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Title page

Stimulation of AMP-activated protein kinase is essential for the induction of drug metabolizing enzymes by phenobarbital in human and mouse liver.

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Running Title page

Running Title: Induction of P450 expression by phenobarbital requires AMPK.

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Abbreviations, AMPK: 5'-AMP-activated protein kinase , AICAR: 5'-phosphoribosyl-5-aminoimidazol-4-carboxamide 1-beta-D-ribofuranoside, TCPOBOP:1,4 bis[2-(3,5-dichloropyridyloxy)]benzene, CITCO: 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime, CAR: Constitutive Active Receptor

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Abstract

Our previous studies have suggested a role for AMP-activated protein kinase (AMPK) in the induction of CYP2B6 by PB (PB) in hepatoma-derived cells (Rencurel et al.,2005). Here, we show in primary human hepatocytes that: 1) 5'-phosphoribosyl-5-aminoimidazol-4-carboxamide 1-beta-D-ribofuranoside (AICAR) and the biguanide metformin, known activators of AMPK, dose-dependently increase the expression of CYP2B6 and CYP3A4 to a similar extent as does PB. 2) Phenobarbital, but not the human nuclear receptor constitutive active/androstane receptor (CAR) ligand 6-(4-chlorophenyl)imidazol[2,1-6][1,3]thiazole-5-carbaldehyde (CITCO), dose-dependently increase AMPK activity. 3) Pharmacological inhibition of AMPK activity with compound C or dominant-negative forms of AMPK blunt the inductive response to phenobarbital. Furthermore, in transgenic mice with a liver-specific deletion of both the α1 and α2 AMPK catalytic subunits, basal levels of Cyp2b10 and Cyp3a11 mRNA were increased but not in primary culture of mouse hepatocytes. However, phenobarbital or 1,4 bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), a mouse CAR ligand, failed to induce the expression of these genes in the liver or cultured hepatocytes from mice lacking hepatic expression of the α1 and α2 subunits of AMPK. The distribution of CAR between the nucleus and cytosol was not altered in hepatocytes from mice lacking both AMPK catalytic subunits. These data highlight the essential role of AMPK in the CAR-mediated signal transduction pathway.

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Introduction

Induction of drug-metabolizing enzymes and drug transporters by drugs and other chemicals is an adptative response of mammals and other organisms to increase the removal of potentially toxic endobiotics and xenobiotics. Phenobarbital (PB) represents a class of inducers where the effect on detoxification is part of a pleiotropic response which includes liver hypertrophy, tumour promotion and induction or repression of multiple genes in addition to genes coding for enzymes or transporters that regulate drug disposition. The molecular mechanism of the induction response remains incompletely understood. The induction of human cytochrome P450 CYP2B6 and its rat and mouse orthologs CYP2B1 and Cyp2b10 by PB is mediated by the nuclear receptor constitutive active/androstane receptor (CAR, NR1I3) (Honkakoski et al.,1998). In untreated primary mouse hepatocytes, CAR is retained in the cytoplasm within a protein complex of chaperones and co-chaperones such as heat shock protein 90 (HSP90) and a protein called cytoplasmic CAR retaining protein (CCRP) (Kobayashi et al.,2003). Exposure to xenobiotics such as PB causes CAR to dissociate from this complex and to transfer into the nucleus where it forms a heterodimer with retinoid X receptor and binds to cognate DNA sequences of target genes (Kawamoto et al.,1999). This process is influenced by phosphorylation and dephosphorylation of unknown proteins (Hosseinpour et al.,2005). In human hepatoma cells such as HepG2, CAR is exclusively found in the nucleus and constitutively active, resulting in CYP2B6 gene expression in cells not exposed to any inducers (Sueyoshi et al.,1999). Co-expression of exogenous CCRP with exogenous CAR in HepG2 cells confirms the cytoplasmic retention of CAR by CCRP but no nuclear transfer is observed upon drug treatment (Kobayashi et al. ,2003). This suggests that additional proteins or reactions are required for drug induced CAR cytoplasmic-nuclear translocations which are missing or non-functional in HepG2 cells. Because hepatoma cells

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do not reciprocate the physiological activation of CAR or its binding to DNA, we decided to focus on CAR in primary human and mouse hepatocytes.

In the liver of fasted mice, CYP2b10 and mCAR expression are induced (Maglich et al.,2004). AMP-activated protein kinase (AMPK) functions as an energy sensor and is activated when cells experience energy depleting stresses such as nutrient starvation (Carling,2004). AMPK is also activated by pharmacological manipulations. 5'-phosphoribosyl-5-aminoimidazol-4-carboxamide 1-beta-D-ribofuranoside (AICAR) is a cell permeant adenosine analogue commonly used to activate AMPK. Although activation of AMPK as a consequence of an increase in the AMP:ATP ratio is the best described mechanism, some studies suggest alternative mechanisms of AMPK activation.

AMPK is a heterotrimeric complex ubiquitously expressed and consists of a catalytic subunit, α, and two regulatory subunits, β and γ (Woods et al.,1996). All three subunits have been identified and each subunit is encoded by two (α1,α2, β1,β2) or three genes (γ1,γ2,γ3) Formation of the trimeric complex is necessary for optimal kinase activity. Changes in the cellular energy state activate AMPK through mechanisms involving an AMP allosteric regulation, and phosphorylation by an upstream kinase on threonine residue 172 within the alpha subunit (Stein et al.,2000). This upstream kinase has recently been identified in liver as LKB1 (Woods et al.,2003).

In a previous study, we have shown that PB can activate AMPK in a HepG2 derived hepatoma cell line, WGA, and that activation of AMPK by AICAR induced CYP2B6 (Rencurel et al. ,2005). However, the activation of AMPK was observed only at high concentrations of PB (above 1mM). Questions thus remain as to whether (a) AMPK activation by PB occurs only in transformed cells (2) or only at high concentrations of the inducer. Thus, it has remained unclear whether activation of AMPK is a necessary component of the PB signalling pathway in fully differentiated liver cells either *in vitro* and or *in vivo*.

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We therefore investigated the relationship between AMPK activation and induction of cytochromes P450 in primary human hepatocytes. Moreover, the recent development of transgenic mice with a deletion of AMPK catalytic subunits α1 and α2 in the liver provides a unique experimental model to address the role of AMPK in PB induction of CYP2b10 and CYP3a11 gene expression *in vivo* and *in vitro*. In primary human hepatocyte culture we now show a concentration-dependent activation of AMPK by PB. Through ablation of AMPK activity by (1) pharmacological approaches (2) or over-expressing a dominant negative form of AMPK or (3) or genetic ablation of AMPK α1 and α2 genes in mice we here provide unequivocal evidence that AMPK activation is a necessary step in the induction of CYP2B6 and Cyp2b10 by PB in both human and mouse hepatocytes.

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Materials and Methods.

Reagents

Phenobarbital (sodium salt) was purchased from Fluka (Buchs, Switzerland). All other chemicals were from Sigma (Buchs, Switzerland). Cell culture media, foetal bovine serum, other tissue culture reagents, and Trizol reagent were from Gibco-BRL Life-Sciences (Basel, Switzerland). Antibodies raised against AMPK α1 and α2 subunits and phospho-acetyl-CoA-carboxylase were purchased from Upstate Biotechnology (Lucern, Switzerland).

Generation of AMPKα1/α2 $^{LS-/-}$ knock out mice.

The liver-specific knock out of both alpha subunits of AMPK has previously been described (Guigas et al.,2006). Briefly, to generate deletion of both catalytic subunits in the liver (α1α2 $^{LS-/-}$), liver specific AMPKα2-null mouse were first generated by crossing floxed AMPKα2 mice (Viollet et al.,2003) and Alfp Cre transgenic mice expressing the Cre recombinase under the control of albumin and α-fetoprotein regulatory elements. A liver-specific AMPKα2 deletion was then produced on an AMPKα1 $^{-/-}$ general knock out background by crossing liver-specific α2$^{-/-}$ mice with AMPKα1-/- general knock out mice (Jorgensen et al.,2004).

