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15-hydroxyeicosatetraenoic acid (15-HETE) is a preferential PPARβ/δ agonist

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Nonstandard Abbreviations: AA, arachidonic acid; ADRP, adipocyte differentiation-related protein; Angptl4, angiopoietin-like 4; DBD, DNA binding domain; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's Minimal Essential Medium; EDBCA, ethyl 3,4-dihydroxy-benzylidene-cyanoacetate; HETE, hydroxy-eicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; LBD, ligand binding domain; LX, lipoxin; NDGA, nordihydroguaiaretic acid; PCR, polymerase chain reaction; PPAR, peroxisome proliferator-activated receptor; PBS, phosphate buffered saline; PPRE, peroxisome proliferator responsive element; RXR, retinoid X receptor; TR-FRET, time resolved fluorescence resonance energy transfer.

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ABSTRACT

Peroxisome proliferator-activated receptors (PPARs) modulate target gene expression in response to unsaturated fatty acid ligands, such as arachidonic acid (AA). Here, we report that the AA metabolite 15-hydroxyeicosatetraenoic acid (15-HETE) activates the ligand-dependent activation domain (AF2) of PPARβ/δ *in vivo*, competes with synthetic agonists in a PPARβ/δ ligand binding assay *in vitro* and triggers the interaction of PPARβ/δ with co-activator peptides. These agonistic effects were also seen with PPARα and PPARγ, but to a significantly weaker extent. We further show that 15-HETE strongly induces the expression of the *bona fide* PPAR target gene *Angptl4* in a PPARβ/δ dependent manner, and conversely, that inhibition of 15-HETE synthesis reduces PPARβ/δ transcriptional activity. Consistent with its function as an agonistic ligand, 15-HETE triggers profound changes in chromatin associated PPARβ/δ complexes *in vivo*, including the recruitment of the coactivator CBP. Both, 15R-HETE and 15S-HETE, are similarly potent at inducing PPARβ/δ coactivator binding and transcriptional activation, indicating that 15-HETE enantiomers generated by different pathways function as PPARβ/δ agonists.

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Introduction

Peroxisome proliferator-activated receptors-β/δ (PPAR β/δ) is a ligand-regulated transcription factor that modulates target gene expression in response to certain fatty acids and fatty acid derivatives (Desvergne et al., 2006; Forman et al., 1997). PPARβ/δ forms heterodimers with the nuclear receptor RXR that bind to peroxisome proliferator response elements (PPREs) in target genes. A major function of the ligand in this context is to induce a conformational change in PPARβ/δ that results in the displacement of interacting corepressors, like SMRT and SHARP, by specific coactivators, such as SRC1 and p300/CBP, resulting in transcriptional activation (Yu and Reddy, 2007; Zoete et al., 2007). PPARβ/δ can regulate genes also by different mechanisms. Thus, PPARβ/δ ligands repress pro-inflammatory gene expression by releasing the hematopoietic transcriptional repressor Bcl-6 from a complex with PPARβ/δ (Lee et al., 2003). In another study it has been reported that PPARβ/δ can repress genes by sequestering RXR from other RXR-dependent nuclear receptors (Matsusue et al., 2006).

PPARβ/δ plays an important role in the regulation of energy homeostasis, lipid catabolism and glucose homeostasis (Desvergne et al., 2006), but also has essential functions in developmental processes, differentiation and wound healing. Mice lacking PPARβ/δ show an aberrant development and malfunction of the placenta (Barak et al., 2002; Nadra et al., 2006; Peters et al., 2000) and exhibit a defect in wound healing (Michalik et al., 2001). PPARβ/δ is critical for the survival, differentiation and proliferation of keratinocytes (Burdick et al., 2006; Di-Poi et al., 2002; Peters et al., 2000), and promotes the differentiation of Paneth cells in the intestinal crypts (Varnat et al., 2006). However, PPARβ/δ also plays a role in cancer

and inflammation: it modulates intestinal tumorigenesis with diverging effects in different mouse models (Barak et al., 2002; Burdick et al., 2006; Di-Poi et al., 2002; Gupta et al., 2004; Peters et al., 2000; Wang et al., 2004), it inhibits chemically-induced skin carcinogenesis (Bility et al., 2008; Kim et al., 2004), it exerts an essential function in the tumor stroma (Abdollahi et al., 2007; Müller-Brüsselbach et al., 2007), and has potent anti-inflammatory activities (Kilgore and Billin, 2008). Therefore, PPARβ/δ represents a highly relevant drug target for the treatment of major human diseases, which has helped lead to the development of several synthetic drug candidates with subtype selectivity and high-affinity binding, such as GW501516 and L165,041 (Peraza et al., 2006).

One of the fatty acids that induces PPARβ/δ activity to a moderate extent is AA. It has been shown that AA is a low-affinity ligand that interacts with the PPARβ/δ LBD (Xu et al., 1999), raising the possibility that the agonistic effect of AA is directly due to its interaction with PPARβ/δ. On the other hand, metabolites of AA may also account for this effect. Prostanoids are major AA metabolites generated by the combined action of cyclooxygenases and prostaglandin or thromboxane synthases. Prostaglandin I_2 (PGI_2; prostacyclin) has indeed been postulated to act as a PPARβ/δ agonist (Gupta et al., 2000; Hatae et al., 2001), but this issue remains controversial (Fauti et al., 2006; Forman et al., 1996; Yu et al., 1995).

Another major group of eicosanoid metabolites is generated by the lipoxygenases (Pidgeon et al., 2007), but lipoxygenase products of AA acting as *bona fide* PPARβ/δ ligands have not been described as yet. 15-hydroxyeicosatetraenoic acid (15-HETE), has been reported to activate PPARβ/δ in a reporter assay in keratinocytes, but it

remains unclear whether this involves a direct interaction with the PPARβ/δ LBD or indirect mechanisms (Thuillier et al., 2002).

In the present study we have systematically addressed this question using mouse fibroblasts as a model system. We show that the agonistic effect of AA is largely due to its lipoxygenase-mediated oxidation to 15-HPETE, and the subsequent enzymatic conversion to 15-HETE. Consistent with this finding, 15-HETE enabled the interaction of PPARβ/δ with co-activator peptides *in vitro*, and interacted with PPARβ/δ in a competitive ligand binding assay. Furthermore, 15-HETE induced the PPARβ/δ target gene *Angptl4* (Mandard et al., 2004) in a clearly PPARβ/δ dependent manner. Both enantiomers of 15-HETE, 15R-HETE and 15S-HETE, showed similar agonistic properties, indicating that different pathways converge on PPARβ/δ. While 15S-HETE is generated by LOX pathways, 15R-HETE is synthesized by cytochrome P450 or acetylated COX-2 (Clària et al., 1996; Clària and Serhan, 1995; Gilroy, 2005; Romano, 2006; Titos et al., 1999). Collectively, our findings demonstrate that 15-HETE enatiomers produced by different signaling pathways function as ligands for PPARβ/δ and induce its transcriptional activity.

Materials and Methods

Chemicals. GW501516, 9-cis-RA and the LOX inhibitors NDGA and EDBCA were purchased from Axxora (Lörrach, Germany) and prostagladins D_2, E_2, and F_2 from Cayman Europe (Tallinn, Estonia). GW1929 was obtained from Cayman Chemical (Biozol; Eching, Germany); LXA4 from Biomol (Hamburg, Germany); and GW7647 and diclofenac from Sigma-Aldrich (Steinheim, Germany). All other eicosanoids

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were from Cayman Chemical (Biozol; Eching, Germany) and Axxora (Lörrach, Germany).

Mouse strains. *Pparb/d* null and wild-type (wt) mice have been described (Peters et al., 2000). Pparb/d^{ck} mice (Barak et al., 2002) harboring a floxed *Pparb/d* exon 4 were kindly provided by R. Evans (The Salk Institute, La Jolla, CA).

Cell culture. *Pparb/d* null, wt and floxed fibroblasts were established from fetal lungs and cultured as described (Müller-Brüsselbach et al., 2007). WPMY-1 cells were obtained from the ATCC. A CHO cell line with a stably integrated pFR-Luc reporter gene (Stratagene/Agilent Technologies, Waldbronn, Germany), which expresses luciferase under the control of five Gal4 DNA binding sites, and stably expressing a Gal4-hPparb fusion protein was generated by successive electroporation of the two vector constructs. After selection in G418, the cells were cloned by limited dilution and positive clones were identified using a CCD camera. The cell clone showing the best response to synthetic PPARβ/δ ligands was chosen for this study. All cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified incubator at 37°C and 5% CO_2.

Plasmids. pCMX-mPparδ (Forman et al., 1997) and Gal4-mPparδ (Shi et al., 2002) were kindly provided by Dr. R. Evans (The Salk Institute, La Jolla, CA). 3xFlag-PPARβ/δ was generated by cloning the coding sequence of mPPARβ/δ N-terminally fused to a triple FLAG tag (Müller-Brüsselbach et al., 2007) into pcDNA3.1(+) zeo (Invitrogen, Karlsruhe, Germany). pCMX-empty has been described (Umesono et al., 1991). LexA-PPARβ/δ, 7L-TATAi and 10xGal4SVGL3 have been described previously (Fauti et al., 2006; Jerome and Muller, 1998). LexA-PPARα and LexA-

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PPARβ/δ were constructed in an analogous way to LexA-PPARβ/δ. pSG5-hRxRa containing the full-length RxRa cDNA was kindly provided by Dr. A. Baniahmad (Jena, Germany). The PPRE-TATAi plasmid was constructed by inserting a PPRE containing fragment of the third intron of the human *ANGPTL4* gene (Mandard et al., 2004) into TATAi-pGL3 (Jerome and Muller, 1998). The pUC18 plasmid was obtained from New England Biolabs (Frankfurt am Main, Germany) and pcDNA3.1 from Invitrogen (Karlsruhe, Germany).

Transfections and luciferase reporter assays. Transfections were performed with polyethylenimine (average MW 25,000; Sigma-Aldrich). Cells were transfected on 12-well plates at 70-80% confluence in DMEM plus 2% FCS with 2.5 µg of plasmid DNA and 2.5 µl of PEI (1:1000 dilution, adjusted to pH 7.0 and preincubated for 15 min in 100µl phosphate-buffered saline for complex formation). Four hours after transfection, the medium was changed and cells were incubated in normal growth medium for 24 hrs. Luciferase assays were performed as described (Gehrke et al., 2003). Values from three independent experiments were combined to calculate averages and standard deviations.

Retrovirally transduced cells expressing FLAG-PPARβ. 3xFLAG-PPARβ/δ was cloned into the retroviral vector pLPCX (Clontech). Phoenix cells expressing ecotropic *env* were transfected with 3xFLAG-mPPARb-pLPCX as described *(http://www.stanford.edu/group/nolan/retroviral_systems/retsys.html)*. Culture supernatant was used to infect *Pparb* null fetal mouse lung fibroblasts that had previously been established from *Pparb* knockout mice by standard procedures. Cells were selected with puromycin (2 µg/ml; Sigma), resulting in a cell population expressing 3xFLAG-mPPARβ/δ at moderate levels.

