Failure of Sodium Benzoate to Alleviate Plasma and Liver Ammonia in Rats

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Received July 15, 1988, and in revised form September 21, 1988

Sodium benzoate 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 some organic acidemias (8). Sodium benzoate conjugates with glycine to form hippurate, which is then excreted. Protection against hyperammonemia is believed to occur through utilization of ammonia to reform glycine that has been removed by benzoate as hippurate.

Although benzoate has been used to combat hyperammonemia in patients, recent reports have shown that it potentiates the lethal effects of hyperammonemia in mice (9,10). Similar results have been obtained by Maswoswe et al. (11) who showed that benzoate sharply increased the mortality in rats challenged subsequently with ammonia. In isolated hepatocytes, benzoate was ineffective in preventing ammonia accumulation (12).

Ammonia is detoxified mainly through the formation of urea and glutamine (13). L-Norvaline interferes with urea formation by inhibiting ornithine transcarbamylase (14,15), whereas L-methionine-SR-sulfoximine is a strong inhibitor of glutamine synthetase (16). In this study, we have administered these compounds to rats ip, either alone or in combination. These treatments caused an increase in ammonia levels in plasma and liver by interfering with the detoxification of ammonia. Treatment of these rats with benzoate not only failed to return ammonia levels to normal, but instead, caused a further increase in ammonia in plasma and liver.

In the course of these studies, we also investigated the effect of administration of benzoate alone. Here, we document that benzoate itself caused an increase
in plasma and liver ammonia levels. The physiological relevance of these results is discussed.

MATERIALS AND METHODS

Sodium benzoate, L-norvaline, MSO,\(^2\) \(\alpha\)-ketoglutarate (lithium salt), ammonium chloride, NADH, and bovine glutamate dehydrogenase (Type II, 500 units/ml) were purchased from Sigma Chemical Co., St. Louis, Missouri.

Male, Sprague-Dawley rats weighing 175–200 g were obtained from Charles River, Wilmington, Massachusetts, and were maintained on Purina Lab Chow ad libitum for at least 1 week before use. Rats weighing 250–300 g were held in restraining cages and through the tail vein, blood (1 ml) was obtained with a heparinized syringe. The rats then received ip injections of saline, L-norvaline (1 mmole/kg bw), MSO (250 \(\mu\)mole/kg), sodium benzoate (varying from 2.5 to 10 mmole/kg) in saline, either alone or in combination as indicated. Whenever benzoate was injected after prior treatment with other compounds, the time interval was 15 min. Blood samples were obtained again 2 hr after the last injection. The plasma was separated and taken for ammonia determination. The plasma samples obtained prior to any treatment served as their own controls for samples obtained after various administrations. In separate experiments, rats were sacrificed by decapitation 2 hr after the injections of various compounds, and the livers were excised and taken for ammonia determination.

The plasma and hepatic ammonia levels were measured fluorometrically using the glutamate dehydrogenase reaction as described (17).

Statistical significance between means was determined by Student’s \(t\) test, and was considered significant when \(P < 0.05\).

RESULTS AND DISCUSSION

The effect of administration of various compounds on ammonia levels in rat plasma is shown in Fig. 1. The blood samples obtained from rats prior to injection of various compounds constitute the control group (\(n = 48\)). Ammonia in plasma prior to any treatment was 60.2 ± 10.1 \(\mu\)mole/liter, remaining unchanged (58.2 ± 10.4) 2 hr after saline injection. Sodium benzoate at a dose of 10 mmole/kg bw caused a significant increase of plasma ammonia when compared with that from saline-treated rats. Ammonia levels of 85.5 ± 10.1 \(\mu\)mole/liter (\(n = 6\)) were obtained at a lower dose of sodium benzoate, 5 mmole/kg (data not shown in figure), that were also significantly higher than control values.

