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Phenobarbital reduces blood glucose and gluconeogenesis through down-regulation of phosphoenolpyruvate carboxykinase (GTP) gene expression in rats

Hiroaki Oda*, Yuji Okuda, Yukiko Yoshida, Noriko Kimura, Atsushi Kakinuma

Laboratory of Nutritional Biochemistry, Department of Applied Molecular Biosciences, Nagoya University, Nagoya 464-8601, Japan

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ABSTRACT

The regulatory mechanism of phosphoenolpyruvate carboykinase (GTP) (EC 4.1.1.32) (PEPCK) gene expression and gluconeogenesis by phenobarbital (PB), which is known to induce drug-metabolizing enzymes, was investigated. Higher level of PEPCK mRNA was observed in spherical rat primary hepatocytes on EHS-gel than monolayer hepatocytes on TIC (type I collagen). We found that PB directly suppressed PEPCK gene expression in spherical hepatocytes on EHS-gel, but not in those on TIC. PB strongly suppressed cAMP-dependent induction of PEPCK gene expression. Tyrosine aminotransferase (TAT), another gluconeogenic enzyme, was induced by cAMP, but not suppressed by PB. Chronic administration of PB reduced hepatic PEPCK mRNA in streptozotocin-induced diabetic and nondiabetic rats, and PB reduced blood glucose level in diabetic rats. Increased TAT mRNA in diabetic rats was not suppressed by PB. These results indicated that PB-dependent reduction is specific to PEPCK. From pyrvate challenge test, PB suppressed the increased gluconeogenesis in diabetic rats. PEPCK gene promoter activity was suppressed by PB in HepG2 cells. In conclusion, we found that spherical hepatocytes cultured on EHS-gel are capable to respond to PB to suppress PEPCK gene expression. Moreover, our results indicate that hypoglycemic action of PB result from transcriptional repression of PEPCK gene and subsequent suppression of gluconeogenesis.

1. Introduction

Glucose homeostasis is maintained by hepatic production of glucose and uptake of glucose by peripheral tissue such as muscle and adipose tissue [1]. Gluconeogenesis mainly in the liver is adapted to the necessity of glucose, and it is induced under fasting and diabetic conditions. Phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) (PEPCK) and glucose 6-phosphatase catalyze the rate-limiting steps of gluconeogenesis. Rate of biosynthesis of PEPCK is well known to be regulated by several hormones such as glucagon, insulin, glucocorticoid and thyroid hormone [2,3]. Since hepatic PEPCK gene expression is expressed under diabetic condition, the induced expression of PEPCK gene is thought to be a good target for treatment of diabetes.

Phenobarbital (PB) is one of the various xenobiotics and induces diverse drug-metabolizing enzymes such as cytochrome P-450 (CYP) [4]. PB-induced CYP2B1/2B2 is suppressed by several protein kinase activators and protein phosphatase inhibitors in rat hepatocyte culture [5]. This indicated that phosphorylation/dephosphorylation steps are critical for the induction of these enxymes by PB. Activity and gene expression of glucose 6-phosphate dehydrogenase and malic enzyme are induced in the livers from PB-treated rats [6]. The increased flux of glucose into the pentose phosphate pathway might subsequently affect intracellular glucose metabolism.

The treatment of PB improves the hyperglycemic state of non-insulin-dependent diabetic patients [7]. It was reported that chronic PB treatment decreased PEPCK activity in isolated rat hepatocytes [8]. A report suggested that constitutive androstane receptor (CAR) was involved in gluconeogenesis and β-oxidation [9].

Abbreviation: apo, apolipoprotein; AUC, area under the curve; CAR, constitutive androstane receptor; CAT, chloramphenicol acetyltransferase; CREB, cAMP response element binding protein; CYP, cytochrome P-450; Dex, dexamethasone; ECM, extracellular matrix; EHS, Engelbreth-Holm-Swan; HNF, hepatocyte nuclear factor; PB, phenobarbital; PEPCK, phosphoenolpyruvate carboxykinase; PGC, peroxisome proliferator activated receptor gamma coactivator; STZ, streptozotocin; TAT, tyrosine aminotransferase; TIC, type I collagen.

