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Short communication

Vitamin E activates gene expression via the pregnane X receptor

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Abstract

Tocopherols and tocotrienols are metabolized by side chain degradation via initial ω-oxidation and subsequent β-oxidation. ω-Oxidation is performed by cytochrome P450 (CYP) enzymes which are often regulated by their substrates themselves. Results presented here show that all forms of Vitamin E are able to activate gene expression via the pregnane X receptor (PXR), a nuclear receptor regulating a variety of drug metabolizing enzymes. In HepG2 cells transfected with the human PXR and the chloramphenicol acetyl transferase (CAT) gene linked to two PXR responsive elements, CAT activity was most strongly induced by α- and γ-tocotrienol followed by rifampicin, δ-, α- and γ-tocopherol. The inductive efficacy was concentration-dependent; its specificity was underscored by a lower response when cotransfection with PXR was omitted. Up-regulation of endogenous CYP3A4 and CYP3A5 mRNA was obtained by γ-tocotrienol, the most potent activator of PXR, with the same efficacy as with rifampicin. This points to a potential interference of individual forms of Vitamin E with the metabolism and efficacy of drugs.

Keywords: Vitamin E; Gene regulation; Pregnane X receptor; Cytochrome P450; Metabolism; Drug interference

1. Introduction

Vitamin E is widely considered as an essential constituent of the antioxidant network. As such it is believed to prevent oxidative-stress related diseases like inflammatory processes, atherosclerosis, and even cancer. However, clinical trials undertaken to prove the potential of Vitamin E to prevent cardiovascular diseases were disappointing. In addition, novel functions of Vitamin E have been detected such as inhibition of cell proliferation [1], protein kinase C activity [2], and NADPH oxidase activation [3], up-regulation of the scavenger receptor CD36 [4] and down-regulation of adhesion molecules [5], functions often restricted to α-tocopherol (for review see [6]). α-Tocopherol is preferentially retained in the body. Degradation of all types of Vitamin E occurs via initial ω-oxidation followed by five cycles of β-oxidation resulting in the respective final products, the carboxyethyl hydroxychromans (CEHCs) [7–12]. Compared to other forms of Vitamin E, α-tocopherol is only poorly metabolized [11]. Metabolism can, however, be enhanced by rifampicin [13], an inducer of a variety of drug metabolizing enzymes. The degradation of tocopherols and tocotrienols by the cellular drug metabolizing system with a preference for those forms not being α-tocopherol has led to the conclusion that Vitamin E might interfere with drug metabolism [6]. The induction of several CYPs by a large number of structurally diverse xenobiotics that often are substrates of the induced cytochromes themselves, is mediated by the nuclear pregnane X receptor (PXR) [14,15]. We here show that individual forms of Vitamin E are also able to activate PXR and in consequence xenobiotics metabolizing enzymes.

2. Materials and methods

HepG2 cells (ATCC HB8065) were grown in 24-well plates in RPMI 1640 containing 5% heat-inactivated fetal calf serum, 2 mM alanyl-glutamine, 100 U/mL penicillin,

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Abbreviations: CAT, chloramphenicol acetyltransferase; CYP, cytochrome P450; PXR, pregnane X receptor; RT-PCR, reverse transcription-polymerase chain reaction; RXR, retinoid X receptor.

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and 100 μg/mL streptomycin (Gibco) at 37° in 5% CO_2 to about 80% confluency. Cells were cotransfected with the human PXR in the pSG5 expression vector [15] (pSG5hPXR; 0.17 μg/well), a CAT reporter plasmid pCATDR3 (0.17 μg/well) containing two copies of the CYP3A1 PXR binding site (DR3, 5′-TGAACTn3T-GAACT-3′) [14], and pCH110 (0.5 μg/well), a β-galactosidase expression vector (Amersham) for normalizing transfection efficiency. In case of PXR negative controls, pSG5hPXR was replaced by pSG5. Transfection was performed with TfX-20 according to manufacturer's instructions (Promega) with 3 μL TfX reagent per microgram DNA to be transfected. After 1 hr of incubation at 37°, medium was added to a final concentration of 10% FCS without Vitamin E (control) or enriched with 50 μM of the indicated type of Vitamin E or 10 μM rifampicin and incubation continued for 48 hr. Vitamin E-containing FCS was prepared by adding the corresponding vitamers from an ethanolic stock solution to the serum followed by an overnight incubation at 4° as described [13]. Rifampicin-containing FCS was prepared accordingly. Respective amounts of ethanol were added to the FCS used for control.

