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The Alcohol-inducible form of Cytochrome P450 (CYP 2E1): Role In Toxicology and Regulation of Expression

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Cytochrome P450 (CYP) 2E1 catalyzes the metabolism of a wide variety of therapeutic agents, procarcinogens, and low molecular weight solvents. CYP2E1-catalyzed metabolism may cause toxicity or DNA damage through the production of toxic metabolites, oxygen radicals, and lipid peroxidation. CYP2E1 also plays a role in the metabolism of endogenous compounds including fatty acids and ketone bodies. The regulation of CYP2E1 expression is complex, and involves transcriptional, post-transcriptional, translational, and post-translational mechanisms. CYP2E1 is transcriptionally activated in the first few hours after birth. Xenobiotic inducers elevate CYP2E1 protein levels through both increased translational efficiency and stabilization of the protein from degradation, which appears to occur primarily through ubiquitination and proteasomal degradation. CYP2E1 mRNA and protein levels are altered in response to pathophysiologic conditions by hormones including insulin, glucagon, growth hormone, and leptin, and growth factors including epidermal growth factor and hepatocyte growth factor, providing evidence that CYP2E1 expression is under tight homeostatic control.

Key words:

INTRODUCTION

Cytochrome P450 (CYP) 2E1 has been identified in hepatic and extrahepatic tissues of humans, rats, rabbits, mice, and every other mammalian species that has been examined (Koop *et al.*, 1985; Ding *et al.*, 1986; Song *et al.*, 1986; Thomas *et al.*, 1987; McCoy and Koop, 1988; Kubota *et al.*, 1988; Davis *et al.*, 1993; Watkins *et al.*, 1985; Wrighton *et al.*, 1986; Shimada *et al.*, 1997; Court *et al.*, 1997). CYP2E1 catalyzes the metabolism of a wide variety of xenobiotics including therapeutic agents, procarcinogens, and low molecular weight halogenated hydrocarbons, as well as endogenous compounds. Substrates for CYP2E1-catalyzed metabolism include benzene, nitrosamines (including those found in cigarettes and food), pyridine, carbon tetrachloride, fluorocarbon refrigerants, acetone, primary alcohols, acetaminophen, isoniazid, anesthetic agents, and numerous low molecular weight halogenated hydrocarbons such as trichloroethylene, vinyl chloride and chloroform (Johansson and Ingelman-Sundberg, 1985; Levin *et al.*, 1986; Johansson and Ingelman-Sundberg, 1988; Ekstrom *et al.*, 1989; Raucy *et al.*, 1989; Guengerich and Shimada, 1991; Guengerich *et al.*, 1991; Yamazaki *et al.*, 1992; Raucy *et al.*, 1993; Snawder *et al.*, 1994; Lee *et al.*, 1996). CYP2E1 also plays a role in the metabolism of endogenous compounds such as fatty acids (including arachidonic acid), and ketone bodies (postulated to be an emergency gluconeogenesis pathway during fasting) (Casazza *et al.*, 1984; Koop and Casazza, 1985; Vaz *et al.*, 1990; Adas *et al.*, 1999a; Adas *et al.*, 1999b).

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Role of CYP2E1 in toxicology and carcinogenesis

Several substrates of CYP2E1 constitute protoxicants (e.g. CCl_4, nitrosamines, benzene, acetaminophen) and, when metabolized by CYP2E1, cause damage to cells or tissues expressing CYP2E1. CYP2E1-mediated toxicity generally results from the generation of free radical metabolites, as has been demonstrated for ethanol (Behrens et al., 1988; Knecht *et al.*, 1990; Albano *et al.*, 1996), CCl_4 (Johansson and Ingelman-Sundberg, 1985; Lindros *et al.*, 1990; Persson *et al.*, 1990), benzene (Johansson and Ingelman-Sundberg, 1988), halothane

(Bentley *et al.*, 1982) and acetaminophen (Potter *et al.*, 1973; Raucy *et al.*, 1989). Pyridine, a constituent of tobacco, has been shown to be a low Km substrate of CYP2E1 and to undergo sequential oxidation, resulting in a product that is capable of redox cycling, generating reactive oxygen species and damaging DNA (Kim and Novak, 1990a).

When CYP2E1 expression is elevated in these cells or tissues, significantly enhanced toxicity in the presence of such protoxicants results (Evarts *et al.*, 1983; Johansson and Ingelman-Sundberg, 1985; Watkins *et al.*, 1988). CYP2E1-catalyzed metabolism of $CC1_4$ results in increased nuclear levels of transcription factors (NF-κB) and transcription factor complexes (AP-1) which stimulate cell proliferation (Gruebele *et al.*, 1996). The presence of DNA damage (oxidative, DNA-adducts) during proliferation substantially increases the potential for cell transformation.

CYP2E1-catalyzed metabolism of ethanol in primary cultured rat hepatocytes or in HepG2 cells transiently or stably expressing CYP2E1 results in cytotoxicity due to apoptosis (Wu and Cederbaum, 1996a; Wu and Cederbaum, 2000). This ethanol-mediated apoptosis is due to the generation of reactive radical species resulting from CYP2E1 metabolism as it was prevented by antioxidants, inhibitors of lipid peroxidation, radical trapping agents, and inhibitors of CYP2E1 metabolism (Wu and Cederbaum, 1996a; Wu and Cederbaum, 2000).

In addition to the enhanced toxicity of exogenous agents, CYP2E1 may play a role in tissue damage and altered endogenous metabolism as a result of CYP2E1-mediated metabolism of endogenous substrates. CYP2E1 plays a role in the enhanced cytotoxicity due to apoptosis resulting from arachidonic acid addition to primary cultured rat hepatocytes or HepG2 cells stably expressing CYP2E1 (Chen *et al.*, 1997; Wu and Cederbaum, 2000). CYP2E1-mediated oxidative stress has also been reported to enhance the expression of collagen type I in rat hepatic stellate cells stably expressing CYP2E1 (Nieto *et al.*, 1999). Hepatic stellate cell proliferation and collagen synthesis occur during fibrogenesis *in vivo* and are stimulated by oxidative stress *in vitro*. Ethanol and arachidonic acid enhanced the induction of collagen type I in these cells, and it is hypothesized that cyclooxygenase-2 expression is enhanced by CYP2E1 and plays a role in the arachidonic acid-mediated increase in collagen type I (Nieto *et al.*, 2000).

CYP2E1 has been implicated in generation of tissue-damaging hydroxyl radicals during diabetes (Ohkuwa *et al.*, 1995), in nonalcoholic steatohepatitis (Weltman *et al.*, 1996; Leclercq *et al.*, 2000a) and in alcohol-induced steatosis (Jarvelainen *et al.*, 2000). CYP2E1 has also been reported to be involved in enhanced lipid peroxidation and free radical generation (Krikun *et al.*, 1984; Albano *et al.*, 1996; Dupont *et al.*, 2000; Navasumrit *et al.*, 2000), and altered fatty acid metabolism (Morimoto *et al.*, 1995), during chronic ethanol consumption.