Recombinant adenoviruses

Adenovirus encoding constitutively active α1 AMPK subunit (ad-CA-α1 ^{312}) or dominant negative mutant α1 AMPK (ad-DNα1) were prepared as described (Diraison et al.,2004). Adenoviruses encoding constitutively active α2 AMPK subunit (ad-CA-α2 ^{312}) or β-galactosidase were also amplified as previously described (Foretz et al.,2005). Adenovirus encoding human CAR in fusion with eGFP (ad-hCAR-GFP) was a kind gift of Dr Ramiro

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Jover (Hospital La Fe, Valencia, Spain). AMPK adenoviruses also express enhanced green fluorescence protein (eGFP), under control of a distinct CMV promoter. Viral particles were purified by cesium chloride density centrifugation and human and mouse hepatocytes were infected 12 hours post seeding with a Multiplicity of Infection (MOI) of 30 to 100.

Culture of primary human hepatocytes.

Primary human hepatocytes were isolated from the resected liver tissue of consented patients undergoing liver surgery. Human hepatocytes were enzymatically dissociated from human liver samples using a two-step enzymatic microperfusion technique with collagenase and kept on ice in suspension (Strain,1994). Hepatocytes were subsequently seeded on rat tail collagen-coated plastic dishes (25μg/cm^2) at a density of 130,000 viable cells/cm^2 and cultured in Dulbeco's Minimum Essential Medium supplemented with 10% heat inactivated foetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin and 1μM dexamethasone.

After overnight culture, the medium was replaced by a serum free Williams'E medium supplemented with 100nM hydrocortisone, penicillin (50 U/ml)/streptomycin (50 μg/ml) and solution ITS+1 containing insulin (5μg/ml), transferin (2.75μg/ml) and selenium (25ng/ml) (Sigma, Buchs, Switzerland). The medium also was supplemented with bovine serum albumin (250μg/ml) and linoleic acid 2.35μg/ml. Twenty four hours after serum deprivation, the human hepatocytes were kept in serum free medium and exposed to various chemicals for 16 hours or less as indicated in the figure legends.

Preparation and culture of primary mouse hepatocytes.

Liver cells were prepared by the two step collagenase method (Berry and Friend,1969) from post-absorptive male mice (25-30g) after anaesthesia with ketamin/xylazin (1 mg/100 g body weight). Hepatocytes were seeded on rat tail collagen type1 coated dishes and cultured

overnight in M199 supplemented with 1% Ultroser G (Biosepra SA, Cergy-Saint-Christophe, France) and penicilline (50 U/ml)/streptomycine (50µg/ml). After overnight culture, the medium was replaced by a serum free William's E medium. Twenty four hours after serum deprivation cells were exposed to chemicals for 16 hours or as indicated in the figure legends, and were maintained in serum free medium.

Real-time PCR assays

One µg of total RNA was reverse-transcribed and used in real-time PCR assays for quantification of different target genes on an ABI PRISM 7700 sequence detection system. Expression levels of these genes were normalized against 18s rRNA for human samples and normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA for mouse samples. The primers sequences are described in table 1.

Western blot analysis

Cultured hepatocytes were washed in ice cold PBS and harvested in 300 µl/6-cm dish of extraction buffer (KCl 100 mM, Hepes 25 mM, $MgCl_2$ 7.5 mM, glycerol 20%, pH 7.4, supplemented with protease inhibitors cocktail tablets (Roche, Rotkreuz, Switzerland) containing dithiothreitol (4 mM), aprotinin (2 mg/ml) and ß-mercaptoethanol (1 mM). The cell suspension was sonicated for 5 sec and cellular debris removed by centrifugation (1000xg for 10 min at 4°C).

Thirty micrograms of total cellular protein were separated by Tris-Tricine glycerol-SDS-PAGE and blotted onto nitrocellulose. The following primary antibodies were employed: anti-AMPK α1 subunit and anti-AMPK α2 subunit, anti-phospho-ACC (Cell Signalling, Allschwill, Switzerland) and anti-Myc (clone 9E10) (Sigma, Buchs, Switzerland). Secondary antibodies anti-rabbit IgG and anti-mouse IgG conjugated to horseradish peroxidase were employed for chemiluminescence immunodetection. Blots were developed using ECL

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reagent (Amersham Biosciences, Otelfingen, Switzerland) and exposure to X-ray films X-OMAT Kodak (Sigma, Buchs, Switzerland).

AMP-activated kinase activity assay

The AMPK assay was performed using the SAMS peptide phosphorylation assay kit from Upstate Biotechnology (Lucerna Chemicals, Lucerne, Switzerland) according to the manufacturer's protocol. Briefly, cells were cultured in serum free medium for 16 hour prior to drug exposure. Chemicals were added straight to cell culture dishes and cells incubated for 1 hour at 37°C. Culture medium was quickly removed, cells washed once with ice cold PBS and harvested in Tris-HCL (50mM, pH 7.5), ethyleneglycol-bis(β-aminoethylether)N,N,N',N'-tetraaceticacid (1mM), ethylenediamine tetra acetic acid (1mM), dithiotreithol (1mM), sodium fluoride (50mM), sodium pyrophosphate (5mM), benzamidine (1mM), soybean trypsin inhibitor (4μg/ml), phenylmethylsulfonylfluoride (1mM), mannitol (250 mM) and protease inhibitor tablets (Roche, Rotkreuz, Switzerland). Cellular debris was removed by centrifugation at 10 000g at 4°C for 20 min and the supernatant snap-frozen in liquid nitrogen. Samples were stored at -70°C prior to AMPK activity assays.

Proteins in the supernatant were concentrated by polyethylene glycol PEG 8000 precipitation and the AMPK reaction was performed for 10 min at 30°C with 20μM SAMS peptide, 10μCi γ-ATP ^{32}P and 10μg protein sample**.** The reaction mixture was then spotted on P81 phosphocellulose paper (Upstate, Lucerna Chemicals, Lucerne, Switzerland), which was washed with 0.75% phosphoric acid and acetone and the radioactivity of phosphor SAMS peptide quantified by scintillation counting.

Measurement of ATP concentration in primary hepatocytes.

Primary human hepatocytes were seeded in 96-well plate as described above. Cells were exposed for 1 hour with different inducers as stated in the figure legend. ATP concentration

was estimated by the luciferase activity using the ATP bioluminescence assay kit (Roche Applied Bioscience, Rothkreuz, Switzerland). Results are expressed as percentage of control cells not exposed to any drugs.

Immunocytochemistry.

Hepatocytes were cultured on glass cover slips coated with rat tail collagen (25µg/cm^2). Mouse hepatocytes were infected 12 hours post seeding in a serum free medium with Ad-hCAR-GFP at a Multiplicity of Infection (MOI) of 30 to 100. Twelve hours after infection, cells were exposed to chemicals for 6 hours then washed twice at room temperature with PBS and fixed for 20 min in 4% (w/v) paraformaldehyde. Cells were visualized in Mowiol mounting medium with a 40x objective (1.40 numerical apertures) by using a Leica TCS NT confocal laser scanning microscope.

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Results.

Induction of CYP2B6 and CYP3A4 by phenobarbital, rifampicin and CITCO in cultured primary human hepatocytes.

Human hepatocytes in primary culture are considered the "gold standard" *in vitro* model to study drug induction of CYP gene expression. Our system of primary human hepatocytes culture therefore was first tested for inducibility of CYP2B6 and CYP3A4 mRNA expression by three typical inducers, i.e phenobarbital (PB), rifampicin (Rif) and CITCO (6-(4-chlorophenyl)imidazol[2,1-6][1,3]thiazole-5-carbaldehyde). Exposure of cells for 16 hours to PB (500 μM) and Rif (10 μM) led to an increase of CYP2B6 mRNA levels by 9.97 $\pm$ 2.44 (PB) and 13.26 $\pm$ 1.35 (Rif) fold, respectively (Fig. 1A), and of CYP3A4 mRNA levels by 12.18 $\pm$ 2.48 (PB) and 25.30 $\pm$ 3.37 fold (Rif) (Fig. 1B). CITCO at 50 nM and 100 nM induced CYP2B6 expression by 2.9 $\pm$ 0.5 and 3.3+0.43 and CYP3A4 mRNA expression by 3.5 $\pm$ 0.38 and 3.6 $\pm$ 0.41 fold, respectively. These results validate our model of primary human hepatocytes culture to study the regulation of CYP2B6 and CYP3A4. Our previous study in a hepatoma derived cell line showed induction of CYP2B6 expression in response to AICAR (Rencurel et al. ,2005). In the present study using primary human hepatocytes, the two AMPK activators AICAR and metformin (Sabina et al.,1985) (Hawley et al.,2002) both induced CYP2B6 and CYP3A4 expression in a dose dependent manner (Fig. 2A-C). This indicates that classical AMPK activators increase expression of drug metabolising enzymes such as CYP2B6 and CYP3A4. In addition, induction of CYP2B6 and CYP3A4 by PB and AICAR is additive suggesting involvement of different mechanism of induction (Fig. 2B-D).

