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Role of vitamin D receptor in the lithocholic acid-mediated CYP3A induction in vitro and in vivo

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Running title page

a) Running title: LCA induction of CYP3A4 through VDR rather than PXR

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d) Abbreviations used in this paper: CA, cholic acid; CDCA, chenodeoxycholic acid; CYP, cytochrome P450; DCA, deoxycholic acid; DMSO, dimethyl sulfoxide; FXR, farnesoid X receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; h, human; LCA, lithocholic acid; MOI, multiplicity of infection; PXR, pregnane X receptor; RIF, rifampicin; RT-PCR, reverse transcription-polymerase chain reaction; RXR, retinoid X receptor; siRNA, small interfering RNA; $TCID_{50}$, 50% titer culture infectious dose; UDCA, ursodeoxycholic acid; VD_3, 1α,25-dihydroxyvitamin D_3; VDR, vitamin D receptor.

Abstract

Lipophilic bile acids are suggested to be involved in the endogenous expression of CYP3A4 in human and experimental animals as ligands of nuclear receptors. To verify the nuclear receptor specificity, the bile acid-mediated induction of CYP3A4 has been studied in vitro and in vivo in the present study. Lithocholic acid (LCA) strongly enhanced the activities of *CYP3A4* reporter gene, which contained multiple nuclear receptor binding elements, in both HepG2 and LS174T cells. The introduction of small interfering RNA for human vitamin D receptor (VDR), but not for human pregnane X receptor, reduced the LCA-induced activation of the reporter gene in these cells, suggesting the major role of VDR in the LCA induction of CYP3A4. Consistently, oral administration of LCA (100 mg/kg/day for 3 days) increased Cyp3a protein levels in the intestine but not in the liver, where negligible level of VDR mRNA is detected. The selective role of VDR was tested in mice with the adenoviral overexpression of the receptor. Oral administration of LCA had no clear influence on the *CYP3A4* reporter activity in the liver of control mice. In mice with the adenovirally-expressed VDR, LCA treatment (100 or 400 mg/kg/day for 3 days) resulted in the enhanced reporter activities and increased levels of Cyp3a proteins in the liver. These results indicate the selective involvement of VDR, but not pregnane X receptor, in the LCA-mediated induction of both human and mouse CYP3As in vivo.

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Introduction

CYP3A4 is expressed in adult human liver and intestine (Obach et al., 2001; Paine et al., 2006; Shimada et al., 1994), and contributes to the metabolism of about one half of drugs prescribed (Li et al., 1995). In fact, it functions as a first-pass filter for orally absorbed chemicals in liver and intestine. Hepatic and/or intestinal levels of CYP3A4 activities are, however, altered by physiological and environmental factors such as nutrition, disease and the exposure to foreign chemicals including therapeutic drugs. Changes of its activities may result in the reduced drug efficacy or cause of the adverse effect in drug therapy. Thus, understanding of the molecular mechanism for the changes in CYP3A4 levels will provide valuable information on the optimization of drug therapy and the development of safe therapeutic drugs.

The CYP3A4 activity is enhanced by up-regulation of CYP3A4 gene transcription. The exposure to chemicals such as rifampicin (RIF), phenobarbital, and dexamethasone, increases CYP3A4 expression (Daujat et al., 1991; Kocarek et al., 1995; Schuetz et al., 1993). Nuclear receptors, mainly, pregnane X receptor (PXR) (Blumberg et al., 1998; Lehmann et al., 1998), vitamin D receptor (VDR) (Schmiedlin-Ren et al., 1997; Thompson et al., 2002), and constitutive androstane receptor (Goodwin et al., 2002) are considered to mediate CYP3A4 induction. These receptors, forming a heterodimer with retinoid X receptor (RXR), up-regulate the CYP3A4 gene transcription through the binding to *cis*-elements of CYP3A4 gene such as proximal PXR response element and distal nuclear receptor binding element

(Goodwin et al., 1999). Furthermore, the bile acid receptor farnesoid X receptor (FXR) has also been reported to activate the CYP3A4 gene transcription (Gnerre et al., 2004).

Bile acids are biosynthesized from cholesterol in liver, and circulate between liver and intestine. Major bile acids found in human body include cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA) and their conjugates. In addition to these compounds, ursodeoxycholic acid (UDCA) is used as a therapeutic drug for cholestasis. These unconjugated bile acids are known to induce CYP3A4 expression in primary culture of human hepatocytes (Schuetz et al., 2001). Although these bile acids are reported to activate nuclear receptors FXR (Makishima et al., 1999), PXR (Staudinger et al., 2001; Xie et al., 2001), and VDR (Makishima et al., 2002), a mechanism by which bile acids mediate the CYP3A4 gene activation has not been fully understood.

To assess the PXR-involvement in the chemical-mediated activation of the CYP3A4 gene, we have established an adenovirus vector expressing human PXR (hPXR)-small interfering RNA (AdhPXR-siRNA) that is able to specifically knock-down the hPXR expression (Matsubara et al., 2007). The adenoviral siRNA system was successfully used to identify a potential hPXR activator causing CYP3A4 induction, and to distinguish the PXR-mediated CYP3A4 gene activation from the VDR-mediated one (Matsubara et al., 2007). In addition, we have also established the in vivo reporter assay system using an adenovirus vector (Furukawa et al., 2002) to identify a hPXR activator (Matsubara et al., 2007).

In the present study, we have assessed the bile acid-mediated induction of hepatic and intestinal CYP3A expression and verified the role of PXR and VDR in the induction using the combination of adenoviral siRNA and in vivo reporter assay techniques. The results presented here indicate the possibility that LCA activates the CYP3A4 gene promoter rather selectively through VDR than PXR.

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Materials and Methods

Materials

Restriction enzymes were purchased from New England BioLabs (Beverly, MA). RIF, LCA and 1α,25-dihydroxyvitamin D_3 (VD_3) were purchased from Sigma-Aldrich (St. Louis, MO). Protein Assay dye reagent concentrate was purchased from Bio-Rad Laboratories (Hercules, CA). Other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) unless otherwise stated. HepG2 and LS174T cells were obtained from Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan).

Construction of recombinant adenovirus

AdhVDR-siRNA: Human VDR (hVDR)-specific siRNA, designed by Takara (Ohtsu, Japan), was amplified by PCR with primers, 5’-CGCGTCGACGTGCCATTGAGGTCATCATTTCAAGAGAAT-3’ and 5’-CGCAAGCTTAAAAAAGTGCCATTGAGGTCATCATTTCAAGAGAAT-3’. The PCR product was digested with SalI and HindIII and cloned into the same restriction sites of the pShuttle-H1 (Kamiyama et al., 2007). The construction of an adenovirus expressing hVDR-siRNA (AdhVDR-siRNA) was performed with AdEasy™ System (MP Biomedicals, Irvine, CA) according to the manufacturer's protocol.

AdhVDR: The DNA encoding hVDR was amplified by PCR with primers,

5'-GTCGACATGGCGGCCAGCACTTCCCTGCCTGACC-3' and

5'-TCTAGATCAGGAGAGATCTCATTGCCAAACACTTCG-3', and cDNA of LS174T cells as a template. The DNA was digested with SalI and XbaI and ligated into the same restriction sites of the pShuttle-CMV (MP Biomedicals). The construction of an adenovirus expressing hVDR was performed with AdEasy™ System.