Treatment with L-norvaline did not cause a significant increase in plasma ammonia levels compared to that of saline injected. However, after MSO, and L-norvaline plus MSO injections, plasma ammonia increased significantly. Rognstad (14) has noted that, at a concentration of 2.5 mM, L-norvaline inhibited urea formation by 40% in isolated rat hepatocytes. MSO has been shown to be an inhibitor both in vivo and in vitro of glutamine synthetase (16,17). Using \([^{15}N]ammonia, Cooper et al. (18) have documented that pretreatment of rats with MSO led to a decrease in the label present in brain glutamine. In our study

\(^2\) Abbreviations used: MSO, L-methionine-SR-sulfoximine; ip, intraperitoneal; bw, body weight.
FIG. 1. The effect of administration of various compounds on plasma ammonia levels in rats. Blood samples (controls) were obtained with a heparinized syringe prior to administration of saline, L-norvaline (N, 1 mmole/kg bw), MS0 (M, 250 pmole/kg), and sodium benzoate (B, 10 mmole/kg), either alone or in combination as indicated. The blood samples obtained prior to any treatment served as controls (n = 48) while samples from any treatment were obtained 2 hr after the injection of compounds, singly or in combination (n = 6 per group). The ammonia was determined in the plasma and results are expressed as means ± SD.

with MS0 (19), using a mixture of both S- and R-isomers, we observed that the lethal dose in rats was 3.7 mmole/kg bw. The lethal dose with L-methionine-S-sulfoximine, the effective isomer that inhibits glutamine synthetase would then be 1.85 mmole/kg. We have used milder conditions, both with L-norvaline and MS0, that would interfere with ammonia detoxification. The combination of L-norvaline and MS0 administration, by inhibiting possibly both urea and glutamine formation, caused a significant increase in plasma ammonia when compared to these compounds injected alone. Treatment with either L-norvaline or MS0 alone probably leaves at least one pathway operable for ammonia detoxification, whereas the combination treatment might interfere with both pathways. The other groups of rats that had received these compounds, on subsequent treatment with benzoate, showed a significant further increase in plasma ammonia when compared with those that did not receive benzoate. To aliquots of plasma samples obtained from rats after various treatments, known amounts of ammonium chloride were added and the estimation of ammonia was carried out. The recovery of added ammonia was 98.1 ± 2.3%, thereby indicating that metabolites present in the plasma after various treatments do not interfere with the assay for ammonia.

We also investigated the effect of injecting ip various amounts of sodium benzoate as well as other compounds on ammonia concentration in rat liver. As shown in Table 1, the ammonia levels increased significantly even in the liver of rats administered 2.5 mmole/kg sodium benzoate. The administration of L-norvaline and MS0 resulted in significant increase in liver ammonia levels.
TABLE 1
Effect of Sodium Benzoate and Other Compounds on Ammonia Levels in Rat Liver

<table>
<thead>
<tr>
<th>No.</th>
<th>Treatment</th>
<th>Dose m mole/kg bw</th>
<th>Ammonia pmole/g liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Saline</td>
<td>—</td>
<td>0.60 ± 0.03</td>
</tr>
<tr>
<td>2</td>
<td>Sodium benzoate</td>
<td>2.5</td>
<td>0.86 ± 0.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0</td>
<td>0.97 ± 0.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.5</td>
<td>0.99 ± 0.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.0</td>
<td>1.19 ± 0.15</td>
</tr>
<tr>
<td>3</td>
<td>Sodium benzoate + glycine</td>
<td>10.0</td>
<td>1.47 ± 0.18</td>
</tr>
<tr>
<td>4</td>
<td>Glycine</td>
<td>10.0</td>
<td>0.67 ± 0.05</td>
</tr>
<tr>
<td>5</td>
<td>L-Norvaline</td>
<td>1.0</td>
<td>0.68 ± 0.05</td>
</tr>
<tr>
<td>6</td>
<td>MSO</td>
<td>0.25</td>
<td>0.93 ± 0.11</td>
</tr>
<tr>
<td>7</td>
<td>L-Norvaline + MSO</td>
<td>10.0</td>
<td>1.11 ± 0.14</td>
</tr>
<tr>
<td>8</td>
<td>L-Norvaline + MSO + sodium benzoate</td>
<td>10.0</td>
<td>1.63 ± 0.15</td>
</tr>
</tbody>
</table>

* Rats were injected ip with saline or various compounds as indicated. There were 6 animals in each group. In Experiment 3, glycine was administered 15 min after sodium benzoate treatment whereas in Experiment 8, benzoate was administered 15 min after injections of L-norvaline and MSO. Rats were sacrificed by decapitation 2 hr after the last injection. Results shown are means ± SD.

