

# 15-Hydroxyeicosatetraenoic Acid Is a Preferential Peroxisome Proliferator-Activated Receptor $\beta/\delta$ Agonist

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## ABSTRACT

Peroxisome proliferator-activated receptor (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 PPAR $\beta/\delta$  in vivo, competes with synthetic agonists in a PPAR $\beta/\delta$  ligand binding assay in vitro, and triggers the interaction of PPAR $\beta/\delta$  with coactivator peptides. These agonistic effects were also seen with PPAR $\alpha$  and PPAR $\gamma$ , 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 PPAR $\beta/\delta$ -dependent manner and, conversely, that inhibition of 15-HETE synthesis reduces PPAR $\beta/\delta$  transcriptional activity. Consistent with its function as an agonistic ligand, 15-HETE triggers profound changes in chromatin-associated PPAR $\beta/\delta$  complexes in vivo, including the recruitment of the coactivator cAMP response element-binding protein binding protein. Both 15R-HETE and 15S-HETE are similarly potent at inducing PPAR $\beta/\delta$  coactivator binding and transcriptional activation, indicating that 15-HETE enantiomers generated by different pathways function as PPAR $\beta/\delta$  agonists.

Peroxisome proliferator-activated receptor- $\beta/\delta$  (PPAR  $\beta/\delta$ ) is a ligand-regulated transcription factor that modulates target gene expression in response to certain fatty acids and fatty acid derivatives (Forman et al., 1997; Desvergne et al., 2006). PPAR $\beta/\delta$  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 PPAR $\beta/\delta$  that results in the displacement of interacting core-

pressors, such as silencing mediator for retinoid and thyroid (SMRT) receptors and SMRT/histone deacetylase-associated repressor protein (SHARP), by specific coactivators, such as sequential recruitment of steroid receptor coactivator-1 (SRC-1) and p300/CBP, resulting in transcriptional activation (Yu and Reddy, 2007; Zoete et al., 2007). PPAR $\beta/\delta$  can regulate genes also by different mechanisms. Thus, PPAR $\beta/\delta$  ligands repress pro-inflammatory gene expression by releasing the hematopoietic transcriptional repressor Bcl-6 from a complex with PPAR $\beta/\delta$  (Lee et al., 2003). In another study (Matsusue et al., 2006), it was reported that PPAR $\beta/\delta$  can repress genes by sequestering RXR from other RXR-dependent nuclear receptors.

PPAR $\beta/\delta$  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

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**ABBREVIATIONS:** PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; PPRE, peroxisome proliferator responsive element; CBP, cAMP response element-binding protein binding protein; GW501516, 2-[2-methyl-4-[(4-methyl-2-[4-(trifluoromethyl)phenyl]-1,3-thiazol-5-yl)methylsulfanyl]phenoxy]acetic acid; L165,041, [4-[3-(4-acetyl-3-hydroxy-2-propylphenoxy)propoxy]phenoxy]acetic acid; AA, arachidonic acid; LBD, ligand binding domain; PG, prostaglandin; 15R-HETE, 15(R)-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid; 15S-HETE, 15(S)-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; COX, cyclo-oxygenase; LOX, lipoxygenase; NDGA, nordihydroguaiaretic acid; EDBCA, ethyl-3,4-dihydroxy-benzylidene-cyanoacetate; GW1929, N-(2-benzoylphenyl)-O-[2-(methyl-2-pyridinylamino)ethyl]-L-tyrosine hydrochloride; LX, lipoxin; GW7647, 2-[[4-[2-[(cyclohexylamino)carbonyl](4-cyclohexylbutyl)amino]ethyl]phenyl]thio]-2-methylpropanoic acid; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; siRNA, small interfering RNA; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid); NP40, nonidet P40; TR-FRET, time-resolved fluorescence resonance energy transfer; LC, liquid chromatography; TATAi, TATA initiator module; PGC1 $\alpha$ , peroxisome proliferator-activated receptor  $\gamma$  coactivator 1 $\alpha$ ; Angptl4, angiopoietin-like 4; Cre, cyclization recombination.

developmental processes, differentiation, and wound healing. Mice lacking PPAR $\beta/\delta$  show an aberrant development and malfunction of the placenta (Peters et al., 2000; Barak et al., 2002; Nadra et al., 2006) and exhibit a defect in wound healing (Michalik et al., 2001). PPAR $\beta/\delta$  is critical for the survival, differentiation, and proliferation of keratinocytes (Peters et al., 2000; Di-Poi et al., 2002; Burdick et al., 2006), and promotes the differentiation of Paneth cells in the intestinal crypts (Varnat et al., 2006). However, PPAR $\beta/\delta$  also plays a role in cancer and inflammation: it modulates intestinal tumorigenesis with diverging effects in different mouse models (Peters et al., 2000; Barak et al., 2002; Di-Poi et al., 2002; Gupta et al., 2004; Wang et al., 2004; Burdick et al., 2006), inhibits chemically induced skin carcinogenesis (Kim et al., 2004; Bility et al., 2008), 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, PPAR $\beta/\delta$  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 PPAR $\beta/\delta$  activity to a moderate extent is AA. It has been shown that AA is a low-affinity ligand that interacts with the PPAR $\beta/\delta$  LBD (Xu et al., 1999), raising the possibility that the agonistic effect of AA is due directly to its interaction with PPAR $\beta/\delta$ . 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<sub>2</sub> (PGI<sub>2</sub>; prostacyclin) has indeed been postulated to act as a PPAR $\beta/\delta$  agonist (Gupta et al., 2000; Hatae et al., 2001), but this issue remains controversial (Yu et al., 1995; Forman et al., 1996; Fauti et al., 2006).

Another major group of eicosanoid metabolites is generated by the lipoxygenases (Pidgeon et al., 2007), but lipoxygenase products of AA acting as bona fide PPAR $\beta/\delta$  ligands have not yet been described. 15-Hydroxyeicosatetraenoic acid (15-HETE) has been reported to activate PPAR $\beta/\delta$  in a reporter assay in keratinocytes, but it remains unclear whether this involves a direct interaction with the PPAR $\beta/\delta$  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 due largely 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 PPAR $\beta/\delta$  with coactivator peptides *in vitro*, and interacted with PPAR $\beta/\delta$  in a competitive ligand-binding assay. Furthermore, 15-HETE induced the PPAR $\beta/\delta$  target gene *Angptl4* (Mandard et al., 2004) in a clearly PPAR $\beta/\delta$ -dependent manner. Both enantiomers of 15-HETE, 15R-HETE and 15S-HETE, showed similar agonistic properties, indicating that different pathways converge on PPAR $\beta/\delta$ . Whereas 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 enantiomers produced by different signaling path-

ways function as ligands for PPAR $\beta/\delta$  and induce its transcriptional activity.

## Materials and Methods

**Chemicals.** GW501516, 9-*cis*-retinoic acid, and the LOX inhibitors NDGA and EDBCA were purchased from Axxora (Lörrach, Germany) and prostaglandins D<sub>2</sub>, E<sub>2</sub>, and F<sub>2</sub> from Cayman Europe (Tallinn, Estonia). GW1929 was obtained from Cayman Europe; LXA4 from BIOMOL (Hamburg, Germany); and GW7647 and diclofenac from Sigma-Aldrich (Steinheim, Germany). All other eicosanoids were from Cayman Europe and Axxora (Lörrach, Germany).

**Mouse Strains.** *Pparb/d*-null and wild-type mice have been described previously (Peters et al., 2000). *Pparb/d*<sup>ck</sup> mice (Barak et al., 2002) harboring a floxed *Pparb/d* exon 4 were kindly provided by R. Evans.

**Cell Culture.** *Pparb/d*-null, wild-type, and floxed fibroblasts were established from fetal lungs and cultured as described previously (Müller-Brüsselbach et al., 2007). WPMY-1 cells were obtained from the American Type Culture Collection (Manassas, VA). 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 expresses 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 charge-coupled device camera. The cell clone showing the best response to synthetic PPAR $\beta/\delta$  ligands was chosen for this study. All cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin in a humidified incubator at 37°C and 5% CO<sub>2</sub>.

**Plasmids.** pCMX-mPpar $\delta$  (Forman et al., 1997) and Gal4-mPpar $\delta$  (Shi et al., 2002) were kindly provided by Dr. R. Evans. 3 $\times$ FLAG-PPAR $\beta/\delta$  was generated by cloning the coding sequence of mPPAR $\beta/\delta$  N-terminally fused to a triple FLAG tag (Müller-Brüsselbach et al., 2007) into pcDNA3.1(+)<sub>zeo</sub> (Invitrogen, Karlsruhe, Germany). pCMX-empty has been described previously (Umesono et al., 1991). LexA-PPAR $\beta/\delta$ , 7L-TATA initiator module (TATAi), and 10 $\times$ Gal4SVGL3 have been described previously (Jérôme and Müller, 1998; Fauti et al., 2006). LexA-PPAR $\alpha$  and LexA-PPAR $\gamma$  were constructed in a fashion analogous to the construction of LexA-PPAR $\beta/\delta$ . pSG5-hRxRa containing the full-length RxRa cDNA was kindly provided by Dr. A. Baniahmad. 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 (Jérôme and Müller, 1998). The pUC18 plasmid was obtained from New England Biolabs (Frankfurt am Main, Germany), and pcDNA3.1 was obtained from Invitrogen (Karlsruhe, Germany).

