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PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL REGULATION OF CYTOCHROME P450

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ABSTRACT:

This article is a report on a symposium sponsored by the American Society for Pharmacology and Experimental Therapeutics and held at the April 1998 Experimental Biology '98 meeting in San Francisco. The presentations focused on the mechanisms of regulation of cytochrome P450 gene expression by developmental factors and by hormones and cytokines, as well as on the interplay be-

tween physiological and chemical regulation. Approaches and systems used to address these questions included conditional gene knockouts in mice, primary hepatocyte cultures, immunofluorescence imaging of cells, and cell lines stably expressing reporter gene constructs.

The expression of cytochrome P450¹ genes in many species is highly regulated during development, by nutritional status, and by hormonal factors, including sex steroids, glucocorticoids, growth hormone, insulin, and inflammatory cytokines. Although the mechanisms and consequences of regulation of P450s by drugs and chemicals have been intensively studied, relatively little is known about the mechanisms by which P450s are regulated by physiological factors. This regulation has documented or potential consequences for pharmacokinetics of drugs in people and animals in different developmental stages, nutritional states, stress and disease states, different stages of the menstrual cycle, and between males and females.

This article summarizes four presentations (F.J.G., R.H.T., C.J.O., E.T.M.) at a symposium sponsored by the American Society for Pharmacology and Experimental Therapeutics at the April 1998 Experimental Biology '98 meeting in San Francisco, chaired by one of the authors (E.T.M.). These articles highlight novel approaches and recent findings in the area of physiological regulation of P450 and in

the interaction of physiological signaling pathways with those utilized by chemical agents.

Mechanisms of P450 Regulation by Inflammation, Cytokines, and Growth Factors (E.T.M., M.B.S., H.I.)

A large number of infectious or inflammatory agents have been shown to depress P450-catalyzed drug metabolism in humans and in experimental animals (Morgan, 1997). Many of these effects are accompanied by suppression of various P450 mRNAs, and this regulation can be mimicked by the action of inflammatory cytokines or interferons (from circulating monocyte/macrophages or from Kupffer cells) on the hepatocyte (Morgan, 1997). In a complex *in vivo* inflammatory response, many other factors may potentially affect P450 expression: for instance, actions of cytokines in the central nervous system affect the hypothalamo-pituitary axis (Bernton *et al.*, 1987), resulting in altered secretion of glucocorticoids, thyroid hormones, and growth hormone, each of which regulates P450 expression (Morgan, 1997). Bacterial endotoxin (LPS) can also act directly on hepatocytes to downregulate P450 expression (Sewer and Morgan, 1997).

Despite the fact that many P450s are suppressed during inflammation or infection, it can be discerned from the literature that some are unaffected and some are induced (Sewer *et al.*, 1997). Thus how an individual P450 is affected depends on the particular stimulus and the tissue being studied (Morgan, 1997). From this, we infer that specific mechanisms must exist for differential regulation of P450s in inflammation and infection. For the purposes of this presentation, we will focus on recent studies in our laboratory on the involvement of two well-characterized intracellular messengers in regulation of P450 expression.

CYP2C11 is the major P450 enzyme expressed in the livers of untreated male rats, and for this reason its regulation has been studied extensively in our laboratory and others. CYP2C11 is downregulated at the mRNA level by a variety of physiological and pathophysiological stimuli, including continuous growth hormone exposure (Morgan *et al.*, 1985), inflammation (Sewer *et al.*, 1997), stress (Merrill *et al.*,

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¹ Abbreviations used are: AdCre, recombinant adenovirus containing the Cre gene; Ah, aryl hydrocarbon; ARNT, Ah receptor nuclear translocator; C/EBP, CAAT enhancer-binding protein; Cre, Cre recombinase; EMSA, electrophoretic mobility shift assay; HNF, hepatocyte nuclear factor; IL, interleukin; LPS, bacterial lipopolysaccharide; NF, nuclear factor; NF-1, nuclear factor 1; P450, cytochrome P450; NOS2, inducible nitric oxide synthase; PB, phenobarbital; PBRU, phenobarbital responsive unit; PKA, cAMP-activated protein kinase; PKC, protein kinase C; PP, protein phosphatase; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TPA, tetradecanoyl phorbol acetate; UDP, uridine diphosphate; UGT, UDP-glucuronosyltransferase.

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1992), fasting (Ma *et al.*, 1989), and diabetes (Favreau and Schenkman, 1988). The effect of diabetes on CYP2C11 is partially due to the decrease in growth-hormone secretion in diabetic rats but also has a growth-hormone-independent component (Donahue *et al.*, 1991). Inflammation, stress, fasting, and diabetes have in common an increase in the circulating glucagon/insulin ratio. Moreover, insulin is required for optimal expression of CYP2C11 in primary hepatocyte cultures, while the effects of glucagon have not been reported. Since glucagon and insulin, in part, exert many of their effects on cells through the modulation of cyclic AMP, we decided to investigate whether 2C11 expression could be regulated by this second messenger.

Incubation of rat hepatocytes cultured on matrigel with the cAMP derivatives dibutyryl cAMP, (S_p)-5,6-dichloro-1- β -D-ribofuranosyl-benzimidazole-3',5'-monophosphorothioate (S_p -cAMPS), and 8-(4-chlorophenylthio)-cAMP each caused a concentration-dependent downregulation of CYP2C11 mRNA. This effect was also seen with glucagon (which stimulates adenylate cyclase through its Gs-coupled membrane receptor) and forskolin (which stimulates adenylate cyclase directly). Together, these results indicate that agents which activate cAMP-dependent protein kinase (PKA) activity in cells can downregulate CYP2C11 expression. R_p -cAMPS is an inhibitor of cAMP activation of PKA by competing for binding to the regulatory subunit of PKA. R_p -cAMPS caused a parallel shift to the right of the concentration-response curve for CYP2C11 suppression by its diastereomer S_p -cAMPS, indicating the specificity of both agents. Furthermore, R_p -cAMPS completely blocked downregulation of CYP2C11 by glucagon and partially inhibited downregulation of CYP2C11 by interleukin (IL)-1. We also found that both the suppression of 2C11 expression and the elevation of cellular cAMP levels by glucagon were sensitive to the glucagon/insulin ratio, such that insulin attenuated the effects of glucagon on both parameters.

