TRANSLATIONAL PHYSIOLOGY

Low myo-inositol and high glutamine levels in brain are associated with neuropsychological deterioration after induced hyperammonemia

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Submitted 9 March 2004; accepted in final form 5 May 2004

Shawcross, D. L., S. Balata, S. W. M. Olde Damink, P. C. Hayes, J. Wardlaw, I. Marshall, N. E. P. Deutz, R. Williams, and R. Jalan. Low myo-inositol and high glutamine levels in brain are associated with neuropsychological deterioration after induced hyperammonemia. Am J Physiol Gastrointest Liver Physiol 287: G503–G509, 2004. 10.1152/ajpgi.00104.2004.—The neuropsychological effect of hyperammonemia is variable. This study tests the hypothesis that the effect of ammonia on the neuropsychological function in patients with cirrhosis is determined by the ability of the brain to buffer ammonia-induced increase in glutamine within the astrocyte by losing osmolytes like myo-inositol (mI) and not by the magnitude of the induced hyperammonemia. Fourteen cirrhotic patients with no evidence of overt hepatic encephalopathy were given a 75-g amino acid (aa) solution mimicking the hemoglobin molecule to induce hyperammonemia. Measurement of a battery of neuropsychological function tests including immediate memory, ammonia, aa, and short-echo time proton magnetic resonance spectroscopy were performed before and 4 h after administration of the aa solution. Eight patients showed deterioration in the Immediate Memory Test at 4 h. Demographic factors, severity of liver disease, change in plasma ammonia, and aa profiles after the aa solution were similar in those that showed a deterioration compared with those who did not. In patients who showed deterioration in the memory test, the mI-to-creatine ratio (mI/Cr) was significantly lower at baseline than those that did not deteriorate. In contrast, the glutamate/glutamine-to-Cr ratio was significantly greater in the patients that deteriorated. The observation that deterioration in the memory test scores was greater in those with lower mI/Cr supports the hypothesis that the neuropsychological effects of induced hyperammonemia is determined by the capacity of the brain to handle ammonia-induced increase in glutamine.

neuropsychological function; amino acid solution; magnetic resonance spectroscopy; hepatic encephalopathy

AMMONIA HAS CONSISTENTLY BEEN shown to be important in the pathogenesis of hepatic encephalopathy (HE) (7, 26). Current hypotheses support the ammonia-glutamine-brain swelling hypothesis of neuropsychological dysfunction in cirrhosis. Astrocytes are the site of ammonia detoxification in the brain and eliminate ammonia by the synthesis of glutamine by amidation of glutamate. Accordingly, accumulation of glutamine in astrocytes induced by hyperammonemia produces osmotic stress and astrocyte swelling (18). In patients with HE, the observation that myo-inositol (mI; a sugar involved in the synthesis of phosphoinositides) is reduced with increasing concentrations of glutamine suggests that mI may be an important osmotic regulator within the astrocyte (13, 22, 34). Recent studies in patients with cirrhosis (11) have shown that the metabolic disturbances are associated with an increase in brain water, the severity of which correlates with worsening of the neuropsychological state.

In keeping with these observations, we recently demonstrated that oral administration of a solution mimicking the amino acid (aa) composition of the hemoglobin molecule led to a significant alteration in neuropsychological function, an increase in brain glutamine/glutamate signal (Glx) and a reduction in mI. The increase in Glx was associated with a reduction in the magnetization transfer ratio (suggesting an increase in brain water) supporting the ammonia-glutamine-brain swelling hypothesis of HE (2). However, the correlation between the aa-induced increase in ammonia concentration was not directly related to the change in neuropsychological function, suggesting that the neuropsychological effects of hyperammonemia may be determined not by the degree of hyperammonemia but by the ability of astrocytes to maintain the osmotic equilibrium by using mI as a compensatory mechanism for the ammonia-induced increase in glutamine. According to this hypothesis, a low mI level before administration of the aa solution would increase the susceptibility of the brain to the effects of induced hyperammonemia. Therefore, the aims of this study were to evaluate the neuropsychological changes after induction of hyperammonemia and to correlate these changes with the brain mI and Glx measured with proton magnetic resonance spectroscopy (1H-MRS).

MATERIALS AND METHODS

Ethical Considerations

Studies were undertaken with the approval of the Hospital Research Ethics Committee and the written informed consent from each patient in accordance with the Declaration of Helsinki (1989) of the

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World Medical Association. The safety of administering 75 g of an aa solution mimicking the composition of the hemoglobin molecule to patients with cirrhosis has been extensively studied (2, 21, 27) without any complication such as the development of overt HE. Similar studies have used other formulations of a combination of aa (15) or glutamine challenge (24, 28, 31), which were shown to produce ammoniagenesis without any significant adverse effects.