CYP2E1, therefore, plays a role in the enhanced toxicity of therapeutic agents such as acetaminophen (Seef *et al.*, 1986; Ishak *et al.*, 1991; Corcoran *et al.*, 1987), halothane (Takagi *et al.*, 1983), and isoniazid (Lieber, 1988), as well as other hepatotoxic agents including $CC1_4$ (Hasumura *et al.*, 1974), benzene (Johansson and Ingel- man-Sundberg, 1988), and cocaine (Smith *et al.*, 1981). CYP2E1 likely also plays a role in the increased incidence of liver disease and cancer in diabetics, alcoholics, and obese individuals (Lieber 1988; Lieber *et al.*, 1979; Blot *et al.*, 1988; Andersen *et al.*, 1984).

Regulation of CYP2E1 expression

CYP2E1 protein levels are elevated 2- to 8-fold in rat hepatic tissue in response to treatment with xenobiotics such as ethanol, acetone, pyridine, pyrazole, and isoniazid (Ryan *et al.*, 1985; Song *et al.*, 1986; Johansson *et al.*, 1988; Palakodety *et al.*, 1988; Kim *et al.*, 1988; Kim and Novak, 1993). Similar increases in CYP2E1 protein levels in response to treatment with these xenobiotics have been reported in primary cultured rat hepatocytes by our laboratory and others (Zangar *et al.*, 1995; Woodcroft and Novak, 1998; Hunt *et al.*, 1991; Eliasson *et al.*, 1988; Perrot *et al.*, 1991; Sinclair *et al.*, 1991; Wu *et al.*, 1997). These elevations in CYP2E1 protein, both *in vivo* and *in vitro*, occur in the absence of a concomitant increase in CYP2E1 mRNA levels (Song *et al.*, 1986; Kim *et al.*, 1988; Kim and Novak, 1993; Johansson *et al.*, 1988; Zangar *et al.*, 1995; Woodcroft and Novak, 1998), indicating that posttranscriptional mechanisms are involved in the regulation of this P450 in response to xenobiotics. Both increased translational efficiency (Kim and Novak, 1990b; Kim *et al.*, 1990; Tsutsumi *et al.*, 1993) and protein stabilization (Song *et al.*, 1989; Roberts *et al.*, 1995; Yang and Cederbaum, 1997a; Goasduff and Cederbaum, 2000) have been implicated in the regulation of CYP2E1 protein expression.

In contrast to the xenobiotic-mediated elevation of CYP2E1, pathophysiologic conditions such as diabetes (spontaneous and chemical-induced), fasting, obesity, high fat diet, and long-term alcohol consumption result in increased CYP2E1 expression at both the mRNA and protein levels (~3- to 8-fold) in experimental animals and humans (Song *et al.*, 1987; Hong *et al.*, 1987; Favreau *et al.*, 1988; Bellward *et al.*, 1988; Johansson *et al.*, 1988; Dong *et al.*, 1988; Song *et al.*, 1990; Raucy *et al.*, 1991; Yun *et al.*, 1992; Ronis *et al.*, 1993; Shimojo *et al.*, 1993; Takahashi *et al.*, 1993; de la Maza, 2000). Elevation of CYP2E1 mRNA levels in the diabetic or fasted state in vivo has been attributed to mRNA stabilization (Song *et al.*, 1987).

As indicated above, several mechanisms have been implicated in the regulation of CYP2E1 expression. These

include transcriptional events [transcriptional activation during development (Song *et al.*, 1986; Umeno *et al.*, 1988; Vieira *et al.*, 1996) and transcriptional suppression by hormones (de Waziers *et al.*, 1995; Chen *et al.*, 1999; Simi and Ingelman-Sundberg, 1999; Morel *et al.*, 2000)]; stabilization of CYP2E1 mRNA (Song *et al.*, 1987; Woodcroft and Novak, 2000a; Peng and Coon, 1998); translational control (Kim and Novak, 1990b, Kim *et al.*, 1990, Tsutsumi *et al.*, 1993); and, post-translational events including altered stability and degradation of CYP2E1 protein (Song *et al.*, 1989; Tierney *et al.*, 1992; Roberts *et al.*, 1995; Yang and Cederbaum, 1997a, 1997b; Goasduff and Cederbaum, 1999; Korsmeyer *et al.*, 1999; Banerjee *et al.*, 2000; Goasduff and Cederbaum, 2000).

Transcriptional activation of CYP2E1 during development

CYP2E1 mRNA or protein cannot be detected in liver of fetal or newborn rats (Song *et al.*, 1986; Umeno *et al.*, 1988; Wu and Cederbaum, 1996b) or humans (Vieira *et al.*, 1996; Wu and Cederbaum, 1996b). However, CYP2E1 mRNA and protein levels rise dramatically during the first few hours after birth in both rat and human liver, and continue to rise slowly over the first week after birth (Song *et al.*, 1986; Umeno *et al.*, 1988; Vieira *et al.*, 1996; Wu and Cederbaum, 1996b). The increase in CYP2E1 mRNA expression during this period is due to transcriptional activation of the CYP2E1 gene, as determined by nuclear run-on analysis (Song *et al.*, 1986). Transcriptional activation of the CYP2E1 gene after birth is coincident with specific demethylation at the 5' end of the CYP2E1 gene in both rat and human liver (Umeno *et al.*, 1988; Vieira *et al.*, 1996). The hepatocyte-specific transcription factor HNF-1 has been implicated in the transcriptional activation of CYP2E1 (Ueno and Gonzalez, 1990; Liu and Gonzalez, 1995). Deletion analysis of the 5' end of the CYP2E1 gene revealed a region between -127 and -89 that was responsible for 90% of the *in vitro* transcriptional activity of liver extracts from adult rats, and protein binding studies indicated this region was equivalent to the binding site for HNF-1 (Ueno and Gonzalez, 1990). The involvement of HNF-1 was later confirmed by transient co-transfection studies using an HNF-1α expression plasmid and a CYP2E1 promoter-chloramphenicol acetyl transferase (CAT) reporter construct (Liu and Gonzalez, 1995; McGehee *et al.*, 1997). HNF-1α transactivated the CYP2E1 promoter (Liu and Gonzalez, 1995; McGehee *et al.*, 1997), and removal or mutation of the HNF-1 binding sequence prevented activation of the CYP2E1 promoter in response to HNF-1α (Liu and Gonzalez, 1995). Moreover, elements upstream of the HNF-1 binding site were found to negatively affect the activation of the CYP2E1 promoter, and DNase I hypersensitivity site mapping revealed a hypersensitive site in this region in adult rat liver but not newborn liver, providing a potential explanation for the lack of transcriptional activity in fetal and newborn liver (Liu and Gonzalez, 1995).

