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Insulin Suppresses the Induction of CYP2B1 and CYP2B2 Gene Expression by Phenobarbital in Adult Rat Cultured Hepatocytes

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The effect of insulin on the phenobarbital (PB)-induced gene expression of CYP2B1 and CYP2B2 (CYP2B1/2B2) in adult rat hepatocytes was investigated. Insulin, which has been regarded as an essential hormone for primary hepatocytes, was found to strongly suppress the induction of CYP2B1/2B2 gene expression in hepatocytes cultured on EHS-gel. Although the induction by PB was not seen in monolayer hepatocytes cultured on type I collagen under standard culture conditions, the induced expression of the CYP2B1/2B2 gene was observed in monolayer hepatocytes by removing insulin from the medium. Further, we succeeded in maintaining the prolonged induction of CYP2B1/2B2 by PB in monolayer hepatocytes by using a medium containing dexamethasone but not insulin. Since the PB-induced UDP-glucuronosyltransferase gene expression was not reduced by insulin, the suppressive effect of insulin was considered to be specific to the CYP2B1/2B2 gene. These results demonstrate that insulin in media masks the PB-induced expression of the CYP2B1/2B2 gene in conventional monolayer hepatocytes and that the use of insulin-free media with primary hepatocytes provides a useful tool for investigating the molecular mechanism of CYP2B1/2B2 gene expression.

Cytochrome P-450 (CYP) comprises a large family of hemoproteins and plays a vital role in the biotransformation of steroids, fatty acids and various kinds of xenobiotics (1). While a significant amount of information is available on the mechanism of transcriptional regulation of 3-methylcholanthrene-induced CYP1A1 gene expression (2), at present knowledge regarding the transcriptional regulation of phenobarbital (PB)-induced CYP2B1/2B2 gene expression is relatively little. One major problem in studying the transcriptional regulation of CYP2B1/2B2 gene is that there are no cell lines in which CYP2B1/2B2 gene is activated by PB (3). Although the primary hepatocyte culture has been useful for studying the mechanism by which xenobiotics induce cytochrome P-450 (4), even in the primary hepatocytes various differentiation functions including the responsiveness to PB are rapidly lost during culture under usual conditions (3-6).

Various efforts to observe enough induction of CYP2B1/2B2 in cultured hepatocytes have been made (4-7), and the most successful ways have revolved around the manipulation of extracellular matrices and tissue culture media. Several studies have established a method whereby primary hepatocytes can be reproducibly cultured on EHS-gel (a reconstituted extracellular basement membrane gel from Engelbreth-Holm-Swarm sarcoma) or in media of highly enriched formulation (4-6,8). We have found that hepatocytes not only attach effectively to

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Abbreviations: apo, apolipoprotein; ANOVA, analysis of variance; CYP, cytochrome P-450; Dex, dexamethasone; EHS, Engelbreth-Holm-Swarm; IRS, insulin responsive sequence; IRS1, insulin receptor substrate 1; PB, phenobarbital; PEPCK, phosphoenolpyruvate carboxykinase; PI-3 kinase, phosphatidylinositol-3 kinase; TAT, tyrosine aminotransferase; TIC, type I collagen; UGT, UDP-glucuronosyltransferase.

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EHS-gel but also maintain responsiveness to PB in media without serum and hormones (unpublished results). Thus, by using EHS-gel, the hormonal regulation of gene expression in hepatocytes can be studied in a serum- and hormone-free medium.

In the present study, we first demonstrate that insulin, which has been regarded as an essential hormone, has a strong suppressive effect on the PB-induced expression of CYP2B1/2B2 gene and that the prolonged induction of CYP2B1/2B2 by PB was achieved in monolayer hepatocytes cultured on type I collagen (TIC).

