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EFFECTS OF THIAMINE DEFICIENCY ON HEPATIC CYTOCHROMES P450 AND DRUG-METABOLIZING ENZYME ACTIVITIES

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Abstract—To elucidate the mechanisms by which thiamine deficiency affects hepatic microsomal monooxygenase activities, the effect of thiamine deficiency on two constitutive cytochrome P450 isozymes, P450IIE1 and P450IIC11, was investigated, using weanling male Sprague–Dawley rats. The clinical signs of thiamine deficiency were apparent after feeding a thiamine-deficient diet for 3 weeks. Thiamine deficiency caused an increase in P450IIE1, which was determined by *N*-nitrosodimethylamine demethylase assay and immunoquantitation of P450IIE1. This increase in the P450IIE1 level was mainly attributed to thiamine deficiency *per se* but not to dietary restriction. Ketone bodies were not elevated in thiamine-deficient rats, whereas ketone bodies were elevated and may have served as inducing factors in calorically restricted pair-fed animals. Injections of pyruvate or pyrithiamine in addition to thiamine deficiency did not potentiate the induction effect. On the other hand, thiamine deficiency did not affect the level of P450IIC11 during the 3 weeks of feeding the thiamine-deficient diet. In addition, thiamine deficiency increased cytosolic glutathione *S*-transferase activity but not steroid isomerase activity. The present study demonstrates the specificity of thiamine deficiency *per se* in the induction of P450IIE1 which does not involve an increase in the ketone body level.

A number of studies have demonstrated that thiamine deficiency, which usually occurs after 2–3 weeks of feeding a thiamine-deficient diet to rats [1, 2], affects the *in vitro* hepatic microsomal metabolism of many compounds. Thiamine deficiency increases the concentrations of cytochromes P450 (P450†) and cytochrome b_5 (b_5), and NADPH:P450 reductase activity [3, 4]. Thiamine deficiency increases the metabolism of aminopyrine, ethylmorphine, zoxazolamine, heptachlor, aniline, *N*-methylaniline, acetanilide, and benzo[*a*]pyrene [3–9], but not of hexobarbital [5, 8]. In addition, increased metabolism of aniline was observed when a thiamine antagonist, pyrithiamine or oxythiamine, was injected into rats fed a thiamine-sufficient diet [6]. The effect of thiamine deficiency on the increased metabolism was not attributed to a caloric restriction due to a loss of appetite observed in the deficient group, because pair feeding did not cause such increase in the metabolism [3, 9]. Thiamine deficiency also increases the *in vivo* metabolism of acetaminophen and *N*-nitrosodimethylamine (NDMA) [1, 2]. The increased metabolism of acetaminophen caused by thiamine deficiency returns to normal when thiamine is given intraperitoneally to deficient animals 24 hr before the administration of the drug [2]. On the other hand, the *in vitro* metabolism of NDMA, measured as a function of the formation of formaldehyde or carbon dioxide, is not increased as a result of thiamine deficiency [1]. This result is rather puzzling and is not consistent with the results obtained by Wade *et al.* [4] for the metabolism of NDMA and aniline. Previous studies showed a good positive correlation between the metabolism of NDMA and aniline [10, 11]. Furthermore, the mechanisms of the enhancing effect of thiamine deficiency on the P450-dependent drug metabolism have not been clearly elucidated.

Our laboratory has studied the induction mechanism of P450IIE1‡ and proposed that ketone bodies may play an important role in P450IIE1 induction [18]. The present work was undertaken to study the mechanisms of P450 induction by thiamine deficiency, by measuring the quantities of specific P450 isozymes immunochemically as well as by determining related microsomal enzyme activities. We report herein that thiamine deficiency *per se* caused increases in NDMA demethylase (NDMAd) activity and in the level of P450IIE1. However, there was no corresponding increase in the serum levels of ketone bodies, indicating the involvement of other factors for P450IIE1 induction under thiamine deficiency. On the other hand, thiamine deficiency did not affect the induction of P450IIC11.

MATERIALS AND METHODS

Chemicals. NDMA was purchased from the Aldrich Chemical Co. (Milwaukee, WI). Benzphet-

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† Abbreviations: P450, cytochromes P450; b_5, cytochrome b_5; NDMA, *N*-nitrosodimethylamine; and NDMAd, NDMA demethylase.

