotic. Culture media were replenished by exchange of 5 ml of medium (of a total of 8 ml/flask) every third day until day 14, and every other day thereafter because of increased metabolic activity in the cells. The cultures were maintained under constant gyratory agitation (80 rpm), at 37 ^{0}C and in an atmosphere of 10 % CO_2 / 90 % humidified air.

Northern blot analysis: PPARβ mRNA was probed with the 1.32 kb BamH1 fragment of the plasmid pSG5-rPPARβ containing the coding region of the rPPARβ.[2] The different ACS mRNAs were probed with DNA fragments isolated from their respective rat cDNA containing plasmids (22). ACS1 mRNA was probed with the 0.96 kb EcoRV fragment of the pRACS15 plasmid. ACS2 mRNA was probed with the 2 kb EcoRI fragment of the pBACS9 plasmid. ACS3 mRNA was probed with the 2.2 kb BglII/SacI fragment of the pACS3 plasmid. L27 mRNA, which was used as an internal control, was probed with the 0.22 kb XbaI/EcoRI fragment of the pKS^{+}/L27 plasmid (25). The above mentioned gel purified DNA fragments of PPARβ (100 ng), the three ACSs (100 ng each) and L27 (25 ng) were used as templates to synthesize [α ^{32}P]-dCTP labeled probes using the High Prime kit (Boehringer Mannheim). Probes were purified on Elutip-d columns according to the manufacturer's protocol (Schleicher and Schuell).

Brain cell aggregates from each flask were carefully transferred to round bottomed 14 ml Falcon tubes, washed with PBS and frozen rapidly by plunging the capped tubes into liquid nitrogen after

aspirating the residual amounts of PBS in the samples. Frozen aggregates were stored at –80 ^{0}C until use. Aggregates were thawed rapidly at 37 ^{0}C in 5 ml of TRIzol (Life Technologies) and total cellular RNA was prepared according to the protocol supplied by the manufacturer.

RNA (25 μg/well) was electrophoresed in a 2.2 M formaldehyde-1% agarose gel in MOPs buffer and transferred onto Zeta Probe GT-membrane (BioRad) by capillary blotting in 10X SSC for about 18 h. The northern blot membranes were baked for 30 min at 80 ^{0}C, pre-hybridized at 65 ^{0}C for 2-6 h in 0.25 M Na_2HPO_4 (pH 7.2) containing 7 % SDS and hybridized at the same temperature with [α^{32}P]-labeled probes (2-3 x 10^6 cpm/ml) for 18 h. After hybridization, the membranes were sequentially washed at 65 ^{0}C for 30 min each in 20 mM Na_2HPO_4 (pH 7.2) containing 5% SDS and in 20 mM Na_2HPO_4 (pH 7.2), 1% SDS and then exposed to pre-flashed films at –70 ^{0}C for 2-3 days for PPARβ and ACSs and 18-24 h for L27. Autoradiographs were quantified in a Elscript 400 AT/SM densitometer (Hirschmann) and the mRNA signals were normalized for the loading error using the L27 mRNA signal as internal control, as described previously (26).

RNase Protection assay: [α^{32}P]-UTP labeled riboprobes were transcribed in vitro with T7 RNA polymerase and RNase protection assay was carried out on 15 μg of total RNA as described previously (25). The PPARα riboprobe was synthesized from the 717 bp TaqI fragment of the pKS$^+$/PPARα plasmid and the L27 riboprobe from the pBS-L27(150) plasmid linearized with EcoRI. Samples were electrophoresed on 6% polyacrylamide gels and quantified using a Storm 840 phosphorimager (Molecular Dynamics).

Subcloning of the acyl-CoA synthetase DNA fragments: Short DNA fragments of the three ACS cDNAs (22) were subcloned into the pBluescript II KS$^-$ vector (pBSII KS$^-$) from Stratagene to use as probes for *in situ* hybridization. For ACS1, the 305 bp HindIII fragment corresponding to nucleotides 1484-1789 in the plasmid pRACS15 was subcloned into the HindIII site of the vector

and the recombinant plasmid was named pBSII KS$^-$-ACS1. For ACS2, the 368 bp BamH1/EcoRI fragment corresponding to nucleotides 143-511 in the plasmid pBACS9 was subcloned into the BamH1/EcoRI site in the bluescript vector and the recombinant plasmid was named pBSII KS$^-$-ACS2. For ACS3, the 364 bp SpeI-XbaI fragment corresponding to nucleotides 682-1047 in the plasmid pACS3 was subcloned into the vector digested with SpeI-XbaI and the recombinant plasmid was named pBSII KS$^-$-ACS3. The ACS1, ACS2 and ACS3 recombinant plasmids contained the insert in such an orientation that the antisense probe was synthesized with T3 RNA polymerase and the sense probe with T7 RNA polymerase.

In situ hybridization: Digoxigenin labeled PPARα and β riboprobes were transcribed in vitro from linearized, gel purified plasmids using T7 RNA polymerase as described previously (17). Antisense probes were synthesized from the plasmids pKS$^+$/PPARα and pSK$^+$/PPARβ while the sense probes were synthesized from the plasmids pSK$^+$/PPARα and pKS$^+$/PPARβ. Digoxigenin labeled ACS riboprobes were *in vitro* transcribed from the plasmids pBSII KS$^-$-ACS1, pBSII KS$^-$-ACS2 and pBSII KS$^-$-ACS3 linearized with XbaI for antisense probes of ACS1 and 2 and NotI for ACS3. Sense probes were synthesized from the same plasmids linearized with XhoI for ACS1 and HindIII for ACS2 and 3.

Aggregates washed with PBS were embedded in tissue freezing medium (Jung, Nussloch), frozen in isopentane cooled with liquid nitrogen as described previously (24) and stored at -80 ^{0}C until use. Cryostat sections (12 μM thickness) were hybridized for 40 h at 58 ^{0}C with digoxigenin labeled probes (400 ng/ml) in 5X SSC containing 50% formamide and 40 μg/ml salmon sperm DNA. Sections were washed and the mRNAs were visualized by alkaline phosphatase staining as previously described (17) and then dehydrated, mounted and photographed on an Axiophot microscope (Carl Zeiss).

