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Induction of Drug Metabolism by Forskolin, the Role of the Pregnane X Receptor and the PKA Signal Transduction Pathway.

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Running Title: PKA Signaling Potentiates CYP3A Induction by PXR

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Figure 5

Figure 6

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Abbreviations: protein kinase A (PKA), adenosine 3',5'-cyclic monophosphate (cAMP), 8-bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP), cytochrome P450 3A (CYP3A), pregnane X receptor (PXR), 7β-acetoxy-8,13-epoxy-1α,6β,9α-trihydroxy-labd-14-en-11-one (Forskolin), 7β-acetoxy-6β-hydroxy-8, 13-epoxy-labd-14-en-11-one (1,9 dideoxyforskolin), pregnenolone 16α-carbonitrile (PCN).

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Abstract

An extract of the plant *Coleus forskohlii* has been used for centuries in Ayurvedic medicine to treat various diseases such as hypothyroidism, heart disease and respiratory disorders. Additionally, complex herbal mixtures containing this extract are gaining popularity in United States for their putative 'fat-burning' properties. The active ingredient in *Coleus forskohlii* extract is the diterpene compound forskolin. Forskolin is a widely used biochemical tool that activates adenyl cyclase, thereby increasing intracellular concentration of cAMP, and thus activating the PKA signal transduction pathway. We show here that both forskolin and its non-adenyl cyclase-activating analog 1,9 dideoxyforskolin induce CYP3A gene expression in primary hepatocytes by functioning as agonists of the pregnane X receptor. We show that activation of PKA signaling potentiates PXR-mediated induction of CYP3A gene expression in cultured hepatocytes and increases the strength of PXR-coactivator protein-protein interaction in cell-based assays. Kinase assays show that PXR can serve as a substrate for catalytically active PKA in vitro. Our data provide important insights into the molecular mechanism of both the PKA-dependent and the PKA-independent effects of forskolin on the expression of drug-metabolizing enzymes in liver. Finally, our data suggest that herbal therapy with *Coleus forskohlii* extract should be approached cautiously due to the potential for herb-drug interactions in patients on combination therapy.

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Introduction

A large number of hormones and neurotransmitters utilize adenosine 3',5'-cyclic monophosphate (cAMP). As an intracellular second messenger, cAMP regulates a number of different cellular processes such as cell growth and differentiation, ion channel conductivity, synaptic release of neurotransmitters, and gene transcription. The principal intracellular target for cAMP in mammalian cells is cAMP-dependent protein kinase (PKA). In the absence of cAMP, PKA is an enzymatically inactive tetrameric holoenzyme consisting of two catalytic subunits bound to a regulatory subunit dimer. Upon binding of cAMP, the PKA enzyme dissociates into a regulatory subunit dimer and two active catalytic subunits that phosphorylate serine and threonine residues on specific substrate proteins, including many transcription factors.

Several widely used pharmacological agents have been developed that activate PKA both directly and indirectly. For example, direct activation of PKA signaling can be achieved by using 8-Br-cAMP (8-bromoadenosine 3',5'-cyclic monophosphate), a cell-permeable cyclic nucleotide derivative that mimics the action of endogenous cAMP by binding to the PKA regulatory subunit. Indirect activation of PKA signaling can be achieved through pharmacological activation of adenyl cyclase, the enzyme responsible for the production of intracellular cAMP. Forskolin (7β-acetoxy-8,13-epoxy-1α,6β,9α-trihydroxy-labd-14-en-11-one) is a diterpene derived from the Indian plant *Coleus forskohlii.* Forskolin is an efficacious activator of adenyl cyclase (Seamon et al., 1981). This characteristic of forskolin led to its extensive use as a biochemical tool that increases intracellular cAMP concentration and activates the PKA signal transduction pathway in cells. *Coleus forskohlii* extract has been used for centuries in Ayurvedic medicine to treat various diseases such as hypothyroidism, heart disease and respiratory disorders [reviewed in (Ammon and Muller, 1985)]. Moreover, complex herbal mixtures that contain *Coleus forskohlii* extract are gaining popularity in United States for use in weight loss regimens due to their putative 'fat-burning' properties and their availability over-the-counter without a prescription.

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Forskolin and the non-PKA activating analogue 1,9 dideoxyforskolin (7β-acetoxy-6β-hydroxy-8, 13-epoxy-labd-14-en-11-one) have cAMP- and PKA-independent biological effects. One of the best-characterized cAMP- and PKA-independent effects of these two compounds is the induction of cytochrome P450 3A (CYP3A) gene expression in cultured primary hepatocytes (Sidhu and Omiecinski, 1996). However, the molecular basis for this phenomenon is currently unknown. The pregnane X receptor (PXR, NR1I2) is a member of the nuclear receptor superfamily of ligand-activated transcription factors. It is now well accepted that PXR is a key regulator of xenobiotic-inducible CYP3A gene expression. In addition, several studies have shown that PXR regulates other genes involved in the metabolism of xenobiotic and endobiotic compounds such as CYP2B10, CYP2C, glutathione S-transferases, sulfotransferases, and UDP-glucuronosyltransferases (Maglich et al., 2002; Sonoda et al., 2002; Wei et al., 2002; Chen et al., 2003). PXR also regulates the expression of the drug transporter genes Oatp2, Mdr1, Mrp2, and Mrp3 (Geick et al., 2001; Kast et al., 2002; Staudinger et al., 2003). Thus, we hypothesized that PXR activation by forskolin and 1,9 dideoxyforskolin represents the mechanism by which these two compounds induce CYP3A gene expression in hepatocytes.

In the present report we show that purified forskolin and 1,9 dideoxyforskolin induce CYP3A gene expression by functioning as PXR ligands. We show that activation of the PKA signal transduction pathway potentiates the PXR-mediated xenobiotic response. We also show that activation of PKA signaling strengthens the interaction between PXR and known co-activator proteins in cell-based assays. Moreover, we demonstrate for the first time that PXR can serve as a substrate of catalytically active PKA in vitro. These studies provide important insights into the molecular mechanism of both PKA-dependent and PKA-independent effects of forskolin and 1,9 dideoxyforskolin. Our data suggest that the therapeutic use of forskolin and herbal extracts containing *Coleus forskohlii* likely increases the metabolism of co-administered drugs in vivo and should thus be approached cautiously. Taken together, these data suggest that PKA signaling likely modulates the xenobiotic response, in part, through altering the phosphorylation status of PXR and may contribute to inter-individual variability in drug metabolism.

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Materials and Methods

Animal Care. Generation of the PXR knockout mice was previously described (Staudinger et al., 2001b). All rodents were maintained on standard laboratory chow and were allowed food and water ad libitum. The studies reported here have 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.

Compounds and Plasmids. Unless otherwise stated, all chemical compounds were purchased from Sigma (St. Louis, MO). The 1,9 dideoxyforskolin was purchased from CalBiochem (San Diego, CA), and the taxol was a kind gift of Dr. Gunda Georg (University of Kansas, Lawrence, KS). The pSG5-hPXR and the GST-hPXR-ligand-binding domain fusion plasmids were previously described (Lehmann et al., 1998). The pSG5-mPXR and the GST-mPXR-ligand-binding domain fusion plasmids were previously described (Kliewer et al., 1998). The pSG5-mCAR plasmid was previously described (Moore et al., 2000). The GAL4-SRC1, GAL4-PBP, and GAL4-NCoR1 expression vectors were previously described (Synold et al., 2001). The GST-p75 neurotrophin receptor plasmid was previously described (Bilderback et al., 1997). The GST-hPXR-DNA-binding domain fusion plasmid was generated using PCR primers that introduced EcoRI sites and the amplimer was subcloned into pGEX4T-1 and encodes amino acid residues 1-107 of human PXR. The full-length human and mouse PXR cDNAs were isolated from the pSG5-hPXR and pSG5-mPXR plasmids and subcloned into pVP16 (BD Biosciences, Palo Alto, CA) in frame with EcoRI and BamHI strategy to create the full-length VP16-hPXR fusion protein. The pFR-LUC reporter gene is commercially available (BD Biosciences, Palo Alto, CA).

Cell Culture and Transient Transfection Analysis. The XREM-LUC reporter gene assays were performed as described (Brobst et al., 2004). The mammalian two-hybrid system analysis was performed in 96-well plates using CV-1 cells at 7,000 cells/well. Transfection mixtures contained pFR-LUC (20ng), GAL4-co-factor (20 ng), VP16-hPXR (10 ng), pSV-β-GAL (40 ng), and pBluescript (20 ng). The next day transfected cells

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were drug-treated for 24 h. All compounds were added to the culture medium as 1000X stocks dissolved in DMSO or ethanol. Luciferase and β-galactosidase activities were determined using the Dual-Light Kit per the manufacturers instructions (Applied Biosystems, Foster City, CA).