MATERIALS AND METHODS

Male rats of the Wistar strain (Japan SLC, Japan), 5-6 weeks of age and weighing about 90g, were used throughout the experiments. The rats were housed individually, and transferred to a semipurified diet (basal diet) (9) after fed a commercial nonpurified diet (CE-2, Japan Clea, Japan) for 3 days. In the experimental diet, 2 g of PB was added per kg of the basal diet. Diabetes was induced by intraperitoneal injection of streptozotocin (60 mg/kg). Rat parenchymal hepatocytes were isolated by perfusing liver with collagenase as described before (10) and cultured in Waymouth's MB 752/1 medium containing penicillin (5 IU/ml) and streptomycin (5mg/ml). Hepatocytes were plated at an initial density of 1×10^7 cells into a TIC coated dish (100mm, IWAKI, Japan) and an EHS-gel coated dish (11). RNA was extracted according to the method of Chomczynski and Sacchi (12), and the RNA containing samples were subjected to Northern blot analysis. The cDNA clones of rat CYP2B1 (13), rat phosphoenolpyruvate carboxykinase (PEPCK) (14), mouse apolipoprotein E (apo E) (15), rat UDP-glucuronosyltransferase 2 (UGT2) (16) and rat tyrosine aminotransferase (TAT) (17) were labeled with [5′α-^{32}P]dCTP using Megaprime DNA labeling system (Amersham, U.S.A.) and used for hybridization. Specific hybridization was quantified with BAS 2000 II (Fuji Film, Japan). The apo E mRNA level was not affected by any treatment, so we used it as a normalization standard. Microsomal fractions isolated by ultracentrifugation were subjected to Western blot analysis. The filters were treated with goat anti-rat CYP2B1 (Daiichi Kagaku, Japan) and rabbit anti-goat IgG with conjugated horseradish peroxidase (E-Y Laboratories, U.S.A.). Immunoreactive proteins were detected using ECL detection system (Amersham, U.S.A.). In animal experiments, the statistical significance of difference among values was analyzed by two-way analysis of variance (ANOVA). When the interaction (PB $\times$ diabetes) was significant, Student's t test was performed. The criterion for significance was $P < 0.05$ or $P < 0.01$, as specified.

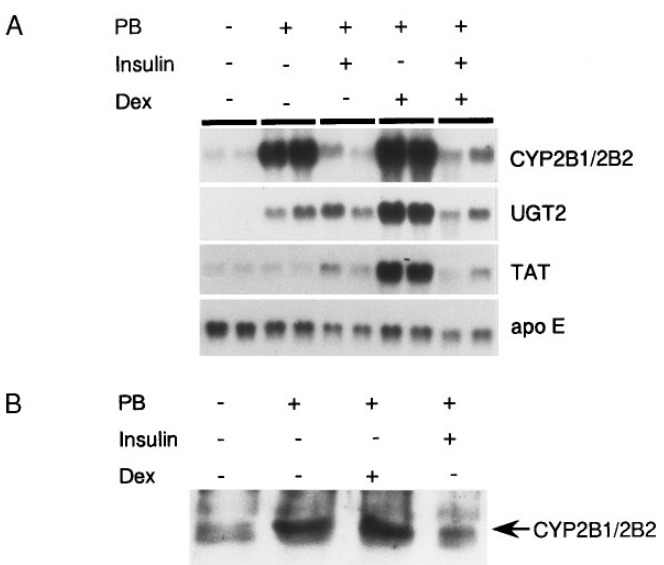

FIG. 1. Insulin suppresses the induction of CYP2B1/2B2 by PB in rat hepatocyte culture. Hepatocytes were cultured on plastic dishes coated with EHS-gel in a serum- and hormone-free medium for 24 h, and then treated with PB (2×10^{-3} M), insulin (5×10^{-8} M) and Dex (1×10^{-6} M) for a further 24 h. (A) Autoradiogram of Northern blot analysis of CYP2B1/2B2, UGT2, TAT and apo E mRNAs. (B) Western blot analysis of microsomal CYP2B1/2B2.