‡ The nomenclature of P450 isozymes follows the recommendation in Refs. 12 and 13: P450IIE1 (ethanol-inducible subfamily) has been referred to as P450ac by Patten *et al.* [14] and as P450j by Ryan *et al.* [15]; P450IIC11 (male specific subfamily) has been referred to as P450 UT-A by Guengerich *et al.* [16] and P450h by Ryan *et al.* [17].

amine·HCl was provided by the Upjohn Co. (Kalamazoo, MI). 1-Chloro-2,4-dinitrobenzene, cytochrome *c*, 2,4-dinitrophenylhydrazine, glucose-6-phosphate (disodium salt), glucose-6-phosphate dehydrogenase (Type XV), glutathione (reduced), NADP, NADPH, pyrithiamine·HBr, pyruvate (sodium salt), and semicarbazide·HCl were obtained from the Sigma Chemical Co. (St Louis, MO). Δ^5-Androstene-3,17-dione was from Steraloids Inc. (Wilton, NH). Reagents for electrophoresis and immunoblotting were obtained from sources described previously [19, 20], and all other chemicals were reagent grade from commercial sources.

Animals, diets, and treatments. Weanling male Sprague–Dawley rats from Taconic, Inc. (Germantown, NY), body weights of 45–50 g, were maintained in individual wire-bottom cages in air-conditioned quarters with a 12-hr light and 12-hr dark cycle. After 5 days of acclimation, twenty-four rats were randomly assigned to six groups (four rats/group). Group 1 (control) received the AIN-76A rodent diet* [21, 22]; Group 2 (pair-fed), the AIN-76A diet pair-fed to Group 3 with a meal feeding during the last 24 hr before the animals were killed; the Group 3 (deficient), a thiamine-deficient diet† for which thiamine was omitted from the AIN-76A diet. Groups 4 to 6 received the following treatments in addition to the thiamine-deficient diet: Group 4, intraperitoneal injection of sodium pyruvate (2 g/kg body weight per day) along with 5% sodium pyruvate in drinking water for 3 consecutive days; Group 5, intraperitoneal injection of pyrithiamine·HBr (1 mg/kg per day) for 3 consecutive days; and Group 6, intraperitoneal injection of thiamine·HCl (10 mg/kg per day) for 1 day, with the last injection given 24 hr before the animals were killed. All six groups received water and their respective diets (except for Group 2) *ad lib.* for 3 weeks, during which time body weight was measured weekly and food intake was measured twice a week. At the end of the 3 weeks of feeding, all the rats were killed, and blood and liver samples were collected. Cytosolic and microsomal fractions were prepared from the liver samples [23].

Enzyme assays. Protein, microsomal P450, and b_5 contents were determined [23, 24]. Microsomal NADPH:P450 reductase and NADH:b_5 reductase activities were assayed at room temperature using cytochrome *c* as an artificial electron acceptor [25]. Microsomal NDMAd and benzphetamine demethylase activities were determined [26, 27]. In brief, the assay mixture contained (in a total volume of 1.0 mL) 50 mM Tris·HCl (pH 7.0 at 37°), 10 mM $MgCl_2$, 150 mM KCl, an NADPH-generating system (0.4 mM NADP, 10 mM glucose 6-phosphate, and 0.4 units glucose-6-phosphate dehydrogenase), microsomes (0.6 to 0.8 mg protein), and substrate (NDMA or benzphetamine, 1 mM final concentration). At termination of a 20-min incubation, the assay mixture was centrifuged, and 0.7 mL supernatant fraction was used for determination of formaldehyde formed [27].

Cytosolic glutathione *S*-transferase activity was determined by measuring the formation of the conjugate of reduced glutathione and 1-chloro-2,4-dinitrobenzene at room temperature according to Warholm *et al.* [28]. Cytosolic steroid isomerase activity was measured at room temperature according to Benson and Talalay [29] using Δ^5-androstene-3,17-dione as substrate.