Immunohistochemistry: Monoclonal antibodies against cell type-specific proteins were used for immunohistochemical analysis of the aggregates. Antibodies against glial fibrillary acidic protein (GFAP), microtubule associated protein 2 (MAP2) (Boehringer Mannheim) and myelin basic protein (MBP) (Boehringer Ingelheim) were used to identify astrocytes, neurons and oligodendrocytes, respectively. Cryosections (12 μM) were fixed for 1 h in 4 % paraformaldehyde-PBS at room temperature, washed in PBS (3 x 5 min.) and incubated overnight at 4 ^{0}C with the primary antibody diluted 1:100 in dilution buffer (PBS containing 1 % bovine serum albumin and 0.3 % Triton X-100). After washing away the primary antibody, sections were first incubated at room temperature for 1 h with an anti-mouse IgG (Sigma) bridging antibody diluted 1:100 in dilution buffer, and then incubated at room temperature for 1 h with the alkaline phosphatase/anti-alkaline phosphatase complex diluted 1:100. Sections were then washed with PBS, stained for alkaline phosphatase for 20 min, dehydrated, mounted and photographed on an Axiophot microscope.

Reporter-gene assay in HeLa cells: Cells were cultured at 37 ^{0}C, 5% CO_2 in DMEM supplemented with antibiotics and 5% fetal calf serum. For transient transfection by the calcium phosphate method, 2.5x10^{5} cells/ well were plated on 6-well plates. After 24 h, DNA mix (200 μl) containing 0.1 μg of PPAR expression plasmid [3], 0.5 μg of the internal control plasmid CMV-βgal (Clontech), 2 μg of the reporter plasmid Cyp2XPalCAT (27) and 5 μg of sonicated salmon sperm DNA was added to each well and cells were transfected for 12 h. The medium was then replaced with DMEM supplemented with 5% charcoal treated fetal calf serum, activators were added and cells were incubated at 37 ^{0}C for 24 h. Cells were then scraped off the dishes and total cell extracts were prepared by 3 cycles of freezing in liquid nitrogen and thawing at 37 ^{0}C. β-Galactosidase and chloramphenicol acetyl transferase (CAT) activities were measured in these extracts by standard methods (28). Relative CAT activity was calculated as the fold increase in CAT activity over the basal activity obtained with the empty vector in untreated cells.

Treatment with activators and marker enzyme assays: Stock solutions of bezafibrate (Sigma) and L-165041 compound (a gift from Parke-Davis, USA) were prepared in ethanol and dimethyl sulfoxide (DMSO) respectively. The effect of activators on marker enzyme activities were measured on reaggregated cultures, equivalent to 1/4th of the original cultures, treated on days 34 or 35 either with 0.05% ethanol (control-bezafibrate) or 0.05% DMSO (control-L-165041) or 10 μM bezafibrate or 5 μM and 10 μM L-165041 for 24 h. After treatment, the samples were homogenized in ground glass homogenizers. The marker enzymes used were glutamine synthetase (GS) for astrocytes, glutamic acid decarboxylase (GAD) for GABAergic neurons and choline acetyl transferase (ChAT) for cholinergic neurons, and were measured using radiometric methods as previously described (24). Determination of protein amounts by the Lowry method was done on replicate cultures representing 1/4th of the original cultures.

RESULTS

PPAR mRNAs in reaggregated brain cell cultures: As PPARs have not been studied so far in the developing postnatal brain, we first characterized the aggregates for the basal level of PPAR mRNAs. We examined these cultures at four ages representing different stages of maturation of neurons and glia (henceforth referred to as differentiation). At day 7 (d7), the cells were undifferentiated; at d21, differentiation of neuron and glia subtypes was ongoing; d35 cultures reached the steady state of differentiation and d49 represented late cultures with the highest level of neuron-specific parameters, although some demyelination may already occur (29;30).

The basal levels of the PPAR isotype mRNAs were different in these aggregates. PPARα mRNA was barely detectable by northern blot analysis, but was detected by RNase protection assay (Fig. 1A). In contrast, PPARβ mRNA was abundant and could be detected easily by northern blot analysis of total RNA (Fig. 1B). Total cellular RNA from adult rat liver and brain was used as positive control for PPARα and β, respectively. PPARγ mRNA was not detected in the aggregates even by RT-PCR (data not shown) and therefore this isotype was not analyzed in subsequent experiments. There was also a different age-dependent pattern of expression of both PPAR isotypes. On the one hand, the PPARβ mRNA basal level was low in undifferentiated cultures (d2, 7), increased 3-5 fold during differentiation (d14, 21, 28), remained high in differentiated (d35) and late (d42, 49) cultures (Fig. 1C). On the other hand, the level of PPARα mRNA remained low at the four developmental stages examined (Figs. 1A and 1C inset). Thus, of the three PPARs, only the two isotypes α and β were present in the aggregates, and the levels of PPARβ increased with the onset of cell differentiation.

Cell type-specific expression of PPAR isotypes in reaggregated brain cell cultures: In order to identify the cells expressing PPARs in the aggregates, we used *in situ* hybridization to detect PPAR transcripts in cells (Fig. 2A), as well as immunohistochemical staining for cell type-specific markers to identify these cells (Fig. 2B). The marker antigens used for immunohistochemistry were

MAP2 for neurons, GFAP for astrocytes and MBP for oligodendrocytes. Since MAP2 and GFAP are cytoskeletal proteins, immunostaining for these antigens is seen in the cell bodies and processes, and the *in situ* hybridization signal of PPAR mRNAs is cytoplasmic. Furthermore, we used thionine staining in parallel sections to visualize the nuclei in the aggregates (Fig. 2A: q-t, 2B: i, k). At d7, a faint signal of PPARα mRNA was detected in most cells of the aggregates (Fig. 2A: compare a with q). At d21, the signal was slightly stronger and restricted to cells with small nuclei (Fig. 2A: compare b with r) which were identified primarily as astrocytes by immunohistochemistry (Fig. 2B: compare c with d, h and l). The pattern was similar at d35 (Fig. 2A: c). At d49, the same cell type was positive for PPARα but with a decreased signal intensity (Fig. 2A: d). The sense probe for PPARα did not stain the sections (Fig. 2A: e-h) indicating that the signal detected with the antisense probe was specific.

