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Metformin represses drug-induced expression of CYP2B6 by modulating the constitutive androstane receptor signaling

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Running title: metformin suppresses CYP2B6 induction by CAR phosphorylation

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Abbreviations:

AMPK, AMP-activated protein kinase; CAR, constitutive androstane receptor; CITCO, [6-(4-chlorophenyl)imidazo[2,1-b]1,3thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime; EGFR, epidermal growth factor receptor; ERK1/2, extracellular signal-regulated kinase-1/2; GRIP1, glucocorticoid receptor-interacting protein 1; MATE, multidrug and toxin extrusion; MET, metformin; OCT, organic cation transporter; p38 MAPK, p38 mitogen-activated protein kinase; PB, phenobarbital; RIF, rifampicin; PXR, pregnane X receptor; SRC1, steroid receptor co-activator 1; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene.

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Abstract

Metformin is currently the most widely used drug for the treatment of type 2 diabetes. Mechanistically, metformin interacts with many protein kinases and transcription factors that alter the expression of numerous downstream target genes governing lipid metabolism, cell proliferation, and drug metabolism. The constitutive androstane receptor (CAR, NR1i3), a known xenobiotic sensor, has recently been recognized as a novel signaling molecule, in that its activation could be regulated by protein kinases in addition to the traditional ligand binding. Here, we showed that metformin could suppress drug-induced expression of CYP2B6 (a typical target gene of CAR) by modulating the phosphorylation status of CAR. In human hepatocytes, metformin robustly suppressed the expression of CYP2B6 induced by both indirect (phenobarbital) and direct [6-(4-chlorophenyl)imidazo[2,1-b]1,3thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime] (CITCO) activators of human CAR. Mechanistic investigation revealed that metformin specifically enhanced the phosphorylation of Threonine-38 of CAR, which blocks CAR nuclear translocation and activation. Moreover, we showed that phosphorylation of CAR by metformin was primarily an AMP-activated protein kinase- and extracellular signal-regulated kinase-1/2-dependent event. Additional two-hybrid and co-immunoprecipitation assays demonstrated that metformin could also disrupt CITCO-mediated interaction between CAR and the steroid receptor co-activator 1 or the glucocorticoid receptor-interacting protein 1. In conclusion, our results suggest that metformin is a potent repressor of drug-induced CYP2B6 expression through specific inhibition of human CAR activation. Thus, metformin may affect the metabolism and clearance of drugs that are CYP2B6 substrates.

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Introduction

Type 2 diabetes is a growing epidemic that is often associated with various comorbidities, and usually requires polypharmacy treatment. As such, patients with type 2 diabetes are at high risk of unwanted drug-drug interactions. Among others, metformin, a biguanide agent, represents the most widely used antidiabetic drug for the treatment of type 2 diabetes (Nathan et al., 2009). As an activator of AMP-activated protein kinase (AMPK), the major anti-hyperglycemic effect of metformin is hepatic suppression of gluconeogenesis and glucose efflux (Viollet et al., 2012). A number of recent studies indicated that in addition to its current clinical application, beneficial effects of metformin may potentially extend to anti-cancer and anti-aging activities (Cufi et al., 2010; Menendez et al., 2012). Thus, the chance of co-exposure of metformin with other remedies is high. Notably, however, metformin undergoes negligible hepatic metabolism, and is primarily eliminated unchanged through the kidneys as substrates of several efflux and uptake transporters including the multidrug and toxin extrusion 1 and 2 (MATE1/MATE2) and the organic cation transporter 1 and 2 (OCT1/OCT2) (Nies et al., 2011; Stocker et al., 2013).

Given the inert nature of metformin in metabolism, drugs affecting the expression and activity of major drug-metabolizing enzymes usually do not influence the pharmacokinetics of metformin. Accumulating evidence, however, revealed that both genetic mutation and drug-induced alteration of MATEs and OCTs could significantly affect the pharmacokinetic and pharmacodynamic profiles of metformin (Higgins et al., 2012; Stocker et al., 2013). Intriguingly, previous studies predominantly focused on how other drugs may affect the pharmacokinetics of metformin, with limited data available regarding how metformin may influence the metabolism and clearance of other co-administered drugs. Although a known activator of AMPK, metformin appears to exert its pharmacological actions both AMPK-dependently and -independently (Do et

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al., 2013; Lee et al., 2012). It also interacts with transcription factors such as the small heterodimer partner, pregnane X receptor (PXR), hepatocyte nuclear factor 4 alpha, and peroxisome proliferator-activated receptor, which disturb the expression of their target genes thereafter (Kim et al., 2008; Krausova et al., 2011; Prieur et al., 2005; Sozio et al., 2011). In particular, the antagonistic effects of metformin on PXR may partly contribute to its speculated anti-cancer activity (Krausova et al., 2011; Wang et al., 2011b).

The constitutive androstane receptor (CAR, NR1i3) is a xenobiotic sensor that governs the transcription of many hepatic drug-metabolizing enzymes and transporters, and influences the metabolism and clearance of both endobiotic and xenobiotic chemicals including drugs (Qatanani and Moore, 2005; Tolson and Wang, 2010). In addition to these well-established roles, recent literature suggests that activation of CAR is potentially involved in cancer development and energy homeostasis in animal models (Dong et al., 2009; Gao et al., 2009; Yamamoto et al., 2004). Unlike prototypical nuclear receptors such as PXR that requires ligand binding for activation (Goodwin et al., 1999), mounting evidence indicates that other than traditional ligand binding, activation of CAR is involved in ligand-independent signaling pathways with the underlying mechanism(s) remaining unclear. For instance, as the prototypical target gene of human (h) CAR, expression of hepatic CYP2B6 can be robustly induced by both hCAR direct activator 6-(4-chlorophenyl)-imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime (CITCO) and the indirect activator phenobarbital (Moore et al., 2000). Most recently, Negishi and colleagues have demonstrated that phosphorylation/dephosphorylation of Thr-38 of CAR is associated with phenobarbital-mediated nuclear translocation and activation of CAR (Mutoh et al., 2009; Osabe and Negishi, 2011); and a number of protein kinases such as the epidermal growth factor receptor (EGFR), the p38

mitogen-activated protein kinase (p38 MAPK), and the extracellular signal-regulated kinase-1/2 (ERK1/2), are differentially involved in the activation of CAR (Koike et al., 2007; Mutoh et al., 2013; Saito et al., 2013). Given that metformin was previously reported as a potential modulator of kinase signaling, we hypothesize that metformin alters the signaling pathways that regulate CAR phosphorylation and affect the inductive expression of CYP2B6.

In this study, we provide experimental evidence to illustrate the molecular mechanism by which metformin suppresses the expression of CYP2B6 induced by both direct (CITCO) and indirect (phenobarbital) CAR activators in human primary hepatocytes. Utilizing experiments including enhanced yellow fluorescence protein (EYFP)-hCAR translocation, luciferase reporter activation, real-time PCR, and western blotting analyses, we investigated how metformin affects the phosphorylation of hCAR through interaction with AMPK, EGFR, p38 MAPK, or ERK1/2 in human primary hepatocytes. Mammalian two-hybrid and co-immunoprecipitation assays were employed to decipher the differential roles of metformin on direct and indirect activation of CAR. Overall, our results showed that metformin potently suppressed phenobarbital- and CITCO-mediated induction of CYP2B6 by phosphorylating hCAR via AMPK- and ERK1/2-dependent signaling pathways.

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Materials and Methods

Reagents

Phenobarbital (PB), metformin (MET), 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio) butadiene (U0126), 4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole (SB202190) and erlotinib were purchased from Sigma-Aldrich (St. Louis, MO). CITCO was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). Matrigel, insulin and ITS+ culture supplements were from BD Biosciences (Bedford, MA). Effectene transfection reagent was purchased from Qiagen (Valencia, CA). XtremeGENE9 transfection reagents were from Roche Diagnostics (Basel, Switzerland). Lipofectamine 2000 transfection reagent was from Invitrogen (Carlsbad, CA). The Dual-Luciferase Reporter Assay System was purchased from Promega (Madison, WI). Antibody against phospho-Thr-38 of CAR was kindly provided by Dr. Masahiko Negishi (National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC). Other antibodies used in this study include human CAR antibody from Perseus Proteomics (Tokyo, Japan), antibodies against phosphorylated AMPK (Thr172), ERK1/2 (Thr202/Tyr204), p38 MAPK (Thr180/Tyr182), and EGFR (Tyr1068) from Cell Signaling Technology (Danvers, MA), antibodies against SRC1, GRIP1, β-actin, and HRP-conjugated antibodies against rabbit or mouse from Santa Cruz Biotechnology (Santa Cruz, CA). Other cell culture reagents were purchased from Invitrogen or Sigma-Aldrich.

