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BREAKTHROUGHS AND VIEWS

Phenobarbital-Elicited Activation of Nuclear Receptor CAR in Induction of Cytochrome P450 Genes

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Phenobarbital (PB) increases metabolic capability of hepatocytes by its ability to activate numerous genes encoding various xenochemical-metabolizing enzymes such as cytochrome P450s and specific transferases. More than 35 years since PB induction was first reported, the key nuclear receptor CAR that mediates the induction has now been identified, and the molecular/cellular mechanism involving multiple signal transduction pathways has begun to be unraveled. In response to PB exposure, CAR in the cytoplasm translocates into the nucleus, forms a heterodimer with the retinoid X receptor, and activates the PB response enhancer element leading to the concerted induction of numerous genes.

Key Words: **phenobarbital; nuclear receptor; cytochrome P450; CYP gene; induction; nuclear translocation; signal transduction; drug metabolism.**

SHORT HISTORY

Evolutionarily, animals and plants have been competing for existence using newly synthesized chemicals as a weapon in "warfare" against each other. To protect themselves against harmful chemicals (phytoalexins) produced by the plants, animals have developed metabolic detoxification pathways as defense mechanisms against their toxicity. These pathways also play the key role in the detoxification of man-made chemicals such as pharmaceutical drugs and environmental pollutants. Hepatic microsomal cytochrome P450s (encoded by the *CYP* gene superfamily) are the first and foremost important enzymes catalyzing the metabolic detoxification.

As a part of the defense mechanism, cells activate transcription of *CYP* genes in response to xenochemical exposure, resulting in the induction of CYP enzymes and metabolic capability. Since the induction phenomenon was first reported in the 1950's, it has been a major driving force attracting many scientists into cytochrome P450 research. There have been two classical groups of xenochemicals that induce the different sets of *CYP* genes: polycyclic aromatic hydrocarbons (PAHs) typified by 3-methylchoranthrene and 2,3,7,8-tetrachlorodibenzo *p*-dioxin that induce CYP1A and CYP1B, and PB that induces CYP2A, CYP2B, CYP2C, CYP2H, and CYP3A. PB and PB-type inducers are characterized by their structural diversity, in sharp contrast to the PAH group of inducers represented by structurally similar compounds. With respect to the organ-specific induction, while PAHs induce CYP1A1 in any exposed organs and cells, PB induction is largely limited to occur in liver, although other organs such as brain can be targets for the induction. Relative simplicity in the chemical properties of PAH-type inducers and their inducibility in any given transformed cell lines *in vitro,* helped to define the Ah receptor-mediated induction mechanism in the 1980s (1). On the other hand, the lack of a PB response *in vitro* systems has hampered discovery of the PB-inducible regulatory mechanism until the mid 1990s. Only the development of suitable primary hepatocyte cultures, transgenic mice, and other gene delivery systems have led to recent progress in defining the PB response elements and identifying the nuclear receptor that regulates the elements. For more general aspects related to the roles of nuclear receptors in the xenochemical induction of *CYP* genes, readers should refer to recent articles (2–5).

PB RESPONSE ELEMENTS

PB exerts a wide spectrum of effects on liver cell homeostasis and metabolism. The highly pleiotropic response to phenobarbital includes proliferation of the

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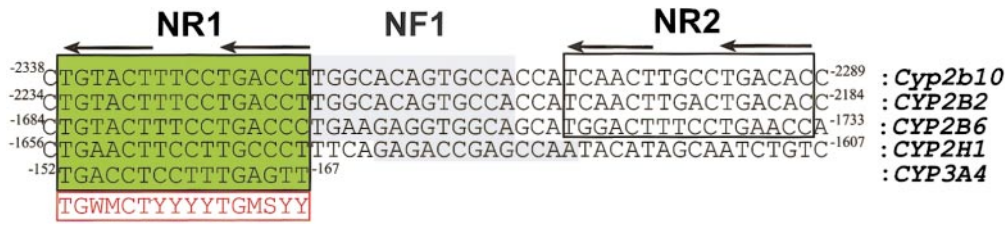

FIG. 1. Phenobarbital-responsive enhancer module (PBREM).

endoplasmic reticulum (6), alteration of cell-cycle checkpoint controls (7), inhibition of apoptosis (8) as well as induction or repression of more than 50 different enzymes including various *CYP* genes (9, 10). Chronic PB administration promotes liver tumor development in rodents.

