Title:
Phenobarbital and insulin reciprocate activation of the nuclear receptor CAR through the insulin receptor

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Running title:
PB inactivates the IR to elicit CAR activation signaling

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The number of
Text pages: 28
Tables: 0
Figures: 5
Legends for Figures: 5
References: 24

The number of words in the
Abstract: 137
Introduction: 475
Discussion: 863

Abbreviations:
ANOVA, analysis of variance; CAR, constitutive androstane receptor; cDNA,
complementary DNA; CITCO,
6-(4-chlorophenyl)imidazo(2,1-b)(1,3)thiazole-5-carbaldehyde
O-(3,4-dichlorobenzyl)oxime; CYP, cytochrome P450; ECL, enhanced
chemiluminescence; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor receptor; EGFR, EGF receptor; ERK1/2, extracellular signal-regulated kinase; FBS, fetal bovine serum; FOXO1, forkhead box protein O1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G6Pase, glucose 6-phosphatase; HRP, horseradish peroxidase; IgG, immunoglobulin G; IgM, immunoglobulin M; IR, insulin receptor; IRS, insulin response sequence; KO, knock out; PB, phenobarbital; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PEPCK1, phosphoenolpyruvate carboxykinase 1; PP2Ac, protein phosphatase 2Ac; PVDF, polyvinylidene difluoride, RACK1, receptor of activated protein kinase C 1; RT-PCR, reverse transcriptase PCR; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBP, TATA-box binding protein; TCPOBOP, 1,4-bis(3,5-dichloro-2-pyridinyloxy)benzene; Thr, threonine; Tyr, tyrosine.

**Recommended section assignment:**

Metabolism, Transport, and Pharmacogenomics.
ABSTRACT

Phenobarbital (PB) antagonized insulin to inactivate the insulin receptor and attenuated both the insulin receptor down-stream ATK-FOXO1 and ERK1/2 signals in mouse primary hepatocytes and HepG2 cells. Hepatic AKT began dephosphorylation in an early stage of PB treatment and blood glucose levels transiently increased in both wild type and CAR KO mice. On the other hand, blood glucose levels increased in wild type but not KO mice in later stages of PB treatment. As a result, PB, acting as an insulin receptor antagonist, elicited CAR-independent increases and CAR-dependent decreases of blood glucose levels at these different stages of treatment, respectively. Reciprocally, insulin activation of the insulin receptor repressed CAR activation and induction of its target CYP2B6 gene in HepG2 cells. Thus, PB and insulin cross talk through the insulin receptor to regulate glucose and drug metabolism reciprocally.
Introduction

Phenobarbital (PB) treatment is known to improve insulin sensitivity and decrease blood glucose levels in patients (Lahtela et al., 1984; Lahtela et al., 1985). Reflecting what was observed in human studies, PB treatment decreased blood glucose levels in rats (Karvonen et al., 1989; Venkatesan et al., 1994). Moreover, utilizing genetically engineered mice, the nuclear receptor CAR (NR1I3) was found to mediate this PB-induced decrease of blood glucose levels (Dong et al., 2009; Jiang and Xie, 2013). Mechanistically, CAR directly interacts with fork head transcription factor 1 (FOXO1) to suppress its binding to an insulin response sequence (IRS) and prevent gluconeogenic genes from being activated (Kodama et al., 2004). PB is also well-known as the classic inducer of hepatic drug metabolism, first reported in 1962 (Kato et al., 1962). CAR regulates not only glucose but also this induction of drug metabolism. In fact, CAR was originally characterized as a PB-activated transcription factor that activates the classic PB-induced cytochrome P4502B (CYP2B) gene (Honkakoski et al., 1998). Reciprocally, insulin is known to repress PB induction of CYP2B (Schenkman, 1991; Yoshida et al., 1996; Woodcroft and Novak, 1997; Kawamura et al., 1999). Here we have examined cross talk between PB and insulin via the insulin receptor to regulate both glucose and drug metabolism and their molecular mechanisms.