For measuring CAT activity the FAST CAT Green (deoxy) assay kit (Molecular Probes) was used. Cells were lysed in 120 μL reporter lysis buffer (Promega) according to the manufacturer's protocol. To 50 μL lysate 10 μL component A (chloramphenicol 1:10) and 10 μL acetyl coenzyme A (7.4 mg/mL H_2O) was added. After 3 hr at 37°, 1 mL ice-cold ethyl acetate was added, mixed and centrifuged at 13,000 *g* for 5 min. Nine hundred and fifty microliters of the upper phase was evaporated to dryness. The residue was redissolved in 100 μL HPLC eluent (55% acetonitrile, 45% H_2O). CAT activity was estimated via the amount of fluorescent-labeled acetylated chloramphenicol quantified by HPLC with fluorescence detection (System 440 and SFM 25, Kontron) and a 250 mm × 3 mm RP18 end caped column (Sepserv). Excitation and emission settings were 495 nm and 525 nm, respectively. A calibration curve made from dilutions of the acetylated chloramphenicol reference standard provided in the FAST CAT kit was linear in a range from 1:600 to 1:20,000. The mean of R^2 was 0.997 ± 0.0046 (N = 5). The inter-day variance was less than 7.4%. β-Galactosidase was assayed by adding 70 μL assay buffer (120 mM Na_2HPO_4, 80 mM NaH_2PO_4, 20 mM KCl, 2 mM $MgCl_2$, 100 mM β-mercaptoethanol, pH 7.3) and 30 μL substrate (4 mg/mL *o*-nitrophenyl-β-D-galactopyranosid, 60 mM Na_2HPO_4, pH 7.5) to 50 μL cell lysate followed by incubation at 37° for 1 hr. Absorption was measured at 405 nm using a 96-well microplate reader Dynatech MR5000 (MTX Lab systems).

For RT-PCR HepG2 cells were seeded into 6-well plates and grown to 80% confluency. After 24 hr serum-deprivation, cells were treated for 48 hr with 10 μM rifampicin or 50 μM γ-tocotrienol added as ethanolic solutions in serum-free medium. Controls contained ethanol at the same concentration (less than 0.3%) as treated cells. Gene specific primers were designed to amplify specific regions of human CYP3A4 (M14096), forward: 5′-GGGAAGCA-GAGACAGGCAAG-3′ (nt 553–572), reverse: 5′-GAGC-GTTTCATTCACCACCA-3′ (nt 1141–1160); CYP3A5 (J04813), forward: 5′-GAAGAAAAGTCGCCTCAAC-3′ (nt 855–873), reverse: 5′-AAGAAGTCCTTGCGTGTC-TA-3′ (nt 1533–1552); and β-actin (X00351), forward: 5′-CAAGAGATGGCCACGGCTGCT-3′, reverse: 5′-TC-CTTCTGCATCCTGTCGGCA-3′ (nt 714–734 and 968–988, respectively). RT-PCR procedures were performed according to standard protocols. PCR samples (3 μg RNA equivalents for CYPs and 0.375 μg for β-actin) were heated to 94° for 4 min and then cycled at 94° for 40 s, 60° for 60 s, and 72° for 120 s. PCR comprised 32 cycles for CYP3A4, 28 cycles for CYP3A5, and 23 cycles for β-actin. β-Actin was used for normalization of CYP3A expression. The degree of induction was calculated relative to untreated controls measured by densitometry of ethidium bromide stained PCR bands (Gel Doc 2000, Biorad).