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Quantitative PCR. cDNA was synthesized from 1 µg of RNA using oligo(dT) primers and the Omniscript kit (Qiagen, Hilden, Germany). qPCR was performed in a Mx3000P Real-Time PCR system (Stratagene, La Jolla, CA) for 40 cycles at an annealing temperature of 60°C. PCR reactions were carried out using the Absolute QPCR SYBR Green Mix (Abgene, Hamburg, Germany) and a primer concentration of 0.2 µM following the manufacturer's instructions. *L27* was used as normalizer. Comparative expression analyses were statistically analyzed by Student's *t*-test (two-tailed, equal variance). The following primers were used: Pparb_for: 5'-CTCCATCGTCAACAAAGACG; Pparb_rev: 5'-TCTTCTTTAGCCACTGCATC; Angptl4_for: 5'-CTC TGG GGT CTC CAC CAT TT; Angptl4_rev: 5'-TTG GGG ATC TCC GAA GCC AT; L27_for; 5'-AAA GCC GTC ATC GTG AAG AAC; L27_rev; 5'-GCT GTC ACT TTC CGG GGA TAG.

siRNA transfections. For siRNA transfection, cells were seeded at a density of $5x10^5$ cells per 6 cm dish in 4 ml DMEM with 10% FCS and cultured overnight. 400 pmol siRNA in 500 µl OptiMEM (Invitrogen) and 10 µl Lipofectamin 2000 (Invitrogen) in 500 µl OptiMEM (Invitrogen) were separately incubated for 5 min at room temperature, mixed and incubated for another 20 min. The siRNA-lipid complex was added to the cells cultured in DMEM without FCS (time = 0), and the medium was changed to normal growth medium after 6 hrs. Cells were passaged and replated 48 hrs post-transfection at a density of $5x10^5$ cells per 6 cm dish. Transfection was repeated 72 hrs after start of the experiment, and cells were passaged after another 24 hrs. Forty-eight hours following the last transfection, cells were stimulated and harvested after 3 hrs for RNA isolation. The following siRNAs were used: Pparb siRNA#1 (CCGCATGAAGCTCGAGTATGA; Qiagen, Hilden, Germany); Pparb

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siRNA#2 (CAAGTTCGAGTTTGCTGTCAA; Qiagen); control siRNA (negative control siRNA 1022563; Qiagen).

Chromatin immunoprecipitation (ChIP) analysis. WPMY-1-1 cells were grown to confluency. After stimulation, the cells were fixed by addition of 37% formaldehyde to a final concentration of 1%. Incubation time was 10 min at room temperature. Glycine was added to a final concentration of 125 mM for 5 min. After two washes with ice-cold PBS, the cells were pelleted for 5 min at 1200 g. Pellets were resuspended in cold hypotonic lysis buffer (5 mM PIPES pH 8.0; 85 mM KCl; 0.5% (v/v) NP40; protease inhibitor cocktail (Sigma)) at a ratio of 1 ml per 10^7 cells and incubated on ice for 20 min. Nuclei were pelleted by centrifugation as before and resuspended in RIPA buffer (10 mM Tris-HCl pH 7.4; 150 mM NaCl; 1% (v/v) NP40; 1% sodium deoxycholate; 0.1% (w/v) SDS; 1 mM EDTA; protease inhibitors) at a ratio of 1 ml per 10^7 nuclei. Soluble chromatin was prepared by sonication with a microtip using a Sonifier S-250D (Branson Ultrasonics) set to 1 s pulse, 2 s pause. 80 pulses were applied. After centrifugation at 16000 g for 15 min in a tabletop centrifuge, the supernatant was collected. An aliquot was incubated overnight with proteinase K and RNAse A at 65 ˚C and loaded on a 1% agarose gel to estimate shearing efficiency. The supernatant was precleared by addition of Protein A sepharose beads (Invitrogen) which were previously blocked in RIPA with 1 mg/ml BSA, 0.4 mg/ml sonicated salmon sperm DNA (Stratagene), and protease inhibitors, coupled to rabbit IgG (Sigma I5006). After rotation for 1 h at 4 ˚C, the beads were removed by centrifugation. The supernatant was used for IPs. 4 µg of antibody (rabbit IgG pool, Sigma; α-PPARβ/δ sc-7197, α-CBP sc-369, Santa Cruz; α-acetylated H4

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06-866, Millipore) were added to 300 µl of precleared chromatin corresponding to $3x10^6$ nuclei and incubated overnight at 4 ˚C with mild rotation. After addition of blocked sepharose beads, incubation time was 1 h at 4 ˚C with mild rotation. The beads were washed once with 1 ml cold mixed micelle buffer (20 mM Tris pH 8.1; 150 mM NaCl; 2 mM EDTA; 0.1% (w/v) SDS; 1% (v/v) Triton X-100), once with buffer 500 (20 mM Tris pH 8.1; 500 mM NaCl; 2 mM EDTA; 0.1% (w/v) SDS; 1% (v/v) Triton X-100), twice with LiCl detergent buffer (10 mM Tris pH 8.1; 250 mM LiCl; 1% (v/v) NP40; 1% (w/v) sodium deoxycholate; 1 mM EDTA), and twice with room-temperature TE. Complexes were eluted twice with 250 µl elution buffer (1% SDS (w/v); 100 mM NaHCO3). Supernatants were pooled, adjusted to 180 mM NaCl, 35 mM Tris pH 6.5, and 9 mM EDTA, and incubated with 20 µg of proteinase K and 10 µg of RNase A for 65˚C overnight. DNA was purified using a PCR purification kit (Qiagen). Eluates were quantified by qPCR employing the deltaCt method relative to an amount equivalent to 1% of DNA used for IP. Standard deviations were calculated from triplicate measurements considering Gaussian error propagation. The following primers were used: *ANGPTL4* PPRE_fw: CCT TAC TGG ATG GGA GGA AAG; *ANGPTL4* PPRE_rv: CCC AGA GTG ACC AGG AAG AC.

Time resolved fluorescence resonance energy transfer (TR-FRET) assays *in vitro*. TR-TRET (Stafslien et al., 2007) was performed with the LanthaScreen™ TR-FRET PPARβ/δ competitive binding assay and the Lanthascreen™ TR-FRET PPARα, PPARβ/δ and PPARγ coactivator assays according to the instructions of the manufacturer (Invitrogen, Karlsruhe, Germany). Incubation time was 60 min for all assays shown in this study. All assays were validated for their robustness by determining the respective Z´-factors (Zhang et al., 1999). Measurements were performed on a VICTOR3V Multilabel Counter (WALLAC 1420; PerkinElmer,

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Rodgau, Germany) with instrument settings as described in the manufacturer's instructions for LanthaScreen™ assays. The following peptides were used for the coactivator recruitment assay: PGC1α, EAEEPSLLKKLLLAPANTQ; C33, HVEMHPLLMGLLMESQWGA; CBP, AASKHKQLSELLRGGSGSS; PRIP, VTLTSPLLVNLLQSDISAG; TRAP220: NTKNHPMLMNLLKDNPAQD.

Quantification of HETEs in cell culture supernatants by LC/MS/MS

Cell culture supernatants were spiked with ~1 ng of deuterated internal standards and acidified with 20 µl saturated NH_4Cl solution containing 1.25 M HCl. The analytes were extracted with 1 ml diisopropyl ether and after complete drying resolved in acetonitrile/water (1:1, *v/v*). For determination, a 10 µl aliquot was injected into the LC/MS/MS. The LC/MS analysis was carried out on a Applied Biosystem API3000 mass spectrometer equipped with two PE Series 200 liquid chromatography micro pumps (PerkinElmer, Waltham, MA, USA), a CTC HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland), a turbo-ion interface and a HSID interface (Ionics Mass Spectrometer Group Inc.; GSG Mess- und Analysengeräte GmbH, Bruchsal, Germany). The flow rate was set to 200 µl/min and a C18 column (5 cm x 2 mm, 3 µm particles) was used. A generic LC gradient of 10 minutes was used for sample separation. Solvents were (A) water : acetonitrile (95:5) and (B) acetonitrile : water (95:5), both containing 0.2% acetic acid. The gradient profile used was 40% solvent A for 0-2 min linearly increasing to 100 percent within 7 min. Mass spectrometer conditions were: Scan type MRM with negative polarity, ionspray voltage -4200V, temperature 350°C, collision gas 4 psi, all potentials (declustering, focusing, entrance and exit potential) were optimized for each ion. Collision energy was 20 eV and the following transitions were used for quantitation: 319.2/114.9 (5-HETE), 327.2/115.9 (d_8-5-HETE), 319.2/154.2 (8-HETE), 319.2/207.8 (12-HETE), 327.2/214.1 (d_8-12-

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HETE), 319.2/218.9 (15-HETE), and 327.2/182.0 (d_8-15-HETE). Dwell time was 300 ms each. All chemicals and solvents were obtained from Merck, Darmstadt, Germany.

Results

Effect of arachidonic acid derivatives on the transcriptional activity of PPARβ/δ. We analyzed the effect of AA and its metabolites on the transcriptional activity of PPARβ/δ in NIH3T3 fibroblasts using luciferase reporter constructs harboring multiple Gal4 or LexA binding sites upstream of a TATA-Initiator module (TATAi). The transcriptional activator in this system is a fusion construct consisting of the PPARβ/δ LBD and a Gal4 (Fig. 1A) or LexA (Fig. 1B) DNA binding domain. With both reporter systems, we observed an approximately 2-fold induction by 20 μM AA. The observed induction by AA was not influenced by the COX inhibitor diclofenac, and no transcriptional activation was seen when specific prostaglandins (PGD_2, PGE_2, PGF_2) were applied (data not shown). This confirms previous findings that AA-derived prostanoids have no detectable effect on the activity of PPARβ/δ in HEK293 and NIH3T3 cells (Fauti et al., 2006; Forman et al., 1996; Yu et al., 1995). PGE_2 has been described as an activator of PPARβ/δ, but this in an indirect effect involving PGE_2-mediated induction of PI_3K-AKT signalling and may therefore be dependent on the cell type and experimental system (Wang et al., 2001). As NIH3T3 cells synthesize 5-, 8-, 12- and 15-HETE at readily detectable levels (up to ~60ng/ml after 6hrs culture, corresponding to ~0.2 μM; Table 1), we tested the effect of LOX inhibitors and found a clear concentration-dependent inhibition on both the basal and AA-induced transcriptional activity of PPARβ/δ by the pan-LOX inhibitor NDGA (Fig. 1A). We also analyzed the 12-/15-LOX inhibitor EDBCA and observed a very

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similar effect compared to NDGA (Fig. 1B). These effects were specific, since no inhibition of transcription by NDGA or EDBCA was observed in the absence of LexA-PPARβ/δ (data not shown).