The fact that glucagon downregulates CYP2C11 expression via the adenylate cyclase/cAMP/PKA pathway and that this effect is modulated by insulin suggest that the increased glucagon/insulin ratio may contribute to the downregulation of this P450 during inflammation, stress, fasting, and diabetes. The observation that downregulation of CYP2C11 by IL-1 can be partially inhibited by R_p -cAMPS indicates that this cytokine utilizes more than one pathway to downregulate CYP2C11. In addition to cAMP, we have suggested that a sphingolipid messenger may also be involved in regulation of this P450 (Nikolova-Karakashian *et al.*, 1997).

Nitric oxide is synthesized in large amounts in monocytes, macrophages, Kupffer cells, and hepatocytes during endotoxemia, because of the induction of inducible nitric oxide synthase (NOS2) by cytokines and LPS. NO has been implicated as a mediator of decreased cytochrome P450 catalytic activities and also in the suppression of P450 gene expression that occurs during endotoxemia, because inhibitors of NOS enzymes can block or attenuate the decreases seen in the mRNA or protein levels, or catalytic activities, of some P450 enzymes after an inflammatory stimulus (Carlson and Billings, 1996; Khatenko and Kikkawa, 1997). We have tested the hypothesis that NO is required for inflammatory downregulation of P450 mRNAs and proteins, using *in vivo* and *in vitro* models to study the effects of NOS inhibitors on P450 suppression caused by LPS or cytokines. We have also studied the suppression of P450s in mice lacking a functional NOS2 gene. These experiments are described below. In contrast to those of other groups, our studies have failed to find a significant role for NO in these phenomena.

In rat hepatocytes cultured on Matrigel, CYP2C11 mRNA and protein expression is suppressed by LPS, IL-1, or by a cocktail of cytokines including LPS, IL-1, tumor necrosis factor- α , and interferon- γ (Sewer and Morgan, 1997). This is accompanied by a large

stimulation of NO production by the cells. The competitive NOS inhibitor *N*-monomethyl-L-arginine completely blocked the induced production of NO but failed to affect the downregulation of CYP2C11 mRNA or protein by any of these stimuli at any time point measured (Sewer and Morgan, 1997). We concluded that NO is not required for CYP2C11 downregulation by IL-1 or LPS in cultured hepatocytes.

To study the role of NO in P450 downregulation in rats *in vivo*, we treated rats with LPS and aminoguanidine, a somewhat selective inhibitor of NOS2. Injections of aminoguanidine every 4 hr completely prevented the rise in plasma NO oxidation products by LPS. Despite this fact, aminoguanidine injections every 4 hr had no effect on the downregulation of CYP2C11, CYP3A2, or CYP2E1 mRNAs or proteins by LPS treatment (Sewer and Morgan, 1998). An interesting aspect of this work was that we observed a decrease in CYP2C11 catalytic activity only 6 hr after LPS treatment when CYP2C11 protein levels were the same as in control livers. One could speculate that this effect is due to inhibition of CYP2C11 catalytic activity by NO, but the aminoguanidine+LPS-treated animals were not informative because aminoguanidine alone caused a decrease in CYP2C11 activity at this time point (Sewer and Morgan, 1998).

Two possible explanations for the lack of agreement between our studies and those of others using NOS inhibitors are (a) that NOS inhibitors have nonspecific effects, or (b) that they may not completely inhibit NO synthesis even when there is no detectable (nitrate + nitrite) in the plasma or culture media. To eliminate both of these concerns, we examined the downregulation of P450 expression and activity in NOS2 knockout mice. These mice are B6,129 hybrids whose NOS2 gene has been disrupted in the calmodulin binding domain, resulting in a frameshift and no detectable NOS2 protein (Laubach *et al.*, 1995). The disruption causes no overt phenotype, but stimulated macrophages from the homozygous knockout ($-/-$) animals fail to produce NO, and there is no elevation of NO in the plasma in response to LPS injection.

When the parental B6,129 mice ($+/+$) were injected with LPS, there was a 50% (approximately) decrease in microsomal levels of CYP3A, CYP2C, and CYP2E proteins measured 24 hr after injection by Western blotting with antibodies to rat CYP3A2, CYP2C11, and CYP2E1 (fig. 1; Sewer *et al.*, 1998). The mRNAs for CYP3A11 and CYP2C29 were also suppressed by about the same magnitude (Sewer *et al.*, 1998). NOS2 $-/-$ mice showed the same changes in these parameters as did the $+/+$ animals, indicating that NO is not required for downregulation of these P450s during endotoxemia (Sewer *et al.*, 1998).

To determine whether NO could be involved in the inhibition of P450 catalytic activity early in the response to LPS (when P450 protein levels are unaffected), we compared the testosterone hydroxylase activities of microsomes from $+/+$ and $-/-$ mice 6 hr after injection of saline or LPS. At this time point, LPS caused decreases in the 6 β - and 16 β -testosterone hydroxylase activities of the microsomes, whereas the 15 α - and 16 α -hydroxylase activities were unaffected. In contrast, testosterone 7 α -hydroxylase activity was actually increased slightly. In the NOS2 knockout mice, there was no significant decrease in testosterone 6 β - or 16 β -testosterone hydroxylase activities, suggesting that NO could be partially responsible for the LPS-evoked declines in these activities at 6 hr (Sewer *et al.*, 1998). However, this is not a firm conclusion because the lack of a significant effect on 6 β - and 16 β -hydroxylase activities in the $-/-$ mice could have been due to the observed decrease in basal activity or the large variability in the control group activities (Sewer *et al.*, 1998), respectively. Of course, the measurement of P450 activities in isolated microsomes would not be expected to detect a reversible inhibition of

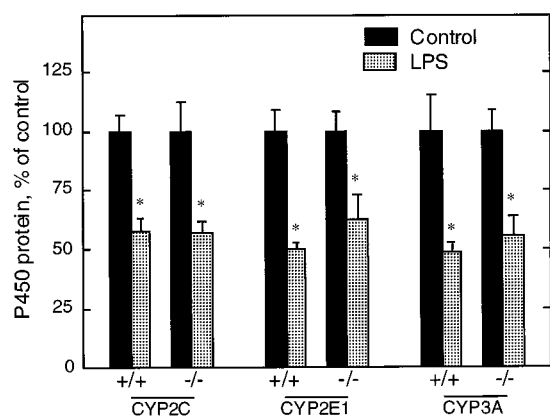


FIG. 1. Downregulation of P450 proteins in NOS2 knockout mice.