3. Results and discussion

To study an activation of PXR by Vitamin E the model of HepG2 cells was chosen because these cells (i) metabolize all forms of Vitamin E [11] and (ii) express CYPs under the control of PXR [16]. HepG2 cells were transiently cotransfected with the human PXR, the CAT reporter gene containing two times the CYP3A1 direct repeat response element (DR3) to which PXR–RXR heterodimers can bind [15], and with a β-galactosidase expression plasmid for transfection control. Then cells were incubated with individual forms of Vitamin E as indicated and with rifampicin as positive control. CAT activity was stimulated by rifampicin and tocols in the following ranking: γ-tocotrienol ≈ α-tocotrienol > rifampicin > δ-tocopherol > *RRR*-α-tocopherol ≥ γ-tocopherol (Fig. 1A), a hierarchy not reflecting the antioxidative capacity of tocols at all [17]. The synthetic *all rac*-α-tocopherol did not show any significant effect. Omitting PXR in the transfected system, significantly decreased CAT-induction (Fig. 1B) indicating that (i) PXR is indeed activated by individual types of Vitamin E and (ii) the content of endogenous PXR present in HepG2 cells is sufficient to achieve some but not maximum induction of CAT. Vitamin E metabolites with a shortened side chain, α-carboxyethyl hydroxychroman (α-CEHC) or its precursor α-carboxymethylbutyl hydroxychroman (α-CMBHC) [11] did not enhance CAT activity (not shown) indicating that rather the authentic tocopherols and tocotrienols than their metabolites exert transcriptional activation.

The dose-dependency of PXR activation is shown for rifampicin as control and γ-tocotrienol, the most effective activator (Fig. 2). γ-Tocotrienol activated PXR at 1–10 μM, concentrations which can be reached in human plasma [18]. Cellular concentrations in intestinal cells or in

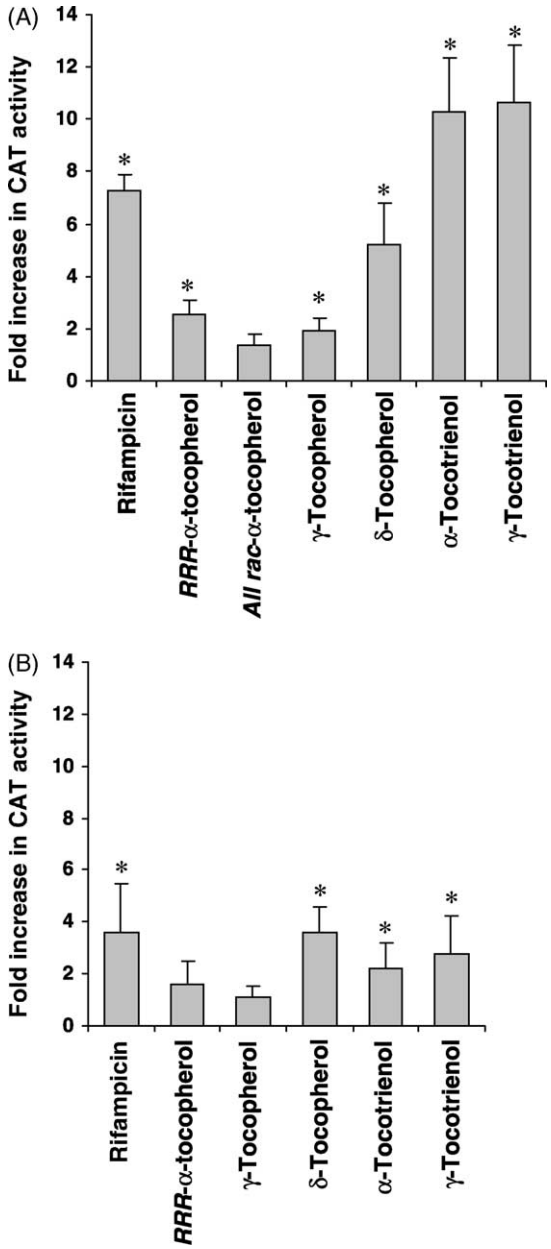

Fig. 1. Induction of PXR-mediated CAT activity by individual forms of Vitamin E. HepG2 cells transiently cotransfected with a human PXR containing pSG5 expression plasmid, a PXR-driven CAT reporter gene, and β-galactosidase expression plasmid (A) or with the PXR gene deleted in the pSG5 (B) were treated with 10 μM rifampicin and 50 μM concentrations of the indicated Vitamin E types. CAT activity was assayed as described in Section 2 and normalized to β-galactosidase. Effects are expressed relative to transfected but untreated controls (fold induction) and represent means ± SD from three to four experiments each performed in triplicate. Asterisk (∗) indicates $P < 0.05$ vs. untreated control.