PB treatment was previously reported to inactivate hepatic insulin receptor in 1986 (Hwang et al., 1986). However, the biological consequences of this insulin receptor inactivation on PB actions to regulate either drug or glucose metabolism and, moreover, CAR activation had never been considered in this context. Recently, the cell
signal-mediated mechanism by which PB, not a CAR ligand, indirectly activates CAR was determined. PB directly binds to epidermal growth factor receptor (EGFR) and represses its down-stream ERK1/2 signaling; this repression becomes a CAR activation signal (Mutoh et al., 2009; Osabe and Negishi, 2011; Mutoh et al., 2013). Given the finding that PB attenuates EGFR signaling that activates CAR, we hypothesized and examined that PB may also interact with other cell membrane receptors and regulate CAR activation.

For this study, the insulin receptor was chosen to examine this hypothesis. CAR KO mice, mouse primary hepatocytes and human hepatoma-derived HepG2 cells were utilized for experimental systems and Western blot and RT-PCR for analytical methods to investigate the insulin receptor phosphorylation and its down-stream signal molecules (AKT, FOXO1, ERK1/2 and RACK1) and the CYP2B, PEPCK1 and G6Pase genes as regulatory targets. Here we have presented experimental results supporting that PB and insulin cross talk via the insulin receptor to mutually regulate glucose and drug metabolism. PB treatment transiently caused CAR-independent increases of blood glucose levels during early stage of treatment and CAR-dependent decreases in later stage. Reciprocally, insulin repressed PB induction of CYP2B. These findings may provide an insight into understanding functional interactions between drugs and insulin and their consequences in drug efficacy and/or toxicity.
Materials and methods

Reagents and Materials. Phenobarbital, TCBOPOP, CITCO and insulin were purchased from Sigma-Aldrich; QuikChange site-directed mutagenesis kit from Stratagene; Protein L resins from Pierce; Dynabeads protein G from Invitrogen; antibodies against RACK1 and mouse normal immunoglobulin M (IgM) from BD Biosciences; antibodies against FOXO1, TBP, HRP conjugated antibodies rabbit or mouse IgG (raised in goat) and normal mouse IgG from Santa Cruz Biotechnology; an antibody against CAR from Perseus Proteomics; antibodies against the insulin receptor, phosphorylated insulin receptor (Tyr1150+1151), AKT, phosphorylated AKT (Ser473), phosphorylated FOXO1 (Ser256), ERK1/2 and phosphorylated ERK1/2 (Thr202/Tyr204) from Cell Signaling Technology; an antibody against phosphorylated insulin receptor (Tyr973) from Abcam; enhanced chemiluminescence reagents from Advansa; polyvinylidene difluoride (PVDF) membranes from GE Healthcare. Antibodies against phosphorylated Tyr52 peptide of RACK1 and an antibody against phosphorylated Thr38 peptide of CAR were produced and characterized in previous work (Osabe and Negishi, 2011; Mutoh et al., 2013). HepG2-derived Ym17 cells, which stably express mouse CAR, were described in our previous report (Swales et al., 2005).

Animal treatments. Nine to 10-week-old Car+/+ C3He males were purchased from Charles River Laboratories (Wilmington, MA, USA). Car−/− C3He males were bred in the NIEHS animal facility. All mice were maintained in a temperature- and light-controlled facility and had free access to water and diet. All animal procedures were approved by the Animal Ethics Committee NIEHS, National Institutes of Health.
In each experiment, mice were randomly divided into two groups and treated with PBS or phenobarbital at a dose of 100 mg/kg body weight. Phenobarbital was given by intraperitoneal injection in all treatment groups.

**Measurement of blood glucose level over time.** Mice were treated with phenobarbital once a day for two days. Immediately after the second injection, mice were fasted and blood was collected from each mouse via tail vein after 0, 6, 12, 18 and 24 hr after fasting. Blood glucose levels were determined using AccuChek Active glucometer (Roche Diagnostics, Indianapolis, IN, USA). The glucometer was calibrated and validated following the manufacturer’s guidelines. To delineate changes over time, data were fit to a cubic equation using GraphPad Prism 5 software (www.graphpad.com).

**Cell culture and transfection.** Mouse primary hepatocytes were isolated from 6- to 8-week-old C57BL/6 male mice (Charles River) using a two-step collagenase perfusion and seeded on collagen-coated 24-well plates (BD Biosciences). Four hours after seeding, culture medium was changed to pre-warmed Williams’ E medium containing 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, penicillin (100 U/ml) and streptomycin (100 μg/ml). HepG2 and Ym17 cells were maintained in Dulbecco’s Modified Eagle’s Medium (Gibco BRL) supplemented with 10% FBS (Atlanta Biologicals), 1 mM sodium pyruvate, penicillin (100 U/ml), and streptomycin (100 μg/ml). These cells (3 x 10^5 cells per well) were placed on a 24-well plate. HepG2 cells plated on 24-well plate or 10 mm dish at a density of 5 x 10^5 cells/ml were transfected with lipofectamine 2000 (Invitrogen) according to the manufacturer’s instruction for 24 h prior to drug treatments.

