2016 Bernard B. Brodie Award Lecture

Phenobarbital meets phosphorylation of nuclear receptors

Masahiko Negishi
Pharmacogenetics, Reproductive and Developmental Biology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709, USA
Running Head: PB and NRs

All corresponds to: Masahiko Negishi at the above address

Telephone, 919-541-2404; Fax, 919-541-0696

E-mail address, negishi@niehs.nih.gov

Number of text pages: 27
Number of tables: 0
Number of figures: 10
Number of words in Abstract: 161
Number of references: 50

Abbreviations: NR, nuclear receptor; CAR, constitutive active/androstane receptor; RXRα, retinoid X receptor α; PXR, pregnane X receptor; ERα, estrogen receptor α; FXR, farenosoid X receptor; ERRα, estrogen receptor related receptor α, RORα, RAR-related receptor α; TRα, thyroid hormone receptorα; HNF4α, hepatocyte-enriched nuclear factor 4α; PPARα, peroxisome proliferator-activated receptor α, EGF, epidermal growth factor, EGFR, epidermal growth factor receptor; GADD45B, growth-arrest and DNA damage induce 45B; VRK1, vaccinia-related kinase 1; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene; CITCO, 6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime; CYP, cytochrome P450; PBREM, phenobarbital-responsive enhancer module; DBD, DNA-binding domain; LBD, ligand-binding domain; XRS, xenochemical response signal; CCRP, cytoplasmic CAR retention protein; KI, knock-in.
Abstract

Phenobarbital (PB) was the first therapeutic drug to be characterized for its induction of hepatic drug metabolism (Remmer and Merker, 1963). Essentially at the same time, cytochrome P450 (CYP), an enzyme that metabolizes drugs, was discovered (Omura and Sato, 1962). After nearly 50 years of investigations, the molecular target of PB induction has now been delineated to phosphorylation at threonine 38 of nuclear receptor, constituitive androstane receptor (CAR) (NR1I3) (Mutoh et al., 2009), a member of the nuclear receptor superfamily. Determining this mechanism has provided us with the molecular basis to understand drug induction of drug metabolism and disposition. Threonine 38 is conserved as a phosphorylation motif in the majority of both mouse and human nuclear receptors, providing us with an opportunity to integrate diverse functions of nuclear receptors. Here I review our works and accomplishment of my laboratory at National Institute of Environmental Health Sciences, NIH, and the future research directions of where our study of CAR might take us.
PB INDUCTION MECHNAISM

To begin, the current cell signal/molecular mechanism of PB induction/CAR activation is depicted in Figure 1. The underlying mechanism of CAR regulation is dephosphorylation of threonine 38, which can be inhibited by EGF to inactivate CAR (Figure 1A) or induced by PB to activate CAR (Figure 1B). Ligands directly bind and enable CAR to be dephosphorylated for its activation (Figure 1C). Here I will detail these regulatory processes.

**PBREM:** There have been a long and intensive investigations to identify a regulatory DNA element in PB-induced cytochrome P450 (CYP2B) genes (Honkakoski and Negishi, 1997). A DNA sequence that responds to PB was first identified within a distal region of the rat CYP2B2 promoter (Trottier et al., 1995) and characterized as the 51-bp phenobarbital responsive enhancer module (PBREM) in the mouse Cyp2b10 promoter (Honkakoski et al., 1998a). PBREM consists of two nuclear receptor binding DR4 motifs (NR1 and NR2) that flanks a Nuclear factor 1-binding site. In mouse primary hepatocytes, PBREM was activated by drugs such as clotrimazole and chlorpromazine in addition to PB and by numerous chemicals including a potent PB-like inducer 1,1,1-trichloro-1,2-bis(o,p'-chlorophenyl)ethane (TCPOBOP), PCBs 2,2’, 4,4’-tetrachlorobiphenyl, 2,2’,5,5’-tetrachlorobiphenyl, 2,3,3’,5,6-hexachlorobiphenyl, but not by AhR ligands 3-methylcholanthrene (3-MC) and 1,4-bis[2-(3-chloro-pyridyloxy)]benzene (TCDD) (Honkakoski et al, 1998b). The PBREM is conserved in the CYP2B genes from rodents to humans, providing us with a cross-species consensus enhancer for activation by PB and PB-type inducers as the experimental basis to subsequent investigations (Figure 2A).
**Nuclear receptor CAR:** Given the caveat that PB induction is a liver-predominant event, various hepatocyte-enriched nuclear receptors were screened. Among them, nuclear receptor CAR activated PBREM (Honkakoski et al., 1998a). In its short history, the cDNA MB67 was cloned as a nuclear receptor that activates an empirical set of retinoic acid response elements (Baes et al., 1994). As its activation occurs spontaneously, the term constitutive activator of retinoic acid response replaced MB67 and, subsequently, was shortened to constitutive active receptor (CAR). Later, androstanes were found to repress constitutive activity of CAR in cell-based assays, which became the basis of “CAR” to mean constitutive androstane receptor (Forman et al., 2001). This trivial name gave rise to the notion that nuclear receptors are ligand-activated transcription factors and should affiliate with so-called endogenous ligands. Although androstanes are useful to identify CAR activators in cell-based re-activation assays (Sueyoshi et al., 1999), there is no biological significance to CAR being repressed by androstanes. In addition, an androstane also activates pregnane X receptor (PXR) (Moore et al., 2000). The true characteristic of CAR resides in its constitutively activated nature that has become the biological basis to understand CAR functions.