Other transcriptional events regulating CYP2E1 expression

The transcriptional regulation of CYP2E1 has not been extensively examined because regulation of CYP2E1 expression under most circumstances has been found to be post-transcriptional. In addition to transcriptional activation during development, however, some other reports of transcriptional regulation of CYP2E1 have been made. Simi and Ingelman-Sundberg (1999) reported that a change of medium in Fao rat hepatoma cells in culture resulted in activation of CYP2E1 gene transcription, a result also observed by our laboratory in primary cultured rat hepatocytes (Woodcroft and Novak, unpublished observations). Depletion of pituitary hormones by hypophysectomy of male rats caused a 6-fold increase in hepatic CYP2E1 expression, which was associated with increased CYP2E1 gene transcription (Chen *et al.*, 1999). Our laboratory has also observed hormonal/growth factor effects on CYP2E1 gene transcription; both insulin and epidermal growth factor have been found to decrease CYP2E1 transcription in primary cultured rat hepatocytes (Woodcroft and Novak, unpublished observations). de Waziers *et al.* (1995) also reported that insulin decreased CYP2E1 transcription in Fao rat hepatoma cells. In contrast, Peng and Coon (1998) observed no effect of insulin or triiodothyronine (T3) on the transcription of the rabbit CYP2E1 5' flanking region with an UTR/luciferase fusion gene in transfected HepG2 cells. In addition to hormonal effects on CYP2E1 transcription, Morel *et al.* (2000) have reported, using a human CYP2E1 promoter/luciferase reporter construct transfected into HepG2 cells, that CYP2E1 promoter activity is decreased by oxidative stress in the form of exogenous H_2O_2 addition, glutathione depletion, or co-transfection of a CYP2E1 expression vector which results in H_2O_2 generation.

Translational control of CYP2E1 protein expression

One very interesting phenomenon associated with CYP2E1 is that, in contrast to other inducible P450s, the increase in CYP2E1 protein levels following treatment with xenobiotic inducers is not accompanied by a corresponding increase in CYP2E1 mRNA. The mechanism(s) governing xenobiotic-mediated increases in CYP2E1 protein levels remain the subject of investigation. Although CYP2E1 protein levels are elevated in response to a variety of structurally diverse compounds, the only consensus regarding the mechanism appears to be that it is post-transcriptional. Even this, however, is not a universal consensus. For example, Kubota *et al.* (1988) reported an increase in translatable CYP2E1 mRNA from

ethanol- and pyrazole-treated hamsters, and Ronis *et al.* (1993) reported increases in CYP2E1 mRNA levels in ethanol-treated rats, albeit at elevated levels of ethanol administration. It has also been reported that acetone increased the level of CYP2E1 mRNA 1.6-fold in cultured rabbit hepatocytes and that α-amanitin inhibited radiolabelling of CYP2E1 protein suggesting that transcription may regulate expression in this system (Kraner *et al.*, 1993).

Our laboratory (Kim and Novak, 1990b; Kim *et al.*, 1990) has provided seminal evidence that xenobiotic induction of hepatic CYP2E1 is associated with translational control. Pyridine treatment of animals resulted in increased CYP2E1 levels in the presence of an inhibitor of transcription, but not in the presence of an inhibitor of translation. In addition, an ~50% decrease in poly(A) RNA was observed following pyridine treatment. Further, increased intensity of a putative CYP2E1 protein was detected in the autoradiograph of an SDS-PAGE gel following a pulse-chase experiment with ^{14}C-leucine. Lastly, a shift in the sucrose density CYP2E1 polysomal distribution profile from the low density fraction to the higher density polysome-containing fractions was noted following pyridine or acetone treatment, suggesting an increase in active translation of pre-existing CYP2E1 mRNA (Kim and Novak, 1990). In addition, elevation in CYP2E1 protein levels achieved through treatment of animals with various nitrogen- and sulfur-containing heterocycles was accompanied by a decrease in poly(A) RNA (Kim and Novak, 1993). When polyribosomal distribution of rat hepatic CYP2E1, CYP2B, CYP3A and CYP4A mRNAs was examined (Fig. 1), only CYP2E1 mRNA exhibited a bimodal distribution, with significant (~30-40%) CYP2E1 mRNA levels detected in the low density sucrose gradient (i.e. 60S-80S fraction) suggestive of an absence of translation, and 60-70% detected in the high density (i.e. actively translated) fraction (Kocarek, *et al.*, 2000). These data suggested not only a translational control component regulating CYP2E1 protein expression but also one which regulates CYP2E1 mRNA turnover.

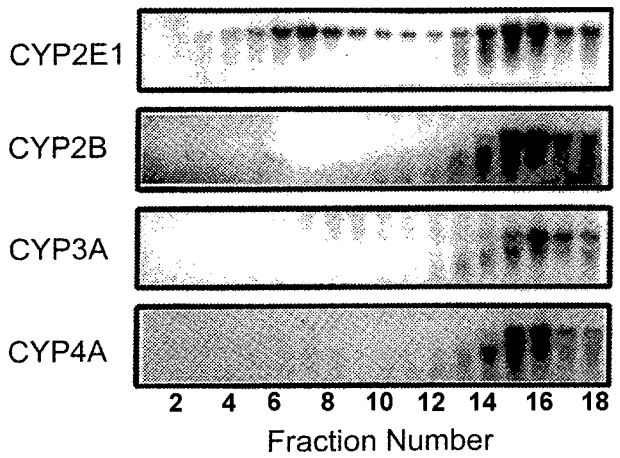

Fig. 1. Polysomal distribution of rat hepatic CYP2E1, CYP2B, CYP3A, and CYP4A mRNAs. Rat liver homogenates were fractionated on sucrose density gradients, and the amounts of CYP2E1, CYP2B, CYP3A, and CYP4A mRNA present in the sucrose fractions were estimated by Northern blot analysis (From Kocarek, T. A., Zangar, R. C., and Novak, R. F., Arch. Biochem. Biophys., 376, 180-190 (2000) with permission.

Recently, Kocarek *et al.* (2000) have provided additional evidence in support of translational control and translation-dependent CYP2E1 mRNA turnover. These experiments, which utilized a series of 5' and 3' untran- slated region (UTR) constructs revealed that the 5' UTR leader sequence, and, in particular, the 3' UTR, could dramatically influence the rate of CYP2E1 mRNA translation. In initial studies designed to examine the CYP2E1 mRNA molecule for sequences/regions that could potentially affect translation, a series of CYP2E1 recombinant RNAs (rcRNAs) with modified 5' or 3' UTRs was translated in vitro using the rabbit reticulocyte lysate system (Fig. 2). Deletion of a majority of the CYP2E1 5' UTR, which was predicted to contain secondary structure, increased CYP2E1 protein synthesis *in vitro*. In contrast, deletion of the poly(A) tail, and partial or complete deletion of the 3' UTR decreased CYP2E1 protein synthesis. CYP2E1 protein synthesis *in vitro* was accompanied by increased degradation of the CYP2E1 rcRNAs. Interestingly, addition of protein synthesis inhibitors (e.g. $HgCl_2$, cycloheximide, puromycin) resulted in decreased degradation of the rcRNAs during *in vitro* translation. In contrast, increased levels of RNase inhibitors failed to affect the degradation of the rcRNAs. These results suggest that secondary structure in the 5' UTR of CYP2E1 mRNA is partially responsible for the inefficient translation of this mRNA. The poly(A) tail and sequences contained in the 3' UTR, however, appear to be important for protecting CYP2E1 mRNA from RNase activity associated with translation (Kocarek, *et al.*, 2000). Furthermore, these studies revealed that CYP2E1 mRNA translation in the cell lysate system is influenced by the 5' and 3' UTRs and that CYP2E1 mRNA turnover is translation dependent. Collectively, these data provide support for the role of translation in regulating CYP2E1 expression.