Phenobarbital activates AMPK in human hepatocytes.

We then tested whether drug inducers such as PB, CITCO and rifampicin were able to change the AMPK activity in primary human hepatocytes. Activity was assessed in cells exposed for

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1 hour to PB, AICAR, CITCO, Rif and the mouse CAR ligand TCPOBOP (Fig. 3A). As expected, a strong increase of AMPK activity was observed with AICAR and most importantly, PB strongly activated AMPK (Fig. 3A) in a concentration dependent manner, the highest activity being reached at 1mM PB (Fig. 3B). PB was as potent as AICAR. Interestingly, no changes in AMPK activity were observed with CITCO (50nM and 100nM), Rif (10μM) and TCPOBOP (250nM) (Fig. 3A). Western blot analysis of crude hepatocyte lysates revealed no changes in expression of both AMPK α1 and α2 catalytic subunits following treatment with PB or AICAR (Fig. 3D). To confirm AMPK activation by PB, phosphorylation of acetyl-CoA carboxylase at serine 79 was visualized by western blot using a phospho-Ser79 specific antibody. An increase in the ACC phosphorylation as a consequence of AMPK activation is clearly shown (Fig. 3D). Finally, we investigated whether PB activation of AMPK is associated with a decrease in ATP concentration. A significant decrease by 40% of ATP levels was determined in cells after 1 hour exposure to 0.5mM PB (Fig. 3C). We conclude that activation of AMPK by PB may be mediated by a decrease in ATP concentration. The mechanism by which PB and PB-like inducers may cause this decrease in ATP is presently being explored in our laboratory.

Pharmacological inhibition of AMPK activity by Compound C lowers PB induction.

To further investigate the functional effects of AMPK activation, we examined the effect of Compound C, a selective AMPK inhibitor, in primary human hepatocytes (Zhou et al.,2001). Compound C (10μM and 40μM) was added to the culture medium 30 min prior to addition of PB. Both concentrations of compound C tested were able to blunt PB induction of CYP2B6 expression whereas compound C alone at a concentration of 10μM induced CYP2B6 (Fig. 4A).This inhibitory effect of Compound C was also observed on PB induction of CYP3A4 expression (Fig. 4B). We thus conclude that inhibition of AMPK activity markedly reduces PB induction.

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Dominant-negative forms of AMP-Kinase blunt the PB response.

It was previously shown that a truncation of AMPK at residue 312 yields a peptide that no longer associates with the β and γ subunits but retains significant kinase activity (Crute et al.,1998). Moreover, replacement of threonine 172 within the α subunit by an aspartic acid mimics the effect of phosphorylation at this site (Stein et al. ,2000). The AMPK mutants used in the present study provide a constitutively active form of AMPK. By contrast, mutation of aspartate 157, an essential residue for MgATP binding within the α1 subunit to alanine, yields an inactive kinase but does not have any effect on the binding to the β and γ subunits (Stein et al. ,2000). Inactive α1 subunit (DNα1) acts as a dominant negative inhibitor by competing with the native α subunits for binding with β and γ which is essential for AMPK activity. In primary rat hepatocytes, expression of the inactive α1 subunit was able to inhibit both α1- and α2 containing complexes to a similar extent (Woods et al.,2000). Adenovirus infection of primary human hepatocytes caused more than 50% to express the AMPK mutant constructs as visualized by enhanced green fluorescence protein (Fig. 5D).

Western (immuno-) blot analysis confirmed the expression of ad-CA-α1, ad-CA-α2, and ad-DNα1 with anti-c-Myc antibody which recognizes the Myc epitope tag contained in the three mutants (Fig. 5C). An adenovirus expressing beta-galactosidase (ad-βgal) was used as control. Twelve hours post infection, cells were induced with 0.5mM PB for 16 hours and mRNA coding for CYP2B6 quantified by real time RT-PCR. Fig. 5A-B show PB induction of CYP2B6 and CYP3A4 expression in ad-βgal infected hepatocytes confirming that adenoviruses do not alter the PB response.

The dominant-negative mutant of α1 AMPK (DN) was not only able to lower the basal expression of CYP2B6 and CYP3A4 genes but completely blocked the PB induction of these

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two genes. Interestingly, the constitutively active mutant, CA-α2, did not significantly change basal levels of the corresponding mRNAs but tended to potentiate the effects of PB effect on CYP2B6 in both donors. More heterogeneous results appear with the CA-α1 where no variations in PB induction of CYP2B6 were observed and even a lower PB induction of CYP2B6 and CYP3A4 was present in Donor 1. These results strongly suggest that AMPK activation is required for PB induction of CYP2B6 and CYP3A4. The specific role played by each AMPK catalytic subunit in such an effect is under further investigation in our laboratory.

Induction of Cyp2b10 and Cyp3a11 expression by phenobarbital depends on AMPK expression in mouse liver and mouse hepatocytes.

To test further the importance of AMPK *in vivo* in drug induction, we tested induction of Cyp2b10 and Cyp3a11 by PB and TCPOBOP in AMPK alpha1/alpha2 liver specific knock out mice ($\alpha1/\alpha2^{LS-/-}$). As expected, intra-peritoneal injection of PB (100mg/Kg body weight) and TCPOBOP (3mg/Kg body weight) in wild type C57Bl/6 mice massively induced Cyp2b10 and Cyp3a11 expression in the liver (Fig 6B). To our surprise, a strong increase of Cyp2b10 (100 fold) and Cyp3a11 (6-fold) expression occurred in untreated $\alpha1/\alpha2^{LS-/-}$ mice. The PB and TCPOBOP induction of Cyp2b10 and Cyp3a11 was decreased from 155-fold to 1.54-fold for Cyp2b10 and from 13.6 fold to 2.42-fold for Cyp3a11 in $\alpha1/\alpha2^{LS-/-}$ knock out mice. To address the question whether the strong upregulation of Cyp2b10 and Cyp3A11 observed *in vivo* in untreated animals is a direct or indirect consequence of the ablation of AMPK catalytic subunits, we chose to perform primary culture of hepatocytes from wild type and $\alpha1/\alpha2^{LS-/-}$ knock-out mice. Primary mouse hepatocytes from wt animals exhibit an induction of Cyp2b10 after exposure to PB (500μM), TCPOBOP (TCP, 250nM), metformin (Metf, 1mM) or AICAR (500μM) (Fig. 6A). Lack of expression of AMPK catalytic subunits α1 and α2 in primary mouse hepatocytes, resulted in the abolition of Cyp2b10 induction by

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either PB, TCPOBOP (TCP), metformin (Metf) or AICAR (Fig. 6A). Importantly, contrary to the *in vivo* situation, the basal expression of Cyp2b10 and Cyp3a11 was not higher in hepatocytes from $\alpha1/\alpha2^{LS-/-}$ mice compared to hepatocytes from wt mice. This implies a role of circulating factors responsible of high basal Cyp2b10 and Cyp3a11 gene expression observed *in vivo*. Moreover, these results provide further evidence for the important role of AMPK in Cyp2b10 and Cyp3a11 induction by PB AICAR and TCPOBOP.

The absence of AMPK catalytic subunits has no effect on CAR cytoplasmic-nuclear transfer induced by PB.

The capacity of PB to trigger CAR cytoplasmic/nuclear transfer was tested in primary mouse hepatocytes expressing the human CAR in fusion with enhanced GFP. After 6 hours exposure to PB, CAR was clearly located predominantly in the nuclei of hepatocytes from wild type mice. An apparent staining close to the plasma membrane and /or cytoskeleton network was also observed in untreated (Control) and PB-treated hepatocytes from both wild type and $\alpha1/\alpha2^{LS-/-}$ mice (Fig.7). Unexpectedly, the cytoplasmic/nuclear shuttling of CAR upon PB treatment was not altered by the absence of AMPK $\alpha1$ and $\alpha2$ catalytic subunits. However, we noted that CAR-GFP fluorescence was located in condensed region of the nucleus in $\alpha1/\alpha2^{LS-/-}$ mice hepatocytes after PB treatment, a phenomenon not observed in hepatocytes from wild-type mice. It is therefore unlikely that the deficiency in the induction of Cyp2b10 and Cyp3a11 by PB observed in $\alpha1/\alpha2^{LS-/-}$ mouse hepatocytes is caused by an alteration in CAR translocation into the nucleus.