While characterizing CAR as a PBREM-activating transcription factor in cell-based assays, in an independent study, we utilized NR1-affinity chromatography to purify a nuclear protein that binds to the PBREM within the Cyp2b10 promoter in mouse livers after PB treatment Western blot and peptide sequence analyses of purified fractions determined this binding protein to be CAR. The results obtained from both cell-based assays and purification implicated CAR as the PB-activated transcription factor (Honkakoski et al., 1998a). Shortly thereafter, as expected, the Car gene was knocked out in mice, confirming that CAR is a nuclear receptor that mediates PB activation of the CYP2B genes in livers (Wei et al., 2000). Subsequently, studies with transgenic...
mice that express either human CAR or PXR in the absence of their mouse counterpart determined CAR as the PB-activated nuclear receptor in mouse liver (Sheer et al., 2008). At that time, CAR, a relatively obscure nuclear receptor for 5 years since it was cloned, became a target for intense investigations in the research field of drug metabolism and toxicity as well as drug-drug interactions.

**Phosphorylation of threonine 38:** CAR is constitutively activated and this activity must be repressed to acquire response capability to PB. Western blot analysis of mouse livers revealed that CAR translocates from the cytoplasm into the nucleus after PB treatment (Kawamoto et al., 1999). Moreover, this nuclear translocation correlated with a PB-induced increase of CYP2B10 mRNA. This observation let us to hypothesize that cytoplasmic retention is the mechanism by which CAR represses its constitutive activity. In mouse primary hepatocytes, treatment with okadaic acid, a protein phosphatase inhibitor, inhibited this nuclear translocation (Kawamoto et al., 1999). In 2009, the target of this dephosphorylation was delineated to threonine 38 of CAR; threonine 38 was phosphorylated and dephosphorylated before and after PB treatment in mouse liver and primary hepatocytes (Mutoh et al., 2009). The topology of threonine 38 and the two functional features, which will be discussed later, is defined in the simulated three-dimensional structure of CAR as including both the DNA binding domain (DBD) and Ligand binding domain (LBD) (Figure 2B). Subsequently, phosphorylation and dephosphorylation was shown to occur in human primary hepatocytes (Yang et al., 2014), confirming that PB-induced dephosphorylation at threonine 38 is conserved in both mice and humans. In fact, the phosphomimetic CAR T38D mutant was unable to bind NR1 in gel-shift assays and retained in the cytoplasm of human hepatoma-derived HepG2 cells. In mouse livers, the CAR T38D mutant
was retained in the cytoplasm even after PB treatment, while the non-phosphomimetic CAR T38A mutant spontaneously accumulated in the nucleus. Although threonine 38 constitutes a protein kinase C(PKC) phosphorylation motif and is phosphorylated by PKC in *in vitro* kinase assays, the kinase that phosphorylates threonine 38 in livers *in vivo* and where it is phosphorylated in the cytoplasm and/or in the nucleus remain vague. Our recent study showed that threonine 38 can be phosphorylated by p38 MAPK in the nucleus of liver cells. p38 MAPK can be the kinase that phosphorylates threonine 38 in the nucleus, exporting phosphorylated CAR into the cytoplasm (Hori et al., 2016). Nevertheless, it is now accepted that the underlying mechanism of CAR activation is dephosphorylation of threonine 38.

**Cell signal regulation:** CAR is activated by dephosphorylation of threonine 38. How is this dephosphorylation regulated? First, it was found that growth factors such as epidermal growth factor (EGF), hepatocyte growth factor (HGF) and insulin repress CAR activation and nuclear translocation in rat and/or mouse primary hepatocytes (Bauer et al., 2004; Koike et al., 2007; Yasujima et al., 2016). Therefore, CAR is, in principle, a cell signal-regulated nuclear receptor (Figure 1A). The enzyme that dephosphorylates threonine 38 is protein phosphatase 2A with which CAR forms a complex in mouse liver in response to PB treatment (Yoshinari et al., 2003). In fact, in *in vitro* assays, PP2Ac utilized the receptor for protein kinase C 1 (RACK1) as a regulatory subunit to dephosphorylate threonine 38. Moreover, shRNA knock-down of either RACK1 or PP2Ac nearly abrogated PB-induced dephosphorylation of CAR and an increase of CYP2B10 mRNA levels in mouse primary hepatocytes (Mutoh et al., 2013). When the EGFR signal is activated, dephosphorylation of CAR at threonine 38 is repressed. Upon activation, EGFR signaling phosphorylates ERK1/2, enabling phospho-ERK1/2 to bind a previously
characterized peptide motif called the xenochemical response signal (XRS) of CAR (Zelko et al., 2002), preventing threonine 38 from being dephosphorylated. In addition, EGFR signaling also phosphorylates RACK1 at tyrosine 52, preventing RACK1 from interacting with CAR and activating PP2Ac to dephosphorylate threonine 38 (Mutoh et al., 2013). Thus, EGF coordinates two different down-stream signals (ERK1/2 and Src1) to repress dephosphorylation and activation of CAR. It was found that inhibition of ERK1/2 phosphorylation by U0126, a MEK1/2 inhibitor is sufficient to activate CAR and activate the Cyp2b10 gene in the absence of exogenous CAR activators in mouse primary hepatocytes (Koike et al., 2007; Osabe and Negishi, 2011). These findings indicate that CAR is truly a cell signal-regulated nuclear receptor and ERK1/2, one of the two signals, may dominate CAR regulation.

