Full Paper

Resveratrol Suppresses the Inducible Expression of CYP3A4 Through the Pregnane X Receptor

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Abstract. The pregnane X receptor (PXR, NR1I2), a member of the nuclear receptor superfamily, is activated by a number of clinically prescribed drugs and herbal extracts. The inducible expression of several important cytochrome P450 (CYP450) enzymes has been shown to be regulated by the activation of PXR in the liver. In the current study, reporter gene-transfected cells were used to identify potential antagonists of PXR. Here, we showed that resveratrol (RES), a natural polyphenolic compound could significantly suppress the rifampicin-induced PXR transactivation of the CYP3A4 promoter. Treatment of hPXR-over-expressed cells with RES reduced the rifampicin-inducible expression of CYP3A4 in a concentration-dependent manner. Moreover, the induction of mRNA and protein expression of CYP3A11 by pregnenolone 16 α -carbonitrile was also significantly reduced when RES was applied in primary cultures of mouse hepatocytes. Taking together, these findings suggest that RES can attenuate the PXR-mediated induction of CYP3A enzyme. Therefore, it would be possible for RES to antagonize the elevation in CYP3A-mediated drug metabolism by identified PXR activators.

Keywords: pregnane X receptor, CYP3A, resveratrol, rifampicin, antagonist

Introduction

Cytochrome P450s (CYPs) belong to the superfamily of hemethiolate isoenzymes that regulate the metabolism of a large number of endogenous compounds, drugs, environmental pollutants, and dietary chemicals. CYP3A4, one of the major isozymes highly expressed in liver and intestine, is the principal enzyme responsible for the biotransformation of nearly 60% of all clinically prescribed drugs in humans (1). Therefore, induction or inhibition of the CYP3A4 enzyme may contribute to the change of the metabolism and eventually the effect of drugs that serve as the substrates of CYP3A4. Evidence has been accumulated to show that the transcriptional activity of CYP450 drug-metabolizing enzymes can

be mediated by members of the nuclear receptor superfamily, such as pregnane X receptor (PXR), constitutive androstane receptor (CAR), glucocorticoid receptor (GR), and vitamin D receptor (VDR) (2). Specifically, CYP3A4 is the best-characterized target gene of PXR in humans (1).

The pregnane X receptor (PXR, NR1I2) was first identified in 1998 as a member of the nuclear receptor superfamily (3). In the past decade, various studies have shown that activation of PXR in the liver and intestine increases the expression of genes involved in the uptake, metabolism and elimination of xenobiotics. It is well established that PXR functions as a master regulator of xenobiotic- and drug-inducible expression of the CYP3A, CYP2B, and CYP2C subfamily of drug-metabolizing enzymes in humans and rodents (4, 5). PXR also regulates the inducible expression of other genes that are involved in the metabolism of xenobiotic compounds including glutathione S-transferase (GST), sulfotransferase, UDP-glucuronosyltransferase (UGT), and carbo-

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xylesterase enzymes in liver (6 – 8). Moreover, additional PXR-target genes encode several important drug transporter proteins, such as organic anion transporting polypeptide 2 (OATP2), multidrug resistance 1 / P-glycoprotein (MDR1), and multidrug resistance proteins 2, 3, 4, and 5 (MRP2/3/4/5) (9 – 11). In this way, PXR-mediated gene activation may lead to profound up-regulation of metabolism, transport, and elimination of potentially toxic compounds from the body. Therefore, activation of PXR not only protects hepatocytes from toxic insults, but also represents the molecular basis for an important class of drug–drug interactions in the clinical setting.

Numerous ligands of PXR have been identified over the years. PXR has been shown to be activated by a broad range of endogenous compounds and xenobiotics, including certain bile acids and a variety of drugs and herbal products (1, 12). For example, previous studies from our laboratory identified several herbal compounds, such as praeruptorin A and C, which induced the PXR transactivation of the CYP3A4 promoter in reporter gene transfected HepG2 and Huh7 cells, and up-regulated the CYP3A4 expression at mRNA and protein levels (13). However, only limited PXR antagonists have been described. Ecteinascidin-743 (ET-743), an antineoplastic agent, repressed PXR activation in reporter gene assays and further inhibited the gene expression of CYP3A4 and MDR1 (11). The antifungal drug ketoconazole (KTZ), which is now forbidden for in vivo use, inhibited the xenobiotic-mediated induction of CYP3A4 and MDR-1 gene transcription through PXR (14). Additional PXR antagonists that have been reported include camptothecin, the topoisomerase I inhibitor, and metformin, the first-line agent against type 2 diabetes, etc. (15, 16). In the current study, we found that resveratrol (RES), a phytoalexin in the skin of red grapes suppressed the rifampicin-induced CYP3A expression through PXR.