CYP2E1 protein stabilization and degradation

Several investigators have provided evidence that protein stabilization and altered degradation represent a mechanism for controlling CYP2E1 expression in response to xenobiotics. Acetone, pyrazole and ethanol have been reported to enhance CYP2E1 expression through protein stabilization (decreased rates of degradation) (Eliasson *et al.*, 1988; Song *et al.*, 1989; Roberts *et al.*, 1995). When rats were treated with acetone for 10 days prior to administration of H14CO3, no change in the rate of CYP2E1 synthesis was monitored relative to untreated

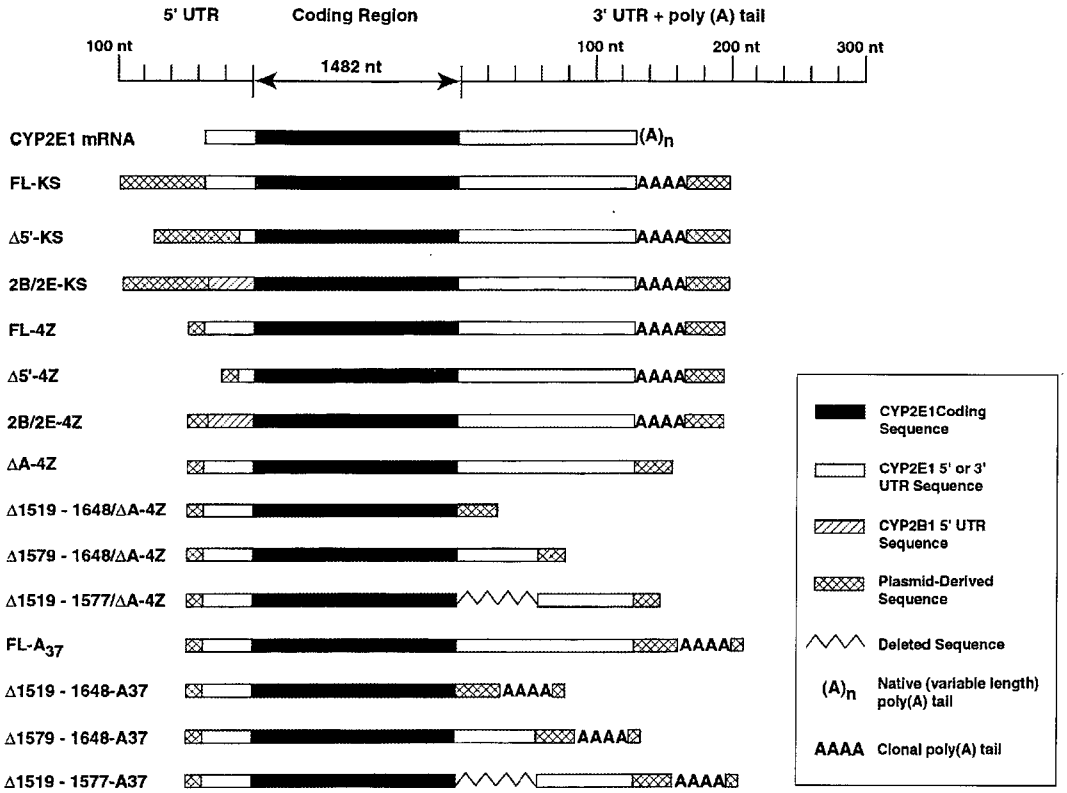

Fig. 2. CYP2E1 rcRNAs used for *in vitro* translation experiments (From Kocarek, T. A., Zangar, R. C., and Novak, R. F., Arch. Biochem. Biophys., 376, 180-190 (2000) with permission.

controls and CYP2E1 degradation was reported to be biphasic with half-lives of 7.5 and 37.5 h (Song *et al.*, 1989). Although CYP2E1 was immunoprecipitated and radiolabel incorporation into CYP2E1 protein determined directly using autoradiography (6 month exposure), several problems plague the interpretation of these data. Firstly, treating animals for 10 days with acetone prior to the administration of H14CO3 fails to take into consideration the early time-dependence of the enhanced expression (linear from 3 to 12 h post administration of acetone, i.p. injection) and hence, the maximal rate of new protein synthesis; secondly, it fails to address the existing pool size of CYP2E1 and how much newly labelled protein might be incorporated into the existing pool, which is already at maximal levels. Moreover, under the conditions of chronic treatment (e.g. acetone for 10 days; ethanol for 15 days), nutritional, hormone, or growth factor levels may be altered, and such alterations may persist for many hours following termination of treatment and present an additional dimension of complexity.

Additional experiments to support the role of protein stabilization in CYP2E1 expression consisted of studies monitoring the effects of ethanol, imidazole, 2-propanol, dimethylsulfoxide and isoniazid on the loss of CYP2E1 in cultured hepatocytes following treatment of the animals with an inducer of CYP2E1 *in vivo* (Eliasson *et al.*, 1988). This study was conducted because CYP2E1 protein levels declined to ~70% of control in 24 h and were below the limit of detection at 72 h in most primary hepatocyte cell culture systems employed. CYP2E1 mRNA levels were below the limit of detection after 24 hr in culture (Eliasson *et al.*, 1988). When hepatocytes from animals treated with inducers in vivo were maintained in culture in the presence of the inducers ethanol, imidazole, 2-propanol and dimethylsulfoxide, the level of CYP2E1 protein was reported to have been maintained relative to untreated cells. After 3 days in culture, ~50% of the CYP2E1 protein present at the time of plating remained in the treated cells, although the levels of mRNA were reported to be reduced to nearly the limits of detection. The level of CYP2E1 protein remaining was dependent on the extent of induction achieved *in vivo* and varied with the inducer used to treat rats *in vivo* (Eliasson *et al.*, 1988). The ability of ethanol, imidazole, 2-propanol and dimethylsulfoxide to maintain CYP2E1 levels was correlated with the spectral binding constant (Kd) for the purified enzyme, suggesting that ligand binding was necessary for stabilization. No data were presented, however, to examine whether other forms of P450 (e.g. CYP2B) were differentially affected by these agents, since nitrogenous bases also bind CYP2B with comparable Kds. Thus, whether the results of this study were unique to CYP2E1,

or more generalized, remains to be established.

Glycerol has also been reported to elevate CYP2E1 protein levels via protein stabilization (Yang and Cederbaum, 1997a). Glycerol (100-200 mM) treatment of HepG2 cells stably expressing CYP2E1 resulted in a 3-fold increase in CYP2E1 protein levels and activity. [^{35}S]Methionine labelling and immunoprecipitation experiments revealed that glycerol treatment increased the half-life of CYP2E1 protein in these cells from 3 h to 11 h. In contrast, glycerol had no effect on cytochrome b5 content or on the activity of NADPH-cytochrome P450 reductase or NADH-cytochrome b5 reductase in these cells (Yang and Cederbaum, 1997a).

