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Alterations in plasma amino acids and hepatic enzymes in the 4-pentenoic acid model of Reye's syndrome

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Reye's syndrome, described in 1963, is characterized by fatty degeneration of the viscera with encephalopathy [1]. While the etiology of this disorder is unknown, Reye's syndrome is usually preceded by a viral illness, and a number of genetic, viral and environmental factors have been implicated, including aspirin ingestion [2–4]. The biochemical abnormalities include hypoglycemia [5], hyperammonemia [6], hyperaminoacidemia [7], and hyperlipidemia [8]. Ultrastructural examination indicates a number of distinctive mitochondrial abnormalities in both hepatocytes and neurons [9]. Many hepatic mitochondrial enzymes show decreased activity and, in this regard, Mitchell *et al.* [10] note an increase in the ratio of cytosolic lactate dehydrogenase [lactate:NAD^+ oxidoreductase, LDH, EC 1.1.1.27] to mitochondrial glutamate dehydrogenase [glutamate:NAD oxidoreductase (deaminating), GDH, EC 1.4.1.3] activity in liver specimens from Reye's patients.

The study of Reye's syndrome is hampered by the absence of a suitable animal model. While many models have been proposed which mimic one or more of the symptoms of Reye's syndrome, most have not been characterized completely in regard to the many clinical, biochemical and histopathological abnormalities associated with this disorder [11]. Glasgow and Chase [12] produced Reye-like symptoms in rats by chronic injection of 4-pentenoic acid, a compound which can inhibit fatty acid oxidation [13]. The rats exhibited hyperammonemia, fatty infiltration of liver, acute encephalopathy as well as hepatic mitochondrial damage [12, 14]. The present study was undertaken to further characterize this 4-pentenoic acid model by determination of serum amino acid concentrations and hepatic LDH and GDH activities.

Methods

4-Pentenoic acid was obtained from ALFA Products (Danvers, MA). A plasma ammonia kit (Bulletin 170-UV), β-NADH (disodium salt), pyruvate, α-ketoglutarate and sodium heparin were purchased from the Sigma Chemical Co. (St. Louis, MO). Acetylsalicylic acid (aspirin) was obtained from J.T. Baker Chemicals (Phillipsburg, NJ).

The protocol of Glasgow and Chase [12] was used with the following modifications. Groups of three to four male, Sprague–Dawley rats (200–300 g) were injected subcutaneously ten times over the course of 3 days with 4-pentenoic acid (12.5 mg/kg body weight), aspirin (50 mg/kg body weight), 4-pentenoic acid plus aspirin, or an equal volume of saline. An additional dose (200 mg/kg) of 4-pentenoic acid was administered 30 min prior to sacrifice to groups receiving 4-pentenoic acid. Blood was collected into heparin-treated tubes and centrifuged to obtain plasma. Livers and brains were removed, weighed and frozen until use.

Plasma ammonia was determined using a commercial kit which employed glutamate dehydrogenase. Additional samples of plasma were deproteinized (4:1, plasma: 10% sulfosalicylic acid) and filtered through a 0.4 μm Millipore filter prior to amino acid analysis on a Waters model 703 trimodular high performance liquid chromatographic automation system equipped with an Interaction IC8011 column [15]. *o*-Phthaldialdehyde derivatization was used [16], and carboxymethylcysteine (2 nmol) served as an internal standard. In triplicate determinations, integrated peak areas for carboxymethylcysteine differed by less than 2.5%. Salicylates were measured by reverse phase HPLC using *N,N*-diethyl-*m*-toluamide as internal standard [17].

Livers were homogenized in 0.1 M potassium phosphate, pH 7.4 (10 mg tissue/ml), and centrifuged at 10,000 *g* for 2.5 hr in a refrigerated Lourdes centrifuge. In some experiments, the liver homogenate was sonicated prior to centrifugation [10]. The supernatant fractions were assayed at 25° for LDH and GDH activity by following the rate of NADH oxidation according to the methods of Schwartz and Bodansky [18] and Strecker [19] respectively. Protein concentration was determined according to the method of Lowry *et al.* [20] using bovine serum albumin as standard. Enzyme activities were expressed as IU per milligram of protein. The data represent the mean of experiments performed in quadruplicate on six to seven different livers per group. Statistical significance was evaluated using Student's *t*-test.