Male B6.129 (+/+) or NOS2 knockout (B6.129-NOS2, -/-) mice were injected with saline or 1 mg *Escherichia coli* LPS per kg body weight, and hepatic microsomal protein was isolated 24 hr later. Equal amounts of protein were subjected to Western blot analysis using antibodies to rat P450 2C11, 2E1, and 3A2, respectively. The graph shows results from densitometric analysis of the blots, with five mice per treatment group (means \pm SE). Results are expressed as a percentage of the saline-treated group mean. *, Significantly different from saline-treated mice, $p < 0.05$. The data are adapted from Sewer *et al.* (1998).

P450 activity caused by heme coordination of the NO (Wink *et al.*, 1993).

In conclusion, our results indicate that NO is not required for the inflammatory downregulation of the mRNA or protein levels of CYP2C11, CYP3A2, or CYP2E1 in the rat or CYP2C9, CYP3A11, or CYP2E1 in the mouse. The possibility remains that NO liberated during inflammation could inhibit P450 catalytic activity *in vivo* independently of an effect on gene regulation.

Conditional Gene Disruption for the Study of Transcription Factors and Regulation of Genes Encoding Xenobiotic-Metabolizing Enzymes (F.J.G., Y.-H.L.)

Most genes encoding xenobiotic-metabolizing enzymes are constitutively expressed in the liver and under control of families of liver-enriched transcription factors, including HNF-1, HNF-3, HNF-4, rat albumin gene D region binding protein, and C/EBP (Cereghini, 1996). Some of these genes are also controlled by ligand-activated receptors, including the aryl hydrocarbon (Ah) receptor and peroxisome proliferator-activated receptor α . Historically, regulation of gene expression has been studied using *in vitro* DNA binding and transfection studies with reporter gene constructs and recombinant transcription factors. These experiments have been used to determine the presence of *cis*-acting regulatory elements and *trans*-acting factors associated with specific genes. In many cases, several *cis*-acting elements and *trans*-acting factors were found to be involved with regulation of certain genes specifically expressed in the liver. For example, the α -fetoprotein gene is expressed in the liver, and its transcription is tightly regulated during the course of development and hepatocarcinogenesis (Bois-Joyeux *et al.*, 1995). Studies have shown that the α -fetoprotein gene has binding sites for the family of C/EBP factors, HNF-1 α and NF-1. The albumin gene also binds to multiple transcription factors, including the C/EBPs, rat albumin gene D region binding protein, NF1, NFY, and HNF-1 (Tronche *et al.*, 1990), and all of these factors can modulate transcriptional activity as assessed by *in vitro* transcription and transfection studies. However, it is not clear whether all of these transcription factors are actually functional in the intact animal or whether a single factor predominates in the adult. This question can be addressed using targeted gene disruption, in which expression of a specific transcription factor can be extinguished.

The standard gene-targeting strategy has been used to disrupt genes encoding transcription factors; however, in many cases the mutations were found to result in embryonic or early postnatal lethality. For example, embryonic lethality was found after disruption of the genes encoding ARNT (Maltepe *et al.*, 1997) and hypoxia-inducible factor 1 α (Iyer *et al.*, 1998), two members of the per and sim/beta helix-loop-helix receptor gene superfamily that includes the Ah receptor (Rowlands and Gustafsson, 1997). Knockout of genes encoding hepatocyte-enriched transcription factors, including HNF-4 (Duncan *et al.*, 1997), HNF-1 (Pontoglio *et al.*, 1996), and C/EBP α (Wang *et al.*, 1995) results in embryonic arrest or early postnatal lethality, indicating that in addition to their regulatory roles in tissue-specific expression of hepatic genes, these factors are critical for embryogenesis and development. Accordingly, it is impossible to study the *in vivo* regulation of gene expression and physiological functions of many transcription factors in adult mice since lethality occurs before the onset of expression of genes encoding xenobiotic-metabolizing enzymes in the mature animal.

To avoid disrupting genes at the germ cell level, using the traditional gene knockout technique, the Cre/loxP conditional gene targeting system (Schwenk *et al.*, 1998; Torres and Kuhn, 1997) was utilized to disrupt genes encoding several liver-enriched transcription factors in a tissue- and age-dependent manner to study their effects on expression of xenobiotic-metabolizing enzymes in the liver. Cre is a bacteriophage DNA recombinase that can delete or invert DNA segments flanked by two loxP sites containing the recognition sequence for the Cre recombinase. LoxP sites were inserted into several liver-enriched transcription factor genes by using the standard gene-targeting technique in embryonic stem cells. These small pieces of DNA are not expected to disrupt gene expression. Cre can be delivered to the liver either by use of recombinant adenovirus (AdCre) or by transgenic mouse lines in which the Cre transgene is driven by an inducible gene promoter that can be activated in the liver at any stage of development (fig. 1). The Cre/loxP conditional gene targeting system was used to study the physiological functions of C/EBP α (Lee *et al.*, 1997) and HNF-1 α (Lee *et al.*, 1998) and their roles in regulating liver-specific expression of genes encoding xenobiotic-metabolizing enzymes.