These observations raised the possibility that 12-/15-LOX products are activators of PPARβ/δ. We therefore tested the effects of 12-HETE and 15-HETE alongside with a number of other eicosanoids on the transcriptional activity of PPARβ/δ. This experiment was performed with CHO cells harboring a stable Gal4-driven luciferase reporter gene and stably expressing a Gal4-PPARb/d fusion protein. Fig. 1C clearly shows that 15-HETE was the only compound that was more potent than AA at activating the transcriptional activity of PPARβ/δ in this cellular system.

15-HETE induces coactivator peptide binding to PPARβ/δ. The observations described above raise the possibility that the LOX products 12- or 15-HPETE and/or their direct metabolites 12- or 15-HETE are agonists of PPARβ/δ. To test this hypothesis we investigated the effect of different LOX products on PPARβ/δ co-activator interaction *in vitro* by TR-FRET. In this assay, the interaction of the PPARβ/δ LBD (indirectly labeled by terbium) with the co-activator peptide C33 (labeled with fluorescein) is determined (Stafslien et al., 2007). C33 was previously identified as a peptide strongly interacting with co-activator binding sites of PPARs in response to agonist binding, similar to PGC1α or SRC coactivator derived peptides (Chang et al., 1999). The readout of this assay is the intensity of terbium-induced fluorescence emission of the fluorescein moiety of the C33 peptide, expressed as the ratio of fluorescein derived and terbium derived fluorescence.

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As shown in Fig. 2A, only AA, 15-HETE and 15-HPETE were able to induce a significant FRET, whereas 8-HETE, 12-HETE and 12-HPETE were unable to do so. These data suggest that the activation of PPARβ/δ by different HETEs reported in a previous study (Thuillier et al., 2002), including 8-HETE and 12-HETE, was due to indirect effects, perhaps involving HETE membrane receptors triggering signaling pathways impinging on PPARβ/δ activated transcription. 15-HETE was clearly the most efficacious compound tested, giving rise to a comparable FRET signal at approximately 25% the concentration of AA or 15-HPETE. Titration analysis of lower concentrations of 15-HETE showed a statistically significant effect already at 0.1 μM and a half-maximum signal at approximately 0.55 μM (Fig. 2B).

15-HETE competes for PPARβ/δ LBD binding. We next analyzed the interaction of AA, 15-HETE and 15-HPETE with the LBD of PPARβ/δ in a TR-FRET based competitive ligand binding assay. In this assay, the terbium-labeled PPARβ/δ LBD interacts with Fluormone Pan-PPAR Green as PPAR ligand, which produces FRET. Displacement of the fluorescent Fluormone Pan-PPAR Green by unlabeled ligand results in a quantifiable attenuation of FRET. The data obtained with this assay are shown in Fig. 3A. In agreement with the results of the coactivator recruitment assay, all three compounds were able to displace the PPARβ/δ bound ligand.

15-HETE induces PPARβ/δ mediated transcription. We next analyzed the effect of 12-HETE, 15-HETE and 15-HPETE on a luciferase reporter construct driven by a PPAR-responsive minimal promoter, i.e., a PPRE containing fragment from the first intron of the *ANGPTL4* gene (Mandard et al., 2004) linked to the TATAi module described above. For this and most of the subsequent experiments we used NIH3T3 fibroblasts, because they essentially lack endogenous PPARα and PPARγ (data not

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shown), which due to their partially redundant function could obliterate PPARβ/δ mediated effects. The data in Fig. 4A show that the basal level activity of the TATAi vector (left panel) was reduced by the presence of a PPRE (center panel), most likely due to the recruitment of PPARβ/δ repressor complexes (our unpublished observations). The addition of AA, 12-HETE, 15-HETE or 15-HPETE led to insignificant changes in basal activity, but a clear induction by both 15-HETE and 15-HPETE was seen with PPRE-TATAi (4.7-fold and 3.7-fold, respectively). In contrast, no significant induction was observed with AA or 12-HETE. Cotransfection of PPARβ/δ and RXRα expression vectors (Fig. 4A, right panel) clearly enhanced the effect. 15-HETE and 15-HPETE strongly induced PPRE-dependent transcription (21-fold and 11-fold, respectively, while the induction by both AA and 12-HETE were comparably weak (2- to 3-fold) though significantly higher than the solvent control (1.3-fold). These data confirm the data obtained by the TR-FRET analysis described above (Fig. 2).

Induction of endogenous PPARβ/δ target genes by 15-HETE. *Angptl4* is an established PPAR target gene (Mandard et al., 2004) and was therefore tested for its inducibility by 15-HETE. As shown in Fig. 5B, *Angptl4* was clearly induced in all three cell lines used for this analysis, i.e, NIH3T3 cells, WPMY-1 myofibroblasts and HaCaT keratinocytes. HaCaT cells showed the strongest response with a 16-fold induction at 5μM, compared to 5.1-fold in WPMY-1 cells and 3.4-fold in NIH3T3 cells. Importantly, a clear induction of *Angptl4* expression was seen already at 1μM 15-HETE (3.1-fold in HaCaT cells; 1.8-fold in NIH3T3 cells). Maximum induction by 15-HETE was comparable to that seen with the synthetic agonist GW501516 at 1μM (Fig. 5A). Since NIH3T3 cells lack other PPAR subtypes, these observations strongly suggest that the observed induction by 15-HETE is mediated by PPARβ/δ.

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Both, GW501516 and 15-HETE did not alter *Pparb/d* expression levels (data not shown), indicating that the observed induction of *Angptl4* expression by these compounds is due to an increased PPARβ/δ activity.

In agreement with these observations, ChIP analyses of human WPMY-1 cells showed recruitment of the coactivator CBP to the PPARβ/δ-bound *ANGPTL4* PPRE upon stimulation with either GW501516 or 15-HETE (Fig. 6A). Concomitant with CBP recruitment to the PPRE, we observed acetylation of histone H4, but not of H3, at both the PPRE (Fig. 6B) and the TSS (data not shown) with either compound. Both, GW501516 or 15-HETE, also led to an increased binding of PPARβ/δ to the *ANGPTL4* PPRE, which is consistent with previous observations made with synthetic ligands (Mandard et al., 2004). In contrast, specific binding of CBP, PPARβ/δ or acetylated H4 to an irrelevant *ANGPTL4* upstream region at -12 kb relative to the TSS was not detectable (data not shown). These similarities in chromatin alterations triggered by both compounds provide further evidence that 15-HETE is an agonistic PPARβ/δ ligand.

Induction of the PPARβ/δ target gene *Angptl4* by 15-HETE is dependent on PPARβ/δ. To obtain unequivocal evidence that *Angptl4* induction by 15-HETE is indeed mediated by PPARβ/δ we performed a series of experiments using genetically modified fibroblasts or siRNA technology. First, we analyzed the effect of 15-HETE on *Angptl4* expression in mouse fibroblasts with disrupted *Pparb/d* alleles and in cells with restored PPARβ/δ expression. For this experiment, fetal lung fibroblasts from *Pparb/d* null mice were infected with a retrovirus expressing FLAG-tagged PPARβ/δ or a control retrovirus (pLPCX). The data in Fig. 7 show a clear PPARβ/δ-dependent induction of the *Angplt4* gene by 0.3μM GW501516 (6.9-fold), 10μM 15-HPETE

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(5.6-fold) and 10μM 15-HETE (10.7-fold), respectively, but only a marginal effect (1.5-fold) by 20 μM AA (Fig. 7B). In the *Pparb/d* null cells infected with the control vector, expression of Angptl4 was not induced by any of the compounds (Fig. 7A). As expected, the PPARα ligand GW1929 and the PPARγ ligand GW7647 showed no detectable regulation in either cell line.

We also performed the converse experiment by making use of fetal lung fibroblasts we established from a mouse strain with a floxed *Pparb/d* allele (Barak et al., 2002). The floxed alleles can be deleted in cell culture by a Cre-expressing retrovirus (Li et al., 1997). In these cells the *Angptl4* gene was clearly inducible by AA (2.7-fold), which was reduced to a 1.5-fold induction after Cre-mediated deletion of *Pparb/d* (Fig. 7D). However, a considerably stronger effect was seen with both 15-HETE and GW501516. These compounds induced *Angptl4* expression in the control cells infected with the empty retroviral vector (pBabe) by 13-fold and 17-fold, respectively. In the Cre virus infected cells these values were reduced to 2.0-fold and 1.9-fold, respectively (Fig. 7D).

Finally, we analyzed the effect of 15-HETE on the *Angptl4* gene after siRNA-mediated knockdown of PPARβ/δ expression in NIH3T3 cells. Fig. 8A shows a 67.4% reduction of *Pparb/d* mRNA in *Pparb/d* siRNA treated cells compared to cells exposed to control siRNA. PPARβ/δ knockdown led to an increased activity of the *Angptl4* gene (Fig. 8A, left), presumably as a consequence of reduced PPARβ/δ repressor complex recruitment (see also above). Treatment with GW501516, 15-HPETE or 15-HETE led to the expected *Angptl4* induction in the control siRNA-treated cells (4.0-fold, 1.9-fold and 3.0-fold, respectively; Fig. 8B), which was reduced by approximately 50% upon exposure to *Pparb/d* siRNA, thus

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confirming *Pparb/d* as the target of GW501516, 15-HPETE and 15-HETE in the regulation of the *Angptl4* gene.

Both enantiomers of 15-HETE activate PPARβ/δ. There are two enantiomers of 15-HETE that are produced by different synthetic pathways, including 15-LOX, cytochrome P450 dependent mechanisms and aspirin-triggered acetylated COX-2 (Clària et al., 1996; Clària and Serhan, 1995; Gilroy, 2005; Romano, 2006; Titos et al., 1999). The 15S-HETE and 15R-HETE enantiomers can be further converted to lipoxins (LXA4, LXB4) and 15-epi-lipoxins (15-epi-LXA4, 15-epi-LXB4), respectively. We therefore sought to investigate these compounds for their ability to induce the transcriptional activity of PPARβ/δ.

We first addressed this question using the TR-FRET based competitive ligand binding assay assay. The data in Fig. 3B clearly show that both 15-HETE enantiomers were able to compete for binding to PPARβ/δ to a very similar extent as the enantiomer mixture [15-HETE; also referred to as (+/-)15-HETE]. In contrast, no significant effect was observed with any of the lipoxins or epi-lipoxins tested.

We next analyzed the inducibility of the transcriptional activity of PPARβ/δ in the LexA-based luciferase assay. Consistent with the data obtained by TR-FRET, both enatiomers induced PPARβ/δ activity to a similar extent (2.5- to 3-fold), while the lipoxin LXA4 showed no effect (Fig. 4B and C). Finally, we tested the effect of 15S-HETE and 15R-HETE on the induction of the PPARβ/δ target gene *Angptl4* in NIH3T3 fibroblasts. Both enantiomers induced *Angptl4* expression to a similar extent as 15-HETE (Fig. 5A). Taken together these observations strongly suggest that both 15S-HETE and 15R-HETE are agonistic ligands of PPARβ/δ.