While protein stabilization (decreased degradation) has been implicated in the enhanced expression of CYP2E1 by xenobiotics, little is known about the proteolytic systems involved in the degradation of CYP2E1 or how CYP2E1 may be targeted for such degradation. Presumably stabilization diminishes the natural turnover of the protein by decreasing its susceptibility to proteolysis caused by heme oxidation or oxidation of amino acid residues by H_2O_2 or other oxidants during normal catalytic activity. Recognition of the labile form of CYP2E1 has been proposed to involve phosphorylation or exposure of a vulnerable amino acid sequence; either or both of these would facilitate degradation by cellular proteases (Watkins *et al.*, 1987; Correia *et al.*, 1989; Tierney *et al.*, 1992; Menez *et al.*, 1993). Several intriguing questions emerge, however, regarding the proposed stabilization of a labile form. The first centers on the wide variety of chemicals that induce CYP2E1; no structure-activity relationship appears to exist, and many serve as substrates. In addition, studies in our laboratory show that no correlation exists between spectral binding constants, inhibition of CYP2E1 activity and enhanced expression (Kim and Novak, 1993). Moreover, if protein adducts are formed, such as those identified with ethanol treatment (Behrens *et al.*, 1988), then one might predict that proteolysis should be enhanced, since the protein has been modified and is no longer recognized as "normal". Indeed, damage to proteins generally enhances proteolysis.

With respect to degradation systems, proteolysis was initially proposed to occur by an autophagosomal/autolysosomal pathway (Ronis and Ingelman-Sundberg, 1989; Ronis *et al.*, 1991). CYP2E1 protein levels and catalytic activity were found to be maximally increased in rat liver 12-24 h following a single dose of acetone. While catalytic activity was reported to have declined to basal levels in 24 h, the levels of immunodetectable CYP2E1 protein remained elevated 96 h following the initial treatment (Ronis *et al.*, 1991). The authors interpreted these results to suggest that degradation of the apoprotein does not proceed concurrently with the loss of catalytic activity, and that CYP2E1 is inactivated prior to its degradation (Ronis and Ingelman-Sundberg, 1989; Ronis *et al.*, 1991). CYP2E1 was detected in the lysosomal compartment in the presence of leupeptin, suggesting that CYP2E1 is degraded by an autophagosomal/autolysosomal pathway (Ronis and Ingelman-Sundberg, 1989; Ronis *et al.*, 1991). Following these reports, however, these authors cited evidence for the involvement of a novel proteinase active in the degradation of CYP2E1 protein (Eliasson *et al.*, 1992; Zhukov *et al.*, 1993). However, the identity, specificity, and activity of this proteinase towards CYP2E1, remains uncertain.

Several reports have provided evidence for decreased protein degradation, and in particular inhibition of ubiquitin-mediated proteolysis, as a mechanism by which xenobiotics elevate CYP2E1 protein levels (Tierney *et al.*, 1992; Roberts *et al.*, 1995; Korsmeyer *et al.*, 1999). CYP2E1-catalyzed metabolism of CCl_4 damages the CYP2E1 protein resulting in rapid ubiquitination and degradation of the protein (Correia, 1991; Tierney *et al.*, 1992). Oxidative modification of CYP2E1 protein as a result of CYP2E1-catalyzed production of reactive oxygen species in human liver microsomes or in HepG2 cells expressing human CYP2E1 has also been suggested to target CYP2E1 for degradation by the proteasome complex (Yang and Cederbaum, 1997b; Goasduff and Cederbaum, 1999). Substrates or ligands of CYP2E1 such as 4-methylpyrazole, ethanol, glycerol, and dimethyl sulfoxide protected against degradation, as did antioxidants, while CCl_4 accelerated CYP2E1 degradation (Yang and Cederbaum, 1997b; Goasduff and Cederbaum, 1999). Inhibitors of the proteasome decreased the degradation of CYP2E1 in these experimental systems (Yang and Cederbaum, 1997b; Goasduff and Cederbaum, 1999). Roberts *et al.* (1995) and others have postulated that substrates such as ethanol protect CYP2E1 against ubiquitin-mediated proteolysis. CYP2E1 was shown to exhibit a biphasic half-life in the presence of ethanol, suggesting a substrate-induced alteration in CYP2E1 conformation and differential rates of protein turnover (Roberts *et al.*, 1995). This observation, however, is challenged by the data reported by Tsutsumi *et al.* (1993) in which labeling of the protein and heme failed to yield biphasic degradation kinetics. Although Roberts (1997) reportedly failed to detect CYP2E1 ubiquitination, Korsmeyer *et al.* (1999) have provided evidence that the CYPs do undergo ubiquitin-dependent 26S proteasomal degradation. To date, however, very little mechanistic information on ubiquitin-mediated CYP degradation was available.

In view of these reports, we initiated research to examine whether ubiquitination and proteasome-mediated degradation of nascent CYP2E1 protein occurred. A computer-derived molecular model of a predicted cytosolic domain of CYP2E1 was constructed and resulted in the identification of a putative ubiquitination target site (residues 317-340) that may also serve as a site for substrate interaction (Banerjee *et al.*, 2000). This region

contains two lysines (Lys^{317}, and Lys^{324}) that may serve as targets for ubiquitination. An affinity-purified CYP2E1 polyclonal antibody reactive to this domain was generated, and this antibody effectively quenched CYP2E1 protein ubiquitination in a concentration-dependent manner in a rabbit reticulocyte lysate-based ubiquitination assay system. Interestingly, this antibody also effectively inhibited rat hepatic microsomal CYP2E1-catalyzed chlorzoxazone 6-hydroxylation. These two observations suggest an association between the CYP2E1 cytosolic domain, which served as a target for ubiquitination, and its involvement in substrate binding or catalysis. This provides a plausible explanation for a mechanism in which the binding of a substrate shields this region of the CYP2E1 protein from ubiquitination and consequent turnover by the 26S proteasome (Banerjee *et al.*, 2000). It should be noted that this antibody should not have inhibited the CYP2E1 activity by affecting the P450 reductase binding site as this site is located ~25A away from the domain (i.e. residues 317-340) of interest based on our computer model (Banerjee *et al.*, 2000).

A very recent report provides evidence that CYP2E1 degradation may be influenced by the molecular chaperone hsp90 (Goasduff and Cederbaum, 2000). These authors stated that it was unclear as to how the CYP2E1 in the microsomal membrane became accessible to the cytosolic 26S proteosome, and hypothesized that molecular chaperones may be involved. The results of experiments employing geldanamycin and molybdate, compounds that modulate hsp90-protein interactions, as well as hsp90 immunodepletion experiments, suggested that hsp90 may be one of the factors interacting with CYP2E1 and/or with the 26S proteasome to promote the degra- dation of microsomal CYP2E1 (Goasduff and Cederbaum, 2000). Our model of CYP2E1(Banerjee *et al.*, 2000), however, would also suggest that a substantial region of CYP2E1 is cytosolic, and is readily available to the 26S proteasome.