Co-activator Receptor Ligind-binding Assays (CARLA). CARLA analysis was performed as described previously (Brobst et al., 2004).

Hepatocyte Cultures, RNA Isolation, and Northern Blot Analysis. Hepatocytes were isolated and cultured as described (LeCluyse et al., 1996). All compounds were added to the culture medium as 1000 x stocks dissolved in DMSO, except forskolin and 1,9 dideoxyforskolin were dissolved in ethanol and 8-Br-cAMP was dissolved in water. Control cells received DMSO and/or ethanol alone. Total RNA was isolated and Northern blotting assays were performed as described (Staudinger et al., 2001a). Blots were hybridized with the ^{32}P-labeled cDNA corresponding to the cDNA sequences for mouse Cyp3a11 (bases 69 to 1609, GenBank NM 007818). The 18S ribosomal RNA probe was amplified from mouse liver cDNA using commercially available primers per the manufacturers instructions (Ambion, Austin, TX).

Thin Layer Chromatography (TLC) Analysis of Forskohlii Ethanol Extract.
Equal volumes of forskolin (10 mM), 1,9 dideoxyforskolin (10 mM) and forskohlii ethanol extract was loaded on a TLC plate. TLC was performed with 4:1 ratio of hexane and ethyl acetate and visualized by standard blue stain.

Forskohlii Ethanol Extract Preparation. A commercial *Coleus forskohlii* root extract was obtained from the PhytoPharmica company (Green Bay, WI). The content of two capsules (50 mg powder/capsule) was extracted with 0.87 ml of absolute ethyl alcohol at 4^{o}C overnight with gentle shaking. The extract was centrifuged at 16,000 X g for 5 min. The ethyl alcohol supernatant was decanted and kept at -20^{o}C until use.

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Real-Time Quantitative PCR (Q-PCR) Analysis for Cyp3a11 Gene Expression. The probe sets and procedures for the quantitative detection of β-actin and Cyp3a11 were previously described (Staudinger et al., 2003)

In Vitro Protein Kinase A Phosphorylation Assay. Purified GST fusion proteins (0.5 μg each) were incubated in 50 μl of PKA reaction buffer [10 mM Tris, pH 7.2; 6.25 mM $MgCl_2$; 0.5 μg of catalytically active PKA (Promega, Madison, WI); 1 μl of γ-ATP^{32}; and 1 μl of 1 mM ATP] at 30^0C for 30 minutes. An equal amount of 2X SDS-loading buffer was added to each reaction and the samples were heated at 100ºC for five minutes. Samples were resolved on 10% SDS-PAGE. Gels were dried at 75ºC for one hour. Radiolabeled proteins were visualized by autoradiography.

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Results

Because forskolin and 1,9 dideoxyforskolin induce expression of CYP3A independent of both cAMP concentration and PKA signaling in hepatocytes (Sidhu and Omiecinski, 1996), we performed a standard cell-based reporter gene assay to investigate whether these compounds would activate human and mouse PXR (Fig. 1A). CV-1 cells were transfected with expression vectors encoding human PXR, mouse PXR, or mouse CAR nuclear receptor proteins together with the PXR- and CAR-responsive luciferase reporter gene XREM-LUC (Goodwin et al., 1999; Goodwin et al., 2001). Forskolin and 1,9 dideoxyforskolin activated the human and mouse PXR with an efficacy comparable to that achieved with the prototypical agonists of human and mouse PXR, rifampicin (RIF) and pregnenolone 16α-carbonitrile (PCN), respectively. In contrast, mouse CAR was not activated by forskolin or 1,9 dideoxyforskolin. The specific PKA activator 8-Br-cAMP was used as a negative control and had little effect on XREM-LUC reporter gene activity. Full concentration-response analysis revealed that forskolin activates human and mouse PXR with EC_{50} values of approximately 400 nM and 900 nM, respectively (Fig. 1B), levels that are one order of magnitude below that required for activation of PKA.

We next tested whether forskolin and 1,9 dideoxyforskolin mediate their effects through direct binding to PXR. CARLA analysis (an in vitro coactivator receptor ligand-binding assay) was performed using the purified mouse PXR ligand-binding domain and the ^{35}S-radiolabeled nuclear receptor co-activator protein SRC1 (Fig. 2A). Treatment with forskolin and 1,9 dideoxyforskolin promoted the interaction between PXR ligand-binding domain and ^{35}S-SRC1 in vitro, suggesting that forskolin and 1,9 dideoxyforskolin interact directly with the PXR ligand-binding domain. PCN treatment was used as a positive control. Similar results were obtained with the human PXR protein (data not shown). In order to further investigate the molecular mechanism whereby forskolin and 1,9 dideoxyforskolin activate PXR in cells, we performed mammalian two-hybrid analysis (Fig. 2B). CV-1 cells were transfected with the expression vectors encoding the receptor interacting domains from the co-repressor protein NCoR1 or the co-activator protein SRC1 fused to GAL4, respectively, together with VP16-tagged human PXR and the GAL4-responsive luciferase reporter gene pFR-LUC. Both forskolin and 1,9

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dideoxyforskolin reduced the interaction between PXR and the co-repressor protein NCoR1 and strengthened the interaction between PXR and the co-activator protein SRC1 in cells. Forskolin and 1,9 dideoxyforskolin also strengthened the interaction between PXR and the co-activator proteins SRC2 and PBP (data not shown). The known human PXR ligands taxol and rifampicin were used as positive controls. Similar results were obtained using the mouse PXR protein in mammalian two-hybrid analysis (data not shown).

To determine whether the induction of CYP3A gene expression by forskolin and 1,9 dideoxyforskolin is mediated through PXR, we took the advantage of our PXR-knockout mouse model. Cultured hepatocytes isolated from wild type and PXR-knockout mice were treated with increasing concentrations of forskolin, 1,9 dideoxyforskolin, and 8-Br-cAMP. Total RNA was isolated and Northern blot analysis was performed using a radiolabeled cDNA probe for Cyp3a11 (Fig. 3A). Treatment with low-concentrations (1 μM) of forskolin produced robust induction of CYP3A gene expression in wild type mouse hepatocytes, but not in PXR-knockout hepatocytes. Treatment of hepatocytes with higher concentrations of forskolin (10 μM and 100 μM) produced very modest levels of CYP3A gene expression in PXR knockout hepatocytes when compared with that achieved in wild type hepatocytes. In contrast to treatment with forskolin, treatment with PKA-inactive 1,9 dideoxyforskolin did not induce CYP3A gene expression at any concentration tested in PXR-knockout hepatocytes, while in wild type hepatocytes 10 μM and 100 μM concentrations induced CYP3A gene expression. When compared with forskolin, 1,9 dideoxyforskolin produced much lower levels of induction in wild type hepatocytes. Interestingly, treatment with 8-Br-cAMP induced CYP3A gene expression in both wild type and PXR-knockout hepatocytes, suggesting the induction of CYP3A in PXR-knockout hepatocytes by high concentrations of forskolin is likely mediated through activation of the PKA signal transduction pathway.

Because significant quantities of both forskolin and 1,9 dideoxyforskolin are contained in the herb *Coleus forskohlii* (Fig. 3B), we investigated whether an extract isolated from this herbal remedy induces CYP3A gene expression in a PXR-dependent manner. Wild type

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and PXR-knockout hepatocytes were treated with *Coleus forskohlii* extract, forskolin, and 8-Br-cAMP. Northern blot analysis was performed using a radiolabeled cDNA probe for Cyp3a11 (Fig. 3C). As expected, *Coleus forskohlii* extract induced CYP3A gene expression in a concentration-dependent manner in wild type mouse hepatocytes. In contrast, induction of CYP3A gene expression by *Coleus forskohlii* extract in PXR-knockout hepatocytes was absent at low doses and minimal at higher doses.