PB, indirect activation mechanism: Our work using isothermal titration calorimetry (ITC), competitive binding assays and dynamic computer simulations of binding corroborated the findings that PB directly binds EGFR. Through this binding, PB repressed EGFR’s down-stream signals ERK1/2 and Src-1 in mouse primary hepatocytes (Mutoh et al., 2013). These observations provided the underlying molecular basis for PB activation of CAR (Figure 1B). By reversing EGFR activation by EGF, PB reverses EGFR signaling. ERK1/2 is dephosphorylated and dissociates from the XRS of phosphorylated CAR. The Src1 signal is attenuated, resulting in dephosphorylation of RACK1 at tyrosine 52. Dissociation of ERK1/12 allows dephosphorylated RACK1 to bind CAR and PP2Ac, dephosphorylating threonine 38 of CAR for nuclear translocation and activation.

The binding site for RACK1/PP2Ac is previously delineated to a loop (from residues 140 to 152) of CAR LBD (Mutoh et al., 2013). CAR utilizes this binding site as a molecular basis to
integrate EGFR signaling into its activation mechanism. Figure 3 depicts the reported homodimer configuration in a CAR LBD crystal structure (Shun et al., 2004). Three loops form the dimer interface, the bottom loop of which bears the RACK1/PP2Ac binding site. Thus, the CAR homodimer buries the RACK1/PP2Ac binding site within this interface. EGF promotes dimerization of CAR to hide the binding site, while PB stimulates monomerization to expose the binding site for RACK1/PP2Ac to dephosphorylate CAR (Shizu et al., 2016). The homodimer-monomer conversion is the molecular base that regulates CAR activation.

**Ligands, direct activation mechanism:** Various chemicals directly activate CAR in cell-based assays among which TCPOBOP and CITCO activate mouse and human CARs, respectively (Sueyoshi et al., 1998; Maglich et al., 2003). Chemicals that activate or repress CAR in cell-based assays are generally considered ligands. They were found to bind the ligand binding domain of CAR in X-ray crystal structures (Suino et al., 2004; Xu et al., 2004). While EGF repressed ligand activation of CAR, such as by ligands such as TCPOBOP and CITCO, unlike PB, these ligands did not repress EGF signaling at the EGFR (Koike et al., 2007; Shizu et al., 2016). Thus, ligands should crosstalk with EGF signaling to activate CAR, but not at the EGFR. Recent studies with human CAR and CITCO have demonstrated that CITCO directly dissociates the CAR homodimer into its monomer for RACK1/PP2Ac to dephosphorylate threonine 38 (Figure 1C) (Shizu et al., 2016). Thus, phosphorylated CAR homodimer is the common target of indirect CAR activators such as PB and ligands such as CITCO. While CAR activation has been investigated from two different angles, the so-called “direct versus indirect activation” approach, when the dust settled, it became clear that both direct and indirect share the same mechanism.
The CAR homodimer can be the target of PB induction which we have been looking for over the last 50 years.

**Regulation in the nucleus:** Nuclear translocation of dephosphorylated CAR is now accepted to be a key process of CAR activation. Nuclear CAR was once thought to elicit constitutive activity to activate transcription. However, it is now known that CAR activation is more complex and tightly regulated in the nucleus. The most unexpected finding of all was that CAR accumulates in the nucleus in mouse primary hepatocytes following treatment with androstanol, a mouse CAR antagonist (Figure 4). Apparently, nuclear translocation is essential but not sufficient for CAR to activate transcription and CAR activation requires distinct processes in the nucleus (Ohno et al., 2014). Recent studies with cytoplasmic CAR retention protein (CCRP) KO mice showed that CCRP does not regulate basal intracellular localization of CAR (Ohno et al., 2014). However, cytoplasmic CAR retention protein (CCRP) appeared to constrain PB-induced nuclear translocation, as indicated by far excess nuclear accumulation of CAR in CCRP KO compared to CCRP WT mice. In response to PB treatment, the Cyp2b10 promoter recruited CAR but not RNA polymerase II in the livers of CCRP KO mice, resulting in an attenuation of PB induction of CYP2B10 mRNA. In addition, CCRP also regulated the CAR-independent but PB-induced demethylation of the Cyp2b10 promoter. Contrary to the Cyp2b10 gene, the other CAR target Cyp2c55 gene was not under CCRP regulations.

CAR requires p38 MAPK activity to activate the CYP2B6 gene in HepG2 cells (Saito et al., 2013). Our recent study with mouse CAR-expressing HepG2 cells and mouse primary hepatocytes demonstrated that, in the nucleus, p38 MAPK recruits CAR to the PBREM of CYP2B promoter for activation and subsequently phosphorylates threonine 38 for inactivation.
(Hori et al, 2016). Through these sequential reactions, p38 MAPK appears to link CAR activation and inactivation as shown in Figure 5.

Experiments with growth arrest and DNA damage induce 45B (GADD45B) KO mice showed that CAR also needs GADD45B to fully activate the Cyp2b10 gene, but not the Cyp2c55 gene (Yamamoto et al., 2008). While CCRP (the co-chaperone DNAJC7) was first characterized for accumulating ectopic CAR in the cytoplasm of HepG2 cells (Kobayashi et al., 2003), CCRP is now known to regulate CAR in the nucleus (Ohno et al., 2014). CAR-mediated transcription is diverse but tightly regulated in the nucleus. It will take novel ideas and efforts to understand their complex mechanisms that involve many protein-protein interactions.