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Discussion

In spite of numerous attempts the molecular mechanism by which PB exerts its effects on gene expression are still incompletely understood and no intracellular protein target of PB has been identified. Here we propose a new mechanism of drug induction, activation of AMPK, which may ultimately explain some of the diverse effects of PB in human and mouse hepatocytes such as its effect on the transcription of CYP genes. We have previously reported that pharmacological activation of AMPK leads to induction of CYP2B6 in a hepatoma derived cell line (WGA) (Rencurel et al. ,2005). However, concentrations of PB above 1mM were required for CYP2B6 induction and AMPK activation in WGA cells; these concentrations are associated with toxic effects in normal hepatocytes. Thus the importance of this mechanism in normal hepatocytes was questioned. We therefore chose two experimental systems, primary culture of human hepatocytes and cultures of hepatocytes from AMPK $\alpha1/\alpha2$ liver specific KO mice ($\alpha1/\alpha2^{LS-/-}$) to investigate the role of AMPK in drug induction. We validated our human hepatocyte culture system by testing classical inducers on CYP2B6 and CYP3A4 gene expression and observed robust and reproducible, dose-dependent induction of CYP2B6 and CYP3A4 when cells were exposed to PB and rifampicin for 16 hours. Interestingly, CITCO, a human CAR agonist (Maglich et al.,2003), was not a potent CYP2B6 inducer when compared to PB. This could be due to the shorter time of exposure than those described by Maglich et al. and to its partially different mode of action, i.e. direct activation of CAR (Maglich et al. ,2003).

The two AMPK activators, AICAR and metformin, induced CYP2B6 and CYP3A4 expression in a dose-dependent manner in primary human hepatocytes, a hallmark of drug induction. An additive effect of PB and AICAR on CYP2B6 expression was observed, suggesting the involvement of distinct mechanism of action of these two drugs. AICAR is the

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most commonly used way to activate AMPK but recently Guigas et al. highlighted an AICAR effect independent of AMPK activation on glucose phosphorylation (Guigas et al. ,2006). To clearly distinguish AMPK-dependent and AMPK-independent effects on CYP induction, we turned to primary hepatocytes from $\alpha1/\alpha2^{LS-/-}$ mice. In these hepatocytes, no induction of Cyp2b10 was observed with PB, TCPOBOP, metformin or AICAR, demonstrating the essential role of AMPK in the induction of CYP2b10. However, minor induction of Cyp3a11 by AICAR was still present in hepatocytes from $\alpha1/\alpha2^{LS-/-}$ mice perhaps reflecting the existence of an AMPK-independent mechanism by which AICAR regulates this gene, possibly acting as a ligand of PXR.

The activation of AMPK by phenobarbital is unique in the sense that other drug inducers so far tested, such as the CAR ligand/activator CITCO, the mouse CAR ligand/activator TCPOBOP and the PXR ligand/activator rifampicin did not change AMPK activity in human hepatocytes. This suggests that PB and PB-like inducers affect transcription of CYP genes by a unique mechanism.

The classical view of AMPK activation is a decrease in cellular energy charge as reflected by the increase in the AMP/ATP ratio. PB is able to significantly lower ATP concentration in hepatocytes within an hour, a finding in agreement with our previous study in hepatoma cells (Rencurel et al. ,2005). The lowering effect of PB on ATP in hepatoma cells was only observed at high concentrations (above 1mM), a level also required for CYP2B6 induction in these cells (Rencurel et al. ,2005). The capacity of PB to induce CYP2B6 expression may thus be related to its efficiency to lower ATP and consequently to activate AMPK. Another interesting observation comes from the CYP2B6 induction by metformin in primary human hepatocytes. The biguanide metformin (N^1, N^1-dimethylbiguanide) is a prescribed to lower fasting blood glucose in patients with Non Insulin Dependent Diabetes Mellitus (NIDDM),

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yet its primary site of action is still uncertain. Only very recently Shaw et al. described the inhibitory effect of metformin on hepatic glucose production to be dependent on the expression of LKB1 kinase (Shaw et al.,2005). Experiments in LKB1 -/- mice have indeed established that LKB1 is the principal AMPK kinase in the liver (Shaw et al. ,2005). Although the molecular mechanisms through which metformin affects cellular energy homeostasis remain disputed, it seems increasingly likely that metformin may act, at least in large part, by inhibiting respiratory chain complex 1 (Owen et al.,2000) **,** hence suppressing mitochondrial ATP synthesis. It is not known yet whether PB activates AMPK via a similar mechanism in the liver, but if so this could explain the known blood glucose lowering effect of PB in NIDDM patients (Sotaniemi,1989).

The most compelling evidence for the role of AMPK in PB-type induction is reflected in our experiments which demonstrate that liver-specific deletion of the AMPKα subunits genes in the mouse abolishes the drug induction of CYP2b10 and CYP3a11. Surprisingly, markedly higher basal levels of the mRNA of these genes were observed. By contrast, no increase of Cyp2b10 and Cyp3a11 basal expression was observed in primary hepatocytes cultured from the livers of $\alpha1/\alpha2^{LS-/-}$ mice. Such a discrepancy between *in vivo* and *in vitro* findings suggested that circulating factors in response to the metabolic changes in the liver may cause the increased basal expression of Cyp2b10. Streptozotocin induced diabetic mouse model exhibits high Cyp2b10 basal expression level which was corrected by insulin treatment to lower hyperglycemia (Sakuma T et al.,2001). Recently, Guigas et al. observed a low glucokinase expression in hepatocytes from $\alpha1/\alpha2^{LS-/-}$ mouse which is associated with low glucose phosphorylation and low glucose uptake (Guigas et al. ,2006). A low rate of glucose phosphorylation was described earlier in primary rat hepatocytes from alloxan-induced diabetic rats (Bontemps et al.,1978) and in fasted animals, where Cyp2b10 and CAR expression is induced (Maglich et al. ,2004). We therefore speculate that a circulating factor

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related to the low carbohydrate turn-over in hepatocytes from $\alpha1/\alpha2^{LS-/-}$ could be responsible for the high basal expression of Cyp2b10 and we are currently testing this hypothesis.

The blunted induction of Cyp2b10 and CYP3a11 by PB in hepatocyte from $\alpha1/\alpha2^{LS-/-}$ mice was not related to an alteration of CAR nuclear translocation upon drug treatment. It is well documented that CAR translocates into the nucleus upon drug treatment to form an active transcriptional complex with the retinoid X receptor (Zelko et al.,2001). Moreover, the translocation and activation of CAR is influenced by phosphorylation/dephosphorylation events (Kawamoto et al. ,1999;Yoshinari et al.,2003). For instance, the protein phosphatase PP2A inhibitor okadaic acid blocks CAR translocation in mouse hepatocytes exposed to either PB or TCPOBOP (Kawamoto et al. ,1999). In addition, okadaic acid induces expression of CYP2B6 in HepG2 cells which were engineered to express the mouse CAR isoform. Recently, again in HepG2 cells, residue Serine-202 of mouse CAR was shown to be phosphorylated and this modification was shown to be important for the retention of CAR in the cytoplasm (Hosseinpour et al. ,2005). On the other hand, despite this preliminary evidence that the serine-202 dephosphorylation of mCAR by protein phosphatase 2A affects the cytoplasmic-nuclear transfer of CAR the identity of the kinase responsible of Serine 202 phosphorylation is unknown (Hosseinpour et al. ,2005). Our own experiments suggest that AMPK does not phosphorylate serine-202 of mouse CAR or the corresponding serine 192 of human CAR (M. Matis and F. Rencurel, unpublished observation). In hepatocytes from $\alpha1/\alpha2$ LS-/- mice hCAR-GFP was located in condensed regions of the nucleus whereas a more homogenous staining was observed in nucleus of wild type mice hepatocytes after PB treatment. These condensed regions are comparable to nuclear speckles described previously in COS-7 cells in which CAR and the coafactor Peroxisome Proliferator-activated Receptor gamma Coactivator-1 alpha (PGC1α) were co-expressed {Shiraki, 2003 #117}. Nuclear speckles contain proteins involved in pre-mRNA splicing but also several kinases (e.g. CLK/STI, PRP4) and phosphatases (e.g. Protein Phosphatase 1: PP1) which can

phosphorylate/dephosphorylate components of the splicing machinery (Handwerger and Gall,2006). Although little or no transcription takes place in nuclear speckles, a set of proteins involved in transcriptional regulation is associated with speckles from where they are shuttled to transcription sites. In PGC1α liver specific knock out mice PB induction of Cyp2b10 was not altered (Handschin et al.,2005). If and how PGC1α interacts with CAR thus is still an open question. A sequestration of CAR in or close to nuclear speckles may control its activity by keeping the nuclear receptor removed or close to the transcription sites. Our data therefore suggest the existence of another control step of CAR signalling independent of CAR intracellular location.