The *in situ* hybridization signal for PPARβ was higher than that for PPARα in all developmental stages of the aggregates (Fig. 2A: compare i-l with a-d). At d7, the PPARβ signal was of medium intensity and was present in most cells of the aggregates (Fig. 2A: i). At d21, the signal was stronger and exhibited a cell type-specific expression pattern. A signal of very high intensity was observed in cells with large nuclei (Fig. 2A: compare j with r) which formed a broad layer in the aggregates. Immunostaining with MAP2 revealed that these cells were neurons (Fig. 2B: compare g with h). PPARβ signal of medium and high intensity was also observed in the same region of the aggregates in cells with smaller nuclei, which were neurons, astrocytes and oligodendrocytes (Fig. 2B: compare g with d, h, and l). At d35 and d49, the pattern of expression of PPARβ mRNA was similar to that of d21, but the signal intensity was lower (Fig. 2A: k, l). The peripheral layer of glial cells were positive for PPARβ at all developmental stages except d7 (Fig. 2B: d, l). Again, the sense probe barely stained the sections (Fig. 2A: m-p) confirming the specificity of the signal obtained for PPARβ. These results showed that PPARβ mRNA was ubiquitous in the aggregates and high in some neurons, while PPARα mRNA was mainly detected in the astrocytes.

Acyl-CoA synthetases in the aggregates: We used a three-step approach to establish whether ACSs were target genes of PPARβ in this model of the developing rat brain. Firstly, we determined the basal levels of the three ACSs in the aggregates. We then examined their cell-type specific expression pattern and lastly studied whether PPAR activators regulate their expression.

All three ACS mRNAs were easily detectable by northern blot analysis of total RNA (Fig. 3). ACS1 mRNA was present at a low level in the four developmental stages examined. At d21, a small but significant increase of 1.8 fold over the level at d7 was observed (*t*-test, $p \leq 0.05$). The basal level of ACS2 mRNA was very low at d7, but increased 5-7 fold in d21 and d35 cultures, and remained steady in the late cultures. In undifferentiated cultures, the basal level of ACS3 mRNA was the highest among the three ACSs and this increased further 2-3 fold until d49. The increase over the basal level at d7 was statistically significant for ACS2 and 3 mRNAs (*t*-test, $p \leq 0.01$).

The comparison of the temporal expression pattern of ACS mRNAs with that of PPARs revealed similarities between ACS1 and PPARα mRNAs on one hand and ACS2 and PPARβ mRNAs on the other hand (Figs. 1 and 3).

Identification of the cell types expressing acyl-CoA synthetases in the aggregates: We examined by *in situ* hybridization the cell-type specific pattern of expression of ACS mRNAs and compared it with the immunohistochemical analysis shown in Fig. 2B. ACS1 mRNA was barely detectable in d7 cultures (Fig. 4A: a). At d21, a signal of medium intensity was observed in small cells in most regions of the aggregates which were predominantly astrocytes (Figs. 4A: b and 2B: d). The intensity of this signal decreased at d35 and d49, but was still primarily astrocytic (Fig. 4A: c, d). The glial cells at the periphery of the aggregates did not stain for ACS1 mRNA. ACS2 mRNA signal was higher than that of ACS1 mRNA in the aggregates, and was observed in most cells at d7 (Fig. 4A: e). At d21, the signal was of high intensity in neurons with large nuclei, which form a broad layer in the aggregates. In the same region a signal of medium intensity was observed in other neurons, astrocytes and oligodendrocytes (Fig. 4A: f). In aggregates of d35 and d49, the ACS2 mRNA signal decreased in intensity (Fig. 4A: g, h), but was present in the same cell types as

observed at d21. ACS3 mRNA was found in most cells of the aggregates at days 7 and 21 (Fig. 4A: i, j) with a few cells showing a very strong signal. At d35 and d49, the ACS3 mRNA signal decreased in intensity and was observed in neurons, astrocytes and oligodendrocytes (Fig. 4A: k, l). Large cells, which stained intensely at d7 and d21, were not apparent in these differentiated cultures. The peripheral layer of glial cells was positive for ACS2 and 3 mRNAs in cultures of all developmental stages except d7. No signal was observed with the sense probes for ACS1, 2 and 3 mRNAs (data not shown).

The *in situ* hybridization analysis of the three ACSs in d21 aggregates was compared with that of PPARβ and is shown at a higher magnification in Fig. 4B. The expression pattern of ACS2 mRNA was similar to that of PPARβ (Fig. 4B: b, c). This finding, taken together with their temporal pattern of expression, suggested that ACS2 might be a target gene of PPARβ.

Activation of rat PPARs in a cell based reporter gene assay: To test whether ACS2 was a PPARβ target gene, a PPAR activator was required, that preferentially activates rPPARβ. In a recent study from our laboratory, several compounds were screened with the coactivator-dependent receptor ligand assay. Fatty acids, eicosanoids and hypolipidemic drugs were identified as ligands for *Xenopus* PPARs (27). Most of the ligands identified for xPPARβ by this assay exhibited similar or greater activity towards xPPARα. As bezafibrate, which was strong on xPPARβ, was the least active on xPPARα, we tested this hypolipidemic drug on rPPARs in a reporter gene assay in HeLa cells. Transfection of the PPARα expression vector resulted in an apparent ligand independent activation of the reporter gene (Fig. 5A), which has been observed previously in HeLa cells (31). In cells treated with 10 and 100 μM bezafibrate, PPARα mediated transcription of the reporter gene was enhanced. In contrast, expression of PPARβ did not affect the basal activity of the reporter, and treatment with the 100 μM bezafibrate activated the reporter gene only weakly. Thus, at a concentration of 10 μM, bezafibrate can be used as a specific activator of rPPARα.

L-165041 compound has been recently identified as a human PPARβ agonist (32). In order to characterize its activity profile on rPPARs, we tested this compound in the reporter gene assay in HeLa cells. In cells treated with 5 and 10 μM L-165041, there was only a small increase in PPARα mediated transcription of the reporter gene, indicating a weak effect of the compound on this isotype (Fig. 5B). After a similar treatment, PPARβ-dependent activity of the reporter gene increased dramatically above its basal level (Fig. 5B). This strong activation contrasts with the absence of background activity of rPPARβ and provide strong evidence for the high L-165041 responsiveness of this isotype. We therefore used this compound as a PPARβ activator on the aggregates.

Effect of bezafibrate and L-165041 on the aggregates: We next tested whether the two activators had any strong and general effect on the aggregates. These cultures are an established model for neurotoxicological studies (33) and the major subtypes of neurons present in the differentiated aggregates are cholinergic, glutamatergic and GABAergic neurons. In the aggregates, the lactate dehydrogenase release assay underestimates the cytotoxic effects of test compounds and therefore estimation of cell-type marker enzyme activities is a more accurate indicator of the effect of chemical treatments. Routine biochemical analysis for the cellular effects of compounds involves measuring GS, ChAT and GAD representing glial cells, excitatory neurons and inhibitory neurons respectively, while the total protein content is used as an indicator of general cytotoxicity. Treatment of the differentiated aggregates with bezafibrate or L-165041 for 24 h did not cause any visible alteration in their size or glucose metabolism. Similarly, there was little effect on the marker enzyme activities and on the total protein content of the aggregates (Table 1). Therefore, we conclude that treatment with the two activators did not modify the neuron and glial cell parameters and did not have general cytotoxic effects on the aggregates. This also suggested that the marker enzymes were probably not regulated by either PPAR.

