A single transient episode of hyperammonemia induces long-lasting alterations in protein kinase A

Carmina Montoliu,1 Blanca Piedrafita,2 Miguel A. Serra,1 Juan A. del Olmo,1 José M. Rodrigo,1 and Vicente Felipo2

1Servicio de Hepatología, Hospital Clínico Universitario, Departamento de Medicina, Universidad de Valencia; and 2Laboratory of Neurobiology, Centro de Investigación Príncipe Felipe, Fundación de la Comunidad Valenciana Centro de Investigación Príncipe Felipe, Valencia, Spain

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Montoliu C, Piedrafita B, Serra MA, del Olmo JA, Rodrigo JM, Felipo V. A single transient episode of hyperammonemia induces long-lasting alterations in protein kinase A. Am J Physiol Gastrointest Liver Physiol 292: G305–G314, 2007; doi:10.1152/ajpgi.00100.2006.—Hepatic encephalopathy in patients with liver disease is associated with poor prognosis. This could be due to the induction by the transient episode of hepatic encephalopathy of long-lasting alterations making patients more susceptible. We show that a single transient episode of hyperammonemia induces long-lasting alterations in signal transduction. The content of the regulatory subunit of the protein kinase dependent on cAMP (PKA-R1) is increased in erythrocytes from cirrhotic patients. This increase is reproduced in rats with portacaval anastomosis and in rats with hyperammonemia without liver failure, suggesting that hyperammonemia is responsible for increased PKA-R1 in patients. We analyzed whether there is a correlation between ammonia levels and PKA-R1 content in patients. All cirrhotic patients had increased content of PKA-R1. Some of them showed normal ammonia levels but had suffered previous hyperammonemia episodes. This suggested that a single transient episode of hyperammonemia could induce the long-lasting increase in PKA-R1. To assess this, we injected normal rats with ammonia and blood was taken at different times. Ammonia returned to basal levels at 2 h. However, PKA-R1 was significantly increased in blood cells from rats injected with ammonia 3 wk after injection. In conclusion, it is shown that a single transient episode of hyperammonemia induces long-lasting alterations in signal transduction both in blood and brain. These alterations may contribute to the poor prognosis of patients suffering hepatic encephalopathy.

hepatic encephalopathy; soluble guanylate cyclase; liver cirrhosis; rat models

CHRONIC LIVER DISEASE LEADS to hemodynamic and neurological alterations. Hepatic encephalopathy is a complex neuropsychiatric syndrome present in patients with liver disease. Hepatic encephalopathy covers a wide range of neuropsychiatric disturbances ranging from minimal changes in personality or altered circadian rhythms (sleep-waking cycle) to alterations in intellectual function, personality, conscience, and neuromuscular coordination. Patients with liver cirrhosis with normal neurological and mental status examination may present minimal forms of hepatic encephalopathy, showing intellectual function impairment that cannot be detected through general clinical examination but can be unveiled by specific neuropsychological and neurophysiological examination (1, 13).

Overt hepatic encephalopathy is usually elicited by a precipitating factor (high protein ingestion, gastrointestinal constipation, bleeding, diuretics) usually associated with increased ammonia levels. The appearance of hepatic encephalopathy is associated with poor prognosis for reasons that are not known. It seems reasonable to assume that this would be due to the induction by the transient episode of hepatic encephalopathy of long-lasting alterations that will predispose the patient to suffer more deleterious effects in response to subsequent injuries. In this work we show that a single transient episode of hyperammonemia levels induces long-lasting alterations in signal transduction.

The content of the regulatory subunit R1 of the protein kinase dependent on cAMP (PKA) is strongly increased in erythrocyte membranes from patients with liver cirrhosis (21). Moreover, PKA-R1 in these membranes binds and retains significant amounts of cGMP. This could contribute to the decrease reported (9) in intracellular cGMP in lymphocytes and erythrocytes of these patients. The decreased intracellular cGMP content and the alteration in PKA-R1 in patients with liver cirrhosis would alter signal transduction modulated by cGMP or cAMP in blood cells, thus altering cellular function. These alterations may be involved in some of the clinical manifestations of the disease, including the alterations in platelet aggregation, the portal hypertension, or the systemic hypertension (21).

To prevent or reverse the alterations in PKA-R1 and cGMP content and the associated clinical manifestations, it is important therefore to identify the factors associated with liver cirrhosis that induce the increase in PKA-R1. This can be done more easily in animal models than in patients.

The initial aim of the present work was to assess whether animal models of chronic liver failure and of chronic hyperammonemia reproduce the alterations in PKA-R1 content found in blood cells of patients with liver cirrhosis. It is shown that both rats with chronic liver failure due to portacaval anastomosis and rats with chronic hyperammonemia without liver failure reproduce the increase in PKA-R1 in blood cells. This suggests that hyperammonemia would be responsible for the increase in PKA-R1.

We then analyzed in patients with liver cirrhosis whether there is a correlation between ammonia levels in blood and the increase in PKA-R1. It is shown that a single transient episode

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of hyperammonemia is enough to induce a sustained increase in PKA-RI.

