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Effect of Pantothenic Acid on Hippurate Formation in Sodium Benzoate-Treated $HepG_2$ Cells

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ABSTRACT

Inborn errors of urea synthesis result in hyperammonemia. Sodium benzoate (SB) therapy has been beneficial in the treatment of hyperammonemia. It conjugates with glycine to form hippurate, which is then excreted. SB has also been used to treat children with nonketotic hyperglycinemia (NKH), where glycine is removed, on conjugation, as hippurate. In mammalian liver mitochondria, SB is activated by an ATP-dependent reaction to its CoA ester, before conjugation with glycine. Pantothenic acid (PA) is the precursor of CoA. In this investigation, increasing the amounts of PA increased CoA levels in $HepG_2$ cells. It also significantly increased formation of hippurate in SB-treated cells. These findings suggest a beneficial effect of PA on the SB therapy in children with NKH as well as hyperammonemia. **(*Pediatr Res* 48: 357–359, 2000)**

Abbreviations

SB, sodium benzoate
NKH, nonketotic hyperglycinemia
PA, pantothenic acid
CSF, cerebrospinal fluid

SB has been administered to patients suffering from hyperammonemia (1–4) due to genetic defects in the urea cycle (5, 6). Hyperammonemia also occurs in serious illnesses such as Reye's syndrome (7) and in some organic acidemias (8). SB conjugates with glycine to form hippurate, which is then excreted.

NKH is an inborn error of glycine metabolism characterized by intractable seizure, lethargy, severe psychomotor retardation, and early death (9, 10). Concentrations of glycine are elevated in plasma and CSF. Oral administration of SB lowered CSF and plasma glycine in these patients accompanied with attenuation of seizures (11–13).

The formation of hippurate from SB in mammalian liver mitochondria proceeds in two steps (14). First, in a reaction requiring ATP, benzyl CoA is formed by conjugation of SB and CoA. Benzyl CoA then reacts with glycine to form hippurate. Diversion of CoA for hippurate formation in isolated rat hepatocytes treated with SB caused depletion of CoA (15). In our studies in treatment of rats with SB, we have shown a significant decrease in liver CoA (16, 17). CoA is synthesized by cells from PA (18). In the absence of dietary PA, hepatic CoA content is significantly reduced (19). We have administered PA to a 4-mo-old patient with NKH who being treated with SB (20). Supplementation of PA resulted in attenuation of seizures, increased urinary excretion of hippurate, and further decrease in glycine concentration in plasma and CSF. $HepG_2$ cells, a human hepatoblastoma derived cell line, display morphology and function similar to that of liver parenchymal cells. $HepG_2$ cells also retain drug-metabolizing capabilities and are used as an *in vitro* model of human hepatocytes (21).

In the present study, therefore, the effect of PA on formation of hippurate is investigated in SB-treated $HepG_2$ cells.

METHODS

Materials. Tissue culture medium, FCS, glycine, calcium pantothenate, and other tissue culture supplies were obtained from Life Technologies, Inc. (Gaithersburg, MD, U.S.A.). SB, CoA, and hippurate were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). $HepG_2$ cells were obtained from American Type Culture Collection (Rockville, MD, U.S.A.).

Cell Culture. $HepG_2$ cells were grown in 75-cm^2 flasks with 10 mL of Eagle's minimum essential medium supplemented with 10% FCS, 100 IU of penicillin/mL, 0.1 mg of streptomycin/mL, and 2 mM glutamine in a 37°C incubator with 5% CO_2. Flasks were subcultured every 7 d with a split ratio of 1:3 using 0.25% trypsin in Ca^{2+} and Mg^{2+} free PBS for 10 min at 37°C. For experiments, cells were seeded in 35-mm dishes (six-well plates) at 10^6 cells/well and were used after 24 h. Medium was removed, cells were washed with PBS, and then 2 mL of medium, which was the same as described earlier but without any glycine, prepared from Hanks' balanced salt so-

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lution, essential amino acid mixture, nonessential amino acids, and vitamin mixture, and containing 10% dialyzed FCS was added. Viability of these cells was typically >95% as determined by trypan blue exclusion. When PA content was 1 μg/L (essentially none) in the medium, viability of cells was <90%.