Hormonal regulation of CYP2E1 expression

As stated previously, conditions such as diabetes (spontaneous and chemical-induced), fasting, obesity, high fat diet, and long-term alcohol consumption result in increased CYP2E1 expression at the mRNA and protein levels (~3- to 8-fold) in animals and humans (Favreau *et al.*, 1988; Bellward *et al.*, 1988; Song *et al.*, 1987; Dong *et al.*, 1988; Shimojo *et al.*, 1993; Hong *et al.*, 1987; Johansson *et al.*, 1988; Raucy *et al.*, 1991; Yun *et al.*, 1992; Ronis *et al.*, 1993; Song *et al.*, 1990; Takahashi *et al.*, 1993). These increases are accompanied by a corresponding increase in enzymatic activity. Diabetes, fasting, obesity, high fat diet, and long term alcohol consumption all result in altered nutritional status and metabolism (increased glucose, ketone body, and fatty acid levels), and altered hormone (insulin, growth hormone, glucagon) secretion. Elevation of CYP2E1 mRNA levels in the diabetic or fasted state in vivo has been attributed to mRNA stabilization (Song *et al.*, 1987). The elevated expression of CYP2E1 in these pathophysiological states has been largely attributed to alterations in metabolism (elevated ketone body levels (Bellward *et al.*, 1988; Dong *et al.*, 1988)) or hormone secretion (decreased growth hormone levels (Yamazoe *et al.*, 1989a, 1989b)). While insulin administration to diabetic rats has been shown to lower CYP2E1 to control levels (Favreau *et al.*, 1988; Dong *et al.*, 1988; Shimojo *et al.*, 1993; Donahue and Morgan, 1990), these effects have been attributed to the normalization of ketone body and/or growth hormone levels. However, a number of observations suggest that ketone body and growth hormone changes during diabetes may not be etiologic factors affecting CYP2E1 expression, and that insulin itself may play a prominent role in regulating CYP2E1 mRNA and protein levels. Insulinoma-bearing rats, which have increased levels of circulating insulin, but normal ketone body levels, exhibit decreased CYP2E1-dependent metabolic activity (Barnett *et al.*, 1992), suggesting that elevating insulin levels in vivo lowers CYP2E1 protein levels. The correlation between ketone body levels and CYP2E1 protein and enzymatic activity levels in a study utilizing spontaneously diabetic rats revealed that the correlation coefficient was only 0.36 for ketone body levels and CYP2E1 protein or aniline hydroxylase activity (Bellward *et al.*, 1988). Treatment of streptozocin-induced diabetic rats with low concentrations of vanadate decreased ketone body levels to near normal but did not increase insulin levels above those in diabetic animals (Donahue and Morgan, 1990). This vanadate treatment failed to decrease elevated CYP2E1 protein levels, suggesting that ketone bodies were not involved in the regulation of CYP2E1 expression. Moreover, CYP2E1 protein and aniline hydroxylase activity reached maximal levels in streptozocin-induced diabetic rats two weeks after streptozocin treatment, whereas ketone body levels continued to increase up to 5 weeks following streptozocin treatment (Shimojo *et al.*, 1993). When these diabetic rats were administered insulin, CYP2E1 protein and aniline hydroxylase activity levels decreased to control levels one week after initiation of insulin treatment, whereas ketone body levels continued to increase for one week following insulin administration and did not return to control levels until 3 weeks following initiation of insulin administration (Shimojo *et al.*, 1993). Thus, *in vivo* studies implicate insulin, rather than ketone bodies, in the regulation of CYP2E1 expression. The role of altered growth hormone levels in the elevation of CYP2E1 during diabetes is also questionable. Thummel and Schenkman (1990) found that both male and female diabetic rats exhibited increased CYP2E1 protein levels, but growth hormone levels were unaltered in the diabetic female rats, and administration of human growth hormone to

the diabetic male rats failed to reverse the diabetes-induced increase in CYP2E1. Son *et al.* (2000) also found that insulin, but not growth hormone, prevented the increase in CYP2E1 mRNA in diabetic rats.

Addressing the role of individual hormones or metabolic factors in regulating CYP2E1 expression *in vivo*, however, is exceptionally difficult given the diversity of hormonal and metabolic responses that occur *in vivo* during diabetes and related pathophysiologic conditions. To this end, our laboratory and others have used primary cultured hepatocytes, hepatoma cell lines, or cell lines stably expressing CYP2E1 to assess the role of individual metabolic factors, hormones, and growth factors in regulating CYP2E1 expression. Experiments in our laboratory have demonstrated that treatment of primary cultured rat hepatocytes with ketone bodies (acetoacetate, 3-hydroxy- butyrate) in the presence of 1 μM insulin did not elevate CYP2E1 mRNA levels (Zangar and Novak, 1997). Moreover, treatment of primary cultured rat hepatocytes cultured in the absence of insulin (more representative of the diabetic state) with concentrations of acetoacetate found during fasting and diabetes actually decreased CYP2E1 mRNA levels by as much as 95% (Woodcroft and Novak, unpublished observations), lending further support to the hypothesis that ketone bodies are not involved in the enhanced expression of CYP2E1 observed during diabetes. Experiments in our laboratory also indicate that treatment of primary cultured rat hepatocytes with growth hormone decreased CYP2E1 mRNA levels (Woodcroft and Novak, unpublished observations), even at the lowest concentration examined (50 ng/ml), which is in the physiological range of serum growth hormone in diabetic rats. These data suggest that insulin may play a more significant role in the regulation of CYP2E1 expression than ketone bodies or growth hormone.

We have further demonstrated that insulin itself, in the absence of other hormonal or metabolic alterations, regulates CYP2E1 mRNA and protein levels in primary cultured rat hepatocytes (Woodcroft and Novak, 1997, 1999a, 1999b). Culturing primary rat hepatocytes in the absence of insulin increased CYP2E1 mRNA and protein levels up to 12- and 7-fold, respectively (Woodcroft and Novak, 1997, 1999a), and replacement of insulin reversed the increase in CYP2E1 expression (Woodcroft and Novak, 1997). The insulin effect was concentration-dependent, with concentrations <10 nM resulting in increased CYP2E1 mRNA levels (Woodcroft and Novak, 1997). Alterations in insulin concentration had little effect on the levels of CYP2B, CYP3A, or CYP4A, suggesting that insulin-mediated regulation is selective for CYP2E1 (Woodcroft and Novak, 1997). Insulin has also been reported to decrease CYP2E1 mRNA and protein levels in Fao rat hepatoma cells (de Waziers *et al.*, 1995), and in HepG2 cells expressing a rabbit CYP2E1 minigene construct (Peng and Coon, 1998).

