

The Effects of Galactosamine-Induced Hepatic Failure Upon Blood-Brain Barrier Permeability

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The role of changes in blood-brain barrier permeability in the pathogenesis of hepatic encephalopathy remains uncertain. To test the hypothesis that brain microvessel permeability is nonselectively increased in hepatic encephalopathy we measured the blood-brain barrier permeability-surface area product in rats with acute liver failure induced by intraperitoneal injection of galactosamine. The permeability-surface area products to the diffusion-limited tracers, sucrose and methylaminoisobutyric acid, were determined as a measure of blood-brain barrier permeability. Animals were examined 24, 36 and 42 hr after injection, at times when they were stuporous, but not comatose. No significant elevations of the permeability-surface area products for either compound were detected in clinically affected experimental animals when compared to controls. Our results indicate there is no generalized increase in brain vascular permeability during hepatic insufficiency in precomatose animals.

The mechanism by which hepatic encephalopathy is produced remains unclear. Several investigators have reported increases in blood-brain barrier (BBB) permeability following experimental liver injury (1-3), and they propose that these changes play a role in the pathogenesis of encephalopathy. Other investigators found no generalized BBB opening (4-6). This discrepancy may be explained, in part, by the low sensitivity of the method used to measure BBB permeability. Most of these studies employed the single-pass, intracarotid injection technique. However, when used to measure brain uptake of poorly diffusible compounds, this method requires large corrections for tracer retained within the intravascular compartment (7). The integrity of the BBB can be meas-

ured more accurately with a technique developed by Ohno et al. (8). The BBB permeability-surface area product (PS) is determined following an intravenous injection of radiolabeled sucrose. The sucrose content in brain can be corrected for the intravascular portion by determining brain blood volume in a separate group of animals. We used this method to test the hypothesis that there is a generalized increase in BBB permeability during galactosamine-induced liver failure.

MATERIALS AND METHODS

General Procedures. Male Sprague-Dawley rats weighing 250 to 350 gm were given an intraperitoneal injection of D+galactosamine hydrochloride (3 gm per kg) as a 0.28 M solution (pH 7.4) in water (9, 10). Control animals were injected with 0.28 M glucose in water. The animals were fed standard laboratory chow and 10 gm% glucose was added to their water.

At 24, 36 and 42 hr after the galactosamine injection, the animals were anesthetized with sodium pentobarbital (50 mg per kg, i. p.) and tracheotomized. Polyethylene catheters were inserted into all four femoral vessels. The left femoral artery catheter was connected to a blood pressure transducer to monitor blood pressure as well as to collect blood-gas samples. The right femoral artery catheter was connected to a peristaltic pump to permit continuous arterial blood withdrawal. One venous catheter was used for intravenous injection of drugs while the other intravenous catheter was used for injection of radioactive tracers.

The animals were paralyzed with gallamine, and ventilated using a volume ventilator set to maintain the arterial pCO₂ between 35 and 45 mm Hg. Temperature was monitored by a rectal probe and maintained at 36.5° to 37.5°C with an incandescent lamp.

Serum enzyme and bilirubin assays were performed in the clinical laboratory of the Columbus Children's Hospital using a Kodak Ektachem-700* analyzer.

Injection of Isotopes. For measurement of both BBB permeability (PS) and cerebral blood volume, the animals were allowed to reach a steady state 15 to 30 min following cannulation. Radioactive tracers were injected intravenously. During PS determinations, arterial blood was withdrawn from the right femoral artery at a known rate. When a predetermined amount of time had elapsed, a terminal arterial blood sample was collected from the left femoral artery, the animal was decapitated and the continuous arterial withdrawal was stopped. The forebrain was separated into right and left halves, then divided into cerebral hemispheres and basal ganglia. The brainstem was separated from the cerebellum. The brain regions were placed in preweighed scintillation vials containing Protosol and

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reweighed. Blood from the continuous arterial withdrawal was expelled into a preweighed vial, weighed and 50 μ l aliquots were placed in scintillation vials containing a 2:1 (volume/volume) mixture of ethanol:Protosol. The terminal arterial blood hematocrit was measured, and 50- μ l aliquots of the blood were also dissolved in ethanol:Protosol. Brain and blood samples were heated at 65°C until they were digested. The blood samples were bleached with 50 μ l 30% hydrogen peroxide. Toluene-based scintillation cocktail was added to all samples, and the amount of radioisotope present was assayed by liquid scintillation using single or double channels as appropriate.