Determination of serum levels of β-hydroxybutyrate and acetone. β-Hydroxybutyrate was measured using a commercially available enzymatic reagent kit (Cat. No. 310-A; Sigma). Acetone was measured by a modification of the method of Farrelly [30] using a high performance liquid chromatographic separation of acetone after the derivatization of acetone with 2,4-dinitrophenylhydrazine [31]. In brief, to 0.2 mL of serum was added 0.02 mL of 50 mM semicarbazide·HCl (pH 7.0), and an aliquot (0.02 mL) of this preparation was added to a tube containing 1 mL H_2O, 0.1 mL of 0.25% 2,4-dinitrophenylhydrazine reagent in 6 N HCl, and 1.5 mL hexane. The tube was capped and shaken for 1 hr. The hexane layer (0.5 mL) was then added to 0.25 mL acetonitrile followed by mixing. An aliquot (0.125 mL) of the acetonitrile layer was transferred to a sample vial which was subsequently loaded onto a WISP autoinjector (Waters Associates, Milford, MA), and 0.05 mL of sample was injected onto a high performance liquid chromatographic column. The column (5 × 100 mm) was Rad-Pak C_{18} on 10-μm silica fitted in an RCM-100 Module (Waters). The mobile phase was 65% acetonitrile (in H_2O) at a flow rate of 1.1 mL/min. The peak of the dinitrophenylhydrazone derivative of acetone had a retention time of 5.4 min. It was monitored by a model 440 UV detector (Waters) at a wavelength of 340 nm and quantified by its peak area which was determined with an integrator. For acetone standard, two concentrations (170 and 340 μM) were run simultaneously with the samples, and the linearity of the acetone standard concentrations was confirmed. Under this assay condition, about 10% of the acetone level measured was derived from acetoacetate present in the sample, but this would not affect the interpretation of results.

Immunoblot analysis. Purified P450IIE1 and polyclonal antibodies against P450IIE1 were prepared as described previously [20]. Purified P450IIC11 and polyclonal antibodies against P450IIC11 were provided by Dr. F. P. Guengerich (Vanderbilt University School of Medicine, Nashville, TN). Immunoblot analysis of liver microsomal proteins using anti-P450 IgG was performed by a modification of the method of Guengerich *et al.* [32] as described previously [20]. Intensities of immunostained bands were measured using a Shimadzu Dual-wavelength Thin-layer Chromato Scanner (model CS-930; Shimadzu Corp., Kyoto, Japan).

* Sources of dietary ingredients are as follows: casein (vitamin-free), DL-methionine, corn starch, sucrose (granular), fiber (cellulose), AIN-76 mineral mix, AIN-76A vitamin mix, and choline bitartrate were purchased from Teklad (Madison, WI). Mazola corn oil (Lot No. 28647) was a gift from Best Foods (Union, NJ).

† AIN-76A vitamin mix (Teklad) was replaced by thiamine-free AIN-76A vitamin mix from ICN Biochemicals (Cleveland, OH).

Table 1. Effects of thiamine deficiency on body weight, food intake, and liver weight

Group	Body weight gain (g)			Food intake (g)			Liver wt (% body weight)
	Week 1	Week 2	Week 3	Week 1	Week 2	Week 3	
1	56.5 (5.8)	55.8 (5.4)	55.5 (5.5)	94.3 (3.4)	139.5 (9.2)	142.5 (11.2)	5.9 (0.3)
2	54.3 (5.9)	22.8* (4.1)	16.0* (4.5)	85.3 (1.0)	86.0* (0.0)	77.3* (1.5)	4.0* (0.3)
3	56.3 (4.8)	3.5* (2.1)	−22.8* (5.6)	92.3 (13.1)	88.3* (2.2)	65.0* (8.6)	4.1* (0.3)
4	62.5 (2.9)	8.8* (2.7)	−25.3* (4.7)	87.5 (3.9)	97.5* (10.0)	39.3* (4.9)	4.3* (0.3)
5	57.8 (7.5)	6.0* (2.9)	−23.3* (6.3)	90.8 (10.1)	94.3* (16.0)	37.5* (3.1)	3.8* (0.2)
6	60.8 (2.4)	9.5* (3.7)	−6.5* (3.1)	89.5 (6.5)	99.8* (7.1)	55.3* (12.3)	6.8 (0.7)

Dietary regimens are as follows: Group 1, control; Group 2, pair-fed; Group 3, deficient; Group 4, deficient + pyruvate; Group 5, deficient + pyrithiamine; and Group 6, deficient + thiamine. Values are means (SD) of four rats.

* Significantly different ($P < 0.01$) from Group 1 by Student's *t*-test.