Culture and treatment of human primary hepatocytes

Human liver tissues were obtained following surgical resection by clinical staff after diagnostic criteria were met and with prior approval from the Institutional Review Board at the University of Maryland School of Medicine. Hepatocytes were isolated from human liver specimens by a

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modification of the two-step collagenase digestion method as described previously (LeCluyse et al., 2005), or obtained from Bioreclamation In vitro Technologies (Baltimore, MD). Demographic information for all human liver donors was summarized in supplementary table 1 (Supplementary Data). Primary hepatocytes were cultured and maintained as described previously (Wang et al., 2003). Thirty-six hours after seeding, hepatocytes were treated with vehicle control (0.1% DMSO), PB (1 mM), CITCO (1 μM), MET (100, 500, 1000 μM) or their combination for another 24 h or between a period of 15 min - 72 h before the detection of mRNA and protein expression, respectively. Cell culture medium was replaced on a daily basis.

Plasmid Constructions

The CYP2B6-PBREM/XREM luciferase reporter plasmid was constructed as described previously (Wang et al., 2003). The adenovirus expressing the EYFP-tagged hCAR (Ad/EYFP-hCAR) was generated as reported earlier (Li et al., 2009). For mammalian two-hybrid assay, pG5-Luc, pACT, and pBind were obtained from Promega. pACT-hCAR, pM-SRC-1 (621-765), and pM-GRIP-1 were kindly provided by Dr. Masahiko Negish (Li et al., 2008; Ueda et al., 2005). The pRL-TK *Renilla* luciferase plasmid used to normalize firefly luciferase activities was from Promega.

Quantitative PCR

Total RNA was isolated from hepatocytes using the TRIzol reagent (Qiagen), and reverse transcribed using the High Capacity cDNA Archive kit (Applied Biosystems, Foster, CA) following the manufacturers' instructions. Primer sequences for real-time PCR assays were as reported previously (Wang et al., 2011a), including: CYP2B6: 5'-AGACGCCTTCAATCCTGACC-3' (forward), 5'-CCTTCACCAAGACAAATCCGC-3'

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(reverse); GAPDH: 5'-CCCATCACCATCTTCCAGGAG-3' (forward), 5'-GTTGTCATGGATGACCTTGGC-3' (reverse). Quantitative real-time PCR assays were performed on an ABI Prism 7000 Sequence Detection System with SYBR Green PCR Master Mix (Applied Biosystems). Fold induction values were calculated according to the equation: fold over control = $2^{\Delta\Delta Ct}$, where ΔCt represents the differences in cycle threshold numbers between the target gene and GAPDH, and ΔΔCt represents the relative change in these differences between control and treatment groups. Expression of CYP2B6 was normalized against that of GAPDH.

Western blotting

Homogenate proteins (40 μg) from treated cells were resolved on SDS-polyacrylamide gels, and electrophoretically transferred onto Polyvinylidenefluoride membranes. Subsequently, membranes were respectively incubated with specific primary antibodies against CYP2B6, phosphorylated hCAR (Thr38), human CAR, phosphorylated forms of AMPK (Thr172), EGFR (Tyr1068), ERK1/2 (Thr202/Tyr204), and p38 MAPK (Thr180/Tyr182). Beta-actin was used for normalization of protein loading. After incubating with HRP-labeled IgG secondary antibodies, membranes were developed with West Pico/Femto chemiluminescent substrate (Thermo, Rockford, IL).

Transient transfection and luciferase reporter assay

Human primary hepatocytes in 24-well plates were co-transfected with CYP2B6-PBREM/XREM firefly and the pRL-TK renilla reporter plasmids using Effectene transfection reagent following the manufacturer's instruction. Twenty-four hours after transfection, cells were treated with solvent (0.1%DMSO), PB (1 mM), CITCO (1 μM), MET (0.1, 0.5, 1 mM) or their

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combination as indicated in the figures for another 24 h. Subsequently, cell lysates were assayed for firefly activities normalized against the activities of renilla using the Dual-Luciferase kit (Promega, Madison, WI). Data are represented as mean ± S.D. of three individual transfections.

Nuclear translocation of CAR

As reported previously (Li et al., 2009), a recombinant adenovirus Ad/EYFP-hCAR has been generated and functionally characterized, in which it infects human hepatocytes with high efficiency and maintains hCAR distribution characteristics in a physiologically relevant manner. Human hepatocytes seeded on collagen coated cover slides in 6-well plates were infected with the AD/EYFP-hCAR for 24 h, and followed by the treatment with DMSO (0.1% v/v), PB (1 mM), CITCO (1 μM), metformin (1 mM) or the combination of metformin with PB or CITCO for 12 h. Subsequently, hepatocytes were fixed with paraformaldehyde and stained with 4,6-diamidino-2-phenylindole (DAPI) for nucleus visualization. The localization of EYFP-hCAR was quantified by counting more than 100 cells per treatment group using Confocal Nikon TE2000 as described previously (Li et al., 2009). For each treatment, hCAR localizations were classified as cytosolic, nuclear, or mixed (cytosolic and nuclear).

Mammalian Two-Hybrid Assay

COS1 cells in 24-well plates were transfected with 110 ng of the reporter plasmid pG5-luc, 80 ng pACT-hCAR, 40 ng pM-SRC-1 or pM-GRIP-1, and 20 ng of reference plasmid pRL-TK using XtremeGENE9. Sixteen hours after transfection, the cells were treated with vehicle control (0.1% DMSO), PB (1 mM), CITCO (1 μM), MET (1 mM) or their combination for another 24 h. Luciferase activities were measured as described above. Data were represented as mean ± S.D. of three individual transfections.

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Co-immunoprecipitation Assays

COS7 cells were transfected with 5 μg hCAR in the presence of 5 μg SRC-1 or GRIP-1 expression plasmid for 24 h. Subsequently, transfected cells were washed with phosphate buffered saline buffer and scraped into Cell Lysis Buffer (Cell Signaling). After incubation on ice for 15 min, the cell lysate was centrifuged at 13,000 x g for 15 min at 4°C. The supernatant fractions were collected and pre-cleared by Protein A-Sepharose beads (Life Technologies). Equal amounts of protein lysate aliquots were then incubated with (0.1% DMSO), PB (0.5, 1, 2 mM), CITCO (0.5, 1, 2 μM), MET (0.1, 1, 2 mM) or their combinations overnight at 4°C, in the presence of Protein A-Sepharose beads and antibody against SRC1 or GRIP1 (Santa Cruz). Corresponding isotope IgG was used as negative control. The precipitated protein complexes were analyzed by western blotting with anti-hCAR antibody.

Statistical analysis

All data represent at least three independent experiments and are expressed as the mean ± S.D. Statistical comparisons were made using one-way analysis of variance (ANOVA) followed by a post hoc Dunnett's test or Student's t test where appropriate. The statistical significance was set at p values < 0.05 (*), or < 0.01 (**).

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Results

Metformin represses PB and CITCO induced CYP2B6 expression

To investigate the effects of metformin on the expression of CYP2B6, human hepatocytes were treated with typical hCAR activators PB or CITCO in the presence or absence of metformin, as described under "*Materials and Methods*". In primary hepatocytes (HL#73 and #74), PB (1 mM) and CITCO (1 μM) treatment increased the expression of CYP2B6 mRNA by over 25-fold compared to the vehicle control. Notably, the CYP2B6 mRNA induction was suppressed by pharmacologically relevant levels of metformin with maximal repression of CYP2B6 mRNA expression reaching 85-98% at 1 mM of metformin (Figure 1A). Consistent with mRNA levels, expression of CYP2B6 protein induced by PB and CITCO was also significantly suppressed by the presence of metformin (Figure 1B). However, metformin alone did not markedly change the basal expression of CYP2B6 in human liver. Given that CITCO and PB are prototypical direct and indirect activators of hCAR, these results suggest that metformin most likely affects CYP2B6 induction through its interaction with CAR.

Metformin suppresses CAR activation in human primary hepatocytes

Different from immortalized cell lines, a more physiologically relevant model of CAR function is demonstrated in primary hepatocytes, in which CAR is predominantly expressed in the cytoplasm under basal condition and translocates into the nucleus upon chemical activation. To determine the effects of metformin on the activity of hCAR, a CYP2B6 reporter construct was transfected into human primary hepatocytes prepared from donors (HL#74 and HL#80). As shown in Figure 2A, both PB and CITCO strongly enhanced, while metformin decreased

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CYP2B6 promoter activity. Notably, co-treatment with 1 mM of metformin completely abolished PB- and CITCO-mediated activation of hCAR, resembling its suppression of CYP2B6 induction at both mRNA and the protein levels.