**Co-immunoprecipitation.** HepG2 cells after drug treatment were lysed by
sonication in lysis buffer [20 mM tris-HCl (pH 7.6), 1 mM EDTA, 200 mM NaCl, 1%
Triton X-100] and centrifuged at 12,000 g for 5 min. The resulting supernatant was
incubated with the indicated antibody overnight at 4 °C. Immunoprecipitates were
prepared by incubation with Dynabeads protein G or protein L resin for 3 h at 4 °C.
The precipitates were washed three times with lysis buffer and then added to sample
buffer for SDS-PAGE.

**Western blots.**  HepG2 cells or mouse primary hepatocytes were lysed by
sonication in 50mM tris-HCl buffer saline (pH 7.6) containing 8 M urea and 0.1% SDS
and centrifuged. Proteins were separated by SDS-PAGE and transferred onto a PVDF
membrane for Western blot analysis. The membrane was blocked in 5% nonfat milk or
5% BSA in 25 mM Tris-HCl (pH 7.5) containing 137 mM NaCl and 0.1% Tween 20
for 1 h and then incubated for overnight at 4 °C with a given primary or
HRP-conjugated Flag antibody. HRP-conjugated anti-rabbit IgG or anti-mouse IgM
were used as a secondary antibody. Finally, protein bands were visualized using ECL
Western blotting detection reagent (Advansta).

**Quantitative real-time PCR.**  Total RNAs were isolated from HepG2 and Ym17
cells or hepatocytes using Trizol Reagent (Life Technologies) and subjected to cDNA
synthesis using High Capacity cDNA Archive Kit (Life Technologies). Real-time PCR
was performed using the ABI prism 7700 sequence detection system (Life
Technologies). Specific detection probe (Life Technologies) for PCR with the TaqMan
PCR Master Mix (Life Technologies) used were human CYP2B6 mRNA
(Hs00167937_m1), human G6Pase mRNA (Hs00609178_m1), mouse CYP2B10
mRNA (Mm00456591_m1) and mouse G6Pase mRNA (Mm00839363_m1). The
TaqMan human and mouse GAPDH probes (Life Technologies) were used as internal controls to normalize expression levels of a given mRNA.

**Statistical analysis.** Blood glucose and real-time PCR data were shown as the mean and analyzed with Student’s *t* test or one-way ANOVA for all groups followed by pair wise comparisons. Significant values are represented as *p < 0.05; **p < 0.01; ***p < 0.001.
Results

**Blood glucose levels after PB treatment.** PB was administered to wild type and CAR KO male mice 24 hr prior to and again at the time fasting began, 10:00am. Blood samples were collected every 6 hr from those mice to determine glucose levels. Consistent with previous observations, 24 hr PB treatment lowered blood glucose levels about 16% in wild type but not KO males (128.1 ± 2.35 in PB and 148.2 ± 6.32 in PBS), although levels remained constant in PBS-treated males (Fig. 1A). After fasting, blood glucose levels began a gradual decrease in both PBS-treated wild type and KO males. PB treatment slowed down this decrease and kept blood glucose levels higher in both wild type and KO males. Wild type males attenuated the levels 18 hr after PB treatment to those observed in the PBS-treated males (75.2 ± 2.77 in PB and 77.3 ± 2.21 in PBS). On the other hand, PB-treated KO males retained higher glucose levels at all-time points and never lowered them to the levels of PBS-treated males (100.7 ± 5.18 in PB and 78.2 ± 3.35 in PBS). These results suggest that PB caused a CAR-independent slow-down of the decrease of blood glucose levels in the early period of treatment as well as the CAR-dependent attenuation in the later period.