AdhPXR-siRNA was reported previously (Matsubara et al., 2007). A non-expressing adenovirus (AdEmpty) was constructed form pShuttle-CMV with AdEasy™ System. AdLacZ (Matsubara et al., 2007) or AdEmpty was used as a control adenovirus. The titer of the recombinant adenovirus, 50% titer culture infection dose ($TCID_{50}$) and multiplicity of infection (MOI), were determined as reported previously (Matsubara et al., 2007).

Animal treatment and Cell culture

Male C57BL/6 mice were purchased from Charles River Laboratories Japan, Inc (Yokohama, Japan) and fed standard rodent chow (CE-2; CLEA Japan, Tokyo, Japan) and water ad libitum. Mice were injected intravenously with AdCYP3A4-362-7.7k (3.4×10^9 $TCID_{50}$/mouse), AdLacZ (0.44×10^9 $TCID_{50}$/mouse), and either AdEmpty or AdhVDR (0.44×10^9 $TCID_{50}$/mouse). Two days after the infection, vehicle (0.5% methyl cellulose/1% ethanol in saline) or LCA (50, 100 or 400 mg/kg/day) was administered orally for 3 consecutive days and the animals were killed 20 h after the last dose. Culture of HepG2 cells

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was performed as described previously (Furukawa et al., 2002). LS174T cells were maintained in Eagle's Minimum Essential Medium (MP Biomedicals, Solon, OH) supplemented with MEM nonessential amino acids (Invitrogen, Carlsbad, CA), Antobiotic-Antimycotic (Invitrogen), and 10% fetal bovine serum (Sigma-Aldrich). Adenovirus infection was carried out as described previously (Furukawa et al., 2002). To investigate bile acid induction of CYP3A4, HepG2 and LS174T cells in a 24-well plate (2.0 x 10^4 cells/well) were infected with AdCYP3A4-362-7.7k at MOI of 50 before the treatment with various bile acids and determination of luciferase activities. To investigate the role of nuclear receptors in LCA induction of CYP3A4, cells were infected with either AdhPXR-siRNA (MOI of 8.3), AdhVDR-siRNA (MOI of 5.0), or AdLacZ (control), in addition to AdCYP3A4-362-7.7k (MOI of 50), where total MOI was adjusted to 100 with AdLacZ.

Determination of mRNA levels

Total RNA was extracted from culture cells or animal tissues by the acid guanidine thiocyanate-phenol-chloroform method. cDNA was synthesized from the total RNA with Ready-To-Go™ You-Prime First-Strand Beads (GE Healthcare). PXR, VDR, FXR, RXRα, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were determined by conventional PCR with Taq DNA polymerase (ABgene, Epsom, UK) and specific primers shown in Table 1. For quantitative PCR analysis, cDNA was synthesized with High Capacity

cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), and VDR, PXR, RXRα, and GAPDH mRNA levels were determined with Power SYBR Green PCR Master Mix (Applied Biosystems) and ABI Prism 7000 (Applied Biosystems) using the primers shown in Table 1.

Immunoblot analysis

Mouse hepatic and small intestinal microsomes were prepared according to the method reported previously (Matsubara et al., 2004), separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was immunostained with the anti-CYP3A antibody (Kawano et al., 1987) and alkaline phosphatase-conjugated goat anti-rabbit IgG, and signals were visualized with 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium.

Luciferase assay

Cell lysates and liver cytosols were prepared for luciferase assays as described previously (Matsubara et al., 2007). Luciferase activities were determined with the Luciferase Assay System (Promega) and Turner Designs TD-20/20 Luminometer (Promega) according to the manufacture's instructions.

Statistical analysis

Statistical differences from the vehicle group were determined by unpaired Student's *t*-test. Statistical significance is indicated by *, **, and *** for $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

Results

Bile acid-mediated induction of CYP3A4 in HepG2 and LS174T cells

Bile acid inducibility of CYP3A4 gene was assessed with the CYP3A4 reporter gene in human hepatoma HepG2 and human colon tumor LS174T cells. Both primary and secondary bile acids such as CA, DCA, CDCA, UDCA, and LCA were tested in this system (Fig. 1). The strongest activations of the CYP3A4 reporter gene were detected in the presence of 10 μM LCA in both HepG2 and LS174T cells. Treatment with 100 μM UDCA also mildly increased the reporter activity in HepG2 cells. Other treatments had minor or little effects on the reporter activities. Because only LCA showed the marked activation of CYP3A4 reporter gene, we hereafter focused on the LCA-mediated induction of CYP3A4.

Assessment of nuclear receptor involved in the LCA-mediated activation of the CYP3A4 gene in HepG2 and LS174T cells

Dose-dependent profiles of the LCA- and RIF-mediated *CYP3A4* activation in HepG2 and LS174T cells were compared (Fig. 2A). LCA-mediated activation of CYP3A4 reporter gene was detected at as low as 1 μM in LS174T cells, whereas only observed at over 10 μM in HepG2 cells. Moreover, clearly higher extent of activation in LS174T cells than in HepG2 cells was detected (35.4- and 9.4-fold, respectively at 10 μM LCA). In contrast, RIF activated the reporter gene more efficiently in HepG2 cells than in LS174T cells both at 1 and 10 μM. These results suggest a possibility that LCA mediates *CYP3A4* activation through a

mechanism distinct from that for RIF.

LCA has been shown to interact with multiple nuclear receptors. As shown in Fig. 2B, PXR, VDR, and RXRα mRNAs were detected both in HepG2 and LS174T cells, although much higher levels of VDR mRNA were detected in LS174T cells than in HepG2 cells. In contrast, FXR mRNA clearly detected in HepG2 but not in LS174T cells. Constitutive androstane receptor mRNA was not detected in either cell line in the present experiment (data not shown).

To verify the involvement of these receptors in LCA induction of CYP3A4, siRNA-expressing adenoviruses were employed. Introduction of siRNA for hPXR by AdhPXR-siRNA did not affect the LCA-mediated activation of the *CYP3A4* reporter in HepG2 (Fig. 2C) and LS174T cells (Fig. 2D), while it attenuated the RIF-mediated activation of the reporter. Introduction of siRNA for hVDR by AdhVDR-siRNA reduced LCA-mediated as well as VD_3-mediated activation of the *CYP3A4* reporter both in HepG2 cells and LS174T cells (Figs. 2C and 2D). These results suggest that VDR rather than PXR is responsible for the LCA-mediated activation of CYP3A4 gene. To confirm the effect of AdhVDR-siRNA on the hVDR expression, nuclear receptor mRNA levels were determined by quantitative reverse transcription-PCR in LS174T cells infected with AdhVDR-siRNA at MOI of 5. mRNA levels of hVDR (63 ± 5% that of control) but not hPXR and hRXRα were significantly decreased in the cells 3 days after adenovirus infection (data not shown).

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Influence of hVDR expression on the LCA-mediated activation of the CYP3A4 gene in mouse liver

To evaluate the role of VDR in the LCA-mediated CYP3A induction in vivo, mice were treated with LCA and Cyp3a protein levels were determined in the liver and intestines. As shown in Fig. 3A, PXR mRNA levels did not differ between liver and intestine of mice, while VDR mRNA was only detected in intestines. Mice, treated with LCA (100 mg/kg/day) for 3 consecutive days, showed increased Cyp3a protein levels in intestines but not in livers (Fig. 3B).