RESULTS

Effects of thiamine deficiency on body weight gain, food intake, and liver weight. Apparent clinical signs for thiamine deficiency were observed at the end of the second week of feeding a thiamine-deficient diet as assessed by body weight gain and food intake (Table 1). Furthermore, a decrease in food intake in the deficient group (Group 3) was remarkable during the third week, which was reflected on actual loss in body weight, whereas the pair-fed group (Group 2) showed slow but steady growth during the 3-week period. These observations for the progress of thiamine deficiency are consistent with those reported in the literature [2, 8, 9]. Injections of pyruvate or pyrithiamine in addition to the thiamine-deficient diet (Groups 4 and 5) did not affect the growth rate, although they appeared to suppress food intake during the injection period. An injection of thiamine 24 hr before sacrifice restored food intake promptly, which was reflected by rapid gains in body weight, food intake, and liver weight. This is in agreement with the reported observation [2].

Effects of thiamine deficiency on serum concentrations of ketone bodies. We reported previously that fasting 1–3 days induces NDMAd activity and P450IIE1 [27, 33]. To avoid a partial fasting effect usually encountered in a pair-feeding experiment, the last daily portion of the diet for the pair-fed group was divided into three aliquots and fed 12, 18, and 24 hr before the animals were killed. This protocol, however, could not abolish the fasting effect as presented by the significantly elevated levels of acetone and β-hydroxybutyrate (Table 2). In contrast, thiamine deficiency (Group 3) did not affect the ketone body levels despite the low food intake. This could be attributed to the fact that the deficient group, because of smaller body size, required lower caloric intake than the pair-fed group, thereby causing the less severe fasting effect. On the other hand, injections of pyruvate or pyrithiamine (Groups 4 and 5) produced slightly higher levels of ketone bodies than the deficient group (Group 3) due to a lower food intake during the injection period (as presented in Table 2).

Effect of thiamine deficiency on cytosolic and microsomal enzymes. Thiamine deficiency (Groups 3–5) induced cytosolic glutathione *S*-transferase activity significantly ($P < 0.01$) but not steroid isomerase activity (Table 3). Thiamine deficiency (Group 3) caused a significant increase ($P < 0.01$) in P450 content over the control group. In addition, cytochrome *c* reductase activities were increased significantly ($P < 0.01$) by thiamine deficiency. However, injections of pyruvate or pyrithiamine (Groups 4 and 5) did not increase P450 content or reductase activities. The reason for this observation is not clear. Although thiamine deficiency (Groups 3–5) appeared to increase b_5 content, such increases were not statistically significant.

Effects of thiamine deficiency on P450 isozymes and their activities. To examine whether the effects of thiamine deficiency on P450 isozymes are specific, we determined microsomal NDMAd and benzphetamine demethylase activities which have been demonstrated to be good substrates for P450IIE1 and P450IIC11 respectively [34, 35]. Thiamine deficiency (Group 3) increased NDMAd activity significantly ($P < 0.01$) over control group (Group 1, Table 4), which is consistent with the observation by Wade *et al.* [4]. The pair-fed group (Group 2), which was fed the diet by the meal-feeding pattern for 24 hr before being killed, also showed a significant increase ($P < 0.01$) in NDMAd activity. However, the increase in NDMAd activity in the deficient group (Group 3) was significantly higher ($P < 0.01$) than that in the pair-fed group. Injections of pyruvate or pyrithiamine (Groups 4 and 5) did not cause a further increase in NDMAd activity. An injection of thiamine 24 hr before the animals were killed (Group

Table 2. Effects of thiamine deficiency on serum concentrations of ketone bodies

Group	Dietary regimen	Acetone (μM)	β-Hydroxybutyrate (μM)
1	Control	33 ± 12	154 ± 35
2	Pair-fed	136 ± 40*	783 ± 201*
3	Deficient	50 ± 17	174 ± 20
4	Deficient + pyruvate	89 ± 49	248 ± 123
5	Deficient + pyrithiamine	106 ± 57	353 ± 191
6	Deficient + thiamine	28 ± 8	111 ± 52

Values are means ± SD of four rats.
* Significantly different ($P<0.01$) from Group 1 by Student's *t*-test.