The C/EBP α gene was disrupted by use of the Cre/loxP system (Lee *et al.*, 1997). The whole C/EBP α gene was flanked by loxP sites and used to generate a homozygous C/EBP α -floxed mouse line. The "floxed" mice were injected through the tail vein with AdCre. This route of injection primarily results in infection of hepatocytes. Several days after injection of the virus, the liver was analyzed by Southern blotting, and the C/EBP α gene was found to be deleted in >90% of the liver DNA. No significant deletion was detected in the lung or kidney, indicating that the virus specifically targeted the liver. Expression of C/EBP α mRNA was also found to be reduced by >90%, while levels of mRNA encoding albumin and β -actin were not different between the C/EBP α conditional-null mice and wild-type mice (fig. 2). However, expression of a form of UDP-glucuronosyltransferase (UGT) designated UGT2B1 mRNA was markedly reduced. In contrast, expression of this gene was not different between the C/EBP β -null mouse and wild-type mice. This result was quite surprising since C/EBP α and C/EBP β bind to the same recognition sequence and activate many of the same promoters in *trans*-activation transfection assays.

The C/EBP α conditional-null mice were also found to have high levels of serum bilirubin, indicating a defect in bilirubin conjugation. Typically, bilirubin is conjugated, and the conjugate is eliminated through the bile. In genetic deficiencies in bilirubin conjugation, UGT1*6, the UGT form responsible for bilirubin conjugation, is

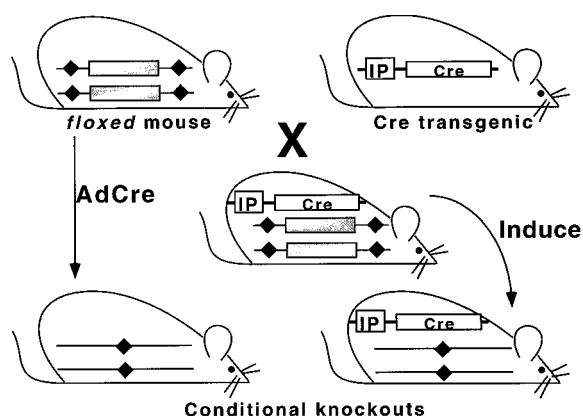


Fig. 2. Schematic diagram of the Cre/loxP conditional gene knockout system.

LoxP sites (◆) are incorporated into the cloned gene. In the diagram, the loxP sites are flanking the gene. Homozygous floxed mice are produced by targeting the modified allele by using the standard method of targeting embryonic stem cells followed by production of mice. Since the small loxP sites are placed in non-essential regions of the gene, gene expression is normal. The Cre gene can be introduced either through a viral vector or by use of a transgenic mouse in which the Cre gene is driven by an inducible promoter (IP). When an inducer is added, it results in expression of Cre and recombination to delete the target gene. The Cre can also be delivered using AdCre.

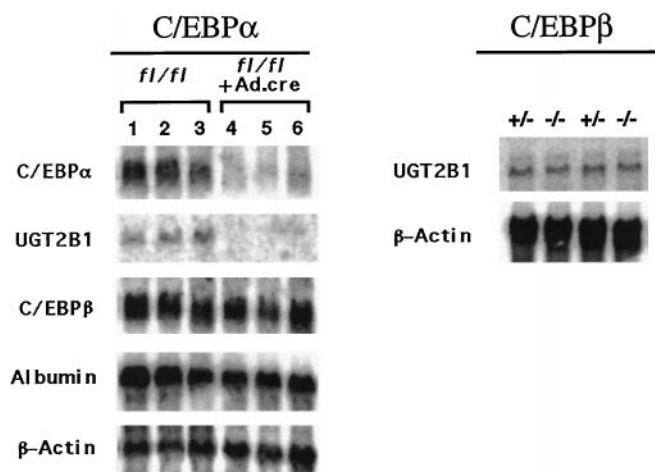


Fig. 3. Northern blot analysis of various mRNAs in the livers of adult *C/EBPα* conditional knockout and *C/EBPβ* knockout mice.

The cDNA probes used are listed. *fl* represents the floxed *C/EBPα* allele.

reduced or eliminated, resulting in high levels of serum bilirubin and the typical yellow-skin jaundiced phenotype (Mackenzie *et al.*, 1997; Owens and Ritter, 1995). The *UGT1*6* mRNA was found to be markedly reduced in the *C/EBPα*-floxed mouse line injected with AdCre (fig. 3), indicating that *C/EBPα* is required for constitutive expression of the *UGT1*6* gene. It is noteworthy that these are the first studies establishing the role of specific transcription factors in control of members of both the *UGT1* and *UGT2* gene families. These studies established that *C/EBPα* is responsible for liver-specific expression of xenobiotic-metabolizing and bilirubin-conjugating genes.

The *HNF-1α* gene was also subjected to gene knockout studies (Lee *et al.*, 1998). When an *HNF-1α* gene was made by use of the Cre/loxP system, in which the gene was disrupted in the germ cell stage by using the adenovirus E11a-Cre transgenic mouse instead of in the adult mouse (Lakso *et al.* 1996), the mice obtained were viable and lived to maturity. This is in contrast to the *HNF-1α*-null mice developed earlier using the standard gene-targeting strategy, which results in early postnatal lethality (Pontoglio *et al.*, 1996). The mice

developed using the Cre/loxP method had no signs of kidney disease and did not die early after birth (Lee *et al.*, 1998). These mice were runty, diabetic, and sterile due in part to low levels of growth hormone and high levels of insulin-like growth factor II and a deficiency in pancreatic insulin production. These phenotypes were not detected in the earlier *HNF-1α*-null mice because of premature death due in part to a kidney disorder. It remains unclear why the phenotypes of the two embryonic knockout mice differ, but it was suggested that the presence of the phosphoribosyltransferase II (Neo cassette) gene and the β -galactosidase gene in the targeted allele of the earlier mouse line (Pontoglio *et al.*, 1996) caused additional phenotypes that are not directly due to loss of *HNF-1α* (Lee *et al.*, 1998). The Cre/loxP method results in removal of the Neo cassette that is used in preparation and selection of targeted embryonic stem cells. The *HNF-1α*-null mice were examined for expression of P450 genes. Earlier studies have indicated that this factor is involved in regulation of *CYP2E1* (Liu and Gonzalez, 1995) and *CYP1A2* (Chung and Bresnick, 1997). Indeed mRNA derived from both of these genes were found to be downregulated in the *HNF-1α*-null mice (unpublished results). These results establish the utility of using gene knockout mice to study gene regulation *in vivo*.