RES (trans-3, 4', 5-trihydroxystilbene) is a diphenolic phytoalexin found in a wide variety of plants, including grapes, berries, and nuts (17). RES is at high concentration in red wine and exerts potent antioxidant and anti-inflammatory effects, which may contribute to the beneficial effects of wine consumption in the prevention of cardiovascular diseases (18). RES has also been suggested to possess chemopreventive potential against cancer by inhibiting cellular events associated with tumor development (19). Besides its potential effects on coronary heart disease and cancer, RES displayed an irreversible inhibition on CYP3A4 activity (20). However, the extent to which RES regulates the mRNA and protein expression of CYP3A4 and the underlying mechanism are largely unknown. Previous studies indicated an involvement of the transcriptional factor

aryl hydrocarbon receptor (AhR) in the inhibition of oltipraz-induced CYP1A1 promoter activity and mRNA expression level by RES (21). Here, we revealed that PXR, the nuclear receptor responsible for the transcriptional activation of CYP3A4 in humans contributes to the suppression of rifampicin-inducible CYP3A4 expression by RES in human cell lines. In addition, RES also attenuated the mRNA and protein expression of CYP3A11 induced by 5-pregnen-3 β -ol-20-one-16 α -carbonitrile (PCN), a prototypical ligand of mouse PXR in primary mouse hepatocytes, confirming the effect of RES on a more physiologically relevant experimental system.

Materials and Methods

Experimental animals

Male BALB/c mice with weight of 18 – 22 g at 6 – 8 weeks of age were purchased from the Medical Experimental Animal Center of Guangdong Province. The animals had free access to standard rodent chow and clean tap water and were kept in a room with a 12/12 dark/light cycle under controlled temperature (22°C $\pm$ 2°C) and 55% – 60% relative humidity. All procedures were in accordance with the Regulations of Experimental Animal Administration issued by the Ministry of Science and Technology of the People's Republic of China. The animal study protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Sun Yat-sen University, Guangzhou, China.

Chemicals and reagents

The pSG5-hPXR expression vector was provided generously by Dr. Steven Kliewer (1) (University of Texas Southwestern Medical Center, Dallas, TX, USA), and the pGL3-CYP3A4-XREM luciferase reporter was provided generously by Dr. Jeff Staudinger (22) (Department of Pharmacology and Toxicology, University of Kansas, Lawrence, KS, USA). Rifampicin (RIF), dimethyl sulfoxide (DMSO), and pregnenolone 16 α -carbonitrile (PCN) were purchased from Sigma-Aldrich (St. Louis, MO, USA). KTZ and RES were purchased from Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Lipofectamine 2000 transfection reagent was purchased from Invitrogen (Carlsbad, CA, USA). Dual-Luciferase Reporter Assay System was obtained from Promega (Madison, WI, USA). RNAiso Plus and PrimeScript RT reagent were supplied by Takara (Kyoto). The primers used in real-time PCR were synthesized by Invitrogen. The anti-CYP3A4 polyclonal antibody was purchased from Santa Cruz (Santa Cruz, CA, USA). The GAPDH antibody, anti-

rabbit IG-HRP antibody, and anti-mouse IG-HRP antibody were purchased from Cell Signaling Technology (Danvers, MA, USA).

Transient transfection and reporter gene assays

LS174T or HepG2 cells were seeded in 96-well plates at 70%–80% confluence. Transfection was performed in phenol red-free RPMI 1640 using Lipofectamine™ 2000. Briefly, cells in each well were transfected with pSG5-hPXR or empty vector (75 ng), CYP3A4-XREM luciferase reporter gene (75 ng), and pRL-TK (15 ng) as an internal control. Six hours after transfection, cells were treated with vehicle (0.1% DMSO) or drugs for an additional 24 h. Luciferase activities were measured with the Dual-Luciferase Reporter Assay System according to the manufacturer's instructions (Promega). Transfection efficiency was determined by Renilla luciferase activity, and the firefly luciferase activity was determined and normalized against Renilla luciferase activity. Values are expressed as the mean of the fold increase in activity compared with that in vehicle-treated hPXR transfected cells.