**MATERIALS AND METHODS**

Patients with Liver Disease and Controls

Forty-four patients with liver disease and forty-four controls were enrolled in the study after informed consent was obtained. The subjects were classified as 1) patients diagnosed with hepatic cirrhosis after histological study, 2) patients diagnosed histologically with chronic hepatitis, or 3) control subjects for whom liver disease was discarded. All patients had previous liver biopsies, which were graded according to the Batts-Ludwig classification (3). Cirrhosis was defined as grade 4 fibrosis (nodules + diffuse fibrosis). All patients with chronic hepatitis had grade 1 (portal), 2 (periportal), or 3 (septal) fibrosis. For cirrhotic patients liver biopsies were performed 4–10 yr before and for patients with chronic hepatitis 1–3 yr before inclusion in the study. All patients with cirrhosis (grade 4 fibrosis) had previously experienced an episode of hyperammonemia or of clinical or minimal encephalopathy, as detected with psychometric tests. The last episode was 1–10 mo before the study. No patients with chronic hepatitis (grades 1–3 of fibrosis) had ever experienced encephalopathy nor had an elevated ammonia level in any of their previous analyses. The composition and characteristics of the different groups are given in Table 1. After a standard history and physical examination, blood was drawn for routine laboratory measures (Table 2). Parallel determinations of blood ammonia and PKA-RI content in blood cells were carried out in two subgroups of 10 controls and 17 cirrhotic patients. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Scientific and Ethical Committees of the Hospital.

Animal Models

Model of chronic liver failure: rats with portacaval anastomosis. Rats were anesthetized, and an end-to-side portacaval anastomosis was constructed under aseptic conditions by a continuous suture technique according to the technique of Lee and Fisher (18). Sham-operated rats had their portal vein and inferior vena cava clamped for 10 min. The anastomosis was examined at the moment of death; the liver was atrophic, and the anastomosis was permeable.

Model of chronic hyperammonemia without liver disease. Male Wistar rats were made hyperammonemic by feeding them an ammonia-containing diet as previously described (2), for 21 days. The comparison of the results obtained with rats with portacaval anastomosis and with this model allows discernment of which effects are due to hyperammonemia and which are due to other factors associated with liver failure.

Preparation of erythrocytes and their membranes. Blood (20 ml) collected in heparin-coated tubes was centrifuged at 500 g for 10 min at 4°C. Erythrocytes and their membranes were obtained as previously described (21). This fraction contains all blood cells, but given that more than 99% of them are erythrocytes we call this fraction “erythrocytes.” The membranes were resuspended in a minimum volume of 0.5 mM NaH2PO4, pH 8.

Isolation of lymphocytes. Lymphocytes were obtained from 10 ml of blood as described by Kimura et al. (17) with the slight modifications described by Montoliu et al. (21). The pellet containing the lymphocytes was resuspended in 0.5 ml 20 mM Tris·HCl pH 7.4, 2 mM EDTA, 0.5 mM EGTA, 0.5 mM DTT, 0.5 PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotonin, 1 mM sodium orthovanadate, 10 mM NaF. The lymphocytes were boiled for 5 min, and protein was determined by the bicinchoninic acid method (Protein Assay Reagent, Pierce). Samples were subjected to SDS-PAGE and immunoblotting as described below.

In vitro treatment of erythrocytes and lymphocytes with ammonia. Erythrocytes (0.5 ml) or lymphocytes (0.5 ml) resuspended in RPMI 1640 medium without l-glutamine (GIBCO-BRL, Life Technologies) containing 10% FBS were treated with 100 μM ammonium chloride for 15 min at 37°C. This concentration was chosen to match the increase in the brain ammonia level in hyperammonemic rats (19). The erythrocytes or lymphocytes were centrifuged at 450 g for 10 min and rinsed two times with cold PBS and resuspended in 0.5 ml of extraction buffer (20 mM Tris·HCl pH 7.4, 2 mM EDTA, 0.5 mM EGTA, 0.5 mM DTT, 0.5 PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotonin, 1 mM sodium orthovanadate, 10 mM NaF). Erythrocytes or lymphocytes were sonicated and homogenized, and protein was determined by the bicinchoninic acid method (Protein Assay Reagent, Pierce). Samples were subjected to SDS-PAGE and immunoblotting as described below.

Primary cultures of astrocytes. Cortical astrocytes were prepared from newborn Wistar rats pups (postnatal day 1). Pups were decapitated, and the head skin was removed. Whole brains were extracted from the skull, and the two cortical hemispheres were dissected from the rest and kept in a petri dish with in basal medium Eagle (GIBCO).

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**Table 1. Composition of the groups of patients with liver disease and controls**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Cirrhosis</th>
<th>Chronic Hepatitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Women</td>
<td>16</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>Men</td>
<td>28</td>
<td>23</td>
<td>5</td>
</tr>
<tr>
<td>Number of patients</td>
<td>44</td>
<td>35</td>
<td>9</td>
</tr>
<tr>
<td>Age</td>
<td>43 ± 12</td>
<td>52 ± 8.3</td>
<td>39 ± 10 (27–61)</td>
</tr>
<tr>
<td>Alcohol</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>HVC</td>
<td>0</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>HVC + alcohol</td>
<td>0</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Other ethologies</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Ascites</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Child Pugh*</td>
<td>0</td>
<td>20 (A)</td>
<td>52 (30–70)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13 (B)</td>
<td>52 (30–70)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 (C)</td>
<td>52 (30–70)</td>
</tr>
</tbody>
</table>

Values are means ± SD. *Child Pugh score: A = 5–6; B = 7–9; C = 10–15. HVC, hepatitis virus C.