Nuclear CAR accumulation was barely observed in the livers 30 min after PB treatment compared with that 18 hr after treatment (Fig. 1B). Consistent with this CAR accumulation, CYP2B10 mRNA induction was not detected 30 min after PB treatment (Fig. 1C). On the other hand, G6Pase mRNA was already increased at 30 min and then declined at 18 hr after PB treatment, respectively (Fig. 1D), which agreed with the blood glucose levels of Fig. 1A.
**PB actions to repress the insulin receptor signaling.** Mouse primary hepatocytes exhibited a concentration dependent insulin activation of the insulin receptor as indicated by phosphorylation of three tyrosine residues (Fig. 2A). PB treatment at 20 µM began to dephosphorylate activated the insulin receptor in the presence of 10 nM insulin. CAR ligand TCPOBOP did not influence dephosphorylation. As the insulin receptor activated, the insulin receptor down-stream AKT-FOXO1 signaling was also activated (i.e. phosphorylated) by insulin treatment (Figs. 2B and C). Activation of this so-called insulin signaling leads to suppression of gluconeogenic genes and gluconeogenesis. As shown in Fig. 2B and C, both phosphorylated AKT and FOXO1 were dephosphorylated after PB but not TCPOBOP treatment; the dephosphorylation by PB appeared to begin at 20 µM. These results indicated that PB antagonized insulin activation of the insulin receptor, thereby offsetting insulin activation of the AKT-FOXO1 signal. In addition, the insulin receptor activation by insulin also resulted in activation of MEK1/2-ERK1/2 signaling as indicated by ERK1/2 phosphorylation. PB, but not TCPOBOP treatment, repressed ERK1/2 phosphorylation in the presence of 10 nM insulin (Fig. 2D). Receptor for activated kinase C 1 (RACK1) is a signal scaffold protein involved in various signal transductions. RACK1 was phosphorylated by insulin and dephosphorylated after PB treatment (Fig. 2E). For both ERK1/2 and RACK1, their significant dephosphorylations began to occur at 200 µM of PB. Dephosphorylation of ERK1/2 and RACK1 was previously characterized as the PB-induced signaling that indirectly activates nuclear receptor CAR.
**Transient upsurge of blood glucose in mice.** PB slowed down a decrease in blood glucose levels within the first 6 hr after treatment in WT and KO mice (Fig. 1). To further dissect this slow down, blood glucose levels were examined 30 min after PB treatment; levels in both WT and KO mice surged from 160 ± 6 to nearly 186 ± 8 mg/dl in the former and from 154 ± 9 to 182 ± 7 mg/dl in the latter (Fig. 3A). At this treatment point, AKT was significantly dephosphorylated in the livers of both WT and KO mice (Fig. 3B). In addition, hepatic levels of G6Pase were elevated in both strains of mice (Fig. 3C). PEPCK1 mRNAs appeared to be increased also, although it was not statistically significant (Fig. 3C). These results indicate that an early event of PB treatment is to repress the AKT signal and increase gluconeogenesis, thereby causing a surge in blood glucose levels in a CAR independent mechanism.

**PB actions to repress the insulin receptor signal in HepG2 cells.** As observed in mouse primary hepatocytes, insulin treatment activated (i.e. phosphorylated) the insulin receptor in a concentration dependent manner in human hepatoma-derived HepG2 cells, whereas co-treatment with PB, but not a human CAR ligand CITCO, inactivated the insulin receptor via dephosphorylating it (Fig. 4A). Competitive binding assays suggested that PB bound the insulin receptor (supplemental Fig. 1) revealed two different affinity values about 21.5 ± 15.5 μM and 2.2 ± 1.4 mM, thereby indicating the possibility that PB may directly bind to the insulin receptor to antagonize insulin. Subsequently, repression of insulin (AKT-FOXO1) signaling was examined (Figs. 4 B and C). Both AKT and FOXO1 were activated (i.e. phosphorylated) after insulin treatment and repressed this phosphorylation after PB, but not CITCO, treatment. Insulin treatment decreased G6Pase mRNA levels in HepG2 cells in which endogenous CAR is barely present and PB co-treatment restored those levels (Fig. 4D). On the
other hand, the co-treatment with PB failed to recover G6Pase mRNA levels decreased by insulin in Ym17 cells (Fig.4E). These results indicate that PB could antagonize insulin signaling to stimulate gluconeogenesis in the absence of CAR.