We have subsequently demonstrated that insulin causes destabilization of CYP2E1, but not CYP2B or CYP3A, mRNA (Woodcroft and Novak, 2000a) and also results in decreased CYP2E1 gene transcription (Woodcroft and Novak, unpublished observations) in primary cultured rat hepatocytes. de Waziers *et al.* (1995) have also reported that insulin increased the turnover of CYP2E1 mRNA and decreased CYP2E1 gene transcription in Fao rat hepatoma cells. Peng and Coon (1998) reported that insulin destabilized CYP2E1 mRNA in HepG2 cells expressing a rabbit CYP2E1 minigene construct; however, they reported no insulin effect on transcription of a rabbit CYP2E1 5' UTR/luciferase reporter construct expressed in HepG2 cells. Thus, insulin appears to regulate CYP2E1 mRNA expression at both transcriptional and post-transcriptional levels. The finding that decreased insulin concentrations enhance xenobiotic-mediated induction of CYP2E1 protein (Woodcroft and Novak, 1999a) suggests that insulin may also have effects on CYP2E1 protein expression unrelated to its effects on CYP2E1 mRNA expression.

Our laboratory has also employed primary cultured rat hepatocytes to demonstrate that glucagon, the physiological antagonist of insulin, elevates CYP2E1 mRNA levels in the absence of insulin, and that insulin and glucagon are mutually antagonistic in their effects on CYP2E1 mRNA expression (Woodcroft and Novak, 1999b).

Recently the expression of hepatic CYP2E1 in obese diabetic *ob/ob* mice and *fa/fa* Zucker rats has been examined (Enriquez *et al.*, 1999). These animals are genetic models of obesity and non-insulin dependent diabetes, and are characterized by insulin resistance, hyperinsulinemia, hyperglycemia, and hepatic steatosis. The *ob/ob* mice are deficient in leptin synthesis, whereas *fa/fa* Zucker rats have defective leptin receptor function. Leptin is a metabolic hormone involved in controlling fat accumulation and energy homeostasis (Pelleymounter *et al.*, 1995; Halaas *et al.*, 1995). CYP2E1 mRNA and protein levels were either unchanged or decreased in both models (Enriquez *et al.*, 1999). This is in contrast to the increase in CYP2E1 protein and activity found in rat dietary models of obesity (Raucy *et al.*, 1991). These results were interpreted to suggest that CYP2E1 is not obligatorily increased by obesity and diabetes, and further, that the absence of leptin or its functional receptor may account for the lack of enhanced CYP2E1 expression in these models (Enriquez *et al.*, 1999).

These authors subsequently reported a more detailed study of the expression of Cyp2e1 in leptin-deficient ob/ob mice (Leclercq *et al.*, 2000b). Cyp2e1 mRNA, protein, and activity levels were decreased in the obese mice as compared to their lean littermates. Leptin treatment of obese mice elevated Cyp2e1 levels to the levels monitored in lean mice. Leptin treatment of lean mice, however, failed to alter Cyp2e1 levels. Another report (Watson *et al.*, 1999) also demonstrated reduced Cyp2e1

catalytic activity in *ob/ob* mice relative to lean controls, which was reversed by leptin administration. Leptin treatment resulted in reduced food intake, and in obese mice pair-fed the reduced amount of food, Cyp2e1 mRNA levels were elevated but no increase was observed in either protein or Cyp2e1 catalytic activity (Leclercq *et al.*, 2000b). Fasting of obese or lean mice for 48 h resulted in elevated Cyp2e1 mRNA and protein levels and catalytic activity in both groups of animals. The authors conclude that full constitutive expression of Cyp2e1 requires the presence of leptin; however, the effects of leptin on Cyp2e1 expression cannot be accounted for solely through the leptin effect on hypothalamic control of food intake (Leclercq *et al.*, 2000b).

It is also possible that the decreased CYP2E1 expression in these obese non-insulin dependent diabetic animals is in part due to the hyperinsulinemia that is characteristic of these animal models, as insulin itself decreases CYP2E1 expression, as noted above (de Waziers *et al.*, 1995; Woodcroft and Novak, 1997, 1999a, 1999b; Peng and Coon, 1998). However, the overall effect of insulin signalling in the ob/ob mouse is unknown as these animals do exhibit insulin resistance. Leptin may restore Cyp2e1 levels by acting as a counter-regulatory hormone to insulin or by causing a reduction in the serum insulin concentration to near that monitored in lean controls (Leclercq *et al.*, 2000b). Whether leptin has direct effects on CYP2E1 expression apart from its influence on insulin levels and dietary regulation remains to be established.

Hypophysectomy has been reported to increase CYP2E1 mRNA and protein expression in rat liver (Chen *et al.*, 1999; Son *et al.*, 2000) and kidney (Chen *et al.*, 1999). Testosterone had no effect on hepatic CYP2E1 levels, but was required in conjunction with growth hormone to restore renal CYP2E1 to control levels in hypophysectomized rats (Chen *et al.*, 1999). Interestingly, hypophysectomized rats exhibited a reduction in plasma glucose and triglyceride levels, and glucose feeding restored CYP2E1 to control levels (Son *et al.*, 2000). Administration of growth hormone resulted in restoration of hepatic CYP2E1 levels to that monitored in sham-operated rats (Chen *et al.*, 1999; Son *et al.*, 2000), and also restored plasma glucose to control levels (Son *et al.*, 2000). In starving hypophysectomized rats, growth hormone administration failed to reduce the elevated CYP2E1 levels, whereas glucose feeding abolished the elevation in CYP2E1 expression (Son *et al.*, 2000). The authors interpreted these results to suggest that the increase in CYP2E1 expression caused by hypophysectomy may result from decreased glucose utilization, and that growth hormone restores CYP2E1 to control levels by enhancing glucose utilization (Son *et al.*, 2000).

Growth factors may also play a role in regulation of CYP2E1 expression. Our laboratory has found that epidermal growth factor (EGF), like insulin and growth hormone, decreases CYP2E1 mRNA levels in primary cultured rat hepatocytes in a concentration-dependent manner (Woodcroft and Novak, 2000b). The negative effect of EGF on CYP2E1 mRNA levels appears to involve both increased CYP2E1 mRNA turnover and decreased CYP2E1 gene transcription (Woodcroft and Novak, unpublished observations), as was found with insulin. Hepatocyte growth factor (HGF) was also found to decrease CYP2E1 mRNA levels, but with greater potency than EGF (Woodcroft and Novak, unpublished observations).

Role of kinase signalling pathways in regulation of *CYP2E1 expression*

The cellular effects of insulin are mediated by specific cell surface receptors which are members of a family of ligand-activated receptor tyrosine kinases (Ullrich and Schlessinger, 1990). Binding of a ligand to the insulin receptor's extracellular (-subunits results in a conformational change leading to autoactivation of the insulin receptor tyrosine kinase and phosphorylation of tyrosine residues in the cytoplasmic receptor α-subunits. Activation of this tyrosine kinase activity in the α-subunits, in addition to causing activation of the receptor via autophosphorylation, also results in the recruitment and phosphorylation of specific intracellular protein substrates which results in sequential activation of downstream signalling enzymes (Fig. 3). Thus, insulin, via the insulin receptor, exerts its downstream metabolic and mitogenic effects through these signalling proteins.