Acyl-CoA synthetase 2 is a target gene of PPARβ in reaggregated brain cell cultures: Specific activators or ligands of PPARα and PPARγ have been successfully used to identify their target genes. Therefore, it was reasonable to expect an effect of L-165041 on ACS2 expression if it was a target gene of PPARβ. Treatment of differentiated aggregates for 24 h with bezafibrate had no effect on either the ACS mRNAs or PPARβ mRNA (Fig. 6A). However, a similar treatment of the aggregates with 5 and 10 μM L-165041 increased the ACS2 mRNA levels significantly (2.4 and 3 fold), but did not affect the ACS1 and 3 mRNAs (Fig. 6B). We conclude that ACS2 is a target gene of PPARβ in the postnatal rat brain since its temporal and cell type-specific expression pattern correlated with that of PPARβ, and its mRNA is induced after treatment with a selective activator of this PPAR isotype. The 2 fold increase in PPARβ mRNA level itself after L-165041 treatment (Fig. 6B) was statistically significant (*t*-test, $p \leq 0.05$).

L-165041 mediated induction of ACS2 mRNA occurs by transcriptional activation and requires protein synthesis: Next, we tested whether the L-165041 effect on ACS2 mRNA expression was at the transcriptional or post-transcriptional level by treating aggregates for 6 and 30 hours with 10 μM L-165041 in the presence of 0.1 μM of the transcriptional inhibitor actinomycin D (ActD, Fig. 7). In the absence of Act. D, the expected L-165041-dependent induction was observed after a 30 hours treatment. However, treatment with ActD prevented induction by L-165041, ruling out an effect of the compound on the stability of the ACS2 mRNA. If L-165041 stabilized the ACS2 mRNA, then higher levels of this mRNA would have been observed in presence of the compound in ActD treated samples. Therefore, regulation is at the transcriptional level and it was then of interest to test whether it requires *de novo* protein synthesis. This possibility was examined in aggregates incubated in the presence of 5 μM of the protein synthesis inhibitor cycloheximide (CHX, Fig. 7). In the presence of CHX, induction of the ACS2 gene by L-165041 was blocked, providing evidence that *de novo* protein synthesis was required for ACS2 mRNA stimulation. The requirement for ongoing protein synthesis suggests that either the PPARβ protein,

or another key factor, is turned over rapidly, or that Merk A induces the *de novo* synthesis of a missing regulatory protein.

DISCUSSION

This is the first report on PPAR expression in reaggregated rat brain cell cultures, which is an *in vitro* experimental model representative of the developing postnatal brain *in vivo*. The finding that PPARβ was the prevalent isotype in these cultures allowed us to test whether ACS1, 2 and 3 were target genes of this PPAR. Using L-165041 as a selective activator of rPPARβ in the aggregates, we have identified ACS2 as the first PPARβ target gene.

The aggregates as an *in vitro* experimental model of the developing postnatal brain: Morphological and biochemical studies have shown that the reaggregated cultures of mechanically dissociated fetal rat telencephalon are able to mimic several morphogenetic events occurring *in vivo*, including cell migration, synaptogenesis and myelination. These three-dimensional cell cultures provide maximum intercellular contacts and interactions which facilitates the maturation-dependent expression and deposition of extracellular matrix components (34). The distinct stages of cell proliferation and differentiation observed are comparable to the tissue *in vivo*. The aggregates also exhibit cell type-specific and development-dependent expression of neuronal and glial cytoskeletal proteins and reproduce the developmental pattern of Na^+-K^+-ATPase gene expression observed *in vivo* (35;36). Therefore, the observations made in this model can be considered to reflect the *in vivo* situation. Since these aggregates were analyzed between 2 to 49 days, this study covers a period of time corresponding to the development of the brain from 18 day embryo to about six weeks postnatal period.

PPARs in the aggregates: The differential expression of PPARs in the aggregates taken together with their cell type-specific expression pattern suggest that the neurons contain a low level of PPARα and a high level of PPARβ, whereas the glia contain a low level of both PPARs. This is in concert with the expression of PPARα and β in monolayer cultures of neonatal neurons (37). However, in astrocyte enriched monolayer cultures, it was observed that PPARβ mRNA levels were

higher than those of PPARα mRNA. Furthermore, in both neuron and astrocyte-enriched monolayer cultures, very low level of PPARγ was present (37). It has also been reported that PPARβ was abundant in monolayer cultures enriched for oligodendrocytes but present only at low levels in primary astrocyte cultures (38). Our *in situ* hybridization results indicate that in the aggregates PPARβ is present at a low level in both these cell types. These differences in PPAR expression between monolayer and aggregate cultures most likely reflect effects of these two modes of culture on gene expression.

The general differential expression of PPAR mRNAs in d49 aggregates (representative of the 6 weeks old rat brain) is consistent with that observed in the adult rodent (14;17;39) and human (40) brain *in vivo*. Furthermore, the cell type-specific expression of PPARβ in these aggregates is also consistent with that found in the rat brain (16;17). Thus, PPARβ expression in the aggregates has more similarities with that *in vivo* than in monolayer cultures. This confirms that reaggregated cultures provide a better model to study the biological role of this PPAR isotype.

Acyl CoA synthetases in the aggregates: The differential expression of ACSs in the aggregates, with ACS2 and 3 being abundant and ACS1 at a very low level, is consistent with that observed in the developing postnatal rodent brain (22;41). Since the cell type-specific expression of ACS1, 2 and 3 mRNAs has not been studied *in vivo*, we analyzed the distribution of these mRNAs in the aggregates and found that ACS1 mRNA is localized mainly in the astrocytes and ACS2 mRNA in astrocytes and neurons. These expression patterns of ACS1 and ACS2 correlate well with PPARα and PPARβ expression, respectively. ACS3 mRNA was present in most cells of the aggregates, a pattern of expression quite different from that of PPARα or β. The similarity in the ACS2 and PPARβ expression patterns identified the former as the product of a potential target gene of the latter. We could confirm this in the differentiated aggregates, which have a high level of PPARβ, using an activator that exhibited a preference for this PPAR isotype.

