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Bumetanide increases manganese accumulation in the brain of rats with liver damage

Sergio Montes [a], Armando Castro-Chávez [b], Circe Florian-Soto [b], Yessica Heras-Romero [c], Camilo Ríos [a], Susana Rivera-Mancía [b,*,1]

[a] Neurochemistry Department, National Institute of Neurology and Neurosurgery "Manuel Velasco Suárez", Insurgentes sur 3877, La Fama, Tlalpan, Mexico 14269, D.F., Mexico
[b] Biological Systems Department, Autonomous Metropolitan University-Xochimilco, Calzada del Hueso 1100, Villa Quietud, Coyoacán, Mexico 04960, D.F., Mexico
[c] Ethology, Wildlife and Laboratory Animals Department, Faculty of Veterinary Medicine and Zootechny, National Autonomous University of Mexico, University City, Coyoacán, Mexico 04360, D.F., Mexico

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ABSTRACT

Hepatic encephalopathy is a common complication in cases of liver damage; it results from several factors, including the accumulation of toxic substances in the brain, e.g. manganese, ammonia and glutamine. We have previously reported that manganese favors ammonia and glutamine accumulation in the brain of cirrhotic rats, and we suggested that such effect could be mediated by manganese-elicited activation of the NKCC1 ($Na^+/K^+/2Cl^-$ cotransporter 1). To test this hypothesis, we used bumetanide, an NKCC1 blocker prescribed to treat ascites in cirrhotic patients; we expected that if NKCC1 was responsible for manganese-mediated ammonia buildup and the subsequent glutamine accumulation, bumetanide could counteract such effect and improve motor coordination. In addition, we considered essential to test the effect of bumetanide on manganese brain levels. We used a model of liver damage in rats, consisting in bile-duct ligation. Animals were exposed to manganese in the drinking water (1 mg/ml) for two weeks and ammonia in the food (20% w/w of ammonia acetate) during the second week after surgery. Bumetanide was administered intraperitoneally in the course of the ammonia treatment. We measured glutamine and manganese in three brain regions: frontal cortex, striatum and cerebellum. Bumetanide produced no effect on glutamine accumulation; however, because of bumetanide treatment, manganese was increased in the brain, and also the activity of gamma-glutamyl transferase in plasma; thus, we consider that the influence of bumetanide and similar diuretics on liver function and manganese homeostasis should be further studied.

1. Introduction

Hepatic encephalopathy is a neuropsychiatric syndrome characterized by cognitive, motor and psychiatric disturbances, which frequently develops as a consequence of chronic liver disease (Butterworth, 2003). Because of liver damage, several toxic substances accumulate in the brain; manganese, ammonia and glutamine are particularly important for the pathogenesis of hepatic encephalopathy (Butterworth, 2008; Rama Rao and Norenberg, 2014). In the brain, ammonia is a substrate for glutamine synthesis, in a reaction catalyzed by glutamine synthetase, an enzyme mainly located in astrocytes, which requires manganese as a cofactor (Martinez-Hernandez et al., 1977; Suárez et al., 2002). The relevance of this chemical reaction relies on the fact that the disequilibrium in the ammonia metabolism in the liver leads to the accumulation of this substance and glutamine in the brain. Glutamine is considered to play a central role in the development of hepatic encephalopathy by acting as a "Trojan horse" in astrocyte mitochondria, where it is hydrolyzed and induces mitochondrial permeability transition and oxidative/nitrosative stress (Rama Rao and Norenberg, 2014).

The relationships between the factors involved in the pathogenesis of hepatic encephalopathy represent a very complex phenomenon. We have previously reported that manganese increases ammonia and glutamine content in the brain of cirrhotic

* Corresponding author.
E-mail addresses: smontes@innn.edu.mx (S. Montes), armado_cruzazul@hotmail.com (A. Castro-Chávez), circevi.floso@gmail.com (C. Florian-Soto), sarehy@gmail.com (Y. Heras-Romero), crios@correo.xoc.uam.mx (C. Ríos), syriverama@conacyt.mx (S. Rivera-Mancía).
[1] Permanent address: Department of Social Medicine, National Institute of Cardiology Ignacio Chávez, Juan Badiano 1, Sección XVI, Tlalpan, Mexico 14080, D.F., Mexico.

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rats; a synergistic effect between manganese and ammonia to increase glutamine in the frontal cortex of rats with chronic liver damage was also observed (Rivera-Mancía et al., 2012). The possibility that manganese increased ammonia transport via the NKCC1 ($Na^+/K^+/2Cl^-$ cotransporter 1) and, consequently, contributed to enhanced glutamine accumulation, was hypothesized (Rivera-Mancía et al., 2012), based in the fact that manganese is able to stimulate NKCC1 via SPAK (STE20/SPS1-related proline/alanine-rich kinase)/OSR1 (oxidative stress response 1) (Gagnon et al., 2006).