RNA isolation and real-time quantitative-polymerase chain reactions

Total RNA was isolated from cells by using the RNAiso™ Plus kit (Takara) according to the manufacturer's directions. After DNase I treatment, 1 µg of RNA was reverse-transcribed into cDNA by using a PrimeScript™ RT reagent Kit (Takara). Equal amounts of cDNA were used in real-time quantitative polymerase chain reactions (RT-QPCRs). The primer sets are shown in Table 1. All the PCR reactions were carried out using SYBR® Premix Ex Taq™ kit (Takara) and followed manufacturer's instructions. Cycling conditions

were 95°C for 30 s followed by 40 cycles of 95°C for 5 s, 60°C for 20 s, and 40°C for 60 s using the Light Cycler 2.0 Real Time Detection System (Roche, Hercules, CA, USA). The fold induction was calculated as described previously (23).

Western blotting

Cells were treated with vehicle (0.1% DMSO) or drug (25 and 50 µM of RES) for 24 h. Ketoconazole (25 and 50 µM) was used as the positive control. Cells were lysed in RIPA buffer and phenylmethylsulfonyl fluoride (PMSF). Total protein was extracted by high-speed centrifugation and quantified using the Pierce® BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Protein expression was measured by western blot analysis. Briefly, protein was resolved on a 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride microporous membranes (Millipore, Bedford, MA, USA) that were probed with anti-CYP3A4 or anti-CYP3A11 antibodies. Immunodetection was performed using the Enlight™ (Engreen Biosystem, China) chemiluminescence detection system. Quantitative densitometric analyses of western blot images were achieved by using densitometry (ImageQuant LAS 4000; GE Healthcare, Waukesha, WI, USA).

Primary mouse hepatocyte culture and treatment

Hepatocytes were isolated from BALB/C mice using a two-step collagenase perfusion method as described previously (24). Hepatocytes were plated in collagen-coate 12-well plates at a density of 2×10^5 live cells/well. At 12 h after plating, hepatocytes were treated with vehicle (0.1% DMSO) and PCN (10 µM) with or without 25 and 50 µM of RES for 24 or 48 h.

Table 1. Primer sequences used for real-time polymerase chain reaction

Gene	5'-3'sequence	PCR condition
<i>CYP3A4</i>		
Forward	TCAGCCTGGTGCTCCTCTATCTAT	Stage 1:
Reverse	AAGCCCTTATGGTAGGACAAAATATTT	Pre-denaturation
<i>GAPDH</i> (human)		(30 s at 95°C)
Forward	GGATTGGTCGTATTGGG	1 Cycle
Reverse	GGAAGATGGTGATGGGATT	
<i>Cyp3a11</i>		
Forward	GGATGAGATCGATGAGGCTCTG	Stage 2:
Reverse	CAGGTATTCCATCTCCATCACAGT	PCR reaction
<i>Gapdh</i> (mouse)		(5 s at 95°C)
Forward	TGAAGCAGGCATCTGAGGG	(20 s at 60°C)
Reverse	CGAAGGTGGAAGAGTGGGAG	40 Cycle
		40°C for 60 s

Statistical analyses

Data are presented as the mean $\pm$ S.D. One-way analysis of variance (ANOVA) was performed for statistical comparison of the results, which was followed by the LSD-*t* test. The criterion of significance was set at $P < 0.05$, and tests were performed using SPSS version 13.0 software (SPSS Inc., Chicago, IL, USA).

Results

RES suppressed the PXR transactivation of the CYP3A4 promoter in reporter gene transfected cells

The pharmacokinetic study of RES showed that the $AUC_{0-\infty}$ was 320 mg min/l when 100 mg/kg RES was administered to rats (25). However, concentrations of RES used to reach biological effects in vitro could be different from those used in in vivo studies. In order to determine the proper concentration of RES to use in the treatment of cultured cells, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to evaluate the cellular viability. RES in combination with RIF did not exhibit significant cytotoxicity on LS174T cells at concentrations up to 100 μ M (Fig. 1).

We then employed dual-luciferase reporter assay to investigate the PXR transactivation of the CYP3A4 promoter in reporter gene-transfected LS174T cells. As shown in Fig. 2A, the reporter activity was not affected by RES treatment in empty plasmid-transfected cells. In hPXR-transfected cells, treatment of RIF significantly activated the CYP3A4 promoter, but no effect was

observed when 1, 5, or 25 μ M of RES were treated alone, with the exception that 50 μ M RES decreased the promoter activity to the similar level as that of the empty plasmid. However, the RIF-induced CYP3A4 reporter activity was markedly decreased when 25 or 50 μ M of RES was co-administered. KTZ was used as a potent CYP3A4 inhibitor for the positive control. Similar results were obtained in reporter gene-transfected HepG2 cells (Fig. 2B). These results indicated that RES functions as a possible antagonist of PXR.