As shown in figure 3A, the specific and potent PKA activator 8-Br-cAMP (1mM) induced modest levels of CYP3A gene expression in mouse hepatocytes. We therefore asked whether PKA activation modulated the induction of CYP3A gene expression by PXR agonists. We performed a comparison of the induction of CYP3A expression following co-administration of increasing concentrations of 8-Br-cAMP (0.01, 0.1, and 1.0 mM) in the presence of a fixed concentration of the PXR agonist taxol (10 μM) (Fig. 4A). Strikingly, treatment with 8-Br-cAMP greatly increased the induction of CYP3A gene expression by taxol in mouse hepatocytes. Moreover, co-treatment with 8-Br-cAMP (1 mM) increased the induction of CYP3A gene expression by both forskolin and 1,9 dideoxyforskolin. As expected, treatment with 1 mM 8-Br-cAMP alone produced only modest induction of CYP3A gene expression. In order to quantify the increase in CYP3A gene expression produced by 8-Br-cAMP we used probes specific for mouse Cyp3a11 and β-actin in real time Q-PCR analysis (Fig. 4B). Treatment with 8-Br-cAMP induced Cyp3a11 gene expression levels by 19.7 $\pm$ 1.5 fold. Treatment of cultured mouse hepatocytes with taxol produced 45.2 $\pm$ 5.8 fold induction of Cyp3a11 gene expression, while co-treatment with taxol and 8-Br-cAMP produced 1202.1 $\pm$ 166.3 fold induction of Cyp3a11 gene expression. Treatment with the PKA-activating forskolin produced 482.9 $\pm$ 70.3 fold induction of Cyp3a11 gene expression, while co-treatment with forskolin and 8-Br-cAMP produced 3651.2 $\pm$ 285.0 fold induction of Cyp3a11 gene expression. Treatment with the non-PKA-activating 1,9 dideoxyforskolin produced 22.4 $\pm$ 3.0 fold induction of Cyp3a11 gene expression, while co-treatment with 1,9 dideoxyforskolin and 8-Br-cAMP produced 868.1 $\pm$ 43.5 fold induction of Cyp3a11 gene expression. Thus, treatment of mouse hepatocytes with the strong PKA activator 8-Br-cAMP potentiates the induction of Cyp3a11 gene expression by PXR agonists.

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To determine whether the potentiation of PXR-mediated induction of CYP3A gene expression is due to the modulation of PXR-co-activator protein-protein interaction, we used the mammalian two-hybrid system reporter gene assay (Fig. 5). Treatment of transfected cells with either taxol or 8-Br-cAMP alone strengthened the interaction between PXR and the receptor-interacting domains of the known PXR-interacting co-activator proteins SRC1 and PBP. Co-administration of taxol and 8-Br-cAMP further strengthened the interaction between PXR and these co-activator proteins.

The potentiation of CYP3A gene expression produced by co-treatment with 8-Br-cAMP in mouse hepatocytes prompted us to determine whether PXR can serve as a direct substrate for PKA. In vitro kinase assays were performed using a catalytically active purified PKA preparation and purified human GST-PXR fusion proteins (Fig. 6). GST alone and GST-p75 neurotrophin were used as negative and positive controls, respectively. Both the PXR DNA-binding domain and ligand-binding domain were phosphorylated by PKA in vitro. The GST alone showed a weak band corresponding to autophosphorylated PKA. The smaller band that appears in the GST-LBD lane occurs through degradation or proteolysis of the GST-LBD protein during the incubation period. Similar results were obtained using purified mouse PXR proteins (data not shown).

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Discussion.

While most of the biological effects of forskolin depend on its ability to increase levels of cAMP, several cAMP-independent effects have been identified through the use of adenyl cyclase-inactive forskolin analogues such as 1,9 dideoxyforskolin. One of the best-characterized cAMP- and PKA-independent effects of forskolin is the induction of CYP3A gene expression in cultured hepatocytes (Sidhu and Omiecinski, 1996). To date, the molecular mechanism of the ability of forskolin and 1,9 dideoxyforskolin to induce CYP3A gene expression has remained obscure. We show here that both forskolin and 1,9 dideoxyforskolin, two ingredients in the herbal remedy *Coleus forskohlii*, activate PXR by functioning as ligands for this receptor, thereby inducing the expression of CYP3A genes in hepatocytes.

In India, practitioners of traditional Ayurvedic medicine have long used an extract of *Coleus forskohlii* to treat various disorders (Ammon and Muller, 1985). Available without a prescription in supplement form containing up to 18% forskolin, *Coleus forskohlii* extract is commonly recommended for treating hypothyroidism. Additional conditions including over weight, asthma, heart disease, high blood pressure, glaucoma, eczema, and psoriasis may respond to *Coleus forskohlii* therapy. Human clinical trial information regarding the effectiveness of forskolin therapy is currently limited. However, treatment with *Coleus forskohlii* extract, although common, is contraindicated in combination with antihypertensives and anticoagulants due to the high potential for herb-drug interactions. Our data further strengthen the notion that herbal remedies should be routinely screened for their ability to interact with prescription medications.

It is interesting to note that forskolin and 1,9 dideoxyforskolin contain a three ring structure that closely resembles the basic steroid ring. Structurally diverse molecules, including certain naturally occurring and synthetic steroids, activate PXR (Moore et al., 2003). Additionally, molecules that bear little structural resemblance to steroids activate PXR, including the antibiotic rifampicin and the antifungal agent clotrimazole (Lehmann et al., 1998). Our studies add forskolin, 1,9 dideoxyforskolin, two compounds found in *Coleus forskohlii*, to the growing list of exogenous PXR ligands and further support the

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notion that PXR evolved, in part, as a xenobiotic sensor that functions to promote the biotransformation and subsequent excretion of potentially toxic compounds we ingest. It is worth noting that forskolin activates PXR with EC_{50} values that are approximately one order of magnitude below that required for activation of PKA. This is important because the therapeutic effect of *Coleus forskohlii* depends on its ability to activate PKA signaling, and therefore, significant PXR-mediated herb-drug interactions are likely to occur even before therapeutic levels of forskolin are achieved in patients using this powerful herbal remedy.

While induction of CYP3A gene expression by both forskolin and *Coleus forskohlii* extract is detectable in cultured hepatocytes isolated from PXR knockout hepatocytes, it is severely diminished. Moreover, higher concentrations of forskolin are required in the cell-free in vitro CARLA analysis to produce modulation of PXR-coactivator interaction when compared with cell-based assays. These data highlight the dual nature of forskolin as both a PXR agonist and an activator of PKA signaling. Moreover, these data indicate that PKA signaling has a profound impact upon the induction of PXR-target gene expression, likely through phosphorylation of the PXR protein. Additionally, we note that activation of PKA signaling by treatment with 8-Br-cAMP produced relatively modest, though roughly equivalent, levels of CYP3A induction in both wild type and PXR null hepatocytes when compared with that produced by forskolin in wild type hepatocytes. These data suggest that PKA signaling also contributes to PXR-independent pathways that produce induction of CYP3A gene expression, perhaps involving the constitutive androstane receptor or the glucocorticoid receptor. These data represent stimulating issues that warrant further analysis in the future.

Activation of PKA signaling with 8-Br-cAMP is known to modulate the interaction of the nuclear receptor co-repressor proteins NCoR1 and NCoR2 with the progesterone receptor (Wagner et al., 1998). In this report we show that when subjected to in vitro kinase assays, the purified PXR protein was phosphorylated by catalytically active PKA. Moreover, we show that activation of PKA signaling with 8-Br-cAMP increases the strength of PXR-coactivator protein-protein interaction in cell-based assays.

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Furthermore, we have shown that activation of the PKA signaling pathway potentiates the induction of CYP3A by PXR ligands in cultured hepatocytes. Taken together, these data suggest that phosphorylation of PXR by PKA may play a key role in regulating the induction of CYP3A gene expression in hepatocytes, in part, through it's ability to modulate PXR-protein co-factor interaction.

The PKA catalytic subunit has been identified in the nucleus and phosphorylates a number of nuclear receptor superfamily members, thereby modulating their *trans*-acting properties (Shao and Lazar, 1999). The activity of the liver enriched hepatocyte nuclear factor 4 (HNF4, NR2A1) is modulated by PKA-mediated phosphorylation in cell-based assays (Viollet et al., 1997). Because HNF4 is required for maximal induction of CYP3A gene expression (Tirona et al., 2003) and is a known substrate for PKA, it likely represents another important determinant of the PKA-dependent effects observed in this study, though it is not currently known how phosphorylation of HNF4 affects CYP3A gene expression.

Our data show that, in addition to evolution of the ligand-binding pocket of PXR, the activation of the PKA signal transduction pathway likely plays a pivotal role in the induction of CYP3A gene expression by PXR agonists. As a master-regulator of xenobiotic-inducible CYP3A gene expression, PXR is well positioned to integrate external cues via phosphorylation together with ligand-dependent induction of target gene expression. Future studies should focus on the identification of sites of PXR phosphorylation by PKA and determining the molecular basis of the modulation of PXR-target gene expression following the activation of the PKA signal transduction pathway.