Results and discussion

Rats that received 4-pentenoic acid showed signs of increased excitability early in the treatment followed by a period of lethargy. While we cannot quantitate this observation, it is reminiscent of the behaviour of children with Reye's syndrome who are often violent and disoriented prior to lapsing into coma [1].

The plasma ammonia concentrations of animals receiving 4-pentenoic acid, aspirin or 4-pentenoic acid plus aspirin were significantly higher than the saline controls (Table 1). Similar results were obtained by Thayer [14] who found that 4-pentenoic acid-treated rats have serum ammonia levels approximately 60% higher than controls. Elevated ammonia levels are a consistent finding in Reye's patients [6].

Salicylate concentrations were determined from plasma samples of the animals receiving aspirin. A significant decrease ($P < 0.002$) in salicylate levels was noted in the aspirin plus 4-pentenoic acid group (8.77 ± 0.14 mg/dl) when compared to the group receiving aspirin alone (11.48 ± 0.71 mg/dl). The reason for this effect is not clear; however, similar decreases were noted from groups receiving 12.5 to 75 mg/kg doses of aspirin and 4-pentenoic acid when compared to those receiving only aspirin (data not shown).

The plasma amino acid concentrations of the saline- and 4-pentenoic acid-treated groups were determined (Table 2). Threonine, serine and glycine levels were significantly lower, whereas isoleucine, leucine and histidine levels were statistically elevated in the treated group. Valine, glutamic

Table 1. Plasma ammonia and hepatic enzyme specific activities in test and control animals

Treatment*	$[NH_3]$ (μg/ml)	Lactate dehydrogenase (IU/mg protein)	Glutamate dehydrogenase (mIU/mg protein)
Saline (N = 6)	3.58 ± 0.20	1.05 ± 0.07	11.6 ± 1.5
4-Pentenoic acid (N = 7)	6.20 ± 0.64†	1.17 ± 0.15	6.7 ± 1.0‡
Aspirin (N = 6)	5.11 ± 0.69§	1.16 ± 0.08	10.9 ± 0.8
Aspirin + 4-pentenoic acid (N = 7)	6.34 ± 0.83‡	0.84 ± 0.19	9.8 ± 1.5

Values are the means ± SE of experiments repeated in duplicate (ammonia) or quadruplicate (enzymes).

* Groups of animals were treated as described in Methods. The number (N) refers to the total number of animals used.

† Significant change from saline-treated group ($P < 0.01$).

‡ Significant change from saline-treated group ($P < 0.02$).

§ Significant change from saline-treated group ($P < 0.05$).

Table 2. Plasma amino acid concentrations in 4-pentenoic acid and saline-treated animals

	Amino acid concentration* (μM)	
	Treatment	
Amino acid	Saline (N = 6)	4-Pentenoic acid (N = 7)
Ala	423.0 ± 21.2	487.1 ± 24.3
Arg	224.0 ± 10.7	184.2 ± 25.3
Asn†	—	—
Asp	17.7 ± 1.5	19.2 ± 1.3
Cys	ND	ND
Gln†	—	—
Glu	157.5 ± 10.7	179.6 ± 11.1
Gly	366.6 ± 12.7	303.6 ± 15.5‡
His	134.2 ± 4.5	235.1 ± 16.4§
Ile	90.1 ± 2.8	108.3 ± 5.2‡
Leu	146.1 ± 3.9	187.7 ± 8.1‖
Lys	377.4 ± 19.9	358.3 ± 24.9
Met	46.8 ± 1.1	49.8 ± 2.6
Phe	73.8 ± 4.2	91.1 ± 15.5
Pro¶	—	—
Ser	242.4 ± 15.8	183.4 ± 10.9**
Thr	875.2 ± 27.8	681.7 ± 26.8§
Trp††	—	—
Tyr	75.8 ± 4.7	78.1 ± 7.2
Val	194.7 ± 4.7	208.4 ± 7.0

* Groups of animals were treated as described in Methods. Values are means ± SE. The number (N) refers to the total number of animals used. ND = not detectable.

† Asn and Gln could not be resolved.

‡ Significant change from saline-treated group ($P < 0.02$).

§ Significant change from saline-treated group ($P < 0.001$).

‖ Significant change from saline-treated group ($P < 0.002$).

¶ Pro does not fluoresce with *o*-phthaldialdehyde.