In conclusion, we demonstrate an essential role of AMPK in induction of CYP2B6 and CYP3A4 by PB in human hepatocytes and of CYP2b10 and CYP3a11 in mouse hepatocytes. This highlights yet another interesting effect of this anticonvulsant and hypnotic drug which also has antidiabetic properties. The role of AMPK in drug induction obviously needs further investigation and its role in the control of CAR activity is of particular interest. As AMP-activated kinase is a target for the development of drugs for type II diabetes and obesity its role in the induction of drug-metabolism deserves close attention.

Acknowledgements: This work has been carried out in accordance with the Declaration of Helsinki and/or with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. Primary human hepatocytes used in this study were from consented patients undergoing liver surgery in accordance with standard procedures approved by an institutional review board, (Approval No. 1.05.01.30.-17). We thank Dr Houchaima Ben Tekaya for help with immunocytochemistry.

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Legends for figures

Figure 1

Regulation of *CYP2B6* and *CYP3A4* in primary human hepatocyte cultures.

Human hepatocytes were exposed for 16 hours to phenobarbital (PB), rifampicin (Rif) or CITCO with the concentrations indicated and *CYP2B6* (A) and *CYP3A4* (B) mRNAs were quantified by real time PCR as described in *Materials and Methods*. Data are expressed as relative units compared to control cells not exposed to any drugs and corrected to 18s rRNA. Results are means of three different donors $\pm$ SD with each determination performed in triplicate. (**$p<0.01$).

Figure 2

AICAR and metformin induce *CYP2B6* and *CYP3A4* in primary human hepatocyte cultures.

Primary human hepatocytes were exposed for 16 hours to different concentrations of metformin (Met) and AICAR as indicated and *CYP2B6* (A) and *CYP3A4* (C) mRNA were quantified by RT- PCR as described in *Materials and Methods*. Hepatocytes were exposed for 16 hours to 500µM PB (PB) alone or in combination with different concentration of AICAR. *CYP2B6* (B) and *CYP3A4* (D) mRNA were quantified by real time PCR. Results are means of 3 different donors $\pm$ SD with each determination done in triplicates. (**$p<0.01$). Values obtained with combinations of AICAR and PB treatment are compared with values obtained from cells treated with PB alone (§ $p<0.05$, §§ $p<0.01$)

Figure 3

Phenobarbital activates AMPK in primary human hepatocyte cultures and lowers ATP.

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Primary human hepatocytes in culture were exposed for 1 hour to different concentrations of phenobarbital (PB) (A-B), AICAR (A-B) and rifampicin (Rif) (B), CITCO (B) and TCPOBOP (B) as indicated. AMP kinase activity was measured as described in *Materials and Methods* and results expressed as fold stimulation compared to values measured in control cells (Control) not exposed to any drugs. Results are means $\pm$ SD of three to 4 different human hepatocyte cultures and each measurement was performed in triplicate. (C) ATP concentration was measured in primary human hepatocytes by luciferase activity as described in *Materials and Methods* after 1 hour exposure to PB. Results are expressed as fold stimulation compared to values obtained in non treated cells (Control). (**$p<0.01$). (D) Human hepatocytes were exposed for 1 hour to PB (0.5mM) or AICAR (2mM) and expression of AMPK alpha1 (α1), AMPK alpha2 (α2) and phosphorylated acetyl-CoA-carboxylase (p-ACC) was estimated by western blot using specific polyclonal antibodies.

Figure 4

Pharmacological inhibition of AMPK activity with compound C lowers phenobarbital induction of *CYP2B6* and *CYP3A4* in human hepatocytes.

Hepatocytes were exposed 16 hours to 500µM PB or compound C (10µM-40µM). Compound C was added to cells 30min prior to addition of PB when both chemicals were used in combination (Comp C+PB). CYP2B6 (A) and CYP3A4 (B) mRNA were quantified by real time PCR and are expressed as relative units corrected to 18s rRNA.

Results represent cultures from 2 different donors (mean $\pm$ SD) with each determination done in triplicates. Comparison with values in control cells (C) not exposed to drugs (**, $p<0.01$). Comparison with values in cells exposed to PB (§§, $p<0.01$)

Figure 5

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Exogenous expression of dominant-negative and constitutively-active mutants of AMPK in primary human hepatocytes affects phenobarbital induction.

Primary human hepatocytes were infected with adenovirus containing beta-galactosidase (β-gal), AMPK alpha1 dominant negative mutant (DN), AMPK constitutively active alpha1 or alpha2 subunits (CA-α1; CA-α2). Twenty four hours post infection, cells were exposed for 16 hours to 0.5mM phenobarbital (PB). Expression of *CYP2B6* (A) and *CYP3A4* (B) was quantified by real time PCR and results expressed as fold stimulation compared to cells infected with ad-βgal (β-gal) and not exposed to drug (C = Controls). Results are expressed as relative units corrected to 18s rRNA Comparison with values in control cells (C) not exposed to drugs and infected with ad-βgal (β-gal) (*, $p<0.05$, **, $p<0.01$). (C) Gene expression in primary human hepatocytes was tested by western blot. Twenty four hours post infection, cells were lysed and 30 μg of total cell lysate were separated in 10% acrylamide gel. The Myc tag of AMPK mutants was detected using a specific monoclonal antibody. The 62kDa band corresponds to the full length α1 dominant negative mutant (DN) and the 35kDa band to the truncated constitutive active α1 and α2 mutants (CA-α1 and CA-α2) as described in the result section. (D) The efficiency of infection was estimated by immunocytochemistry in primary human hepatocytes 24hours post infection. Each adenoviral construct expressed green fluorescent protein (GFP) under the control of a CMV promoter as described in *Material and Methods*.

Figure 6

Liver specific deletion of AMPK α1 and α2 subunits in mouse impairs induction of *Cyp2b10* and *Cyp3a11 in vivo* and *in vitro*.

Primary hepatocytes from wild type (wt, black bars) mice or from mice with liver-specific deficiencies of AMPK catalytic subunit α1 and α2 ($\alpha_1/\alpha_2^{LS-/-}$, grey bars) were exposed for 16

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hours to PB(500μM) (PB), TCPOBOP (500nM) (TCP), metformin (1mM) (Metf) or AICAR (500μM) (AICAR). Expression of *Cyp2b10* (A top) and *Cyp3a11* (A bottom) was quantified by real time PCR and results expressed as fold stimulation compared to non drug-exposed cells (C=Controls) from wild type mice (wt). Results represent two different cell preparations. (** $p<0.01$ unpaired Student's t-Test). Mice deleted of AMPK α1 and α2 in liver ($\alpha_1/\alpha_2^{LS-/-}$,grey bars) and wild type mice (wt, black bars) were injected intraperitonealy with saline solution (C=Controls), phenobarbital (PB) or TCPOBOP (TCP) and sacrificed 16 hours later as described in Material and Methods. *Cyp2b10* (B, top) and *Cyp3a11* (B, bottom) expression was quantified by RT-PCR and standardized to GAPDH. Results are expressed as fold stimulation compared to values obtained in saline injected animals (C=controls) of each group of Wt and $\alpha_1/\alpha_2^{LS-/-}$ mice. The inset results are expressed as fold induction compared to values obtained in saline injected (C) wild type animals (wt). Results are expressed as means $\pm$ SD, *$p<0.05$ and **$p<0.01$ determined by unpaired Student's t-Test.

Figure 7

Human CAR cytoplamic/nuclear transfer induced by phenobarbital is not altered by the deletion of AMPK α1 and α2 catalytic subunits in primary mouse hepatocytes.