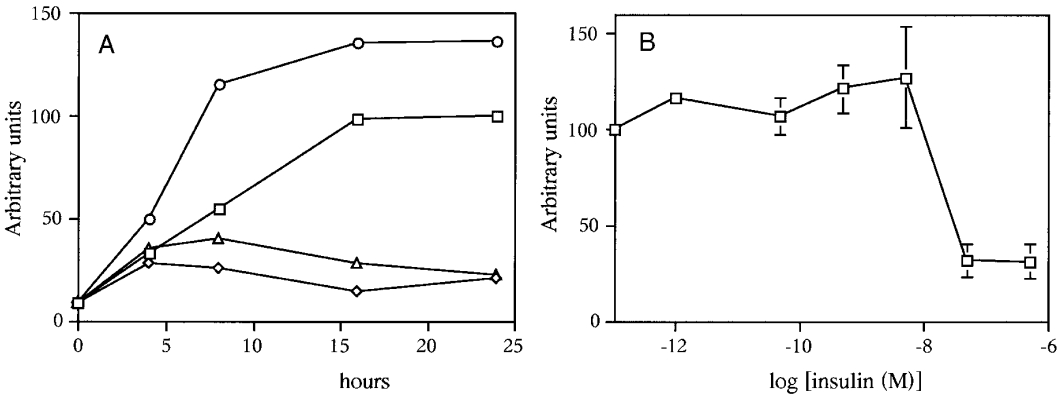

FIG. 2. Time-course and dose-response of the insulin effect on PB-induced CYP2B1/2B2 gene expression in rat hepatocyte culture. Hepatocytes were cultured on plastic dishes coated with EHS-gel in a serum- and hormone-free medium. (A) Hepatocytes were treated with PB (2×10^{-3} M), insulin (5×10^{-8} M) and Dex (1×10^{-6} M) for indicated time periods, and the cells were cultured for 48 h totally. (□) PB alone; (◇) PB plus insulin; (○) PB plus Dex; (△) PB plus insulin and Dex. (B) Hepatocytes were cultured on plastic dishes coated with EHS-gel in a serum- and hormone-free medium for 24 h, and the cells were treated with various concentration of insulin (1×10^{-12} to 5×10^{-7} M) in the presence of PB (2×10^{-3} M) for a further 24 h. Quantitative analysis of specific hybridization was performed with BAS 2000 II. Values of CYP2B1/2B2 mRNA were normalized with those of apo E mRNA. The CYP2B1/2B2 mRNA levels are expressed as percentage relative to the values of hepatocytes treated with PB alone for 24 h. Each values is expressed as means of two (A) or three (B) independent duplicates.

RESULTS

In contrast to hepatocytes cultured on EHS-gel, the induction of CYP2B1/2B2 by PB was barely detectable in the conventional monolayer hepatocytes cultured on TIC (4,5). We have already found that spherical hepatocytes cultured on EHS-gel maintain high responsiveness to PB in a serum- and hormone-free medium (unpublished results). Thus, we can now investigate the molecular mechanism of CYP2B1/2B2 gene expression by several hormones which have so far been added to culture media routinely. Insulin which is added to the conventional media used for hepatocyte culture is regarded as one of the essential hormones for primary hepatocytes. As shown in Fig. 1A, however, we found that this insulin strongly suppressed the PB-induced expression of CYP2B1/2B2 gene. The suppressive effect of insulin was also observed at the protein level (Fig. 1B). As demonstrated previously (6), dexamethasone (Dex) displays a synergy with PB in inducing CYP2B1/2B2 gene expression (Fig. 1A). To our surprise, insulin dramatically suppressed the PB- and Dex-induced CYP2B1/2B2 gene expression. UGT2 which is known to be a phase 2 drug-metabolizing enzyme is induced by PB (18). The induction of UGT2 by PB was not inhibited by insulin, although it inhibited the enhancement by Dex of PB-induced UGP2 gene expression. In monolayer hepatocytes cultured on TIC, PB-induced UGT2 gene expression was enhanced by insulin (data not shown). These results suggest that insulin dose not blunt the signal transduction pathway of PB. Rather, the suppressive effect of insulin seems to be specific to CYP2B1/2B2 gene.