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**Table 2. Analytical data of the patients with hepatic failure**

<table>
<thead>
<tr>
<th></th>
<th>Range</th>
<th>Control</th>
<th>Cirrhosis</th>
<th>Chronic Hepatitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOT, μmol/l</td>
<td>(1–37)</td>
<td>20 ± 4</td>
<td>42 ± 22*</td>
<td>68 ± 32*</td>
</tr>
<tr>
<td>GPT, μmol/l</td>
<td>(1–41)</td>
<td>17 ± 6</td>
<td>35 ± 24*</td>
<td>106 ± 53*</td>
</tr>
<tr>
<td>GGT, μmol/l</td>
<td>(10–49)</td>
<td>47 ± 10</td>
<td>96 ± 60*</td>
<td>64 ± 56*</td>
</tr>
<tr>
<td>Uric acid, mg/dl</td>
<td>(2.5–7)</td>
<td>40 ± 10</td>
<td>80 ± 50</td>
<td>120 ± 60</td>
</tr>
<tr>
<td>Creatinine, mg/dl</td>
<td>(0.5–1.3)</td>
<td>30 ± 40</td>
<td>90 ± 12</td>
<td>210 ± 100</td>
</tr>
<tr>
<td>Cholesterol, mg/dl</td>
<td>(140–200)</td>
<td>172 ± 22</td>
<td>181 ± 55</td>
<td>162 ± 42</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>(40–160)</td>
<td>87 ± 30</td>
<td>110 ± 57</td>
<td>86 ± 39</td>
</tr>
<tr>
<td>Bilirubin, mg/dl</td>
<td>(0.1–1)</td>
<td>0.6 ± 0.3</td>
<td>1.5 ± 0.6*</td>
<td>1.0 ± 0.8</td>
</tr>
<tr>
<td>Albumin, g/dl</td>
<td>(3.5–5)</td>
<td>4.4 ± 0.2</td>
<td>3.8 ± 0.6</td>
<td>4.4 ± 0.6</td>
</tr>
<tr>
<td>Prothrombin time, s</td>
<td>13 ± 1.3</td>
<td>22 ± 4*</td>
<td>32 ± 5*</td>
<td>21 ± 5*</td>
</tr>
<tr>
<td>Fibrinogen, g/l</td>
<td>(2–4)</td>
<td>3.1 ± 1.0</td>
<td>3.5 ± 1.5</td>
<td>3.0 ± 0.6</td>
</tr>
<tr>
<td>Alk. phosphatase, μmol/l</td>
<td>(50–250)</td>
<td>162 ± 61</td>
<td>273 ± 72*</td>
<td>190 ± 76</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>(4.2–6.1)</td>
<td>4.6 ± 0.4</td>
<td>4.2 ± 0.5</td>
<td>4.6 ± 0.7</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>(4.8–10.8)</td>
<td>6.7 ± 1.3</td>
<td>6.2 ± 2.4</td>
<td>6.6 ± 1.3</td>
</tr>
<tr>
<td>Neutrophils, %</td>
<td>(55–75)</td>
<td>55 ± 7.4</td>
<td>60 ± 9.8</td>
<td>55 ± 10</td>
</tr>
<tr>
<td>Lymphocytes, %</td>
<td>(17–45)</td>
<td>35 ± 5.0</td>
<td>26 ± 10</td>
<td>35 ± 5.0</td>
</tr>
<tr>
<td>Monocytes, %</td>
<td>(2–8)</td>
<td>6.0 ± 1.3</td>
<td>9.2 ± 3.5*</td>
<td>8.3</td>
</tr>
<tr>
<td>Eosinophils, %</td>
<td>(1–4)</td>
<td>3.3 ± 2.0</td>
<td>2.2 ± 1.1</td>
<td>3.1 ± 2.4</td>
</tr>
<tr>
<td>Basophils, %</td>
<td>(0.05–0.5)</td>
<td>0.5 ± 0.2</td>
<td>0.6 ± 0.2*</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>Ammonia, μM</td>
<td>(45–75)</td>
<td>67 ± 26</td>
<td>145 ± 16*</td>
<td>77 ± 15</td>
</tr>
</tbody>
</table>

Values are means ± SD. GOT, glutamate-oxaloacetate transaminase; GPT, glutamate-pyruvate transaminase; GGT, gamma-glutamyl transferase; Alk phosphatase, alkaline phosphatase. *Significantly different from controls, *P < 0.05.
The brain was sliced with a sterile blade and transferred into a vial with basal medium Eagle containing 10% FBS (GIBCO). Tissue was then homogenized and vortexed for 1 min to destroy cortical neurons. The cell suspension was filtered through a mesh with a pore size of 90 μm and the cellular pellet was resuspended in Dulbecco’s modified Eagle’s medium containing 10% FBS, glutamine 2 mM, Fungizone 5 μg/ml, 20 U/ml penicillin, and 20 μg/ml streptomycin. Cells were seeded (~600,000 cells/ml) on untreated plastic petri dishes (60-mm diameter). Cells were incubated at 37°C in 5% CO₂ atmosphere. Culture medium was changed every 5 days. Astrocyte cultures were used at 12 days; 100 μM NH₄Cl was added to the culture medium and the astrocytes were homogenized 15 or 30 min later and subjected to immunoblotting as described below.