To further assess the role of VDR, the influence of hVDR introduction on the LCA-induced CYP3A4 reporter activity and Cyp3a expression in mice was investigated. To confirm adenovirus-mediated hVDR expression, hVDR mRNA in mouse livers was amplified. As expected the mRNA was detected in the liver of mice infected with AdhVDR but not with AdEmpty (Fig. 3C). Results of in vivo reporter assays were shown in Fig. 3D. LCA treatment did not affect the levels of CYP3A4 reporter activity significantly in control mice. However, introduction of hVDR into mouse liver markedly enhanced the LCA-mediated activation of the CYP3A4 reporter (18.4-, 34.6- and 32.4-fold at 50, 100, and 400 mg/kg/day, respectively). In addition to the reporter activity, microsomal Cyp3a protein levels were determined in the liver used for the reporter assay (Fig. 3E). Consistent with the results with the *CYP3A4* reporter, LCA treatment did not increase microsomal Cyp3a protein levels in control mouse livers. With the overexpression of hVDR, hepatic Cyp3a protein levels were increased after

the administration of LCA at 100 mg/kg/day (2.3-fold).

Discussion

Nuclear receptor selectivity of bile acid-mediated CYP3A4 induction was studied with human-derived cells and *in vivo* in mice. Among the bile acids tested, LCA showed the most drastic enhancement of the CYP3A4 reporter activities both in HepG2 and LS174T cells (Fig. 1). LCA is known to activate PXR (Staudinger et al., 2001; Xie et al., 2001), VDR (Makishima et al., 2002), and FXR (Makishima et al., 1999). In further, these nuclear receptors have been reported to mediate CYP3A induction (Blumberg et al., 1998; Gnerre et al., 2004; Lehmann et al., 1998; Schmiedlin-Ren et al., 1997). In the present study, a typical FXR ligand CDCA weakly activated the *CYP3A4* reporter in HepG2 but not in LS174T cells. CA activated in neither of cells (Fig. 1). These data supported a minor, if any, role of FXR on the LCA-mediated induction of CYP3A4 in vivo. Thus the possible involvement of PXR and/or VDR in the LCA-induced expression of CYP3A4 gene was further examined. Introduction of siRNA for hVDR but not for hPXR was found to drastically reduce the LCA activation of the CYP3A4 reporter gene both in HepG2 and LS174T cells (Figs. 2C and 2D). The higher CYP3A4 reporter activities were detected in LS174T cells than in HepG2 cells. These results suggest that LCA distinctively activates CYP3A4 gene transcription, compared to other bile acids tested, via VDR rather than PXR at least in culture cells.

LCA has been reported to increase liver and intestinal Cyp3a11 mRNA levels (Makishima et al., 2002; Staudinger et al., 2001; Xie et al., 2001). Both PXR and VDR are activated by LCA, and thus it remains obscure whether both PXR and VDR mediate this

phenomenon in vivo. In this study, influence of LCA treatment on Cyp3a levels was determined in both small intestine and liver. Cyp3a induction was observed only in small intestine (Fig. 3B). Furthermore, no clear induction of hepatic Cyp3a was detected even after the administration of 400 mg/kg of LCA. Adenoviral introduction of hVDR into mouse livers, however, evoked LCA-mediated Cyp3a induction (Fig. 3E). These results are consistent with the previous report on LCA-mediated increase of CYP3A11 mRNA levels in small intestine of PXR-null mice (Makishima et al., 2002) and support the idea that LCA-induced Cyp3a expression in vivo is mediated selectively by VDR. In this study, we further demonstrated that the *CYP3A4* reporter was activated by LCA in mouse liver only in the presence of hVDR (Fig. 3D). These results, together with the data obtained with culture cells, suggest that LCA activates intestinal CYP3A4 gene expression through VDR in human as well as mice.

LCA is known as a liver toxicant in mice (Hofmann, 2004). Treatment of LCA-treated mice with a strong murine PXR activator, pregnenolone 16α-carbonitrile, reversed its hepatotoxicity through the expression of LCA-metabolizing sulfotransferase St2a enzymes (Miyata et al., 2006a). These results are consistent with the idea that LCA hardly acts as a PXR ligand in mouse livers.

FXR is a possible mediator for the LCA induction of CYP3As. Recently, we have reported that LCA treatment of mice increases CDCA levels in the liver (Miyata et al., 2006b). CDCA is a strong FXR activator and is shown to enhance CYP3A4 expression in human hepatocytes (Schuetz et al., 2001). It is, thus, possible that FXR activated with CDCA

increases the Cyp3a gene expression. This is, however, unlikely because FXR also increases the hepatic level of SHP (Goodwin et al., 2000; Lu et al., 2000), which inhibits the CYP3A gene transcription (Ourlin et al., 2003). This idea is supported by our results showing that CDCA barely activated the CYP3A4 reporter gene in culture cells (Fig. 1).

Recently LCA was reported to function as a substitute for vitamin D in rats fed a vitamin D-deficient diet, although its action was weaker than that of VD_3 (Nehring et al., 2007). VD_3 was reported to induce CYP3A4 expression through the c-Jun N-terminal kinase pathway in Caco-2 cells (Yasunami et al., 2004). Therefore, LCA may be able to stimulate the c-Jun N-terminal kinase pathway as in the case of VD_3. Moreover, LCA and its conjugates are known to interact with M3 muscarinic receptor (Raufman et al., 2002) and G protein-coupled plasma membrane receptor TGR5 (Kawamata et al., 2003). These factors remain to be investigated.

CYP3A4 levels are known to show large inter-individual differences (Wolbold et al., 2003). In addition, hepatic CYP3A4 activities do not necessarily correlate with intestinal CYP3A4 activities within individuals (Lown et al., 1994). The exact mechanisms causing these differences have not been well understood. Our results suggest that LCA is an intestine-specific CYP3A4 inducer. Inter-individual differences in the serum LCA levels in humans are reported (Campbell et al. 1975; Cowen et al., 1977). Moreover, it is reported that average serum concentrations of total bile acids in healthy subjects and hepatitis patients are 5.3 μM (1.1 – 16.4 μM) and 44.9 μM (2.7 – 80.3 μM), respectively, and that LCA accounts

for 13% (0-32%) and 18% (0-53%) of the total bile acid in healthy subjects and in hepatitis patients, respectively (Campbell et al. 1975). Because LCA undergoes enterohepatic circulation, its concentration in intestines could be high enough to activate VDR. These facts suggest that LCA is a possible factor causing inter-individual differences in the CYP3A4 expression levels in intestines.

In conclusion, we have investigated the bile acid inducibility of hepatic and intestinal CYP3A4 expression and the role of PXR and VDR in the CYP3A4 induction. With combined use of the siRNA expression system and the CYP3A4 reporter gene assay, we have demonstrated that LCA can enhance the CYP3A gene expression in intestines through VDR rather than PXR. Because LCA-mediated CYP3A induction was observed in mouse livers with the ectopic expression of hVDR, our present results further suggest that the expression profile of transcription factors is a major determinant for the tissue-dependency of CYP3A induction in response to a xenobiotic/endogenous compound.

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Footnotes

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Legend to Figures

Fig. 1. Bile acid-induced activation of CYP3A4 reporter gene in HepG2 and LS174T cells

HepG2 (A) or LS174T (B) cells in a 24-well plate (2.0 x 10^4 cells/well) were infected with AdCYP3A4-362-7.7k (MOI of 50) and were treated with vehicle (0.1% DMSO) or 1 to 100 μM of bile acids (only 10 μM for LCA) for 48 h, and then luciferase activities were determined as described in Materials and Methods. The activities normalized by protein concentration are expressed as ratio to those in the vehicle-treated cells. Data represent the mean ± SD (n = 4).