Nuclear translocation of CAR has been established as the requisite and initial step of CAR activation (Kawamoto et al., 1999). Utilizing a recently generated Ad/EYFP-hCAR, the effect of metformin on hCAR translocation was investigated in human primary hepatocytes infected with this EYFP-hCAR expressing virus as described under "*Materials and Methods*". Confocal microscopy analysis showed that EYFP-hCAR expression was predominantly cytoplasmic under vehicle control (0.1% DMSO) and translocated to the nucleus upon PB and CITCO treatment, which could be abrogated by the presence of metformin (Figure 2B). Collectively, these results indicate that the repression of CYP2B6 expression in human hepatocytes by metformin could be attributed to its effect of suppressing CAR activation and nuclear translocation elicited by typical activators.

Metformin disrupts interaction between hCAR and co-activators

The nuclear transactivation of CAR featured by the recruitment of co-activators is a crucial step that differs between direct and indirect activation of hCAR (Auerbach et al., 2005; Chen et al., 2010). In that, although both direct and indirect activators efficiently translocate CAR from the cytoplasm to the nucleus, only agonistic binding of CAR can enhance the recruitment of co-activators. Here, we further investigated the effects of metformin on interactions between CAR and steroid receptor co-activators including SRC1 and GRIP1. As shown previously, transfected CAR spontaneously accumulates in the nucleus of immortalized cell lines, which differs from that in primary hepatocytes (Kawamoto et al., 1999). Therefore, our mammalian two-hybrid and

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co-immunoprecipitation assays in COS1 and COS7 cells were not intended to reflect the effects of metformin on CAR nuclear translocation. In mammalian two-hybrid assays, interaction between hCAR and SRC1 or GRIP1 was enhanced by the hCAR agonist CITCO, but not affected by PB the indirect activator (Fig. 3A, 3B). Importantly, the increased luciferase activity mediated by CITCO was significantly antagonized by metformin (Figure 3A, 3B), suggesting that metformin could disrupt the ligand-dependent interaction between CAR and SRC1 or GRIP1.

To confirm this correlation, next we overexpressed hCAR and co-activator proteins in COS7 cells and investigated their interactions under the treatment of PB, CITCO and metformin as outlined in "*Materials and Methods*". As shown in figure 3C and 3D, metformin decreased the amount of CAR protein precipitated by SRC1 or GRIP1 in Co-IP assays, supporting that metformin was able to disrupt the binding of CAR with co-activators. Moreover, dissociations of hCAR and SRC1/GRIP1-mediated by metformin were restored by increasing amount of CITCO through direct ligand-binding competition, but not by PB the indirect activator of CAR. Together, these results suggest that metformin affects multiple stages of hCAR activation that are differentially associated with direct and indirect CAR activators.

Metformin enhances the phosphorylation of CAR

Previous studies have shown that dephosphorylation of CAR (Thr-38) is required for nuclear translocation and activation of CAR (Mutoh et al., 2009). To investigate whether metformin alters the phosphorylation status of CAR in human primary hepatocytes, firstly we confirmed the effects of PB and CITCO on CAR dephosphorylation. As shown in Figure 4A, both PB and CITCO decreased CAR phosphorylation 24 h after treatment, while the expression level of total CAR was unchanged. In agreement with previous reports, our result indicated that

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dephosphorylation is associated with CAR activation mediated by both direct and indirect activators.

The effects of metformin on CAR phosphorylation were evaluated in both time- and concentration-dependent manners. As shown in Figure 4B, metformin (1 mM) time-dependently enhanced the phosphorylation of CAR up to 24 h in hepatocytes prepared from three liver donors (HL#77, #78, #80). In separate experiment, hepatocytes were exposed to metformin at the concentrations of 0.1, 0.5 and 1 mM for 24 h. Concentration-dependent enhancement of CAR phosphorylation was clearly observed in liver donor HL#73 with low basal phosphorylation levels of CAR (Figure 4C), while metformin had minimal effects in hepatocytes from donor HL#74 with high basal levels of CAR phosphorylation. Importantly, PB-mediated dephosphorylation of CAR was efficiently restored by metformin in a concentration-dependent manner (Figure 4D). In the case of CITCO, co-treatment of metformin increased the phosphorylation status of CAR but not in a clearly concentration-dependent fashion (Figure 4E). Overall, these findings parallel with the observed effects of metformin on CAR activation and CYP2B6 transactivation, suggesting that metformin suppresses human CAR activation by enhancing its phosphorylation.

Metformin promotes CAR phosphorylation by activating AMPK

Given that metformin is a well-established activator of AMPK, we subsequently evaluated the role of AMPK in metformin-mediated CAR phosphorylation and CYP2B6 repression. As shown in Figure 5A, both PB- and CITCO-induced CYP2B6 mRNA and protein were significantly repressed by AICAR, a prototypical activator of AMPK. Conversely, the metformin-mediated repression of CYP2B6 induction (by PB or CITCO) was partially restored in a concentration-

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dependently manner by compound C, a known inhibitor of AMPK (Figure 5B). In phosphorylation experiments, in contrast to the effects of metformin, compound C alone dramatically decreased the phosphorylation of AMPK as well as hCAR (Figure 5C). Metformin enhanced phosphorylation of both AMPK and CAR was efficiently disrupted by the presence of compound C (Figure 5C). Moreover, both PB and CITCO decreased the phosphorylation of AMPK at 24 h after treatment (Figure 5D). Collectively, these results suggest that metformin enhancing CAR phosphorylation is at least partly dependent on AMPK activation.

ERK1/2 and p38 MAPK signaling in metformin-mediated CAR phosphorylation

It was reported previously that both ERK1/2 and p38 MAPK signaling pathways were involved in the mechanism for CAR phosphorylation and activation (Koike et al., 2007; Osabe and Negishi, 2011; Saito et al., 2013). Here, we further investigated whether these two kinases are important for metformin-mediated CAR phosphorylation and CYP2B6 repression. Figure 6A indicates that treatment with metformin (1 mM) increased the active phosphorylated forms of ERK1/2 but decreased the phosphorylated-p38 MAPK in human hepatocytes from three donors. Consistent with these observations, induction of CYP2B6 expression by a known inhibitor of ERK1/2 activation (U0126) was concentration-dependently repressed by metformin (Figure 6B). In contrast, PB- and CITCO-mediated induction of CYP2B6 was repressed by metformin but not by SB202190, a known inhibitor of p38 MAPK (Figure 6C). These results suggest that while ERK1/2 activation may partially contribute to metformin-mediated suppression of CYP2B6, dephosphorylation of p38 MAPK alone is not sufficient to alter PB and CITCO induced expression of CYP2B6.

EGFR signaling does not affect metformin-induced hCAR deactivation

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Most recently, EGFR was identified as the initial target for PB-mediated CAR activation, where PB directly binds and disrupts EGFR signaling to elicit CAR dephosphorylation (Mutoh et al., 2013). Therefore, potential contribution of the EGFR signaling in metformin-induced CAR deactivation was evaluated in human primary hepatocytes. As expected, co-treatment of EGF (25 and 100 ng/ml) potently reduced the mRNA expression of CYP2B6 induced by PB and CITCO (Figure 7A); while the known EGFR inhibitor, erlotinib decreased EGFR phosphorylation and induced CYP2B6 expression at both mRNA and protein levels (Figure 7B). Notably, as shown in Figure 7C, the phosphorylation status of EGFR at Tyr-1068 was largely unaffected by metformin treatment. Moreover, metformin-mediated repression of CYP2B6 expression was not influenced by the presence of increasing doses of erlotinib (Figure 7D); and metformin did not affect CYP2B6 expression induced by erlotinib (Figure 7E). These findings suggest that although EGFR signaling plays an important role in the phosphorylation and activation of CAR, metformin-mediated deactivation of hCAR is most likely an EGFR-independent event.

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Discussion

Our study identified that metformin, a widely used anti-diabetic drug, suppressed CYP2B6 gene transactivation by inhibiting the activation of CAR in human hepatocytes. Mechanistic investigation demonstrated that metformin enhanced the phosphorylation of CAR at Thr-38 thereby inhibiting its nuclear translocation. Particularly, we characterized the signaling regulation of CAR phosphorylation. Our data indicated that metformin induces CAR phosphorylation and deactivation primarily through the AMPK and ERK1/2 pathways, with the p38 MAPK and EGFR signaling showing negligible involvement. Additionally, metformin also disrupts the interaction between CAR and co-activators, which was enhanced by CITCO, the direct activator of hCAR (Figure 8).