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

**Retrovirally Transduced Cells Expressing FLAG-PPAR $\beta$ .** 3 $\times$ FLAG-PPAR $\beta/\delta$  was cloned into the retroviral vector pLPCX (Clontech, Mountain View, CA). Phoenix cells expressing ecotropic *env* were transfected with 3 $\times$ FLAG-mPPARb-pLPCX as described elsewhere ([http://www.stanford.edu/group/nolan/retroviral\\_systems/retsys.html](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  $\mu\text{g}/\text{ml}$ ; Sigma), resulting in a cell population expressing 3 $\times$ FLAG-mPPAR $\beta/\delta$  at moderate levels.

**Quantitative PCR.** cDNA was synthesized from 1  $\mu\text{g}$  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  $\mu\text{M}$  according to 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: forward, 5'-CTCCATCGTCAACAAAGACG; reverse, 5'-TCTTCTTTAGCCACTGCATC; Angptl4: forward, 5'-CTC TGG GGT CTC CAC CAT TT; reverse, 5'-TTG GGG ATC TCC GAA GCC AT; *L27*: forward, 5'-AAA GCC GTC ATC GTG AAG AAC; reverse, 5'-GCT GTC ACT TTC CGG GGA TAG.

**siRNA Transfections.** For siRNA transfection, cells were seeded at a density of  $5 \times 10^5$  cells per 6-cm dish in 4 ml of DMEM with 10% FCS and cultured overnight. Four hundred picomoles of siRNA in 500  $\mu\text{l}$  of OptiMEM (Invitrogen) and 10  $\mu\text{l}$  of Lipofectamine 2000 (Invitrogen) in 500  $\mu\text{l}$  of OptiMEM 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 h. Cells were passaged and replated 48 h after transfection at a density of  $5 \times 10^5$  cells per 6-cm dish. Transfection was repeated 72 h after start of the experiment, and cells were passaged after another 24 h. Forty-eight hours after the last transfection, cells were stimulated and harvested after 3 h for RNA isolation. The following siRNAs were used: Pparb siRNA1 (CCG-CATGAAGCTCGAGTATGA; QIAGEN); Pparb siRNA2 (CAAGTTC-GAGTTTGCTGTCAA; QIAGEN); control siRNA (Alexa Fluor 488-labeled nonsilencing duplex siRNA; QIAGEN).

**Chromatin Immunoprecipitation Analysis.** WPMY-1 cells were grown to confluence. 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 phosphate-buffered saline, 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, and 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 radioimmunoprecipitation assay 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, and 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 Corporation, Danbury, CT) set to 1-s pulse, 2-s pause. Eighty pulses were applied. After centrifugation at 16,000g 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 radioimmunoprecipitation assay buffer with 1 mg/ml bovine serum albumin, 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 immunoprecipitations. Four micrograms of antibody [rabbit IgG pool, Sigma;  $\alpha$ -PPAR $\beta/\delta$ ,  $\alpha$ -CBP, Santa Cruz Biotechnology (Santa Cruz, CA);  $\alpha$ -acetylated H4, Millipore (Billerica, MA)] were added to 300  $\mu\text{l}$  of precleared chromatin corresponding to  $3 \times 10^6$  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 of chilled

mixed micelle buffer [20 mM Tris, pH 8.1, 150 mM NaCl, 2 mM EDTA, 0.1% (w/v) SDS, and 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, and 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, and 1 mM EDTA), and twice with room-temperature Tris-EDTA. Complexes were eluted twice with 250  $\mu\text{l}$  of elution buffer [1% SDS (w/v) and 100 mM NaHCO<sub>3</sub>]. Supernatants were pooled; adjusted to 180 mM NaCl, 35 mM Tris, pH 6.5, and 9 mM EDTA; and incubated with 20  $\mu\text{g}$  of proteinase K and 10  $\mu\text{g}$  of RNase A for 65°C overnight. DNA was purified using a PCR purification kit (QIAGEN). Eluates were quantified by qPCR employing the  $\Delta C_t$  method relative to an amount equivalent to 1% of DNA used for immunoprecipitation. Standard deviations were calculated from triplicate measurements considering Gaussian error propagation. The following primers were used: *ANGPTL4*: PPRE, forward: CCT TAC TGG ATG GGA GGA AAG; reverse, CCC AGA GTG ACC AGG AAG AC.

**Time-Resolved Fluorescence Resonance Energy Transfer Assays in Vitro.** TR-FRET (Staflieni et al., 2007) was performed with the LanthaScreen TR-FRET PPAR $\beta/\delta$  competitive binding assay and the LanthaScreen TR-FRET PPAR $\alpha$ , PPAR $\beta/\delta$ , and PPAR $\gamma$  coactivator assays according to the instructions of the manufacturer (Invitrogen). 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 Life and Analytical Sciences, 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: PGC1 $\alpha$ , EAEEPSLLKLLLPANTQ; C33, HVEMHPLLMLLMESQWGA; CBP, AASKHKQLSELLRGGSGSS; PPAR $\gamma$ -interacting protein, VTLTSPLLVNLQSDISAG; TRAP220, NTKNHPMLMNLKDNPAQD.

**Quantification of HETEs in Cell Culture Supernatants by Liquid Chromatography/Tandem Mass Spectrometry.** Cell culture supernatants were spiked with  $\sim 1$  ng of deuterated internal standards and acidified with 20  $\mu\text{l}$  of saturated NH<sub>4</sub>Cl solution containing 1.25 M HCl. The analytes were extracted with 1 ml of diisopropyl ether and, after complete drying, resolved in acetonitrile/water [1:1 (v/v)]. For determination, a 10- $\mu\text{l}$  aliquot was injected into the LC/tandem mass spectrometry. The LC/MS analysis was carried out on a mass spectrometer (API3000; Applied Biosystems, Foster City, CA) equipped with two Series 200 LC Micro Pumps (PerkinElmer Life and Analytical Sciences, Waltham, MA), a CTC HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland), a turbo-ion interface and a HSID interface (Ionics Mass Spectrometer Group Inc., Bolton, ON, Canada). The flow rate was set to 200  $\mu\text{l}/\text{min}$ , and a C18 column (5 cm  $\times$  2 mm, 3- $\mu\text{m}$  particles) was used. A generic LC gradient of 10 min was used for sample separation. Solvents were water/acetonitrile (95:5) (A) and acetonitrile/water (95:5) (B), both containing 0.2% acetic acid. The gradient profile used was 40% solvent A for 0 to 2 min linearly increasing to 100% within 7 min. Mass spectrometer conditions were: scan type, multiple reaction monitoring with negative polarity; ion-spray voltage,  $-4200$  V; 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*<sub>8</sub>-5-HETE), 319.2/154.2 (8-HETE), 319.2/207.8 (12-HETE), 327.2/214.1 (*d*<sub>8</sub>-12-HETE), 319.2/218.9 (15-HETE), and 327.2/182.0 (*d*<sub>8</sub>-15-HETE). Dwell time was 300 ms each. All chemicals and solvents were obtained from Merck (Darmstadt, Germany).