Fig. 2. Role of VDR in the LCA-induced activation of CYP3A4 reporter gene in vitro

A. HepG2 or LS174T cells in a 24-well plate (2.0 x 10^4 cells/well) were infected with AdCYP3A4-362-7.7k (MOI of 50) and were treated with vehicle (0.1% DMSO) or 0.001 to 10 μM of LCA or RIF for 48 h, and then luciferase activities were determined as described in Materials and Methods. The activities normalized by protein concentration are expressed as ratio to those in the vehicle-treated cells. Data represent the mean ± SD (n = 4).

B. HepG2 or LS174T cells were seeded at density of 5.0 x 10^4 cells/well in a 6-well plate and total RNA was prepared 4 d later. RT-PCR was carried out with pooled RNA (n = 3) as described in Materials and Methods.

C and D. HepG2 (C) or LS174T (D) cells in a 24-well plate (3.3 x 10^4 cells/well)

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were infected with AdCYP3A4-362-7.7k (MOI of 50) and either of AdhPXR-siRNA (MOI of 8.3), AdhVDR-siRNA (MOI of 5.0), or AdLacZ (control). Total MOI was adjusted to 100 with AdLacZ. Three days after the infection, the cells were treated with vehicle (0.1% DMSO), 10 μM RIF, 10 nM VD_3, or 10 μM LCA, and luciferase activities were determined as described in Materials and Methods. The activities are expressed as ratio to those in the vehicle-treated cells. Data represent the mean ± SD (n = 4).

Fig. 3. Role of VDR in the LCA-mediated CYP3A induction in mouse livers

A. Mouse PXR, VDR and GAPDH mRNAs in the liver and small intestine were amplified by RT-PCR as described in Materials and Methods. In lane N, PCR was carried out without cDNA solution.

B. Male mice (7-week-old) were orally administered vehicle (0.5% methyl cellulose/1% ethanol in saline) or LCA (100 mg/kg/day) for 3 consecutive days. Mouse Cyp3a proteins were detected by immunoblot analyses as described in Materials and Methods. A portion of microsomal proteins (5 μg for liver or 30 μg for small intestine) was loaded on each lane. Bands indicated by arrows showed the same electrophoresis mobility.

C-E. Control group mice (AdEmpty) were infected with AdCYP3A4-362-7.7k (3.4×10^9 $TCID_{50}$/mouse), AdEmpty (0.44×10^9 $TCID_{50}$/mouse), and AdLacZ (0.44×10^9 $TCID_{50}$/mouse), while hVDR group mice (AdhVDR) were infected with AdCYP3A4-362-7.7k (3.4×10^9 $TCID_{50}$/mouse), AdhVDR (0.44×10^9 $TCID_{50}$/mouse), and

AdLacZ (0.44 x 10^9 $TCID_{50}$/mouse). AdLacZ expressing β-galactosidase was used to normalize the infection efficiency. Two days after the infection, mice were orally treated with vehicle (0.5% methyl cellulose/1% ethanol in saline) or LCA (100 mg/kg/day) for 3 consecutive days. In C, hepatic total RNA was prepared and cDNA was synthesized individually. hVDR and moue GAPDH mRNAs were amplified from pooled cDNA (n = 3). In D, luciferase activities in the liver were determined and normalized by β-galactosidase activities as described in Materials and Methods. In E, mouse Cyp3a protein levels were determined by immunoblot analyses as described in Materials and Methods. The levels in vehicle-treated mice were set at 1. Columns represent the mean ± SD (n = 3).

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Table 1. Primer sequences for RT-PCR

	Sense/antisense	Sequence
Human		
PXR	sense	5´-CAACACAGTGTTCAACGCG-3´
	antisense	5´-GAAGAGGGAGATGGCCTGC-3´
VDR	sense	5´-CTCTTCAGACATGATGGACTCG-3´
	antisense	5´-GGATGCTGTAACTGACCAGG-3´
FXR	sense	5´-ACAGAGCCTCTGGATACCAC-3´
	antisense	5´-TGTACATATCCATCACACAGTTGC-3´
RXRα	sense	5´-AGCTTGTGTCCAAGATGCG-3´
	antisense	5´-ACTTGTGCTTGCAGTAGGCC-3´
GAPDH	sense	5´-AACAGCCTCAAGATCATCAGC-3´
	antisense	5´-GGATGATGTTCTGGAGAGCC-3´
Mouse		
PXR	sense	5´-GCTGATGGACGCTCAGATGC-3´
	antisense	5´-GAAGCTCACAGCCACTGTGG-3´
VDR	sense	5´-CATCACTGATGTCTCCAGAG-3´
	antisense	5´-CCAGCTTAGCATCCTGTACC-3´
GAPDH	sense	5´-TGCATCCTGCACCACCAACTG-3´
	antisense	5´-GTCCACCACCCTGTTGCTGTAG-3´