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Effect of 15-HETE on different PPAR subtypes. Several previous studies have reported an activation of PPARγ by 15-HETE (Chen et al., 2003; Huang et al., 1999; Nagy et al., 1998; Schild et al., 2002). It was therefore of interest to compare the effects of 15-HETE on PPARγ and PPARβ/δ in some of the assays used in the present study. We first addressed this question by analyzing the effect of 15-HETE on the transcriptional activation by the PPARγ and PPARβ/δ LBDs fused to the LexA DBD. As shown in Fig. 9, 15-HETE significantly activated all PPAR subtypes, but the induction of PPARβ/δ was stronger (~11-fold) compared to the activation of PPARγ (~3-fold) and PPARα (~2-fold). Consistent with this finding, *in vitro* recruitment of the TRAP coactivator peptide to PPARα and PPARγ was detectable, but weaker than the interaction with PPARβ/δ (Fig. 10A). However, a precise quantitative comparison in this assay is difficult, since the exact concentrations of functional recombinant PPARs and their occupation by lipid ligands cannot be determined and may thus differ among the three subtypes.

We also analyzed the 15-HETE triggered interaction of PPARβ/δ and PPARγ with C33 and three other coactivator peptides (PGC1α, CBP, PRIP). Again, the 15-HETE induced interaction with PPARγ was much weaker compared to the recruitment of all coactivator peptides to PPARβ/δ (Fig. 10B, C). As expected, the synthetic ligands GW1929 and GW501516 included as positive controls strongly induced the recruitment of all four peptides to PPARγ and PPARβ/δ, respectively (Fig. 10B, C).

Discussion

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Several synthetic drugs with strong agonistic properties and high subtype selectivity have been described for PPARβ/δ (Peraza et al., 2006), but its regulation by natural ligands remains unclear. The description of PGI_2 (prostacyclin) (Gupta et al., 2000; Hatae et al., 2001) and all-trans retinoic acid (atRA) (Schug et al., 2007) as PPARβ/δ agonists are in conflict with data published by others (Borland et al., 2008; Forman et al., 1996; Yu et al., 1995) and our own laboratory (Fauti et al., 2006; Rieck et al., 2008). Unsaturated fatty acids have been reported to interact with the LBD PPARβ/δ (Forman et al., 1996; Xu et al., 1999), but their effect on the transcriptional activity of PPARβ/δ is weak (Rieck et al., 2008). Among the fatty acids with a clearly activating effect on PPARβ/δ is AA, but it is unknown whether this results from a direct interaction with the PPARβ/δ LBD or the formation of a more potent metabolite. In the present study, we addressed this question and have identified a novel AA-dependent but Cox-independent pathway regulating the transcriptional activity of PPARβ/δ.

Using fusion constructs of the PPARβ/δ LBD and heterologous DBDs (LexA, Gal4) we could show that the agonistic effect of AA is LOX-dependent. The pan-LOX inhibitors NDGA (Fig. 1A) and esculetin (data not shown), and the 12- and 15-LOX inhibitor EDBCA (Fig. 1B) clearly diminished the induction of PPARβ/δ by AA. These data demonstrate that the agonistic effect of AA is not merely due to its direct interaction with the PPARβ/δ LBD.

To identify the active metabolite(s) generated by lipoxygenase pathway(s) we analyzed by TR-FRET various LOX-generated AA metabolites for their potential to trigger the interaction of recombinant PPARβ/δ with coactivators peptides *in vitro* (Fig. 2). This experiment identified 15-HETE as the only metabolite tested that was

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clearly superior to non-metabolized AA. However, in an in vitro ligand binding assay 15-HETE was only marginally more efficient than AA (Fig. 3A), suggesting that the stronger coactivator binding is not attributable to a higher affinity for the PPARβ/δ LBD but rather to a specific conformational change induced by 15-HETE.

The agonistic effect of 15-HETE was selective for PPARβ/δ, since (i) the interaction of PPARβ/δ with the direct precursor of 15-HETE, the 12/15-LOX product 15-HPETE, was considerably weaker; (ii) HETE-derived lipoxins LXA4 and LXB4 showed a weaker binding to recombinant PPARβ/δ *in vitro* than 15-HETE itself (Fig. 3); and (iii) the 15-HETE triggered activation of a LexA-PPARβ/δ and the 15-HETE induced coactivator peptide recruitment to PPARβ/δ were significantly stronger relative to PPARα and PPARγ (Figs. 9, 10). This subtype selectivity is in apparent contrast to published data suggesting that 15-HETE activates the transcriptional activity of PPARγ (Chen et al., 2003; Huang et al., 1999; Nagy et al., 1998; Schild et al., 2002), which was also seen in our study, albeit to a lesser extent compared to PPARβ/δ (Fig. 9). In contrast to our work, the studies quoted above did not show a direct interaction of 15-HETE and PPARγ. It can therefore not be excluded that the described effects of 15-HETE on PPARγ are, at least in part, indirect. In addition, the effect of 15-HETE on PPARβ/δ was not assessed in the published studies so that a direct comparison with our data is not possible.

We next performed a series of experiments confirming 15-HETE as an efficient inducer of endogenous PPARβ/δ target genes using the *Angptl4* gene as a verified model (Mandard et al., 2004). At a concentration of 10μM 15-HETE induced the *Angptl4* gene to a similar extent as the synthetic agonist GW501516 at 0.3μM, a concentration leading to maximal induction by this compound (Fig. 5A). Consistent

with a function as a PPAR agonist 15-HETE triggered the recruitment of PPARβ/δ and the coactivator CBP to PPRE in the *ANGPTL4* gene in the human myofibroblast cell line WPMY-1 (Fig. 6A), and triggered the acetylation of histone H4 at the TSS of the gene (Fig. 6B). These alterations were basically indistinguishable from those induced by the established PPARβ/δ ligand GW501516. Furthermore, we were able to demonstrate the PPARβ/δ dependence of the 15-HETE mediated induction of the *Angptl4* gene by comparing PPARβ/δ expressing cells, *Pparb/d* null cells and siRNA treated cells (Figs. 7 and 8). Taken together these data provide compelling evidence that 15-HETE functions as an activating ligand for PPARβ/δ.

Another important question concerns the physiological relevance of the proposed 15-HETE/PPARβ/δ signaling pathway. The highest concentration of 15-HETE measured in the culture supernatant of NIH3T3 cells was approximately 200 nM (Table 1), which is close to the concentration that elicits an effect on *ANGPTL4* in HaCAT cells (Fig. 5B). However, such comparisons must be considered with great caution, since the concentration reached in the culture medium clearly depends on the time of culture, the cell number and the volume of the medium. In addition, cell types other that NIH3T3 may synthesize higher amounts of 15-HETE. Moreover, we do not yet know in which physiological scenario the 15-HETE/PPARβ/δ signaling pathway plays a role: is signaling paracrine and what would be the interacting cell types? Or does this pathway not involve the release of 15-HETE and rather functions intracellularly? What is the role of fatty acid binding proteins, which may increase local concentrations of 15-HETE in a particular subcellular microenvironment? For these reasons the issue of physiological relevance cannot be completely clarified at present, but this applies to most other studies addressing the role of endogenous

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PPAR ligands. However, the response sensitivity of the response of HACaT cells to 15-HETE concentrations referred to above provides a strong argument to believe that the regulation of PPARβ/δ by 15-HETE is indeed physiologically relevant.

The 15-HETE used for all experiments discussed so far is a mixture of two enantiomers, 15S-HETE and 15R-HETE, that are produced by different pathways. 15S-HETE is formed by a physiological two-step process, involving the 15-LOX catalyzed oxydation of AA to 15S-HPETE, which is then enzymatically converted to 15S-HETE. 15R-HETE, on the other hand, is produced from AA by a cytochrome P450 dependent mechanism or by aspirin-triggered acetylated COX-2 (Clària et al., 1996; Clària and Serhan, 1995; Gilroy, 2005; Romano, 2006; Titos et al., 1999). In view of these differences in the synthetic pathways it was important to show that both enantiomers are able to interact with PPARβ/δ (Fig. 3B) and to induce its transcriptional activity (Fig. 4B,C and 5A). These results clearly indicate that 15-HETE enatiomers generated by different pathways function as agonistic ligands for PPARβ/δ, which provides further evidence for a central role of PPARβ/δ in eicosanoid regulated signaling. In this context it tempting to speculate that PPARβ/δ may also play a role in mediating the anti-inflammatory effects of aspirin, which would be in agreement with the reported anti-inflammatory function of 15-HETE (van Dijk et al., 1993). This hypothecial connection between aspirin and PPARβ/δ through 15R-HETE will be addressed in future studies.

Eveṇthoughits role in tumorigenesis is controversial, PPARβ/δ and synthetic PPARβ/δ agonists have been reported to inhibit cancer cell proliferation in multiple studies (reviewed in(Müller et al., 2008a; Müller et al., 2008b; Peters and Gonzalez, 2009). Interestingly, 15-HETE has also been linked to cell proliferation, both as in

mitogenic and anti-mitogenic compound (Moreno, 2009), but is effect on tumor cell proliferation is invariably inhibitory, including prostate cancer (Shappell et al., 2001), lung cancer (Clària et al., 1996) and myeloid leukemia cells (Mahipal et al., 2007), which may point to another link between 15-HETE and PPARβ/δ. In this context it is also noteworthy that the agonistic effect of 15-HETE on PPARβ/δ was particular high is keratinocytes (HaCaT cells; Fig. 5B), where a growth inhibitory role for PPARβ/δ is well established (Borland et al., 2008; Burdick et al., 2007; Chong et al., 2009; Girroir et al., 2008; Kim et al., 2006; Michalik et al., 2001).

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Footnotes

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Figure legends

Fig. 1. Effects of lipoxygenase inhibitors and specific eicosanoids on the transcriptional activity of the PPARβ/δ ligand binding domain. NIH3T3 were transiently transfected with an expression vector encoding a Gal4-PPARβ/δ (A) or LexA-PPARβ/δ fusion protein (B) together with a LexA or Gal4 binding luciferase reporter plasmid. Cells were preincubated with the pan-LOX inhibitor NDGA (A) or the 12/15-LOX inhibitor EDBCA (B) at the indicated concentrations for 24 hrs, and then for another 24 hrs with 20 μM AA prior to harvesting. Control cells (-) received solvent only. C, CHO cells stably expressing a Gal4-PPARβ/δ fusion protein and haboring a stably integrated Gal4 responsive luciferase reporter gene were treated with the indicated compounds for 20 hrs. Values represent the average of triplicates; error bars show the standard deviation. Significant differences between control cells and cells treated with inhibitor are indicated by an asterisk (paired t-test; $P < 0.001$).