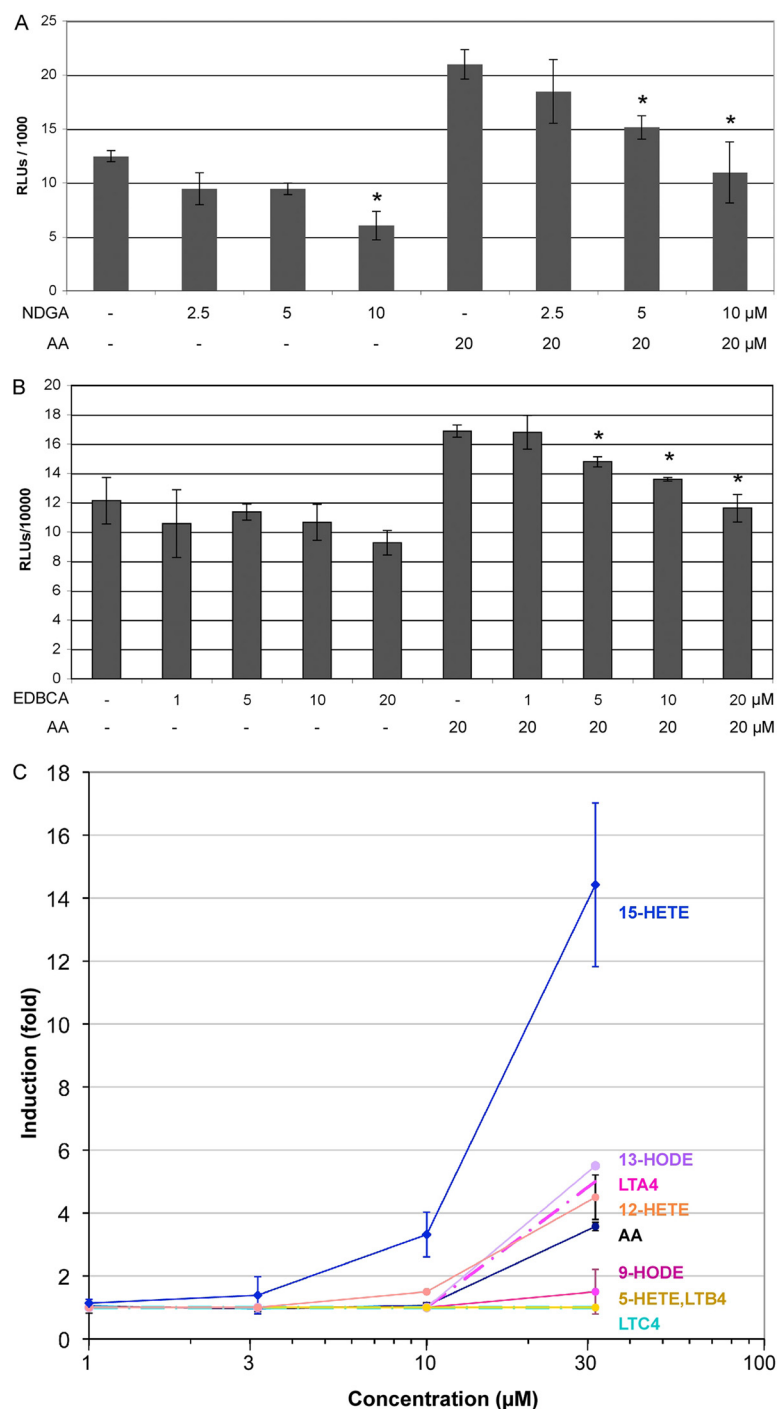
## Results

**Effect of Arachidonic Acid Derivatives on the Transcriptional Activity of PPAR $\beta/\delta$ .** We analyzed the effect of AA and its

metabolites on the transcriptional activity of PPAR $\beta/\delta$  in NIH3T3 fibroblasts using luciferase reporter constructs harboring multiple Gal4 or LexA binding sites upstream of a TATAi. The transcriptional activator in this system is a fusion construct consisting of the PPAR $\beta/\delta$  LBD and a Gal4 (Fig. 1A) or LexA (Fig. 1B) DNA binding domain. With both reporter systems, we observed an induction of approximately 2-fold by 20  $\mu$ M AA.

The observed induction by AA was not influenced by the COX inhibitor diclofenac, and no transcriptional activation was seen when specific prostaglandins (PGD<sub>2</sub>, PGE<sub>2</sub>, PGF<sub>2</sub>) were applied (data not shown). This confirms previous findings that AA-derived prostanoids have no detectable effect on the activity of PPAR $\beta/\delta$  in human embryonic kidney 293 and

NIH3T3 cells (Yu et al., 1995; Forman et al., 1996; Fauti et al., 2006). PGE<sub>2</sub> has been described as an activator of PPAR $\beta/\delta$ , but this is an indirect effect involving PGE<sub>2</sub>-mediated induction of PI<sub>3</sub>K-AKT signaling and may therefore be dependent on the cell type and experimental system (Wang et al., 2001). Because NIH3T3 cells synthesize 5-, 8-, 12-, and 15-HETE at readily detectable levels (up to ~60 ng/ml after 6-h culture, corresponding to ~0.2  $\mu$ M; 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 PPAR $\beta/\delta$  by the pan-LOX inhibitor NDGA (Fig. 1A). We also analyzed the 12-/15-LOX inhibitor EDBCA and observed a



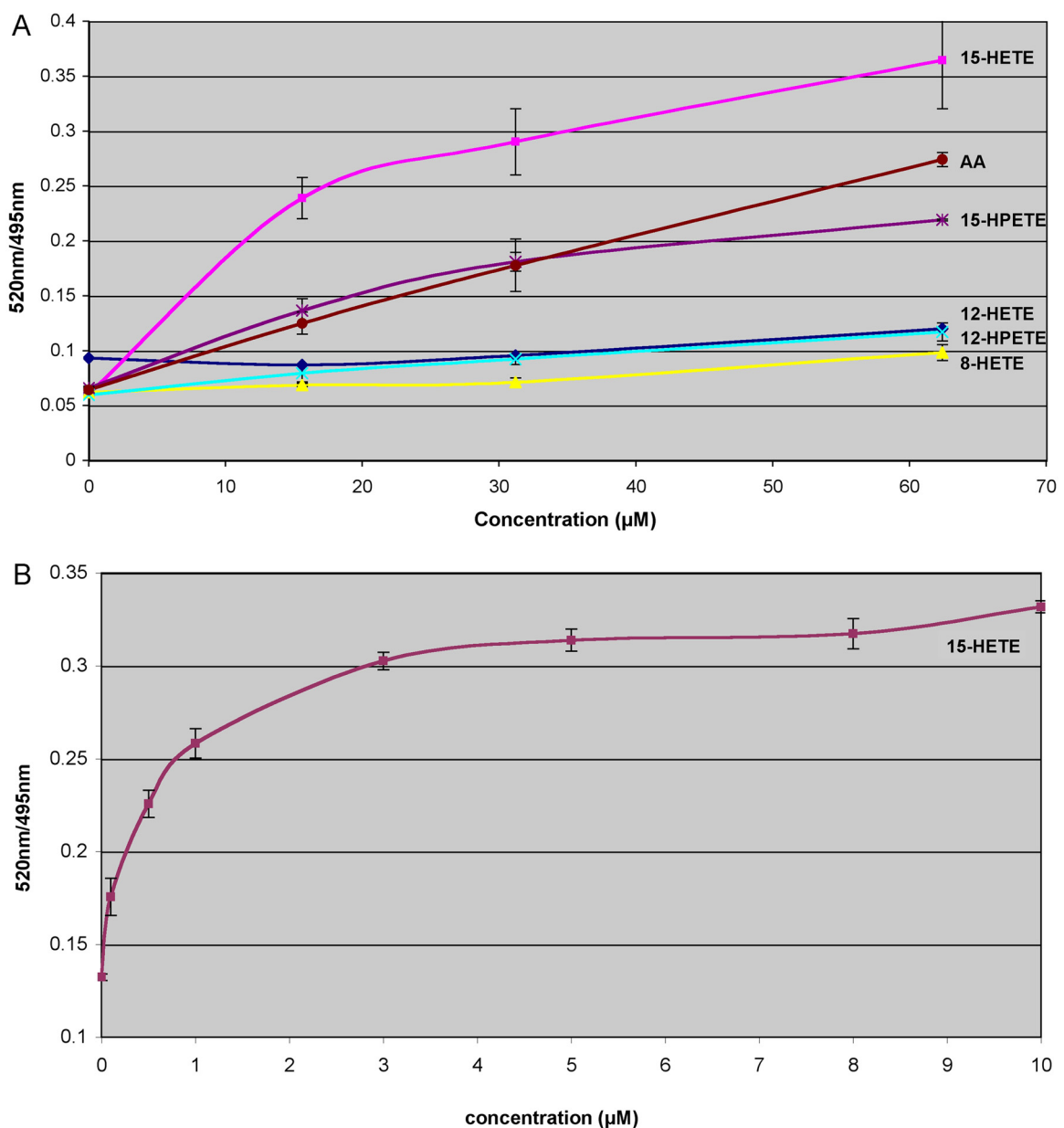
**Fig. 1.** Effects of lipoxygenase inhibitors and specific eicosanoids on the transcriptional activity of the PPAR $\beta/\delta$  ligand binding domain. NIH3T3 cells were transiently transfected with an expression vector encoding a Gal4-PPAR $\beta/\delta$  (A) or LexA-PPAR $\beta/\delta$  fusion protein (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 h and then for another 24 h with 20  $\mu$ M AA before harvesting. Control cells (-) received solvent only. C, CHO cells stably expressing a Gal4-PPAR $\beta/\delta$  fusion protein and harboring a stably integrated Gal4-responsive luciferase reporter gene were treated with the indicated compounds for 20 h. 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).

TABLE 1  
HETE synthesis by NIH3T3 fibroblasts  
Concentrations shown are those in the culture medium 6 h after addition of arachidonic acid.

HETE Synthesis	Concentration <i>ng/ml</i>
25 $\mu$ M AA	
5-HETE	8.5 $\pm$ 0.5
8-HETE	6.5 $\pm$ 0.5
12-HETE	4.4 $\pm$ 0.9
15-HETE	11.2 $\pm$ 1.3
100 $\mu$ M AA	
5-HETE	58.0 $\pm$ 1.0
8-HETE	25.5 $\pm$ 3.5
12-HETE	12.6 $\pm$ 0.3
15-HETE	62.8 $\pm$ 1.0

very similar effect compared with NDGA (Fig. 1B). These effects were specific, because no inhibition of transcription by NDGA or EDBCA was observed in the absence of LexA-PPAR $\beta/\delta$  (data not shown).