PB most effectively induces the *CYP2B* genes, therefore PB response elements have first been defined in these genes (Fig. 1). PB response activity was associated with a 163-bp distal DNA sequence of the *CYP2B2* gene using rat primary hepatocytes (11) and was independently confirmed using an *in situ* injection of the

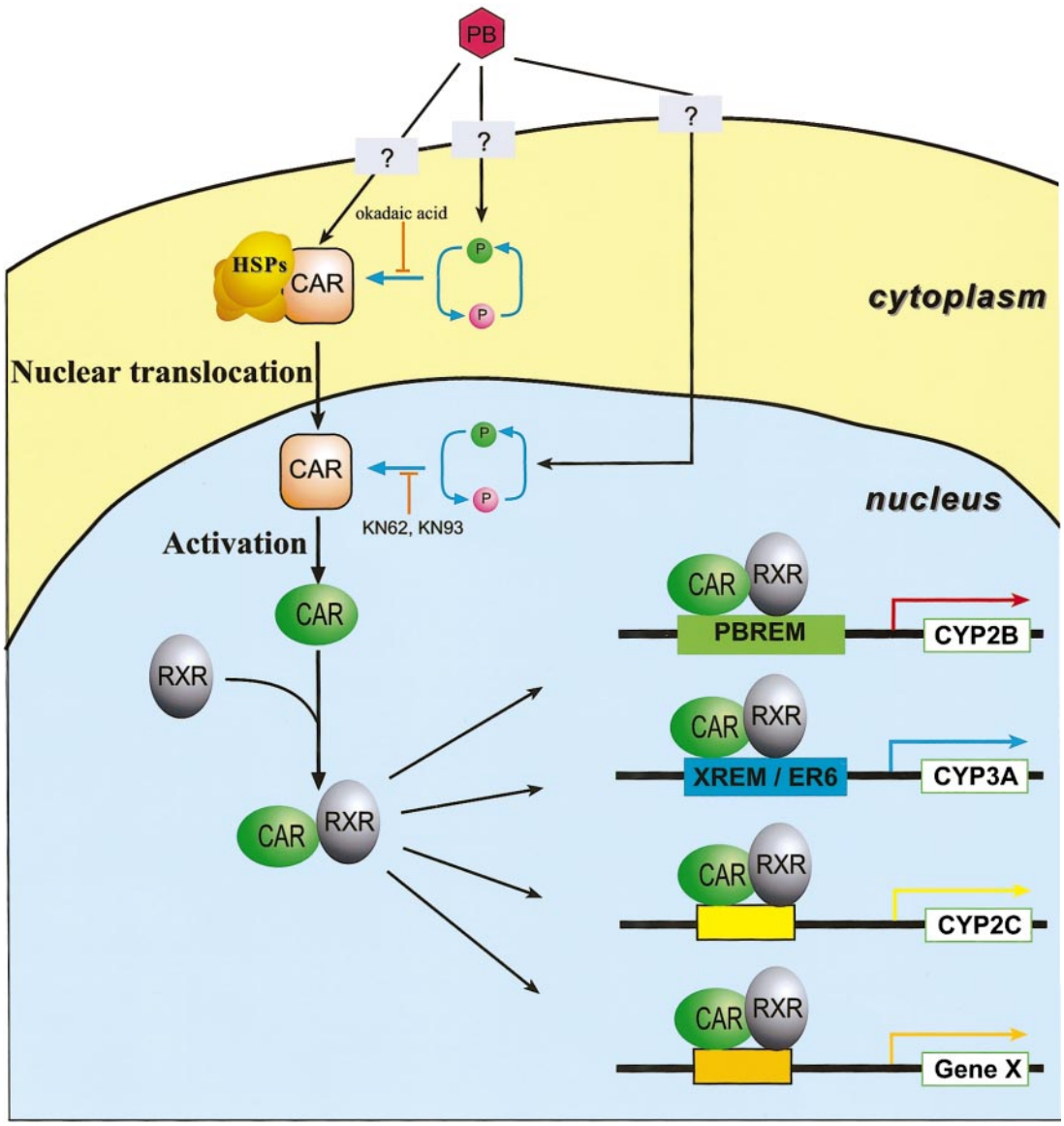

FIG. 2. Proposed mechanism of PB induction.

reporter gene constructs into rat livers (12). Using the mouse PB-inducible *Cyp2b10* gene, the PB response element was delineated to a 51-bp minimum sequence now called the phenobarbital-responsive enhancer module or PBREM (13). Finally studies with transgenic mice bearing a PBREM-containing 5′-flanking sequence of the *CYP2B2* gene showed that PBREM exclusively is the enhancer element that is required for PB induction (14). The PBREM sequences are conserved in the mouse, rat, and human CYP2B genes (15).