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Figure Legends

Figure 1. Forskolin and 1,9 dideoxyforskolin are PXR Ligands. (A) CV-1 cells were transfected with expression vectors encoding human PXR, mouse PXR and mouse CAR together with the XREM-LUC reporter gene. Cells were treated with vehicle (0.1% DMSO and 0.1% EtOH) or 10 μM of the indicated compounds (forskolin, F; 1,9 dideoxyforskolin, F1,9; rifampicin, RIF; 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene, TCPOBOP, PCN) , except 8-Br-cAMP which was used at 1mM. The reporter gene activity of human PXR with RIF, mouse PXR with PCN, and mouse CAR with TCPOBOP is defined as 100%, respectively. **(B)** CV-1 cells were transfected with expression vectors encoding human PXR or mouse PXR together with the XREM-LUC reporter gene. Transfected cells were treated with increasing concentrations of forskolin. RLU: relative light unit (Luciferase activity normalized to β-galactosidase activity).

Figure 2. Forskolin and 1,9 dideoxyforskolin Modulate PXR-Coactivator Interaction. (A) CARLA analysis was performed with vehicle (0.1% ethanol and 0.1 % DMSO), forskolin (F, 100 μM), 1,9 dideoxyforskolin (F1,9, 100 μM) and PCN (10 μM) together with mouse GST-PXR ligand-binding domain and ^{35}S-radiolabeled steroid receptor coactivator 1 protein. **(B)** CV-1 cells were transfected with the GAL4-responsive pFR-LUC reporter gene and the expression vectors encoding GAL4-co-factors and VP16-hPXR and treated with vehicle or 10 μM of the indicated compounds. Data points in reporter gene assays represent the mean $\pm$ SEM (n=4).

Figure 3. Forskolin, 1,9 dideoxyforskolin, and Coleus Forskolii Extract Induce CYP3A Gene Expression through Activation of PXR. (A) Primary hepatocytes isolated from wild type or PXR-knockout mice were treated with the indicated concentrations of compounds for 24 h before RNA isolation. Total RNA (10 μg) was used for Northern blot analysis. The blots were probed sequentially with ^{32}P-labled cDNA fragments of Cyp3a11, and 18S RNA. **(B)** Thin layer chromatography was performed using equal volumes of forskolin (F), 1,9 dideoxyforskolin (F1,9), and the ethanol extract of *Coleus forskohlii*. **(C)** Primary hepatocytes isolated from wild type and PXR-knockout mice were treated with indicated dilutions of *Coleus forskohlii* extract, 10

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μM Forskolin (F) or 1 mM 8-Br-cAMP for 24 h before RNA isolation. Total RNA (10 μg) was used for Northern blot analysis. The blots were probed sequentially with ^{32}P-labled cDNA fragments of Cyp3a11 and 18 S.

Figure 4. Activation of PKA Signaling Potentiates Induction of CYP3A Gene Expression by PXR Agonists. (A) Primary hepatocytes isolated from wild type and PXR knockout mice were treated with taxol (10 μM) in combination with the indicated concentrations of 8-Br-cAMP, forskolin (F, 100 μM) and 1,9 dideoxyforskolin (F1,9, 100 μM) alone or in combination with 8-Br-cAMP (8-Br, 1 mM) for 24 h before RNA isolation. Total RNA (10 μg) was used for Northern blot analysis. The blots were probed sequentially with ^{32}P-labled fragments of Cyp3a11 and 18S. **(B)** Total RNA isolated from wild type hepatocytes treated with taxol (10 μM), forskolin (F, 100 μM), or (F1,9, 100 μM) alone or in combination with 8-Br-cAMP (8-Br, 1 mM) was used to generate cDNA for use in Q-PCR analysis to determine the relative expression levels of Cyp3a11. The data are normalized to β-actin levels and are expressed as average values (n=4) $\pm$ the standard error of the mean.

Figure 5. Activation of the PKA Signaling Pathway with 8-Br-cAMP Increases the Strength of Interaction Between PXR and Coactivator Proteins SRC1 and PBP. CV-1 cells were transfected with expression vectors encoding VP-16-tagged mouse PXR and the GAL4-fusions of the receptor-interacting domains derived from the coactivator proteins SRC1 and PBP together with the pFR-Luc reporter gene. Twenty-four hr post-transfection, cells were treated with vehicle (0.1% DMSO), taxol (10 μM), 8-Br-cAMP (1 mM), or a combination of taxol and 8-Br-camp. Data points represent the mean $\pm$ SEM (n=4).

Figure 6. PXR is Phosphorylated by Protein Kinase A In Vitro. Human PXR GST-fusion proteins were expressed and isolated from E. coli (BL21DE3) and resolved on 10% SDS-PAGE gel. Proteins were subjected to Coomassie blue staining (left). The in

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vitro PKA assay was performed for 30 minutes and the purified proteins were resolved on SDS-PAGE gel for autoradiography (right).

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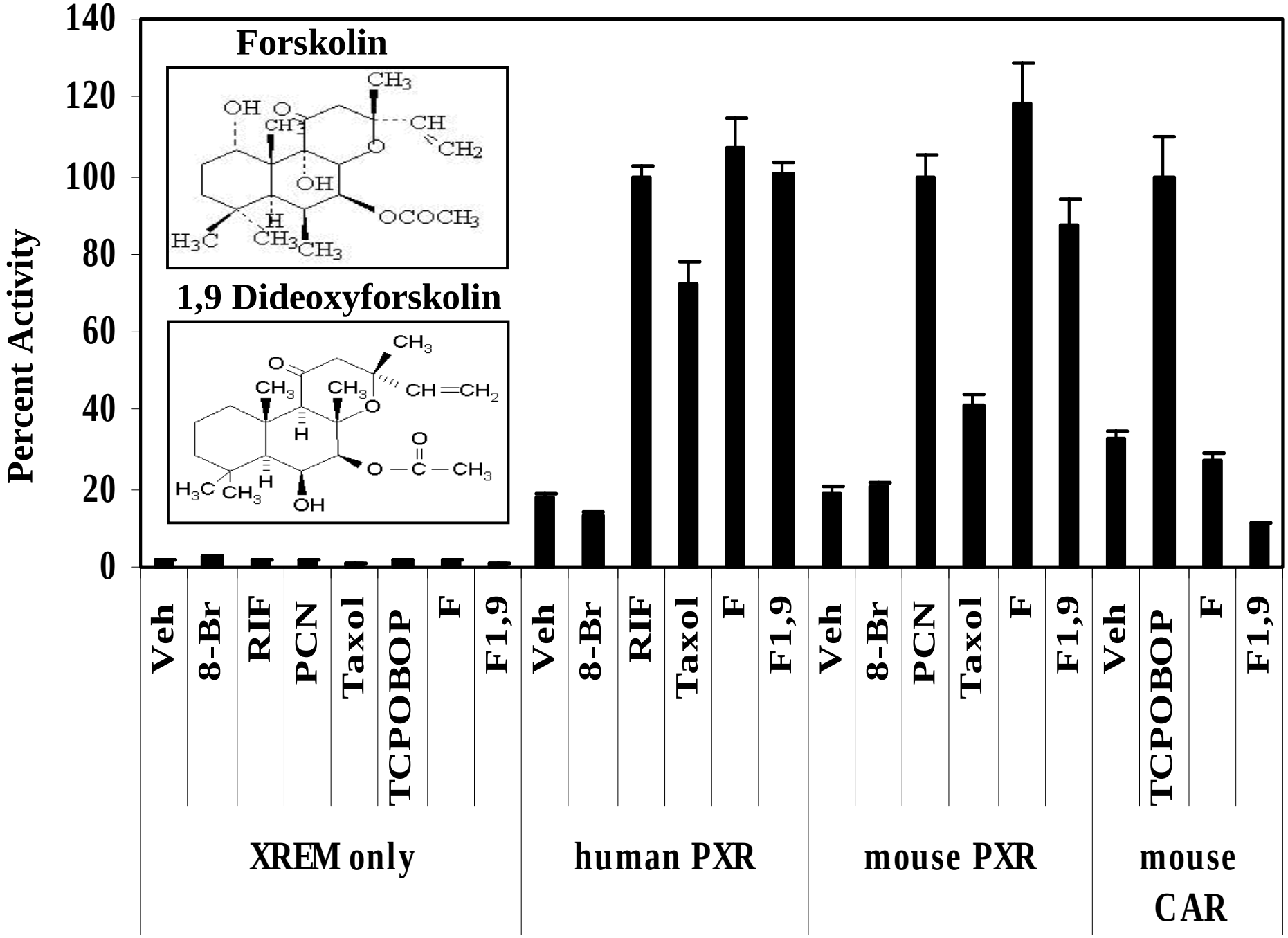