NKCC1 is able to transport ammonia instead of potassium (See Ott and Larsen, 2004 for a review) and is considered as a mediator of brain edema in several neurological conditions (See Jayakumar and Norenberg, 2010). Bumetanide (3-*n*-butylamino-4-phenoxy-5-sulphamoyl benzoic acid), a specific NKCC1 inhibitor at low concentrations (2–10 μm) (Kahle et al., 2009), has been intensively used for the study of several pathologies in which this transporter seems to be involved (Kahle et al., 2008) and it is one of the diuretic drugs recommended to treat ascites, a common complication in cirrhotic patients (Moore and Aithal, 2006; Moore and Van Thiel, 2013). Bumetanide is used instead of furosemide when patients do not respond to high doses of the latter, because of its potency (40 to 60 times higher than furosemide) and similar side effects (Marcantonio et al., 1983; Moore and Van Thiel, 2013).

In this work, we evaluated the effect of bumetanide on glutamine accumulation, under conditions of manganese and ammonia accumulation in liver-damaged rats. In addition to glutamine levels, we evaluated the consequences of bumetanide treatment on manganese brain concentration and motor coordination.

2. Materials and methods

2.1. Animals and treatments

Ninety-eight male Wistar rats, weighing 250–280 g, from the animal care facility of the National Institute of Neurology and Neurosurgery, were used for the experiments. We used the same model previously reported (Rivera-Mancía et al., 2012), adding bumetanide as another factor to be tested. Sixteen groups resulted from the combination of the factors studied (bile duct ligation (L), manganese (M), ammonia (A) and bumetanide (B)): S, SA, SM, SAM, L, LA, LM, LAM, SB, SAB, SMB, SAMB, LB, LAB, LMB, LAMB (see Table 1). L groups were liver-damaged rats; liver damage was induced using a previously reported procedure (Montes et al., 2002). Briefly, rats were anesthetized with sodium pentobarbital (35 mg/kg i.p.), before making a midline incision and exposing the bile duct; three ligations were made to the conduct, leaving two of them on the side coming from the liver and a single ligation on the side attached to the intestine. The same procedure was carried out in the S (sham) rats, except for the obstruction of biliary flux. In M (manganese) groups, drinking water was replaced by a $MnCl_2$ solution (1 mg of manganese/ml) during the two weeks of treatment. Along the second week of the treatments, rats in A (ammonia) groups received 20% w/w ammonium acetate in their food (Jover et al., 2006; Rivera-Mancía et al., 2012), and rats in B groups were treated with bumetanide (3.8 mg/kg daily); so that, LAMB rats were bile-duct ligated and treated with ammonia, manganese and bumetanide. Bumetanide solution was prepared according to Brandt et al. (2010) and the corresponding vehicle was administered to the rats not receiving bumetanide. Seven surgery sessions (6–18 animals each) spaced two weeks apart, were performed until complete three animals per group (24 animals per factor). In each surgery session, every rat was given a consecutive number; then, each number was randomly assigned to the different treatments, considering mortality data from previous sessions. Animals were killed by decapitation two weeks after surgery. One brain hemisphere was used to determine glutamine and the other one was used to measure manganese levels.

Throughout the duration of the study, animals were housed on a 12 h light/dark cycle. This study was made in accordance with our institutions guidelines and the Mexican regulation regarding the care and use of laboratory animals (NOM-062-ZOO-1999). All efforts were made to minimize the number of animals and their suffering.

2.2. Liver function tests

Blood was collected at the moment of sacrifice to determine alanine aminotransferase and gamma-glutamyl transferase activities in plasma by standard procedures (Glossmann and Neville, 1972; Reitman and Frankel, 1957).

2.3. Brain dissection

Brain dissection was performed following the procedures suggested by Glowinski and Iversen (1966) and Chiu et al. (2007). Briefly, to obtain frontal cortex from each hemisphere, the genu of corpus callosum was taken as a point of reference, that is +1 mm from bregma (Paxinos and Watson, 2007); brain cortex was cut at

Table 1
Experimental design.

Number of factors combined	Factor				Resulting group
	Bile duct ligation	Ammonia	Manganese	Bumetanide	
Zero	No	No	No	No	Sham (S)
One	Yes	No	No	No	Liver damage (L)
	No	Yes	No	No	Sham + ammonia (SA)
	No	No	Yes	No	Sham + Mn (SM)
	No	No	No	Yes	Sham + bumetanide (SB)
Two	Yes	Yes	No	No	Liver damage + ammonia (LA)
	No	Yes	Yes	No	Sham + ammonia + Mn (SAM)
	No	No	Yes	Yes	Sham + Mn + bumetanide (SMB)
	No	Yes	No	Yes	Sham + ammonia + bumetanide (SAB)
	Yes	No	Yes	No	Liver damage + Mn (LM)
	Yes	No	No	Yes	Liver damage + bumetanide (LB)
Three	Yes	Yes	Yes	No	Liver damage + ammonia + Mn (LAM)
	No	Yes	Yes	Yes	Sham + ammonia + Mn + bumetanide (SAMB)
	Yes	Yes	No	Yes	Liver damage + ammonia + bumetanide (LAB)
	Yes	No	Yes	Yes	Liver damage + Mn + bumetanide (LMB)
Four	Yes	Yes	Yes	Yes	Liver damage + ammonia + Mn + bumetanide (LAMB)