the liver that are first hit by all ingested tocols may, however, be more relevant. In liver, the α-tocopherol transfer protein (α-TTP) sorts out α-tocopherol from all incoming tocols for the incorporation into VLDL and transfer into the plasma [19]. In view of the selective binding of α-tocopherol by α-TTP [20] γ-tocotrienol concentrations in liver cells might even be higher than those measured in plasma which, however, needs to be investigated. In rats fed

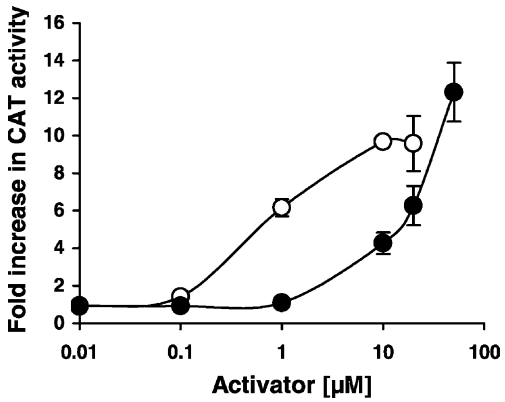

Fig. 2. Dose response of rifampicin and γ-tocotrienol for the activation of the PXR-driven CAT reporter. HepG2 cells were cotransfected as described in legend to Fig. 1, treated with indicated concentrations of rifampicin (open circles) or γ-tocotrienol (black circles), and assayed as described in Section 2. Values are means ± SD from three experiments performed in triplicate.

a tocotrienol-rich fraction from palm oil, α- and γ-tocotrienol could not be detected in liver [21]. Comparable human studies are not available so far.

A most reliable indicator of PXR activation is the induction of CYP3A-type enzymes [14,15]. In human hepatocytes the relevant subtypes are CYP3A4 and CYP3A5. The PXR response elements (PXRE) in the promoters of these enzymes are imperfect averted repeats separated by six nucleotides (ER6) [15,22]. Binding of the human PXR–RXR heterodimer was efficiently competed by the direct repeat (DR3) PXRE, which also is used in this study, indicating that the human PXR can bind to both, the PXRE of the rodent CYP3A1 as well as the human CYP3A4 promoter [15]. Thus, the species-specific induction of CYP3A genes is rather conferred by species-specific transcription factors than by response elements present in CYP3A promoters [22]. The induction of CYP3A4 and CYP3A5 by γ-tocotrienol as the most effective activator of PXR in HepG2 cells was therefore tested in comparison to rifampicin. Fig. 3A and B shows that γ-tocotrienol induces the transcription of both CYP3A4 and CYP3A5 to the same extent as rifampicin. Induction was small but significant and reflects the degree of induction of CAT activity mediated by the endogenous PXR content of HepG2 cells (see Fig. 1B). The induction of endogenous CYPs, thus, underscores the physiological relevance of the reporter gene experiments. Up-regulation of CYP3A enzymes by vitamins might be more common than considered at first glance. For example, Vitamin D has been shown to induce CYP3A4 in intestinal cells [23]. The pathway was different from the one presented here where PXR was required for the gene activating function of individual tocols. Although dependent on a functional PXRE in the *CYP3A4* promoter, induction was achieved via the Vitamin D receptor (VDR) and not via PXR [23]. The proposed regulation of intestinal CYP3A4 by VDR was, thus, suggested to be complementary to the role of PXR in regulating hepatic CYP3A4.