Fig. 1. Cell survival of LS174T cells. LS174T cells were treated with RES at 1, 2.5, 5, 10, 25, 50, and 100 μ M in the presence of RIF (10 μ M) for 24 h. Cell survival was determined and normalized against control vehicle-treated cells. Results are presented as the mean $\pm$ S.D. ($n = 3$).

Fig. 2. Effect of RES on the PXR transactivation of the CYP3A4 promoter in reporter gene-transfected cells. LS174T (A) and HepG2 (B) cells were transfected with the pGL3-CYP3A4-XREM-Luc reporter gene construct and expression vector encoding hPXR. Cells were treated with either vehicle (0.1% DMSO) or RIF (10 μ M) with or without RES (25 and 50 μ M) or KTZ (25 and 50 μ M) for 24 h. Luciferase activity was then determined. Data are expressed as fold change over the control group in hPXR-transfected cells. * $P < 0.05$ and ** $P < 0.01$ indicate statistically significant effects compared to RIF-treated cells in the hPXR-transfected group ($n = 3$).

RES suppressed the RIF-induced mRNA and protein expression of CYP3A4 through PXR

Next, we investigated the effects of RES on the RIF-induced mRNA and protein expression of CYP3A4 in LS174T cells. Cells were treated with RIF alone or in combination with different concentrations of RES for 24 h, and total RNA and protein was isolated for subsequent analysis of gene expression and protein levels, respectively. Treatment of RIF largely increased the gene expression of CYP3A4 as expected, whereas co-administration of RES suppressed the RIF-induced CYP3A4 mRNA expression only in cells over-expressing PXR protein (Fig. 3: A and B). Proteins isolated from treated cells were resolved on an SDS-polyacrylamide

electrophoresis gel for western blot analysis. It was interesting to notice that the protein expression of CYP3A4 was increased by 50 μ M of KTZ when compared to that of 25- μ M group only in hPXR-non-transfected LS174T cells. However, the underlying mechanism is currently unknown, which requires further investigation. It was noteworthy that RES suppressed the RIF-induced CYP3A4 protein expression in a concentration-dependent manner regardless of the over-expression of PXR protein (Fig. 3: C and D). These data indicate that PXR protein plays a critical role in the suppression of RIF-induced CYP3A4 expression by RES.

Fig. 3. Effect of RES on the expression of CYP3A4 in LS174T cells. Non-transfected LS174T cells (A and C) and LS174T cells transfected with hPXR expression plasmid (B and D) were treated with vehicle (0.1% DMSO) or RIF (10 μ M) with or without RES (25 and 50 μ M) or KTZ (25 and 50 μ M) for 24 h, and then CYP3A4 mRNA and protein levels were analyzed. Data are expressed as fold change over the control group. * P < 0.05 and ** P < 0.01 indicate statistically significant effects compared to RIF-treated cells (n = 3).

RES suppressed the PCN-induced mRNA and protein expression of CYP3A11 through PXR

In order to explore the suppressive effect of RES on RIF-induced CYP3A4 expression in a more physiologically relevant experimental system, primary mouse hepatocytes were isolated and cultured, considering the scarcity of primary human hepatocytes. PCN, a prototypical ligand of mouse PXR was applied to the primary mouse hepatocytes for 24 or 48 h, and total RNA was then isolated for Q-PCR analysis. Treatment with PCN significantly induced the mRNA level of CYP3A11, the mouse ortholog of human CYP3A4. Co-treatment

with RES suppressed the PCN-induced CYP3A11 mRNA expression (Fig. 4: A and B). Similar effect of RES was observed when protein expression of CYP3A11 was evaluated by western blotting analysis (Fig. 4: C and D). These results were consistent with what we observed in human cell lines, suggesting the non-species-specific effect of RES on suppressing drug-inducible expression of CYP3A4 enzyme. Taken together, these data again indicated the involvement of PXR in mediating the suppressive effect of RES on drug-inducible expression of CYP3A enzyme.

Fig. 4. Effect of RES on the expression of CYP3A11 in the primary mouse hepatocytes. Primary cultures of mouse hepatocytes were treated with vehicle (0.1% DMSO) or PCN (10 μM) with or without RES (25 and 50 μM) for 24 or 48 h. Message RNA (A and B) and protein (C and D) expression of CYP3A11 was monitored. Data are expressed as fold change over the control group. * $P < 0.05$ and ** $P < 0.01$ indicate statistically significant effects compared to PCN-treated cells ($n = 3$).