Figure 1A

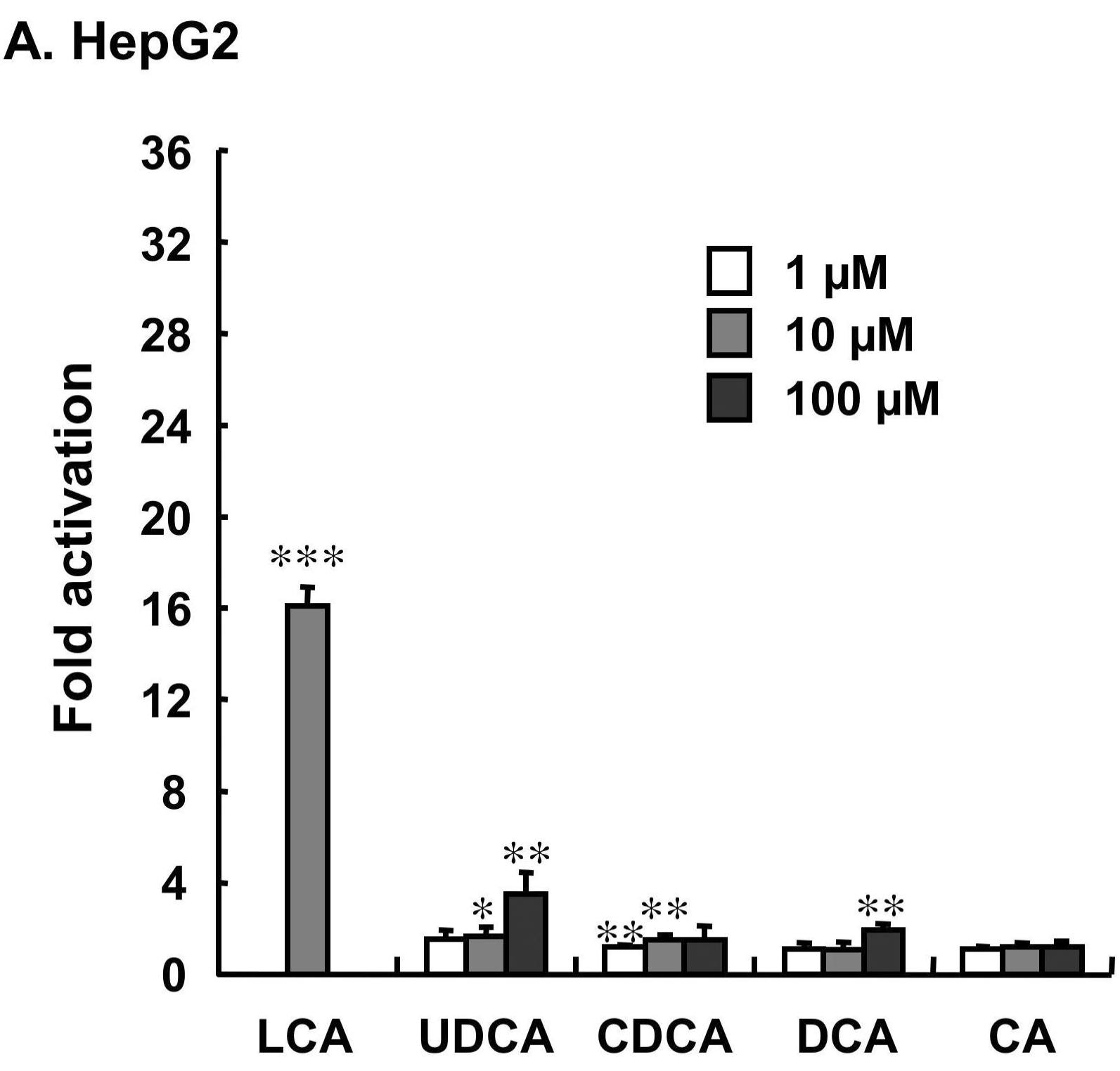

A. HepG2
Fold activation
36
32
28
24
20
16
12
8
4
0
1 μM
10 μM
100 μM
***
*
**
**
**
**
LCA
UDCA
CDCA
DCA
CA

Figure 1B

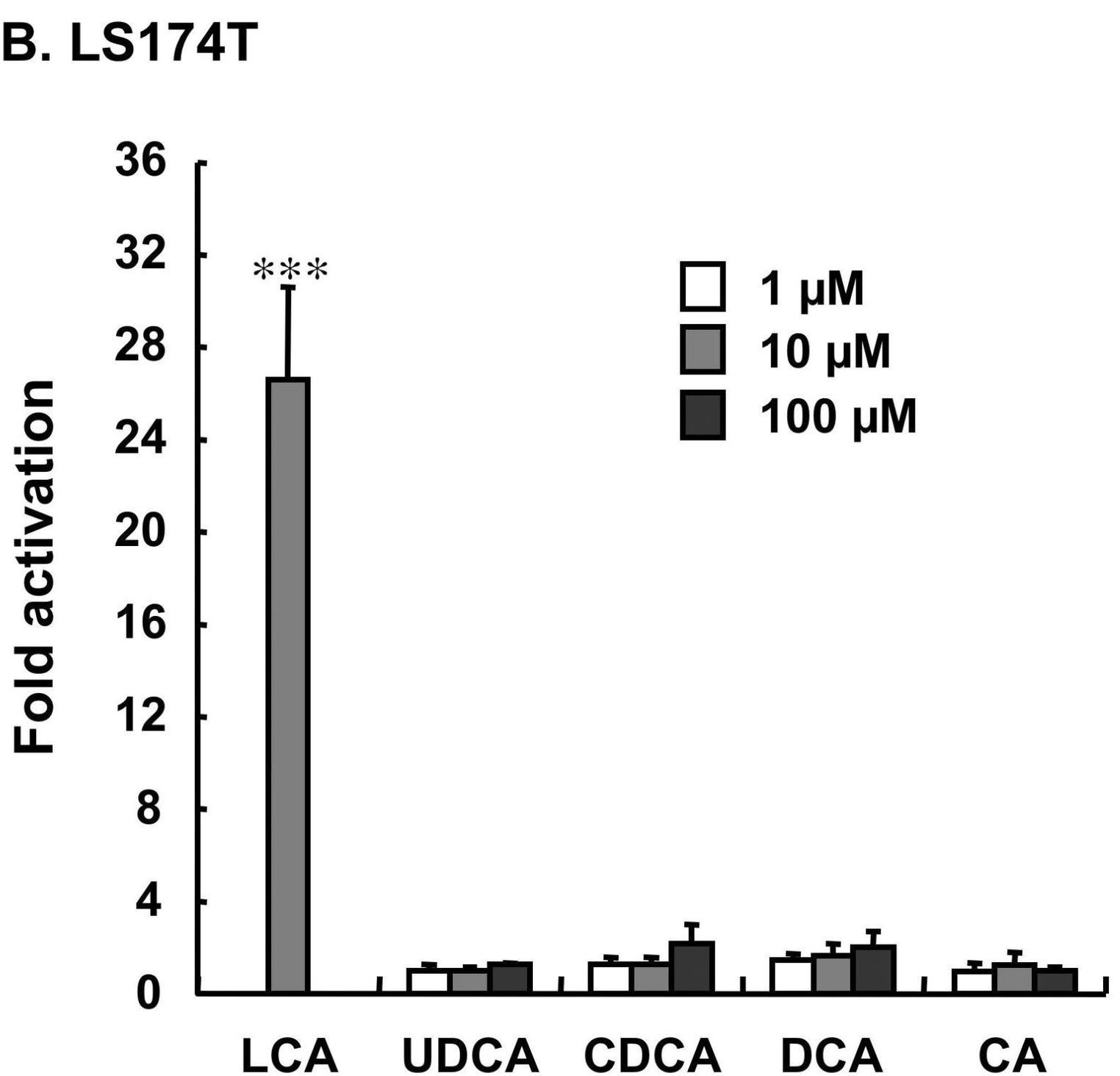

B. LS174T
Fold activation
36
32
28
24
20
16
12
8
4
0
***
1 μM
10 μM
100 μM
LCA
UDCA