**Beyond PB and drug metabolism:** NIEHS was developing its own cDNA microarray system around 2000. This array allowed us to analyze PB-induced and CAR-dependent genes using CAR KO mice, although it was a far smaller array than currently available arrays. Our array experiments generated two significant findings, one is that CAR regulates PB induction of not only cytochrome P450 but also various enzymes that orchestrate drug metabolism, and the other was the fact that CAR mediates PB-induced repression of hepatic gluconeogenesis (Ueda et al., 2002). As to the mechanism, activated CAR directly interacts with a forkhead transcription factor FOXO1, preventing it from activating gluconeogenic genes (Kodama et al., 2004). Since insulin stimulates phosphorylation of FOXO1 to inactivate it, PB and insulin share the same target to repress gluconeogenesis. Recently, it was also suggested that CAR forms a complex with PGC-1 to stimulate its degradation, thereby repressing gluconeogenesis (Gao et al., 2015). CAR activation may benefit humans to repress hepatic gluconeogenesis as well as associated diseases such as Type 2 diabetes and steatosis.
**CAR as a signal transducer:** CAR directly interacts with and regulates cell signal molecules to determine cell fate, such as cell growth and death and inflammation. For example, CAR represses stress-induced signaling. CAR forms a complex with a growth arrest and DNA damage inducible beta GADD45B to repress JNK1 signaling in mouse primary hepatocytes, attenuating apoptosis (Yamamoto et al., 2010). Recently, it was also found that CAR-GADD45B complex suppresses MKK6-catalized phosphorylation of p38 MAPK and its down-stream signaling in mouse livers (unpublished observations). CAR activation is essential for PB to promote non-genomic development of hepatocellular carcinoma (HCC) in rodents as shown in Figure 6 (Yamamoto et al., 2004), although this promotion mechanism remains undetermined. Repression of these JNK 1 and p38 MAPK signal pathways may be the foundation of the molecular mechanism by which PB promotes HCC. An epidemiological study with epileptic patients suggested no direct link between PB treatment with HCC development (Clemmesen and Jensen, 1978). However, defining the underlying process of cell signaling that leads to HCC development in rodents is critically important for us to investigate and understand HCC in humans.

**INTEGRATING NUCLEAR RECEPTORS**

**Threonine 38 conserved in nuclear receptors:** Threonine 38 is in the loop between two zinc fingers within the DBD and conserved as a PKC phosphorylation motif in both mouse and human nuclear receptors and in 41 out of the 46 total human nuclear receptors as shown in Figure 7 (Hashiguchi et al., 2016). Despite that this phosphorylation motif is the most conserved in nuclear receptors, it has been ignored in nuclear receptor research. This scorn may have arisen
from the idea that the DBD was only considered for DNA binding and as the entity regulated by the LBD and/or N-terminal domain (NTD/AF1). In addition, the fact that phosphorylation of this motif was not confirmed in endogenous nuclear receptors in tissues in vivo may have perpetuated this ignorance. Besides CAR, three more nuclear receptors (ERα, at serine 216, FXR at serine 154 and RXR at threonine 162) are phosphorylated in mice in vivo (Shindo et al., 2013; Hashiguchi et al., 2016; unpublished observation). Further investigations may establish this phosphorylation in numerous nuclear receptors, shedding a light to the conserved motif of the DBD and providing us with an opportunity to integrate nuclear receptors.

**Phosphorylated DBD as a NR degradation signal:** This conserved phosphorylation motif has only sporadically been the subject of investigations with a limited number of nuclear receptors such as VDR, HNF4α and FXR (Hsieh et al., 1991; Sun et al., 2007; Gineste et al., 2008). Our current work systematically examined 11 different human nuclear receptors by expressing them in COS-1 cells (Hashiguchi et al., 2016). Among these 11 nuclear receptors, upon a phosphomimetic mutation at their conserved motifs, eight nuclear receptors (FXR, CAR, VDR, TRα, Rev-erb α, RORα, HNF4α and PPARα) degraded through ubiquitination and proteasomes in COS-1 cells. These observations have now defined the phosphorylated DBD as a minimum structural signal for nuclear receptors to degradation. Interestingly, whether or not and how nuclear receptors utilize this degradation signal appeared to be determined by other subdomains such as the Activation Function 1 (AF1) and/or the LBD. For example, AF1 enabled FXR to degrade through ubiquitination and proteasomes, while the LBD enabled RORα to evade degradation and accumulate. Thus, nuclear receptors can diverge their functions and regulations by utilizing this conserved phosphorylation differently.
Control constitutive activity: One major question that has remained persistent around many nuclear receptors is how their constitutive activity is controlled. The idea of so-called endogenous ligands does not appear to have helped us much in answering this question, as indicated by the following quote, “Estrogen-related receptors (ERRs) are founding members of the orphan nuclear receptor (ONR) subgroup of the nuclear receptor superfamily: Twenty-seven years of study have yet to identify cognate ligands for the ERRs, though they have firmly placed ERRα (ESRRA) and ERRγ (ESRRG) at the intersection of cellular metabolism and oncogenesis.” (Divekar et al., 2016). Alternative to the hypothesis of “endogenous ligands”, the conserved phosphorylation of DBD presents an experimental basis for us to determine the underlying mechanism by which nuclear receptors control their constitutive activities.