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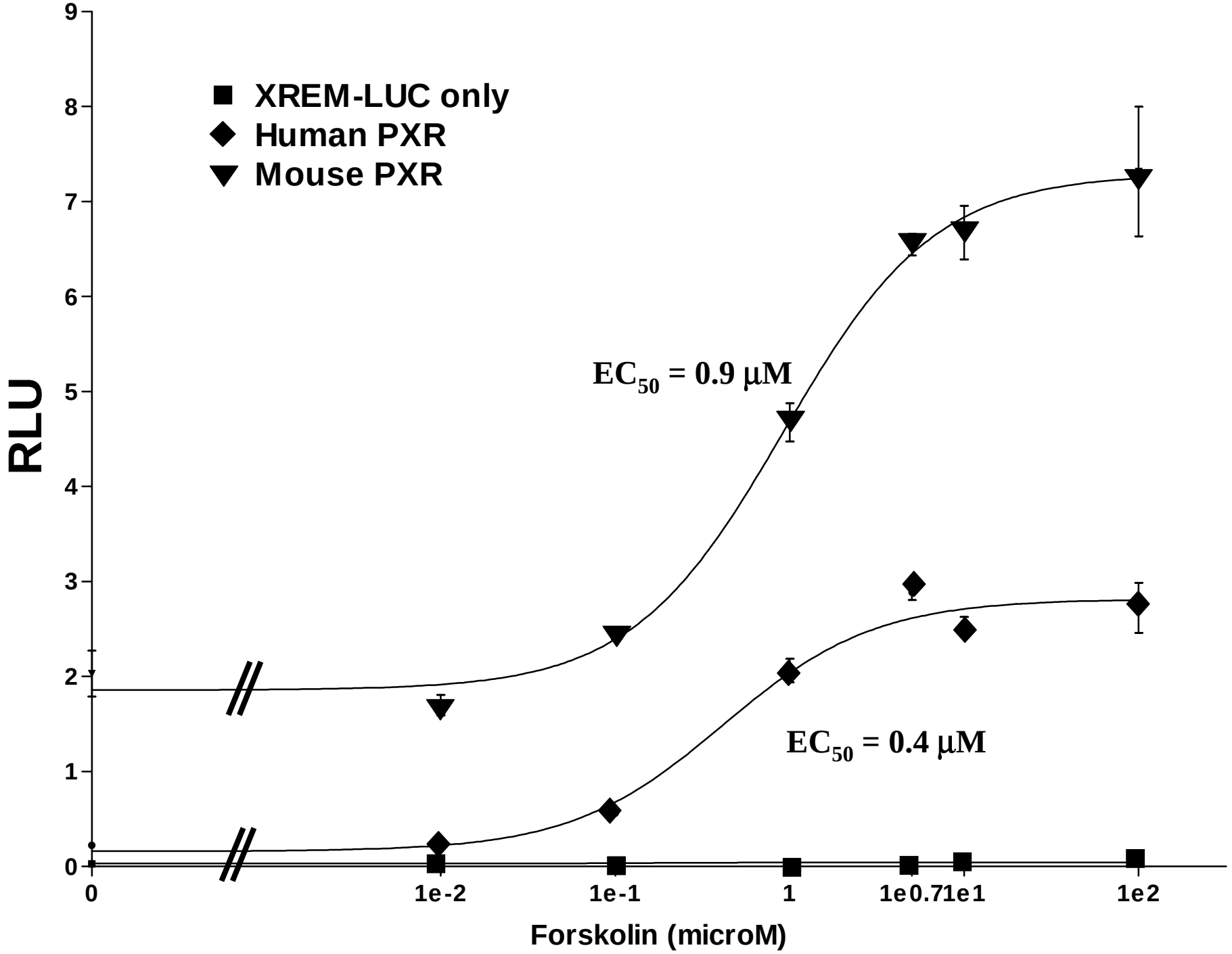

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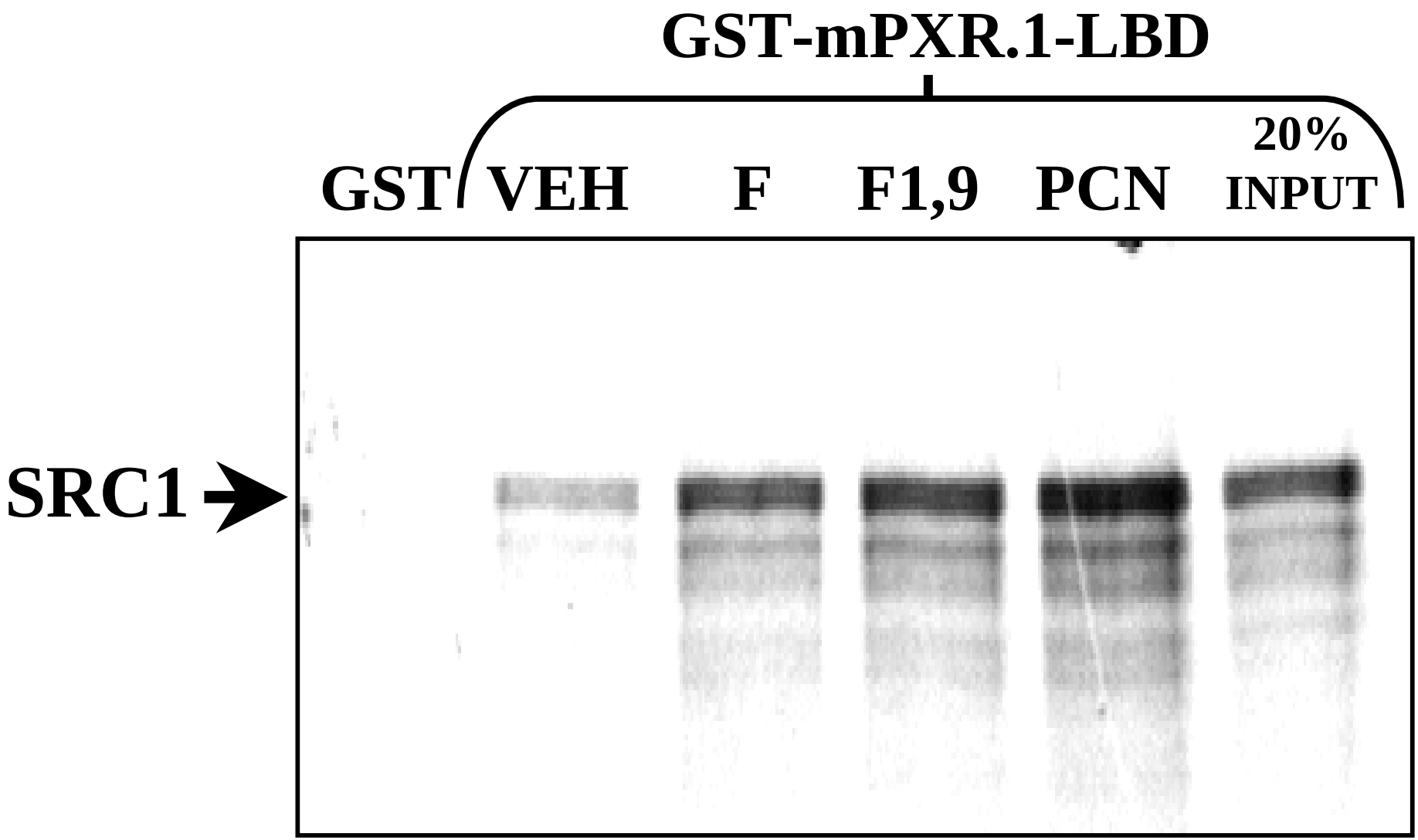