Next, we determined the time-course and dose-response of the insulin effect on PB-induced CYP2B1/2B2 gene expression. The maximal induction of CYP2B1/2B2 gene expression by PB alone or PB plus Dex was obtained 16 h after the treatment (Fig. 2A). On the other hand, the suppressive effect of insulin was observed throughout the treatment (Fig. 2A). Enhancement of the induction by Dex was completely inhibited by insulin. The PB-induced expression of CYP2B1/2B2 gene was suppressed by insulin in a concentration-dependent manner (Fig. 2B).

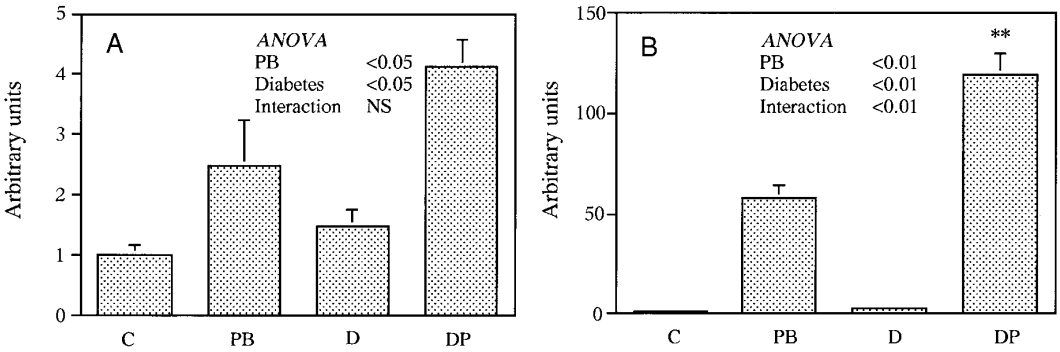

FIG. 3. Enhanced induction of CYP2B1/2B2 gene expression in streptozotocin-induced diabetic rats. Diabetes was induced by intraperitoneal injection of streptozotocin (60 mg/kg). (A) Diabetic and nondiabetic rats were treated with either PB (75 mg/kg, administered intraperitoneally) or saline. Livers of the rats were removed 2 h after PB-injection. (B) Diabetic and nondiabetic rats were fed a PB-diet (2 g PB/kg diet). Livers of the rats were removed 7 d after PB-feeding. Fifteen micrograms of RNA were used for Northern blot analysis. Quantitative analysis of specific hybridization was performed with BAS 2000 II. Values of CYP2B1/2B2 mRNA were normalized with those of apo E mRNA, and values of the control group (C) were set to 1. Vertical bars and lines are expressed as means and SEMs of 4-5 rats, respectively. Statistical significance of differences among values was analyzed by two-way ANOVA. NS, not significant ($P > 0.05$). **, the value differed significantly ($P < 0.01$) from the PB group using Student's *t* test. C, the control group; PB, the PB-treated group; D, the diabetic group; DP, the PB-treated diabetic group.

Treatment with 5×10^{-8} M insulin resulted in more than 70% reduction of the CYP2B1/2B2 mRNA level as compared to the values in the absence of insulin. Higher concentration of insulin no longer reduced the CYP2B1/2B2 mRNA level. The concentration of insulin necessary to suppress the PB-induced CYP2B1/2B2 gene expression is a little higher than its physiological concentration (1.5-5 nM) (19). In our conditions, however, the mRNA level of PEPCK, which is as well known inhibited by insulin transcriptionally in rat liver, was dramatically reduced at the same concentration of insulin (5×10^{-8} M) (data not shown). Therefore, it is conceivable that the physiological level of insulin inhibits the PB-induced CYP2B1/2B2 gene expression in rat liver.