Blood Volume Measurement. Regional brain plasma volume and red blood cell volume were determined using a variation of a method previously described (11, 12). Homologous rat red blood cells were incubated with 300 μ Ci ^{51}Cr per 1.2 ml packed cells for 30 min at 37°C. The labeled red cells were then washed three times with iced phosphate-buffered saline (PBS) containing 10 mM glucose and kept on ice. Just prior to use, a 200- μ l aliquot of cells was washed with PBS and suspended with 100 μ l 1% bovine serum albumin (BSA) in PBS containing 10 μ Ci ^{125}I -labeled BSA (1.39 μ Ci per mg BSA). The suspension was injected via the left femoral venous line. The labeled cells and plasma were allowed to circulate for 2 min, then a terminal arterial blood sample was rapidly withdrawn and the animal was decapitated. The samples were subsequently handled as described in the general procedures. The radioactive content was determined in a two-channel liquid scintillation counter that was calibrated for simultaneous counting of the Auger and conversion electrons emitted by ^{51}Cr and ^{125}I (13). Typical counting efficiencies were 20 and 20% for channel A, and 1 and 33% for channel B, for ^{51}Cr and ^{125}I , respectively.

PS. A 10 μ Ci aliquot of [^{14}C]sucrose (560 μ Ci per mmole) or [^{14}C]methyl-aminoisobutyric acid (MAIB), 40 to 60 mCi per mmole in PBS was injected intravenously. Arterial blood was continuously withdrawn for 10 min, then a terminal blood sample was obtained and the animal was killed. Tissues and blood were handled as in general procedures.

Calculations. Assuming that the PS for sucrose or MAIB is much less than the regional cerebral blood flow, and that the early brain concentration of either isotope is negligibly small (7), PS may be determined by the following relationship:

$$PS = \frac{C_b}{\int_0^t C_a dt}$$

where C_b = the amount of isotope in the brain region and C_a = the amount of isotope in the arterial blood at a given time, t . The integral of $C_a dt$ between the time of isotope injection and termination of the experiment can be estimated by using a continuous arterial withdrawal at a known rate using the following equation:

$$\int_0^t C_a dt = \frac{C_0}{F_0}$$

where C_0 = total amount of isotope in the arterial blood sample and F_0 = the withdrawal rate of the pump (14). Knowing the tissue plasma volume (PV), the following relationship can be made:

$$PS = \left[\frac{\text{brain dpm}}{\text{brain weight}} - (C_t \cdot PV) \right] \cdot F_0 \cdot (1 - \text{Hct}_a) / C_0$$

where C_t = the terminal arterial concentration of isotope and Hct_a = the arterial hematocrit. PV is calculated from the following equation using Hct_a and values for regional blood volume (BV) and the ratio (R) of tissue to peripheral hematocrits determined in a separate group of animals:

$$PV = BV \cdot (1 - \text{Hct}_a \cdot R)$$

Thus, the plasma volume term takes into account any variability in peripheral hematocrits between groups of animals.

Materials. Animals were purchased from Harlan Sprague-Dawley, Inc. (Haslett, Mich.). Chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.) and were reagent grade. Protosol and α -[^{14}C]MAIB (48.4 mCi per mmole) were purchased from DuPont-NEN (Boston, Mass.). [^{14}C]Sucrose (10.0 mCi per mmole) and ^{125}I -BSA (0.8 mCi per mg) were purchased from ICN (Irvine, Calif.).

Statistical Analysis. Results were assumed to be random samples that were mutually independent under the experimental conditions. Because of small sample sizes, a normal distribution was not assumed, and nonparametric statistical tests were selected. For the data in Tables 1 to 4, results from three experimental animal groups were compared simultaneously with controls by the Kruskal-Wallis test using $\alpha = 0.01$. For comparisons of the single MAIB uptake group to controls, the Mann-Whitney rank sum test was employed using the same $\alpha = 0.01$ (15, 16).