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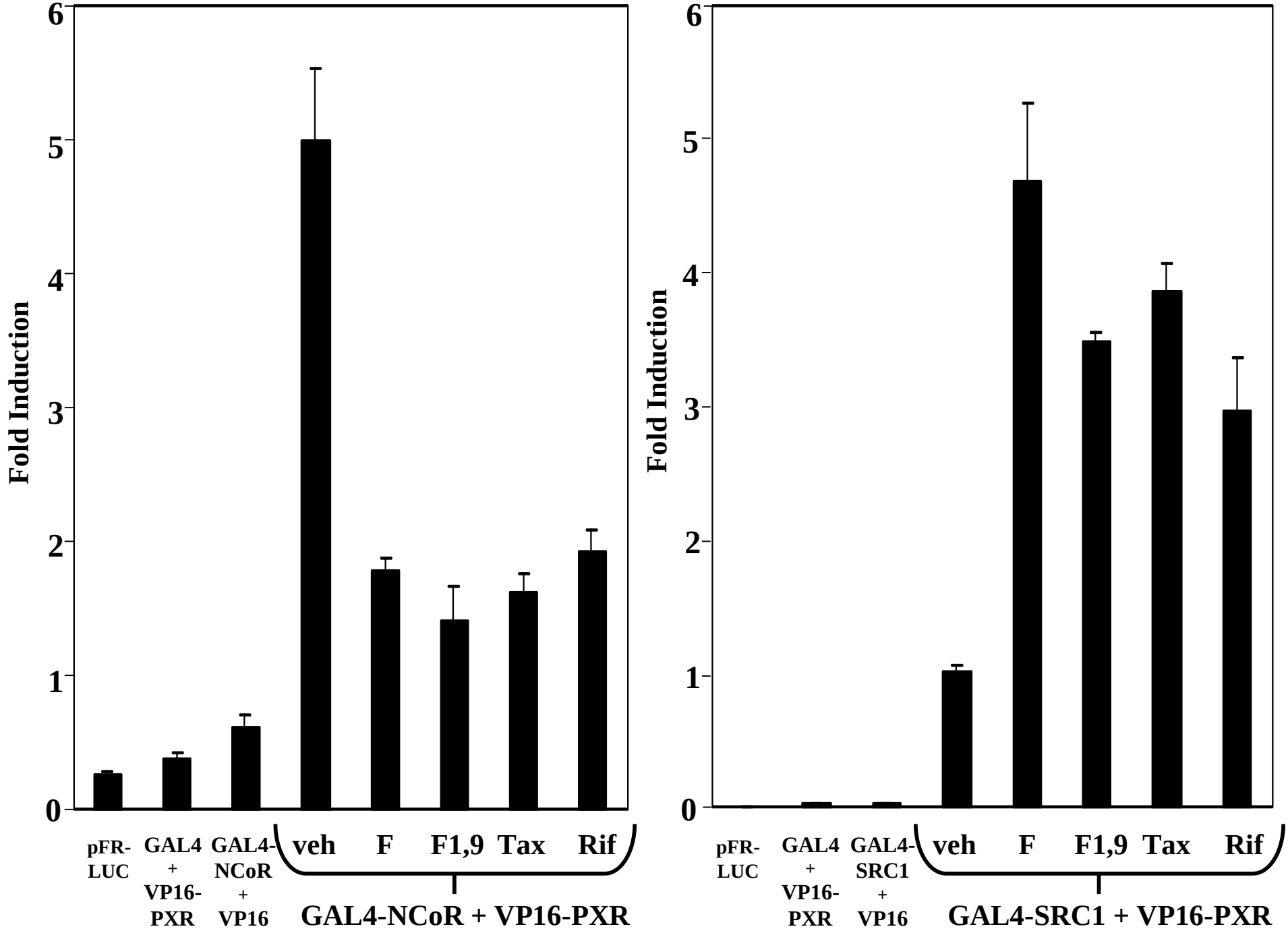

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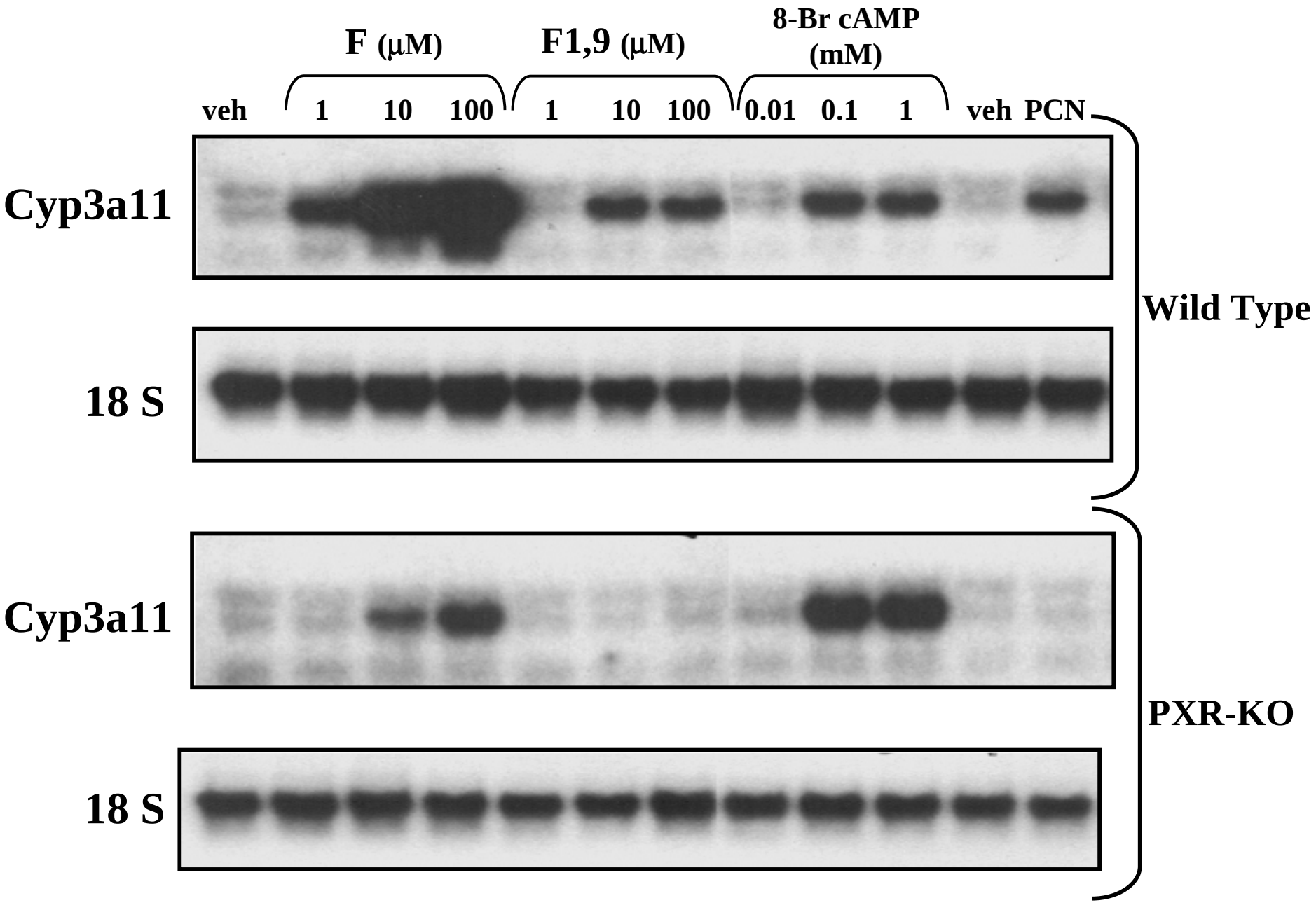

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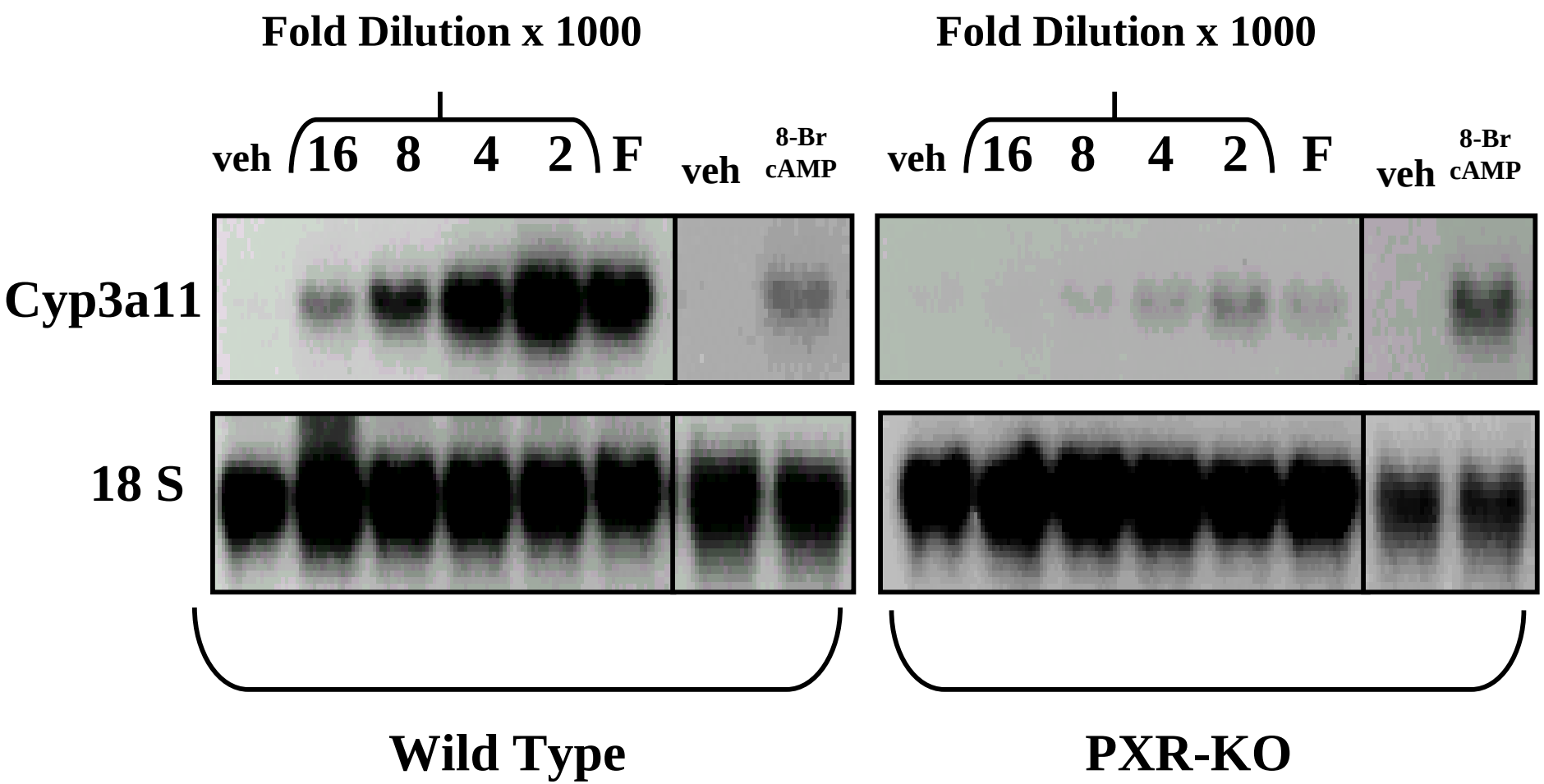