Threonine 38, directing its sidechain to be opposite from the surface of CAR binding DNA, does not directly interact with DNA. Phosphorylation of threonine 38 distorts the C-terminal side helix of the two helices flanking the loop bearing threonine 38, disabling CAR from binding and activating PBREM (Mutoh et al., 2009). Phosphorylation of threonine 87 of HNF4α is suggested to alter interactions between its DBD and LBD to inactivate DNA binding (Chandra et al., 2013). In general, phosphomimetic mutations at the conserved motif inactivates nuclear receptors in both constitutive and ligand-based activities in cell-based reporter assays. Thus, this conserved phosphorylation motif promotes nuclear receptors’ability to regulate both constitutive and response activities. Moreover, this motif enables nuclear receptors to coordinate their activation and inactivation. For example, threonine 38 is dephosphorylated by PP2A in the cytoplasm to translocate CAR into the nucleus. In the nucleus, p38 MAPK forms a complex with CAR enabling it to bind to the PBREM and activate the Cyp2b10 promoter. Subsequently, p38
MAPK phosphorylates threonine 38 and dissociates CAR from the promoter, thereby exporting it back to the cytoplasm (Hori et al., 2016). Not only PB, but also ligands such as CITCO and TCPOBOP also utilize this phosphorylation to coordinate CAR activation and inactivation. For this matter, threonine 38 links CAR activation and inactivation as well as nuclear import and export through phosphorylation and dephosphorylation, probably for recycling CAR. Further investigations with additional nuclear receptors may conceptualize phosphorylation of the conserved motif of the DBD as a common regulatory mechanism for nuclear receptors.

**Nuclear receptor crosstalk via phosphorylation:** Nuclear receptors crosstalk to co-regulate CYP expression and drug metabolism (Gotoh et al., 2015), the molecular mechanism of which remains nebulous. The conserved phosphorylation motif within the DBD can be used as a foundation for nuclear receptors to crosstalk with each other. As described, CAR forms its homodimer through the interactions between two LBDs by utilizing a surface (Surface A) opposite from the surface (Surface B) that forms a heterodimer with RXR (Figure. 8). Nuclear receptors should be able to form their homodimers in the same configuration as observed with the CAR homodimer. In addition to homodimerization, nuclear receptors should also heterodimerize with another nuclear receptor through Surface A in the cytoplasm in the absence of RXR. In the presence of RXR, it may be possible for two nuclear receptors to form a tetramer (RXR-NR1-NR2-RXR) in cases such as crosstalk between CAR and VDR to repress the CYP24A1 gene by locking the co-repressor SMRT onto its promoter in the nucleus (Konno et al., 2009). Although this crosstalk can be a molecular basis for investigating metabolic bone diseases caused by chronic drug therapy with PB and/or phenytoin which activate CAR, the mechanism of this locking is not understood yet. Phosphorylation of the conserved motif could determine
functionality of nuclear receptors’ crosstalk by altering formations of their complexes. When it becomes available in the future, a KI mouse library bearing a non-phosphomimetic mutation in nuclear receptors will help us to investigate nuclear receptor’s crosstalk as well as correlate regulation of phosphorylation and dimerization and indicate their pharmacological significances.

**Biology of conserved phosphorylation:** In addition to CAR, two nuclear receptors FXR and ERα are now confirmed to be phosphorylated at their conserved motifs *in vivo* in mice. FXR becomes phosphorylated at serine 154 in the nucleus of livers of ligand-treated mice, in which the nuclear kinase vaccinia-related kinase 1 (VRK1) appears to phosphorylate serine 154 (Hashiguchi et al., 2016). As suggested by the FXR S154D mutant, phosphorylated FXR does not bind or activate FXRE and it is degraded by proteasomes either in the nucleus or cytoplasm. Thus, FXR utilizes the conserved phosphorylation motif to link its activation, inactivation, and degradation. This FXR linking mechanism resembles that observed with CAR and p38 MAPK (Figure 5). The lack of this phosphorylation may prolong the activated status of FXR, dysregulating liver functions such as bile acid, fatty acid and glucose metabolism and developing liver diseases.

ERα, which conserves this phosphorylation residue at serine 212 in humans and serine 216 in mice, was found to be phosphorylated in mouse immune cells (Shindo et al., 2013). Immunohistochemistry with an anti-phospho-S216 peptide antibody (αP-S216) first revealed staining of certain cells but not uterine cells of the mouse uterus (Figure 9A). Double fluorescence immunostaining confirmed that these cells were infiltrating neutrophils. Neutrophils infiltrate the uterus from blood stream (Figure 5B), about 20% of which express phosphorylated ERα. Only blood neutrophils expressing phosphorylated ERα migrated in *in vitro* migration assays and
infiltrated the uterus of female mice as shown in Figure. 9 (Shindo et al., 2013). Phosphorylation of the conserved motif enables neutrophils to migrate and infiltrate. ERα is also phosphorylated in mouse microglia, the resident macrophages, and only immune cells in the brain (Shindo et al., 2016). Knock-in (KI) mice bearing a single mutation of serine 216 to alanine (Esr1S216A) were generated and began to reveal anti-inflammatory and anti-apoptotic functions of phosphorylated ERα in immune cells. Esr1S216A mice, obese but fertile in both sexes, can be an excellent animal model for us to study inflammation-related diseases including obesity and neuronal degenerative diseases such as Alzheimer’s and Parkinson’s Disease. Again, the majority of nuclear receptors contain this conserved phosphorylation motif within their DBDs in both humans and mice. It is important to generate a library of knock-in (KI) mouse lines which bear the non-phosphomimetic mutation of this motif in each nuclear receptor for us to determine the biological roles of the conserved phosphorylation of nuclear receptors.