Fig. 2. Ligand-induced binding of a coactivator derived peptide to the PPARβ/δ LBD *in vitro*. Interaction of fluorescein labeled coactivator peptide and recombinant GST-Pparb bound by a terbium-labeled anti-GST antibody was determined by TR-FRET. AA and the indicated metabolites or synthetic ligands were used at the indicated concentrations. Panel B shows a titration analysis of lower concentrations of 15-HETE. Results are expressed as the ratio of fluorescence intensity at 520 nm (fluorescein emission excited by terbium emission) and 495 nm (terbium emission). All data points represent averages of triplicates (± S.D). In panel B all FRET ratios obtained for concentrations ≥0.1μM are stastistically significant (Bonferroni-Holm adjusted t-test).

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Fig. 3. Competitive *in vitro* ligand binding assay for PPARβ/δ. Displacement of the Fluormone™ Pan-PPAR Green PPAR ligand from recombinant GST-Pparb was determined by TR-FRET. AA and the indicated metabolites were used at the concentrations shown in the graph. A, Binding of 15-HETE and 15-HPETE compared to that of AA. B, Binding of 15-HETE enantiomers and their metabolites. Results are expressed as the ratio of fluorescence intensity at 520 nm (fluorescein emission excitated by terbium emission) and 495 nm (terbium emission). All data points represent averages of triplicates (± S.D).

Fig. 4. Effects of different HPETEs and HETEs on the transcriptional activity of PPARβ/δ. A, NIH3T3 cells were transiently transfected with a PPRE-lacking control plasmid (left bars) or a PPRE-driven luciferase reporter plasmid (middle and right bars) together with either empty vector (left bars and middle) or expression vectors encoding PPARβ/δ and RXRα (right bars). The concentration of 12-HETE, 15-HETE and 15-HPETE was 10μM, AA was 20 μM. B and C, Analysis of 15-HETE enantiomers and their metabolites in the LexA-based system described in Fig. 1. In panel B the empty vector pcDNA3.1 was cotransfected instead of the LexA-Pparb expression plasmid (C). Values represent the average of triplicates; error bars show the standard deviation. Significant differences between solvent (EtOH) and ligand treated cells are indicated by an asterisk (paired t-test; $P < 0.001$).

Fig. 5. Inducibility of the PPARβ/δ target gene *Angptl4*. (A) Regulation of *Angptl4* by 15-HETE and its enantiomers 15S-HETE and 15R-HETE in comparison to GW501516 (0.3μM) in NIH3T3 cells. (B) Regulation of *Angptl4* by 15-HETE at different concentrations in NIH3T3, HaCaT and WPMY-1 cells. Cells were treated for 3 hrs, RNA was isolated and analyzed by qPCR using *L27* for normalization.

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Induction values were calculated relative to solvent (EtOH) treated cells and represent averages of triplicates (± S.D) normalized. *: values significantly different ($P < 0.001$) between solvent (EtOH) and ligand treated cells.

Fig. 6. Altered transcription factor binding and histone acetylation on the *ANGPTL4* promoter following treatment with 15-HETE of WPMY-1 myofibroblasts. Cells were treated with 15-HETE (10 μM), GW501516 (0.3 μM) or solvent (ethanol for 15-HETE, DMSO for GW501516) for 1 h (A) or 3 h (B). ChIP was carried out using antibodies against CBP (A), or PPARβ/δ and acetylated histones H3 and H4 (B). An unspecific IgG pool from rabbit was used as a negative control. DNA was amplified with primers encompassing the *ANGPTL4* PPRE. Relative amounts of amplified DNA in immunoprecipitates were calculated by comparison with 1% of input DNA. Results are expressed as % input and represent averages of triplicates (± S.D).

Fig. 7. Expression of the PPARβ/δ target gene *Angptl4* in fibroblasts lacking PPARβ/δ (A), in the same cells with restored PPARβ/δ expression (B) and after the retrovirus-mediated deletion of floxed *Pparb/d* alleles (C, D). Fetal lung fibroblasts from *Pparb/d* null mice (Müller-Brüsselbach et al., 2007; Peters et al., 2000) were infected with a control retrovirus (pLPCX; A) or a retrovirus expressing FLAG-tagged PPARβ/δ (3xFlag-Pparb; B). C and D, fetal lung fibroblasts established from a mouse strain with a floxed *Pparb/d* allele (Barak et al., 2002) were infected with either the empty retroviral vector (pBabe; left) or a Cre-expressing retrovirus (pBabe-Cre; right) (Li et al., 1997). C, reduction of *Pparb/d* expression after deletion of the floxed alleles. D, effect of *Pparb/d* deletion on *Angptl4* expression. Cells were treated with solvent (EtOH), AA (20μM), 15-HPETE (10μM), 15-HETE (10μM), GW501516 (GW; 0.3μM), the selective PPARγ ligand GW1929 (0.3μM) or the

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selective PPARα ligand GW7647 (0.3μM) for 6 hrs and analyzed for expression of *Angptl4* (A, B, D) and *Pparb/d* (C) by qPCR. Induction values were calculated relative to solvent treated cells and represent averages of triplicates (± S.D) normalized. *: values significantly different ($P < 0.001$) to vector control (A) or pBabe (C), or between solvent (EtOH) and ligand treated cells (D)

Fig. 8. Effect of siRNA mediated PPARβ/δ knockdown on *Angptl4* inducibility in NIH3T3 cells. A, efficacy of siRNA treatment. Cells exposed to *Pparb/d* specific siRNA show a 67% reduction of *Pparb/d* expression and an increased basal *Angptl4* mRNA expression (presumably due to the removal of repressor complexes). B, effect of control and *Pparb/d* siRNA treatment on *Angptl4* induction by GW501516 (0.3 μM), 15-HPETE (10μM) and 15-HETE (10μM). Cells were treated for 3 hrs and RNA levels were quantified by qPCR. Induction values were calculated relative to solvent treated cells and represent averages of triplicates (± S.D) normalized. *: values significantly different ($P < 0.001$) between control and *Pparb/d* siRNA treated cells.

Fig. 9. PPAR subtype selective transcriptional activation by 15-HETE. NIH3T3 were transiently transfected with an expression vector encoding a LexA-PPARα, LexA-PPARβ/δ or LexA-PPARγ fusion protein together with a LexA responsive luciferase reporter plasmid (7L-TATAi). Cells were incubated with 15-HETE at the indicated concentrations or with synthetic ligands (1 μM) for 24 hrs prior to harvesting. Control cells (-) received solvent only. Values represent the average of triplicates; error bars show the standard deviation. Significant differences between control cells and 15-HETE treated cells are indicated by an asterisk (Bonferroni-Holm adjusted t-test).

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Fig. 10. PPAR subtype selective coactivator peptide recruitment *in vitro* by 15-HETE. A, Comparison of 15-HETE induced binding of TRAP220 coactivator peptide to the PPARα, PPARβ/δ and PPARγ LBDs. Data are shown as the ratio of fluorescence intensity at 520/495 nm relative to samples without added ligand. The results thus reflect the factor of increased coactivator peptide binding following 15-HETE addition and allow for a direct comparison of the results obtained with different PPAR subtypes. B and C, Ligand-induced binding of four different coactivator derived peptides to the PPARβ/δ and PPARγ LBD. GW501516 and GW1929 were used at a concentration of 0.3 μM, 15-HETE at 31.2 μM. Results are expressed as the ratio of fluorescence intensity at 520/495 nm (as in Fig. 2). All data points represent averages of triplicates (± S.D). Significant differences between solvent and ligand treated cells are indicated by an asterisk (paired t-test; $P < 0.05$).

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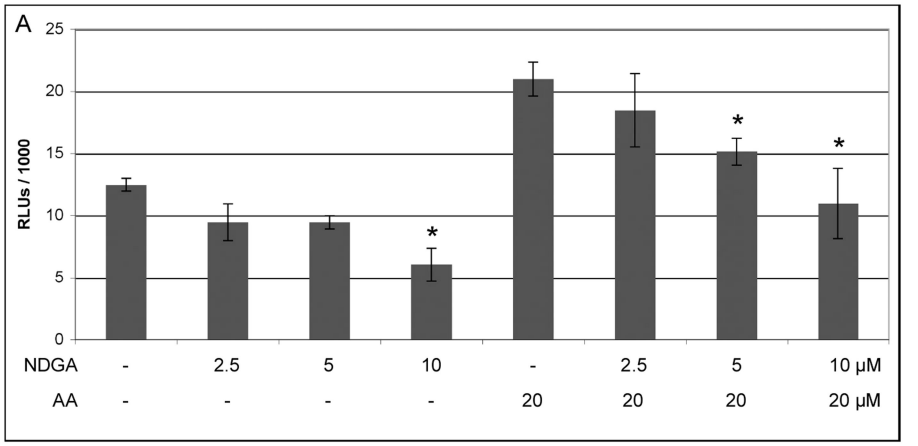
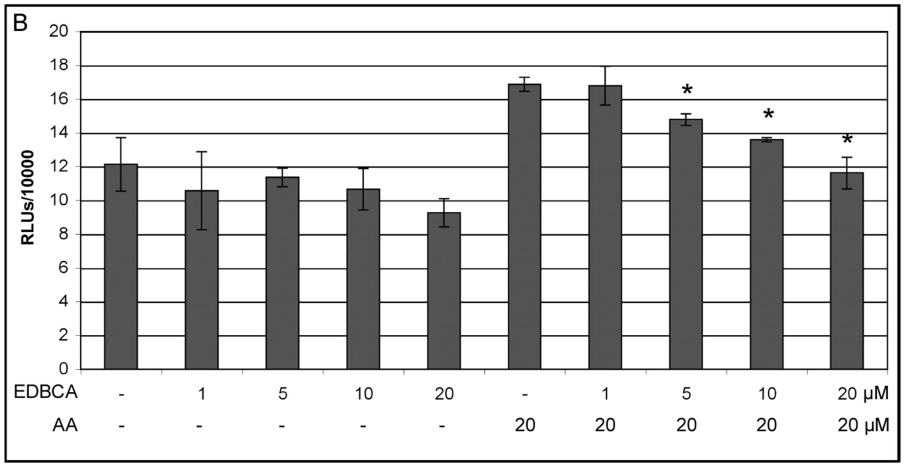
Table 1: HETE synthesis by NIH3T3 fibroblasts.

A. HETE synthesis (25μM AA)	**ng/ml***
5-HETE	8.5 ± 0.5
8-HETE	6.5 ± 0.5
12-HETE	4.4 ± 0.9
15-HETE	11.2 ± 1.3
B. HETE synthesis (100μM AA)	**ng/ml***
5-HETE	58.0 ± 1.0
8-HETE	25.5 ± 3.5
12-HETE	12.6 ± 0.3
15-HETE	62.8 ± 1.0

*HETE concentrations in the culture medium 6 hrs after addition of 25 μM arachidonic acid.