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

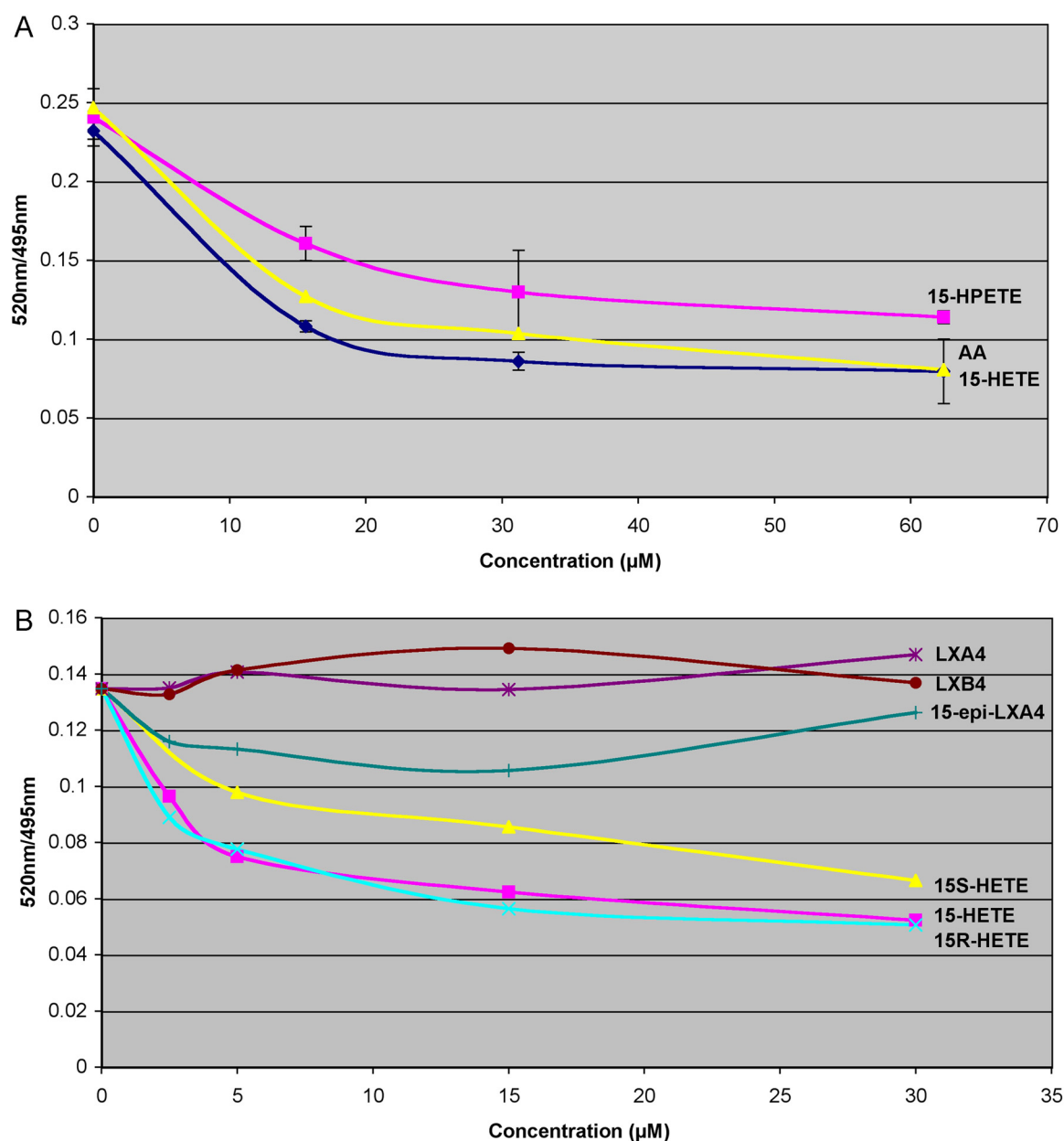


**Fig. 2.** Ligand-induced binding of a coactivator-derived peptide to the PPAR $\beta/\delta$  LBD in vitro. Interaction of fluorescein-labeled coactivator peptide and recombinant GST-Ppar $\beta$  bound by a terbium-labeled anti-GST antibody was determined by TR-FRET. A, the indicated metabolites or synthetic ligands were used at the indicated concentrations. 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 ( $\pm$  S.D.). In B, all FRET ratios obtained for concentrations  $\geq 0.1$   $\mu$ M are statistically significant (Bonferroni-Holm adjusted *t* test).

**15-HETE Induces Coactivator Peptide Binding to PPAR $\beta/\delta$ .** 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 PPAR $\beta/\delta$ . To test this hypothesis we investigated the effect of different LOX products on PPAR $\beta/\delta$  coactivator interaction in vitro by TR-FRET. In this assay, the interaction of the PPAR $\beta/\delta$  LBD (indirectly labeled by terbium) with the coactivator peptide C33 (labeled with fluorescein) is determined (Stafslien et al., 2007). C33 was previously identified as a peptide strongly interacting with coactivator binding sites of PPARs in response to agonist binding, similar to PGC1 $\alpha$ - or steroid receptor coactivator-derived peptides (Chang et al., 1999). This assay measures the intensity of terbium-induced fluores-

cence emission of the fluorescein moiety of the C33 peptide, expressed as the ratio of fluorescein- and terbium-derived fluorescence.

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 PPAR $\beta/\delta$  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 PPAR $\beta/\delta$ -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 analy-



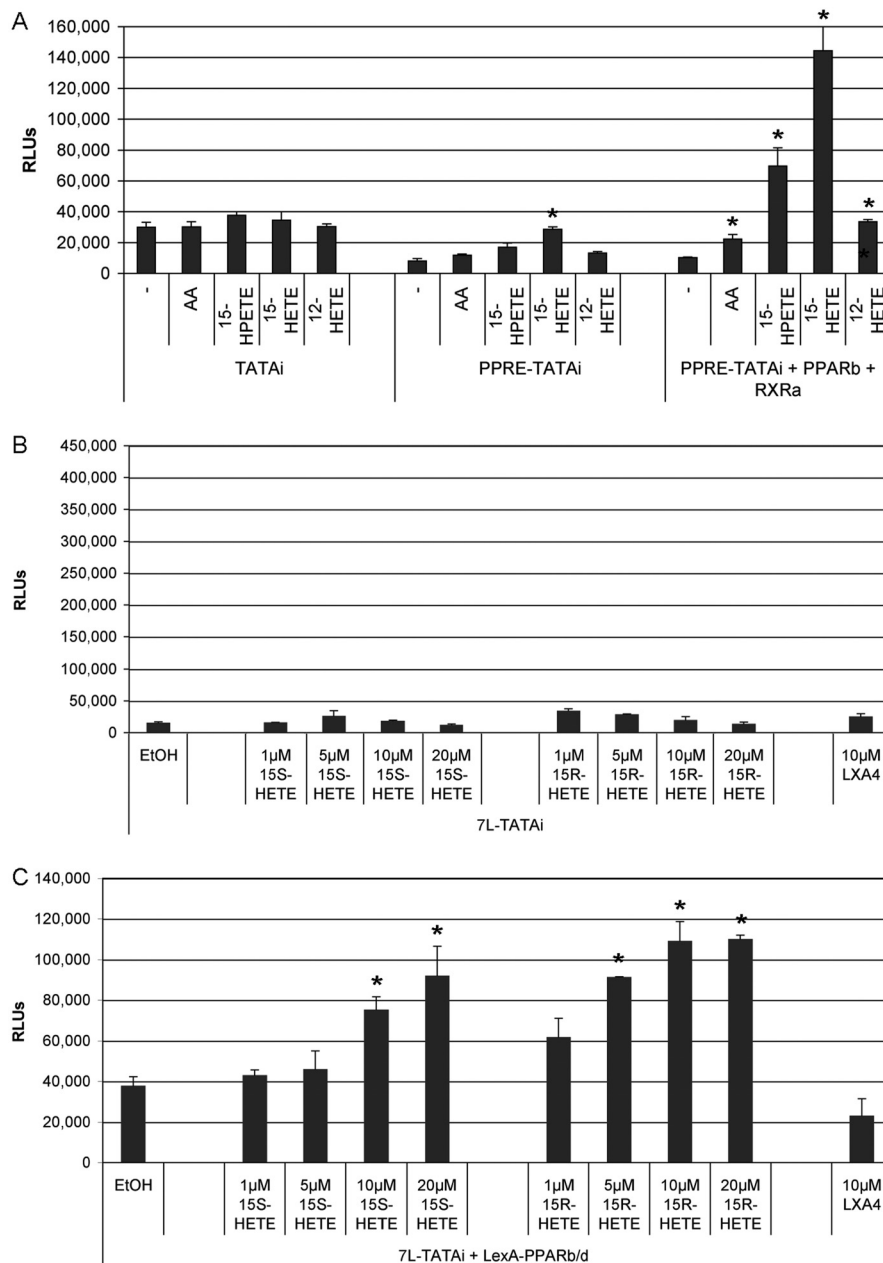
**Fig. 3.** Competitive in vitro ligand binding assay for PPAR $\beta/\delta$ . Displacement of the Fluormone Pan-PPAR Green PPAR ligand from recombinant GST-Pparb was determined by TR-FRET. AA and the indicated metabolites were used at the concentrations shown in the graph. A, binding of 15-HETE and 15-HPETE compared with 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 excited by terbium emission) and 495 nm (terbium emission). All data points represent averages of triplicates ( $\pm$  S.D.).

sis of lower concentrations of 15-HETE showed a statistically significant effect already at 0.1  $\mu\text{M}$  and a half-maximal signal at approximately 0.55  $\mu\text{M}$  (Fig. 2B).