Epilogue

A single residue matters: Mammalian cytochrome P450s (CYPs) consist of about 500 amino acid residues. Mouse CYP2A4 and CYP2A5 specifically catalyze testosterone 15α-hydroxylation and coumarin 7-hydroxylation yet they differ by only 11 amino acid residues from their total 494 residues (Negishi et al., 1989). Later, it was later found that CYP2A6, the human homologue of mouse CYP2A5, also catalyzes coumarin 7-hydroxylation. The residue at position 209 is leucine in CYP2A4 and phenylalanine in CYP2A5. A single mutation of phenylalanine 209 to leucine converted the catalytic activity of CYP2A5 from coumarin to testosterone hydroxylation (Lindberg and Negishi, 1989). Furthermore, mutation of phenylalanine 209 to asparagine conferred corticosterone 15α hydroxylase activity to CYP2A5 (Iwasaki et al., 1993).
Subsequently, using a dynamic simulation study, with the only available crystal structure of bacterial P450cam (now CYP101) at that time, F and G helices and the loop between them were suggested to be structurally flexible, enabling CYP2A5 to determine substrate specificity (Iwasaki et al., 1993). In addition, our mutagenesis work suggested that CYP2A5 may have two different substrate binding sites (Lindberg and Negishi, 1989). The idea of two substrates in one substrate binding pocket was first proposed more than 30 years ago by Alan Conney’s group: flavonoids stimulate benzo(a)pyrene hydroxylation activity of rat liver microsomes (Huan et al., 1981). Since the first mammalian P450 crystal structure was solved with CYP2C5 (Johnson, 2002), a large number of the crystal structures has been determined, which confirm our early predictions in the 90’s that single amino acid, along with these helices, loop and the two substrate binding sites enable P450s to be flexible and diverse in their catalytic activities.

In retrospect, this belief that a single residue matters sustained my laboratory throughout our ordeal to discover phosphorylation of threonine 38 of CAR in PB induction and has been extended beyond CAR by us. It is still thought that phosphorylation of a single residue cannot and should not regulate nuclear receptors, since there are many potential phosphorylation sites in any given nuclear receptor. As it turns out, threonine 38 is not one of these sites; it is the most conserved phosphorylation site of nuclear receptors. Moreover, this conserved phosphorylation motif has renewed research interests in the DBD as a regulatory determinant of nuclear receptor functions beyond being the simple DNA binding entity of a nuclear receptor.

Many thanks: I started my career in science as a graduate student in Professor Ryo Sato’s laboratory at Institute for Protein Research, Osaka University, under the supervision of Professor Tsuneo Omura in 1968. From the beginning to the presence, Omura sensei (mentor) has never
stopped helping me develop my carrier, although I was his worst student in many ways. For 5 years after I obtained my degree, I worked at Kansai Medical University under Professor Yutaka Tashiro who provided me with an opportunity to work in the United States in 1976. Since 1983, I have been a group leader at National Institute of Environmental Health Sciences, NIH and it has been my privilege to work with more than one hundred postdoctoral fellows, graduate students and guest researchers (Figure. 10). I am sincerely thankful for all of these mentors and colleagues who made my life happy and fruitful. Now I would like to finish my review by “entrusting the next 50 years to the next generation of young scientists and students.”

Acknowledgements

My deepest appreciation is due to Dr. Joyce Goldstein who has been my friend and collaborator for the last 33 years at NIEHS. I also thank Drs. Lalith Perera, Lars Pedersen, Ryo Shizu, Takeshi Hori and Mack Sobhany for helping me to write this review.

Authorship Contributions

Wrote: Negishi
References


Remmer h, and Merker HJ, (1963). Drug-induced changes in the liver endoplasmic reticulum:
association with drug-metabolizing enzymes. 142: 1657-1658.


binding domain of nuclear receptors alters intracellular localization. Mol. Endocrinol. 21:
1297-1311.
Ueda A, Hamadeh HK, Webb HK, Yamamoto Y, Sueyoshi T, Afshari CA, Lehmann Negishi M,
(2002). Diverse roles of the nuclear orphan receptor CAR in regulating hepatic genes in
drug-induced expression of CYP2B6 by modulating the constitutive androdstane receptor
receptor constitutive /androstane receptor is essential for liver tumor promotion by
TNFα-induced cell death by interacting with anti-apoptotic GADD45B. PLOS ONE
45β regulates the nuclear receptor constitutive active/androstane receptor-mediated
activation of the nuclear constitutive androstane receptor through the insulin receptor. J.


Footnote

This work was supported by the Intramural Research Program of the National Institutes of Health and National Institute of Environmental Health Sciences [Grant Z01ES71005-01]
Figure Legends:

Figure 1. The molecular mechanism of phenobarbital induction. A. EGF activates EGF receptor (EGFR), preventing CAR from being dephosphorylated and keeping CAR inactivated. B. PB binds EGFR and represses its signaling, dissociating ERK1/2 from XRS and allowing RACK1/PP2Ac to dephosphorylate threonine 38 for CAR activation. C. Ligands such as CITCO directly bind phosphorylated CAR, dissociating P-ERK1/2 from XRS. Blue arrows indicate signals to inactivate CAR and red and orange arrow to activate CAR.

Figure 2. A. The PBREM sequences are conserved in the mouse (Cyp2b10), rat (CYP2B1 and CYP2B2) and human (CYP2B6) genes (Sueyoshi et al., 1999). NR1 and NR2 denote nuclear receptor binding site 1 and 2, respectively. NF1 is for nuclear factor 1. A response element in the human CYP3A4 promoter was reported as ER6, which can also be DR4. B. Three-dimensional model of CAR structure is depicted from our previous publication (Mutoh S, Sobhany M, Perera L, Pedersen L, Sueyoshi T, Negishi M, (2013). Phenobarbital indirectly activates the constitutive active androstane receptor (CAR) by inhibition of epidermal growth factor receptor signaling (from [Mutoh S, Sobhany M, Perera L, Pedersen L, Sueyoshi T, Negishi M, (2013)] Sci. Signal. 6: ra31); reprinted with permission from AAAS. The DBD is in light blue; the hinge in yellow; the LBD in white. Green and red indicate xenochemical response signal (XRS) and RACK1/PP2Ac binding site, respectively.