A
RLUs / 1000
25
20
15
10
5
0
*
*
*
NDGA - 2.5 5 10 - 2.5 5 10 µM
AA - - - - 20 20 20 20 µM


B
RLUs/10000
20
18
16
14
12
10
8
6
4
2
0
*
*
*
EDBCA - 1 5 10 20 - 1 5 10 20 µM
AA - - - - - 20 20 20 20 20 µM

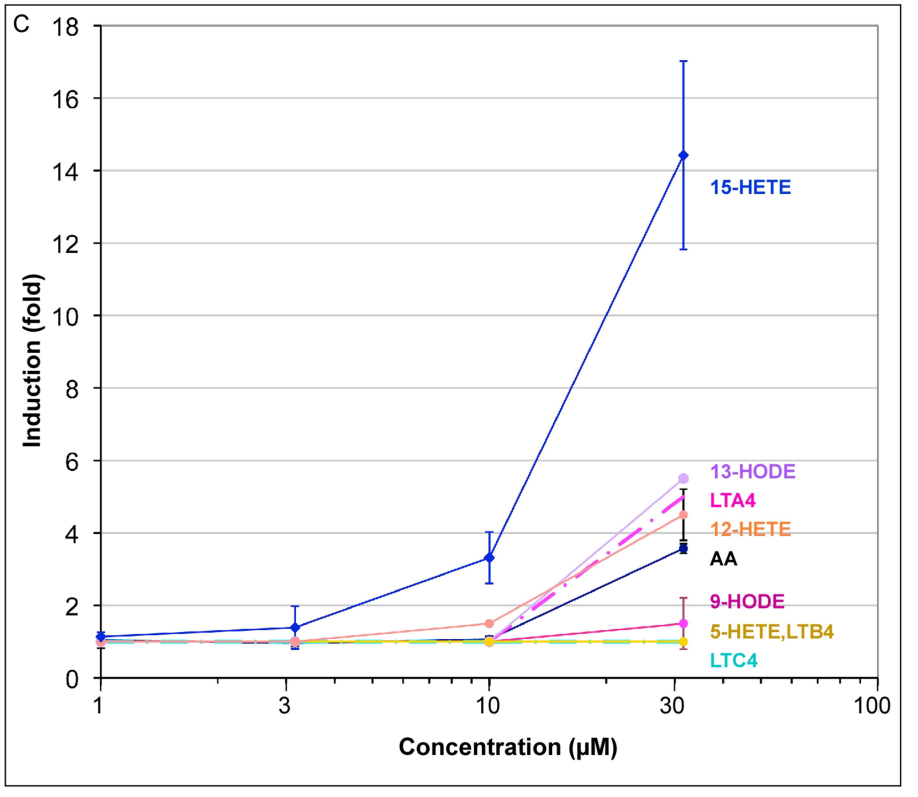

C
Induction (fold)
18
16
14
12
10
8
6
4
2
0
1
3
10
30
100
Concentration (µM)
15-HETE
13-HODE
LTA4
12-HETE
AA
9-HODE
5-HETE,LTB4
LTC4

FIG 1

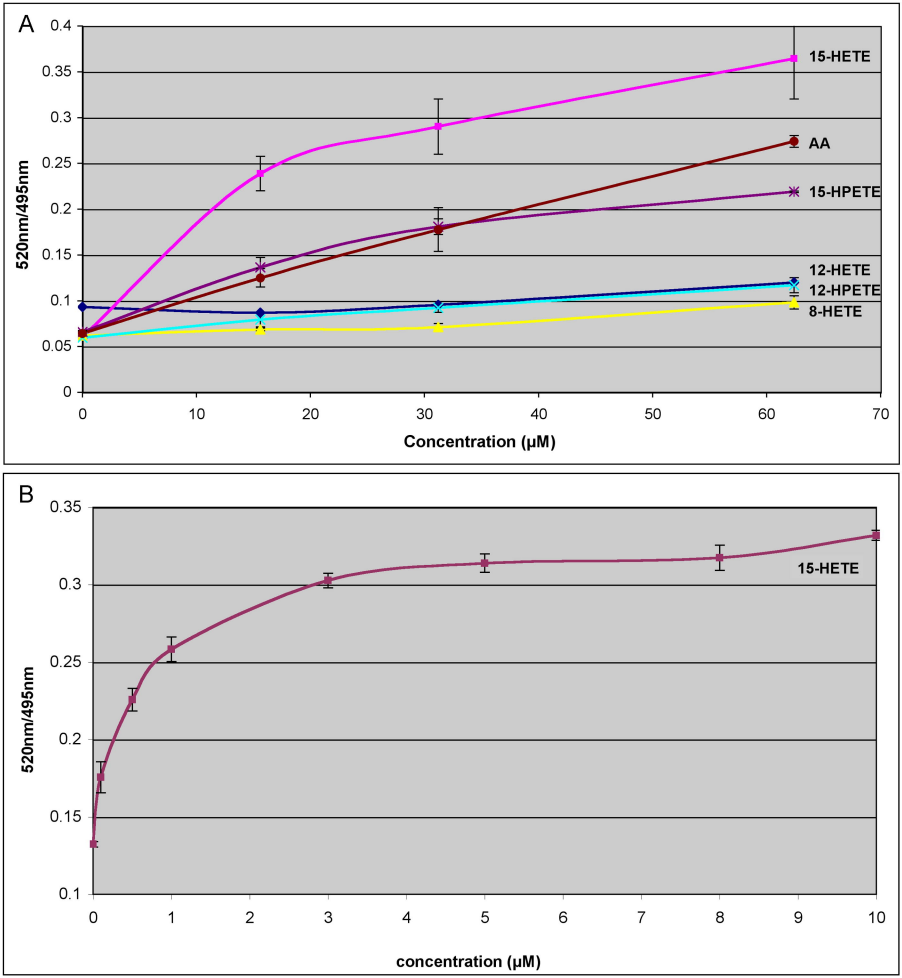
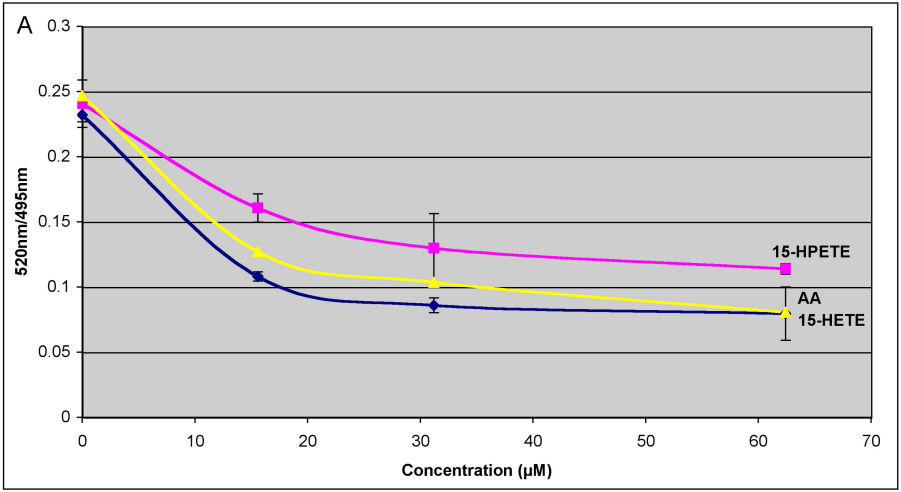

A
520nm/495nm
Concentration (μM)
15-HETE
AA
15-HPETE
12-HETE
12-HPETE
8-HETE
B
520nm/495nm
concentration (μM)
15-HETE

FIG 2


A
520nm/495nm
0.3
0.25
0.2
0.15
0.1
0.05
0
0
10
20
30
40
50
60
70
Concentration (μM)
15-HPETE
AA
15-HETE

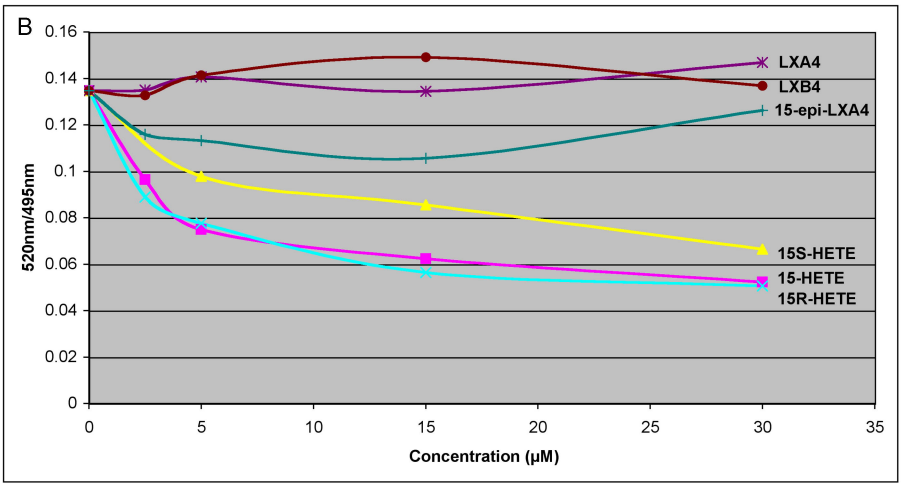
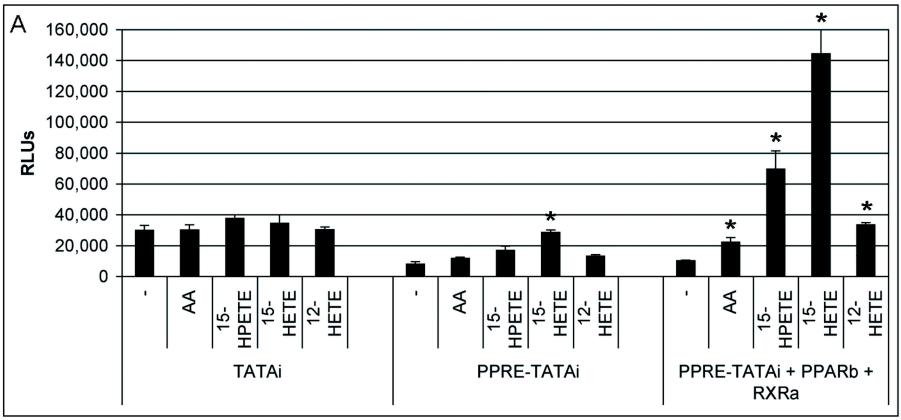
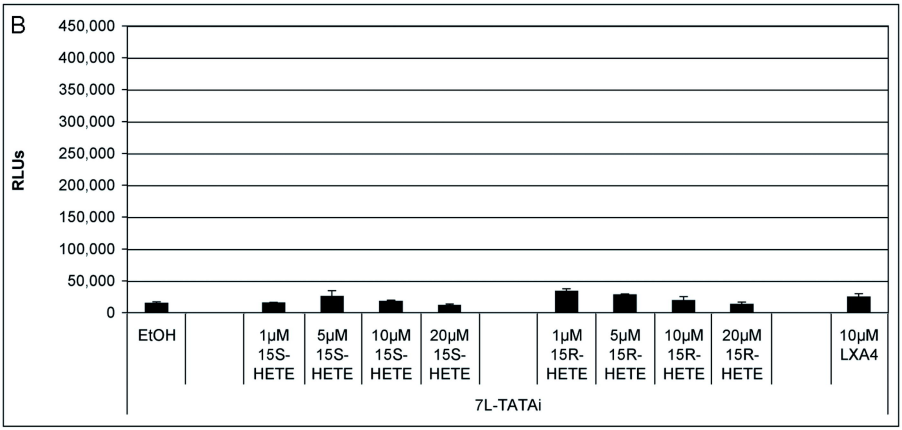

