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Brainstem Bilirubin Toxicity in the Newborn Primate May Be Promoted and Reversed by Modulating P_{CO_2}

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ABSTRACT. The auditory brainstem response (ABR) was monitored during infusion of bilirubin in six ventilated newborn rhesus monkeys (138–145 d gestation) while acute changes in pH were produced by varying inspired CO_2. Prolonged respiratory acidosis without bilirubin infusion produced minimal changes in the ABR (one animal). CO_2 exposure, usually initiated when the bilirubin level reached ~20 mg/dL, decreased arterial pH to values ranging from 6.85 to 7.10. ABR changes, including prolongation of the wave II–IV peak to peak intervals and decreased wave amplitudes, first developed 2–4 h after initial exposure to CO_2. Total and unbound bilirubin levels at this time ranged from 376 to 564 μmol/L (22–33 mg/dL) and 38 to 65 nmol/L (2.5–3.8 μg/dL), respectively. Correction of respiratory acidosis produced partial to complete reversal of ABR changes within 3 to 20 min. Reexposure to CO_2 immediately reproduced the ABR abnormality. Production and reversal of the abnormal ABR was obtained through two to three cycles in three animals. Thus, when the brainstem bilirubin level was near the threshold for toxicity, the effect of changes in P_{CO_2} on the ABR were immediate, suggesting that auditory pathway toxicity is initially mediated by a reversible pH-dependent bilirubin-membrane complex. In contrast to humans, in monkeys auditory toxicity appeared to be a late manifestation of bilirubin toxicity, inasmuch as all monkeys were obtunded and apneic 30–70 min before ABR abnormalities appeared. Notwithstanding these limitations, the results support the hypothesis that bilirubin toxicity can be both promoted and reversed by modulating brain pH. (*Pediatr Res* 34: 6–9, 1993)

Abbreviations

ABR, auditory brainstem evoked response

Bilirubin is a dicarboxylic acid and is thought to exist primarily as the dianion in plasma (1). The nearly insoluble free bilirubin acid concentration increases with decreasing pH and with increasing total bilirubin concentration, creating a supersaturated unbound bilirubin level in hyperbilirubinemic infants (2, 3). The uptake of bilirubin by tissue also increases with decreasing pH (4–9), whereas serum binding remains unchanged (9, 10), and Brodersen and coworkers (1–3) have proposed that bilirubin encephalopathy results from the deposition of insoluble bilirubin acid aggregates in or on susceptible neuronal membranes (1–3).

Although precipitation of bilirubin in membranes may represent the final stage of kernicterus (11), there is increasing evidence that early bilirubin toxicity and possibly the chain of events leading to lethal toxicity may be initiated by quite a different mechanism. It is unlikely that precipitation of a supersaturated bilirubin solution is responsible for changes in the ABR (12–18), because ABR abnormalities may be corrected during exchange transfusion even when the free bilirubin concentration remains above proposed levels of solubility (2, 12, 13). Furthermore, we have found that nearly all binding of bilirubin to red blood cells or mitochondria is due to a reversible pH-dependent mechanism involving a single proton addition (4). This suggested to us that the soluble monovalent anion and not the insoluble bilirubin acid is responsible for initial partitioning between plasma and tissue.

These observations led us to postulate that early toxicity might involve reversible pH-dependent bilirubin-membrane complexes. If true, dissolution as well as formation of toxic complexes in brain should occur rapidly after a sudden change in brain pH and should be reflected in a measurable expression of neurotoxicity. To test this hypothesis, we examined the effects of pH jumps on brainstem function as measured by the ABR of jaundiced premature primates. We modified pH by CO_2 inhalation because CO_2 equilibrates rapidly across the blood-brain barrier, immediately altering cerebral pH. The premature newborn rhesus monkey was selected as an experimental model because it is born with an immature bilirubin processing system resulting in unconjugated hyperbilirubinemia (19) and has a threshold for developing bilirubin brainstem toxicity similar to that seen in human newborn infants (20).

MATERIALS AND METHODS

Six newborn rhesus monkeys (*Macaca mulatta*) were delivered by cesarean section at 138–145 d gestation. The monkeys were immediately intubated and stabilized using an Infant Star ventilator (Infrasonics Corp., San Diego, CA). Arterial lines were placed for sampling, blood pressure monitoring, and glucose infusion. Blood was replaced with heparinized placental blood and maternal blood. Venous lines were established in the inferior vena cava for bilirubin infusion. The animals were initially sedated with ketamine. Blood gases were modulated by infusing CO_2 into the gas humidifier. Oxygen and compressed air were blended in an attempt to maintain normal arterial P_{O_2} values.

