Hyperammonemia Induces Neuroinflammation That Contributes to Cognitive Impairment in Rats With Hepatic Encephalopathy

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BACKGROUND & AIMS: Hyperammonemia and inflammation cooperate to induce neurological alterations in hepatic encephalopathy. Recent studies in animal models suggest that chronic hyperammonemia and neuroinflammation impair learning ability by the same mechanism. Chronic hyperammonemia might induce inflammatory factors in the brain that impair cognitive function. We sought to determine whether hyperammonemia itself induces neuroinflammation, whether ammonia-induced neuroinflammation mediates cognitive impairment, and whether neuroinflammation also occurs in rats with bile duct ligation (BDL rats)—a model of chronic liver injury that results in hyperammonemia and hepatic encephalopathy. **METHODS:** Chronic moderate hyperammonemia was induced by feeding male Wistar rats an ammonium-containing diet or performing BDL. Rats that received a standard diet or a sham operation were used as controls. Neuroinflammation was assessed by measuring activation of microglia and inflammatory factors. Brain samples were collected from hyperammonemic and BDL rats; microglial activation was determined by immunohistochemistry and quantification of inflammatory markers (ie, inducible nitric oxide synthase, interleukin-1β, and prostaglandin E2). Learning ability and motor activity were assessed in hyperammonemic and BDL rats given ibuprofen as an anti-inflammatory agent. **RESULTS:** Chronic moderate hyperammonemia or BDL activated the microglia, especially in cerebellum; increased inducible nitric oxide synthase, interleukin-1β, and prostaglandin E2 levels; and impaired cognitive and motor function, compared with controls. Ibuprofen reduced microglial activation and restored cognitive and motor functions in the hyperammonemic and BDL rats. **CONCLUSIONS: Chronic hyperammonemia is sufficient to induce microglial activation and neuroinflammation; these contribute to the cognitive and motor alterations that occur during hepatic encephalopathy.**

Keywords: Hyperammonemia; Neuroinflammation; Hepatic Encephalopathy; Bile Duct-Ligation.

Hepatic encephalopathy (HE) is a neuropsychiatric syndrome seen in patients with liver diseases. HE covers a wide range of neuropsychiatric disturbances from minimal changes in personality or altered sleep–waking cycle to altered cognitive function and motor activity and coordination.

Recent reports support the idea that hyperammonemia and inflammation cooperate in inducing the neurological alterations in HE. Shawcross et al[1] proposed that systemic inflammation exacerbates the neuropsychological alterations induced by hyperammonemia. They showed that hyperammonemia deteriorates neuropsychological test scores during inflammatory state, but not after its resolution. Serum levels of the inflammatory cytokines interleukin (IL)-6 and IL-18 are increased in cirrhotic patients with minimal HE and correlate with the grade of minimal HE, while hyperammonemia is present in both groups.[2] Hyperammonemia increases the sensitivity to immune challenges.[3] Injection of lipopolysaccharide (LPS) increases cytokine production similarly in normal or hyperammonemic mice. However, the cognitive deficits induced by LPS were stronger and long-lasting in hyperammonemic mice. These reports support that hyperammonemia and inflammation cooperate in inducing cognitive deficits.

The mechanisms by which cognitive function is impaired in HE are beginning to be clarified in animal models. The ability to learn a Y-maze task is impaired in rats with HE because of portacaval shunts (PCS), which are a result of impaired function of the glutamate–nitric oxide (NO)–cyclic guanosine monophosphate (cGMP) pathway in brain. Learning ability may be restored in PCS rats by treatment with inhibitors of phosphodiesterase 5, which restores the function of this pathway and learning ability.[4] PCS rats are hyperammonemic and show neuroinflammation.[5] Treatment with ibuprofen, an anti-in-

Abbreviations used in this paper: BDL, bile-duct ligation; cGMP, cycle guanosine monophosphate; HE, hepatic encephalopathy; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MHCII, major histocompatibility complex class II; NO, nitric oxide; PCS, portacaval shunts; PGE2, prostaglandin E2.

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flammatory, reduces neuroinflammation but not hyperammonemia, and also restores the function of the glutamate-NO-cGMP pathway and learning ability in PCS rats,[5] supporting that neuroinflammation plays a main role in the impairment of the pathway and of cognitive function in HE.

However, rats with "pure" hyperammonemia without liver failure induced by feeding an ammonium-containing diet also show impaired function of the glutamate–NO–cGMP pathway[6] and reduced ability to learn the Y-maze task,[7] which is also restored by treatment with phosphodiesterase inhibitors.[8] This suggests that hyperammonemia and neuroinflammation impair learning ability by the same mechanism. All these data lead us to hypothesize that hyperammonemia would induce some inflammatory factor in the brain, which would be responsible for impairment of the glutamate–NO–cGMP pathway and of cognitive function.