Primary hepatocytes from wild type (Wt) mice or from mice in which the AMPK catalytic subunits α1 and α2 were deleted in the liver ($\alpha_1/\alpha_2^{LS-/-}$) were infected with adenovirus coding for human CAR in fusion with enhanced green fluorescence protein (ad-hCAR-GFP) as described in *Materials and Methods*. Twelve hours after infection, cells were exposed to phenobarbital (PB) for 6 hours, controls were not exposed to any drugs. Human CAR-GFP was visualized in cells in Mowiol mounting medium with a 40x objective (1.32 numerical aperture) by using a Leica TCS NT confocal laser scanning microscope. White bar scale corresponds to 10μm.

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Table 1: Primers and probes sequences used in quantitative Real-Time PCR *(TaqMan®)*

TAMRA: tetramethylrhodamine labelled probe, FAM: Fluoresceine labelled probe

Human genes	Human sequences
CYP2B6 Forward	5'-ACATCGCCCTCCAGAGCTT-3'
CYP2B6 Reverse	5'-GTCGGAAAATCTCTGAATCTCATAGA-3'
CYP2B6 Probe	6FAM-5'-ACCGAGCCAAAATGCCATACACAGAGG-3'TAMRA
CYP3A4 Forward	5'-CATTCCTCATCCCAATTCTTGAAGT-3'
CYP3A4 Reverse	5'-CCACTCGGTGCTTTTGTGTATCT-3'
CYP3A4 Probe	6FAM-5'-CGAGGCGACTTTCTTTCATCCTTTTTACAGATTTTC-3'TAMRA
18s Forward	5'-AGTCCCTGCCCTTTGTACACA-3'
18s Reverse	5'-CGATCCGAGGGCCTCACTA-3'
18s Probe	6FAM-5'-CGCCCGTCGCTACTACCGATTGG-3'TAMRA
Mouse genes	**Mouse sequences**
Cyp2b10 Forward	5'-CAATGTTTAGTGGAGGAACTGCG-3'
Cyp2b10 Reverse	5'-CACTGGAAGAGGAACGTGGG-3'
Cyp2b10 Probe	6FAM-5'-CCCAGGGAGCCCCCCTGGA-3'TAMRA
Cyp3a11 Forward	5'-AGAACTTCTCCTTCCAGCCTTGTA-3'
Cyp3a11 Reverse	5'-GAGGGAGACTCATGCTCCAGTTA-3'
Cyp3a11 Probe	6FAM-5'-CTAAAGGTTGTGCCACGGGATGCAGT-3'TAMRA
GAPDH Forward	5'-CCAGAACATCATCCCTGCATC-3'
GAPDH Reverse	5'-GGTCCTCAGTGTAGCCCAAGAT-3'
GAPDH Probe	6FAM-5'-CCGCCTGGAGAAACCTGCCAAGTATG-3'TAMRA

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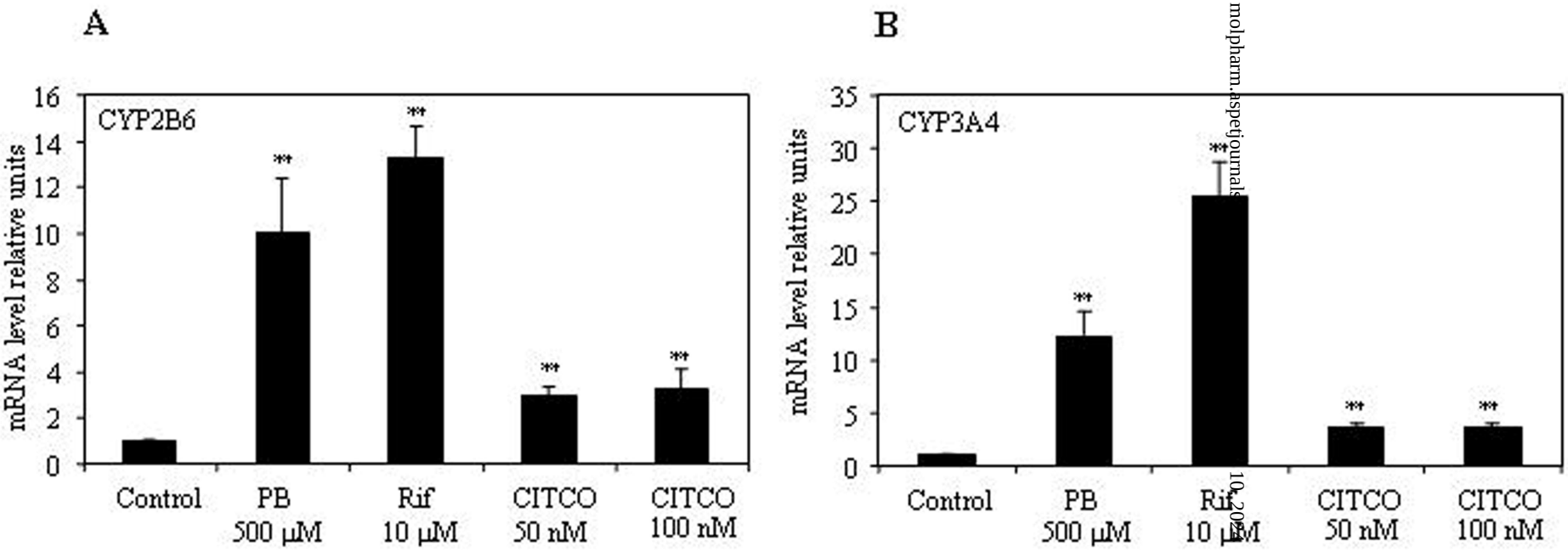