TABLE 1. Physiological parameters

	Control	24 hr	36 hr	42 hr
Regional blood volume determination animals				
Weight (gm)	314 \pm 37	342 \pm 23	260 \pm 29	240 \pm 19
Blood pressure (mm Hg)	118 \pm 13	81 \pm 20	97 \pm 23	77 \pm 21
Temperature ($^{\circ}\text{C}$)	37.6 \pm 0.6	37.5 \pm 0.6	37.2 \pm 0.3	37.4 \pm 0.9
pH	7.39 \pm 0.04	7.37 \pm 0.04	7.28 \pm 0.09	7.33 \pm 0.13
pCO ₂ (mm Hg)	39 \pm 3	39 \pm 4	40 \pm 4	39 \pm 2
pO ₂ (mm Hg)	94 \pm 15	125 \pm 9	86 \pm 12	104 \pm 12
Hematocrit	0.44 \pm 0.03	0.53 \pm 0.03	0.53 \pm 0.02	0.51 \pm 0.04
PS determination animals				
Weight (gm)	240 \pm 48	212 \pm 31	232 \pm 24	182 \pm 49
Blood pressure (mm Hg)	102 \pm 16	66 \pm 10	80 \pm 10	85 \pm 12
Temperature ($^{\circ}\text{C}$)	37.6 \pm 0.9	3.68 \pm 0.6	37.4 \pm 0.5	37.8 \pm 0.6
pH	7.35 \pm 0.05	7.36 \pm 0.07	7.35 \pm 0.05	7.30 \pm 0.1
pCO ₂ (mm Hg)	41 \pm 5	40 \pm 5	38 \pm 3	39 \pm 2
pO ₂ (mm Hg)	94 \pm 17	115 \pm 28	114 \pm 28	111 \pm 14
Hematocrit	0.39 \pm 0.05	0.41 \pm 0.04	0.47 \pm 0.03	0.45 \pm 0.02

Each value is the mean \pm S.D. of determinations from five animals.

TABLE 2. Blood chemistry values in galactosamine-injected rats

	Control	24 hr	36 hr	42 hr
AST (IU/liter)	120 ± 37	9,499 ± 9,383	15,234 ± 6,545	7,753 ± 3,973
ALT (IU/liter)	53 ± 6	4,395 ± 5,264	13,454 ± 6,559	5,687 ± 3,128
Total bilirubin (mg/dl)	0.3 ± 0.1	1.1 ± 0.8	3.3 ± 1.6	2.4 ± 0.6
Direct bilirubin (mg/dl)	0.1 ± 0.1	0.8 ± 0.6	1.5 ± 1.0	2.3 ± 0.6

Each value is the mean ± S.D. of determinations from 5 to 7 animals.

TABLE 3. Regional volume and hematocrit determinations

	Control	24 hr	36 hr	42 hr
Tissue plasma volume ^a				
Cortex	5.1 ± 0.6	5.5 ± 0.6	5.6 ± 0.2	5.3 ± 1.1
Basal ganglia	6.6 ± 1.4	6.8 ± 0.8	7.0 ± 0.8	7.0 ± 0.6
Cerebellum	7.7 ± 1.0	8.4 ± 0.9	8.9 ± 1.0	8.5 ± 0.5
Brainstem	6.7 ± 0.8	7.1 ± 0.9	7.4 ± 0.4	7.6 ± 0.4
Tissue red cell volume ^a				
Cortex	1.8 ± 0.2	2.4 ± 0.2	2.1 ± 0.2	2.1 ± 0.7
Basal ganglia	3.0 ± 0.5	4.3 ± 0.9	3.0 ± 0.2	4.1 ± 1.5
Cerebellum	3.0 ± 0.3	3.8 ± 0.4	3.9 ± 1.0	3.6 ± 0.8
Brainstem	2.3 ± 0.3	2.9 ± 0.2	2.9 ± 0.6	3.1 ± 0.6
Tissue blood volume ^a				
Cortex	7.0 ± 0.8	7.8 ± 0.8	7.7 ± 0.2	7.67 ± 1.7
Basal ganglia	9.6 ± 1.3	11.1 ± 1.6	10.1 ± 0.9	11.1 ± 1.6
Cerebellum	10.7 ± 1.1	12.2 ± 1.2	12.9 ± 1.9	12.1 ± 0.9
Brainstem	9.0 ± 1.0	10.0 ± 1.0	10.3 ± 0.9	10.7 ± 0.6
Tissue hematocrit				
Cortex	0.26 ± 0.01	0.30 ± 0.02	0.27 ± 0.02	0.28 ± 0.05
Basal ganglia	0.32 ± 0.07	0.38 ± 0.03	0.30 ± 0.02	0.36 ± 0.09
Cerebellum	0.28 ± 0.03	0.31 ± 0.02	0.30 ± 0.03	0.30 ± 0.05
Brainstem	0.26 ± 0.02	0.29 ± 0.02	0.28 ± 0.04	0.29 ± 0.05