The Role of Signal Transduction and Ah Receptor-Mediated Transcriptional Activation of the *CYP1A1* Gene (R.H.T., S.O., T.V., Y.-H.C.)

The dioxin or aryl hydrocarbon (Ah) receptor is a nuclear transcriptional factor that controls the expression of a host of different genes whose functions are linked to the metabolism of dietary constituents, drugs and potentially hazardous agents through exposure to environmental contaminants. Several of the enzymes involved in this process include a number of the heme-thiolate proteins (cytochrome P450s) (Tukey *et al.*, 1981; Okino *et al.*, 1992), the rodent UDP-glucuronosyltransferase identified as *UGT1A6* (Lamb *et al.*, 1994; Iyanagi *et al.*, 1986), the NAD(P)H:quinone oxidoreductases (Jaiswal, 1991) and several of the glutathione S-transferases (Telakowski-Hopkins *et al.*, 1988; Pimental *et al.*, 1993). These enzymes are located in abundance in tissues that play an important role in first-pass metabolism, digestion, and drug metabolism, such as those of the gastrointestinal tract and the liver. Regulation of gene expression by the Ah receptor results in the induction of proteins involved in both Phase I and Phase II drug metabolism, a process that facilitates the channeling of hydrophilic substances to more polar oxidized compounds, as well as the processing of agents through conjugation reactions to water-soluble metabolites. For the most part, these enzymatic reactions could be considered to play an important role in drug metabolism by targeting these compounds for elimination since the result of metabolism is a substantial increase in the partition coefficient of these agents for water. However, the induction of certain heme-thiolate proteins, such as *CYP1A1*, is considered to be potentially counterproductive to this process since *CYP1A1* is capable of producing epoxides and dihydrodiol epoxides from aromatic and halogenated hydrocarbons. These metabolites have been shown in a number of experimental systems to be mutagenic (McManus *et al.*, 1990). Therefore, it is important to understand the cellular and molecular processes that contribute to the induction of gene expression after activation of the Ah receptor. As a result of the significant levels of induction of the *CYP1A1* gene, an appreciation of the regulatory potential of the Ah receptor has been obtained through experiments examining the ability of the receptor to activate *CYP1A1* transcription.

An understanding of the cellular signaling events involved in the activation of the Ah receptor, a process that stimulates its nuclear uptake and dimerization with ARNT, has been eloquently elucidated

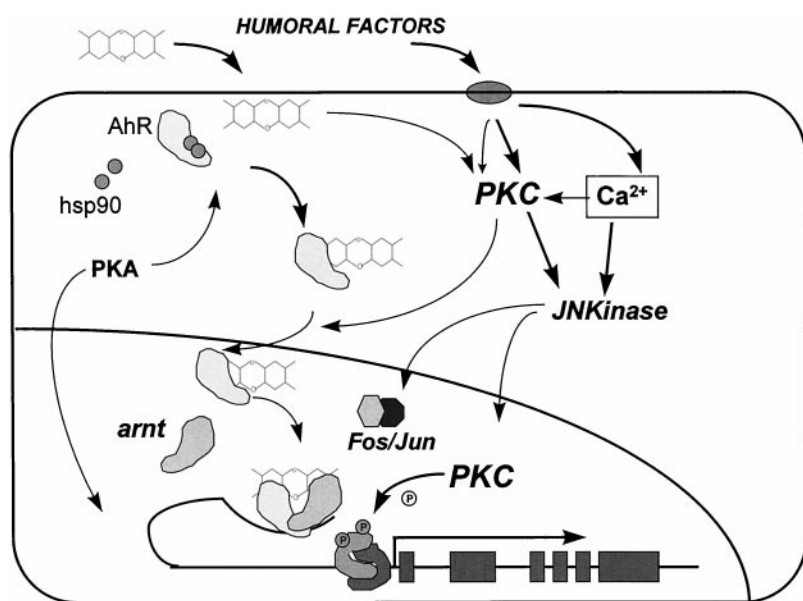


FIG. 4. Flow diagram showing the potential involvement of PKC in the regulation of *CYP1A1* gene transcription and Ah receptor regulation.

PKC is regulated by Ah receptor ligands, humoral factors, and cellular calcium, which influences Ah receptor uptake to the nucleus as well *CYP1A1* gene transcription. The mechanisms of action of PKC on transcription and Ah receptor uptake are unknown but could potentially involve Jun kinase (JNK kinase), AP-1 (Fos/Jun), as well as phosphorylation events that might impact directly on the assembly of proteins that promote transcription. Ligand binding to the Ah receptor involves hsp90 and tyrosine kinase (PKA), and there is evidence that PKA may also effect transcriptional events.

by several researchers (Gradin *et al.*, 1994; Jain *et al.*, 1994; Fujisawa-Sehara *et al.*, 1987; Pollenz *et al.*, 1994; Okino and Whitlock, 1995; Reyes *et al.*, 1992). A general flow diagram of some of these events is shown in fig. 4. The activation of the Ah receptor by ligand is coupled with the release of hsp90, a process that is linked to tyrosine kinase activity. Ligand association is followed by rapid uptake of liganded complex into the nucleus (Tukey *et al.*, 1982), where it pairs with the nuclear ARNT protein (Whitelaw *et al.*, 1993). In mice, the intraperitoneal administration of 3-methylcholanthrene has been shown to fully simulate nuclear uptake in less than 30 min, as measured by EMSA (Pendurthi *et al.*, 1993). Similar studies have detected the Ah receptor in the nucleus as early as 5 min after exposure of HepG2 cells to TCDD. While this is certainly an active transport process, there is very little known about the cellular events that underlie nuclear uptake of the Ah receptor.