In contrast to most nuclear receptors, CAR can be activated through both direct and indirect mechanisms (Moore et al., 2000). Nevertheless, nuclear translocation of CAR from the cytoplasm is the initial and essential step for its activation regardless of the mechanisms involved (Kawamoto et al., 1999). Previous studies demonstrated that phosphorylated CAR is predominantly retained in the cytoplasm of primary hepatocytes and de-phosphorylation of Thr-38 initiates the nuclear translocation and activation of CAR (Mutoh et al., 2009; Osabe and Negishi, 2011). In this report, we showed that both PB and CITCO decreased the Thr-38 phosphorylation of CAR in primary human hepatocytes, correlating well with the apparent nuclear translocation and marked CYP2B6 induction. Notably, co-treatment with metformin at concentrations bracketing its pharmacologically relevant levels dramatically repressed both PB- and CITCO-induced expression of CYP2B6, and clearly enhanced CAR phosphorylation in both time- and concentration-dependent manners. This finding has led to the interrogation of how

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metformin modulates the signaling pathways involved in CAR phosphorylation.

AMPK activation is an important mechanism by which metformin suppresses hepatic gluconeogenesis and glucose efflux. Recent studies have suggested that AMPK was involved in the PB-mediated induction of CYP2B gene expression (Rencurel et al., 2005), however, its precise role in the regulation of CAR activation is yet contradictory. Rencurel et al. showed that liver-specific deletion of AMPK catalytic subunits in mouse impaired PB-induced expression of Cyp2b10 and Cyp3a11, without affecting the nuclear translocation of CAR (Rencurel et al., 2006). Therefore, the authors presumed the existence of an additional control step of CAR signaling elicited by AMPK that is independent of translocation. In a different study, Shindo et al. demonstrated that metformin and AICAR induced CAR nuclear translocation but failed to induce hepatic CYP2B gene in mouse and rat livers (Shindo et al., 2007). In contrast, another study revealed AICAR but not metformin prevented nuclear translocation of CAR and repressed PB-induced CYP2B expression in rat primary hepatocytes (Kanno et al., 2010). Although AMPK appears to be involved in the CAR signaling, these studies provide contradictory outcomes when connecting CYP2B transactivation and CAR translocation to AMPK activation, which may be partially attributed to the known species-specific feature of CAR. Using human primary hepatocytes, we consistently showed in the current study that metformin robustly repressed PB/CITCO-induced CYP2B6 expression through inhibiting the de-phosphorylation and translocation of CAR. Importantly, AICAR treatment mimicked the effect of metformin on CYP2B6 suppression, while this suppression was partially but concentration-dependently recovered by the AMPK inhibitor compound C. However, it is noteworthy to mention that the maximal recovery of metformin-mediated CYP2B6 mRNA repression by the compound C was approximately 7-fold, and accounted only around 10% of uninhibited PB induction, suggesting

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the involvement of additional signaling pathways. Although activation of AMPK may not directly phosphorylate CAR at Thr-38, signaling molecules downstream of the AMPK pathway may function as the switch controlling the status of CAR phosphorylation. In addition, we also found that PB and CITCO both notably decreased AMPK phosphorylation at 24 h after treatment, which was in line with CAR de-phosphorylation. Taken together, these data indicate that metformin enhances CAR phosphorylation in human hepatocytes in part through an AMPK-dependent signaling pathway.

In addition to AMPK signaling, the epidermal growth factor and MAPK pathways have also been suggested to be involved in the regulation of CAR phosphorylation (Bauer et al., 2004; Koike et al., 2007; Mutoh et al., 2013). In mouse primary hepatocytes, ERK1/2 activation by growth factors such as EGF and HGF effectively repressed CAR de-phosphorylation by PB and 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), and sequestered CAR in the cytoplasm (Bauer et al., 2004; Osabe and Negishi, 2011), while inhibition of the ERK1/2 pathway by U0126 induced the Cyp2b10 gene and enhanced the CAR-regulated promoter activity (Koike et al., 2007). Our results showed that metformin enhanced the activity of ERK1/2, and deactivation of ERK1/2 by U0126 was associated with induced CYP2B6 expression in human hepatocytes. Importantly, U0126 induced CYP2B6 expression was efficiently repressed by metformin, suggesting ERK1/2 activation may represent another important route by which metformin represses CAR activation. Recently, p38 MAPK was identified as a required factor in direct activation of CAR by ligands such as CITCO and TCPOBOP in hepatoma cell lines (Saito et al., 2013). Nevertheless, our observation does not support a role of p38 MAPK in metformin-mediated deactivation of CAR in human hepatocytes. Although metformin can potently decrease

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the phosphorylation of p38 MAPK, inhibition of p38 MAPK alone was not sufficient to affect either PB- or CITCO-induced expression of CYP2B6.

Most recently, Negishi and colleagues identified EGFR as a PB-responsive receptor mediating CAR de-phosphorylation and activation in mice (Mutoh et al., 2013). In this study, we also confirmed the role of EGFR signaling in the regulation of CYP2B6 in human hepatocytes. However, the effect of metformin on EGFR phosphorylation at Tyr-1068, which mediates Grb2 adaptor protein binding and Ras-ERK1/2 pathway activation, was modest. More importantly, inhibition of EGFR by erlotinib failed to restore metformin-mediated repression of CYP2B6; and metformin did not repress erlotinib induced CYP2B6 expression. This result indicates that metformin-induced CAR phosphorylation was independent of the EGFR activation in human hepatocytes.

Translocation of CAR into the nucleus is essential but may not be sufficient for the activation of this receptor (Shindo et al., 2007). Although metformin can rapidly increase the phosphorylation status of CAR at Thr-38 within 1 h (Figure 4B), we were unable to assess whether this event could lead to a direct and rapid repression of CAR nuclear accumulation, given that PB- and CITCO-induced nuclear translocation of the Ad/EYFP-hCAR did not occur until at least 3 h and was maximized at 12 h after treatment in human primary hepatocytes (Data not shown). Once inside the nucleus, CAR undergoes heterodimerization with retinoid X receptor (Auerbach et al., 2005; Frank et al., 2003) and recruitment of co-activators such as SRC1 (Muangmoonchai et al., 2001) and GRIP1 (Miao et al., 2006) to stimulate target gene transcription. Thus, a multi-stage CAR activation process was proposed, where the activity of nuclear localized CAR can be further regulated by its interaction with co-regulators, which is often influenced by direct activators. For example, the transcriptional activity of CAR can be

promoted by agonists such as CITCO and TCPOBOP with enhanced interaction with SRC1 and GRIP1 (Miao et al., 2006; Muangmoonchai et al., 2001), while competitively inhibited by PK11195, clotrimazole or KN-62 , without affecting the nuclear accumulation of CAR (Li et al., 2009; Li et al., 2008; Yamamoto et al., 2003). Our data from two-hybrid and Co-IP assays show that metformin also attenuated interactions between CAR and co-activators, which could be restored by CITCO but not PB, indicating different roles of metformin in affecting direct and indirect activation of CAR.

In conclusion, our results have shown that the anti-diabetic agent metformin is a potent repressor for CYP2B6 transcription through both direct and indirect inhibition of CAR activity. Specifically, metformin mediates CAR deactivation at the initial nuclear translocation stage by enhancing the phosphorylation of CAR, and the nuclear activation stage by disrupting ligand-dependent recruitment of co-activators. Among protein kinases investigated, AMPK and ERK1/2 pathways appear to be important for the effects of metformin on the activity of CAR, with EGFR and p38 MAPK negligibly involved. Meanwhile, we do realize that neither AMPK nor ERK1/2 signaling alone was sufficient to cope with the metformin-mediated robust suppression of CYP2B6 induction, suggesting additional signaling molecules may contribute to this specific metformin response, which warrants further investigation.

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Authorship Contributions

Participated in research design: H. Yang, P. Shapiro, and H. Wang

Conducted experiments: H. Yang, and B. Garzel

Contributed new reagents or analytic tools: P. Shapiro, S. Heyward, and T. Moeller

Performed data analysis: H. Yang, P. Shapiro, and H. Wang

Wrote or contributed to the writing of the manuscript: H. Yang, B. Garzel, S. Heyward, T. Moeller, P. Shapiro, and H. Wang

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FOOTNOTES

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Figure legends

Figure 1 Metformin represses PB and CITCO induced CYP2B6 expression. Human hepatocytes (HL#73, #74) were treated with DMSO (0.1%), PB (1 mM), CITCO (1 μM), MET (0.1, 0.5, 1 mM), or their combinations as indicated for 24 h or 72 h for analysis of mRNA and protein expression, respectively. Total RNA extracted from hepatocytes was subjected to real-time PCR analysis of CYP2B6 expression (A). Homogenate proteins (20 μg) from each group were prepared for CYP2B6 and β-actin immunoblotting analysis (B). Data represent the mean ± S.D. of three independent transfections (**, $p < 0.01$).