Fig. 1

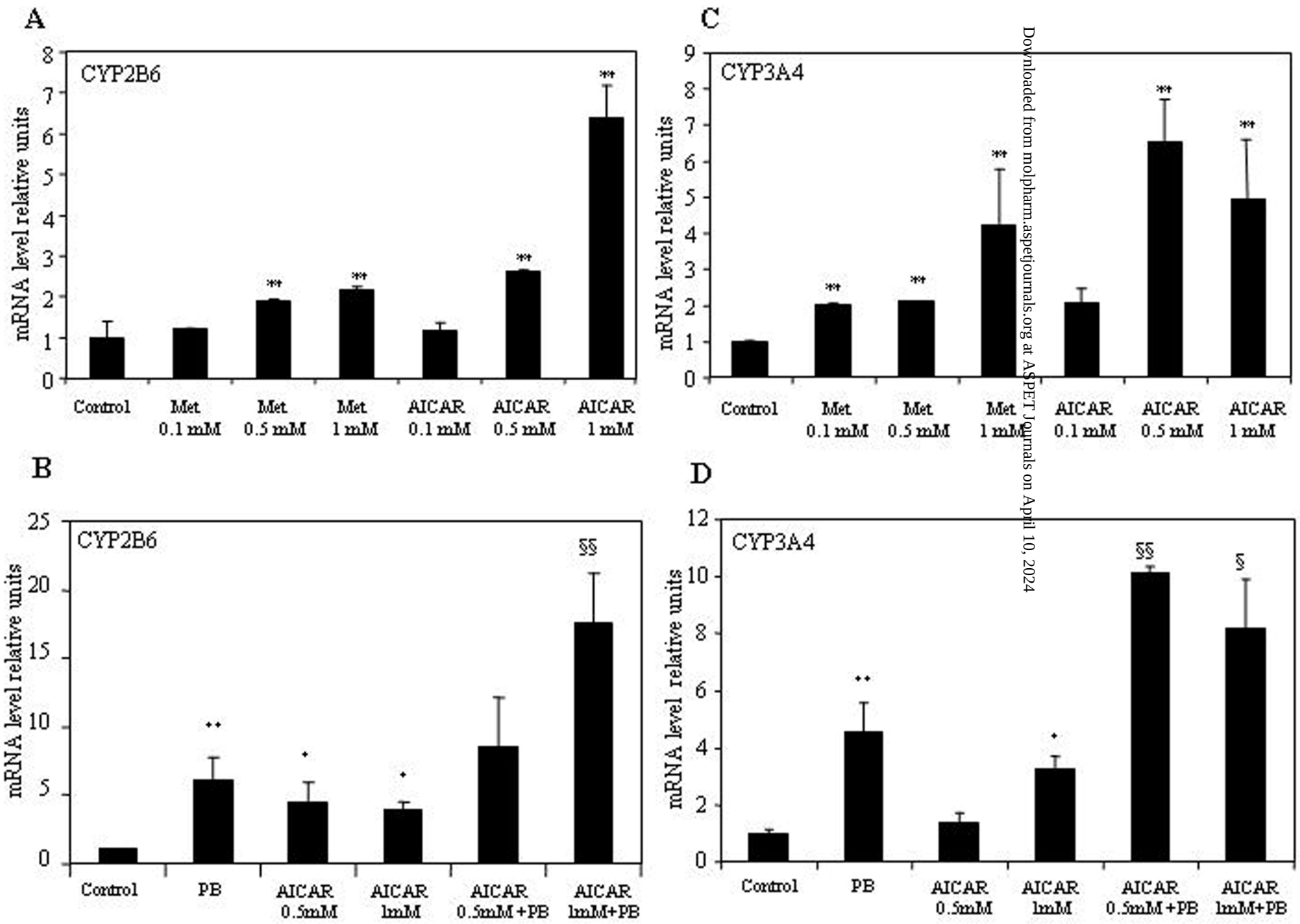

Fig. 2

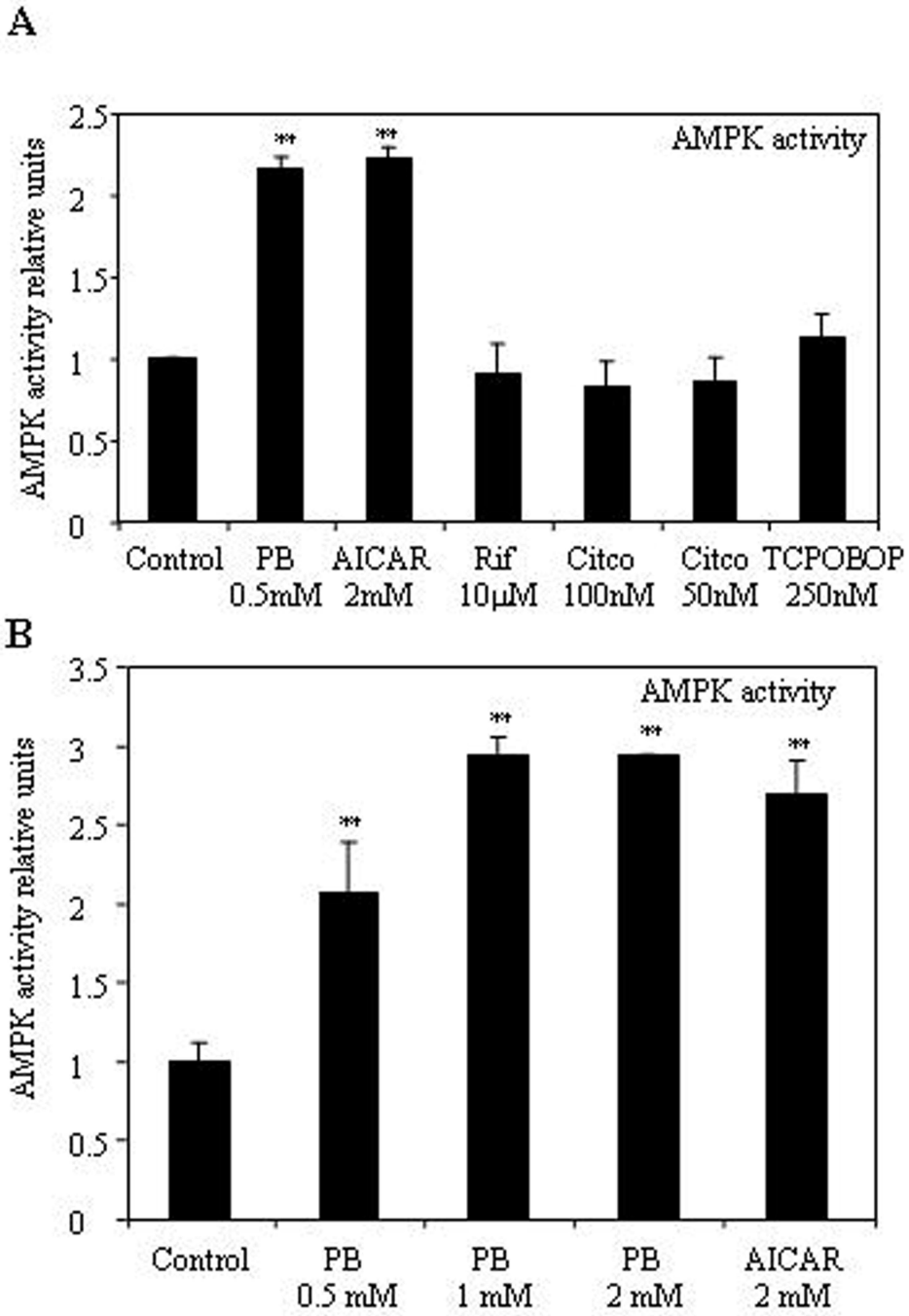

Fig. 3

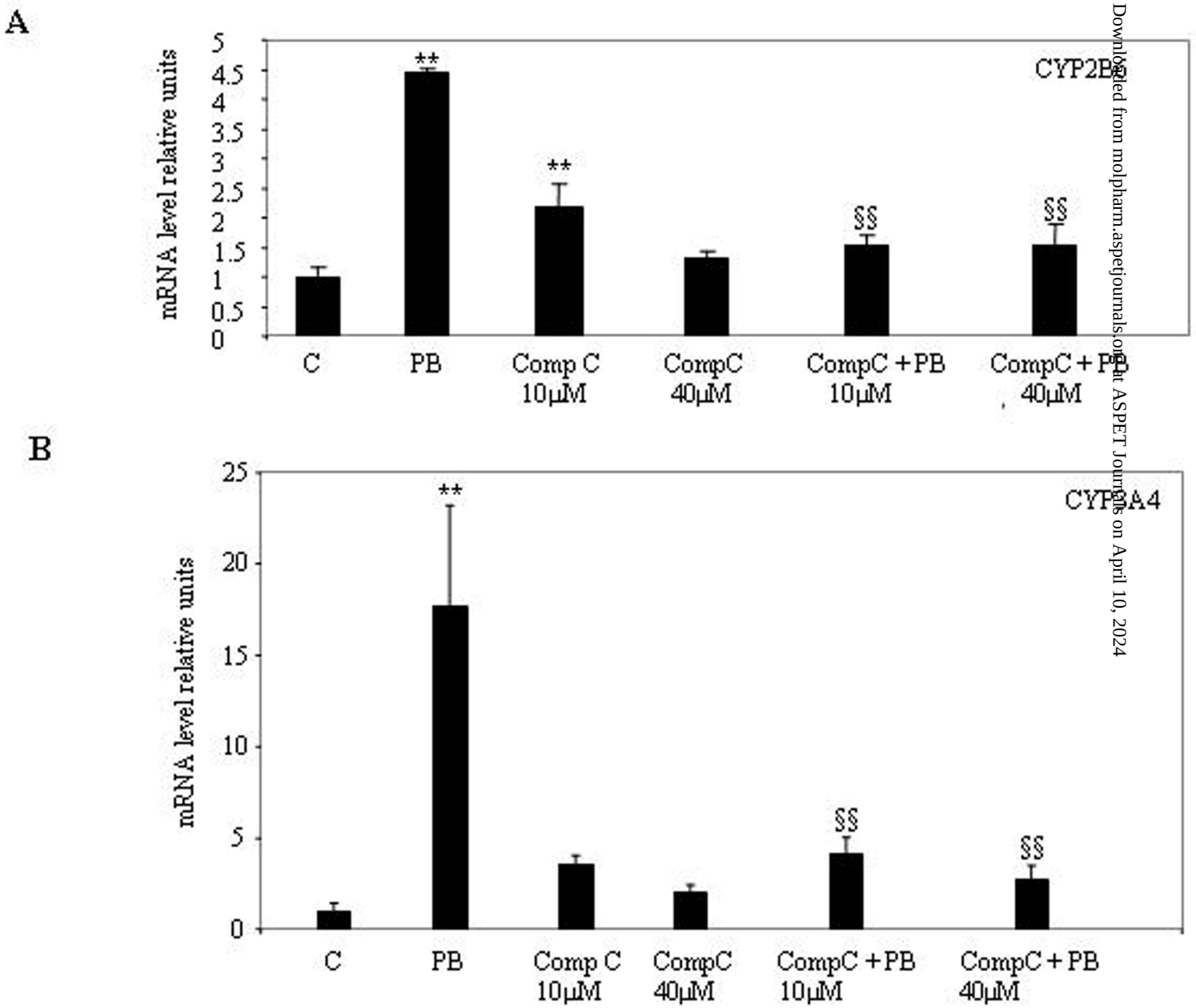

