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Peroxisome proliferator-activated receptor δ is a specific sensor for teratogenic valproic acid derivatives ☆

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Abstract

The antiepileptic drug valproic acid (2-propylpentanoic acid) is a potent teratogen in both humans and mice. Valproic acid can induce differentiation of F9 teratocarcinoma cells and stimulate peroxisome proliferator-activated receptor (PPAR) activity. In this study, the structure–activity relationship between valproic acid, its teratogenic and non-teratogenic analogues (branched small- and medium chain fatty acids) and the three PPAR subtypes α, γ or δ was investigated. PPAR-α and PPAR-γ were activated by some valproic acid-derivatives; however, no correlation between teratogenicity and receptor activation could be observed. In contrast, only valproic acid and exclusively its teratogenic analogues were able to activate PPAR-δ in different cellular systems. However, valproic acid appears not to be a direct ligand of PPAR-δ, since in contrast to carbaprostacyclin (cPGI), valproic acid showed not to be able to induce complex formation of PPAR-δ–retinoid X receptor (RXR) heterodimers on DNA. In conclusion, in contrast to PPAR-α and PPAR-γ, PPAR-δ shows to be a specific sensor for teratogenic valproic acid-derivatives.

Keywords: PPAR (peroxisome proliferator-activated receptor); Valproic acid; Nuclear receptor; Fatty acid; Teratogenicity

1. Introduction

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily that are activated by micromolar concentrations of a variety of fatty acids, including the hypolipidemic fibrate class of drugs (Isseman and Green, 1990; Göttlicher et al., 1992; Krey et al., 1997). PPARs belong to those nuclear receptors that function as heterodimers with the retinoid X receptor (RXR) (Mangelsdorf and Evans, 1995), and contain a highly conserved DNA-binding domain (DBD) and a carboxy-terminal ligand-binding domain (LBD). These receptors regulate the expression of target genes by binding as heterodimers with retinoid X receptor to specific DNA sequences, which are referred to as PPAR response elements (PPREs) (Schoonjans et al., 1997). The three mammalian PPAR subtypes, designated PPAR-α (NR1C1), PPAR-γ (NR1C3) and PPAR-δ (NR1C2), have a distinct tissue expression profile: PPAR-α is the main subtype in the liver; in adipose tissue, PPAR-γ is the main subtype, and PPAR-δ is expressed in many tissues (Braissant et al., 1996). PPAR-α and PPAR-γ both play an important role in lipid and carbohydrate metabolism, whereas the physiological role of PPAR-δ is not clearly defined. A recent study found that non-steroidal anti-inflammatory drugs are able to inhibit tumorigenesis through the inhibition of PPAR-δ (He et al., 1999), which suggests that PPAR-δ has a function in the regulation of cellular growth. In addition, the identification of a PPA-Rδ-selective agonist allowed to link the action of PPAR-δ with reverse cholesterol transport (Oliver et al., 2001).

The antiepileptic drug valproic acid (VPA; for structure see Fig. 1) has recently been shown to activate PPAR-δ (Lampen et al., 1999; Werling et al., 2001). Valproic acid has proven to be particularly useful for the treatment of absence seizures, as well as partial and generalized tonic–clonic seizures and migraine prophylaxis (Sörensen, 1988; Cutrer et al., 1997). However, valproic acid was found to be teratogenic in human and mice (Robert, 1988; Nau et al., 1991; Narotski et al., 1994). In humans, the most striking malformations are neural tube defects (spina bifida aperta), whereas exencephaly can be induced by valproic