 **CYP2B10 induction through the insulin receptor.** The insulin receptor activation resulted in an increased phosphorylation of both RACK1 and ERK1/2 and, as expected, the insulin receptor inactivation by PB caused them to be dephosphorylated in Ym17 cells, a line that stably expresses mouse CAR (Figs.5A and 5B). Our previous works determined RACK1 and ERK1/2 as two essential signal molecules that regulate CAR activation; phosphorylated ERK1/2 binds inactivated CAR, while dephosphorylated RACK1 binds CAR to activate it (Osabe and Negishi, 2011; Mutoh et al., 2013). Consistent with the ways by which insulin or PB regulates the insulin receptor and its down-stream RACK1 and ERK1/2, insulin treatment decreased interactions between CAR and RACK1 while PB co-treatment increased this interaction. On the other hand, CAR interaction with ERK1/2 was increased and decreased after insulin treatment and PB co-treatment, respectively (Fig. 5C). These patterns of interactions were suggestive of insulin-PB cross talk to regulate CAR-mediated transcription of *CYP2B6* gene. We utilized HepG2-derived Ym17 cells to examine this cross talk and found that insulin repressed PB-induced CYP2B10 mRNA levels in a concentration dependent manner (Fig. 5D). However, insulin didn’t repress levels induced by TCPOBOP. These results indicate that insulin activates the insulin receptor to suppress CYP2B10 mRNA while PB, but not TCPOBOP, inactivates the insulin receptor to induce it.
Discussion

PB is now found to bind and antagonize the insulin receptor signal, similar to what our previous study demonstrated with EGF and EGFR (Mutoh et al., 2013). A role for EGF in the repression of CAR-mediated trans-activation of phenobarbital responsive enhancer module was first described in rat primary hepatocytes (Meyer et al., 1989). Subsequent works identified ERK1/2 as an essential down-stream molecule of the EGFR signal that represses CAR activation and CYP2B induction (Koike et al., 2007; Mutoh et al., 2009; Osabe and Negishi, 2011). In order to activate CAR indirectly, PB binds to EGFR and antagonistically attenuates the EGF-ERK1/2 signal (Mutoh et al., 2013). Since PB also represses the insulin-ERK1/2 signal, PB and insulin reciprocally regulating CAR activation should have been expected. In fact, PB treatment was found to induce CYP2B6 mRNA in Ym17 cells, whereas insulin co-treatment diminished this induction in a concentration dependent manner.

RACK1 is another signal molecule involved in CAR activation; upon PB treatment RACK1 is dephosphorylated at threonine 57 to bind and activate PP2Ac on CAR for CAR activation (Mutoh et al., 2013). Insulin increases RACK1 phosphorylation, which prevents it from binding to CAR. PB reverses these insulin actions to elicit CAR activation signal. Thus, insulin can regulate not only ERK1/2 but also RACK1 signal to repress CAR activation in the cytoplasm. Insulin also regulates FOXO1, which co-activates CAR-mediated transcription (Kodama et al., 2004). Consistent with this co-activation, CYP2B10 expression is up-regulated in the liver of FOXO1 transgenic mice (Zhang et al., 2006). Since the insulin-activated insulin receptor-AKT signal stimulates FOXO1 phosphorylation to eliminate FOXO1 from the nucleus (Biggs et al.,
1999; Rena et al., 2001; Zhang et al., 2002), insulin could repress CYP2B10 expression via FOXO1. Therefore, PB and insulin cross talk could also occur in the nucleus; insulin-induced depletion of FOXO1 reduces CAR to transcriptionally activate its targets such as CYP2B genes. Thus, PB and insulin cross talk via the insulin receptor reciprocally regulate down-stream ERK1/2, RACK1 and FOXO1 signals that activate CAR and induces CYP2B. This insulin receptor-mediated cross talk mechanism has provided the molecular basis for understanding a long-standing question of how insulin represses hepatic drug metabolism.

In our present study, whereas blood glucose levels gradually declined after fasting in both CAR wild and KO mice, they remained higher 18 hr after PB treatment in CAR KO mice, compared with those in CAR wild type mice. This finding is consistent with an already established notion that CAR interacts with FOXO1, preventing it from binding and enhancing IRS, thereby decreasing gluconeogenesis and blood glucose levels in mice (Kodama et al., 2004). FOXO1 activates IRS to induce gluconeogenic genes and insulin stimulates FOXO1 phosphorylation to inactivate and exclude it from the nucleus and suppress gluconeogenesis during feeding. After prolong fasting, FOXO1 is gradually imported back into the nucleus as insulin levels decline. Under conditions such as an 18 hr PB treatment, CAR-FOXO1 complex greatly increased in the liver nuclear fractions (supplemental Fig. 2); CAR accumulates in the nucleus, catches in-coming FOXO1 and represses its transcriptional activity. Therefore, the function of CAR to repress gluconeogenesis is insulin independent in nature.