The treatment of mice by the intraperitoneal administration of phorbol esters such as TPA dramatically reduced the TCDD directed transcriptional activation of the *Cyp1a1* gene (Okino *et al.*, 1992). Analysis of Ah receptor activation and nuclear binding to enhancer sequences indicated that the reduction in TCDD-initiated *Cyp1a1* transcriptional activity by TPA was in part attributed to a reduction in nuclear migration of the Ah receptor to the nucleus. This was documented directly by following the uptake and migration of ³H-TCDD-receptor complex from the cytosol to nucleus, a result that confirmed that the actions of TPA were interfering with nuclear transport processes. Since one of the actions of TPA results in the stimulation of PKC, an early event in many of the signaling events, experiments were undertaken to examine the actions of PKC on *CYP1A1* transcription.

A clonal HepG2 cell, called 101L, was developed, which carries the *CYP1A1* regulatory and promoter elements driving the firefly luciferase gene (Postlind *et al.*, 1992). With the ease of analyzing luciferase activity, the cells serve as an ideal biological tool for analysis of Ah receptor agonists as determined by transcriptional activation of the reporter gene. Ah receptor ligands such as TCDD, 3MC, polychlorinated biphenyls, and benzodiazepines activate *CYP1A1* luciferase

activity (Quattrochi and Tukey, 1993; Chen and Tukey, 1996). Dose-dependent increases in transcriptional activity appear to be concordant with the accumulation of the Ah receptor in the nucleus, as measured by EMSA. To examine the effect of PKC activation on *CYP1A1* transcription, 101L cells were treated with several receptor ligands in the presence of TPA. While Ah receptor agonists induced *CYP1A1* transcription, phorbol esters increased transcriptional activity two- to threefold (fig. 5). The ability of PMA to synergize TCDD-mediated induction is an early event, since longer-term exposure of cells to PMA leads to a reduction in this response. Interestingly, the actions of PMA appear to facilitate the accumulation of Ah receptor in the nucleus with longer exposure times, as measured by EMSA, but this increase is not concordant with enhanced transcriptional events. The early synergistic actions of PMA are specific since 4 α -phorbol 12,13-didecanoate, an inactive phorbol ester derivative, showed no effect on TCDD-induced transcriptional activation. Combined, the direct actions of PMA on HepG2 cells indicate that PKC activity plays a role in both the nuclear transport as well as in the ligand-induced actions of the Ah receptor on *CYP1* gene transcription.

The specific activation of transcription by PKC activity elicited by phorbol esters could be monitored by treating cells with PKC-specific inhibitors such as staurosporine and chelerythrine chloride. Interestingly, PKC inhibitors not only abrogated the synergistic actions of PMA on *CYP1A1* transcriptional activity, but they also completely abolished Ah receptor ligand-mediated induction of reporter gene activity. In the experiment shown in fig. 6, 1 nM TCDD can generate a 40-fold increase in luciferase activity within 3 hr. Thirty minutes' pre-treatment with PKC inhibitors blocks TCDD induction. The induction of HepG2 *CYP1A1* mRNA by TCDD is also blocked when cells are treated with PKC inhibitors (not shown). The rapid inhibition of *CYP1A1* gene transcription in the presence of PKC inhibitors suggests that cellular PKC activity plays an important role in the functional properties of the Ah receptor or the catalytic mechanisms that underlie *CYP1A1* transcription.

The role of PKC could not be directly linked to the functional properties of the Ah receptor. Treatment of 101L cells with PKC

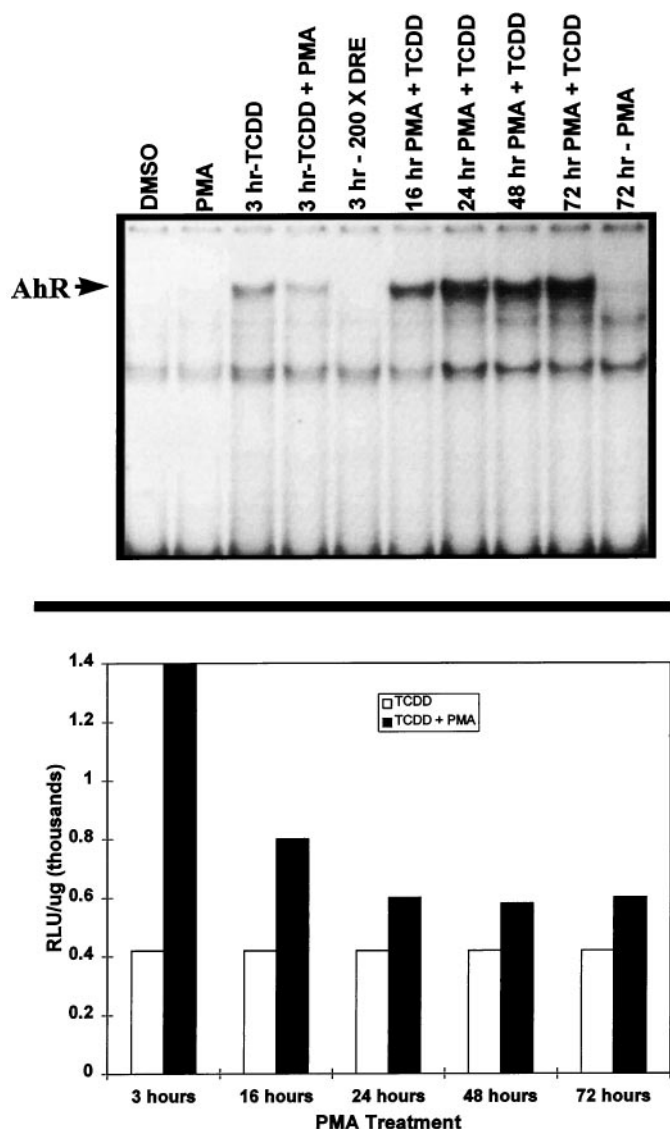


FIG. 5. The actions of phorbol esters on CYP1A1 gene transcription.