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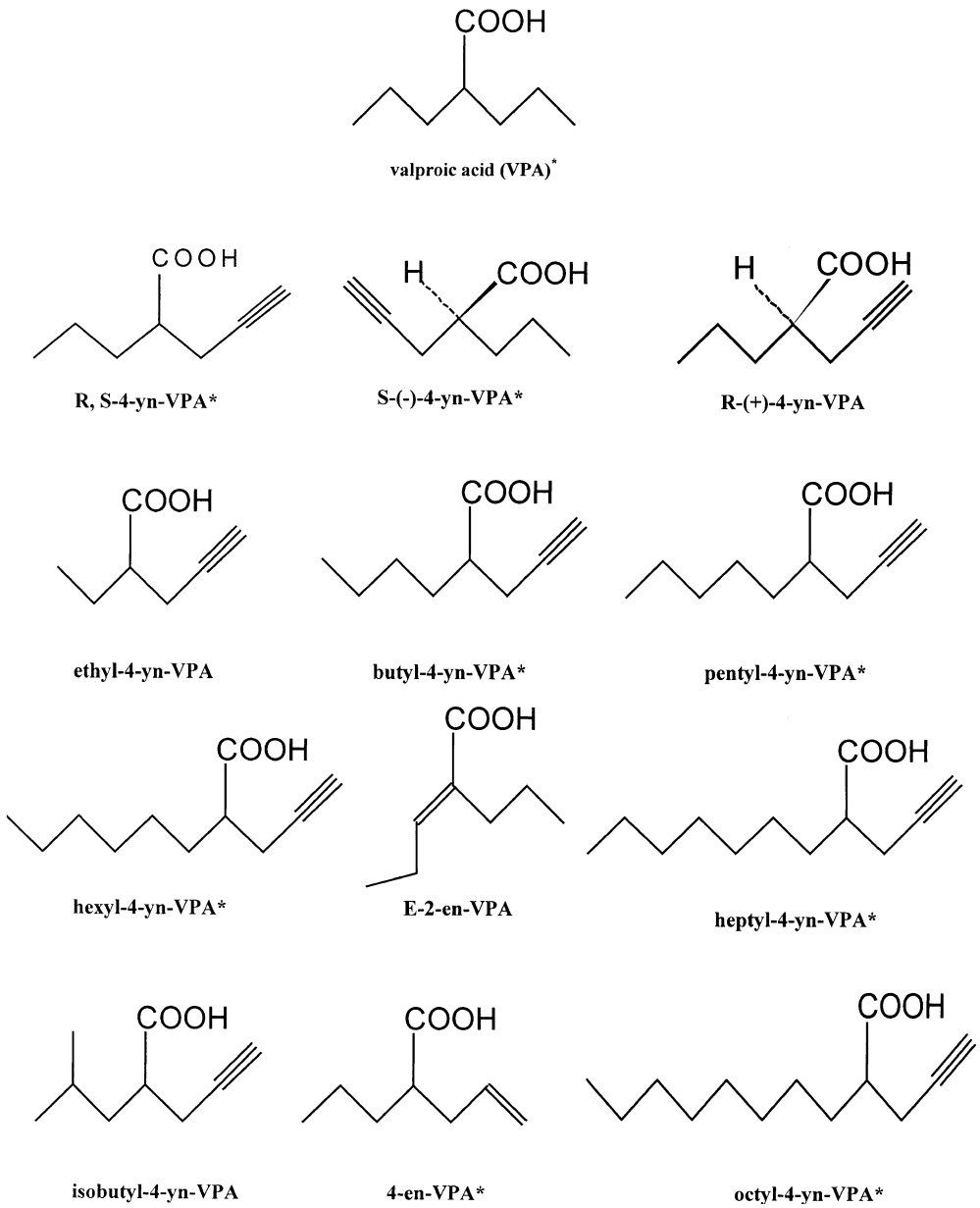

Fig. 1. Structure of valproic acid and valproic acid-derivatives. Teratogenic compounds are indicated by an asterisk.

acid administration to mice and is the main externally visible neural tube defect. Valproic acid-analogues (Fig. 1) with varying teratogenic activity have been synthesized and evaluated in vivo in mice (Nau et al., 1991; Ehlers et al., 1992) and in vitro in F9 mouse embryonic carcinoma cells (Lampen et al., 1999). Studies with these valproic acid-analogues indicate that the teratogenic potency depends strictly on the structure of the administered compound. In vitro teratogenic, but not non-teratogenic valproic acid-derivatives induce differentiation of F9 cells, which reflects events in the embryonal development (Hogan et al., 1983). The molecular mechanisms behind the teratogenic effects of valproic acid are not clear, but the activation of PPAR-δ by valproic acid and its teratogenic analogues suggests an involvement of the receptor in valproic acid teratogenicity.

In this study, the structure–activity relationship between valproic acid, its teratogenic and non-teratogenic analogues and the three PPAR subtypes α, γ or δ were investigated. Moreover, the direct binding of valproic acid to PPAR-δ–retinoid X receptor complexes was evaluated.

2. Materials and methods

2.1. Cell culture

Chinese Hamster Ovary (CHO) cells were cultured in Ham's F12 medium, and Human Embryo Kidney

(HEK293) cells were grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal calf serum.

2.2. Synthesis of valproic acid-derivatives

Valproic acid was obtained from Sigma (Deisenhofen, Germany). *E*-2-en-valproic acid was from Desitin (Hamburg, Germany) or synthesized by U. Gravemann (Tierärztliche Hochschule, Hannover). The other valproic acid-derivatives and the pure enantiomers *R*- and *S*-4-yn-valproic acid were synthesized as previously described (Hauck and Nau, 1992; Bojic et al., 1996, 1998).

2.3. Hybrid receptor activation assay

In the mouse mammary tumor virus promoter driven expression constructs of the chimeric receptors GR/PPAR-α, GR/PPAR-γ and GR/PPAR-δ, the amino-terminal domain and the DNA-binding domain (DBD) of the glucocorticoid receptor (GR) were fused to the ligand-binding domain (LBD) of mouse PPAR-α, -γ or -δ, respectively. The control glucocorticoid receptor (GR)/- receptor contained only the amino-terminal domain and the DNA-binding domain (Göttlicher et al., 1992; Werling et al., 2001). A glucocorticoid receptor response element-driven alkaline phosphatase (AP) gene was used as a reporter. Expression and reporter constructs were stably transfected into CHO cells as described previously by Göttlicher et al. (1992).

2.4. Alkaline phosphatase reporter gene assay

Stable transfected CHO cells (GR/PPAR-α, GR/PPAR-γ, GR/PPAR-δ and GR/-) were seeded on 24-well plates at a density of 20,000 cells per culture well (Greiner, Nürtingen, Germany) in 1 ml of medium. In transactivation experiments, the medium was replaced with new medium containing the test compounds. After 48 h of incubation, cell culture supernatants were heated to 65 °C for 30 min and alkaline phosphatase activity was determined as the increase of absorption (A_{405}) at 30 °C in a 1 ml reaction mixture containing 0.75 ml of supernatant, 200 mM Tris (pH 8.8), 275 mM NaCl, 0.5 mM $MgCl_2$, and 5 mM *p*-nitrophenyl phosphate (final concentrations). Treatment with WY14643 (4-chloro-6-(2,3-xylidino)-2-pyrinidinyl[thio]acetic acid), bromopalmitate and indomethacin served as positive controls for the activation of PPAR-α, -δ, or -γ, respectively (Kliewer et al., 1997).