Contrary to what was observed at later stages of PB treatment, blood glucose levels transiently increase shortly after treatment in both CAR wild type and CAR KO mice. The fact that G6Pase, but not CYP2B10, mRNA levels increase indicates that although
PB already attenuates the gluconeogenic signal, the CAR activation signal has not been elicited in CAR wild type mice within this short time of treatment. It is now understood that PB can either increase or decrease blood glucose levels depending on treatment conditions.

TCPOBOP and CITCO are agonistic ligands of mouse and human CAR, respectively. Our previous experiments indicate that CITCO is unable to activate and translocate human CAR T38D mutant in mouse liver (Mutoh et al., 2009). Therefore, CAR must be dephosphorylated prior to ligand binding or ligands promote dephosphorylation. These ligands have now been found that, unlike PB, they neither attenuate the insulin receptor signaling nor insulin represses CYP2B6 induction. Similarly, these ligands didn’t also inactivate EGFR (unpublished observation). Thus, CAR ligands do not utilize the cell membrane receptor-initiated CAR activation mechanism. Whether or not ligand binding recruits RACK1 and PP2Ac to CAR for dephosphorylation remains unknown at the present time. If it does, deciphering its molecular mechanism is an urgent target of future investigations.

In conclusion, PB treatment decreases blood glucose levels in mice and humans. The molecular basis underlying this PB action is PB interacting with the insulin receptor and antagonizing its signaling. PB attenuates the insulin receptor-ERK1 signal and this attenuation elicits CAR activation. Once CAR is activated and accumulates in the nucleus, CAR forms a complex with FOXO1 to attenuate the gluconeogenic insulin receptor-AKT signal and decreases blood glucose levels in fasting mice in later stages of PB treatment. On the other hand, in early PB treatment stages where CAR is not activated, attenuation of the insulin receptor-AKT signal causes a transient upsurge of blood glucose levels. Our present study provides new insights into understanding how
PB and CAR regulate gluconeogenesis and drug metabolism and may have clinical implications as well as for metabolic diseases caused by xenobiotic exposures.
Acknowledgements

We thank CMB and DNA Sequence Core at NIEHS for their assistances.
Authorship Contributions

Participated in research design: Negishi, Yasujima.

Conducted experiments: Yasujima, Saito, Moore.

Contributed new reagents or analytic tools: Yasujima, Saito, Moore.

Performed data analysis: Yasujima, Saito.

Wrote or contributed to the writing of the manuscript: Negishi, Yasujima, Saito.
References


Footnotes

This work was supported by the Intramural Research Program of the NIH and NIEHS [Z01ES71005-01].

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Figure legends

**Fig. 1.** Time-dependent changes of blood glucose levels in PB treated fasting mice. (A) Bloods were collected from fasting CAR\(^{+/+}\) and CAR\(^{-/-}\) mice at various times indicated after PB or PBS treatment and subjected to glycometer. Values express mean S.E. (n=9 or 10). The data of blood glucose level were fit to cubic equation. ***, p < 0.01; and ***, p < 0.005 for significance in difference between PBS- and PB-treated samples at each time point. (B) Nuclear extracts prepared from the livers of CAR wild type mice after PB treatment were subjected to Western blot analysis with an anti-CAR or anti-TBP antibodies. Hr denotes hours after treatment. (C) Hepatic RNA samples were prepared from CAR wild type mice at various time points after PB treatment and were subjected to real time PCR analysis. The relative expression values of CYP2B10 or G6Pase mRNAs were normalized to endogenous glyceraldehyde-3-phosphate dehydrogenase mRNA levels using the comparative cycle threshold method. Values express mean ± S.D. (n = 4). *, p < 0.05; and ***, p < 0.005 for significance in difference compared to PB injected mice at 0 hr.