** Significant change from saline-treated group ($P < 0.01$).

†† Trp overlaps buffer-change peak.

acid and alanine were elevated slightly, although not significantly, in the test group. These alterations may be the consequence of the mitochondrial damage observed in rats treated with 4-pentenoic acid [14]. Reye's syndrome patients have elevated concentrations of glutamic acid, glutamine, isoleucine, valine, alanine and lysine [7]. Thus, the 4-pentenoic acid-treated group exhibited some of the same alterations in plasma amino acid levels as noted in Reye's patients, although more dramatic increases in amino acid concentrations were noted in the patients [7]. However, the highest elevations in plasma amino acids correlated with the sickest patients [7] and, in the present study, the amounts of 4-pentenoic acid (12.5 mg/kg) chronically administered to the rats was well below the lethal dosage (100–200 mg/kg).

Hepatic GDH and LDH activities were determined for all groups (Table 1). A statistically significant decrease in GDH activity was noted in those rats receiving 4-pentenoic acid. In animals treated with aspirin and 4-pentenoic acid, a slight, although not statistically significant, decrease in GDH activity was found. Thus, aspirin did not potentiate the inhibition of GDH by 4-pentenoic acid. Regardless of the treatment, LDH activity was unchanged. To assure that the decrease in GDH activity observed in the 4-pentenoic acid-treated group was not due to incomplete release of mitochondrial matrix enzymes, liver homogenates from saline- and 4-pentenoic acid-treated animals were sonicated prior to GDH assay. A significant decrease ($P < 0.05$) in the specific activity of GDH was still observed in the 4-pentenoic acid-treated group (15.0 ± 1.4 mIU/mg) when compared to the control group (21.8 ± 3.2 mIU/mg). These results parallel those obtained from liver specimens of Reye's patients where GDH activity is decreased significantly while LDH activity is unaltered [10]. Mitchell *et al.* [21] ruled out the presence of a GDH inhibitor in the hepatocytes of Reye's syndrome patients. The units of GDH activity in a mixture of liver homogenates from control and 4-pentenoic acid-treated animals equalled the sum of the GDH activity in the individual homogenates (Table 3). Therefore, an inhibitor of GDH does not appear to be present in the livers of the 4-pentenoic acid-treated animals. In both this animal model as well as Reye's syndrome patients, a generalized liver mitochondrial defect may be the basis for the loss of GDH but not cytoplasmic LDH activity. It is not surprising that LDH and GDH activities were not altered in the group receiving only aspirin because the dehydrogenases generally are reversibly inhibited by only very high levels (mM) of salicylates [22]. While liver salicylate levels were not determined in the animals, plasma salicylate concentrations did not exceed 1 mM.

Table 3. GDH activity in liver homogenates

Homogenate*	GDH activity (mIU)
Saline	2.88 ± 0.41
4-Pentenoic acid	2.59 ± 0.17
Saline + 4-pentenoic acid	5.17 ± 0.36

Values are the means ± SE of triplicate determinations.
* Liver homogenates from the animals were sonicated prior to centrifugation and GDH assay.

In addition to the encephalopathy, fatty infiltration of the liver, and other abnormalities reported by various researchers [12, 14], the results of this study indicate that the 4-pentenoic acid model meets several additional criteria of Reye's syndrome. These include the elevation of certain plasma amino acid concentrations and an increase in the LDH:GDH ratio due to a loss of GDH activity. While this model appears to mimic Reye's syndrome rather well, it suffers from the same drawbacks as other animal models, namely, a lack of one or more of the major clinical and pathological criteria of Reye's syndrome or an incomplete characterization of all of the parameters of this complex disorder. In this model serum glutamic oxaloacetic transaminase activity and free fatty acid and amino acid concentrations were not elevated to the extent noted in Reye's patients [12]. Nonetheless, the major advantage of the 4-pentenoic acid model is that the main biochemical action of 4-pentenoic acid is known to be the inhibition of fatty acid β-oxidation by the CoA derivative of this molecule [13]. In the other prominent animal models such as the margosa oil model of Sinniah *et al.* [23] or the ferret model of Deshmukh *et al.* [24], the primary biochemical defect is less clearly understood. Thus, the 4-pentenoic acid model is useful in the study of hepatic disorders such as Reye's syndrome when the inability to efficiently metabolize fatty acids is a major biochemical defect.

In summary, chronic administration of 4-pentenoic acid to rats produced an increase in serum concentrations of leucine, isoleucine and histidine, plus an increase in the hepatic LDH:GDH ratio due to a decrease in GDH activity. These results demonstrate that the 4-pentenoic acid model meets several additional criteria for Reye's syndrome.

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