2.5. Subcutaneous pentylenetetrazole (PTZ) seizure threshold test

Anticonvulsant activity was measured by the subcutaneous pentylenetetrazole seizure threshold test. A dose of the sodium salt of the acid of 0.5, 1.0 or 1.5 mmol/kg of body weight was applied initially. The test has been previously described in detail by Elmazar et al. (1993). However, in brief, the compounds were injected intraperitoneally as the sodium salt in groups of 5–8 animals. After 15 min, pentylenetetrazole at 65 mg/kg (0.65% solution in saline) was applied in a loose fold of skin on the back of the neck, the animals were then observed for 30 min. The number of animals protected, those showing an absence of a single 5-s episode of clonic spasms; threshold seizure was recorded and compared with the control (dosed only with pentylenetetrazole) and valproic acid-treated mice.

2.6. Luciferase reporter gene assay

SV-40-driven expression vectors for PPAR-δ and retinoid X receptor (RXR)-α, a synthetic PPAR response element response element (PPRE)-driven luciferase reporter construct (He et al., 1999) and β-galactosidase reference plasmid (pCMV-β-galactosidase vector for normalization) were transiently transfected into HEK293 cells, which were stimulated after transfection with the indicated ligands. Twenty-four hours after transfection, cells were lysed and luciferase and β-galactosidase activities were determined.

2.7. Gel shift and gel shift clipping assays

The full-length proteins of PPAR-δ and retinoid X receptor-α were generated by in vitro transcription-coupled translation of their respective cDNAs using the TNT system (Promega, Mannheim, Germany). A PPAR response element response element probe was formed by annealing the oligonucleotides 5′-ATTTCTCAAATA-T**AGGCCA**T**AGGTCA**TCTAGACCC-3′ and 5′-GG-GTCTAGA-3′ and labelled using ^{32}P-dCTP and Klenow-Polymerase (Roche, Mannheim, Germany). Gel shift and gel shift clipping assay were performed as described previously (Quack and Carlberg, 2000). Equal amounts of in vitro translated PPAR-δ and retinoid X receptor proteins were mixed and incubated in the presence of the indicated concentration of valproic acid, carbaprostacyclin (cPGI) (or demethylsulfoxid [DMSO] as control) for 15 min at room temperature in a total volume of 20 μl binding buffer (10 mM Hepes/HCl, pH 7.9, 1 mM dithiothreitol, 0.2 μg/μl poly(d(I–C)), 5% glycerin). The buffer was adjusted to a final concentration of 150 mM monovalent ions. Approximately 1 ng of labelled PPAR response element response element (50,000 cpm) was added to the receptor/ligand mixture and the incubation was continued for 20 min. In gel shift clipping assays, trypsin was added to a final concentration of 33 ng/μl and mixture was incubated for a further 15 min at room temperature. In both cases, protein–DNA complexes were resolved on 8% non-denaturing polyacrylamide gels in 0.5 × TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3). Gels were dried and exposed to a Fuji MP2040S imager screen