Bilirubin (Sigma Chemical Co., St. Louis, MO) was dissolved in 0.1 N NaOH, and diluted with saline containing essentially

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fat-free human serum albumin (Sigma) to obtain a final bilirubin concentration of 1 mg/mL and a bilirubin:albumin molar ratio of 20:1. Bilirubin-albumin solutions were refreshed hourly, shielded from light, and infused through a 0.45-μm Millipore filter.

ABR recordings were made immediately after stabilizing the preparation and at 5- to 10-min intervals throughout the study using a Nicolet Compact IV electro-diagnostic system (Nicolet Biomedical Instruments, Madison, WI). Subcutaneous needle recording electrodes were placed over each mastoid and at the vertex. A ground electrode was placed on the forehead. Earphones (Nicolet, TDH-39) were placed 1 cm from the ear and produced clicks at 75 dB intensity relative to threshold in a group of adult humans with normal hearing. Masking noise of 45 dB intensity was given to the opposite ear. Responses to 2000 rarefaction click stimuli of 0.1 ms duration were averaged at a rate of 11.4/s, stored on disk, and displayed on hard copy. The latencies and amplitudes from baseline of waves I, II, and IV were measured, and the I–IV and II–IV peak to peak intervals were calculated for each response (21–23). In the monkey, wave II arises from the cochlear nucleus and wave IV is generated by the lateral lemniscus (23); therefore, the II–IV peak to peak interval was used to measure brainstem conduction.

Total bilirubin levels were assayed by absorption at 460 nm, correcting for Hb. In five of six animals, unbound bilirubin levels were estimated using the peroxidase method (24) (UB Analyzer, Arrow Co., Osaka, Japan). Blood gases and electrolytes were monitored using a Nova Stat Profile 5 analyzer. Data were statistically analyzed using analysis of variance and paired t test. This research was approved by the animal use committee of the University of California, Davis, and conformed to NIH guidelines for the care and use of laboratory animals.

RESULTS

Bilirubin was initially infused at a rate of 25 mg/kg/h, producing serum bilirubin levels ranging from 273 to 376 μmol/L (16–22 mg/dL) by 1 h. The infusion rate was then slowed and CO_2 was introduced, raising the P_{CO_2} to 12.4–16.7 kPa (93–125 mm Hg) and decreasing arterial blood pH to 6.85–7.10. In one animal, CO_2 was introduced 2.4 h before bilirubin infusion, producing a minimal pH of 6.89. This produced minimal changes in the ABR: a slight increase in wave I latency (2.60 to 2.84 ms) and an increase in wave IV amplitude (0.46 to 0.68 μV).

Initially, the animals responded to the elevated P_{CO_2} with tachypnea and agitation, requiring ketamine sedation. After exposure to hyperbilirubinemia and hypercarbia for 1.5–2 h, the animals became depressed, with minimal response to stimulation and only reflex inspiration during positive pressure ventilation. Changes in the ABR first appeared 30–70 min after general neurologic depression was observed.

Maximum bilirubin levels during the procedure ranged from 410 to 564 μmol/L (24–33 mg/dL) and usually coincided with the onset of neurologic depression. Bilirubin levels ranged from 376 to 530 μmol/L (22–31 mg/dL) when ABR changes first appeared. At this time, unbound bilirubin levels ranged between 38 and 65 nmol/L (2.2–3.8 μg/dL). Serum binding deteriorated in most animals as signs of toxicity progressed, and the unbound bilirubin levels generally remained high despite a decrease in total serum bilirubin concentration.

ABR changes initially consisted of prolongation of II–IV interval, followed by a decrease in amplitude in waves II or IV. Wave I remained intact in five of six animals. When the amplitude in any wave form decreased 50% or more, CO_2 infusion was discontinued and minute ventilation was increased. In all animals, there was immediate recovery in the ABR, with the ABR approaching baseline values within 10 min in three of six animals (Fig. 1, Table 1). Two monkeys had partial improvement and one animal had only transient improvement. The most