Table 3. Effects of thiamine deficiency on cytosolic and microsomal enzymes

Group	Dietary regimen	Glutathione *S*-transferase (units)	Steroid isomerase (units)	Cytochrome (nmol/mg protein)		Cytochrome *c* reductase (units)	
				P450	b_5	NADPH	NADH
1	Control	479 ± 48	52.6 ± 10.9	0.70 ± 0.08	0.41 ± 0.11	220 ± 26	1155 ± 100
2	Pair-fed	553 ± 100	51.2 ± 14.9	0.90 ± 0.12	0.45 ± 0.06	181 ± 21	776 ± 59*
3	Deficient	654 ± 64*	43.8 ± 17.8	0.96 ± 0.07*	0.77 ± 0.18	316 ± 19*	1483 ± 105*
4	Deficient + pyruvate	615 ± 35*	39.2 ± 12.5	0.82 ± 0.04	0.73 ± 0.15	231 ± 13	1201 ± 124
5	Deficient + pyrithiamine	675 ± 38*	57.6 ± 10.5	0.73 ± 0.09	0.71 ± 0.14	267 ± 32	1126 ± 103
6	Deficient + thiamine	561 ± 79	38.6 ± 14.5	0.60 ± 0.10	0.68 ± 0.12	241 ± 20	1362 ± 144

One unit of glutathione *S*-transferase, steroid isomerase, and cytochrome *c* reductase activities corresponds to 1 nmol product formed/min/mg protein. Values are means ± SD of four rats.
* Significantly different ($P < 0.01$) from Group 1 by Student's *t*-test.

Table 4. Effects of thiamine deficiency on P450 isozymes and their activities

Group	Dietary regimen	NDMAd (nmol/min/mg)	P450IIE1 (units)	Benzphetamine demethylase (nmol/min/mg)	P450IIC11 (units)
1	Control	0.86 ± 0.16	100	9.04 ± 0.32	100
2	Pair-fed	1.45 ± 0.22*	228	8.90 ± 0.50	91
3	Deficient	2.17 ± 0.22†	496	9.36 ± 0.26	97
4	Deficient + pyruvate	1.91 ± 0.20*	422	9.94 ± 0.90	87
5	Deficient + pyrithiamine	2.33 ± 0.25†	503	10.18 ± 0.84	94
6	Deficient + thiamine	1.25 ± 0.19	241	9.14 ± 0.52	95

Final concentrations of NDMA and benzphetamine were 1 mM. Values are means ± SD of four rats for NDMAd and benzphetamine demethylase activities. Units of P450IIE1 and P450IIC11 are relative densitometric intensities of immunostained bands of pooled microsomal samples with the value of Group 1 set at 100.
* Significantly different ($P < 0.01$) from Group 1 by Student's *t*-test.
† Significantly different ($P < 0.01$) from Groups 1, 2, and 6 by Student's *t*-test.

6) did not abolish the induction effect of thiamine deficiency on NDMAd activity, but diminished it significantly ($P < 0.01$) from the deficient group (Group 3). The significant increase in NDMAd activity in deficient groups (Groups 3–5) was attributed to the actual induction of P450IIE1 isozyme as measured by immunoblotting (Table 4 and lanes D–F of Fig. 1). A high correlation ($r = 0.986$, $P < 0.01$) between the NDMAd activity and the P450IIE1 content confirms our previous observation (Table 4) [31]. On the other hand, thiamine deficiency did not affect benzphetamine demethylase

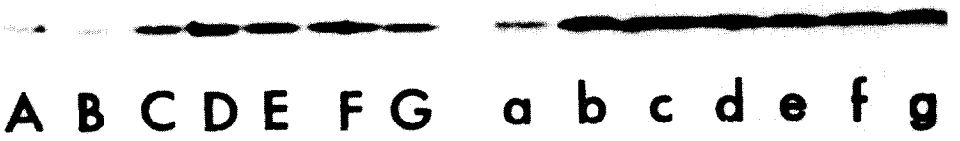

Fig. 1. Immunoblots of rat liver microsomal proteins. Lanes A–G, immunoblots using polyclonal antibodies against P450IIE1 and lanes a–g, immunoblots using polyclonal antibodies against P450IIC11; A, purified P450IIE1 (0.13 μg protein/lane) and a, purified P450IIC11 (0.34 μg/lane); B to G and b to g, rat liver microsomal samples (8.0 μg/lane) from Groups 1 to 6 (Table 1) in order.

activity or P450IIC11 content as determined by immunoblotting (Table 4 and lanes b–g of Fig. 1). However, the benzphetamine demethylase activity rather appeared to increase with development during the 3-week experimental period regardless of dietary regimen (data not shown).