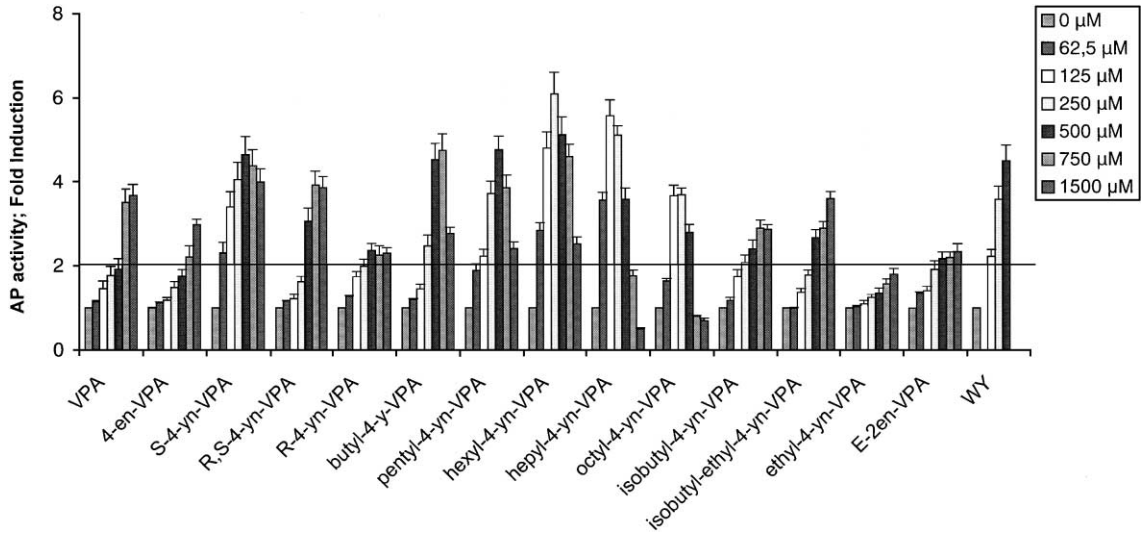

Fig. 2. Activation of the GR/PPAR-α hybrid receptor by valproic acid or valproic acid-derivatives. Relative alkaline phosphatase activity (AP) in culture supernatants was measured by increase in A_{405} per hour per culture. CHO cells containing stably expressed GR/PPAR-α were seeded at a density of 20,000 cells per culture well and grown for 24 h. Cells were grown for an additional 48 h in the presence of test compounds prior to assaying the culture supernatants for alkaline phosphatase activity. Values represent means from four determinations. Line indicates two-fold effect concentration ($EC_{2\times}$).

overnight. The ratio of free probe to protein-probe complexes was quantified on a Fuji FLA2000 reader using Image Gauge software. Each condition was analyzed, at least, in triplicate.

2.8. Statistics

Values for concentrations and concentration ratios were expressed as means ± standard deviations (SD). Test of significances of differences between mean values were made using the SigmaStat™ analysis of variance (ANOVA) procedure.

3. Results

Thirteen antiepileptic branched small- and medium chain fatty acids (valproic acid-derivatives, for structure see Fig. 1) with comparable antiepileptic potency but variable toxicity were investigated: eight are known teratogens (*S*-4-

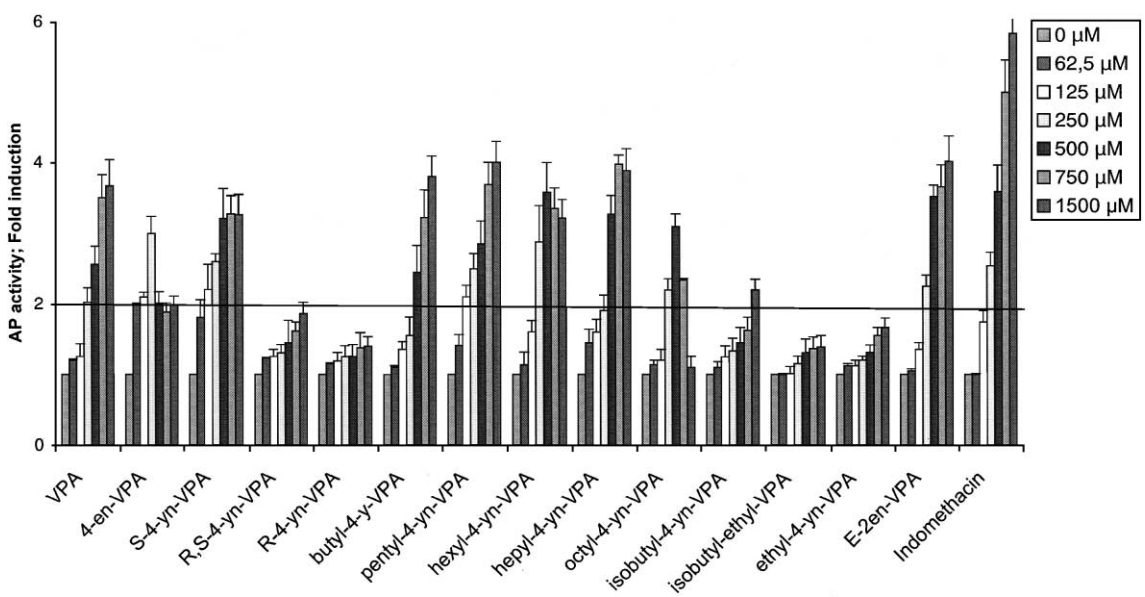

Fig. 3. Activation of the GR/PPAR-γ hybrid receptor by valproic acid or valproic acid-derivatives. Relative alkaline phosphatase activity (AP) in culture supernatants was measured by increase in A_{405} per hour culture. CHO cells containing stably expressed GR/PPAR-γ were seeded at a density of 20,000 cells per culture well and grown for 24 h. Cells were grown for an additional 48 h in the presence of test compounds prior to assaying the culture supernatants for alkaline phosphatase activity. Values represent means from four determinations. Line indicates two-fold effect concentration ($EC_{2\times}$).