The PBREM is characterized as a composite element consisting of two nuclear receptor-binding sites (NR1 and NR2) and the NF1 binding site. Both NR1 and NR2 are DR-4 motifs, a direct repeat of imperfect half sites separated by 4 bases. Only the NR sites are essential for the PB response, although the NF1 site may be required to confer full PBREM activity. Within the PBREM sequences NR1 is most conserved in the *CYP2B* genes and similar DR-4 motifs have been found in other *CYP3A* (15) and *CYP2H* (16) genes that are also PB-inducible. In addition to PB, PBREM is capable of responding to various PB-type inducers while neither 3-methylcholanthrene (CYP1A1 inducer), dexamethasone (CYP3A inducer), nor clofibrate (CYP4A inducer) activated PBREM (17). Thus, PBREM has emerged as a versatile response element that is capable of responding specifically to PB and various other PB-type inducers.

NUCLEAR RECEPTOR CAR

Nuclear receptor binding motifs are usually represented by two half-sites AGGTCA oriented as direct (DR), inverted (IR) or everted (ER) repeats divided by a 1 to 7 bp space, and the receptors select the binding sequences based on the characteristics of these motifs. Given the fact that both NR1 and NR2 are DR-4 motifs and that PB induction is a liver-specific phenomenon, various liver-specific nuclear receptors were screened for their ability to activate the PBREM-tk reporter gene in cotransfected HepG2 and HEK 293 cells. The screening resulted in identifying the nuclear orphan receptor CAR (*c*onstitutive *a*ctive *r*eceptor) as a candidate receptor that activates the PBREM (18). This receptor was originally characterized as a constitutive activator of an empirical set of retinoic acid response elements, meaning that CAR can activate the elements in the absence of retinoic acid (19).

We simultaneously concentrated our efforts to characterize biochemically a nuclear protein binding to NR1 site, since the NR1 is the most conserved region within the known PBREMs, and is alone capable of responding to PB. Affinity purification using NR1 oligomers coenriched CAR with retinoid X receptor (RXR) in fractions derived from the PB-induced nuclear extracts, but not from the control nuclear extracts (18). Both immunochemical and protein microsequencing analyses proved that the enriched protein was CAR. In fact, the *in vitro* translated CAR and RXR heterodimer bound to NR1 as well as NR2 in gel-shift mobility assays. Western blot analysis of the nuclear extracts revealed a rapid time-dependent increase of CAR in the nucleus that preceeds the induction of Cyp2b10 mRNA in the PB-treated mouse livers. Thus, these results have indicated the following processes of PB induction: in response to PB exposure, CAR accumulates in the nucleus, forms the heterodimer with RXR, binds to both NR1 and NR2 sites of PBREM, and activates the transcription of *CYP2B* genes (Fig. 2).

NUCLEAR TRANSLOCATION OF CAR

As CAR was named after its characteristic of being activated without the binding of agonistic ligand, this receptor activates NR1 in the absence of PB or PB-type inducers in HepG2 cells. The expression of fluorescent protein-tagged CAR revealed that the receptor spontaneously accumulated in the nucleus of the transfected HepG2 cells. In liver *in vivo*, however, this constitutive activity of CAR must be repressed in order to acquire the PB responsiveness. One of the regulatory mechanisms to control CAR activity is to alter its intracellular compartmentalization after PB treatment. Our earlier finding that CAR accumulated in the liver nucleus only after PB treatment, suggested that CAR is sequestered in the cytoplasm of control livers (18). In fact, immunohistochemical analysis on the control liver sections showed that only few nuclei near vessels were stained and nearly all other nuclei were devoid of staining compared with the cytoplasmic regions, whereas practically all nuclei were heavily stained on the liver sections of PB-induced mice (20). The receptor CAR, thus, is retained in the cytoplasm of the control mouse livers and the cytoplasmic retention prevents the receptor from activating PBREM and the *Cyp2b10* gene.