Each value is the mean ± S.D. of determinations from five animals.

^a In $\mu\text{l/gm}$ tissue weight.

TABLE 4. Sucrose PS^a determination

	Control	24 hr	36 hr	42 hr
Cortex	8.2 ± 1.5	10.8 ± 3.2	7.4 ± 1.2	8.5 ± 1.9
Basal ganglia	10.6 ± 1.7	11.3 ± 0.4	10.9 ± 0.6	10.3 ± 2.6
Cerebellum	11.1 ± 0.7	10.2 ± 0.8	9.2 ± 1.6	9.7 ± 2.1
Brainstem	9.6 ± 2.4	10.6 ± 2.2	8.8 ± 1.9	10.4 ± 2.3

Each value is the mean ± S.D. of determinations for five animals.

^a In $\text{ml/gm/min} \times 10^4$.

RESULTS

At 24 hr after administration of galactosamine, the urine and plasma of the experimental rats were icteric, but the animals showed no behavioral difference from control animals. By 36 hr, the animals were listless, and they startled easily. At 42 hr, the rats were awake, but poorly responsive to the environment. By this time, two animals had died after a period of irritability followed by stimulus-induced tonic seizures. When physiological parameters were compared, animals at 36 to 42 hr were more acidotic and hypotensive than the 24-hr experimental rats (Table 1).

We measured serum aminotransferases, and total and

direct bilirubin to characterize the extent of liver damage. In animals injected with glucose, values for AST, ALT, total bilirubin and direct bilirubin remained constant for at least 48 hr after injection (data not shown). In animals injected with galactosamine, serum aminotransferases steadily rose to a maximum at 36 hr after galactosamine injection and declined by 42 hr, although the aminotransferase levels remained greater than control values (Table 2). Serum direct bilirubin steadily increased above control values after galactosamine injection at all time points measured. All values at 24, 36 and 42 hr were significantly different from controls ($p < 0.01$). The pronounced rise in serum aminotransferases and the progressing hyperbilirubinemia point to the severity of galactosamine-induced hepatic injury.

No significant increases from control values were noted in tissue plasma volumes, red cell volumes, blood volumes and tissue hematocrits in the cortex, basal ganglia, cerebellum and brainstem of galactosamine-treated animals at 24, 36 or 42 hr (Table 3). Brain blood volumes, expressed as microliters per gram of brain weight, did not vary with body weight in the animals examined (comparison not shown). The only multiple sample comparisons that approached significance were an increase in tissue red cell volumes for the cortex ($p = 0.037$) and the brainstem ($p = 0.049$) when results at different time points were compared to controls. The differences were most notable between controls and 24-hr animals than at other times.

PS was calculated for sucrose using the blood volumes shown in Table 2. At all times following galactosamine injection, PS for affected animals did not differ significantly from controls (Table 4). This was true at 24 hr when animals were behaviorally unaffected as well as at 36 and 42 hr when their behavior was distinctly abnormal.

Other investigators have reported significant increases in the regional PS product for aminoisobutyric acid in galactosamine-treated rabbits (2). In order to determine whether this observation was the result of a specific change in brain uptake of this type of amino acid analog, we determined the PS product for MAIB in rats 24 hr after galactosamine administration. We found no increase in PS for MAIB compared to controls. In fact, the MAIB-PS in all regions was lower than control (Table 5). While MAIB enters the brain very poorly, it is possible that a portion of its brain uptake is mediated by an amino acid transport system. Reduced brain uptake of MAIB in galactosamine-treated rats might, therefore, be explained by competition for transport from the elevated plasma concentrations of other neutral amino acids during hepatic failure (17).