101L cells were treated with phorbol 12-myristate 13-acetate (PMA) for either 3, 24, 48 or 72 hr, followed by 3 hr with TCDD. Electrophoretic mobility shift analysis (EMSA) using oligonucleotides to the DRE sequence are shown in the upper panel. The effects of TCDD and PMA on CYP1A1 transcription are shown in the lower panel.

inhibitors did not affect the ability of TCDD to bind to the cytosolic Ah receptor or to activate a cytosolic receptor into a DNA-binding form. In addition, TCDD-initiated uptake of the Ah receptor to the nucleus, as determined by the ability of the activated nuclear receptor to bind to xenobiotic response element sequences, was not altered by pretreatment of HepG2 cells with PKC inhibitors. While PKC has been shown to promote the nuclear accumulation of Ah receptor, its actions appear to be secondary to additional signals that promote the ligand-induced uptake of the receptor. While PKC inhibitors efficiently block ligand-mediated induction of CYP1A1 gene transcription, the inability of these inhibitors to impact on the functional properties of the Ah receptor (*i.e.* ligand binding, nuclear uptake, activation to a DNA-binding form) indicates that PKC activity is involved in cellular or molecular events that play a crucial role in facilitating the ability of the receptor to promote transcription. It is unclear whether PKC activity or additional signaling mechanisms utilize the Ah receptor as substrate or whether its actions are more

general in nature by supporting the transactivation potential of the receptor. The latter would appear to be the case, since PKC inhibition blocks transactivation of reporter genes by ARNT as well as by TCDD-directed induction of reporter genes by the Ah receptor.

In summary, the potential role of cellular signaling pathways on the regulation and control of the CYP1A1 gene are outlined in fig. 4. Cellular mechanisms that modulate PKC activity have been demonstrated to influence the nuclear uptake of the Ah receptor as well as control the ability of the receptor to promote transcriptional activation of the CYP1A1 gene (Chen and Tukey, 1996; Berghard *et al.* 1993; Carrier *et al.*, 1992). While exposing rodents to phorbol esters impairs Ah receptor uptake to the nucleus and a reduction in transcriptional activation of the Cyp1a genes, phorbol esters dramatically stimulate Ah receptor-mediated transcriptional activation of the CYP1A1 gene in HepG2 cells. This contradiction in response could be attributed to the role or activation by TPA of humoral factors *in vivo* that are not present in an isolated cell system, which in turn influence cellular signaling processes that cannot be duplicated in tissue culture. The accelerated rate of CYP1A1 transcription in HepG2 cells and this linkage to PKC activity most likely involves cellular signaling events that do not dramatically influence Ah receptor activation or function. It is certainly possible that PKC could be initiating signals through phosphorylation patterns that impact on the assembly of the transcriptional complex, a priority that may be necessary for protein-protein interactions between the Ah receptor and other transcriptional factors. Since it has been shown that Ah receptor ligands are capable of activating PKC (Weber *et al.*, 1994; Bombick *et al.*, 1985), ligand-induced activation of the Ah receptor, as well as the promotion of PKC-directed signaling events, may occur in concert to promote the molecular changes necessary to stimulate CYP1A1 gene transcription.

Signaling and Receptor Pathways Modulating Gene Induction by Phenobarbital (J.S.S., C.J.O.)

Phenobarbital (PB) is a prototype agent for other barbiturates and a variety of other xenobiotic compounds such as chlordane, dichlorodiphenyl-trichloroethane, certain polychlorinated biphenyls, *etc.*, that exhibit profound inductive effects on a variety of biotransformation systems, including particular glutathione-S-epoxide transferases, UDP-glucuronyl transferases, aldehyde dehydrogenases, and the cytochrome P450 monooxygenases (Honkakoski and Negishi, 1998; Waxman and Aronoff, 1992). The PB induction response occurs in many species, including humans (Waxman and Aronoff, 1992).

Although it was demonstrated previously that PB stimulates these genes at the transcriptional level (Hardwick *et al.*, 1983), relatively little is known regarding the underlying molecular mechanisms governing the PB induction response or cell- or stage-specific expression of P450 genes. Previous reports from our laboratory (Omiecinski, 1986), and many others (Wojcik *et al.*, 1988; Bars *et al.*, 1992) have established that the liver is a primary PB-responsive organ in mammalian organisms.

A 15-bp "barbie box" promoter element was characterized as an important regulator of PB induction in *Bacillus megaterium* (He and Fulco, 1991; Shaw and Fulco, 1993); however, an accumulating body of evidence now suggests that critical regulatory elements for PB induction in mammalian liver cells exist independently of the barbie box (Shaw *et al.*, 1998). Studies using gene-transfection analyses in primary rat and mouse hepatocytes (Stoltz *et al.*, 1998; Honkakoski *et al.*, 1998) and direct gene injections in rat liver (Park *et al.*, 1996) demonstrated that proximal promoter barbie box sequences were not required for PB responsiveness in these systems. Rather, experimental results have implicated a PB-responsive unit (PBRU) upstream of the barbie box element as modulating PB activation. Anderson and co-