Insulin effects on cells can be broadly categorized into metabolic (stimulation of glucose transport, protein and glycogen synthesis, inhibition of lipolysis, and regulation of gene transcription and translation) and mitogenic (promotion of DNA synthesis and cell division) (Proud, 1996). The mitogenic effects of insulin are mediated primarily via activation of the Grb2-Sos/Ras/Raf/MEK/MAPK signalling pathway (White and Kahn, 1994). The metabolic effects of insulin are mediated primarily by activation of phosphatidylinositol 3-kinase (PI3-kinase) which results in the production of 3-phosphorylated phosphatidylinositides, which then serve to participate in the activation of a variety of downstream enzymes including the protein serine/threonine kinase Akt/PKB and p70 S6 kinase, a serine/threonine kinase which serves as an effector of some, but not all, of insulin actions mediated by PI3-kinase (Proud, 1996; Shepherd *et al.*, 1998). Src kinase ($pp60^{src}$) is also tyrosine phosphorylated in response to insulin (Luttrell *et al.*, 1989) and can serve as an activator of PI3-kinase activity (Hamaguchi *et al.*, 1993). The EGF receptor is a member of the same family of ligand-activated receptor tyrosine kinases as the insulin receptor (Ullrich *et al.*, 1990) and EGF-mediated mitogenic effects are usually mediated through a Ras/Raf/MEK/MAPK pathway; however, cross-talk does occur

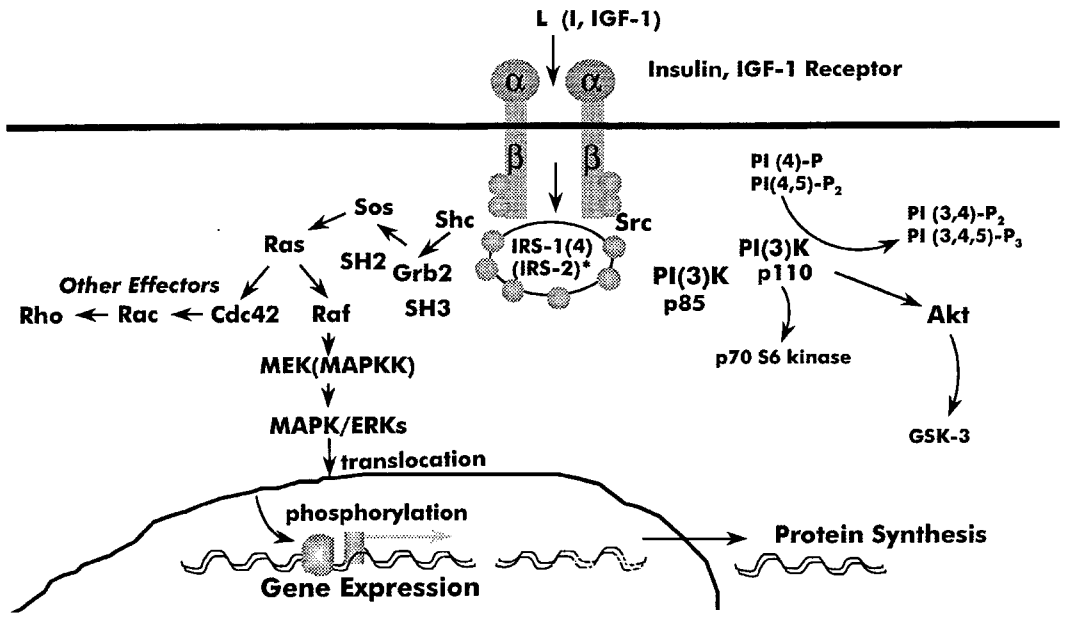

Fig. 3. Insulin receptor-mediated signalling pathways.

between most cellular signalling pathways.

Insulin signalling is regulated not only by a cascade of protein phosphorylation via tyrosine kinases and serine/threonine kinases, but also by dephosphorylation of many insulin-regulated enzymes by tyrosine phosphatases and serine/threonine phosphatases (Cohen, 1985).

Glucagon also plays a significant physiological role in the regulation of glucose and ketone body metabolism by regulating the expression of a number of enzymes involved in these functions (Granner *et al.*, 1986; Burcelin, *et al.*, 1996). Glucagon and insulin are physiological antagonists, and several enzymes important in cellular metabolism are regulated in an opposing manner by these hormones. The actions of glucagon are mediated by a membrane receptor, leading to increased cellular levels of cAMP and activation of protein kinase A (PKA) (Agati *et al.*, 1998).

Our laboratory has investigated, using primary cultured rat hepatocytes, the signalling pathways involved in insulin, EGF, and glucagon regulation of CYP2E1 expression. The PI3-kinase inhibitor wortmannin, or the Src kinase inhibitor geldanamycin, prevented the insulin-mediated decline in CYP2E1 mRNA. The MEK inhibitor PD98059, however, failed to inhibit the insulin-mediated decrease in CYP2E1 mRNA levels (Woodcroft and Novak, 1999b). Identical results were monitored for the EGF-mediated decrease in CYP2E1 expression; wortmannin and geldanamycin attenuated the EGF effect, whereas PD98059 failed to affect the EGF-mediated decrease in CYP2E1 mRNA levels (Woodcroft and Novak, 2000b). These results suggest that the negative regulation of CYP2E1 expression by insulin or EGF is mediated by the metabolic signalling pathway involving PI3-kinase and Src kinase, and not by the mitogenic pathway involving the MAP kinases.

Our laboratory has also observed that the phosphatase inhibitors okadaic acid and sodium orthovanadate caused a decrease in CYP2E1 expression in primary cultured rat hepatocytes equivalent to that caused by insulin. Moreover, these phosphatase inhibitors prevented the positive effect of geldanamycin on CYP2E1 expression (Woodcroft and Novak, unpublished observations). These findings further confirmed the involvement of kinase/phosphatase signalling pathways in insulin regulation of CYP2E1 expression.

Treatment of primary cultured rat hepatocytes with cell-permeable cAMP analogues resulted in the same positive effect on CYP2E1 mRNA levels as did glucagon treatment, and the PKA inhibitor H89 attenuated the glucagon-mediated increase in CYP2E1 mRNA levels (Woodcroft and Novak, 1999b). These findings support the involvement of cAMP and PKA as mediators of the positive effect of glucagon on CYP2E1 expression.

What is abundantly clear from the above discussions is that CYP2E1 mRNA and protein levels are regulated in response to pathophysiologic conditions and by a variety of pathways in response to different stimuli. The regulation of CYP2E1 expression is quite complex, involving transcriptional, post-transcriptional, translational, and post-translational mechanisms. CYP2E1 appears to be quite unique among CYPs in the complexity of its regulation of expression. CYP2E1 protein expression is enhanced in response to a variety of xenobiotics, many of which are CYP2E1 substrates and targets for bioac- tivation to hepatotoxic or carcinogenic products. CYP2E1 expression is also regulated, both positively and negatively, by hormones and growth factors, which

provides evidence that CYP2E1 expression is under tight homeostatic control.

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