The aims of this work were to assess whether hyperammonemia induces neuroinflammation; whether ammonia-induced neuroinflammation is responsible for the cognitive impairment; and whether neuroinflammation is also present in a model of chronic liver injury resulting in hyperammonemia and HE. We used a rat model of pure chronic moderate hyperammonemia without liver failure induced by feeding rats an ammonium-containing diet.[9] As a model of chronic liver injury resulting in hyperammonemia and HE, we used rats with bile duct ligation (BDL), a model recommended by the International Society for Hepatic Encephalopathy.[10]

A main mechanism leading to neuroinflammation is activation of microglia, which releases inflammatory factors. We determined in the brains of these rats, the activation of microglia by immunohistochemistry and presence of inflammatory markers (inducible nitric oxide synthase [iNOS], IL-1β, and prostaglandin 2 [PGE2]).

To assess whether neuroinflammation is responsible for the cognitive and motor impairments, we tested whether treatment with the anti-inflammatory ibuprofen restores learning ability and motor activity in hyperammonemic or BDL rats.

Materials and Methods

Model of Pure Chronic Hyperammonemia in Rats Without Liver Failure

Male Wistar rats (120–140 g) were made hyperammonemic by feeding them an ammonium-containing diet for 1, 7, or 28 days, as in Felipo et al.[9]

Model of BDL in Rats

Male Wistar rats (175–200 g) were used. Liver injury was induced by common BDL, as in Jover et al.[11] Control rats were sham-operated. Animal experiments were approved by the Center and carried out in accordance with the European Communities Council Directive (86/609/EEC).

Treatment With Ibuprofen

Rats were treated daily with S-(+)ibuprofen (Fluka, Seelze, Germany) or saline. Treatment with ibuprofen started 2 weeks later than diet, after the rats had chronic hyperammonemia, or 2 weeks after surgery for BDL rats. Ibuprofen treatment was maintained until sacrifice. Ibuprofen in sterile saline was injected intraperitoneally, 30 mg/kg per day in 0.5 mL/100 g body weight. Control rats were injected with saline. Analysis of learning ability (Y-maze test) and motor activity were performed 10 and 21 days after starting ibuprofen treatment, respectively. Microdialysis studies were performed at days 24–25 of ibuprofen treatment.

Motor Activity

Motor activity was determined using an actimeter with arrays of infrared motion detectors (Med Associates, St Albans, VT), as in Cauli et al.[12] Motor activity is given as ambulatory counts in 60 minutes. Assessment of motor activity started 1 hour after ibuprofen or saline injection.

Y-Maze Learning Test

The ability to learn a conditional discrimination task in a Y maze was tested as in Aguilar et al.[7] Rats were trained for 10 trials per day until the completion of a criterion of 10 correct responses on the same day.

Determination of Extracellular Concentrations of Ammonia, PGE2, and IL-1β by In Vivo Brain Microdialysis

Rats were anesthetized using halotane and a microdialysis guide was implanted in the cerebellum (anteroposterior −10.2, mediolateral −1.6, and dorsoventral −1.2), as in Hermenegildo et al.[6] After 48 hours, a microdialysis probe was implanted. Probes were perfused (3 μL/min) with artificial cerebrospinal fluid: NaCl mM, 145 mM; KCl, 3.0 mM; $CaCl_2$, 2.26 mM; buffered at pH 7.4 with 2 mM phosphate. After a 2–3-hour stabilization period, 30-minute samples were collected. Concentrations of ammonia, PGE2, and IL-1β were determined in the microdialysis samples. PGE2 was measured using the ELISA Biotrak System (Amersham Biosciences, Buckinghamshire, UK); IL-1β using an enzyme-linked immunosorbent assay kit from Pierce (Rockford, IL) and ammonia as described in the Ammonia Determination section.

Determination of IL-1β in Cerebellum

Cerebella were homogenized in 5 volumes of 20 mM Tris-HCl (pH 7.4), 10 mM ethylenediamine tetraacetic acid, and 0.2 mM phenylmethylsulfonylfluoride using an Ultra-Turrax (IKA-Werke, Wilmington, NC). Samples were centrifuged at 10,000*g* at 4°C for 10 minutes. IL-1β was measured in the supernatants using an enzyme-linked immunosorbent assay kit from Pierce. Protein concentration was measured by the bicinchonic acid procedure.