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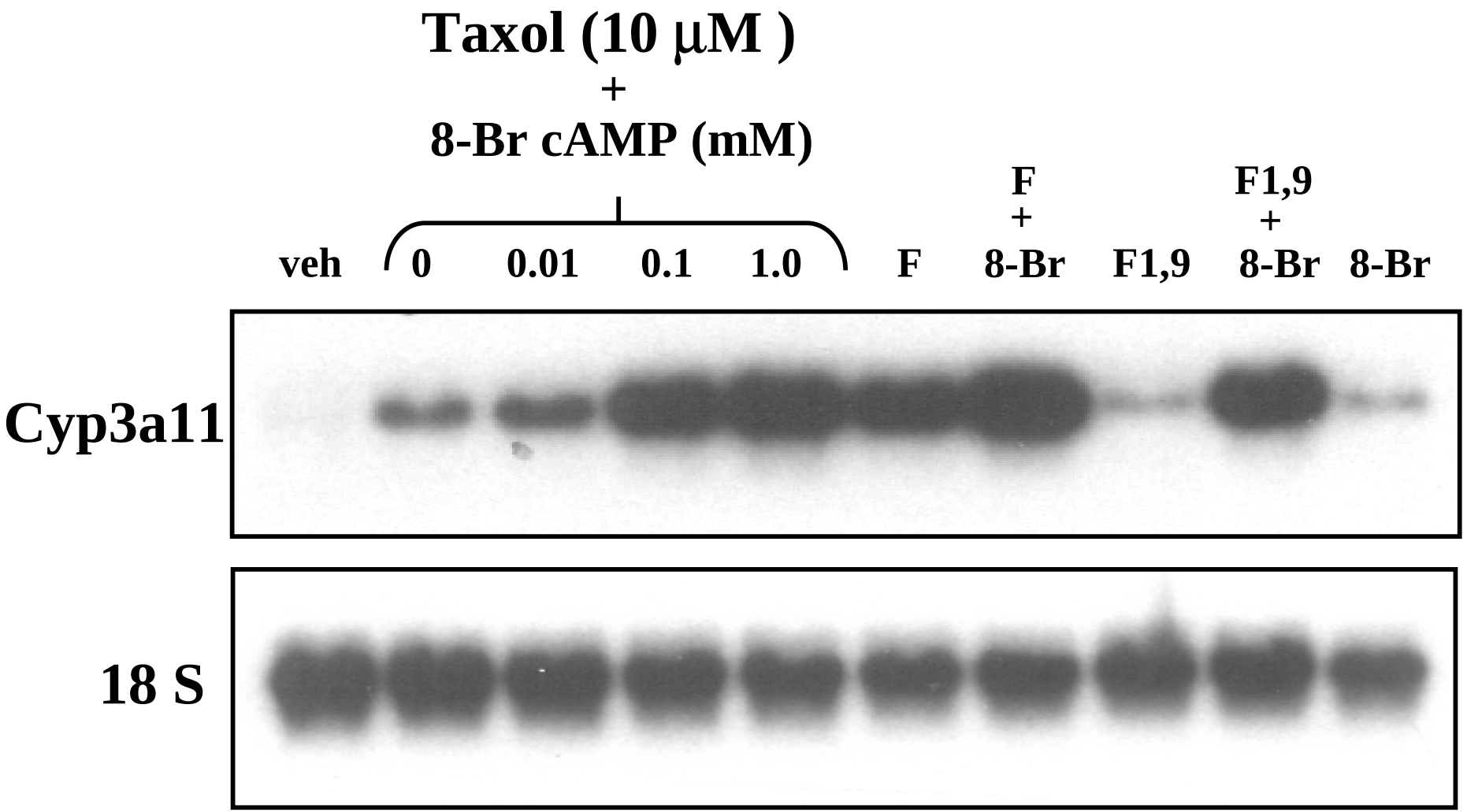

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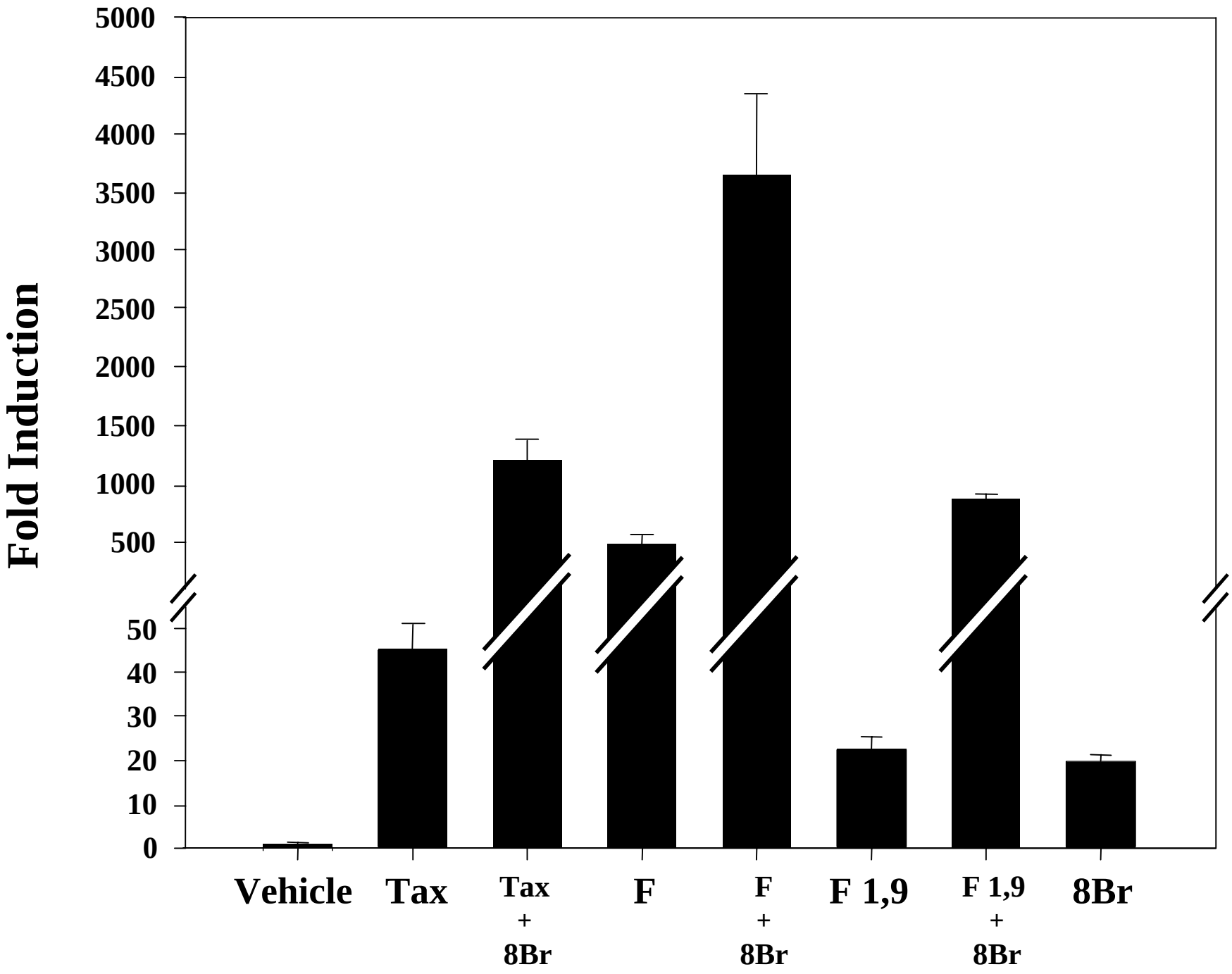