In order to examine whether the inhibition of PB-induced CYP2B1/2B2 gene expression by insulin is observed in rat liver or not, we then measured the CYP2B1/2B2 mRNA level in streptozotocin-induced diabetic rats treated with PB. As indicated previously (20), the basal level (untreated with PB) of CYP2B1/2B2 mRNA was higher in diabetic rats than in normal rats (Fig. 3). The level of both acute and chronic inductions of CYP2B1/2B2 gene expression as determined after 2 h injection and 7 d PB-feeding, respectively, was greater in diabetic rats than in normal rats (Fig. 3). As expected, insulin-deficiency enhanced PB-induced CYP2B1/2B2 gene expression *in vivo*. These results led us to conclude that the physiological level of insulin inhibits the PB-induced expression of CYP2B1/2B2 gene.

Conventionally, insulin is added to culture media as an essential hormone for primary hepatocyte culture. We postulated that insulin in the media masked the PB-induced expression of CYP2B1/2B2 gene in monolayer hepatocytes. To investigate this possibility, we cultured monolayer hepatocytes on TIC in a medium deprived of insulin. A strong induction of CYP2B1/2B2 gene expression by PB was observed in the absence of insulin until 48 h, although the induction was not detected during the prolonged culture period (Fig. 4A). To establish a culture system that enables the prolonged induction of CYP2B1/2B2, we again determined the enhancing effect of Dex on CYP2B1/2B2 induction in the cells cultured on different matrices. Although Dex enhanced the induction on both TIC and EHS-gel, it is noteworthy