The nuclear translocation elicited by PB of CAR appears to be an initial step of the induction process. In WKY rats, PB induces the *CYP2B* genes in the males while the females respond only poorly to the PB induction (21). While there are no significant sex differences in the cytoplasmic levels of CAR, the CAR levels in the nucleus are extremely low in the PB-induced females compared with the corresponding males (22), reiterating that the nuclear translocation may be the essential step in CAR-mediated induction. Finding the regulatory mechanisms of the cytoplasmic retention and nuclear accumulation remains a main focus of our current investigation. Glucocorticoid, progesterone, and vitamin D3 receptors (GR, PR, and VDR) also translocate into the nucleus following the binding to angonists (23–25). These receptors are sequestered in the cytoplasm as the multiprotein complexes with such as

TABLE 1

Nuclear Receptors Involved in CYP Gene Regulation

Nuclear receptors	Chemicals	Regulated CYP genes
CAR	PB, TCPOBOP, chlorpromazine, 16-androstenes, progesterone, androgens, estrogens	CYP2B, 3A, 2C, 2H
PXR/SXR/PAR	Rifampicine, dexamethasone, pregnenolone 16α-carbonitrile	CYP3A
FXR	Bile acids, farnesol metabolites, forskolin	CYP7A
LXR	Derivatives of cholesterol, mevalonic acid	CYP7A
VDR	Vitamin D, calcitriol	CYP2D, CYP24, CYP27A1, CYP27B1
PPAR	Fatty acids, fibrate drugs, phthalate esters	CYP4A
SF-1/Ad4BP	Oxysterols	Steroidogenic CYPs

heat-shock proteins (hsp), immunophilins, protein phosphatase, and possibly additional proteins (26). Learning from this well-established model, we used mouse primary hepatocytes and found that the PB-elicited nuclear accumulation was repressed by treatment with okadaic acid (OA) at the concentration specifically inhibiting protein phosphatases PP1A, PP2 and PP5 (20). Since OA did not inhibit the CAR-mediated activation of the NR1-tk-luciferase gene in the co-transfected HepG2 cells, the repressive effect of OA appeared to occur specifically at the nuclear translocation step. Upon PB exposure, the liver cells may initiate a signal transduction pathway in which dephosphorylation is a critical step for eliciting the nuclear translocation of CAR. The site of the PB-induced dephosphorylation is not known at the present time. However, since the protein phosphatases such as PP5 are copurified with the steroid receptor-hsp-immunophilin complexes (27), the cytoplasmic complex of CAR may be the site to look for the dephosphorylation occurring upon PB treatment. Nevertheless, the previous observation that OA inhibited the PB induction of *CYP2B* genes in the rat and mouse primary hepatocytes (28, 29) can now be understood as the consequence of the OA-dependent repression of the receptor nuclear translocation of CAR (Fig. 2).

NUCLEAR RECEPTOR CROSS-TALK

Various nuclear receptors are involved in the regulations of *CYP* genes (Table 1). Because of yet unknown structural reasons, the nuclear receptors may share an identical binding site or different sites of the same enhancer elements, resulting in either activation or repression of these elements. Two different PB-induced up-stream enhancers PBREM (in *CYP2B* genes) and XREM (in *CYP3A4* gene) have been characterized. PXR activates XREM in the co-transfected HepG2 cells in response to PB (30, 31), while CAR is also capable of activating XREM and the proximal ER-6 element of *CYP3A* genes (15, 32). The question arose as to whether there are redundant mechanisms in which both CAR and PXR regulate PB induction of the *CYP3A* or the *CYP2B* genes. Our recent studies using obese Zucker rats have suggested that this may not be the case (Yoshinari and Negishi, in preparation). PB poorly induced both *CYP2B* and *CYP3A* genes in accordance with the extremely low levels of CAR in the obese rats compared with the lean Zucker rats in which PB effectively induces these *CYP* genes. The levels of PXR, however, were indistinguishable between the obese and lean Zucker rats and the PXR-specific activator pregnenolone 16α-carbonitrile (PCN) induced the *CYP3A* gene in the lean as well as obese Zucker rats. Moreover, PXR was unable to activate PBREM in presence or absence of PB in cotransfected HepG2 cells (unpublished observation). A recent report from Moore's group has confirmed that all PB induction was basically abrogated in the CAR-null mice (33). Thus, CAR appears to be the receptor that mediates all PB inductions, at least, in rodents.

Xenochemical response elements are often composite elements that contain multiple binding sites for transcription factors. PBREM, for example, also shares homology with consensus binding sequences for estrogen receptor-related receptor (ERR), COUP-TF, TR2, PXR, and probably other nuclear receptors. In fact, the NR1-affinity purification co-enriched ERR and TR2 receptors (but not PXR) in addition to CAR-RXR from the PB-treated mouse liver nuclear extracts (34). Interestingly, the recovery of ERR in the purified fractions was decreased after PB treatment and transient transfection assay showed ERR repressed the CAR-RXR mediated activation of PBREM in the HepG2 cells. This is consistent with the hypothesis that ERR may be a repressor of *CYP2B* genes: the repressor ERR is released from PBREM and is replaced by the activator CAR upon PB induction. More general roles of the heterodimerization partner RXR in the regulation of basal and induced expression of the *CYP* genes have been recently suggested using the RXR•-null mice (35).