**15-HETE Competes for PPAR $\beta/\delta$  LBD Binding.** We next analyzed the interaction of AA, 15-HETE, and 15-HPETE with the LBD of PPAR $\beta/\delta$  in a TR-FRET-based competitive ligand binding assay. In this assay, the terbium-labeled PPAR $\beta/\delta$  LBD interacts with Fluormone Pan-PPAR Green (Invitrogen) 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 PPAR $\beta/\delta$  bound ligand.

**15-HETE Induces PPAR $\beta/\delta$ -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 PPAR $\alpha$  and PPAR $\gamma$  (data not shown), which, because of their partially redundant function, could obliterate PPAR $\beta/\delta$ -mediated effects. The data in Fig. 4A show that the basal level activity of the TATAi vector (left) was reduced by the presence of a PPRE (center), most likely as a result of the recruitment of PPAR $\beta/\delta$  repressor complexes (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- and 3.7-fold, respectively). In contrast, no significant induction was observed with AA or 12-



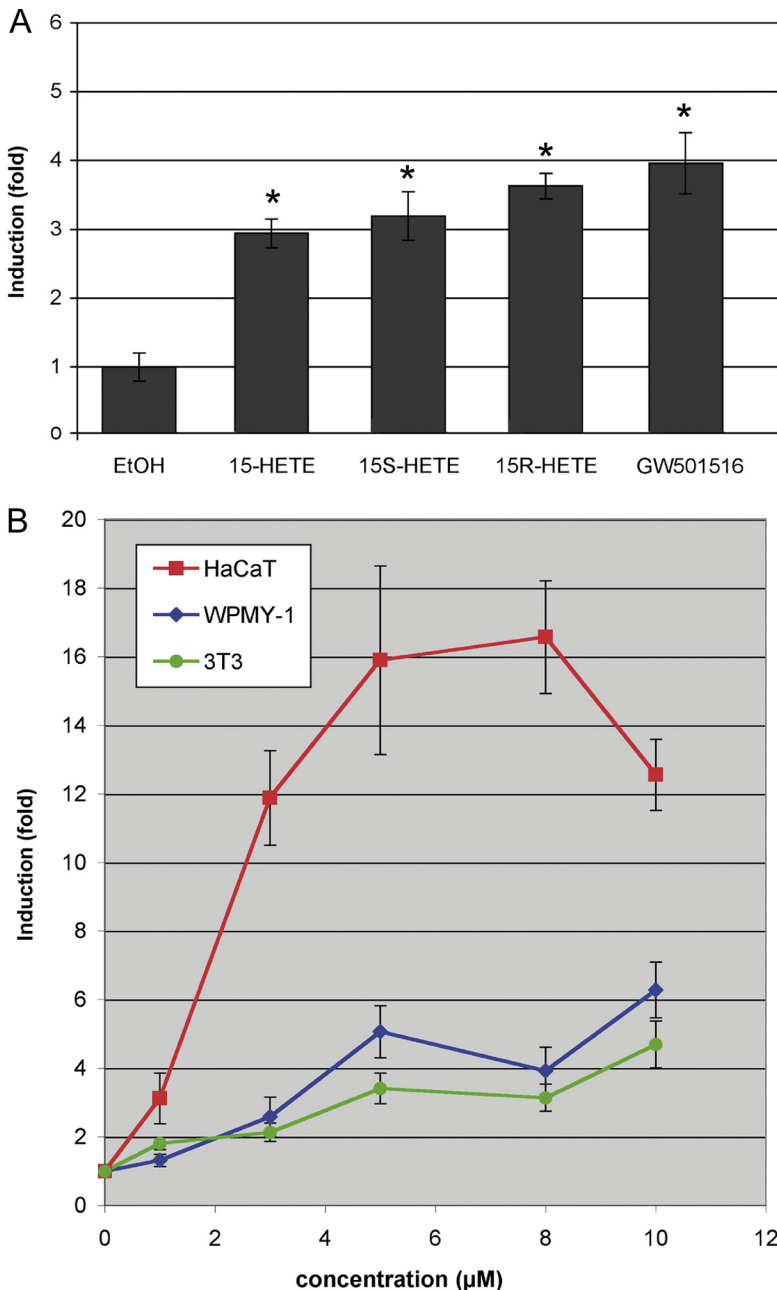
**Fig. 4.** Effects of different HPETEs and HETEs on the transcriptional activity of PPAR $\beta/\delta$ . A, NIH3T3 cells were transiently transfected with a PPRE-lacking control plasmid (left bars) or a PPRE-driven luciferase reporter plasmid (middle and right bars) together with either empty vector (left bars and middle) or expression vectors encoding PPAR $\beta/\delta$  and RXR $\alpha$  (right bars). The concentration of 12-HETE, 15-HETE, and 15-HPETE was 10  $\mu\text{M}$ , AA was 20  $\mu\text{M}$ . B and C, analysis of 15-HETE enantiomers and their metabolites in the LexA-based system described in Fig. 1. In B, the empty vector pCDNA3.1 was cotransfected instead of the LexA-PPAR $\beta$  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;  $P < 0.001$ ).

HETE. Cotransfection of PPAR $\beta/\delta$  and RXR $\alpha$  expression vectors (Fig. 4A, right) clearly enhanced the effect. 15-HETE and 15-HPETE strongly induced PPRE-dependent transcription (21- and 11-fold, respectively), whereas the induction by both AA and 12-HETE was comparably weak (2- to 3-fold) albeit 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 PPAR $\beta/\delta$  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 5  $\mu$ M, compared with 5.1-fold in WPMY-1 cells and 3.4-fold in NIH3T3 cells. It is noteworthy

that a clear induction of *Angptl4* expression was seen already at 1  $\mu$ M 15-HETE (3.1-fold in HaCaT cells; 1.8-fold in NIH3T3 cells). Maximum induction by 15-HETE was comparable with that seen with the synthetic agonist GW501516 at 1  $\mu$ M (Fig. 5A). Because NIH3T3 cells lack other PPAR subtypes, these observations strongly suggest that the observed induction by 15-HETE is mediated by PPAR $\beta/\delta$ . Neither GW501516 nor 15-HETE altered *Pparb/d* expression levels (data not shown), indicating that the observed induction of *Angptl4* expression by these compounds is due to an increased PPAR $\beta/\delta$  activity.

In agreement with these observations, chromatin immunoprecipitation analyses of human WPMY-1 cells showed recruitment of the coactivator CBP to the PPAR $\beta/\delta$ -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

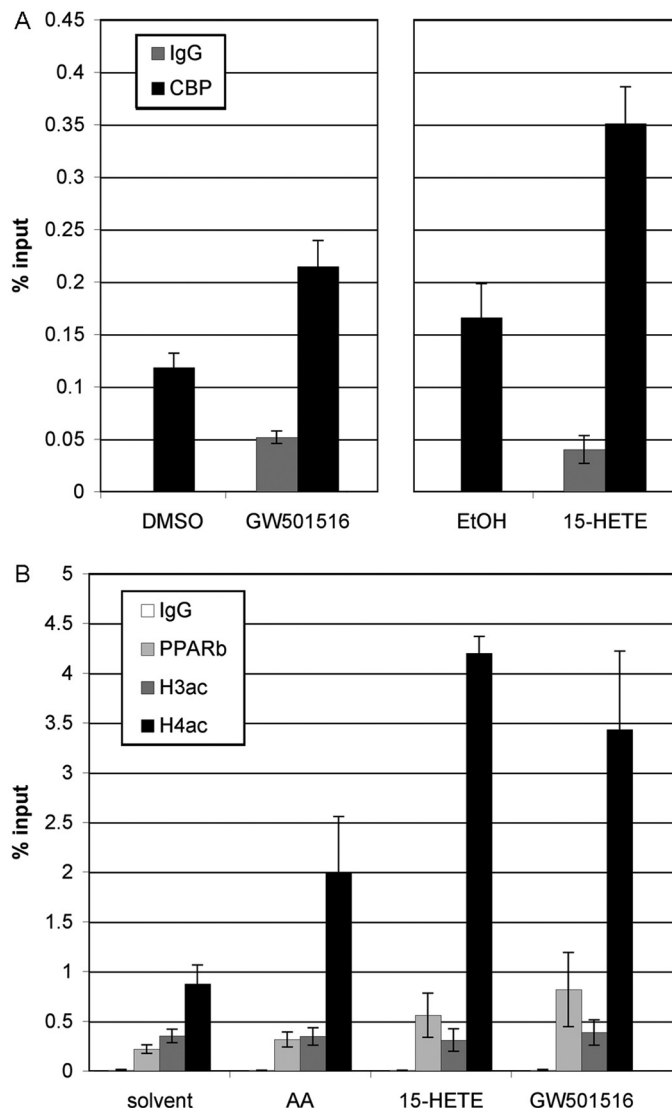


**Fig. 5.** Inducibility of the PPAR $\beta/\delta$  target gene *Angptl4*. A, regulation of *Angptl4* by 15-HETE and its enantiomers 15S-HETE and 15R-HETE in comparison to GW501516 (0.3  $\mu$ M) in NIH3T3 cells. B, regulation of *Angptl4* by 15-HETE at different concentrations in NIH3T3, HaCaT, and WPMY-1 cells. Cells were treated for 3 h, RNA was isolated and analyzed by qPCR using *L27* for normalization. Induction values were calculated relative to solvent (EtOH)-treated cells and represent averages of triplicates ( $\pm$  S.D) normalized. \*, values significantly different ( $P < 0.001$ ) between solvent (EtOH) and ligand treated cells.