Figure 2 Metformin suppresses CAR activation in human primary hepatocytes. (A) Human primary hepatocytes (HL#74, and #80) were transfected with the CYP2B6 reporter and the pRL-TK plasmids using Effectene reagent as described in "*Materials and Methods*". Subsequently, transfected hepatocytes were treated with DMSO (0.1%), MET (1 mM), PB (1 mM), CITCO (1 μM), or their combinations for 24 h before the determination of luciferase activities. Data represent the mean ± S.D. of three independent transfections (*, $p < 0.05$; **, $p < 0.01$). (B) Human hepatocytes were infected with Ad/EYFP-hCAR for 24 h followed by treatment with DMSO (0.1%), MET (1 mM), PB (1 mM), CITCO (1 μM), or their combinations as indicated for 12 h. Confocal images of hepatocytes from a representative donor (HL#77) were shown for the localization and translocation of EYFP-hCAR. Over 100 EYFP-hCAR-expressing cells per treatment group were counted and classified as cytosolic, nuclear, or mixed (cytosolic and nuclear) cellular localizations.

Figure 3 Metformin disrupts interaction between hCAR and co-activators. Mammalian two-hybrid assays were performed in COS1 cells transfected with expression plasmids encoding

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VP16-AD/hCAR fusion proteins and GAL4-DBD/co-activator fusion proteins as indicated (A, SRC1 and B, GRIP1) together with the reporter gene plasmid pG5*luc* for 16 h. Cells were treated with vehicle control (0.1% DMSO), PB (1 mM), CITCO (1 μM), MET (1mM) or their combination for another 24 h before the determination of luciferase activities. Data represent the mean ± S.D. of three independent transfections (**, $p < 0.01$). Co-immunoprecipitation assays were performed using COS7 cells transfected with expression plasmids encoding hCAR and co-activator proteins (C, SRC1 and D, GRIP1). The whole cell lysate was collected and incubated with DMSO (0.1%), MET (0.1, 1, 2 mM), PB (0.5, 1, 2 mM), CITCO (0.5, 1, 2 μM), or their combination overnight at 4°C. Proteins were immune-precipitated and analyzed by western blotting as outlined in "*Materials and Methods"*.

Figure 4 Metformin enhances phosphorylation of CAR. Homogenate proteins from treated human hepatocytes were used for western blotting analysis of phosphorylated Thr-38 hCAR, total hCAR and β-actin as described in "*Materials and Methods*". Specific treatments in this study include, DMSO (0.1%), PB (1 mM) or CITCO (1 μM) for 24 h in human hepatocytes (HL#69, #77, and #81) **(**A); MET (1 mM) for 0, 15 min, 1 h, 4 h, and 24 h in human hepatocytes (HL#77, #78, and #80) (B); MET (0.1, 0.5, 1 mM) (C), and the combination of MET with PB (1 mM) (D) or CITCO (1 μM) (E) for 24 h in human hepatocytes (HL#73, #74, and #82).

Figure 5 Metformin promotes CAR phosphorylation by activating AMPK. (A) Human hepatocytes (HL#78, and #81) were treated with DMSO (0.1%), AICAR (100, 250 μM), PB (1 mM), CITCO (1 μM), or their combinations as indicated. CYP2B6 expression was assayed by real-time PCR and western blotting. **(**B) Human hepatocytes (HL#74 and #83) were treated with 0.1% DMSO, PB (1 mM), CITCO (1 μM), MET (1 mM), Compound C (5, 10, 20 μM), or their combinations as outlined in "*Materials and Methods*". Total RNA from each group was analyzed

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by real-time PCR for CYP2B6 expression. Homogenate proteins from each treated hepatocytes were used for western blotting of CYP2B6, hCAR (Thr-38), and β-actin. **(**C) Human hepatocytes (HL#81) were treated with MET (1 mM), Compound C (20 μM), or their combination for 0, 15 min, 1 h, 4 h, and 24 h. Homogenate proteins from treated hepatocytes were used for western blotting analysis of hCAR (Thr-38), AMPK (Thr172), and β-actin. (D) Phosphorylation of AMPK (Thr172) was also measured in human hepatocytes (HL#78, #82, and #83) after the treatment of vehicle control (0.1% DMSO), PB (1 mM), or CITCO (1 μM) for 24 h. Data represent the mean ± S.D. of three independent transfections (**, $p < 0.01$).

Figure 6 ERK1/2 and p38 MAPK signaling in metformin-mediated CAR phosphorylation. Human hepatocytes (HL#77, #78, and #80) were treated with MET (1 mM) for 0, 15 min, 1 h, 4 h, and 24 h. Homogenate proteins from each treatment group were used for western blotting analysis of AMPK (Thr172), ERK1/2 (Thr202/Tyr204), p38 MAPK (Thr180/Tyr182), and β-actin (A). Real-time PCR was employed to measure CYP2B6 expression in human hepatocytes (HL#74, and HL#80) treated for 24 h with 0.1% DMSO, MET (0.1, 0.5, 1 mM), U0126 (10 μM), PB (1 mM), CITCO (1 μM), SB212190 (1, 5, 10 μM) or their different combinations as indicated in (B) and (C). Dephosphorylation of ERK1/2 by U0126 (10 μM) and p38 MAPK by SB212190 (10 μM) were analyzed in treated hepatocytes (HL#82). Data represent the mean ± S.D. of three independent transfections (*, $p < 0.05$; **, $p < 0.01$).

Figure 7 EGFR signaling does not affect metformin-induced hCAR deactivation. (A) Human hepatocytes (HL#69) were treated with 0.1% DMSO, PB (1 mM), CITCO (1 μM), EGF (100, 250 ng/ml), or their combinations for 24 h. CYP2B6 expression was analyzed by real-time PCR. (B) Effects of erlotinib (10 μM) on the expression of CYP2B6, and phosphorylation of

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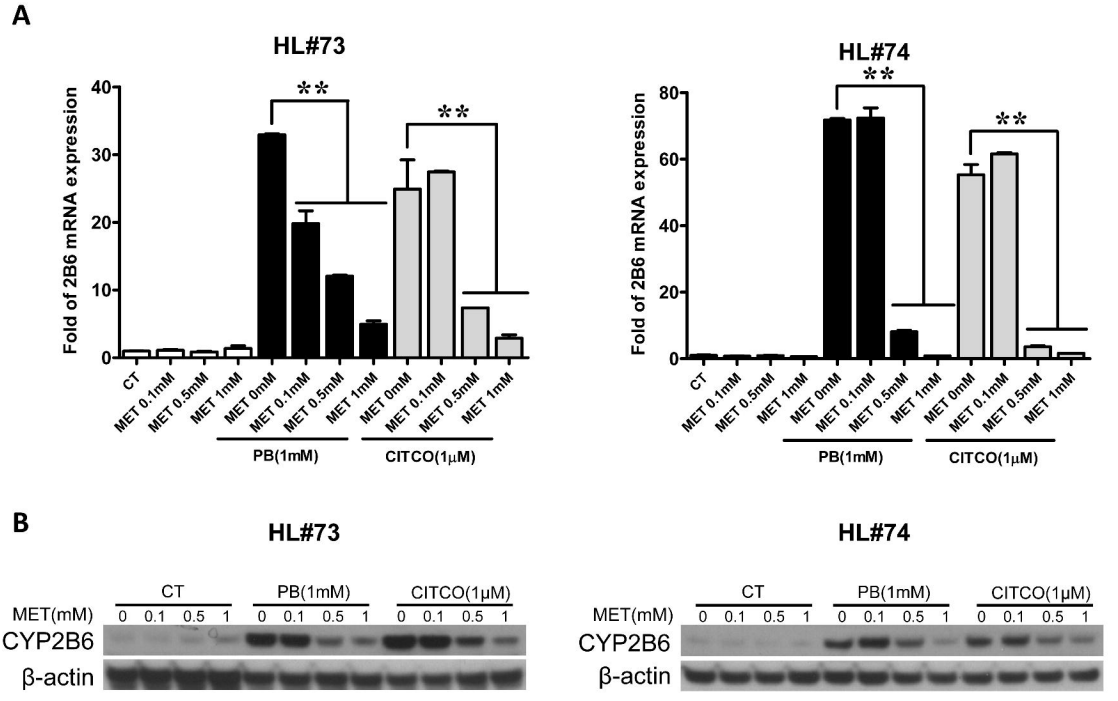
EGFR (Tyr1068) were determined by real-time PCR and western blotting in human hepatocytes (HL#65, #66, #76 or #77) as detailed in "*Materials and Methods*". (C) Immuno-blotting analysis of EGFR (Tyr1068), total EGFR, and β-actin was carried out in human hepatocytes (HL#77 and #81) treated with MET (1 mM) for 0, 15 min, 1 h, 4 h, and 24 h. CYP2B6 mRNA expression was measured in human hepatocytes (HL#80 and #82) treated for 24 h with 0.1% DMSO, MET (0.1, 0.5, 1 mM), PB (1 mM), CITCO (1 μM), Erlotinib (1, 5, 10 μM), or their combinations as indicated in (D) and (E). Data represent the mean ± S.D. of three independent transfections (**, $p < 0.01$).