**Immunoblotting.** Samples were subjected to SDS-PAGE and immunoblotting as previously described (21) by using antibodies against the regulatory subunit of PKA (PKA-RI, BD Transduction Laboratories, St. Louis, MO) and alkaline phosphatase color development (Sigma). Images were captured with a Hewlett-Packard Scanjet 5300C. The intensities of the bands were measured with the 1D Main program from AAB Software (Advanced American Biotechnology, 1166E).

**Injection of control rats with ammonia.** For studies in blood cells, groups of 9 to 18 male Wistar rats weighing 200–250 g were used in each experiment. One group (ammonia group) was injected with 5 mmol/kg ip of ammonium acetate, and control rats were injected with saline. Rats were killed at different times (from 20 min to 3 wk) after injection. The rats were generally anesthetized by injection of 40 mg/kg ip pentobarbital sodium (Eutea-Lender, Normon, Spain). Then 0.4 ml of heparin was injected into the femoral vein and blood was taken from the cava vein and diluted with 1 volume of PBS for isolation of lymphocytes and erythrocytes.

To isolate the cytosol and membrane fractions, the lymphocytes were centrifuged at 840 g for 10 min and washed three times with PBS solution (NaCl 137 mM, KCl 2.68 mM, Na₂HPO₄ 6.4 mM, KH₂PO₄ 1.47 mM; pH 7.4) and suspended in 1 ml of buffer A (Tris-HCl 20 mM, EDTA 2 mM, EGTA 0.5 mM, leupeptin 50 μg/ml, aprotinin 50 μg/ml, PMSF 100 mM, NaF, 2 mM, Na₃P₂O₇ 1 mM, Na₃VO₄ 2 mM, DTT 0.25 mM). Samples were sonicated in a Branson sonicator and centrifuged at 100,000 g for 1 h at 4°C (Optima XL100 Centrifuge, Coulter). The supernatant was removed and stored at −80°C as the cytosol fraction. The pellet was suspended in 200 μl of buffer B (Tris-HCl 20 mM, Igepal 1%, EDTA 2 mM, EGTA 0.5 mM, leupeptin 10 μg/ml, aprotinin 5 μg/ml, PMSF 100 mM, Benzamidine 1 mM, DTT 0.5 mM) and centrifuged at 18,890 g for 15 min at 4°C. The pellet was discarded, and the new supernatant containing the membrane proteins was stored at −20°C as the membrane fraction.

**Determination of ammonia in blood.** Ammonia was measured as described previously (14). Duplicate 150-μl aliquots of arterial blood samples were added to 150 μl of ice-cold 10% trichloroacetic acid. After centrifugation at 12,000 g for 10 min at 4°C, the supernatant was collected and neutralized with 15 μl of KHCO₃ to pH 7. Duplicate 50-μl samples were mixed with 32.5 μl of the mixture (phosphate buffer 0.2 M, pH 8; α-ketoglutarate 0.6 M pH 7; NADH 10 mM) and 10 μl of glutamate dehydrogenase. Ammonia was measured by means of the Fluoroskan Ascent using excitation and emission filters of 355 and 460 nm, respectively.

**Statistical Analysis**

Results were analyzed by two-way ANOVA followed by post hoc Newman-Keuls test using the GraphPad PRISM version 3.0. The probability level accepted for significance was P < 0.05. To analyze the correlation between the different parameters, data were processed with the software package SPSS version 10.0 (SPSS, Chicago, IL), and two-sided P values < 0.05 were considered significant.

The experimental procedures were approved by the Center and met the guidelines of the European Union for treatment and use of experimental animals. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

**RESULTS**

We first analyzed whether the content of PKA-RI is increased in blood cells in animal models of chronic liver failure or of chronic hyperammonemia without liver failure.

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Fig. 1. The content of the regulatory subunit of cAMP-dependent protein kinase (PKA-RI) is increased in erythrocytes and in their membranes from rats with portacaval anastomosis. Erythrocytes and erythrocyte membranes were isolated from rats with portacaval anastomosis, and the content of PKA-RI was analyzed by immunoblotting as described in MATERIALS AND METHODS. A: typical immunoblottings corresponding to erythrocyte membranes from 2 different controls (C1–C2) and 2 different rats with portacaval anastomosis (PCS1–PCS2). The images were captured, and the intensities of the bands were measured and are shown for erythrocyte membranes (B) and for erythrocytes (D). Values are means ± SD of 5 samples in B and of 4 samples in D. Significantly different from controls: *P < 0.05; **P < 0.005.
As shown in Fig. 1, A and B, the content of PKA-RI was significantly increased (163 ± 43% of control) in membranes from erythrocytes from rats with portacaval anastomosis. The content of PKA-RI was also significantly increased (184 ± 42% of control) in whole erythrocytes from these rats (Fig. 1, C and D). Moreover, the content of PKA-RI was also significantly increased (178 ± 40% of control) in lymphocytes from rats with portacaval anastomosis (Fig. 2).