Activators of PPARβ: One of the key issues in analyzing PPARβ function is that of the availability of PPAR isotype selective ligands. All compounds that have been reported to activate PPARβ so far in various types of assays are also active on PPARα (7;14;27;42-44). Species differences in PPAR ligand affinity is another factor that needs to be taken into account. For example, bezafibrate was identified as a ligand for xPPARβ (27), but in reporter gene assay in HeLa cells, we found that bezafibrate was an activator of rPPARα but not of rPPARβ. In the same type of assay, L-165041 preferentially activated rPPARβ, which enabled us to identify ACS2 as the first target gene of this isotype.

ACS2 as a PPARβ target gene: In this study we provide evidence that ACS2 is a target gene of PPARβ, indeed the first one identified. The L-165041 mediated induction of ACS2 mRNA occurs by transcriptional activation of the gene and is not a consequence of stabilization of the mRNA itself. In the presence of a ligand, a direct target gene of PPARβ would be activated via a PPRE present in its promoter, even in the absence of protein synthesis, while an indirect response gene would require protein synthesis for its activation. It is not known whether the promoter of the ACS2 gene contains a PPRE. However, it is noteworthy that a functional PPRE has been identified in the promoter of the ACS1 gene (45). The requirement of *de novo* protein synthesis to achieve L-165041 mediated induction of ACS2 mRNA suggests that the ACS2 gene might be an indirect target of PPARβ. However, at this stage we cannot exclude that a short half-life of the PPARβ protein itself is the limiting parameter. This possibility will be tested when a PPARβ selective antibody becomes available.

Possible biological functions of ACS2 in the aggregates: ACS2 mRNA levels increase significantly in the differentiating aggregates, which coincides with the maturation of neurons, onset of myelination and high metabolic activity (29). Since no specific roles have yet been attributed to ACS2 in the brain, we present below several functions in the modulation of which a

regulated expression of ACS2 might be beneficial. Maturation of neurons, that is their cytodifferentiation and the formation of neuronal connectivity, involves extensive lipid biosynthesis and turnover. Biochemical analysis of ACS2 has revealed that its preferred substrates are arachidonic, eicosapentaenoic and docosahexaenoic acids (46), which are major components of the neural membranes. Another characteristic of neuron maturation is the production of neurotransmitters, their vesicular transport, storage and release. Recently, ACS1 has been localized in Glut 4 containing vesicles prepared from rat adipocytes (47), and palmitoyl-CoA appears to be required for vesicular transport *in vitro* (48). With respect to signal transduction pathways that play key roles in the brain, there are several examples of the involvement of acyl-CoA esters (reviewed in 49), such as in the stimulation of Ca^{2+} release from intracellular compartments (50-52) and stimulation of ion channels (53;54). Finally, acylation is a common post-translational modification of myelin proteins (reviewed in 55), whose bound fatty acids are turned over very rapidly (3). ACS activity has been reported in myelin (21), but the type involved not yet determined. It will be the aim of future work to investigate whether and how ACS2 is involved in the above-described processes.

Conclusions: In reaggregated brain cell cultures PPARs are expressed differentially with a cell-type specific pattern. PPARβ is the prevalent isotype and it is ubiquitous in the aggregates. PPARα is present at a low level and is mainly astrocytic while PPARγ is absent. Among the three ACSs analyzed in this study, the temporal and cell type-specific expression pattern of ACS2 correlates very well with that of PPARβ. The identification of this ACS as a PPARβ target gene demonstrates a role of this receptor in lipid metabolism in the brain. The diversity of the cellular processes in which acyl-CoA esters are known to be involved may partly explain why PPARβ appears early during development and is ubiquitous in adult tissues. A systematic analysis to correlate PPARβ directly with the cellular processes regulated by acyl-CoA esters will help to identify other target genes of this receptor.

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Footnotes:

[1] The abbreviations used are : ACS, acyl-CoA synthetase; ActD, actinomycin D; CAT, chloramphenicol acetyl transferase; ChAT, choline acetyl transferase; CHX, cycloheximide; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; GAD, glutamic acid decarboxylase; GFAP, glial fibrillary acidic protein; GS, glutamine synthetase; MAP2, microtubule associated protein 2; MBP, myelin basic protein; PPAR, peroxisome proliferator-activated receptor.

[2] P.Escher, S.Basu-Modak, O.Braissant, B.Desvergne, and W.Wahli, unpublished data.

[3] P.Escher, S.Basu-Modak, O.Braissant, B.Desvergne, and W.Wahli, unpublished data.

FIGURE LEGENDS

Figure 1: PPARs in reaggregated brain cell cultures. (A) Basal level of PPARα mRNA was determined by RNase protection assay of total RNA from reaggregated brain cell cultures of 7, 21, 35 and 49 days. Adult rat liver was used as positive control. **(B)** Northern blot analysis of PPARβ mRNA in aggregates of 7, 21, 35 and 49 days in which total RNA from adult rat brain was used as a positive control and L27 mRNA as an internal loading control. **(C)** Northern blots of RNA from 2, 7, 14, 21, 28, 35, 42 and 49 days old cultures were probed for PPARβ and L27 mRNAs. Mean ± SD, n=3-8. Inset: PPARα and L27 mRNA signals obtained by RNase protection assay. The data is a mean of two sets of samples.

Figure 2: Cell type-specific expression of PPARα and β mRNAs in the reaggregated brain cell cultures. (A) *In situ* hybridization for PPARα (a-h) and PPARβ (i-p) on aggregates of 7, 21, 35 and 49 days (columns 1-4 respectively from left). Panels a-d are sections of the aggregates hybridized with the antisense probe for PPARα mRNA (PPARαAS) while panels e-h are those hybridized with the sense probe (PPARαS). Panels i-l are sections hybridized with the antisense probe for PPARβ mRNA (PPARβAS) and panels m-p are those for the sense probe (PPARβS). Panels in the bottom row are sections of aggregates stained with thionine to visualize the general morphology of the aggregates. (Bar: 100 μM). **(B)** Immunohistochemical analysis of 7 and 21 days old aggregates (columns 2 and 4 respectively from left). Immunostaining for GFAP (b, d), MAP2 (f, h) and MBP (j, l) to identify astrocytes, neurons and oligodendrocytes respectively. For panel h, aggregates were fixed overnight in Carnoy, dehydrated and embedded in paraffin. Sections of 12 μM thickness were rehydrated and stained for MAP2 as described for cryosections. *In situ* hybridization with the antisense probe for PPARα (a, c) and PPARβ (e, g) in aggregates of 7 and 21 days (columns 1 and 3 respectively from left). Panels i and k are sections of aggregates stained with thionine. (Bar: 100 μM).