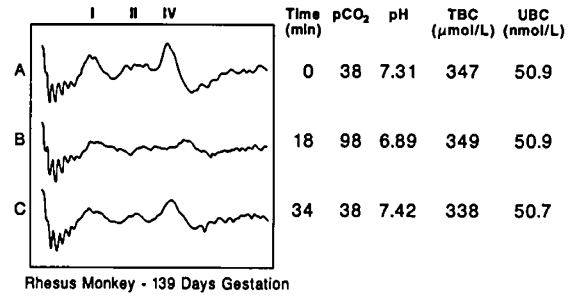

Fig. 1. Response of ABR to hyperbilirubinemia and a second episode of hypercarbia in a 139-d gestation primate. *A*, ABR obtained 20 min after termination of initial CO_2 exposure. Changes associated with initial hypercarbia (not shown) have reversed, and the ABR is nearly identical with the baseline ABR obtained before bilirubin infusion. *B*, ABR 16 min after reintroduction of CO_2 into the ventilator circuit. *C*, Recovery of ABR 14 min after cessation of CO_2 supplementation. *TBC*, total bilirubin concentration; *UBC*, unbound bilirubin concentration.

Table 1. *Conditions of first ABR change and reversal*

	II–IV interval (ms)	Wave IV amplitude (μV)	Blood pH	P_{CO_2} (kPa)	Bilirubin (μmol/L)	UBC (nmol/L)*
Animal 1						
Baseline	2.02	0.59	7.42	7.5		
1st change	2.72	0.00	6.91	16.7	393	
Reversal	1.98	0.25	6.97	10.7	393	
Animal 2†						
Baseline	1.72	0.29	7.36	4.5	410	44
1st change	2.12	0.04	6.99	15.2	342	53
Reversal	1.68	0.24	7.45	4.0	342	52
Animal 3						
Baseline	2.10	0.43	7.39	4.8		
1st change	2.44	0.24	6.97	12.4	427	65
Reversal	1.80	0.31	7.31	5.1	359	52
Animal 4						
Baseline	2.16	0.25	7.38	4.4		
1st change	2.84	0.14	6.93	15.5	376	38
Reversal	2.24	0.13	7.27	5.1	342	36
Animal 5						
Baseline	2.44	0.58	7.35	4.8		
1st change	2.56	0.36	7.02	13.1	461	43
Reversal	2.50	0.53	7.37	5.3	495	39
Animal 6						
Baseline	2.09	0.25	7.35	5.7		
1st change	2.54	0.09	7.05	12.9	529	55
Reversal	2.22	0.26	7.60	2.4	564	50

* UBC, unbound bilirubin concentration.

† Represents 2nd change. This monkey was exposed to CO_2 for 2.4 h before adding bilirubin. Before CO_2 exposure, II–IV interval was 2.20 ms, and IV amplitude was 0.46 μV. Wave IV amplitude *increased* to 0.68 with CO_2 exposure. When bilirubin reached 410 μmol/L, IV amplitude decreased to 0.29 μV and II–IV *decreased* to 1.96 ms. Normalization of pH reduced II–IV further to 1.72 ms, but IV amplitude remained unchanged. These values are used as baseline for this primate.

significant change in the ABR in response to pH manipulation was in the II–IV peak to peak interval. Data from the first cycle in each of the six animals demonstrated that acidosis in the presence of hyperbilirubinemia prolonged the II–IV interval from 2.13 ± 0.17 ms (mean ± SD) to 2.54 ± 0.25 ms. With a return to a less acidotic pH, this interval recovered to 2.06 ± 0.30 ms ($p = 0.008$, $F_{2,15} = 6.71$) (Table 2). The data were also analyzed by paired t test, because the large SD in baseline values might obscure changes when comparing group means. Significant differences between CO_2 exposure and both baseline and recovery values were found in wave IV latency and both I–IV and II–IV peak to peak intervals.

Table 2. *Initial changes and reversal of ABR latencies (±SD) after introduction and removal of CO_2 supplementation*

	Latency (ms)				
	Wave I*	Wave II	Wave IV	I–IV interval*	II–IV interval
Baseline	2.28 (±0.523)	3.45 (±0.421)	5.59 (±0.461)	3.31 (±0.431)	2.13 (±0.170)
Bilirubin + CO_2	2.44 (±0.605)	3.74 (±0.405)	6.19 (±0.552)	3.84 (±0.433)	2.54 (±0.249)
Bilirubin − CO_2	2.37 (±0.521)	3.64 (±0.509)	5.78 (±0.413)	3.43 (±0.322)	2.06 (±0.299)
n	5	6	6	5	6
ANOVA (*F*, *p*)†	0.13, 0.883	0.65, 0.536	2.46, 0.119	2.81, 0.094	6.71, 0.008
Paired *t* test (*p*)					
+CO_2 *vs* baseline	NS	<0.02	<0.02	<0.01	<0.01
+CO_2 *vs* −CO_2	NS	NS	<0.03	<0.02	<0.01

* Wave I not obtainable in one animal with bilirubin + CO_2.