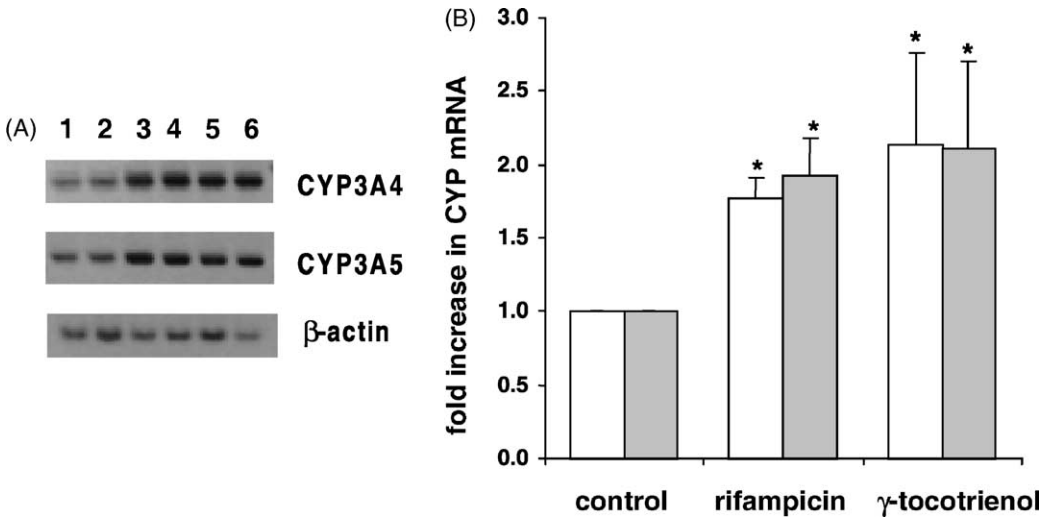

Fig. 3. Induction of endogenous CYPs by rifampicin and γ-tocotrienol. (A) RT-PCR products of CYP3A4, CYP3A5 and β-actin obtained from HepG2 cells either left untreated (lanes 1 and 2) or stimulated with 10 μM rifampicin (lanes 3 and 4) or 50 μM γ-tocotrienol (lanes 5 and 6) for 48 hr. Shown are data from two representative experiments each. (B) Fold induction over control of CYP3A4 (white bars) and CYP3A5 (dark bars) by rifampicin or γ-tocotrienol. Data represent β-actin standardized means ± SD of four individual experiments. Asterisk (∗) indicates $P < 0.05$ vs. control. For experimental details see Section 2.

The efficacy of PXR activation does not necessarily reflect the degree by which different forms of Vitamin E are metabolized. Metabolism of *RRR*-α-tocopherol is generally low, the metabolism of *all rac*-α-tocopherol being three to four times higher [24]. It can be enhanced about 3-fold in HepG2 cells by rifampicin, an inductor of CYP3A4 but not by clofibrate, an inductor of type 4 CYPs [13]. γ-Tocopherol is metabolized to a much higher extent *in vivo* [25,26] as well as in cultured cells [11,27] but it is only a weak activator of PXR. In contrast to α-tocopherol, side chain degradation of γ-tocopherol was not stimulated by rifampicin [28]. Tocotrienols are moderately metabolized *in vivo* [10] but extensively in HepG2 cells [11]. They are the most potent PXR activators (Fig. 1). Recently CYP4F2 has been identified as the enzyme most efficient in ω-hydroxylating γ-tocopherol with a much lower activity towards α-tocopherol [12]. Sesamin and ketoconazole, inhibitors for both CYP3A4 and CYP4F2, inhibited the metabolism of α- and γ-tocopherol [12,27,29]. Taken together the available data suggest that more than one form of CYP might be involved in Vitamin E metabolism.

In summary, we have for the first time demonstrated a direct transcriptional gene activation by tocopherols and tocotrienols. Gene activation is mediated by the nuclear receptor PXR that regulates a variety of cytochromes P450 [30], glutathione *S*-transferase A2 [31], sulfotransferase 2 [32] and ABC transporters including the multidrug resistance-associated protein 2 [33]. Quantitatively, the inducing activity is similar to that of rifampicin that is known to substantially accelerate the metabolism of drugs and hormones via PXR activation. It is also similar to the recently reported effect of hyperforin, a constituent of St. John's wort, known to stimulate the metabolism of immunosuppressants and oral contraceptives [34,35]. Clinical trials will have to demonstrate at which alimentary or supplementary dosages analogous effects can be exerted by individual forms of Vitamin E.

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