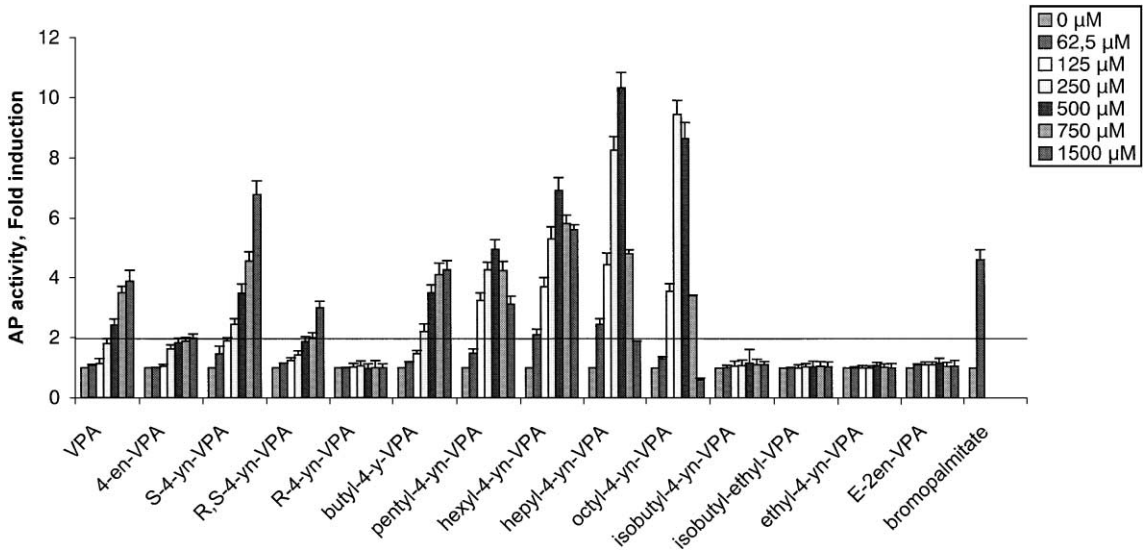

Fig. 4. Activation of the GR/PPAR-δ hybrid receptor by VPA or VPA-derivatives. Relative alkaline phosphatase activity (AP) in culture supernatants was measured by increase in A_{405} per hour per culture. CHO cells containing stably expressed GR/PPAR-δ were seeded at a density of 20,000 cells per culture well and grown for 24 h. Cells were grown for an additional 48 h in the presence of test compounds prior to assaying the culture supernatants for alkaline phosphatase activity. Values represent means from four determinations. Line indicates two-fold effect concentration ($EC_{2\times}$).

yn-valproic acid, *R,S*-4-yn-valproic acid, 4-en-valproic acid, butyl-4-yn-valproic acid, pentyl-4-yn-valproic acid, hexyl-4-yn-valproic acid, heptyl-4-yn-valproic acid and octyl-4-yn-valproic acid), and five are non-teratogenic (*R*-4-yn-valproic acid, isobutyl-4-yn-valproic acid, isobutyl-ethyl-valproic acid, ethyl-4-yn-valproic acid and *E*-2-en-valproic acid). The teratogeinic effects were stereospecific in vivo; *S*-4-yn-valproic acid was teratogen, whereas the *R*-4-yn-valproic acid enantiomer showed no teratogenicity at all (Hauck and Nau, 1992). Elongation of the aliphatic side chain from butyl-4-yn-valproic acid to octyl-4-yn-valproic acid increased the teratogenic effects in vivo (Bojic et al., 1996).

3.1. Activation of the PPAR-α ligand-binding domain

In alkaline phosphatase reporter gene assays performed with CHO cells stably transfected with chimeric GR/PPAR-α receptor expression vectors, all teratogenic and non-teratogenic derivatives of valproic acid activated the PPAR-α ligand-binding domain (Fig. 2). Most potent PPAR-α activation was observed after incubation with the teratogen hexyl-4-yn-valproic acid. Moreover, elongation of the aliphatic side chain from butyl-4-yn-valproic acid to hexyl-4-yn-valproic acid was found to increase the activation of the PPAR-α ligand-binding domain. Valproic acid, 4-en-valproic acid, *R,S*-4-yn-valproic acid, isobutyl-ethyl-4-yn-valproic acid and ethyl-4-yn-valproic acid showed a dose-dependent increase of PPAR-α activation with a maximal level at approximately 1500 µM, whereas at concentrations higher than 1500 µM, *S*-4-yn-valproic acid, butyl-4-yn-valproic acid, pentyl-4-yn-valproic acid, hexyl-4-yn-valproic acid, heptyl-4-yn-valproic acid, and octyl-4-yn-valproic acid showed a decreased PPAR-α activation, which is due to cytoctoxic effects (data not shown). Interestingly, both stereoisomers *S*-4-yn-valproic acid and R-4-yn-valproic acid were able to activate PPAR-α. Overall, the activation of PPAR-α showed no correlation to the teratogenicity of valproic acid-derivatives.

3.2. Activation of the PPAR-γ ligand-binding domain

In alkaline phosphatase reporter gene assays performed with CHO cells stably transfected with chimeric GR/PPAR-γ receptor expression vectors, all teratogenic and

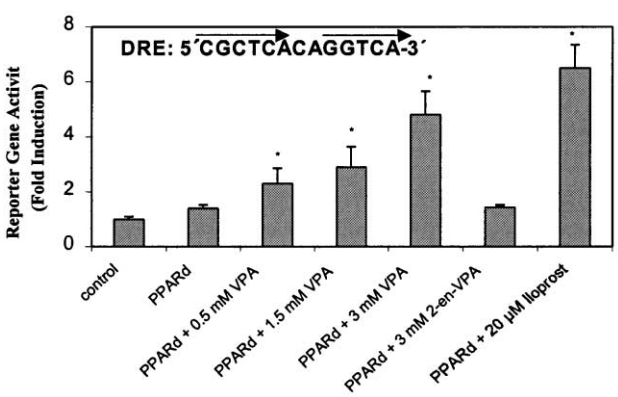

Fig. 5. Activation of the specific PPAR-δ-responsive (DRE)-reporter gene by VPA. HEK293 cells were transfered with PPAR-δ-responsive (DRE) luciferase reporter construct (He et al.,1999), with 1.0 µg of either pBV-luc Vector (control), or pBV-PPAR-δ expression vector, and a transfection control vector (β-galactosidase). Where indicated, cells were treated with the PPAR-δ ligand iloprost (20 µM), valproic acid or 2-en-valproic acid. Luciferase activity is shown as relative luciferase activity (fold induction) compared to solvent represent means ± SD from six determinations; asterisks indicate a significant difference ($p < 0.005$; ANOVA) from untreated cultures [PPARd = PPARδ].