Figure 8 Model of Metformin-mediated CAR phosphorylation and deactivation. The schematic figure illustrates that metformin inhibits both the nuclear translocation and nuclear activation of CAR, and represses CYP2B6 expression induced by PB and CITCO. In the cytoplasm, metformin enhances CAR phosphorylation thereby blocks its nuclear translocation, primarily through the AMPK and ERK1/2 pathways, with the p38 MAPK and EGFR signaling negligibly involved. Inside the nucleus, metformin can disrupt the interaction between CAR and co-activators, which was enhanced by the direct hCAR activator CITCO. The solid and dash lines indicate strong and weak interactions, respectively. The arrows show activation and the blunt-head lines demonstrate inhibition.

Figure 1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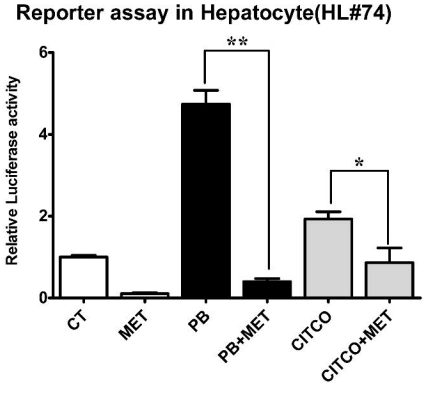
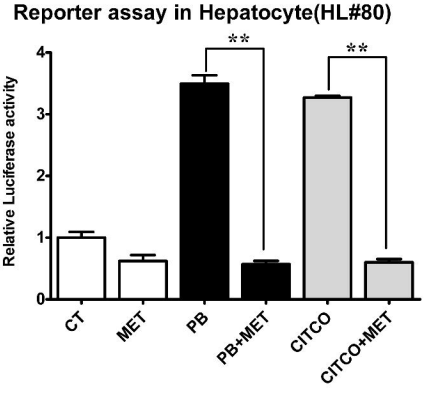
CYP2B6
β-actin

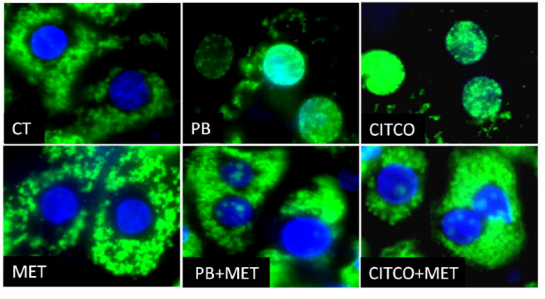
Figure 2

A





B



Quantification of CAR localization

Group	Cytosolic	Nuclear	Mixed	Total
CT	119(93.7%)	2(1.6%)	6(4.7%)	127(100%)
MET	111(98.2%)	0(0%)	2(1.8%)	113(100%)
PB	12(10.0%)	81(67.5%)	27(22.5%)	120(100%)
PB+MET	99(93.4%)	4(3.8%)	3(2.8%)	106(100%)
CITCO	22(18.6%)	42(35.6%)	54(45.8%)	118(100%)
CITCO+MET	80(78.4%)	8(7.8%)	14(13.7%)	102(100%)

Figure 3

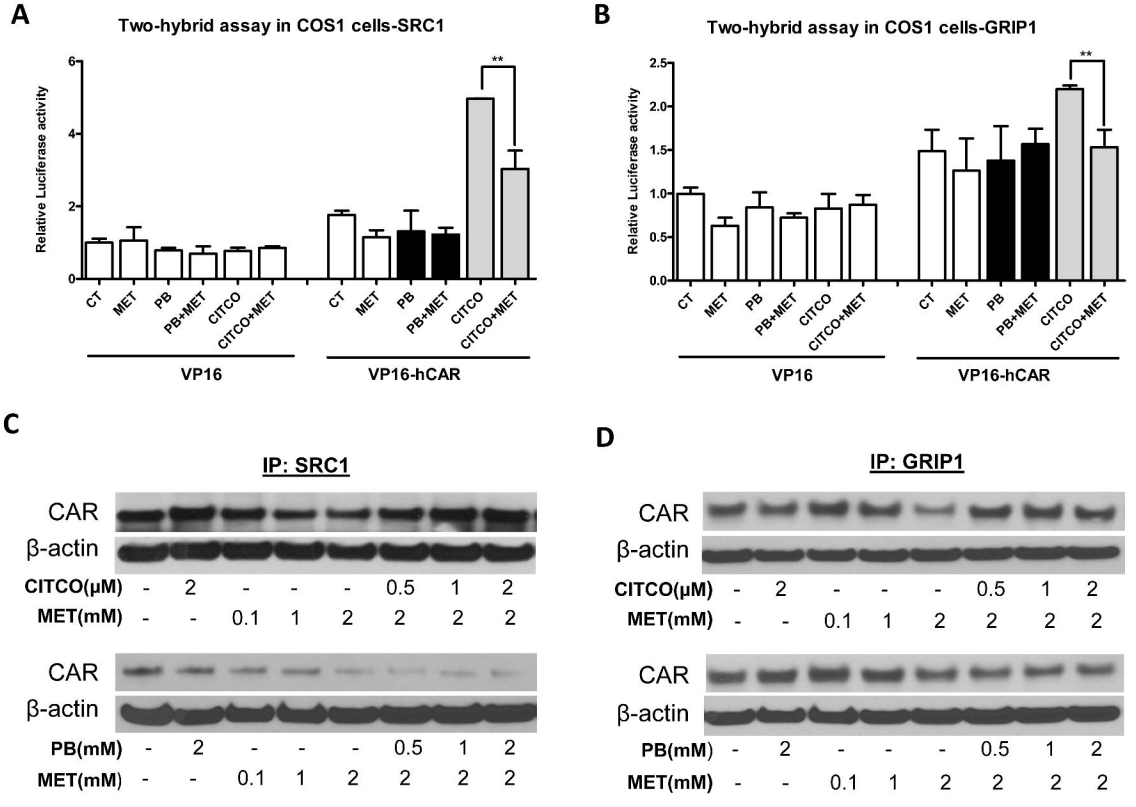

A
Two-hybrid assay in COS1 cells-SRC1
Relative Luciferase activity
CT
MET
PB
PB+MET
CITCO
CITCO+MET
VP16
VP16-hCAR
B
Two-hybrid assay in COS1 cells-GRIP1
C
IP: SRC1
CAR
β-actin
CITCO(μM)
MET(mM)
PB(mM)
D
IP: GRIP1