Additions of glycine, PA, and SB were carried out as recognized in different experimental conditions. After addition of PA and/or glycine, cells were incubated for 4 h, washed with PBS, two to four wells pooled together by trypsin treatment, and then taken for CoA estimation as described by us (17) fluorometrically by the method of Williamson and Corkey (22).

In other experiments, SB was added to the medium and further incubation was carried out for 2 h. The whole contents of the wells were used for estimation of hippurate. After adjusting to pH 4.0 with hydrochloric acid, it was extracted with ethyl acetate and the organic phase was evaporated to dryness. Under these conditions, only hippurate is extracted in ethyl acetate, leaving glycine in aqueous phase. The residue was dissolved in 6 N HCl, hydrolyzed, and taken for estimation of glycine (formed from hippurate) on a Beckman amino acid analyzer.

RESULTS

Effect of addition of PA on CoA levels in $HepG_2$ cells was studied as shown in Table 1. The concentration of PA was varied in the culture media from 1 to 5 mg/L at 1 mM glycine. CoA concentration was not determined at PA concentration of 1 μg/L, as under these conditions, viability of cells was <90%. At 1 mg/L of PA, CoA was 27.2 ± 2.8 pmoles/10^6 HepG2 cells (mean ± SD), which increased significantly ($p < 0.01$) to 45.1 ± 3.9 at 2 mg/L and leveled off at 57.3 ± 4.3 to 59.6 ± 3.8 pmoles/10^6 $HepG_2$ cells between 3 and 5 mg/L. The results indicate that increasing PA from 1 to 3 mg/L caused an increase of 100% in CoA levels.

Effect of PA on formation of hippurate in $HepG_2$ cells at varying glycine concentration of 0.5–5.0 mM is shown in Figure 1. The concentration of PA studied was 1, 2, or 4 mg/L in the media whereas SB in all experiments was 0.5 mM. The experimental conditions, order of additions, and incubation times are described in "Methods." Under these conditions, at 0.5 mM SB, less than 3% of it was converted to hippurate and the viability of the cells was >95%. Formation of hippurate

Table 1. *Effect of PA on coenzyme A levels in $HepG_2$ cells*

Pantothenic acid mg/L	Coenzyme A pmoles/10^6 $HepG_2$ cells
1	27.2 ± 2.8*
2	45.1 ± 3.9
3	57.3 ± 4.3
4	59.8 ± 4.0
5	59.6 ± 3.8

Values are expressed as means ± SD from six experiments. Cells were incubated in medium containing 1 mM glycine for 4 h at 37°C, after addition of PA. Cells were harvested and pooled together from two to four wells for the estimation of coenzyme A.

* Coenzyme A level at 1 mg/L PA was compared against other concentrations and was significantly lower ($P < 0.01$) by t test.

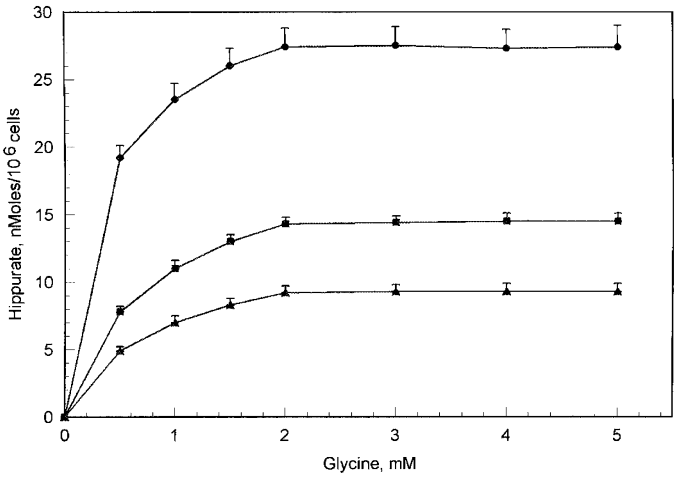

Figure 1. Effect of PA on hippurate formation in $HepG_2$ cells. $HepG_2$ cells containing PA at 1 mg/L ([trif]), 2mg/L ([squlf]), or 4 mg/L ([circf]) in the medium were incubated with glycine (0.5–5.0 mM) for 4 h at 37°C. SB was added to give a concentration of 0.5 mM. After further incubation for 2 h, hippurate formed in the wells was determined. Each point is an average of five determinations, and bars represent mean ± SD.