DISCUSSION

During the past several years, we have studied P450IIE1 extensively and have demonstrated the following points: (a) P450IIE1 is not inducible by traditionally known inducers such as phenobarbital, 3-methylcholanthrene, and Aroclor 1254, but is rather inducible several-fold by pathophysiological conditions such as fasting and diabetes, and by pretreatment with compounds such as acetone, ethanol, and isopropanol [34]; and (b) P450IIE1 has high affinity for NDMA and aniline, and is primarily responsible for the bioactivation of NDMA to an alkylating and mutagenic species [11, 36]. We have also studied the different molecular mechanisms involved in the induction of P450IIE1 [31, 37–39].

The availability of specific antibodies has allowed us to measure specific P450 isozymes of interest by using an immunoblotting technique. Immunoquantitation of specific P450 isozymes in addition to related enzyme activities with high specificities will thus provide us with better understanding of the data. To test whether thiamine deficiency induces P450 isozymes selectively, we chose two constitutive P450 isozymes, P450IIE1 and P450IIC11. Our present data demonstrate that thiamine deficiency elevates P450IIE1 as indicated by increases in NDMAd activity and immunochemically detected P450IIE1. However, no corresponding increases in ketone body levels were observed in the thiamine-deficient group, suggesting the involvement of factors other than ketone bodies as inducers for P450IIE1 in thiamine deficiency. In contrast, an increase in P450IIE1 by pair-feeding was attributed to a partial fasting effect as confirmed by the elvation of ketone body levels. Injections of pyruvate or pyrithiamine in addition to thiamine deficiency did not potentiate the increase of P450IIE1. It could be speculated that the effect of thiamine deficiency had already reached at plateau after 3 weeks. We believe that the increase in P450IIE1 in thiamine-deficient rats was mainly due to thiamine deficiency, because an injection of thiamine 24 hr before sacrifice to the deficient group diminished NDMAd activity and immunochemically detected P450IIE1 significantly from those of the deficient group. Although a high correlation ($r = 0.986$, $P < 0.01$) was observed between the NDMAd activity and the immunochemically detected P450IIE1 content, the magnitude of the increase of the latter by thiamine deficiency was almost 2.5-fold higher than that of the former. The reason for this difference is not clearly understood yet, but could be due to the fact that immunoblotting detects apoenzyme as well or due to overestimation of enzyme by densitometry as band intensities increase. Nevertheless, the high correlation with differences in the magnitude of increases between the two parameters confirms our previous observation [31]. On the other hand, thiamine deficiency did not affect benzphetamine demethylase activity and immunochemically detected P450IIC11. P450IIC11 appeared to be developmentally regulated, which is consistent with results in the literature [40, 41]. The present data thus clearly demonstrate the differential effects of thiamine deficiency on the regulation of P450IIE1 and P450IIC11.

Cytosolic glutathione *S*-transferases are a family of isozymes which catalyze the conjugation of glutathione to electrophilic ligands generated by the metabolism of various xenobiotics. Glutathione *S*-transferase B, a heterodimer comprised of two

closely related subunits, Ya and Yc, is the most abundant form in rat liver [42]. Two other major isozymes are glutathione *S*-transferases A and C which are both homodimers comprised of two Yb subunits [42]. Benson *et al.* [43] reported substrate specificities for different glutathione *S*-transferase isozymes: isozyme B has an exclusive activity for steroid isomerase whereas isozyme A has a high activity for glutathione *S*-transferase using 1-chloro-2,4-dinitrobenzene as substrate. Our present data showed different effects on the two enzyme activities by thiamine deficiency: the deficiency significantly increased glutathione *S*-transferase activity but not steroid isomerase activity. This may indicate some degree of specificity in the induction of glutathione *S*-transferase isozymes by thiamine deficiency, but further investigation is needed to substantiate this point.

It has been reported that P450IIE1 is very active in catalyzing the metabolism of not only NDMA and aniline but many other compounds such as ethanol, other alcohols, acetone, enflurane, ether, acetaminophen, carbon disulfide, carbon tetrachloride, and benzene [34, 44]. The present observation, the significant increase of P450IIE1 by thiamine deficiency, therefore provides mechanistic insight of possible potentiation of hepatotoxicities caused by some chemicals under thiamine deficiency. It remains to be examined whether the regulation of P450IIE1 by thiamine deficiency occurs at the mRNA level.

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