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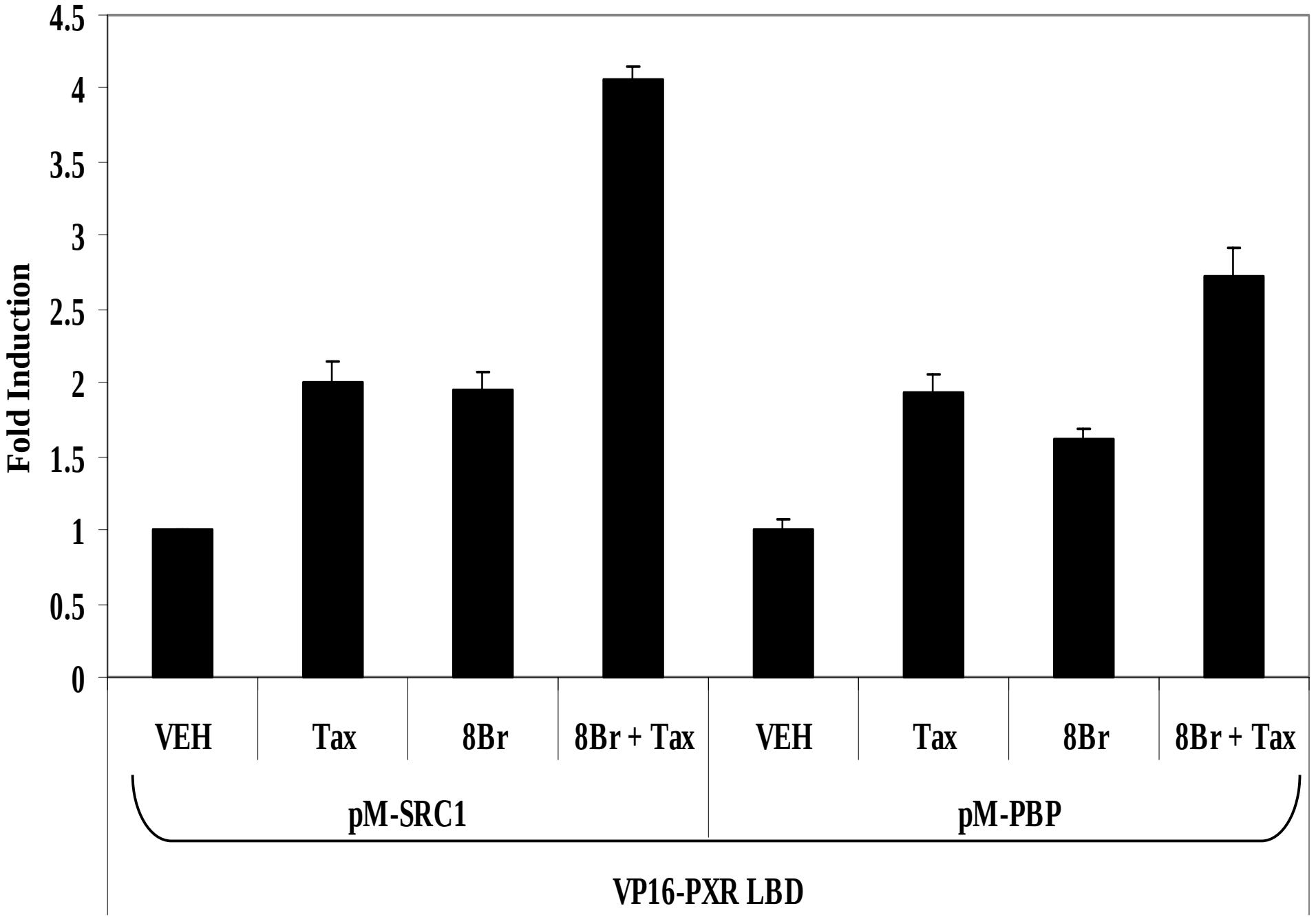

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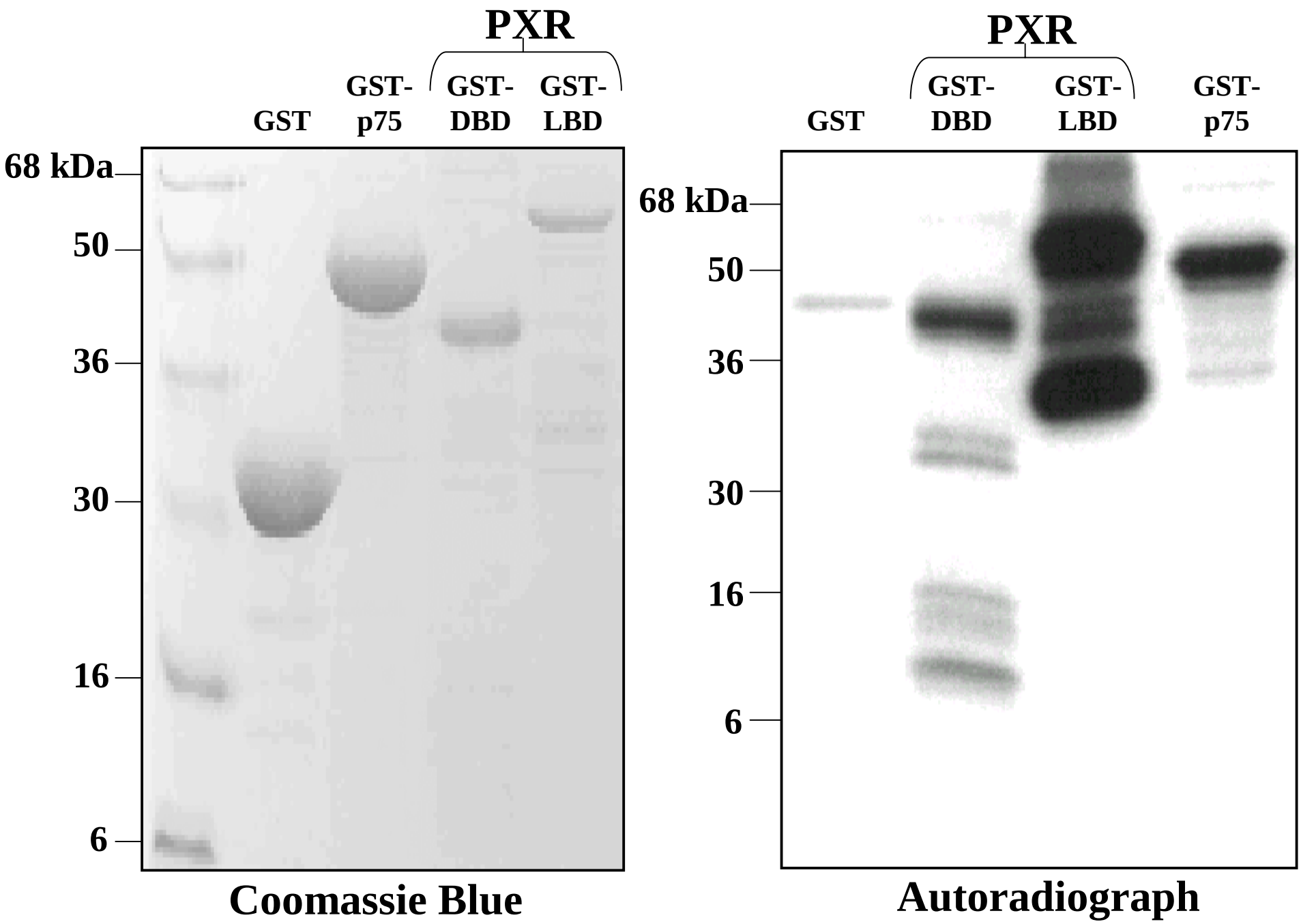

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