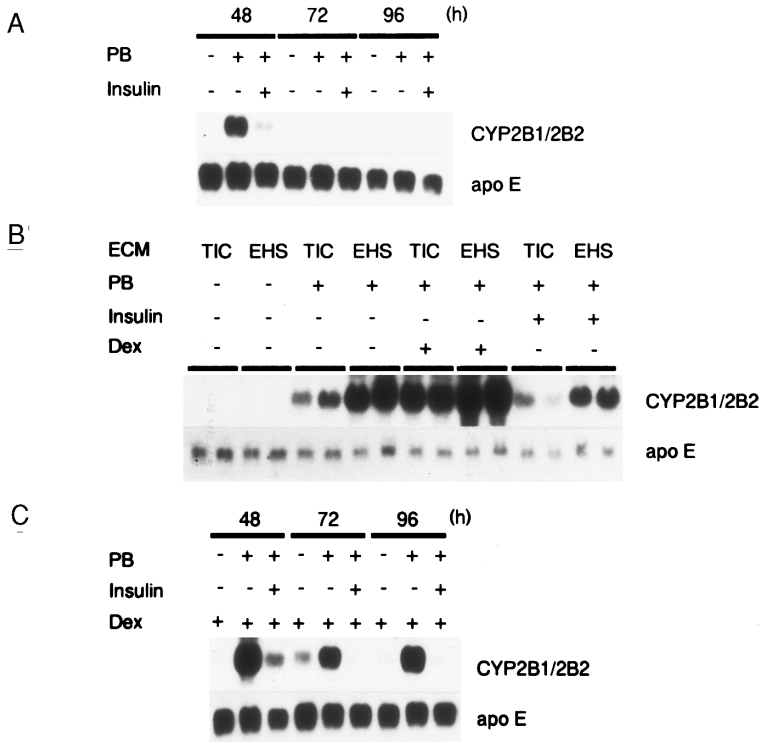

FIG. 4. Prolonged induction of CYP2B1/2B2 gene expression by PB in monolayer hepatocytes. (A) Hepatocytes were cultured on plastic dishes coated with TIC for 24, 48 or 72 h. Then the cells were treated with PB (2×10^{-3} M) and insulin (5×10^{-8} M) for a further 24 h. CYP2B1/2B2 and apo E mRNA levels are presented. (B) Hepatocytes were cultured on plastic dishes coated with TIC or EHS-gel in a serum- and hormone-free medium for 24 h. Then the cells were treated with PB (2×10^{-3} M), insulin (5×10^{-8} M) and Dex (1×10^{-6} M) for a further 24 h. (C) Hepatocytes were cultured on plastic dishes coated with TIC in a medium containing Dex (1×10^{-6} M) for 24, 48 or 72 h. Then the cells were treated with PB (2×10^{-3} M) and insulin (5×10^{-8} M) for a further 24 h. Fifteen micrograms of RNA was used for Northern blot analysis.

that the extent of the enhancement by Dex was much greater on TIC than on EHS-gel (Fig. 4B). The level of CYP2B1/2B2 mRNA induced by PB plus Dex on TIC was comparable to that induced by PB on EHS-gel. Then we investigated whether the addition of Dex enabled the long-term induction of CYP2B1/2B2 gene expression in the cells on TIC. When Dex was added, the induction of CYP2B1/2B2 gene expression was observed until 96 h (Fig. 4C).

DISCUSSION

Insulin is known to be potent in maintaining cultured hepatocytes, and the cells cultured in insulin-containing media are good at adherence and extension (21). In the present study, we have demonstrated for the first time that insulin has a strong suppressive effect on PB-induced CYP2B1/2B2 gene expression. Moreover, even in monolayer hepatocytes on TIC, the long-term induction was observed in media containing Dex but not insulin. There are some advantages in culturing hepatocytes on TIC: 1) TIC is easy to be coated and is inexpensive compared to EHS-gel and 2) the monolayer hepatocytes on TIC are able to be transfected with exogenous genes (22), while EHS-gel inhibits the transfection of primary hepatocytes (23).

The insulin signal-transduction pathway is highly complicated exhibiting an interesting

variation from activated insulin receptor. Binding of insulin results in phosphorylation of its receptor and of insulin receptor substrate 1 (IRS1). Phosphorylated IRS1 binds to the SH2 domains of several proteins including Grb2, phosphatidylinositol-3 kinase (PI-3 kinase) and Syp (24). From each signal transducer, the subsequent signaling pathway continues. In our preliminary experiments, an inhibitor of PI-3 kinase, wortmannin, did not affect the PB-induced CYP2B1/2B2 gene expression, indicating that PI-3 kinase appears not to be linked to the suppressive action of insulin (to be published).

As shown in Fig. 1A, insulin did not inhibit the induction of UGT2 by PB. This suggests that the ability of insulin to inhibit the PB-induced gene expression is specific to CYP2B1/2B2 gene. Insulin is well known to regulate the transcription of several genes either negatively or positively. An insulin responsive sequence (IRS) of ten base pairs (5′-TGGTGTTTTG-3′) in the promoter region of PEPCK gene mediates a part of the insulin response (25), and sequences with similarities to this PEPCK IRS are present in such other genes regulated by insulin as those encoding glucokinase, malic enzyme and apo CIII (26,27). It is interesting to note that two or three PEPCK IRS-like elements are found between −500 and +1bp of CYP2B2 or CYP2B1 gene, respectively. We are now examining whether these IRS-like elements are functional or not. Recently Waziers *et al.* (28) reported that insulin down-regulated the gene expression in Fao hepatoma cells in which CYP2B1/2B2 gene expression was not induced by PB, and concluded that insulin destabilized CYP2B1/2B2 mRNA. Transformed hepatoma cells have been demonstrated to be different from primary hepatocytes in many aspects. We speculate that insulin suppresses PB-induced CYP2B1/2B2 gene expression at both transcriptional and post-transcriptional levels as the case of PEPCK inhibition by insulin, and are currently investigating the effect of insulin on CYP2B1/2B2 gene expression at the both levels.

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