Figure 4

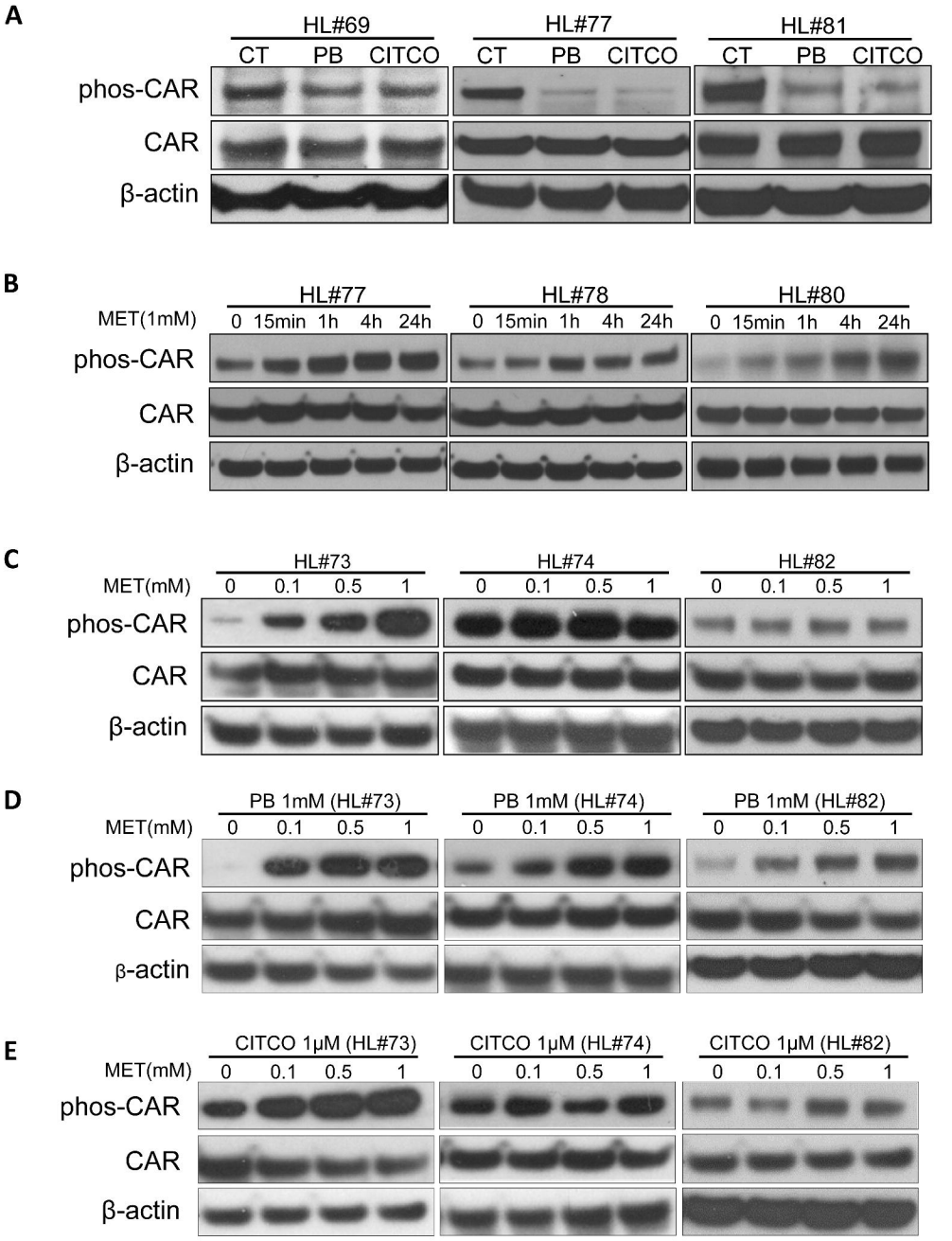

A
HL#69
HL#77
HL#81
CT
PB
CITCO
phos-CAR
CAR
β-actin
B
HL#77
HL#78
HL#80
MET(1mM)
0
15min
1h
4h
24h
phos-CAR
CAR
β-actin
C
HL#73
HL#74
HL#82
MET(mM)
0
0.1
0.5
1
phos-CAR
CAR
β-actin
D
PB 1mM (HL#73)
PB 1mM (HL#74)
PB 1mM (HL#82)
MET(mM)
phos-CAR
CAR
β-actin
E
CITCO 1μM (HL#73)
CITCO 1μM (HL#74)
CITCO 1μM (HL#82)
MET(mM)
phos-CAR
CAR
β-actin

Figure 5

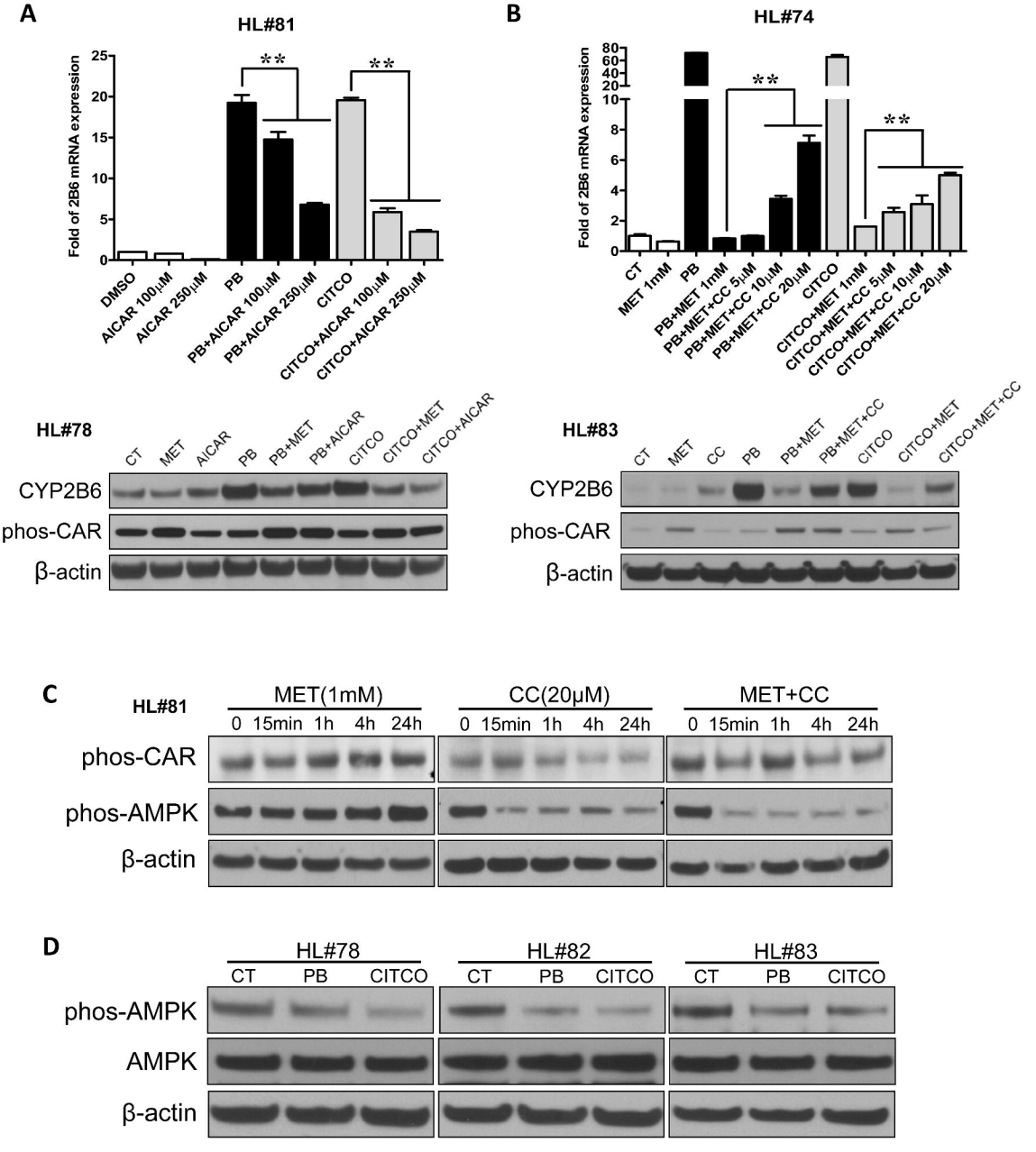

A
HL#81
Fold of 2B6 mRNA expression
DMSO
AICAR 100μM
AICAR 250μM
PB
PB+AICAR 100μM
PB+AICAR 250μM
CITCO
CITCO+AICAR 100μM
CITCO+AICAR 250μM
B
HL#74
Fold of 2B6 mRNA expression
CT
MET 1mM
PB
PB+MET 1mM
PB+MET+CC 5μM
PB+MET+CC 10μM
PB+MET+CC 20μM
CITCO
CITCO+MET 1mM
CITCO+MET+CC 5μM
CITCO+MET+CC 10μM
CITCO+MET+CC 20μM
HL#78
CT
MET
AICAR
PB
PB+MET
PB+AICAR
CITCO
CITCO+MET
CITCO+AICAR
CYP2B6
phos-CAR
β-actin
HL#83
CT
MET
CC
PB
PB+MET
PB+MET+CC
CITCO
CITCO+MET
CITCO+MET+CC
CYP2B6
phos-CAR
β-actin
C
HL#81
MET(1mM)
CC(20μM)
MET+CC
0 15min 1h 4h 24h
phos-CAR
phos-AMPK
β-actin
D
HL#78
HL#82
HL#83
CT PB CITCO
phos-AMPK
AMPK
β-actin