non-teratogenic derivatives of valproic acid activated the PPAR-γ ligand-binding domain (Fig. 3). The most potent derivatives were *S*-4-yn-valproic acid, pentyl-4-yn-valproic acid, hexyl-4-yn-valproic acid, heptyl-4-yn-valproic acid, 4-en-valproic acid and *E*-2-en-valproic acid, whereas isobutyl-ethyl-valproic acid, ethyl-4-yn-valproic acid and R-4-yn-valproic acid were only weak activators of the PAR-γ ligand-binding domain. Also, there was no correlation between the activation of PPAR-γ and the teratogenicity of valproic acid-derivatives. Interestingly, the non-teratogen *E*-2-en-valproic acid was a potent activator of PPAR-γ, and only a moderate activator of PPAR-α, yet failed to activate PPAR-δ. This suggests that *E*-2-en-valproic acid is a PPAR-γ specific activator (compare Figs. 2–4).

3.3. Activation of the PPAR-δ ligand-binding domain

In alkaline phosphatase reporter gene assays performed with CHO cells being stably transfected with chimeric GR/PPAR-δ receptor expression vectors, only teratogenic valproic acid-derivatives activated the PPAR-δ ligand-binding domain (Fig. 4). The level of PPAR-δ activation increased with the length of the side chain again from butyl-4-yn-valproic acid up to octyl-4-yn-valproic acid (Fig. 4). This ranking of the efficacy was not observed for the activation of PPAR-α or PPAR-γ (Figs. 2 and 3). Interestingly, only the teratogenic *S*-4-yn-valproic acid, but not the non-teratogenic *R*-4-yn-valproic acid was found to activate PPAR-δ (Fig. 4), the activation of PPAR-α or PPAR-γ showed no stereoselectivity (Figs. 2 and 3). Octyl-4-yn-valproic acid, the valproic acid-analogue with the longest carbon chain, was the most potent PPAR-δ activator. However, PPAR-δ activation decreased at octyl-4-yn-valproic acid concentrations higher than 750 μM suggesting cytotoxic effects.

3.4. Anticonvulsivity

Both teratogenic and non-teratogenic valproic acid-derivatives showed anticonvulsive activities. Treatment with