increased as the amount of PA increased from 1 to 2 and 4 mg/L when the glycine concentration was the same. At 1 mg/L of PA, hippurate formation per 10^6 $HepG_2$ cells, was 4.9 ± 0.3 nmoles at 0.5 mM glycine, increasing to 9.3 ± 0.5 nmoles at 2.0 mM glucine. Further increase in glycine had no effect. At 2 mg/L of PA it was 7.8 ± 0.6 nmoles at 0.5 mM glycine, leveling off to 14.3 ± 0.7 nmoles at 2.0 mM glycine. Similarly, at 4 mg/L of PA, 19.2 ± 1.0 nmoles of hippurate was formed at 0.5 mM glycine, increasing to 27.4 ± 1.4 at 2.0 mM glycine. The increase in glycine concentration from 2.0 to 5.0 mM had no further effect on hippurate formation at all the levels of PA studied. Similarly, increasing PA from 4 to 5 mg/L did not cause any further increase in hippurate formation.

DISCUSSION

Recent advances in the treatment of inborn errors of urea synthesis have significantly decreased mortality (23). Urea cycle disorders result in nitrogen accumulation, manifested by hyperammonemia. Treatment with SB results in conjugation with glycine to form hippurate and is excreted as waste nitrogen. Evidence of clinical toxicity from SB at recommended dosage of 1.73 mmol/kg/d (250 mg/kg/d) has been rare. Green *et al.* (3) have shown that neonates form hippurate as effectively as older children (4). Beliveau and Brusilow (24) have suggested that glycine availability limits hippurate synthesis *in vivo*, leading to some toxic effects of SB. Studies with hepatocytes have shown (25) that glycine antagonizes the inhibition of ureagenesis by SB. Recently we have reported (16) that administration of SB to rats at a dose of 2.5 μmol/kg dropped plasma glycine levels from 310 ± 30 to 175 ± 25 μmol/L at 2 h, and at higher dosage, plasma glycine dropped to much lower levels and for more prolonged time.

NKH is an inborn error of glycine degradation in which large quantities of glycine accumulate in plasma and CSF (26). The primary biochemical defect is in the glycine cleavage system (EC 2.1.2.10), an intramitochondrial enzyme system

present in the liver, kidney, and brain. High concentration of glycine in brain and CSF points to a defect mainly in the brain enzyme complex (27). No effective treatment has been reported consistently. SB has been used in the treatment because it reacts with glycine to form hippurate, which is then excreted (12, 13, 20, 26). Although benzoate doses as high as 3.5–5.2 mmol/kg/d (500–750 mg/kg/d) (26) have been used, they were not without toxicity (20, 28). When patients were treated with SB at 1 mmol/kg orally, serum concentration of SB reached 1.4–2.4 mM (29), and in patients treated at 5.2 mmol/kg/d it reached a concentration of 2.5–3.0 mM in serum and sometimes as high as 9.8 mM (28). We have used PA supplementation in the treatment of a patient with NKH in conjunction with SB. Because PA is a precursor of CoA, required for formation of hippurate, we were able to increase excretion of hippurate without increasing SB dose (20).

Other enzymes involved in the formation of hippurate may play a role in treatment with SB. Newborn children, due to a delayed development of hepatic and renal glycine-N-acyltransferase (EC 2.3.2.13), which conjugates benzyl CoA with glycine, may be more vulnerable to benzyl CoA toxicity (30).

The present study shows that increasing PA in the media caused an increase in the CoA levels in $HepG_2$ cells. It also caused an increase in the formation of hippurate at 0.5–2.0 mM glycine. PA supplementation may result in increased formation of hippurate *in vivo* by increasing CoA levels. This therapy may result in using lower doses of SB and thereby minimizing its toxic effects. Although increased hippurate formation might have been the result of CoA formation, other possibilities, such as direct transcriptional effect of PA on enzymes involved in hippurate synthesis, cannot be eliminated. Further experience will be required to determine the optimal relationships among dosages of PA and SB in the treatment of NKH and hyperammonemia.

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