Figure 6

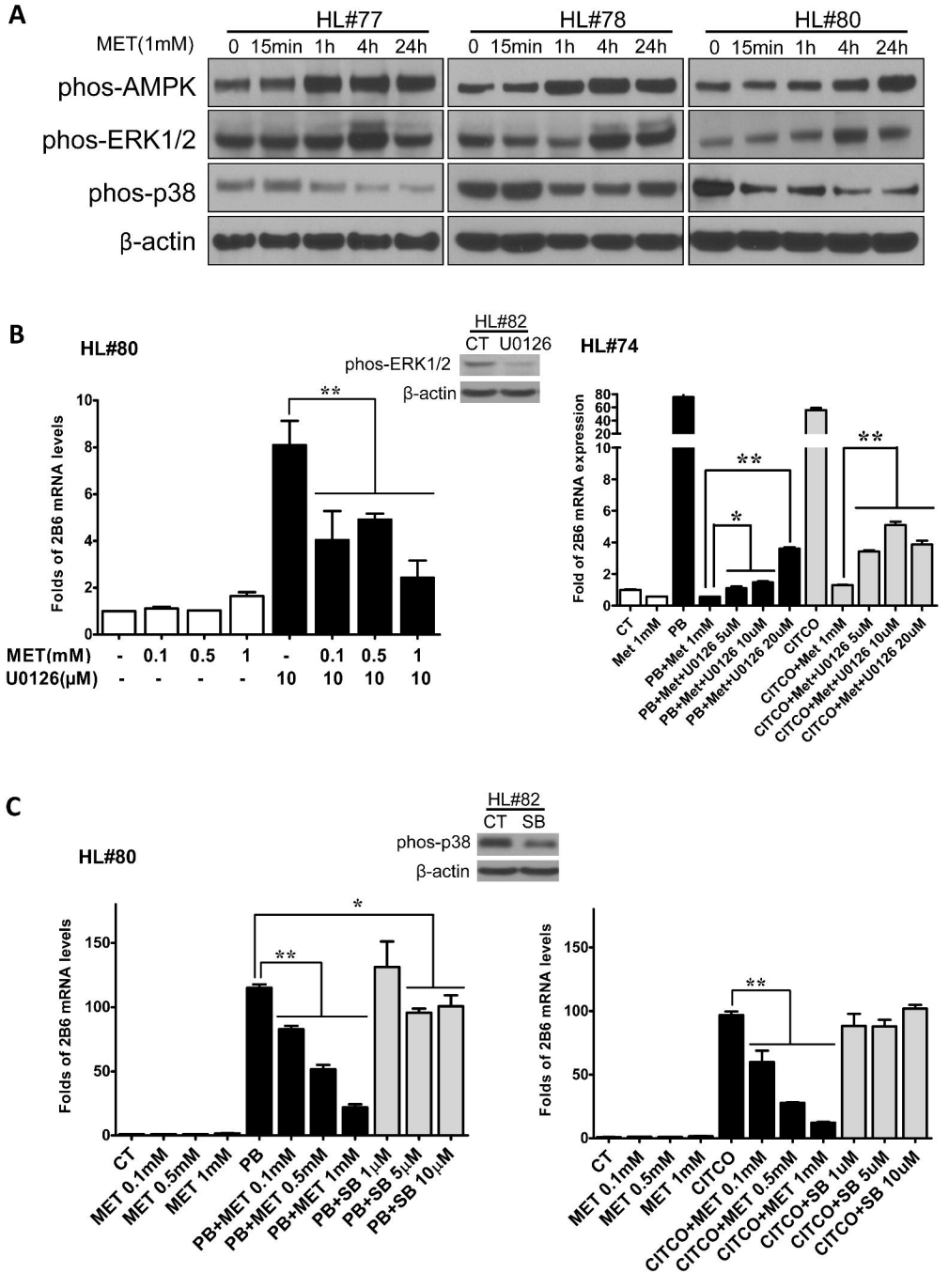

A
HL#77
HL#78
HL#80
MET(1mM)
0 15min 1h 4h 24h
phos-AMPK
phos-ERK1/2
phos-p38
β-actin
B
HL#80
HL#82
CT U0126
phos-ERK1/2
β-actin
Folds of 2B6 mRNA levels
MET(mM) - 0.1 0.5 1 - 0.1 0.5 1
U0126(μM) - - - - 10 10 10 10
HL#74
Fold of 2B6 mRNA expression
CT
Met 1mM
PB
PB+Met 1mM
PB+Met+U0126 5uM
PB+Met+U0126 10uM
PB+Met+U0126 20uM
CITCO
CITCO+Met 1mM
CITCO+Met+U0126 5uM
CITCO+Met+U0126 10uM
CITCO+Met+U0126 20uM
C
HL#80
HL#82
CT SB
phos-p38
β-actin
Folds of 2B6 mRNA levels
CT
MET 0.1mM
MET 0.5mM
MET 1mM
PB
PB+MET 0.1mM
PB+MET 0.5mM
PB+MET 1mM
PB+SB 1μM
PB+SB 5μM
PB+SB 10μM
CITCO
CITCO+MET 0.1mM
CITCO+MET 0.5mM
CITCO+MET 1mM
CITCO+SB 1uM
CITCO+SB 5uM
CITCO+SB 10uM

Figure 7

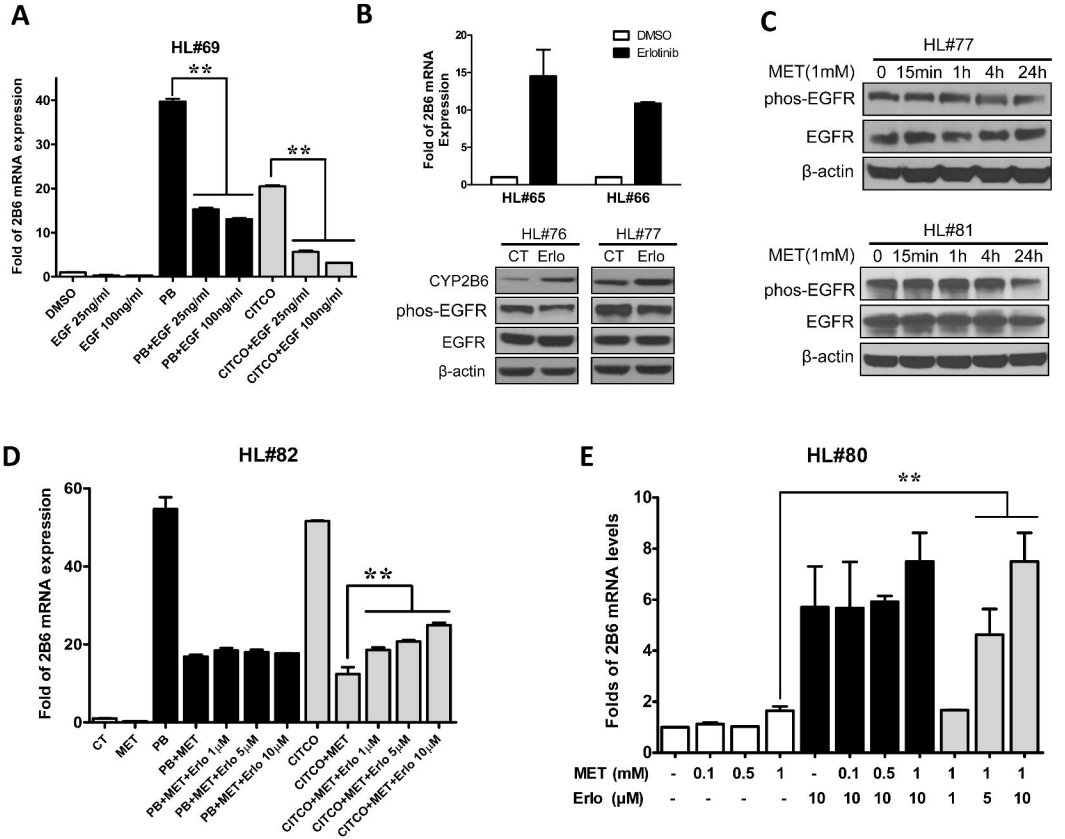

A
HL#69
Fold of 2B6 mRNA expression
**
**
DMSO
EGF 25ng/ml
EGF 100ng/ml
PB
PB+EGF 25ng/ml
PB+EGF 100ng/ml
CITCO
CITCO+EGF 25ng/ml
CITCO+EGF 100ng/ml
B
Fold of 2B6 mRNA Expression
DMSO
Erlotinib
HL#65
HL#66
HL#76
HL#77
CT Erlo
CT Erlo
CYP2B6
phos-EGFR
EGFR
β-actin
C
HL#77
MET(1mM)
0 15min 1h 4h 24h
phos-EGFR
EGFR
β-actin
HL#81
MET(1mM)
0 15min 1h 4h 24h
phos-EGFR
EGFR
β-actin
D
HL#82
Fold of 2B6 mRNA expression
**
CT
MET
PB
PB+MET
PB+MET+Erlo 1μM
PB+MET+Erlo 5μM
PB+MET+Erlo 10μM
CITCO
CITCO+MET
CITCO+MET+Erlo 1μM
CITCO+MET+Erlo 5μM
CITCO+MET+Erlo 10μM
E
HL#80
Folds of 2B6 mRNA levels
**
MET (mM) - 0.1 0.5 1 - 0.1 0.5 1 1 1 1
Erlo (μM) - - - - 10 10 10 10 1 5 10

Figure 8