H3, at both the PPRE (Fig. 6B) and the TSS (data not shown) with either compound. Either GW501516 or 15-HETE also led to an increased binding of PPAR $\beta/\delta$  to the *ANGPTL4* PPRE, which is consistent with previous observations made with synthetic ligands (Mandard et al., 2004). In contrast, specific binding of CBP, PPAR $\beta/\delta$ , or acetylated H4 to an irrelevant *ANGPTL4* upstream region at  $-12$  kilobase pairs relative to the transcription start site was not detectable (data not shown). These similarities in chromatin alterations triggered by both compounds provide further evidence that 15-HETE is an agonistic PPAR $\beta/\delta$  ligand.

**Induction of the PPAR $\beta/\delta$  Target Gene *Angptl4* by 15-HETE Is Dependent on PPAR $\beta/\delta$ .** To obtain unequiv-



**Fig. 6.** Altered transcription factor binding and histone acetylation on the *ANGPTL4* promoter after treatment with 15-HETE of WPMY-1 myofibroblasts. Cells were treated with 15-HETE (10  $\mu$ M), GW501516 (0.3  $\mu$ M) or solvent [ethanol for 15-HETE, dimethyl sulfoxide (DMSO) for GW501516] for 1 h (A) or 3 h (B). Chromatin immunoprecipitation was carried out using antibodies against CBP (A) or PPAR $\beta/\delta$  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 percentage input and represent averages of triplicates ( $\pm$  S.D.).

ocal evidence that *Angptl4* induction by 15-HETE is indeed mediated by PPAR $\beta/\delta$ , 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 PPAR $\beta/\delta$  expression. For this experiment, fetal lung fibroblasts from *Pparb/d*-null mice were infected with a retrovirus expressing FLAG-tagged PPAR $\beta/\delta$  or a control retrovirus (pLPCX). The data in Fig. 7 show a clear PPAR $\beta/\delta$ -dependent induction of the *Angptl4* gene by 0.3  $\mu$ M GW501516 (6.9-fold), 10  $\mu$ M 15-HPETE (5.6-fold), and 10  $\mu$ M 15-HETE (10.7-fold), respectively, but only a marginal effect (1.5-fold) by 20  $\mu$ M 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 PPAR $\alpha$  ligand GW1929 and the PPAR $\gamma$  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- and 17-fold, respectively. In the Cre virus-infected cells, these values were reduced to 2.0- and 1.9-fold, respectively (Fig. 7D).

Finally, we analyzed the effect of 15-HETE on the *Angptl4* gene after siRNA-mediated knockdown of PPAR $\beta/\delta$  expression in NIH3T3 cells. Figure 8A shows a 67.4% reduction of *Pparb/d* mRNA in *Pparb/d* siRNA-treated cells compared with cells exposed to control siRNA. PPAR $\beta/\delta$  knockdown led to an increased activity of the *Angptl4* gene (Fig. 8A, left), presumably because of reduced PPAR $\beta/\delta$  repressor complex recruitment (see also Fig. 4A). Treatment with GW501516, 15-HPETE, or 15-HETE led to the expected *Angptl4* induction in the control siRNA-treated cells (4.0-, 1.9-, and 3.0-fold, respectively; Fig. 8B), which was reduced by approximately 50% upon exposure to *Pparb/d* siRNA, thus confirming PPAR $\beta/\delta$  as the target of GW501516, 15-HPETE, and 15-HETE in the regulation of the *Angptl4* gene.

#### Both Enantiomers of 15-HETE Activate PPAR $\beta/\delta$ .

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 PPAR $\beta/\delta$ .

We first addressed this question using the TR-FRET-based competitive ligand binding assay. The data in Fig. 3B clearly show that both 15-HETE enantiomers were able to compete for binding to PPAR $\beta/\delta$  to an extent very similar to that of the enantiomer mixture [15-HETE; also referred to as ( $\pm$ )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 PPAR $\beta/\delta$  in the LexA-based luciferase assay. Consistent with the data obtained by TR-FRET, both enantiomers induced PPAR $\beta/\delta$  activity to a similar extent (2.5- to 3-fold), whereas the lipoxin LXA4 showed no effect (Fig. 4, B and C). Finally, we tested the effect of 15S-HETE and 15R-HETE on the induction of the PPAR $\beta/\delta$  target gene *Angptl4* in NIH3T3 fibroblasts. Both enantiomers induced *Angptl4* expression to an extent similar to that of 15-HETE (Fig. 5A). Taken together, these observations strongly suggest that both 15S-HETE and 15R-HETE are agonistic ligands of PPAR $\beta/\delta$ .

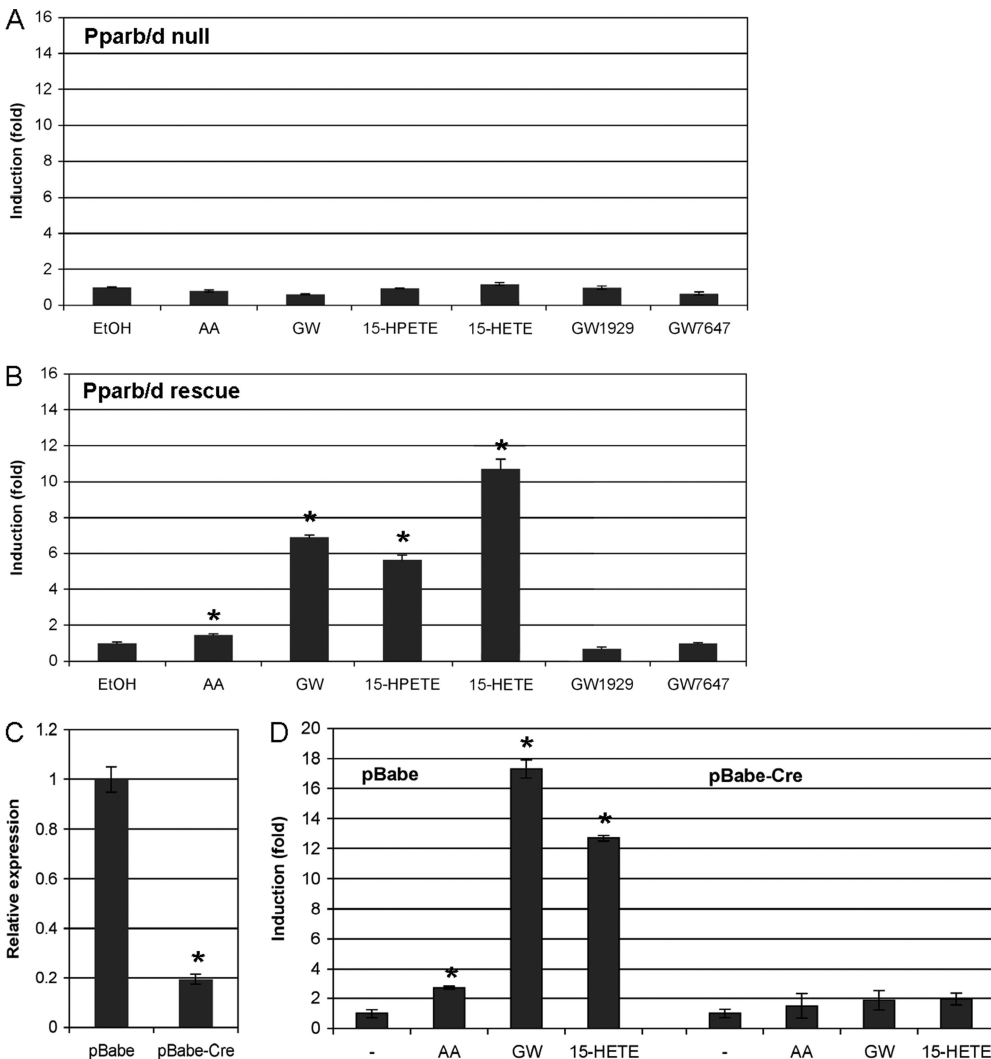
**Effect of 15-HETE on Different PPAR Subtypes.** Several previous studies have reported an activation of PPAR $\gamma$  by 15-HETE (Nagy et al., 1998; Huang et al., 1999; Schild et al., 2002; Chen et al., 2003). It was therefore of interest to compare the effects of 15-HETE on PPAR $\gamma$  and PPAR $\beta/\delta$  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 PPAR $\gamma$  and PPAR $\beta/\delta$  LBDs fused to the LexA DNA binding domain. As shown in Fig. 9, 15-HETE significantly activated all PPAR subtypes, but the induction of PPAR $\beta/\delta$  was stronger (~11-fold) com-

pared with the activation of PPAR $\gamma$  (~3-fold) and PPAR $\alpha$  (~2-fold). Consistent with this finding, in vitro recruitment of the thyroid hormone receptor-associated protein coactivator peptide to PPAR $\alpha$  and PPAR $\gamma$  was detectable but weaker than the interaction with PPAR $\beta/\delta$  (Fig. 10A). However, a precise quantitative comparison in this assay is difficult, because the exact concentrations of functional recombinant PPARs and their occupation by lipid ligands cannot be determined and may thus differ among the three subtypes.