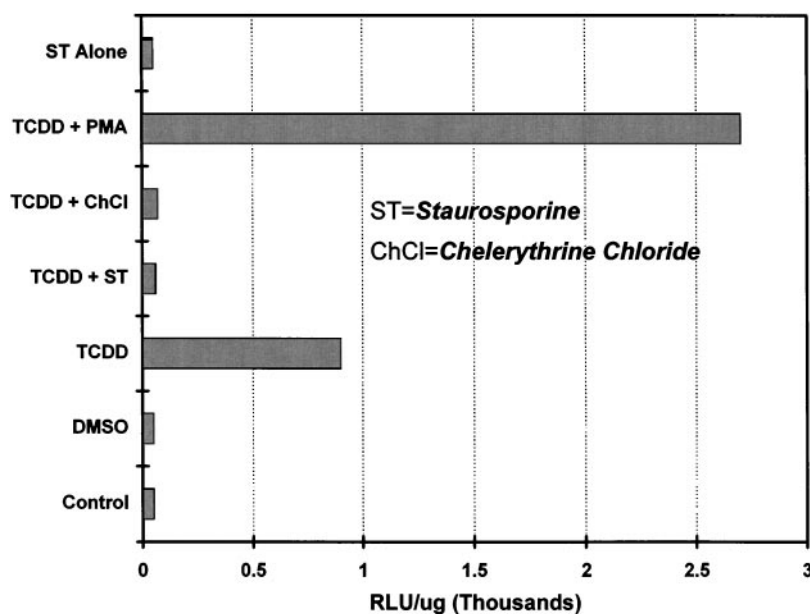


FIG. 6. Effects of PKC inhibitors on *CYP1A1* gene transcription. 101L cells were pretreated with staurosporine (ST) or chelerythrine chloride (ChCl) for 1 hr, followed by the addition of 1nM TCDD and/or 125 nM PMA for 3 hr. After incubation, the cells were harvested and luciferase activity was determined. DMSO, dimethyl sulfoxide; RLU, relative light units.

workers delineated a PBRU from -2318 bp to -2155 bp upstream of the transcription start site of the *CYP2B2* gene (Trottier *et al.*, 1995; Stoltz *et al.*, 1998). In the mouse *Cyp2b10* gene, a region from -2170 to -2258 bp was identified by functional studies as conferring PB responsiveness (Honkakoski and Negishi, 1997) and was more recently narrowed to a 51-bp element centering on an NF-1 site (Honkakoski and Negishi, 1998). Of interest, this 51-bp region also contains putative half-sites for nuclear receptors (Honkakoski and Negishi, 1998). In another recent study, *in vivo* footprinting approaches were used to define PB-induced chromatin alterations in the -2.2 kb region of the rat *CYP2B1/2* genes, also centering around a putative NF-1 site (Kim and Kemper, 1997). This positioning of a PB-responsive region is consistent with functional experiments we reported previously, which used transgenic mouse lines that demonstrated that gene sequences residing 5' of -800 bp upstream of the transcription initiation site were required for PB-inducible expression of *CYP2B2* (Ramsden *et al.*, 1993).

For some years it has been assumed that PB induction required *de novo* protein synthesis. However, evidence we had accumulated that indicated that post-transcriptional processes, *i.e.* protein kinase/phosphatase cascades, are regulators of PB signaling in hepatocytes did not appear to be consistent with these perceptions (Sidhu and Omiecinski, 1995 and 1997). These studies, which examined the role of cAMP/PKA activators and PP1/PP2A inhibitors on the PB signaling process, indicated that an upstream phosphatase pathway may participate as an activator of PB induction (Sidhu and Omiecinski, 1995 and 1997). Furthermore, results from DNase I hypersensitivity determinations, DNA affinity purification experiments, electrophoretic mobility shift assays, and *in vitro* DNase I footprinting studies all failed to reveal differences between nuclear protein extracts derived from control or PB-induced liver (Stoltz *et al.*, 1998; Sommer *et al.*, 1996). In a recent study, we demonstrated that *de novo* protein synthesis was not required for PB induction (Sidhu and Omiecinski, 1998). Although certain protein synthesis inhibitors did inhibit the response, the latter effect appeared to be non-specific since inactive analogs of protein synthesis inhibitors, *e.g.* puromycin aminonucleoside, also were potent negative modulators of PB induction. Other

pathways are perturbed in hepatocytes by application of a variety of protein synthesis inhibitors, including the stress-activated protein kinase cascade (Sidhu and Omiecinski, 1998). Therefore, in view of the collective data, it appears likely that pre-existing regulatory factors interact with the PBRU constitutively and then are subjected to post-transcriptional alterations in the presence of PB. These alterations in turn may enable positive interaction of the PBRU with the transcriptional initiation complex.

A broad array of pharmacological agents effective in modulating various cell-signaling pathways have been tested in our laboratory for their effects on the PB-induction process. Examples are illustrated in table 1. The data illustrate the caution that needs to be exercised before deriving conclusions from effects of "specific" modulators. For example, we observed that staurosporine, a broad-band kinase inhibitor that has been used by investigators as a "specific" PKC inhibitor, profoundly inhibited the PB-induction response in cultured hepatocytes. Similar effects were noted with KN64, an inhibitor of Ca²⁺ calmodulin kinase II. However, when additional modulators of the same pathways were tested, contrasting results were obtained. Use of inactive structural analogs of the specific effector agents has provided an important resource for dissecting the actual effects. With these agents, we have demonstrated that, contrary to initial indications,

TABLE 1

Summary of effects of various pharmacological agents and analogs on PB induction in primary rat hepatocyte cultures^a

Agent ^b	Pathway	Phenobarbital Induction Response
Staurosporine	Broad-band PK inhibitor	Inhibition
Calphostin C	PKC inhibitor	No effect
PMA	PKC activator/inhibitor	No effect
KN62	CAM kinase II inhibitor	Inhibition
KN04	Inactive analog of KN62	Inhibition
KN93	CAM kinase II inhibitor	Inhibition
KN92	Inactive analog of KN93	Inhibition
PD 98059	MAPKK inhibitor	No effect

^a PKC, protein kinase C; CAM kinase II, Ca²⁺-calmodulin kinase II; MAPKK, mitogen-activated protein kinase kinase.

^b All agents listed were tested over a range of concentrations.

neither the PKC nor Ca²⁺ calmodulin kinase II pathways appear to be involved in PB signaling. The lack of effect of the specific mitogen-activated protein kinase inhibitor, PD 98059, also appears to rule out the mitogen-activated protein kinase pathway as integral to PB signaling. Further experiments are in progress to delineate the exact nature of the PB-signaling response. It is likely that the downstream targets of these pathways will impinge on the nuclear regulatory proteins interacting with the PBRU and basal transcriptional machinery.

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