CDCA
DCA
CA

Figure 2A

A

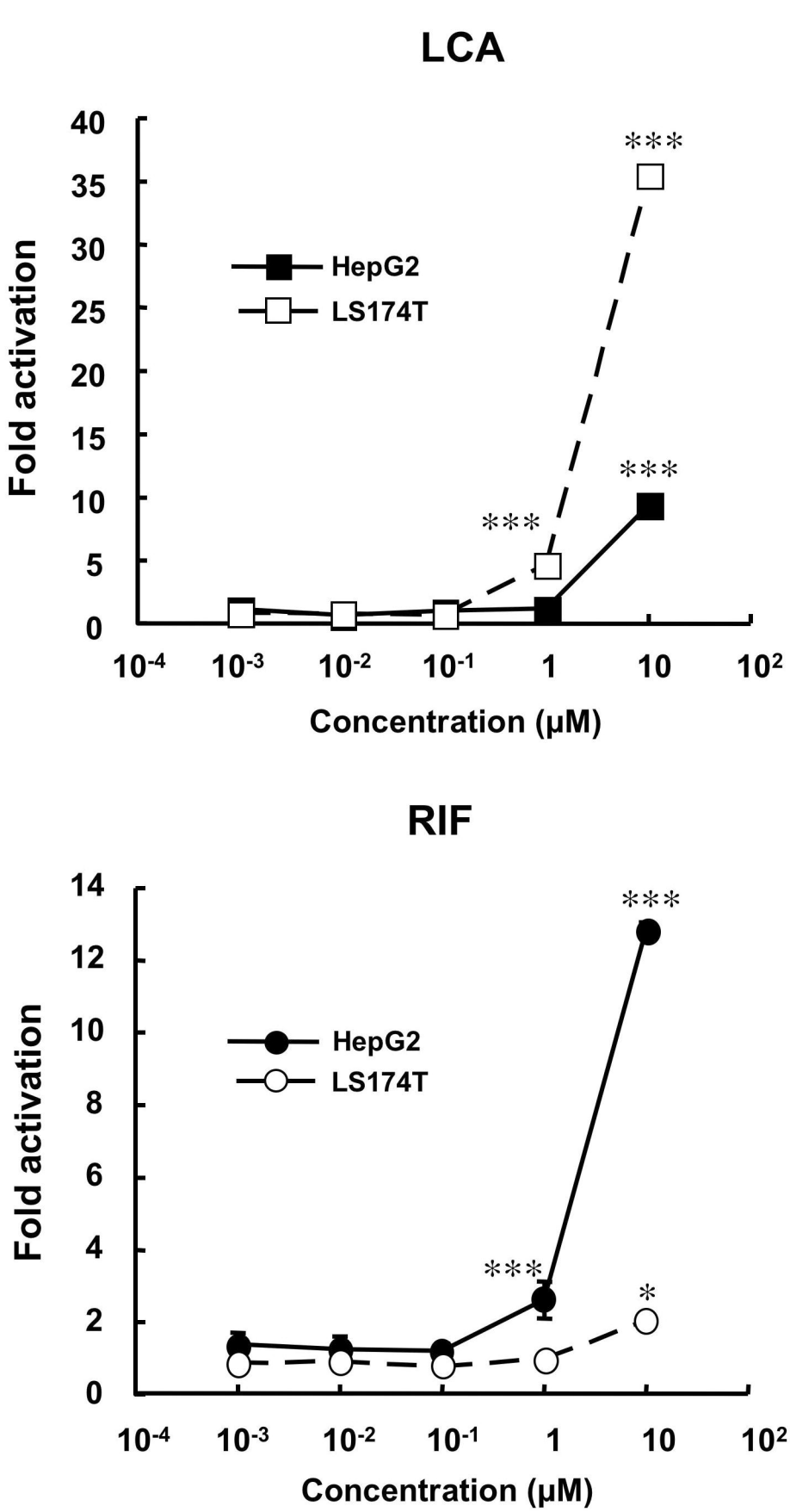

LCA
Fold activation
40
35
30
25
20
15
10
5
0
HepG2
LS174T
***
***
***
10-4
10-3
10-2
10-1
1
10
102
Concentration (μM)
RIF
Fold activation
14
12
10
8
6
4
2
0
HepG2
LS174T
***
***
*
10-4
10-3
10-2
10-1
1
10
102
Concentration (μM)