Figure 3. Novel configuration of CAR homodimer. The mouse CAR dimer from the x-ray crystal structure (pdb ID = 1XNX). The RACK1 binding motif (residues 150-162) are in red and the XRS motif (ERK1/2 binding site; residues 312-319) in green.
Figure 4. CAR antagonist accumulates CAR in the nucleus. Mouse hepatocytes were prepared from C3H mice, pre-cultured for 3 h and treated with 10 μM of androstenol (Andro) and/or 250nM of TCPOBOP (TC) for 2h. Hepatocytes were fractionated using NE-PER (Thermo), a nuclear and cytoplasmic extraction kit, and subjected to Western blot analysis with anti-CAR (PP-N4111-00, Perseus proteomics), TATA-box binding protein (TBP) (sc-273, Santa Cruz Biotechnology) or Hsp90 antibodies (610419, BD Biosciences). Protein bands were visualized with chemiluminescence reagent WesternBrightTM ECL reagents (GE Healthcare) and quantified using Image Studio™ Software with C-DiGit® Blot Scanner (LI-COR). For detection of extremely low levels CAR in cytosols, Western blots were scanned in a condition of high contrast.

Figure 5. p38 MAPK phosphorylates CAR at threonine 38 to link PB activation, inactivation and intracellular localization, as shown in our previous publication (Hori et al., 2016). PB/TC is used to indicate dephosphorylation in response to phenobarbital (PB) or TCPOBOP (TC).

Figure 6. No PB-promoted HCC in CAR KO mice. Car−/− (CAR KO) and Car+/+ (CAR WT) mice with C3H/HeNCrlBR background were treated with a single dose of diethylnitrosamine (DEN) for tumor initiation and chronically with phenobarbital (PB) for tumor promotion according to a standard protocol of the two-step hepatocarcinogenesis (Yamamoto et al., 2014). After 32-week PB treatment, eight out of 20 Car+/+ mice developed carcinoma, while none of the 20 Car−/− mice developed carcinoma.

Figure 7. Threonine 38 is conserved as a PKC phosphorylation motif in nuclear receptors. Only four members of the subfamily 3C and NR1I2 (PXR) do not conserve this motif. Some data from a previous publication (Hashiguchi et al., 2016). These conserved phosphorylation residues are in red and boxed. Letters shadowed with blue are amino acid residues that are conserved among nuclear receptors.
Figure 8. Two different dimer surfaces of nuclear receptors. Surfaces A and B are in the opposite sides of the CAR molecule. Three loops comprise surface A for homodimerization and may also for heterodimerization of two different nuclear receptors, such as CAR and VDR. Surface B is well established as heterodimerizing with RXR. ERα was suggested to form its homodimer through surface B.

Figure 9. ERα phosphorylated at serine 216 in neutrophils infiltrating the mouse uterus. A. Immunohistochemistry (IHC); an anti-Ser216 peptide antibody (αP-S216) was used to stain sections of the mouse uterus. B. Double immunostaining with an anti-P-S216 (green) and anti-Ly6G (a neutrophil marker, in red). DAPI stained nuclei. These pictures are assembled from our previous publication (Shindo et al., 2013).

Figure 10. Thank you, all lab members past, present and constant
Figure 1

A. Cell signal regulation

- EGF → P-RACK1 → RACK1
- EGF → ERK1/2 → P-ERK1/2

B. PB activation

- PB → P-RACK1 → RACK1
- PB → ERK1/2 → P-ERK1/2

C. Ligand activation

- Ligand activation pathway involving DBD, LBD, ERK1/2, and RACK1.
Figure 2

A

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2B6</td>
<td>AGTACTTTTCTGACCGCTGAAGGTGGTGGCAAGCTTACGTTTTCTGACCCCA</td>
</tr>
<tr>
<td>Cyp2b10</td>
<td>TCTGTACCTCTGTTCCTTGGCACCTTGACCTTGACCCCA</td>
</tr>
<tr>
<td>CYP2B1</td>
<td>TCTGTACTTTTCTGACCTTGACCTTGACCCCA</td>
</tr>
<tr>
<td>CYP2B2</td>
<td>TCTGTACTTTTCTGACCTTGACCTTGACCCCA</td>
</tr>
<tr>
<td>CYP3A4 ER6</td>
<td>TGACCTCCCTTGAGTTCA</td>
</tr>
</tbody>
</table>