† ANOVA, analysis of variance.

Reinstatement of CO_2 immediately reproduced the ABR abnormalities in all animals. Production and complete or partial reversal of the ABR abnormalities were obtained through two cycles in two animals and three cycles in one animal before irreversible clinical deterioration (hypotension, metabolic acidosis) occurred. The severity of ABR changes usually increased with each CO_2 exposure.

Oxygen requirements increased during the procedure. In one animal, reversal of the ABR abnormality occurred after increasing pH even with a P_{O_2} value of 3.7 kPa (28 mm Hg). Elevation of the P_{CO_2} produced an increase in blood pressure and a decrease in heart rate in all animals. Conversely, normalization of the P_{CO_2} resulted in a decrease in blood pressure. Three animals became hypotensive when the P_{CO_2} was reduced (mean arterial pressure 16–22 mm Hg), but this did not prevent improvement in the ABR.

DISCUSSION

We report herein evidence that modulation of brain P_{CO_2} and presumably brain pH acutely alters the threshold of bilirubin toxicity as measured by the ABR. When the tissue bilirubin level is near threshold for toxicity, the effect of changes in P_{CO_2} on the ABR are immediate and dramatic, suggesting that auditory pathway toxicity is initially mediated by a reversible pH-dependent bilirubin-membrane complex. The change in the II–IV peak to peak interval suggests that bilirubin primarily affects central conduction rather than the peripheral component of the ABR (23). Wave I, reflecting peripheral 8th nerve function, was attenuated in only one animal.

Several studies have previously demonstrated that hypercapnia will increase brain bilirubin content (25–28), and this has been attributed to either alteration of pH or increased cerebral blood flow (28, 29). This study confirms previous suggestions that hypercarbia should increase bilirubin toxicity, but further demonstrates that reducing P_{CO_2} can rapidly reverse bilirubin toxicity. This observation suggests that alteration of cerebral pH must play a more important role in the effect of hypercarbia on bilirubin toxicity, inasmuch as a decrease in cerebral blood flow with correction of hypercarbia would not be expected to rapidly remove bilirubin from brain. Although we hypothesized that increasing brain pH would reverse toxic bilirubin acid-membrane complexes, this study does not preclude the possibility that hypercarbia and bilirubin have independent but synergistic effects on brain function. In the absence of bilirubin, however, the ABR has been reported to be very resistant to hypoxic and hypercarbic conditions. Sohmer *et al.* (30) found a small but statistically significant increase in wave IV latency in cats under severe hypercarbic conditions (25% CO_2, pH 6.8–6.9) but no effect on wave amplitude. Hypoxia had no effect on the ABR, although either hypoxia or hypercapnia severely depressed EEG activity. In this study, only one animal received prolonged exposure to CO_2 with minimal changes in the ABR. CO_2 effects would be expected to occur fairly acutely, and all animals were exposed to CO_2 for 2 h or longer in the presence of bilirubin before an effect was observed. Thereafter, changes were immediate. The delay in the onset of ABR changes is more likely due to the slow equilibration of bilirubin between plasma and brain compartments than to a delayed response to hypercarbia.

In contrast to humans, in the premature primate auditory toxicity appeared to be a late manifestation of bilirubin toxicity, at least under the experimental conditions used. All monkeys were obtunded and apneic before ABR abnormalities appeared. These manifestations of bilirubin poisoning were not reversed by eliminating respiratory acidosis and may or may not be mediated by a pH-dependent mechanism.

These studies are too preliminary to recommend the use of hyperventilation in treating patients with hyperbilirubinemia and signs of bilirubin encephalopathy, but they do emphasize the need to be attentive to brain pH and particularly to arterial P_{CO_2} in patients at risk for kernicterus. Newborns with respiratory disease and hypercarbia associated with hyperbilirubinemia might be expected to have lower thresholds for neurotoxicity. These findings suggest that evaluation and treatment of patients with severe hyperbilirubinemia would be enhanced by measuring the arterial blood gas and monitoring acid-base status. This may be particularly important in patients undergoing exchange transfusion.

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