Figure 2B

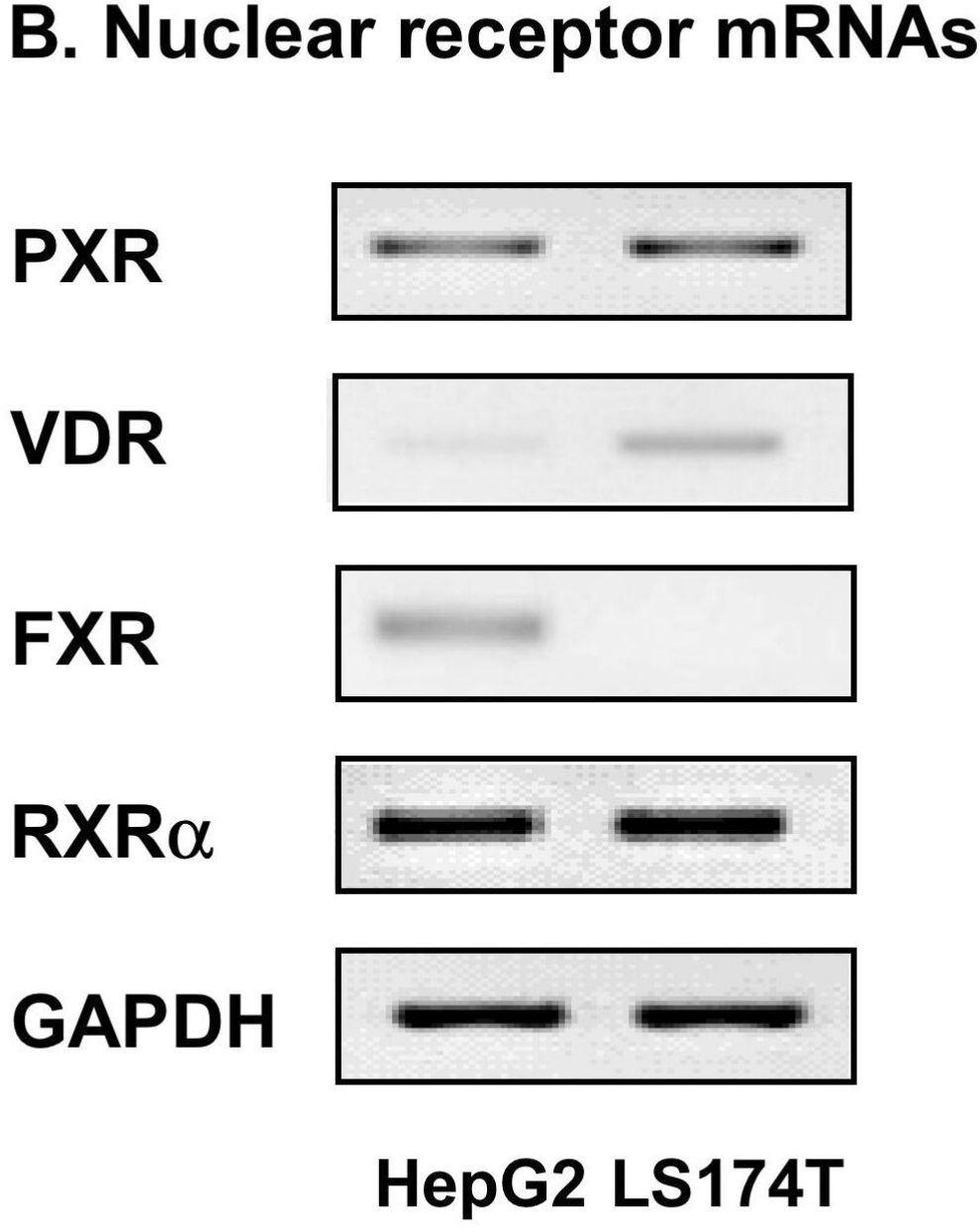

B. Nuclear receptor mRNAs
PXR
VDR
FXR
RXRα
GAPDH
HepG2 LS174T

Figure 2C and 2D

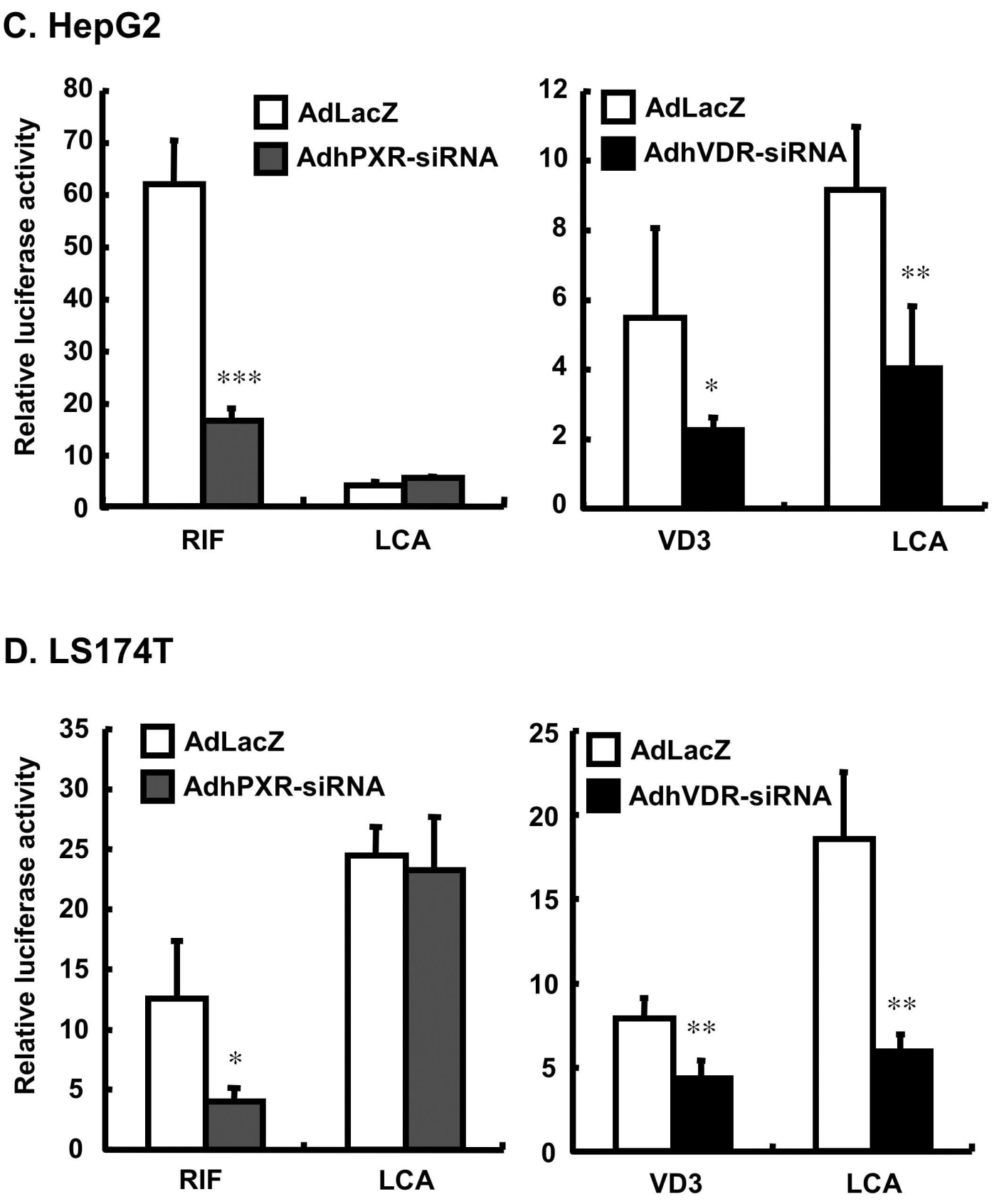


Figure 3A, 3B, and 3C

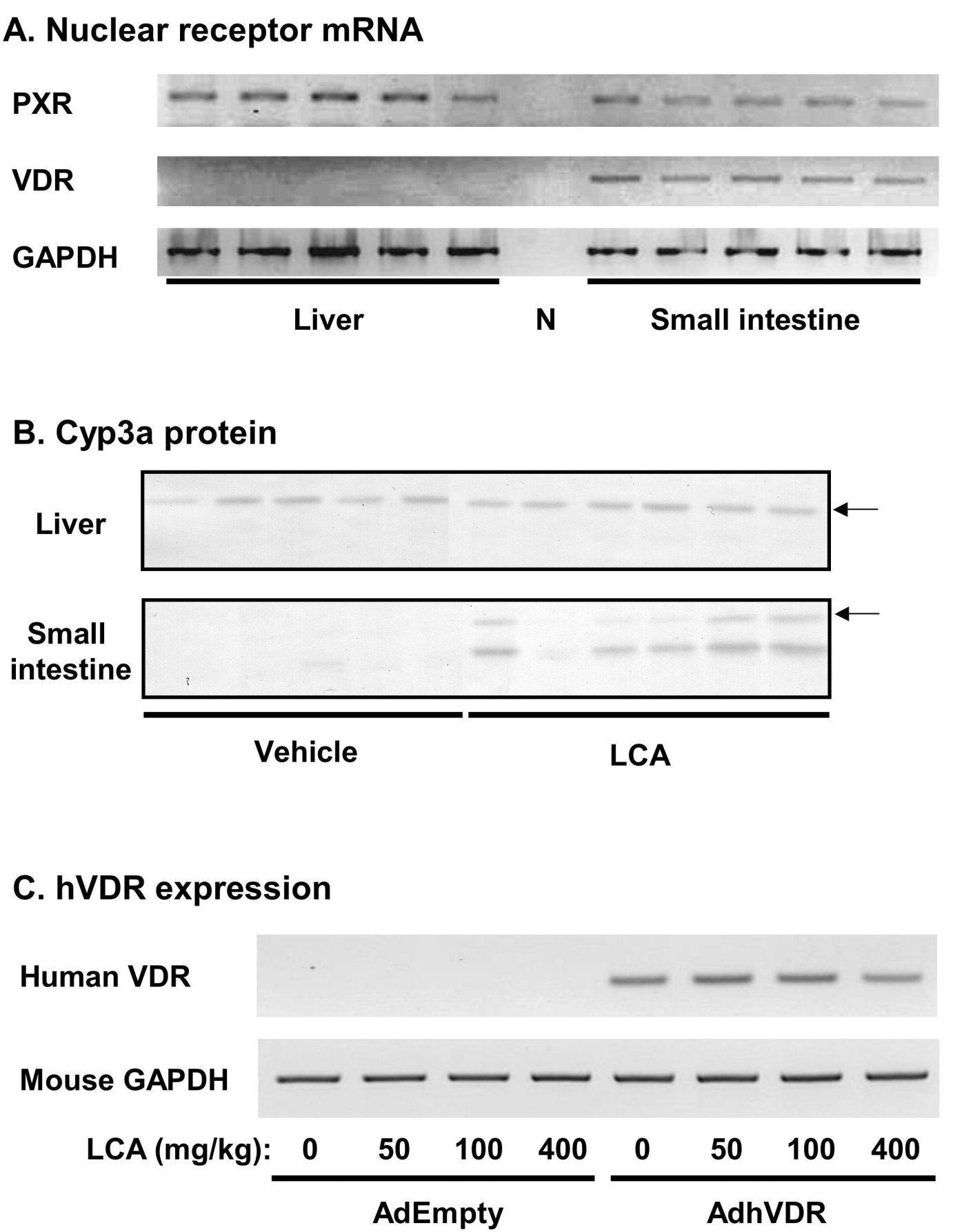


Figure 3D

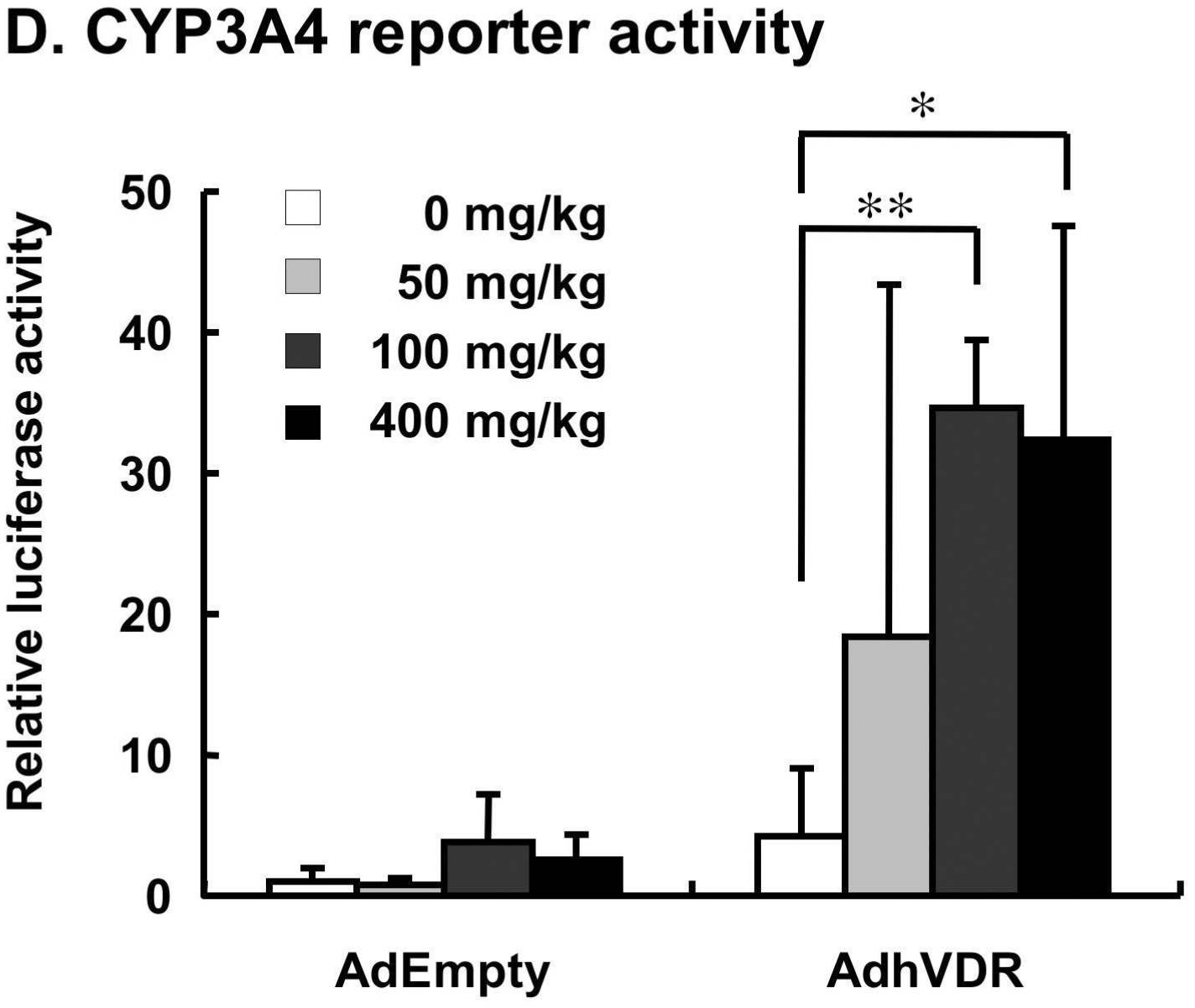