Figure 3: Acyl-CoA synthetases in the reaggregated brain cell cultures. Northern blot analysis of ACS mRNAs in 7, 21, 35 and 49 days old cultures. The total RNA was blotted in duplicate. One blot was probed in the order ACS1, L27 and ACS3, while the other was probed in the order PPARβ, L27 and ACS2. Lane B in each panel corresponds to total RNA from adult rat brain. Mean ± SD, n=3-5.

Figure 4: *In situ* hybridization for acyl-CoA synthetases in reaggregated brain cell cultures. **(A)** Sections of aggregates of 7, 21, 35 and 49 days (columns 1-4 respectively from left) were hybridized with the antisense probes for ACS1 (a-d), ACS2 (e-h) and ACS3 (i-l). (Bar: 100 μM). **(B)** *In situ* hybridization with the antisense probes for ACS1 (a), ACS2 (b), PPARβ (c) and ACS3 (d) in aggregates of 21 days. (Bar: 100 μM).

Figure 5: Activation of rat PPARs in cell based reporter-gene assay. HeLa cells were cotransfected with reporter plasmid Cyp2XPalCAT and either the empty expression vector (pSG5) or rPPARα (pSG5-rPPARα) or rPPARβ (pSG5-rPPARβ). After transfection cells were treated with bezafibrate (Bz) **(A)** and L-165041 **(B)** for 24 h. Relative CAT activities for the two activators are plotted for the empty expression vector and PPARα and β. Mean ± SD, n=3-5.

Figure 6: L-165041 treatment increases ACS2 and PPARβ mRNA levels. Reaggregated brain cell cultures were treated on day 35 for 24 h with 0.05% ethanol (control) or 10 μM bezafibrate **(A)**, or 0.05% DMSO (control) or 5 μM and 10 μM L-165041 **(B)**. Total RNA was northern blotted in duplicate. One blot was hybridized in the order ACS1, L27 and ACS3, while the other was probed in the order PPARβ, L27 and ACS2. Mean ± SD, n=3.

Figure 7: L-165041 mediated increase in ACS2 mRNA occurs at the transcriptional level and requires protein synthesis. Reaggregated brain cell cultures were treated on day 34 with 0.05%

DMSO (control) or 10 μM L-165041 for 6 and 30 h. Cycloheximide (CHX, 5 μM) and actinomycin D (ActD, 0.1 μM) were used to inhibit protein synthesis and transcription respectively. Total RNA was northern blotted and probed for ACS2 and L27 mRNAs. Treatments with CHX or ActD for 30 h had an effect on the internal control itself and therefore the non-normalized data for these samples are plotted. Mean ± SD, n=3.

TABLE 1: Effect of activators on marker enzyme activities in reaggregated cultures

Treatment	[a]Relative activity			Protein [mg]
	[a]Glutamine Synthetase	[a]Glutamic Acid Decarboxylase	[a]Choline Acetyl Transferase	
[b]Untreated	100 ± 16	100 ± 23	100 ± 13	2.46 ± 0.29
Control bezafibrate	103 ± 12	110 ± 20	103 ± 18	2.41 ± 0.22
10 μM bezafibrate	105 ± 14	111 ± 15	118 ± 13	2.41 ± 0.30
Control L-165041	87 ± 11	74 ± 19	93 ± 10	2.54 ± 0.20
5 μM L-165041	83 ± 15	114 ± 15	103 ± 8	2.61 ± 0.16
10 μM L-165041	84 ± 12	78 ± 11	88 ± 18	2.59 ± 0.35

a: Activities expressed relatively to untreated samples taken as 100%. Mean ± SD, n=4-5 samples. ChAT, choline acetyl transferase, GAD: glutamic acid decarboxylase, GS: glutamine synthetase.

b: Average total activities in untreated samples: GS 228±36 nmoles/min; GAD 2370±540 pmoles/min; ChAT 912±114 pmoles/min.

Figure 1 :

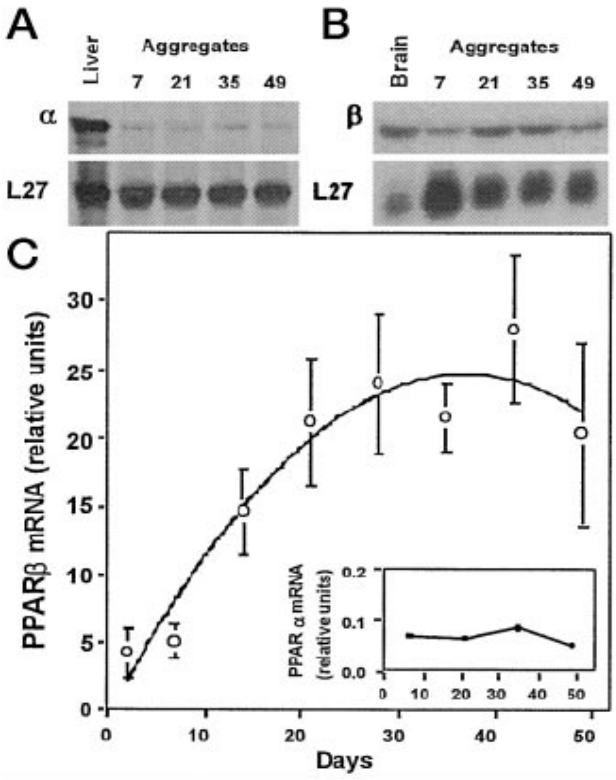

A
Liver
Aggregates
7
21
35
49
α
L27
B
Brain
Aggregates
7
21
35
49
β
L27
C
PPARβ mRNA (relative units)
Days
PPAR α mRNA (relative units)

Figure 2 :