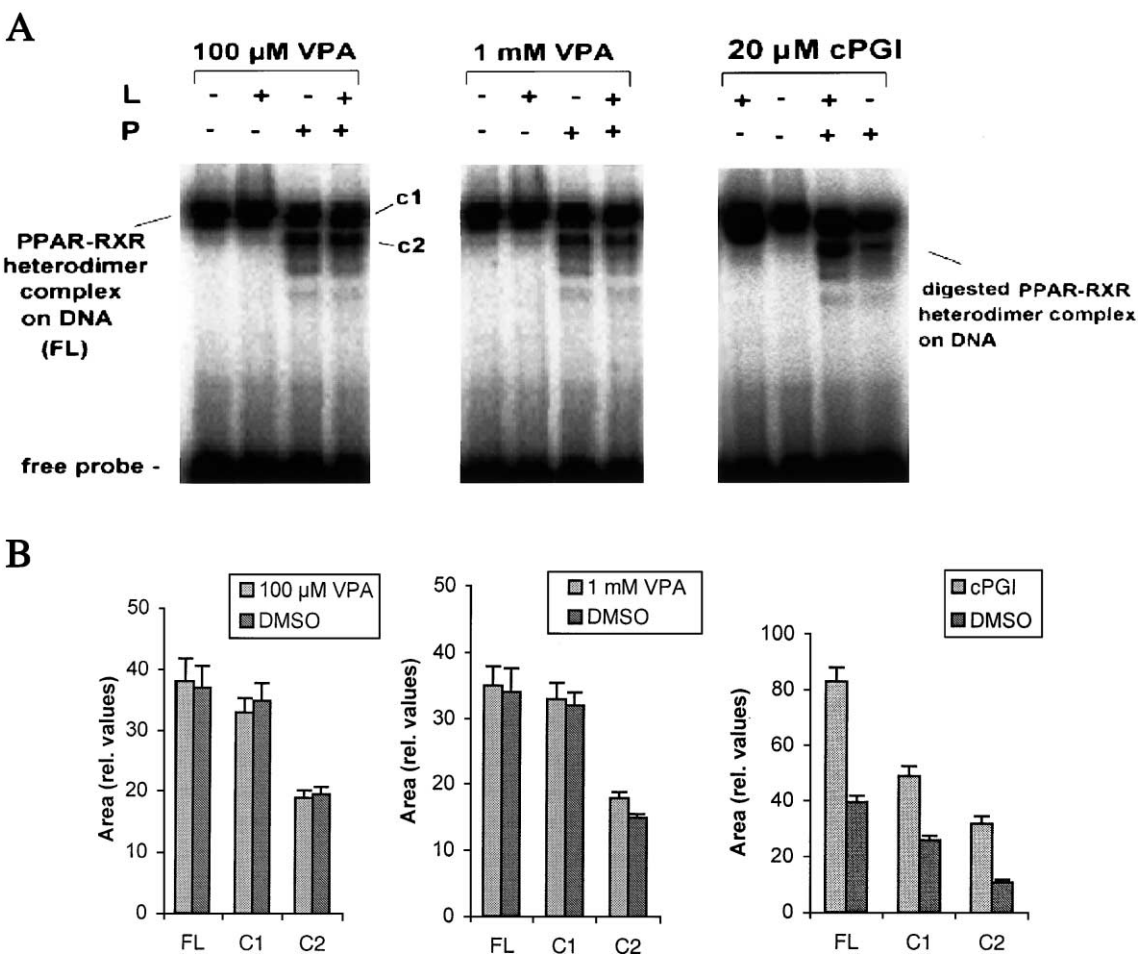

Fig. 6. Ligand inducibility of PPAR–retinoid X receptor heterodimers in vitro. Heterodimers formed by in vitro translated PPAR-δ and in vitro translated retinoid X receptor were preincubated with 100 μM valproic acid, 1 mM valproic acid, 20 μM carbapprostacyclin (cPGI), or demethylsulfoxid (DMSO). L = ligand added; P = protease treated probes (50 ng/μl trypsin). PPAR–retinoid X receptor heterodimers were separated from free probe on an 8% non-denaturing polyacrylamide gel; (A) representative gels are shown; (B) the amounts of digested PPAR–retinoid X receptor heterodimer–PPAR response element complexes (c1 and c2) as well as non-digested PPAR–retinoid X receptor heterodimer (FL) were densitometrically analysed. Each column represents the average of three determinants and bars indicate SD.

0.5 and 1.5 mmol/kg valproic acid resulted in 38% and 100% protection, respectively, in the subcutaneous pentylenetetrazole test. *E*-2-en-valproic acid and isobutyl-4-yn-valproic acid (each with 1.5 mmol/kg) showed 63% and 38% protection, respectively, in the pentylenetetrazole test. Increasing of the aliphatic chain length resulted in an increase of the anticonvulsive activity. Butyl-4-yn-valproic acid (1.0 mmol/kg and 1.5 mmol/kg) showed 20% and 100% protection, respectively. Pentyl-4-yn-valproic acid (0.5 mmol/kg and 1.0 mmol/kg) showed 25% and 100% protection, respectively. Hexyl-4-yn-valproic acid (0.5 mmol/kg, and 1.0 mmol/kg) showed each 100% protection. Additionally, heptyl-4-yn-valproic acid (0.5 mmol/kg) and octyl-4-yn-valproic acid (0.5 mmol/kg) showed each 100% protection in the pentylenetetrazole test.

3.5. Activation of PPAR-δ–retinoid X receptor heterodimers

In luciferase reporter gene assays, performed with HEK cells (transiently transfected with expression vectors of full-length PPAR-δ and retinoid X receptor-α (RXRα) and a PPAR response element-driven luciferase reporter gene; He et al., 1999), valproic acid showed a dose-dependent activation of PPAR-δ–retinoid X receptor heterodimers, whereas the non-teratogenic *E*-2-en-valproic acid was inactive (Fig. 5). This indicates that the results from the activation of the PPAR-δ ligand-binding domain within a GR–PPAR fusion protein (Fig. 4) can be transferred to naturally occurring PPAR-δ–retinoid X receptor heterodimers.

3.6. PPAR-δ–retinoid X receptor complex formation

Gel shift clipping assays combine the well established gel shift assay as a detection method for protein–DNA interactions and the limited protease digestion assay as a method for the analysis of functional nuclear receptor conformation (Quack and Carlberg, 2000). These assays were performed with in vitro translated PPAR-δ–retinoid X receptor-α heterodimers bound to the PPAR response element of the fatty acid binding protein gene promoter (Juge-Aubry et al., 1997; Desvergne et al., 1998). Separation of the protein–DNA complexes on non-denaturing polyacrylamide gels provided two complexes that migrated faster than non-digested PPAR-δ–retinoid X receptor heterodimers (Fig. 6). Neither the non-digested PPAR-δ–retinoid X receptor heterodimers (gel shift) nor the PPAR-δ–retinoid X receptor heterodimer core complexes (gel shift clipping) showed valproic acid-dependent complex formation. The strong activator heptyl-4-yn-valproic acid also failed to induce any ligand-dependent protein–DNA complex formation (data not shown). The induction of complex formation by the known PPAR-δ ligand, carbaprostacyclin served as a positive control and demonstrated the functionality of the experimental system. This suggests that neither valproic acid nor its heptyl-4-yn-derivative interact directly with PPAR-δ.