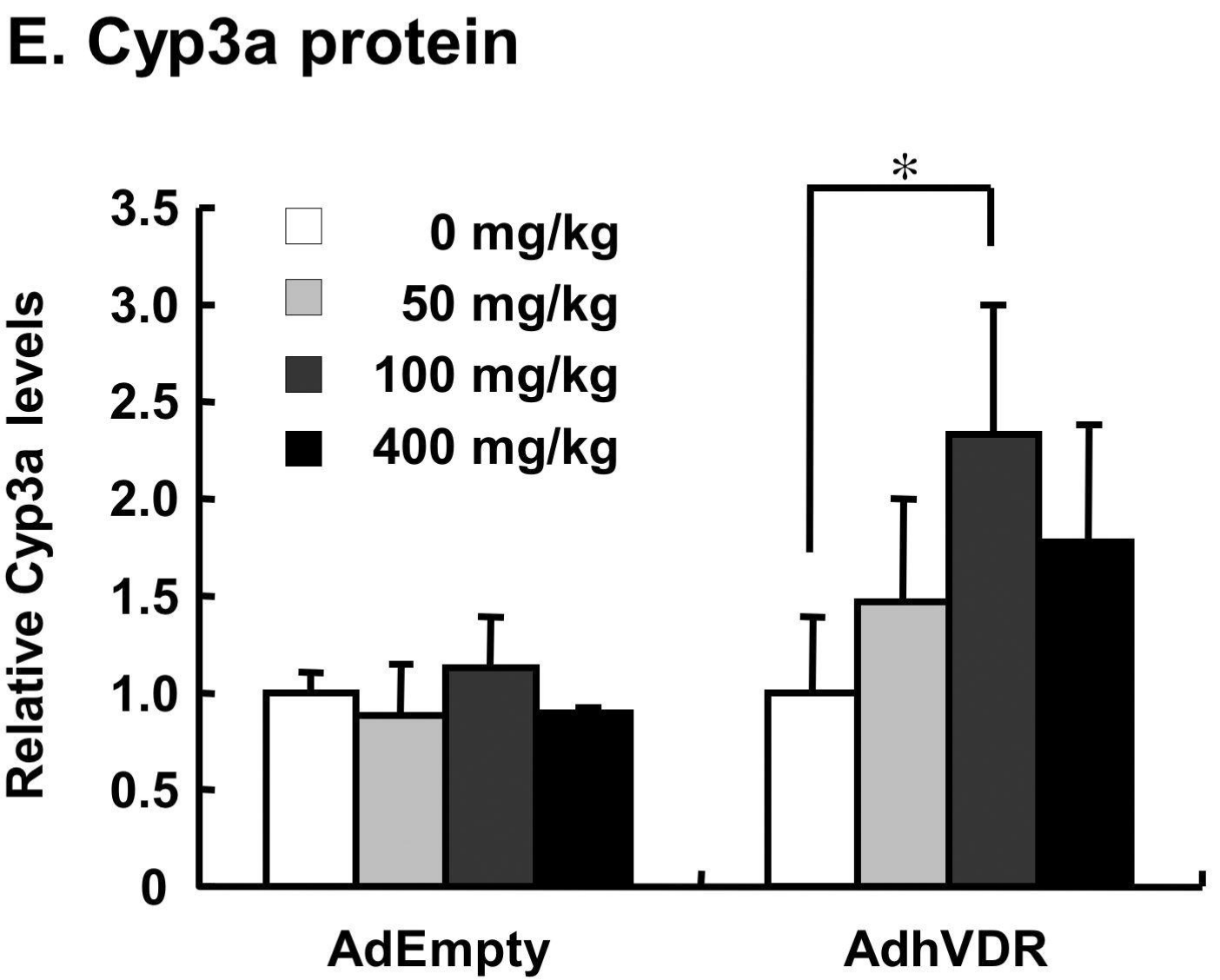

D. CYP3A4 reporter activity
Relative luciferase activity
50
40
30
20
10
0
0 mg/kg
50 mg/kg
100 mg/kg
400 mg/kg
*
**
AdEmpty
AdhVDR

Figure 3E


E. Cyp3a protein
Relative Cyp3a levels
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
0 mg/kg
50 mg/kg
100 mg/kg
400 mg/kg
*
AdEmpty
AdhVDR