Fig. 4

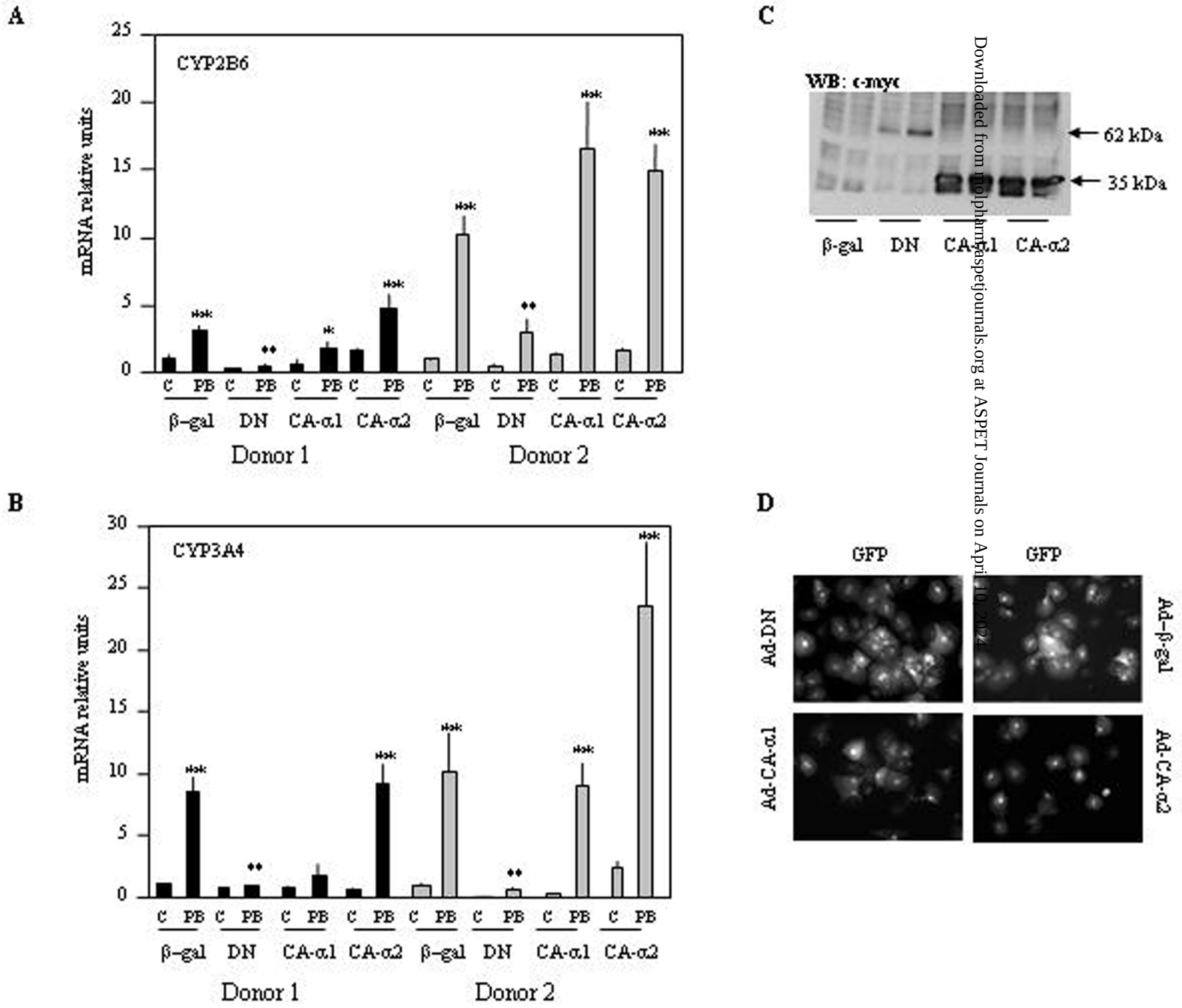

Fig. 5

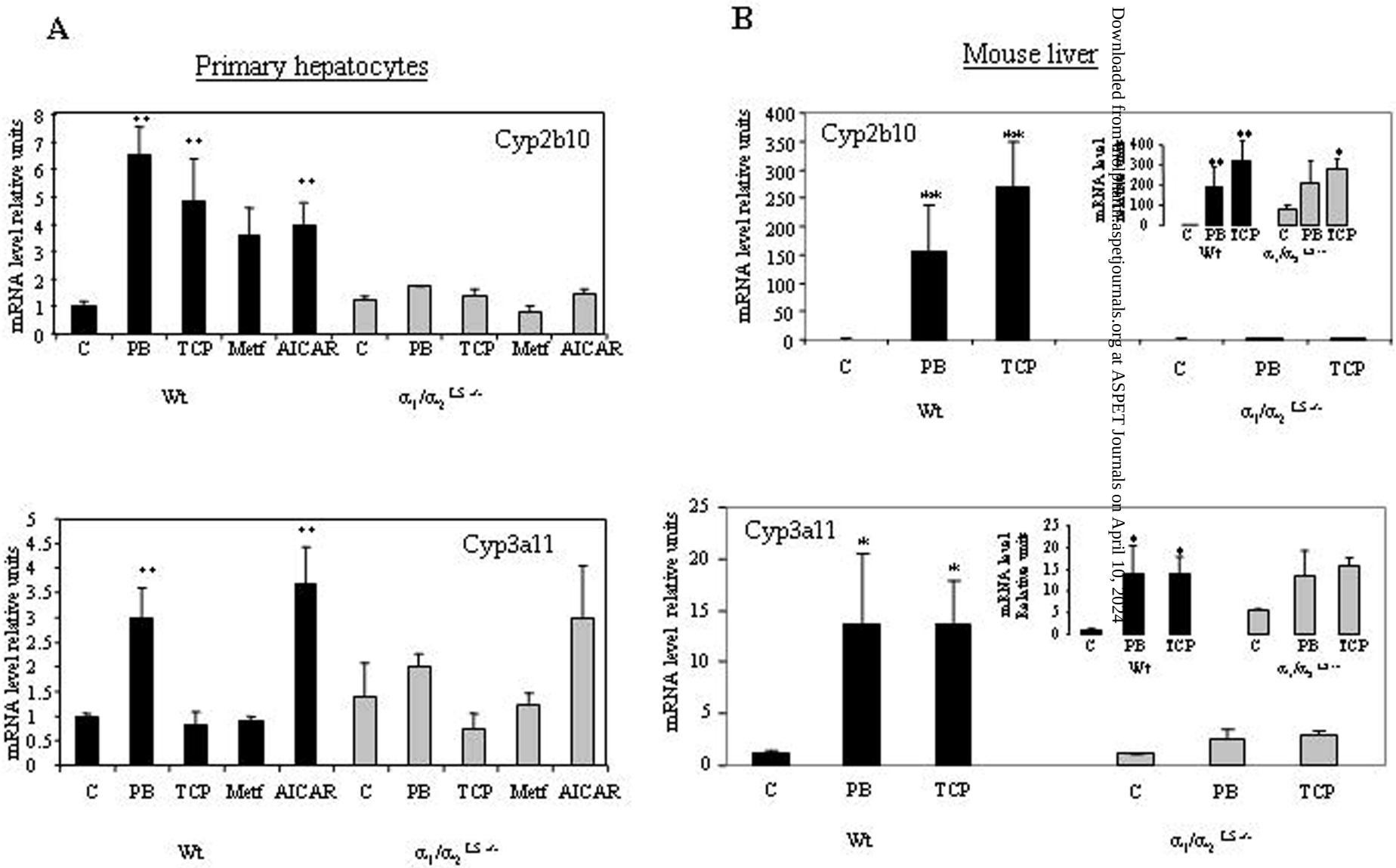

Fig. 6

Fig. 7