In rats with chronic moderate hyperammonemia without liver failure (induced by feeding an ammonium-containing diet), the content of PKA-RI was also significantly increased (254 ± 52% of control) in erythrocyte membranes (Fig. 3, A and B) as well as in whole erythrocytes (148 ± 11% of control, P < 0.0001) (Fig. 3, C and D).

Moreover, PKA-RI was also significantly increased (215 ± 51% of control) in lymphocytes from rats with chronic hyperammonemia without liver failure (Fig. 4).

These results indicate that hyperammonemia is enough to induce the increase in PKA-RI and suggest that the increase in PKA-RI in patients with liver cirrhosis should be also due to the increase in ammonia levels.

To assess this possibility, we analyzed in groups of patients with liver cirrhosis and of control subjects the content of PKA-RI in blood cells and ammonia levels in blood. The content of PKA-RI was significantly increased in erythrocytes (187 ± 46% of control, P < 0.0001) (Fig. 5, A and B) and lymphocytes (178 ± 32% of control, P < 0.0001) from these patients (Fig. 5, C and D).

The concentration of ammonia in blood of these patients and controls is shown in Fig. 6. Some patients were hyperammonemic whereas other had normal levels of ammonia in blood.

We analyzed whether there is a correlation between ammonia levels and PKA-RI content. As shown in Fig. 7, all cirrhotic patients had increased content of PKA-RI, but only some of them showed increased ammonia levels. When Fig. 7A is divided into four areas (A1–A4), it can be seen that all controls (10 of 10) are in the area A3, with normal levels of ammonia and of PKA-RI. Eight of 17 cirrhotic patients are in area A2, with increased ammonia levels and PKA-RI content. However, 9 of 17 patients are in area A1, with increased PKA-RI content but normal ammonia levels. This indicates that there is no correlation between ammonia concentration in blood and...
PKA-RI content in erythrocytes or lymphocytes (Fig. 7). However, patients in area A1 have had previously episodes of hyperammonemia. Moreover, it can be seen that area A4 of Fig. 7 is empty, indicating that all individuals with increased ammonia levels have increased PKA-RI content. This suggested to us that maybe a single transient exposure to high ammonia levels could induce the increase in PKA-RI that then will be maintained for a long period of time.

To assess this possibility we injected normal rats intraperitoneally with a non lethal dose of ammonia (5 mmol/kg) and killed groups of rats at different times after injection (from 20 min up to 3 wk) and analyzed the content of PKA-RI, which was significantly increased in erythrocytes from rats injected with ammonia at all times tested (Fig. 8). The increases were as follows: at 20 min (0.33), 159 ± 11%, P = 0.002; at 1 h, 164 ± 11%, P = 0.002; at 4 h, 188 ± 20%, P = 0.05; at 24 h, 300 ± 68%, P = 0.033; at 4 days (98 h), 419 ± 68%, P = 0.0039; at 7 days (168 h), 428 ± 73%, P = 0.020; at 14 days (336 h), 428 ± 33%, P = 0.0006; and at 21 days (504 h), 389 ± 51%, P = 0.030.

In lymphocytes from rats injected with ammonia the content of PKA-RI was significantly increased from 24 h to 3 wk (Fig. 9). The increases were as follows: at 24 h, 171 ± 25%, P = 0.035; at 4 days (98 h), 209 ± 20%, P = 0.011; at 7 days (168 h), 261 ± 41%, P = 0.017; at 14 days (336 h), 228 ± 25%, P = 0.0068; and at 21 days (504 h), 237 ± 58%, P = 0.038.

Figure 10 shows the time course of ammonia levels in blood and of PKA-RI content in erythrocytes and in lymphocytes. Ammonia levels return to basal values 2 h after ammonia injection. However, the content of PKA-RI increases continuously during 4 days and then remains at high levels during at least 3 wk.

These results show that a single transient increase in ammonia levels is enough to induce a long-lasting increase in the content of PKA-RI in erythrocytes and lymphocytes and support the idea that the increase of PKA-RI in blood cells of patients with liver cirrhosis is a consequence of a previous episode of hyperammonemia.

To further support this idea we carried out two additional types of experiments: 1) we analyzed the content of PKA-RI in blood cells of patients with chronic hepatitis who had not suffered any episode of hyperammonemia, and 2) we tested whether addition of ammonia in vitro to erythrocytes freshly isolated from controls or patients induces an increase in PKA-RI.
As shown in Fig. 5, the content of PKA-RI is not altered in erythrocytes (126 ± 26% of controls, \( P = 0.218 \)) nor in lymphocytes (116 ± 16% of controls, \( P = 0.107 \)) in patients with chronic hepatitis that have not suffered episodes of hyperammonemia. Blood ammonia levels in these patients (77 ± 15 \( \mu M \)) were not different than in controls (67 ± 26 \( \mu M \) (Fig. 6 and Table 2).