* Corresponding author. Tel.: +81 52 789 4124; fax: +81 52 789 5050.
E-mail address: hirooda@agr.nagoya-u.ac.jp (H. Oda).

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In CAR-null mice, PB-repressed genes, PEPCK and carnitine parmitoyltransfease and so on, were not suppressed [9]. However, precise mechanism for suppressing PEPCK and changes in gluconeogenesis by PB is still unclear.

In the present study, we examined the effects of PB treatment on PEPCK gene expression in rat primary hepatocytes and type 1 diabetic rats. The suppressive action of PB on PEPCK gene expression reduced the blood glucose and gluconeogenic activity.

2. Materials and methods

Male Wistar rats weighing about 90 g (Japan SLC, Hamamatsu, Japan) were maintained at 24C with a 12-h light and dark cycle. Rats were fed a casein diet (control diet, 250 g/kg diet [5]). Diabetes was induced by intraperitoneal injection of streptozotocin (STZ, 60 mg/kg). PB (2 g/kg diet) was supplemented to the control diet for 7d. To estimate the rate of gluconeogenesis *in vivo*, the pyruvate challenge test was performed as described [10]. In the experiment of acute PB-treatment, after developing diabetic state, rats were injected with PB (75 mg/kg) and insulin (5 IU/kg) intraperitoneally. All rats were killed by decapitation 2 h after the injection. The procedures of animal experiments were approved by the Animal Research Committee of Nagoya University and were conducted according to the Regulations for Animal Experiments at Nagoya University.

Rat hepatocytes were prepared by perfusing liver with collagenase as described before [11], and were plated into a type I collage (TIC) coated dish or an EHS (Engelbreth Holm Swan)-gel coated dish [11]. Total cellular RNA was extracted and was subjected to Northern blot analysis. PEPCK-CAT (chloramphenicol acetyltransferase) reporter plasmid [12] and a vector containing RSV-β-galactosidase gene were transfected to HepG2 cells.

Statistical significance of treatment was analyzed by ANOVA. Then Duncan's multiple range test or Student's t-test were performed. *P* values of 0.05 were considered significance.

3. Results

We at first examined PEPCK gene expression in rat primary cultured hepatocytes. PEPCK mRNA level rapidly declined after hepatocytes were disaggregated (Fig. 1A). TAT gene expression also showed a similar to PEPCK gene expression (Fig. 1B). Although the level of PEPCK mRNA became under detectable in spread monolayer hepatocytes cultured on TIC without any hormones, spherical hepatocytes cultured on EHS-gel substantially expressed PEPCK gene. Since PEPCK gene expression was observed in cultured hepatocytes without hormones, we next investigated the effect of PB on PEPCK gene expression. As spherical hepatocytes cultured on EHS-gel maintain high responsiveness to PB in CYP-induction [13,14], we used this 3-dimensional culture system to investigate PEPCK gene expression by PB. PB was found to suppress PEPCK gene expression in hepatocytes on EHS-gel (Fig. 1C). But PB did not suppress TAT gene expression (Fig. 1D). It is well known that cAMP and Dex activate gene transcription of PEPCK and TAT genes [15]. Although cAMP increased PEPCK mRNA, Dex did not increase PEPCK mRNA in primary cultured hepatocytes (Fig. 1E). PB completely suppressed the cAMP-dependent induction of PEPCK gene expression. TAT gene expression was induced by Dex and cAMP, but not suppressed by PB (Fig. 1F). These results demonstrated that PB specifically suppresses the basal and cAMP-induced PEPCK gene expression in rat hepatocytes.