4. Discussion

PPAR-α and -γ have clearly established roles of in lipid metabolism and PPAR-δ has a putative role in lipid homeostasis. Moreover, all three PPAR subtypes bind with individual preference to fatty acids, eicosanoids and their derivatives (Kersten and Wahli, 2000). This gives the members of the PPAR subfamily an important function as lipid sensors. Since valproic acid and its derivatives are also lipids, it leads to the question concerning the way in which these synthetic compounds may interfere with the natural lipid sensing system of PPARs. This study demonstrates that all three PPAR subtypes are activated by valproic acid and some of its analogues in a structure-dependent manner. PPAR-α and -γ were not able to differentiate between teratogenic and non-teratogenic valproic acid-derivatives, whereas PPAR-δ demonstrated selectivity for teratogenic valproic acid-analogues. This suggests that PPAR-δ has a role as a sensor for teratogenic valproic acid derivatives.

No stereoselectivity was observed between the *S*-4-yn-valproic acid and *R*-4-yn-valproic acid enantiomers concerning their ability to activate PPAR-α and PPAR-γ. Interestingly the activation of PPAR-α was increased when the aliphatic carbon chain was lengthened from butyl-4-yn-valproic acid to heptyl-4-yn-valproic acid. Elongation of the aliphatic carbonchain of valproic acid derivatives resulted in an enhanced activation of PPAR-δ, but not of PPAR-γ. The non-teratogen *E*-2-en-valproic acid was found to preferentially activate PPAR-γ, whereas the other valproic acid-derivatives showed no receptor selectivity. Structure–activity relationships indicate that activation of PPAR-δ is not involved in the antiepileptic activity of valproic acid-derivatives, since teratogenic as well as non-teratogenic valproic acid-derivatives exhibited anticonvulsive activities. Elongation of the aliphatic C-chain (from butyl-4-yn-valproic acid to hexyl-4-yn-valproic acid) resulted in an enhanced anticonvulsivity. The same increase was seen in the activation of PPAR-α, which allows speculating that PPAR-α may be related to anticonvulsive effects. However, further investigations with a greater number of valproic acid -derivatives are required to prove this.

Valproic acid induces neural tube defects causing spina bifida aperta in 1–2% of human fetuses exposed to the compound during early pregnancy (Robert, 1988). This event and the teratogenic potency depend strictly on the structure of the administered compound (Bojic et al., 1996). The same structure–activity relationships have been observed in this study with the activation of PPAR-δ. The sensitive period of NMRI mice for the development of neural tube defects induced by valproic acid is between

gestational days 8 and 9, i.e. very short (Nau et al., 1991). At this early stage of development only PPAR-δ is expressed, whereas PPAR-α and PPAR-γ are detectable much later on day 13.5 of gestation in mice (Kliewer et al., 1994). Therefore, it could be speculated that PPAR-δ, but not PPAR-α and PPAR-γ, may have a significant role in the mechanism of the valproic acid-induced malformations.

The concentrations of fatty acids required for efficient activation of PPARs have been found in general to fall within a super-physiological range and it has proven difficult to demonstrate that fatty acids directly bind to PPARs. Therefore, it was repeatedly suggested that the physiological ligands for these receptors are metabolites of fatty acids rather than the fatty acids themselves (Keller et al., 1993; Lin et al., 1999). PPAR activation involves the conversion of the receptor to a transcriptionally active form. Activation of PPARs is initiated by lipophilic ligands that interact with the ligand-binding domain.

Subsequent research efforts that focused on identifying compounds that may serve to modulate the activity of PPARs in vivo resulted in the reports that several naturally occurring fatty acids and some arachidonic acid metabolites can bind to PPARs and activate them in the context of transactivation assays. However, a persistent problem in accepting these conclusions has been that the reported binding affinities between PPARs and these putative ligands were too weak to account for efficient interactions in vivo (Lin et al., 1999). These compounds are poorly soluble in water and distribute in vivo mainly between biological membranes and various binding proteins. In blood, long-chain fatty acids are bound to serum albumin, and in cells they are tightly associated with cellular fatty acid binding protein (Spector and Fletchter, 1978; Ockner and Manning, 1974). The concentrations of free fatty acids dissolved in cytosol are difficult to assess, but available estimates indicate that the upper limit of the abundant fatty acid, palmitate, in intracellular aqueous phases is on the order of 50 nM (Noy et al., 1986).

In this study, valproic acid was shown to be an activator of the PPAR-δ ligand-binding domain in CHO cells and of PPAR-δ–retinoid X receptor heterodimers in HEK cells in a concentration range of more than several 100 μM. In comparison, EC_{50} for binding of fatty acids ($C > 14$) to PPARs are on the order of 10 μM (Krey et al., 1997; Forman et al., 1997).

However, valproic acid was not able to induce complex formation of PPAR-δ–retinoid X receptor heterodimers on a PPAR response element, such as other nuclear hormones do to their specific receptors (Quack and Carlberg, 2000). Only the PPAR-δ ligand, carbaprostacyclin (Forman et al., 1997) showed an induction in complex formation of PPAR-δ–retinoid X receptor heterodimers on a PPAR response element, which has already been shown earlier by He et al. (1999). However, according to PPAR-δ ligand-binding domain crystal structure analysis (Xu et al., 1999), short fatty acids ($C < 14$) cannot make the hydrophobic interactions that are required for stable ligand binding. This leaves the conclusion that valproic acid is not a direct PPAR-δ ligand.

In conclusion, PPAR-α and PPAR-γ are nonspecific sensors for valproic acid-derivatives, whereas PPAR-δ is a specific sensor for teratogenic valproic acid-derivatives. However, there is no direct binding of valproic acid to PPAR-δ.

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