We then tested whether addition of ammonia in vitro to erythrocytes from control subjects without liver disease results in increased PKA-RI content. Addition of 10 \( \mu M \) ammonia did not affect PKA-RI content whereas addition of 30 \( \mu M \) or more ammonia increased PKA-RI significantly (Fig. 11). This confirms that hyperammonemia increases PKA-RI content.

To assess whether this effect is selective for blood cells or it occurs also, for example, in brain cells, we tested whether

![Fig. 6. Ammonia concentration in blood of patients with liver cirrhosis, chronic hepatitis and controls. The concentration of ammonia was analyzed as described in MATERIALS AND METHODS using blood from patients with liver cirrhosis or from healthy individuals. The mean was significantly higher \( (P = 0.0018) \) in patients with liver cirrhosis than in controls. ○, Controls (n = 13); ●, patients with chronic hepatitis (n = 9); ▽, patients with liver cirrhosis (n = 17).](image1)

![Fig. 7. There is no correlation between ammonia concentration in blood and the content of PKA-RI in erythrocytes or lymphocytes. The values for ammonia concentration in blood and the content of PKA-RI in erythrocytes (A) or lymphocytes (B) corresponding to the same individual are represented. There is no significant correlation between ammonia and PKA-RI levels in erythrocytes or lymphocytes. ○, Controls (n = 10); *, patients with chronic hepatitis (n = 9); ●, patients with liver cirrhosis (n = 17).](image2)

![Fig. 8. Injection of ammonia induces a sustained increase in the content of PKA-RI in rat erythrocytes. A: immunoblottings corresponding to PKA-RI in erythrocytes under basal conditions and at different times after injection of 5 mmol/kg of ammonium acetate. B: the images were captured and the intensities of the bands were measured. Values are means ± SD of 5 experiments. All values are significantly higher than basal (*\( P < 0.05 \); **\( P < 0.01 \); ***\( P < 0.001 \)). n, No. of rats in each group.](image3)

![Fig. 9. Injection of ammonia induces a sustained increase in the content of PKA-RI in rat lymphocytes. A: immunoblottings corresponding to PKA-RI in lymphocytes under basal conditions and at different times after ip injection of 5 mmol/kg of ammonium acetate. B: the images were captured and the intensities of the bands were measured. Values are means ± SD of 4 experiments. All values from 24 to 336 h are significantly higher than basal (*\( P < 0.05 \); **\( P < 0.01 \)). n, No. of rats in each group.](image4)
addition of ammonia also increases PKA-RI content in cultured astrocytes. As shown in Fig. 12, addition of 0.1 mM ammonia increased significantly (P < 0.05) PKA-RI content in astrocytes to 121 ± 7 and 135 ± 9% at 15 and 30 min, respectively.

The above data show that a single exposure to high ammonia levels is enough to induce a long-lasting increase in PKA-RI content in blood cells and indicate that its increase in patients with liver cirrhosis would be a consequence of previous transient episode(s) of hyperammonemia.

We hypothesized that ammonia may induce a translocation of PKA-RI from cytosol to the membrane and that this will increase PKA-RI synthesis (see DISCUSSION). To assess this possibility, we treated erythrocytes from controls or cirrhotic patients with 0.1 or 1 mM ammonia for 15 min and analyzed the content of PKA-RI in whole erythrocytes, in cytosol, and in the membrane.

In erythrocytes from controls addition of ammonia increased significantly (P < 0.05) the content of PKA-RI in whole erythrocytes to 186 ± 61 and 246 ± 85% for 0.1 or 1 mM ammonia, respectively (Fig. 13, A and B). The content of PKA-RI in cytosol was not significantly affected (Fig. 13C) and the content in the membrane fraction increased significantly from controls: *P < 0.01; **P < 0.05.

In vitro exposure to ammonia increases the content of PKA-RI in cultured astrocytes. Cultured astrocytes were treated with 0.1 or 1 mM ammonium chloride for 15 min at 37°C. The amount of PKA is expressed as percentage of the amount in astrocytes under basal conditions. A: a typical immunoblotting. B: the images were captured and the intensities of the bands were measured. Values are means ± SD of 4 experiments. Significantly different from controls: *P < 0.01; **P < 0.05.

![Time course of the changes in blood ammonia and in the content of PKA-RI in erythrocytes and lymphocytes in rats injected with ammonia. Experiments were carried out as in Figs. 8 and 9. Values are means ± SD of 5 different experiments. Values that are significantly different from basal are indicated by asterisks: *P < 0.05; **P < 0.01. (see legends to Figs. 8 and 9).](#)
significantly (P < 0.01) to 417 ± 97 and 427 ± 153% for 0.1 or 1 mM ammonia, respectively (Fig. 13, A and B). The content of PKA-RI in cytosol was not significantly affected (Fig. 13C), and the content in the membrane fraction increased significantly (P < 0.01) to 328 ± 98 and 581 ± 156% for 0.1 or 1 mM ammonia, respectively (Fig. 13D).