In order to examine the inhibitory effect of PB on PEPCK gene expression in rat liver, we treated STZ-induced diabetic rats with PB. The increased level of blood glucose in diabetic rats was significantly reduced by PB on d 2 and 4 (Fig. 2A). Hepatic PEPCK mRNA was higher in diabetic rats than in nondiabetic rats. Chronic treatment of PB (7d) dramatically induced CYP2B gene expression (data not shown) [13] and repressed PEPCK gene expression in both diabetic and nondiabetic rats (Fig. 2B). However, TAT gene expression in the liver was not affected by PB (Fig. 2C). Acute treatment of PB (2 h), which is enough to induce CYP2B gene expression (data not shown) [13], did not suppress PEPCK gene expression, although insulin effectively suppressed it (Fig. 2D). TAT was not also suppressed by acute treatment of PB (Fig. 2E). These results obtained in rat liver indicated that chronic treatment of PB specifically suppressed PEPCK gene expression in rat liver. These results led us to postulate that PB-dependent suppression of PEPCK gene expression might be responsible for the reduction of blood glucose by PB. Moreover, the mechanism by which PEPCK gene expression was repressed was specific for PEPCK gene.

To elucidate the mechanism for the reduction of plasma glucose in rats fed PB, we investigated if PB suppressed gluconeogenic activity from pyruvate (pyruvate challenge test). Since pyruvate is preferentially used by the liver as a substrate for gluconeogenesis, pyruvate challenge is good tool to examine gluconeogenic activity *in vivo*. Blood glucose concentration in nondiabetic rats did not so much increase after pyruvate injection and returned to the basal levels within 120 min (Fig. 3A). This is probably because rats were fasted in daytime before pyruvate challenge In diabetic rats, blood glucose concentration increased compared with nondiabetic rats in 30 min after pyruvate injection. Administration of PB to diabetic rats suppressed the rapidly increased blood glucose level, blood glucose increased gradually until 90 min (Fig. 3A). To compare the gluconeogeneic activity from pyruvate quantitatively, we measured the incremental glucose area under the curve (AUC) (Fig. 3B). The AUC of pyruvate challenge in diabetic rats was larger than that in nondiabetic rats. The gluconeogenesis was significantly suppressed by PB treatment. These result suggests that PB-dependent suppression of gluconeogenesis from pyruvate is responsible for the reduced blood glucose by PB.

Finally, in order to examine effect of PB on transcription of PEPCK gene, PEPCK gene promoter linked to CAT was transfected HepG2 cells. The reporter construct which contains −490 to +73 of rat PEPCK gene promoter can respond to extracellular stimuli such as cAMP, insulin and glucocorticoid [12]. In the present study cAMP induced CAT activity, and PB suppressed the cAMP-induced CAT activity (Fig. 4). This result showed that PB directly suppressed PEPCK gene transcription induced by cAMP.

4. Discussion

Increased hepatic glucose output is a central factor for hyperglycemia in both type 1 and type 2 diabetes [16], and it is reported that increased gluconeogenesis in the liver results in glucose intolerance in diabetic animal models [17]. PEPCK, which is one of the rate-limiting enzymes of gluconeogenesis, is regulated by various hormones at a transcription level [18]. It is thought that suppression of gluconeogenesis or PEPCK gene might be an effective target to ameliorate hyperglycemia in diabetic animals. Insulin is known to be a unique hormone to reduce blood glucose level.

At the present study, we succeeded that substantial amount of PEPCK mRNA was observed in primary hepatocytes cultured on EHS-gel in a medium without any hormones. Hepatocytes cultured on EHS-gel exhibit a spherical morphology and have higher mRNAs for liver-specific genes such as HNF-4 (hepatocytes nuclear factor 4), albumin, apo A-I, TAT and induction of CYP2B1/2B2 as compared with monolayer hepatocytes [11,14]. Higher amount of HNF-4 mRNA would be responsible for the higher amount of PEPCK mRNA, since HNF-4 is known to be a positive regulator of PEPCK gene expression [19]. Therefore, PB-dependent reduction of PEPCK