Discussion

Drug–drug interactions (DDIs) have been implicated as an important clinical issue accounting for about 7% of hospitalizations every year. As the number of medications a patient takes increases, the potential for DDIs increases, which may eventually lead to potentially fatal adverse drug reactions. Many DDIs are due to alterations in drug metabolism. One notable system involved in metabolic DDIs is the enzyme system comprising the cytochrome P450 oxidases. It has been well established that regulation of the nuclear receptor PXR serves as the molecular basis for the cytochrome P450-mediated DDIs. For example, if one drug activates or antagonizes PXR, it can be predicted that administration of this drug will promote or inhibit the metabolism and elimination of other co-administrated drugs that are also metabolized and eliminated by PXR-target gene–encoded proteins. Therefore, the efficacy of many drug therapies will be reduced or enhanced in patients on combination therapy. In the current study, we provided evidence for the first time that RES attenuates the rifampicin-inducible expression of CYP3A4 through PXR. As high concentration of RES is found in red wine and RES is also used as an anti-aging supplement, the time and amount of wine and RES supplement consumption should be paid attention to for those patients under combination therapy with drugs functioning as PXR ligands. The expected elevation in the CYP3A-mediated drug metabolism by identified PXR activators would be therefore possibly antagonized by RES.

Previous studies revealed the effect of RES on the expression and activity of CYP3A4 without elucidating the underlying mechanism (26 – 28). Here, we displayed the pivotal role of PXR in suppressing the rifampicin-inducible expression of CYP3A4 by RES. First, we determined the proper concentration of RES used in the treatment of cultured cells by evaluating the cellular viability through MTT assay. It should be noted that there may be differences in RES concentration applied to reach biological effect between *in vitro* and *in vivo* studies. For example, plasma protein binding of RES *in vivo*, which is currently unknown, might alter the effect that we observed *in vitro*. Next, we employed a reporter gene assay to investigate the suppressive effect of RES on RIF-induced PXR transactivation of CYP3A4 promoter. Yu et al. identified RES as a PXR activator by a similar reporter gene assay (29). On the other hand, Jacobs et al. showed no effect of RES on PXR activation (30). However, the conclusions of these results were only based on the comparison of reporter activity between cells treated with RES and RIF, without showing the basal level. A different CYP3A4 promoter construct

was employed to reveal the activation of PXR by RES by Kluth et al. (31). As reporter gene assay is a highly artificial technique, so the results could not properly reflect the effect of RES without showing the gene and protein expression of CYP3A4. In our study, treatment of RES induced the reporter activity in hPXR-transfected cells, but no big change was observed when compared with the vehicle-treated group, indicating that RES might not be a PXR activator (Fig. 2A). The mRNA and protein expression of CYP3A4 was then examined. It was interesting to notice that co-administration of RES suppressed the RIF-induced CYP3A4 mRNA expression only in cells over-expressing PXR protein, whereas the suppressive effect of RES on RIF-induced CYP3A4 protein expression was detected in cells regardless of the over-expression of PXR protein. Post-transcriptional events and alteration in CYP3A4 protein stability may be responsible for the discrepancy. Last but not least, the effect of RES on suppressing drug-inducible expression of CYP3A was also confirmed by employing primary mouse hepatocytes.

To elucidate the underlying mechanisms of the observed suppressive effect of RES on PXR-mediated CYP3A4 induction, further studies should be conducted. The complete function of PXR depends on the binding of ligands to the ligand-binding domain (LBD) of the receptor, interacting with protein cofactors, and subsequent heterodimerizing with retinoid X receptor α (RXR α) and binding to the responsive element in the promoter of the target genes. In the absence of ligands, PXR forms a complex with co-repressor proteins such as nuclear receptor co-repressor (NCoR) that inhibits the transcriptional activity. However, ligand-binding of PXR results in dissociation from the co-repressor and recruitment of co-activator proteins such as steroid receptor co-activators (SRC1 and SRC2) (3). It has been shown that KTZ disrupts the interaction of PXR with co-activator SRC1 (32). Furthermore, coumestrol inhibits PXR and SRC1 interactions regardless of the ligand presented (33). Therefore, it is of particular interest to determine 1) whether RES exerts its suppressive effects through the competition of binding to LBD with ligands, 2) whether RES inhibits ligand-induced dissociation from co-repressor and recruitment of co-activator proteins to PXR, and 3) whether RES interferes with the binding of PXR: RXR α heterodimer to CYP3A4 promoter.

In summary, our results revealed the suppressive effects of RES on the inducible expression of CYP3A4 enzyme through PXR signaling pathway. Therefore, it may be possible for RES to antagonize the expected elevation in the CYP3A-mediated drug metabolism by identified PXR activators.

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Conflicts of Interest

The authors have declared that there is no conflict of interest.

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