B
520nm/495nm
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
5
10
15
20
25
30
35
Concentration (μM)
LXA4
LXB4
15-epi-LXA4
15S-HETE
15-HETE
15R-HETE

FIG 3


A
RLUs
160,000
140,000
120,000
100,000
80,000
60,000
40,000
20,000
0
-
AA
15-HPETE
15-HETE
12-HETE
TATAi
PPRE-TATAi
PPRE-TATAi + PPARb + RXRa
*


B
RLUs
450,000
400,000
350,000
300,000
250,000
200,000
150,000
100,000
50,000
0
EtOH
1μM 15S-HETE
5μM 15S-HETE
10μM 15S-HETE
20μM 15S-HETE
1μM 15R-HETE
5μM 15R-HETE
10μM 15R-HETE
20μM 15R-HETE
10μM LXA4
7L-TATAi

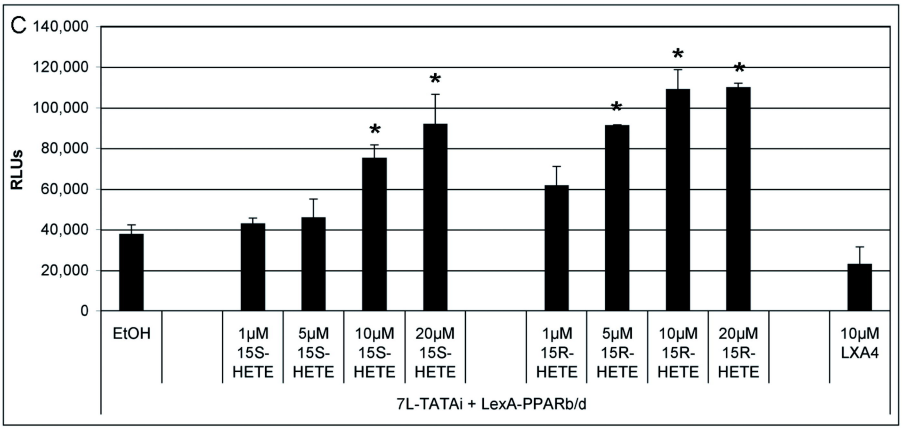
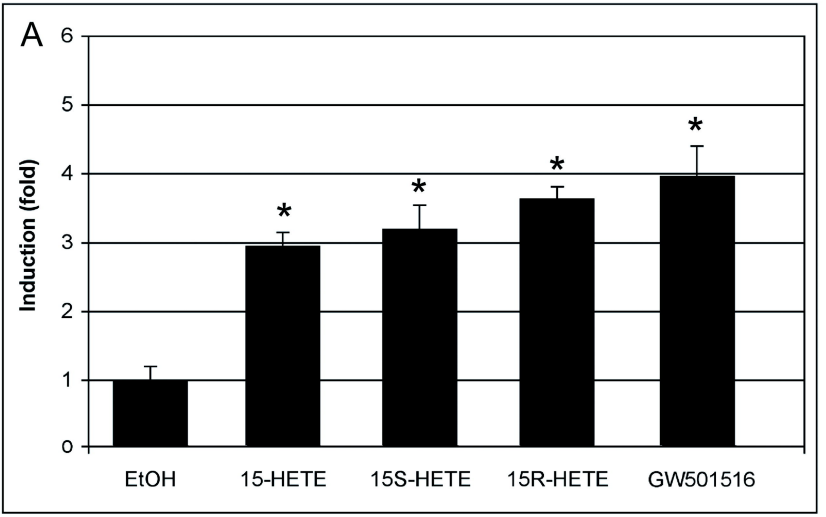

C
RLUs
140,000
120,000
100,000
80,000
60,000
40,000
20,000
0
EtOH
1μM 15S-HETE
5μM 15S-HETE
10μM 15S-HETE
20μM 15S-HETE
1μM 15R-HETE
5μM 15R-HETE
10μM 15R-HETE
20μM 15R-HETE
10μM LXA4
7L-TATAi + LexA-PPARb/d
*

FIG 4


A
Induction (fold)
0
1
2
3
4
5
6
*
*
*
*
EtOH
15-HETE
15S-HETE
15R-HETE
GW501516

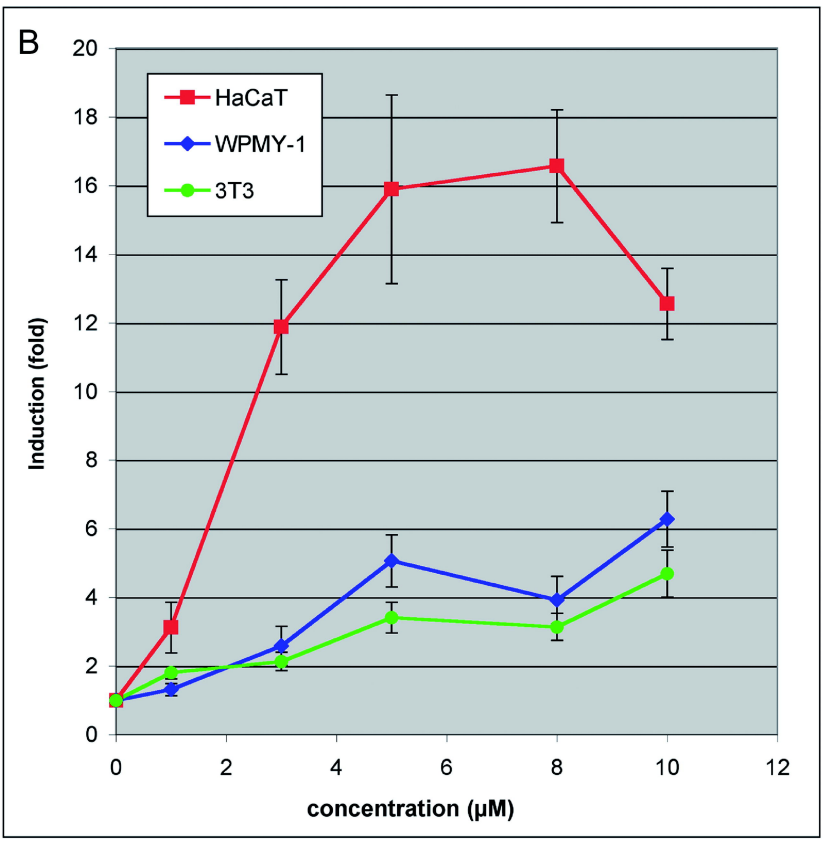
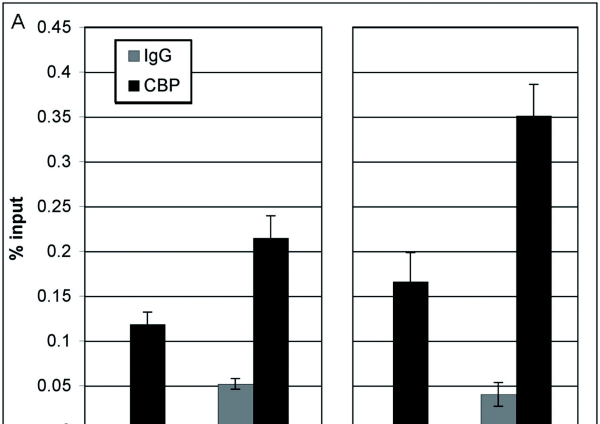

B
Induction (fold)
0
2
4
6
8
10
12
14
16
18
20
HaCaT
WPMY-1
3T3
0
2
4
6
8
10
12
concentration (μM)

FIG 5


A
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
% input
IgG
CBP
DMSO
GW501516
EtOH
15-HETE

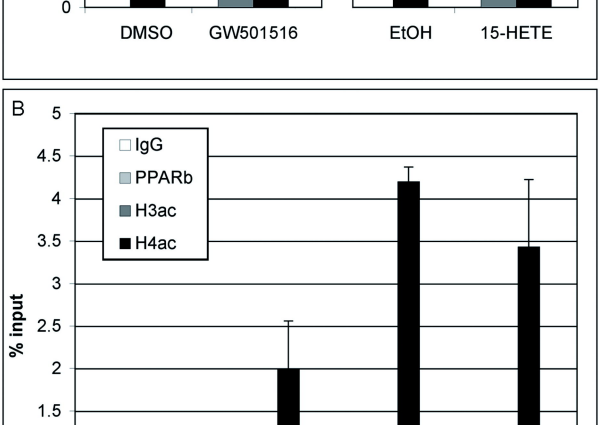
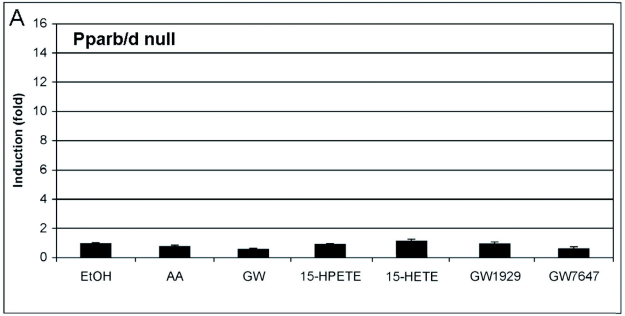
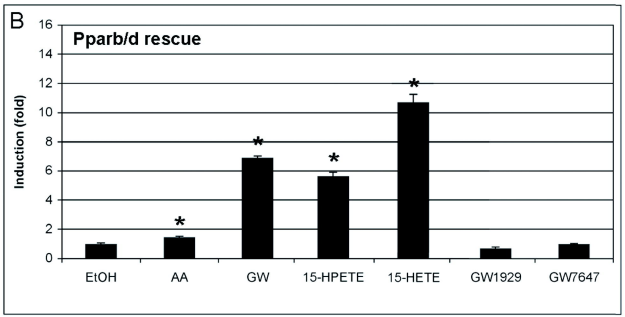
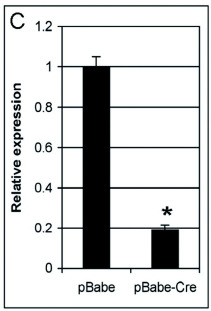

B
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
% input
IgG
PPARb
H3ac
H4ac
solvent
AA
15-HETE
GW501516

FIG 6


A
Pparb/d null
Induction (fold)
16
14
12
10
8
6
4
2
0
EtOH
AA
GW
15-HPETE
15-HETE
GW1929
GW7647


B
Pparb/d rescue
Induction (fold)
16
14
12
10
8
6
4
2
0
*
*
*
*
EtOH
AA
GW
15-HPETE
15-HETE
GW1929
GW7647