This supports the idea that ammonia induces a translocation of PKA-RI to the membrane that, in turn, stimulates PKA-RI synthesis. We then analyzed whether a similar process occurs in lymphocytes of rats injected with ammonia. We injected normal rats intraperitoneally with a nonlethal dose of ammonia and killed groups of rats at different times after injection (as for experiments shown in Figs. 8 and 9) and analyzed the content of PKA-RI in cytosol and membrane fractions of lymphocytes.

In lymphocytes from rats injected with ammonia, PKA-RI was significantly reduced in cytosol at 20 min, 1 h, and 4 h after injection of ammonia; was not different from basal from 1 to 14 days; and was slightly but significantly increased (132 ± 10% of basal) at 21 days (Fig. 14).

In the membrane fraction of the same lymphocytes, PKA-RI was not altered at 20 min, increased not significantly to 154% at 1 h, and was significantly increased (P < 0.001) from 4 h to 21 days. The increases were as follows: at 4 h, 205 ± 27%; at 24 h, 254 ± 32%; at 4 days (96 h), 327 ± 41%; at 7 days (168 h), 465 ± 46%; at 14 days (336 h), 451 ± 43%; and at 21 days (504 h), 440 ± 30% (Fig. 14).

DISCUSSION

The results reported show that a single transient episode of hyperammonemia leads to long-lasting alterations in the content of PKA-RI in blood cells. This indicates that transient hyperammonemia may result in long-lasting alterations in signal transduction modulated by PKA in blood cells and likely in other signal transduction processes in blood and other tissues such as brain.

This finding may be very relevant for the understanding of the evolution of the disease and of hepatic encephalopathy in patients with liver cirrhosis. The episodes of hepatic encephalopathy are reversible; however, the appearance of hepatic encephalopathy (usually associated with episodes of hyperammonemia) is not. This indicates that transient hyperammonemia may result in long-lasting alterations in signal transduction modulated by PKA in blood cells and likely in other signal transduction processes in blood and other tissues such as brain.

The effects were similar in erythrocytes from patients with liver cirrhosis. The basal content of PKA-RI was already increased (186 ± 85%, P = 0.008). Addition of ammonia in vitro further increased PKA-RI content in whole erythrocytes to 402 ± 130% (P = 0.01) and 431 ± 178% (P = 0.0068) of basal for 0.1 and 1 mM ammonia, respectively (Fig. 13, A and B).
monemia) in these patients is associated with poor prognosis (5, 15). This indicates that these transient episodes of hepatic encephalopathy (and of hyperammonemia) induce some permanent changes that make the patients more susceptible to subsequent injuries. This is not an unusual fact in physiology and pathology. A similar induction of susceptibility occurs in allergy. The first exposure to an allergen does not induce deleterious effects but renders the individual susceptible to subsequent exposures to the allergen.

The reasons by which a first episode of hepatic encephalopathy is associated with poor prognosis are not known. It seems reasonable to assume that this would be due to the induction by the transient episode of hepatic encephalopathy of long-lasting alterations in some molecules leading to altered modulation of some physiological processes that will predispose the patient to suffer more deleterious effects in response to subsequent injuries or exposure to pathological agents or situations that before the hepatic encephalopathy episode induced no or mild damage.

The episodes of hepatic encephalopathy are usually associated with hyperammonemia. There is a good correlation between arterial blood ammonia concentration and the symptoms of hepatic encephalopathy (e.g., Clemmensen et al., Ref. 8).

It is likely that hyperammonemia would be responsible for the induction of the long-lasting alterations associated with the transient episode of hepatic encephalopathy. Here we show that in fact transient hyperammonemia induces such kind of long-lasting alterations in a key molecule in signal transduction, PKA-RI.

We also show that PKA-RI is not altered in blood cells of patients with chronic hepatitis that have suffered the disease for more than one year but have not had episodes of hyperammonemia. This reinforces the idea that the alterations in PKA-RI in patients are due to the episodes of hyperammonemia and not to other factors associated with hepatitis such as alterations associated with inflammation. The results shown in patients strongly support, but do not demonstrate definitely, that the increase in PKA-RI is due to hyperammonemia. The results in the experiments with rats injected with ammonia completely confirm that hyperammonemia is responsible for the increase. Moreover, this is further corroborated by the fact that addition of ammonia to human erythrocytes in vitro increases PKA-RI.

Figure 11 shows that an increase of 10 μM ammonia does not affect PKA-RI content and significant increases of PKA-RI requires an increase in ammonia of 30–50 μM, which represents a 50–100% increase over normal values (67 μM in controls, Table 2). The effect of ammonia increases rapidly with ammonia concentration up to 100 μM and then increases more slowly (Fig. 11).

The increase in PKA-RI seems independent of the etiology of liver disease. The etiology is different for different individuals within the group of cirrhotic patients (Table 1), and all of them have increased PKA-RI.