**Fig. 2.** PB antagonizes insulin-elicited the insulin receptor phosphosphorylation and its down-stream signals in mouse primary hepatocytes. Hepatocytes were prepared from C3H male mice and cultured as described in the Experimental Procedures section and were treated with various concentrations of insulin or co-treated with insulin (10 nM) and increased concentrations of PB or TCPOBOP for 30 min. Total extracts from these hepatocytes were subjected to Western blot analysis with anti-phospho-Try972 or anti-phospho-Tyr1150+1151 antibody for the insulin receptor (A), anti-phospho-Ser473

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antibody for AKT (B), anti-phospho-Ser256 for FOXO1 (C),
anti-phospho-Thr202/Tyr204 antibody for ERK1/2 (D) and anti-phospho-Tyr52
antibody for RACK1 (E). Antibody which reacts both phosphorylated and
non-phosphorylated protein were used to observed total amount of each protein (A, B,
C, D and E).

Fig. 3. Transient up-surge of blood glucose shortly after PB treatment in mice. (A)
Bloods were corrected from fasting males 30 min after PB or PBS treatment and
subjected to glycometer. Values of blood glucose levels express mean S.E. (n = 4), *, p
< 0.05 for significance differences between PBS- and PB-treated mice. From the same
mice, total liver extarcts and RNAs were prepared for Western blot (B) and real time
PCR (C) analysis, respectively, for AKT and P6Pase and PEPCK1 mRNA as described
in the Experimental Procedures section and the legends of Fig. 1 and 2. Values of
PCR express mean ± S.D. (n = 4)*, p < 0.05 for significance in difference compared to
PBS treatment.

Fig. 4. PB antagonizes insulin-elicited the insulin receptor phosphosphorylation and
its down-stream signals in HepG2-derived Ym17 cells stably expressed mouse CAR.
Ym17 cells were treated with insulin at various concentrations as indicated or co-treated
with 10 nM insulin and either PB or CITCO at increasing concentrations as indicated
for 30 min (A, B, C and E). In D, HepG2 cells were treated with PB, insulin or PB or
cotreated with PB and insulin. Total extracts and RNAs from these cells were subjected
to Western blot and real time PCR analysis, respectively, as described in the legends of
Figs. 1 and 2. Values of PCR express mean ± S.D. (n = 4), *, p < 0.05 for significance in difference compared to PBS treatment.

**Fig. 5.** PB antagonizes insulin activating the insulin receptor to elicit CAR activation signal. Ym17 cells were pretreated with insulin (left) for 30 min prior to the co-treatment with insulin (10 nM) and PB or CITCO at indicated concentrations for an additional 30 min. Phosphorylation of RACK1 and ERK1/2 was confirmed by Western blot as described in the Experimental Procedures section and legend of Figure 2 (A and B). (C) HepG2 cells were transfected with Flag-CAR expression plasmid and treated with PBS or insulin or co-treated insulin and PB as above-mentioned, from which total extracts were prepared for subsequent co-immunoprecipitation and Western blots. Co-immunoprecipitation was performed for RACK1 (upper) or ERK1/2 (lower) as described in the Methods section. Western blot is described in the legend of Figure 2. (D)Ym17 cells were co-treated with PB (2 mM) or TCPOBOP (250 nM) and various concentrations of insulin for 24 hr, from which RNAs were prepared for real time PCR for CYP2B6 mRNA as described in the legend of Figure 1. CYP2B6 expression levels were calculated relative to its levels without insulin as the one. Values express mean ± S.D. (n = 4); *, p < 0.05 for significance in difference compared to mRNA level in absence of insulin.
Fig. 1
**Fig. 2**

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<th>Insulin (nM)</th>
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Fig. 4

A

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p-Tyr972

p-Tyr1150+51

IR

B

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<th>CITCO (μM)</th>
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p-AKT

AKT

C

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p-FOXO1

FOXO1

D

In HepG2 cells

E

In m17 cells

Graphs showing different experiments and results.
Fig. 5

**A**

<table>
<thead>
<tr>
<th>Insulin (nM)</th>
<th>PB (µM)</th>
<th>CITCO (µM)</th>
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**B**

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**C**

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<tr>
<td>Experimental</td>
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**D**

Bar graph showing the effect of insulin concentration on cellular parameters. The x-axis represents insulin concentration (µM) ranging from 0 to 1000, and the y-axis represents the concentration of a specific protein. Two conditions are compared: 2 nM P53 versus 200 µM TCP533OP.