Subsequent treatment with benzoate to rats that had received L-norvaline and MSO caused a further increase in ammonia concentration in the liver.

Although sodium benzoate has been used therapeutically to remove blood ammonia in children suffering from urea cycle disorders (1–4), studies by several investigators using whole animals and isolated hepatocytes indicate that benzoate, on the contrary, potentiates ammonia toxicity (9–12). These investigators have used ammonium acetate to increase ammonia in vivo or in isolated hepatocytes. Here, we have caused a mild increase in plasma ammonia by administering L-norvaline which interferes with urea formation. Higher ammonia levels were obtained when MSO was injected. Sodium benzoate administration under these conditions did not alleviate plasma ammonia, but, on the contrary, caused a further significant increase. When both pathways of ammonia detoxification were interfered with, a subsequent administration of benzoate increased plasma ammonia concentration to 330 ± 40 pmole/liter. Similar results were obtained on ammonia levels in liver.

As proposed (1,2), the protection against ammonia toxicity is thought to result from utilization of ammonia to replenish the glycine spent in the conjugation with benzoate to form hippurate. The nitrogen atom of hippurate is thought to be derived from de novo synthesis of glycine via transamination from glutamate or directly from ammonium via the glycine cleavage pathway (1). Although the glycine cleavage system reaction that forms ammonia and carbon dioxide is reversible in vitro (20), the equilibrium of the reaction is overwhelmingly in favor of the degradation of glycine. The glycine cleavage reaction, leading to the formation of glycine, in vivo has not been documented (21). However, it is possible that formation of glycine required for hippurate synthesis can take place...
from glutamate via glyoxalate transamination. Qureshi et al. (22), using $[^3]H$glycine and $[^4]C$serine in mice, have documented that the glycine transported from body pools may be the primary source in hippurate formation in benzoate treatment, and that the de novo synthesis of glycine may have a secondary effect.

Recently, Beliveau and Brusilow (23) have suggested that glycine availability limits hippurate synthesis in vivo leading to some of the toxic effects of benzoate. In their studies with hepatocytes, Cyr et al. (24) have shown that glycine accelerates the conversion of benzoyl-CoA to hippurate and thereby, antagonizes the inhibition of ureagenesis by benzoate. However, when we administered glycine ip to rats that had received benzoate, the liver ammonia levels were higher in comparison with those that had received benzoate alone. Injecting rats with glycine by itself did not change ammonia levels, compared to those in saline injected rats.

The studies carried out by other investigators (10,11) have indicated that benzoate decreases N-acetylglutamate synthesis in liver mitochondria and thus impairs urea formation. The results of Tremblay’s group (11) have suggested that increased formation of benzoyl-CoA might deplete acetyl-CoA, thereby impairing N-acetylglutamate synthesis. The ammonia-dependent carbamyl phosphate synthetase requires N-acetylglutamate as an essential activator. Recently, we have observed (25) that benzoate administration consistently produces a dose-dependent depletion of hepatic ATP. Apart from the utilization of ATP by benzoate to form hippurate, it is essential for N-acetylglutamate, urea, and glutamine formation. In the light of these findings, we believe this may be another mechanism through which benzoate potentiates ammonia toxicity.

**SUMMARY**

The intraperitoneal administration of L-norvaline and L-methionine-SR-sulfoximine to rats caused an increase in the concentration of ammonia in plasma as well as in liver. These compounds interfere with urea and glutamine formation, respectively. Subsequent injection of sodium benzoate failed to alleviate ammonia levels, and on the contrary, caused a further increase. Sodium benzoate itself, when administered, resulted in higher levels of ammonia in plasma and liver of the rats. Administration of glycine to rats treated with benzoate did not lower ammonia levels indicating that other factors besides glycine may also be necessary for the removal of sodium benzoate.

**ACKNOWLEDGMENT**

This work was supported by the Meadowbrook Medical Education and Research Foundation, East Meadow, New York.

**REFERENCES**