C
Relative expression
1.2
1
0.8
0.6
0.4
0.2
0
*
pBabe
pBabe-Cre

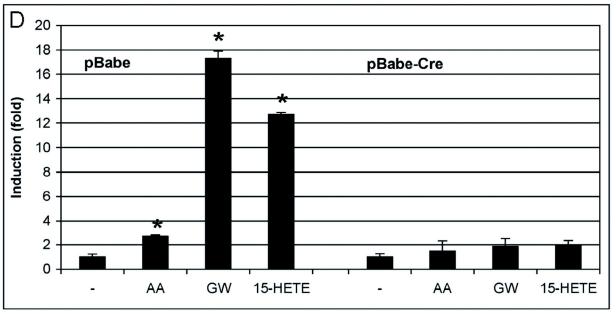

D
Induction (fold)
20
18
16
14
12
10
8
6
4
2
0
pBabe
pBabe-Cre
*
*
*
-
AA
GW
15-HETE
-
AA
GW
15-HETE

FIG 7

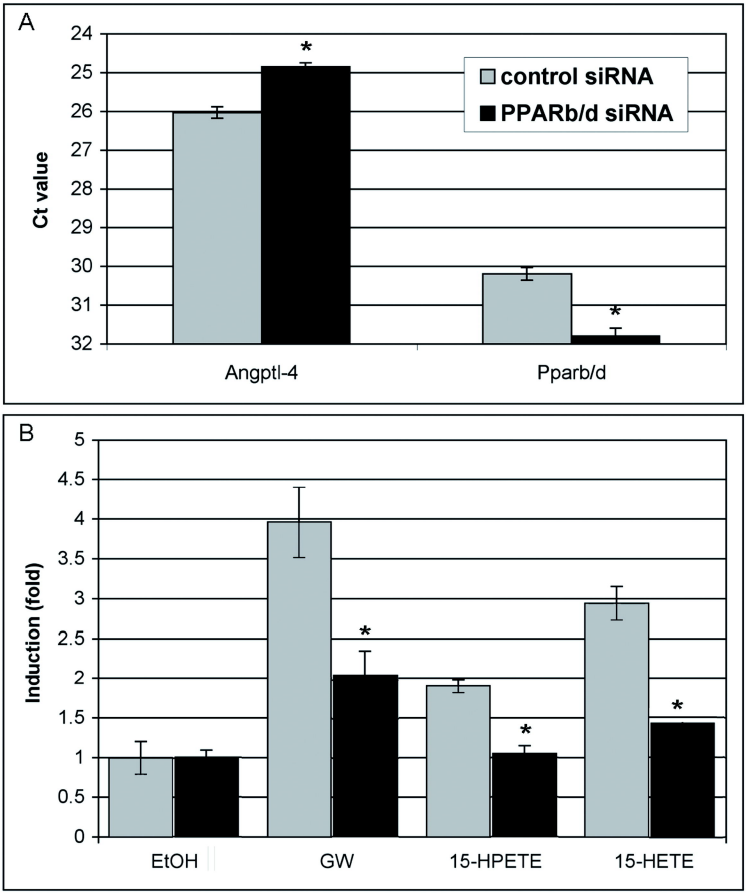

A
24
25
26
27
28
29
30
31
32
Ct value
*
*
control siRNA
PPARb/d siRNA
Angptl-4
Pparb/d
B
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Induction (fold)
*
*
*
EtOH
GW
15-HPETE
15-HETE

FIG 8

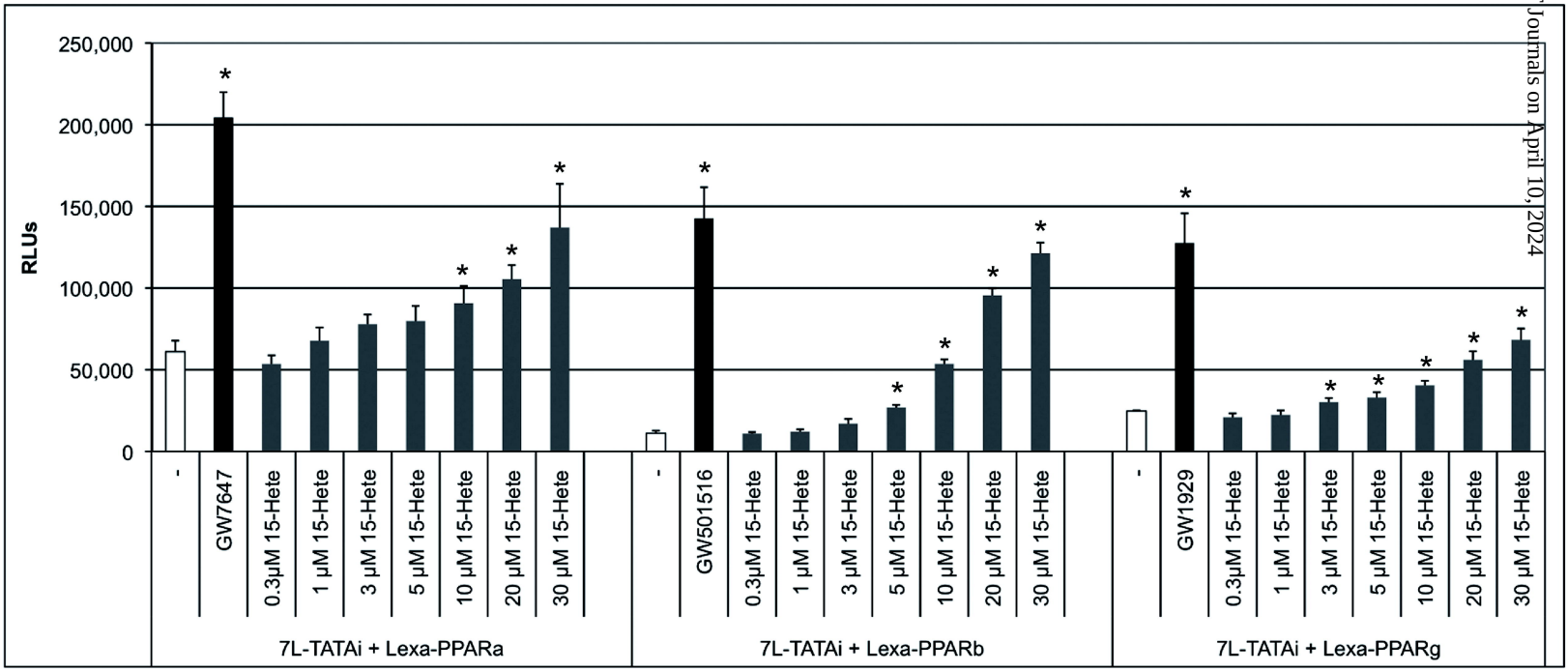


FIG 9

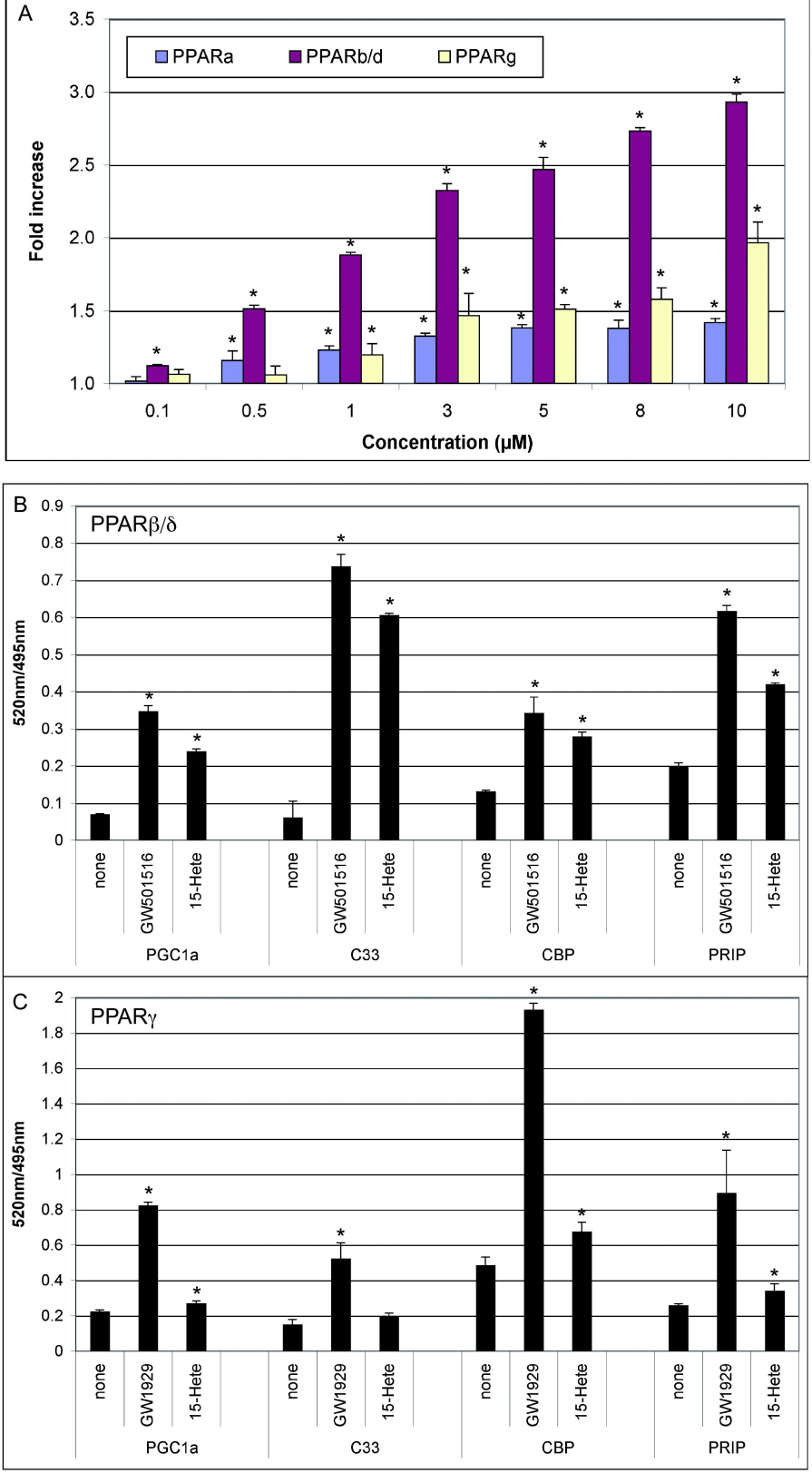

A
PPARa
PPARb/d
PPARg
Fold increase
3.5
3.0
2.5
2.0
1.5
1.0
0.1
0.5
1
3
5
8
10
Concentration (μM)
B
PPARβ/δ
520nm/495nm
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
none
GW501516
15-Hete
PGC1a
C33
CBP
PRIP
C
PPARγ
520nm/495nm
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
none
GW1929
15-Hete
PGC1a
C33
CBP
PRIP

FIG 10