TABLE 5. MAIB PS Product*

	Control	24 hr	p value ^b
Cortex	8.1 ± 1.1	6.5 ± 0.9	0.012
Basal ganglia	6.7 ± 2.7	3.1 ± 1.3	0.07
Cerebellum	10.9 ± 1.7	7.2 ± 1.3	0.018
Brainstem	11.1 ± 2.7	7.2 ± 2.1	0.012

Each value is the mean ± S.D. for six control and three experimental animals.

* In ml/gm/min × 10⁴.

^b One-tailed test.

DISCUSSION

During the course of galactosamine-induced acute hepatic failure, we found no change in BBB permeability to sucrose despite the increasing duration of hepatic injury and progressive alterations in behavior. During the times studied, all animals showed evidence of severe liver damage. By 36 hr, they were less active and by 42 hr they were either lethargic or hyperirritable, but not comatose.

Our results are in agreement with other studies which found no change in BBB permeability following liver injury. In one study, rats with chronic portacaval shunts were given ammonium acetate to acutely induce encephalopathy (4). There was no difference in the brain uptake of sucrose compared with controls. A second study used a similar method without ammonia loading; no change in the BBB permeability to inulin was found (5). A third study found no change in brain uptake of ²²Na and ⁵¹Cr-EDTA in rats examined 3 weeks after the creation of a portacaval shunt (6). These three studies used the intracarotid bolus injection method to measure the brain uptake index, although this technique is relatively insensitive to changes in BBB permeability for poorly diffusible compounds. In a fourth study, Mans et al. (18) used the technique of quantitative autoradiography, which is more sensitive than the brain uptake index technique to determine BBB permeability to sucrose. They found no change in PS in rats examined 7 to 8 weeks after portacaval shunting. Mans et al. did not examine BBB changes during acute hepatic encephalopathy.

Our findings conflict with the observations of Zaki et al. (1) who employed the brain uptake index technique in galactosamine-induced hepatic failure and hepatic devascularization. They detected a 3- to 4-fold increase in the uptake of inulin and a 3-fold increase in the uptake of sucrose and L-glucose. The animals were examined in deep coma and were, therefore, more severely affected than the animals in the present study. It is possible, as those authors point out, that the detected changes in BBB permeability reflected the moribund state of the animals.

Also, in contrast to our findings are the results of Horowitz et al. (2) who detected localized increases in BBB permeability in a rabbit model of galactosamine-induced hepatic failure. Using autoradiographic techniques to identify regional changes in isotope concentrations, the investigators examined the equilibrium transfer constant for ¹⁴C-labeled aminoisobutyric acid. At a time before the animals became encephalopathic, in-

creases in the aminoisobutyric acid transfer constant occurred in the medial geniculate body, superior colliculus, central grey substance, area retrosplenius and lateral thalamic nucleus. Less pronounced changes were noted in the limbic regions, basal ganglia and temporal cortex. Other cortical regions and white matter tracts were unaffected.

One possible explanation for the differences between our results and those of Horowitz et al. (2) is that the permeability change they observed is specific for aminoisobutyric acid and not sucrose. Aminoisobutyric acid normally has a low BBB permeability because the carrier for which this amino acid analog has the highest affinity is found on the antiluminal but not the luminal membrane of the brain capillary endothelial cell (19). Redistribution of this transport protein to the luminal membrane could result in a selective increase in BBB permeability to aminoisobutyric acid with no change in permeability to sucrose (20). The fact that BBB permeability to MAIB, a similar amino acid analog, did not increase in our model, makes this redistribution of carriers unlikely. Alternatively, it is possible that the highly localized increases in BBB permeability detected by the autoradiographic method of Horowitz et al. could not be resolved by our method, which required analysis of larger pieces of brain containing many brain structures.

We conclude that galactosamine-induced hepatic injury does not produce a generalized breakdown of the BBB in pre-moribund animals. However, it remains possible that localized changes in BBB permeability may occur in specific brain regions. Future research should attempt to confirm that BBB permeability does increase in brain structures, like the medial geniculate body and the lateral thalamus, since an analysis of the causes and consequences of BBB breakdown in those regions could provide important insights into the pathogenesis of hepatic encephalopathy.

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