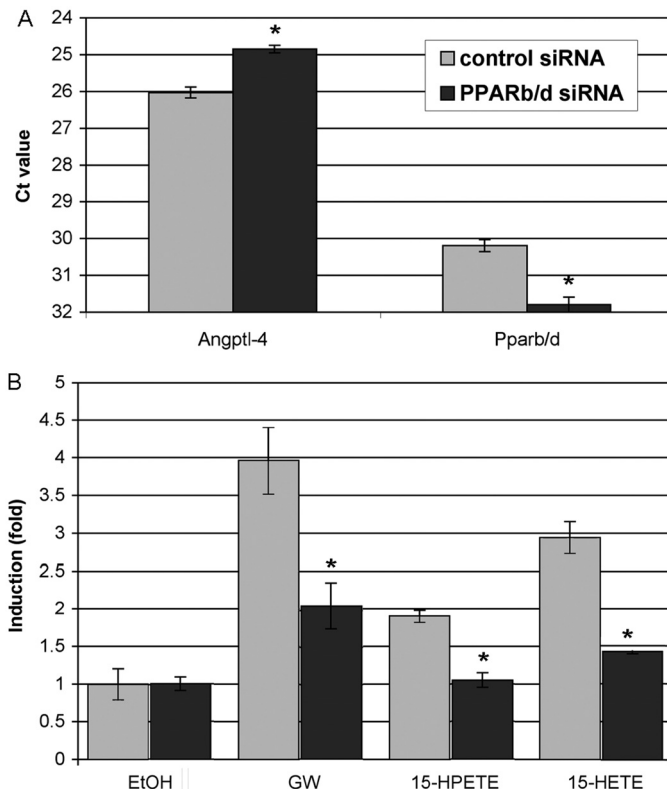
We also analyzed the 15-HETE-triggered interaction of PPAR $\beta/\delta$  and PPAR $\gamma$  with C33 and three other coactivator peptides (PGC1 $\alpha$ , CBP, PPAR $\gamma$ -interacting protein). Again, the 15-HETE induced interaction with PPAR $\gamma$  was much weaker compared with the recruitment of all coactivator peptides to PPAR $\beta/\delta$  (Fig. 10, B and C). As expected, the synthetic ligands GW1929 and GW501516 included as positive controls strongly induced the recruitment of all four peptides to PPAR $\gamma$  and PPAR $\beta/\delta$ , respectively (Fig. 10, B and C).

## Discussion

Several synthetic drugs with strong agonistic properties and high subtype selectivity have been described for PPAR $\beta/\delta$  (Peraza et al., 2006), but its regulation by natural ligands re-



**Fig. 7.** Expression of the PPAR $\beta/\delta$  target gene *Angptl4* in fibroblasts lacking PPAR $\beta/\delta$  (A), in the same cells with restored PPAR $\beta/\delta$  expression (B) and after the retrovirus-mediated deletion of floxed *Pparb/d* alleles (C and 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 PPAR $\beta/\delta$  (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 (20  $\mu$ M), 15-HPETE (10  $\mu$ M), 15-HETE (10  $\mu$ M), GW501516 (GW; 0.3  $\mu$ M), the selective PPAR $\gamma$  ligand GW1929 (0.3  $\mu$ M), or the selective PPAR $\alpha$  ligand GW7647 (0.3  $\mu$ M) for 6 h and analyzed for expression of *Angptl4* (A, B, and D) and *Pparb/d* (C) by qPCR. Induction values were calculated relative to solvent-treated cells and represent averages of triplicates ( $\pm$  S.D) normalized. \*, values significantly different ( $P < 0.001$ ) from vector control (A) or pBabe (C), or between solvent (EtOH) and ligand-treated cells (D).



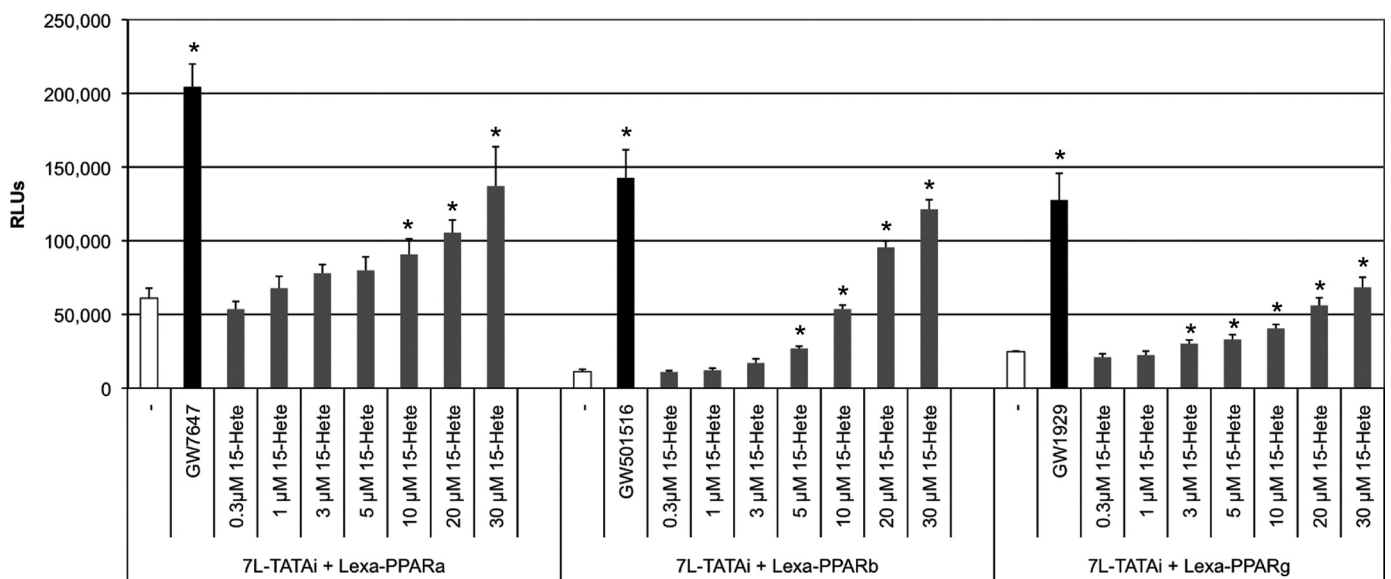
**Fig. 8.** Effect of siRNA-mediated PPAR $\beta/\delta$  knockdown on *Angptl4* inducibility in NIH3T3 cells. **A**, efficacy of siRNA treatment. Cells exposed to *Pparb/d*-specific siRNA show a 67% reduction of *Pparb/d* expression and an increased basal *Angptl4* mRNA expression (presumably due to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

mains unclear. The description of PGI<sub>2</sub> (prostacyclin) (Gupta et al., 2000; Hatae et al., 2001) and all-*trans* retinoic acid (Schug et al., 2007) as PPAR $\beta/\delta$  agonists are in conflict with data published by us (Fauti et al., 2006; Rieck et al., 2008) and by others (Yu et al., 1995; Forman et al., 1996; Borland et al., 2008). Unsaturated fatty acids have been reported to interact with the PPAR $\beta/\delta$  LBD (Forman et al., 1996; Xu et al., 1999), but their effect on the transcriptional activity of PPAR $\beta/\delta$  is weak (Rieck et al., 2008). Among the fatty acids with a clearly activating effect on PPAR $\beta/\delta$  is AA, but it is unknown whether this results from a direct interaction with the PPAR $\beta/\delta$  LBD or from 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 PPAR $\beta/\delta$ .

Using fusion constructs of the PPAR $\beta/\delta$  LBD and heterologous DNA binding domains (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 PPAR $\beta/\delta$  by AA. These data demonstrate that the agonistic effect of AA is not due merely to its direct interaction with the PPAR $\beta/\delta$  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 PPAR $\beta/\delta$  with coactivators peptides in vitro (Fig. 2). This experiment identified 15-HETE as the only metabolite tested that was clearly superior to nonmetabolized 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 PPAR $\beta/\delta$  LBD but rather to a specific conformational change induced by 15-HETE.