STEROID HORMONES AS CAR MODULATORS

Endocrine factors such as steroid hormones, growth hormones, and growth factors may not regulate PB

response elements such as PBREM and XREM, but modulate PB induction *in vivo*. In this respect, it is important to understand the regulation of CAR by endogenous factors, if in fact, they directly modulate the CAR function. Previously, 16-androtenes were found to act as negative ligands repressing the constitutive activity of mouse CAR in the transfected cells (36). The repressed CAR, then, could be re-activated by PB or other PB-type inducers (15). Our studies and others, however, suggested that 16-androstenes may not be true endogenous modulators since treatment with high doses of androstenol did not affect CAR activity in the mouse primary hepatocytes as well as in rats. Recently, we screened various steroids using the CAR-mediated activation of NR1-tk-luciferase reporter gene in the transfected HepG2 cells (37). Progesterone and androgens turned out to CAR repressors at μM orders of magnitude. Active estrogens (estradiol and estrone at μM concentrations) are effective activators of the repressed CAR. In mice, the endogenous levels of estrogen appeared not to regulate CAR in the female mice, but treatment with estradiol at pharmacological doses caused accumulation of CAR in the nucleus. Endogenous levels of androgens prevented the nuclear accumulation of CAR in the male mice. The regulation of CAR by androgens, estrogens, and progesterone brings new insight into the roles of the receptor in the endocrine modulation of PB induction in livers *in vivo*.

FUTURE PROSPECTIVES

1. Mechanism of nuclear translocation: Except for evidence that OA represses the nuclear translocation of CAR, the cellular and molecular mechanism of the translocation has not been understood. Our recent studies have indicated that the AF2 (ligand-dependent activation function domain) of the CAR molecule is not required for the PB-induced nuclear translocation. Instead, a Leu-X-X-Leu-X-X-Leu motif on the helix 10 appears to be essential for the translocation (Zelko *et al.*, submitted). This is in sharp contrast to the nuclear translocation of steroid receptors: for instance, mutation of the AF2 domain abrogated the agonist-dependent nuclear translocation of VDR (38). Through efforts to define the roles of the motif, we may be able to better understand the nuclear translocation of CAR in response to PB exposure.

2. Nuclear activation of CAR: Is CAR always active once the receptor is localized in the nucleus? The answer to this question may be NO. We often observed that the presence of nuclear CAR is not accounted for by an equivalent increase of CYP2B mRNA in livers. For example, the nuclear levels of CAR in control lean Zucker (*fal*+) rats were higher than that in PB-treated obese Zucker (*falfa*) rats. However, no CYP2B mRNA was detectable in the control lean rats, while the mRNA was detectable in the PB-induced obese rats (Yoshinari and Negishi, in preparation). Treatment with KN-93 and KN-62, both calcium/calmodulin-dependent kinase (CaMK) inhibitors inhibited the CAR-mediated transactivation of the NR1-tk-luciferase reporter gene in the PB-treated primary hepatocytes, while they did not repress the nuclear translocation of CAR in the hepatocytes. Moreover, treatment with KN-93 alone accumulated CAR in nucleus of the mouse primary hepatocytes, without inducing CYP2B10 mRNA (39). The nuclear activation of CAR is a distinct step in the PB induction process that may be regulated by CaMK (Fig. 2).

3. Roles of human CAR: Whereas human CAR shares many common characteristics with its rodent counterparts, it also displays its own specificity. Similar to rodent receptors, human CAR translocates into nucleus in response to PB and binds to PBREM. Unlike the rodent receptors, human CAR is not inhibited by the known repressors: androstenol, progesterone, androgens, and CaMK inhibitors. Because of this, PB responsiveness of human CAR has not been demonstrated convincingly in the transfected cells *in vitro*. Finding the mechanism by which human CAR can be regulated in response to PB is an urgent issue in order to determine its role in PB induction.

4. PB binding site: Although the nuclear receptor CAR is now known to regulate PB induction, the key question has not yet been answered. Does PB bind directly to the CAR? Even if, in fact, the direct binding occurs, is only binding necessary for the CAR-mediated induction by PB? Fluorescence resonance energy transfer and coactivator binding assays have failed to demonstrate the direct PB binding to mouse and human CARs (31, 32). There is ample evidence that PB might act on other proteins than CAR in order to elicit the multiple signal transduction pathways that regulate CAR. If CAR turns out to be not the direct target of PB, various membrane receptors such as G-protein-coupling receptors may be alternate places to look for PB binding sites.

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