B

Thr38
Figure 4
Figure 6

CAR KO

CAR WT
<table>
<thead>
<tr>
<th>Code</th>
<th>Name</th>
<th>Amino Acid Sequence</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR1A1</td>
<td>(TRα)</td>
<td>EGCCKFFRRTIQKNLHPTYSCKY125</td>
<td>147</td>
</tr>
<tr>
<td>NR1A2</td>
<td>(TRβ)</td>
<td>EGCCKFFRRRTIQKNLHPSYSCKY125</td>
<td>147</td>
</tr>
<tr>
<td>NR1B1</td>
<td>(RARα)</td>
<td>EGCCKFFRRRTIQKNMIVYTHCRRD125</td>
<td>147</td>
</tr>
<tr>
<td>NR1B2</td>
<td>(RARβ)</td>
<td>EGCCKFFRRRTIQKNMIVYTHCRRD125</td>
<td>147</td>
</tr>
<tr>
<td>NR1B3</td>
<td>(RARγ)</td>
<td>EGCCKFFRRRTIQKNMIVYTHCRRD125</td>
<td>147</td>
</tr>
<tr>
<td>NR1C1</td>
<td>(PPARα)</td>
<td>EGCCKFFRRTIRKLEYECKCERS147</td>
<td>147</td>
</tr>
<tr>
<td>NR1C2</td>
<td>(PPARβ)</td>
<td>EGCCKFFRRTIRKLEYECKCERS147</td>
<td>147</td>
</tr>
<tr>
<td>NR1C3</td>
<td>(PPARγ)</td>
<td>EGCCKFFRRTIRKLEYECKCERS147</td>
<td>147</td>
</tr>
<tr>
<td>NR1D1</td>
<td>(Rev-erbα)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR1D2</td>
<td>(Rev-erbβ)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR1F1</td>
<td>(RORα)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR1F2</td>
<td>(RORβ)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR1F3</td>
<td>(RORγ)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR1H2</td>
<td>(LXRβ)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR1H3</td>
<td>(LXRα)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR1I1</td>
<td>(VDR)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR1I2</td>
<td>(PXR)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR1I3</td>
<td>(CAR)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR2A1</td>
<td>(HNF4α)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR2A2</td>
<td>(HNF4γ)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR2B1</td>
<td>(RXRα)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR2B2</td>
<td>(RXRβ)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR2B3</td>
<td>(RXRγ)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR2C1</td>
<td>(TRα)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR2C2</td>
<td>(TRβ)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR2C3</td>
<td>(TRγ)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR2F1</td>
<td>(COUP-TFI)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR2F2</td>
<td>(COUP-TFII)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR2F3</td>
<td>(EAR2)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR3A1</td>
<td>(ERα)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR3A2</td>
<td>(ERβ)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR3B1</td>
<td>(ERRα)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR3B2</td>
<td>(ERRβ)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR3B3</td>
<td>(ERRγ)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR4A1</td>
<td>(NGFIB)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR4A2</td>
<td>(NURR1)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR4A3</td>
<td>(NOR1)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR5A1</td>
<td>(SF1)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR5A2</td>
<td>(LRH1)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR5B1</td>
<td>(GCN1)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR112</td>
<td>(PXR)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR3C1</td>
<td>(GR)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR3C2</td>
<td>(MR)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR3C3</td>
<td>(PR)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
<tr>
<td>NR3C4</td>
<td>(AR)</td>
<td>EGCCKFFRRTIQKELKYNVYRKY125</td>
<td>125</td>
</tr>
</tbody>
</table>
Figure 8

Homodimer

Monomer

Surface A

Surface B

Heterodimer

DBD   DBD

Helix 11

DBD

Helix 11

Surface A

Surface B

DBD

Helix 11

LBD

LBD

DBD

Helix 11

LBD

LBD

DBD   DBD

Helix 11

RXR

LBD

LBD
Figure 9

A. IHC with $\alpha$P-S216

B. Double immunofluorescence staining

- $\alpha$P-S216
- $\alpha$Ly6G
- Merged/DAPI
Figure 10

Thank you

Aida Kaoru
Allen Devon
Allen Camila
Antonsson David
Arakawa Shingo
Burkhart Barbara
Beth Doerflein
Chang Annie
Chang Thomas
Devore Kathy
Dong Jian
Fashe Muluneh
Franco Lea
Ferguson Steve
Gotoh Saki
Green Will
Harper Deborah
Harada Nobuhiro
Hashiguchi Takuyu
Hess Holly
Hempel Nadine
Honkakoski Paavo
Hori Takeshi
Hosseinpour Fardin
Hu Hao
Ikeda Mari
Inoue Kaoru
Itakura Takao
Ichikawa Takeshi
Iwasaki Masahiko
Jonathan Jackson
Juvonen Risto
Kakizaki Satoru
Kakuta Yoshimitsu
Kamino Hiroki
Kanayama Tomohiko
Karami Sohrab
Kato Yukio
Katoh Yoshinobu
Kawajiri Kaname
Kawamoto Takeshi
Kobayashi Kaoru
Kodama Susumu
Koike Chika
Kojima Hiroyuki
Konno Yoshihiro
Lakso Merja
Lang Matti
Lee Karen
Lee Su-Jun
Leaven Lauren
Li Shuanfang
Lindberg Raija
Masaki Ryuichi
Matsui Kenji
Matsumura Yonehiro
Miyauchi Yuu
Moore Rick
Mutoh Shingo
Nakamura Kouich
Nakanishi Nobu
Nishio Koji
Noshiro Mitsuhide
Ohno Marumi
Ohyama Toru
Osabe Makoto
Park Linda
Parkish Sudip
Pascussi Jean-Marc
Pedersen Lars
Peterochenko Jenya
Powell Chiquita
Ready Joe
Salahudeen Ameen
Sakuma Tsutomu
Saitou Kosuke
Shizu Ryota
Shevtsov Serguei
Shindo Sawako
Song Wen-Chao
Squires James
Sueyoshi Tatsuya
Sobhany Mack
Sugatani Junko
Suzuki Tomohiro
Swales Karen
Tien Eric
Timsit Yoav
Ueda Akiko
Uno Tomohide
Usanov Sergey
Yamamoto Yukio
Yamazaki Yuichi
Yasujima Tomoya
Yasuoka Akihito
Yi Myeongji
Yokobori Kosuke
Yokomori Norihiro
Yoshinari Kouich
Yoshioka Hidefumi
Vinal Kellie
Wall Francis
Washburn Kimberly
Wong Garry
Wong Hongbing
Woodrum Tyler
Zelko Igor
Zuch Michael

Downloaded from dmd.aspetjournals.org on October 14, 2017.

DMD Fast Forward. Published on March 29, 2017 as DOI: 10.1124/dmd.116.074872