The agonistic effect of 15-HETE was selective for PPAR $\beta/\delta$ , because 1) the interaction of PPAR $\beta/\delta$  with the direct precursor

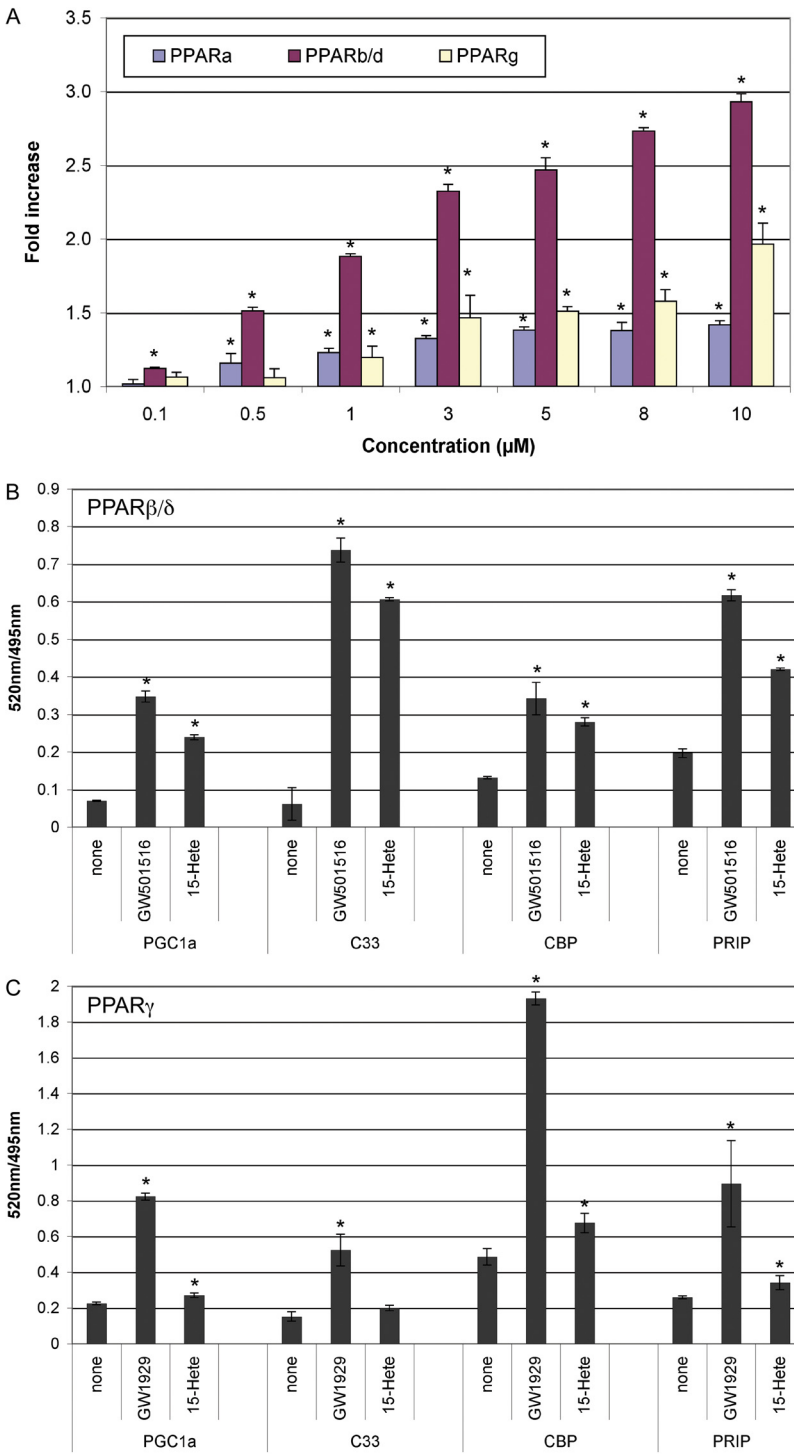


**Fig. 9.** PPAR subtype-selective transcriptional activation by 15-HETE. NIH3T3 were transiently transfected with an expression vector encoding a LexA-PPAR $\alpha$ , LexA-PPAR $\beta/\delta$ , or LexA-PPAR $\gamma$  fusion protein together with a LexA-responsive luciferase reporter plasmid (7L-TATAi). Cells were incubated with 15-HETE at the indicated concentrations or with synthetic ligands (1  $\mu$ M) for 24 h before 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).

sor of 15-HETE, the 12/15-LOX product 15-HPETE, was considerably weaker, 2) HETE-derived lipoxins LXA4 and LXB4 showed a weaker binding to recombinant PPAR $\beta/\delta$  in vitro than 15-HETE itself (Fig. 3), and 3) the 15-HETE-triggered activation of a LexA-PPAR $\beta/\delta$  and the 15-HETE-induced coactivator peptide recruitment to PPAR $\alpha$  and PPAR $\gamma$  (Figs. 9 and 10). This subtype selectivity is in apparent contrast to published data suggesting that 15-HETE activates the transcriptional activity of PPAR $\gamma$  (Nagy et al., 1998; Huang et al., 1999; Schild et al., 2002; Chen et al., 2003), which was also

seen in our study, albeit to a lesser extent compared with PPAR $\beta/\delta$  (Fig. 9). In contrast to our work, the studies quoted above did not show a direct interaction of 15-HETE and PPAR $\gamma$ . It can therefore not be excluded that the described effects of 15-HETE on PPAR $\gamma$  are, at least in part, indirect. In addition, the effect of 15-HETE on PPAR $\beta/\delta$  was not assessed in the published studies so that a direct comparison with our data are not possible.

We next performed a series of experiments confirming 15-HETE as an efficient inducer of endogenous PPAR $\beta/\delta$  target genes using the *Angptl4* gene as a verified model



**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 PPAR $\alpha$ , PPAR $\beta/\delta$ , and PPAR $\gamma$  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 after 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 PPAR $\beta/\delta$  and PPAR $\gamma$  LBD. GW501516 and GW1929 were used at a concentration of 0.3  $\mu$ M and 15-HETE at 31.2  $\mu$ M. Results are expressed as the ratio of fluorescence intensity at 520/495 nm (as in Fig. 2). All data points represent averages of triplicates ( $\pm$  S.D). Significant differences between solvent and ligand treated cells are indicated by an asterisk (paired *t* test; *P* < 0.05).

(Mandard et al., 2004). At a concentration of 3  $\mu\text{M}$ , 15-HETE induced the *Angptl4* gene to an extent similar to that of the synthetic agonist GW501516 at 0.3  $\mu\text{M}$ , a concentration leading to maximal induction by this compound (Fig. 5). Consistent with a function as a PPAR agonist 15-HETE triggered the recruitment of PPAR $\beta/\delta$  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 transcription start site of the gene (Fig. 6B). These alterations were basically indistinguishable from those induced by the established PPAR $\beta/\delta$  ligand GW501516. Furthermore, we were able to demonstrate the PPAR $\beta/\delta$  dependence of the 15-HETE-mediated induction of the *Angptl4* gene by comparing PPAR $\beta/\delta$ -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 PPAR $\beta/\delta$ .

Another important question concerns the physiological relevance of the proposed 15-HETE/PPAR $\beta/\delta$  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, because 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 than NIH3T3 may synthesize higher amounts of 15-HETE. Moreover, we do not yet know in which physiological scenario the 15-HETE/PPAR $\beta/\delta$  signaling pathway plays a role: Is signaling paracrine? What would be the interacting cell types? Or does this pathway not involve the release of 15-HETE and instead 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 PPAR ligands. However, the sensitivity of the response of HaCaT cells to 15-HETE referred to above provides a strong argument to believe that the regulation of PPAR $\beta/\delta$  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 oxidation 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 PPAR $\beta/\delta$  (Fig. 3B) and to induce its transcriptional activity (Figs. 4, B and C, and 5A). These results clearly indicate that 15-HETE enantiomers generated by different pathways function as agonistic ligands for PPAR $\beta/\delta$ , which provides further evidence for a central role of PPAR $\beta/\delta$  in eicosanoid-regulated signaling. In this context, it is tempting to speculate that PPAR $\beta/\delta$  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 hypothetical connection between aspirin and PPAR $\beta/\delta$  through 15R-HETE will be addressed in future studies.

Even though their role in tumorigenesis is controversial, PPAR $\beta/\delta$  and synthetic PPAR $\beta/\delta$  agonists have been reported to inhibit cancer cell proliferation in multiple studies (for review, see Müller et al., 2008a,b; Peters and Gonzalez, 2009). It is noteworthy that 15-HETE has also been linked to cell proliferation, both in mitogenic and antimitogenic compounds (Moreno, 2009), but its 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 PPAR $\beta/\delta$ . In this context, it is also noteworthy that the agonistic effect of 15-HETE on PPAR $\beta/\delta$  was particularly high in keratinocytes (HaCaT cells; Fig. 5B), where a growth-inhibitory role for PPAR $\beta/\delta$  is well established (Michalik et al., 2001; Kim et al., 2006; Burdick et al., 2007; Borland et al., 2008; Girroir et al., 2008; Chong et al., 2009).

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