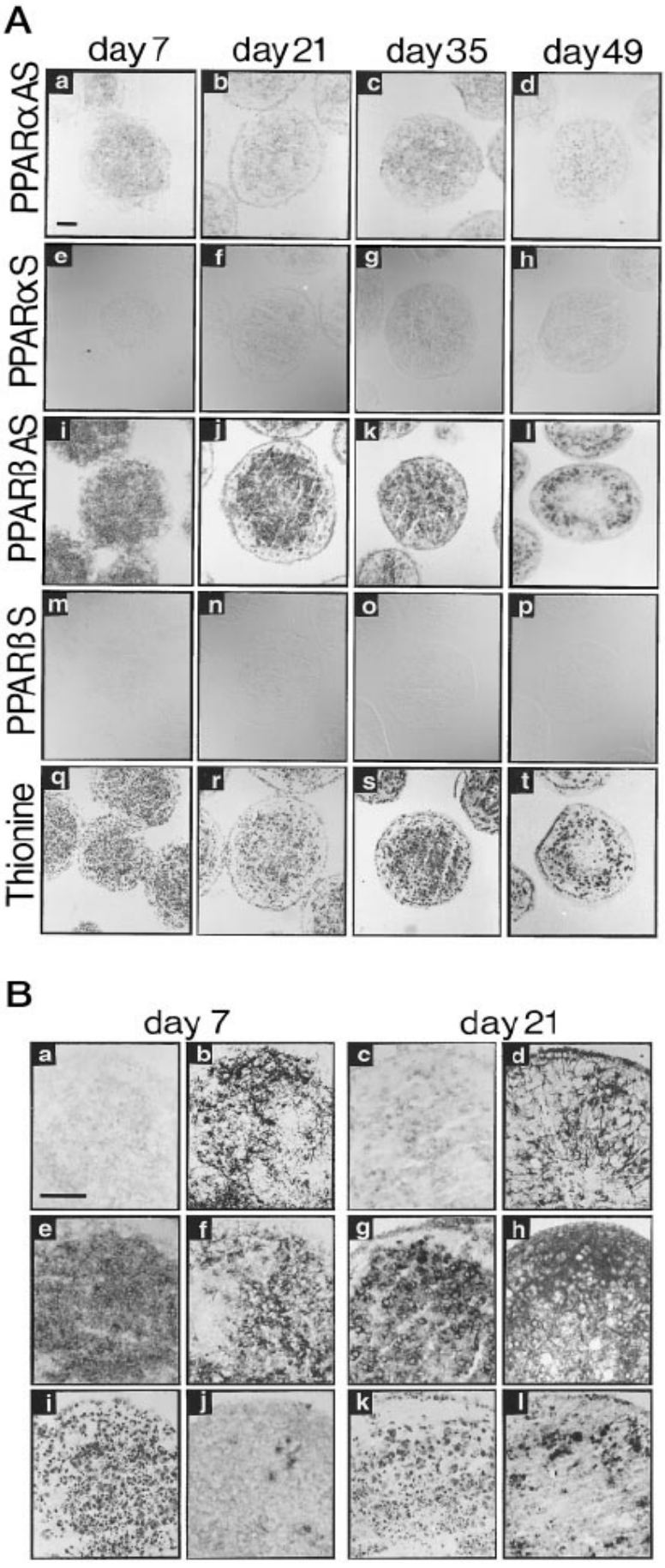

A
day 7
day 21
day 35
day 49
PPARαAS
PPARαS
PPARβAS
PPARβS
Thionine
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
s
t
B
day 7
day 21
a
b
c
d
e
f
g
h
i
j
k
l

Figure 3 :

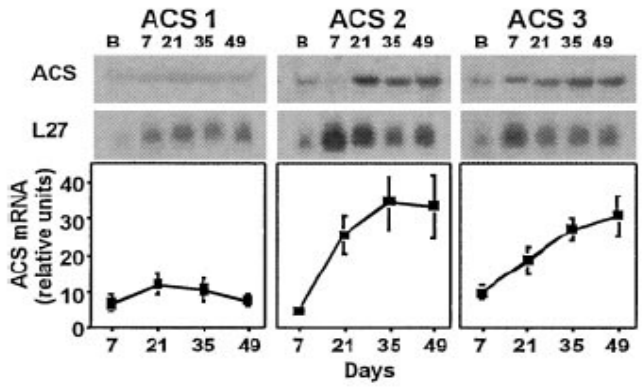


Figure 4 :

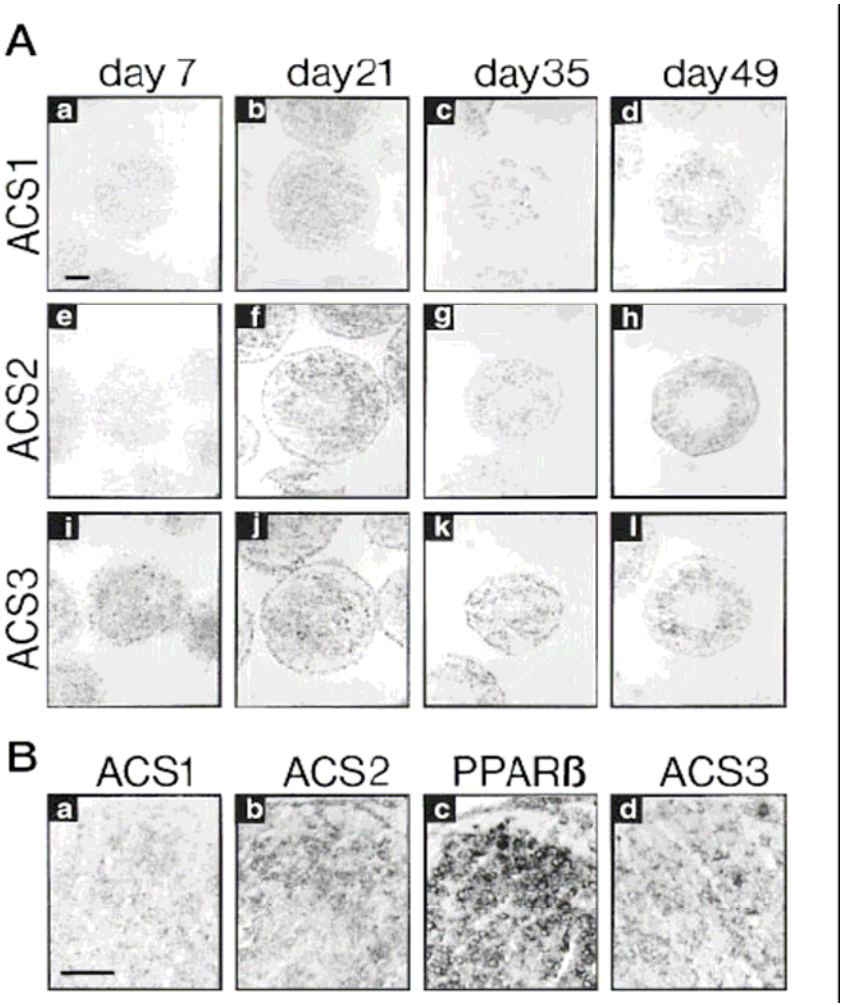


Figure 5 :