Moreover, the effect is not a general effect on protein kinases; it is selective for PKA-RI whereas the contents of other protein kinases such as protein kinase C or MAPK are not affected in the same erythrocyte samples from patients in which the content of PKA-RI is increased more than twice (21).

The increase in PKA-RI induced by ammonia occurs very rapidly. As shown in Fig. 8, injection of ammonia increased PKA-RI in erythrocytes already at 20 min. Moreover, addition of ammonia to isolated erythrocytes or cultured astrocytes increases PKA-RI already at 15 min (Fig. 12). This rapid increase in subsequently maintained for long periods of time either in the presence or the absence of hyperammonemia.

We tried to shed some light on the possible mechanisms by which ammonia may induce the increase in PKA-RI. We first tested whether this could be a consequence of ammonia-induced cell swelling. We analyzed cell volume by flow cytometry and found that cell volume in erythrocytes or lymphocytes was not affected by addition of 0.1, 1, or 5 mM ammonia (data not shown). Addition of 10 mM ammonia was necessary to induce cell swelling in lymphocytes (data not shown). This indicates that ammonia-induced increase in PKA-RI is not due to cell swelling.

We then hypothesized that ammonia may induce a translocation of PKA-RI from cytosol to the membrane and that this will increase PKA-RI synthesis. A similar mechanism of regulation of protein synthesis has been demonstrated for tubulin. Tubulin is present in the cells in an equilibrium between free and polymerized tubulin (microtubules). The level of free tubulin modulates tubulin synthesis. The increase in free tubulin inhibits tubulin synthesis while a decrease of free tubulin enhances its synthesis (4). Factors that stimulate tubulin polymerization (e.g., some microtubule-associated proteins) decrease free tubulin and increase tubulin synthesis. Also factors that depolymerize microtubules (e.g., colcemid) increase free tubulin and reduce tubulin synthesis (e.g., Ref. 6). Moreover, this modulation is independent of transcription and of nuclear factors as shown by the fact that enucleated cells retain the ability to turn off tubulin protein synthesis in response to microtubule depolymerization (6). These effects are also very rapid, and significant changes in tubulin synthesis in 30 min have been reported (7).

In a similar way, it is possible that the levels of cytosolic PKA-RI modulate the synthesis of PKA-RI. The transport of PKA-RI to the membrane will reduce transiently the level of PKA-RI in cytosol. This occurs for example in lymphocytes of rats between 20 min and 4 h after injection of ammonia (Fig. 14). This reduction will increase PKA-RI synthesis. It seems therefore that ammonia induces a translocation of PKA-RI from cytosol to membrane and this leads to the increase in PKA-RI synthesis and amount.

It is noteworthy that hyperammonemia also increases tubulin synthesis in brain by stimulating its polymerization (10–12, 19, 20). Hyperammonemia decreases protein kinase C-dependent phosphorylation of microtubule-associated protein 2 and increases its binding to tubulin, resulting in increased polymerization and transient decrease in free tubulin (12). This leads in turn to increased mRNA translation and tubulin synthesis and amount (10–12, 19, 20).

In the case of PKA-RI, hyperammonemia could modulate some factor (e.g., phosphorylation) regulating the transport of PKA-RI to the cellular membrane, and the decrease in cytosolic PKA-RI would stimulate PKA-RI synthesis. The increase in PKA-RI synthesis does not necessarily requires transcription of new mRNA. It is well known that protein synthesis may increase by increasing the efficiency of translation of preexisting mRNA; for example, insulin, amino acids, or leucine increase protein synthesis by stimulating initiation of translation (16, 22). Also the mRNA for some proteins (e.g., for
TNF-α contains elements (e.g., adenine-uridine-rich elements) which modulate the translational efficiency in response to some agents (e.g., Ref. 25). Maybe ammonia could modulate one of this elements in the mRNA for PKA-RI. This process can occur therefore also in cells (erythrocytes) without nucleus but with mRNA. Another possible explanation for the increase in the amount of PKA-RI protein could be that its degradation may be reduced by ammonia, resulting in increased amount of the protein without changes in its synthesis. An inhibition of protein synthesis by ammonia in hepatocytes has been previously reported (23, 24). It is possible that in blood cells ammonia could preferentially inhibit PKA-RI degradation.

The increase in PKA-RI in patients with liver disease may have functional consequences. PKA-RI increases essentially in the membrane fraction. As previously shown, this PKA-RI in the membrane retains a large amount of cGMP (21), which could be the reason for the reduced intracellular cGMP concentration in lymphocytes and erythrocytes of patients with liver cirrhosis (6, 13). This PKA-RI-induced decrease in intracellular cGMP content in patients may be involved in some of the clinical manifestations of the disease, including the alterations in platelet aggregation, the portal hypertension, and the systemic hypotension. It may be also involved in the reduced formation of atheroma plaques observed in patients with liver cirrhosis.

The long-lasting alterations in PKA (and likely in other signal transduction molecules and processes) induced by transient hyperammonemia may contribute to the poor prognosis in patients that have suffered an episode of hepatic encephalopathy. The identification of the factors contributing to this poor prognosis may allow design of treatments to reverse the long-lasting alterations and hopefully to improve the prognosis of patients.

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