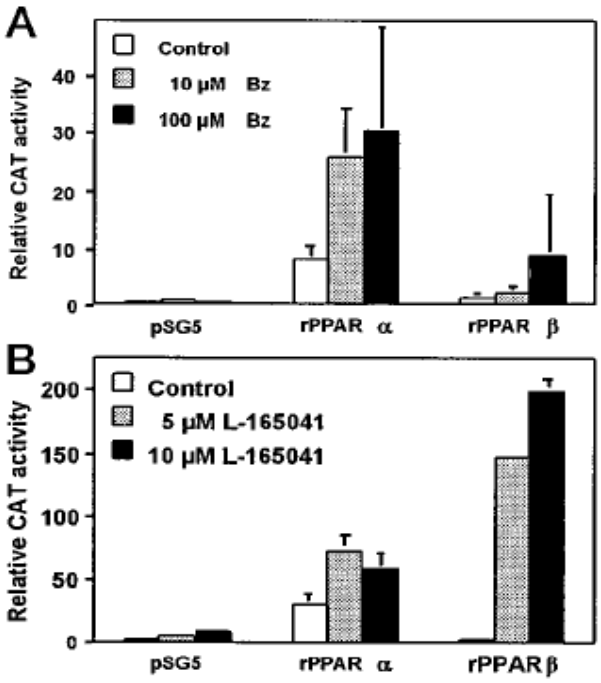

A
Control
10 µM Bz
100 µM Bz
Relative CAT activity
0
10
20
30
40
pSG5
rPPAR α
rPPAR β
B
Control
5 µM L-165041
10 µM L-165041
Relative CAT activity
0
50
100
150
200
pSG5
rPPAR α
rPPARβ

Figure 6 :

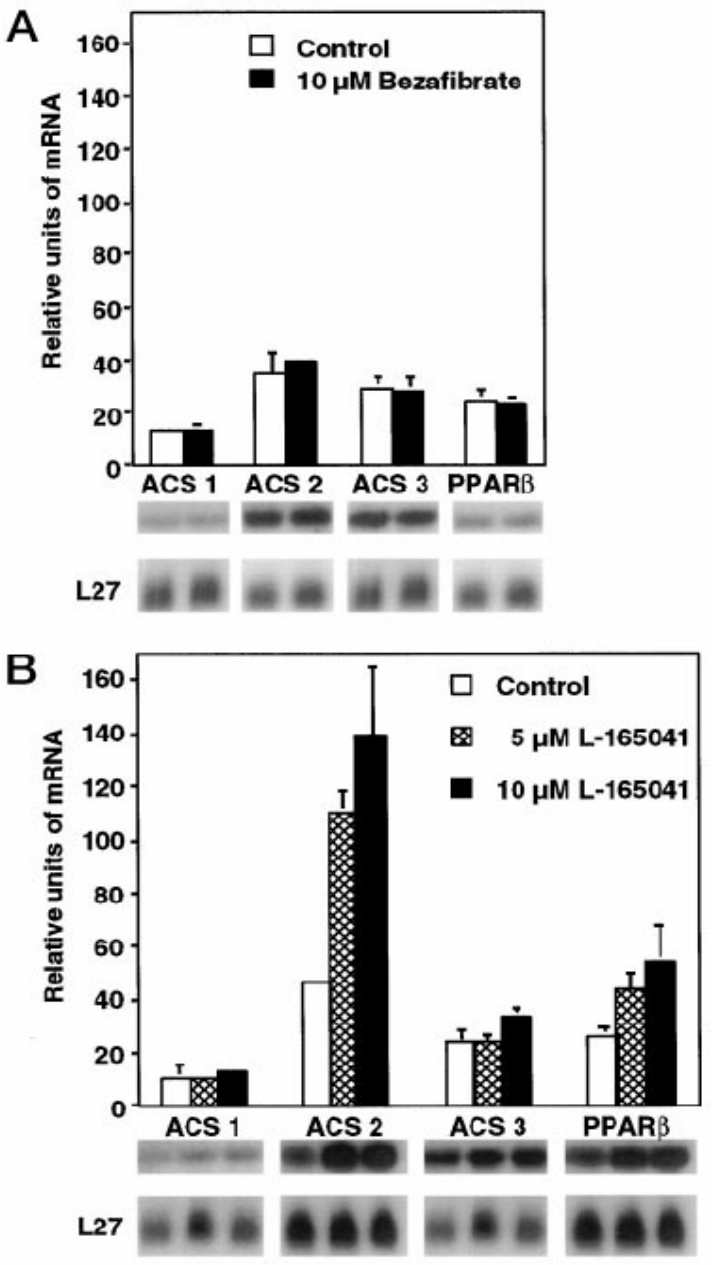
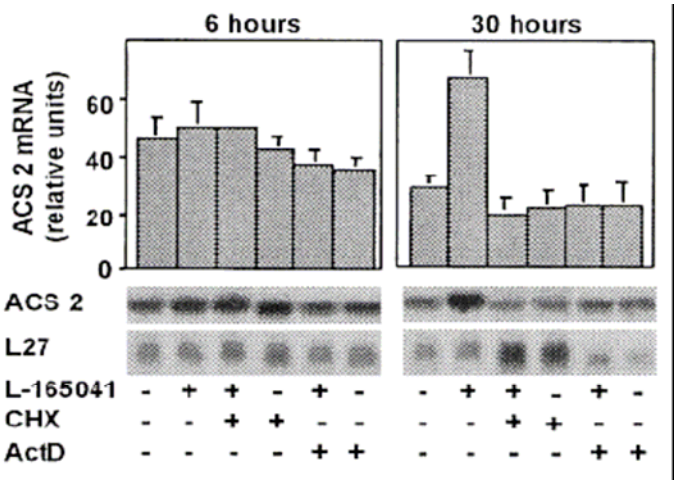

A
Control
10 µM Bezafibrate
Relative units of mRNA
160
140
120
100
80
60
40
20
0
ACS 1
ACS 2
ACS 3
PPARβ
L27
B
Control
5 µM L-165041
10 µM L-165041
Relative units of mRNA
160
140
120
100
80
60
40
20
0
ACS 1
ACS 2
ACS 3
PPARβ
L27

Figure 7 :


6 hours
30 hours
ACS 2 mRNA (relative units)
60
40
20
0
ACS 2
L27
L-165041 - + + - + - - + + - + -
CHX - - + + - - - - + + - -
ActD - - - - + + - - - - + +