Patients

Sixteen hemodynamically stable patients with biopsy-proven cirrhosis who were enrolled from the outpatient clinic were studied. Patients were excluded if they had clinical evidence of overt HE (10), diabetes, cardiovascular disease, renal dysfunction (serum creatinine >150 μM), serum sodium <130 mM, serum potassium <3.2 mM or >5 mM, concomitant neurological disease, recent gastrointestinal bleeding (within the previous 4 wk), malignancy, or pregnancy. Patients had to be abstinent from alcohol and benzodiazepines for at least 1 mo before the study. Patients were studied after an overnight fast. Patient details are summarized in Table 1.

Oral Amino Acid Solution

Simulation of the upper gastrointestinal bleed was by administration of an oral bolus of 75 g of a specially prepared solution (product no.: 24143; Nutricia, Cuijk, The Netherlands) that mimics the aa composition of the hemoglobin molecule (in mmol/g of aa solution: 99.8 leucine, 0 isoleucine, 85.9 valine, 55.4 glycine, 8.3 tryptophan, 44.4 threonine, 61 lysine, 33.3 glutamate, 27.7 asparagine, 11.1 glutamine, 8.3 methionine, 16.6 arginine, 16.6 tyrosine, 38.8 proline, 41.6 aspartate, 99.8 alanine, 8.3 cysteine, 44.4 serine, 41.6 phenylalanine, and 52.7 histidine) (19). The solution was freshly made in 200 ml water, and xanthum gum was added to prevent sedimentation.

Measurement of Neuropsychological Function

A construct-driven neuropsychological test battery was used to test concentration, memory, visuospatial-construction skills, and motor function. This consisted of: Trails B Test (14), the Digit Symbol Substitution Test (DSST) (20), the Immediate Story Recall Subtest of the Randt Test Battery (30), and Choice Reaction Time Test Time (16). This battery of tests was performed immediately before the administration of the aa solution and 4 h afterward. The total time taken to perform this battery was <20 min. The same investigator performed the neuropsychological tests. All patients had one practice session for each test. All of the tests were well validated, and parallel forms were used. The Trails B Test (14) is a derivative of the Trail Making Test that measures visual conceptual and visuomotor tracking. The test must be completed in 420 s. The DSST (20) is part of the Wechsler Adult Intelligence Schedule and is used to assess visuomotor coordination and vigilance. The test score was the number of symbols correctly substituted in 90 s. The Immediate Story Recall Subtest of the Randt Test Battery (30) measures immediate memory function. The subject is asked to recall 20 words from a paragraph read to him or her that has an emotionally charged substance and includes fire and disaster. One point was awarded for each word that was recalled immediately after presentation (acquisition). The Choice Reaction Time (16) is part of the Continuous Performance Task and measures motor function, sustained concentration, and the ability to suppress inappropriate responses. The task was to press the space bar of the computer as quickly as possible every time the letter X appeared, except when it was immediately preceded by the letter Y. The mean reaction time and the number of observations were recorded.

Proton MR Spectroscopy

The patients underwent proton MR spectroscopy (1H-MRS) immediately before and 4 h after administration of the aa solution to determine changes in the brain osmolytes Glx and ml. This was performed by using an Elscint Prestige scanner (GE Medical Systems, Haifa, Israel) in the Scottish Higher Education Funding Council (SHEFC) Brain Imaging Research Centre for Scotland, operating at 1.9 Tesla. With the use of T2-weighted axial images for positioning, PRESS-localized spectra were acquired from 15-mm cubical volumes of interest including both gray and white matter in the left basal ganglia and the left temporal cortex. We chose to study the dominant hemisphere, and in the case of the patients reported, the left hemisphere was dominant in all. The subthalamic structures were chosen to be studied because they are critical to memory and learning (9), which are important in executive attention. The repetition time was 1,500 ms, and the echo time was 36 ms. After localized shimming and water-suppression calibration for each volume of interest, 200 acquisitions with water suppression were collected. Eight acquisitions without water suppression were also collected to serve as a phase reference. The volume-of-interest positions within the head coil were noted, as was the scanner radio frequency calibration figure to adjust for the effects of head-coil loading. Spectroscopy data were transferred to a Sun workstation for analysis. Analysis consisted of phase correction using the water reference data (29) and removal of the residual water signal using Hankel-Lanczos singular value decomposition (8). Spectral peak areas were quantified by using the Advanced Method for Accurate, Robust, and Efficient Spectral Fitting (AMARES) method within the Magnetic Resonance User Interface software (Lyon, France) (25). A model consisting of 12 Gaussian peaks was developed with reference to in vitro measurements on healthy volunteers on the same scanner with the same sequences, and literature values for peak assignments. Three observers studied the fitted results independently. The observers discussed any peak assignments on which they did not all agree. In this way, a consensus was reached for all of the spectra in the study. The after cerebral metabolites were quantified: N-acetyl aspartate (NAA), choline (Cho), Cr, ml, and Glx. Cho resonance reflects changes in phospholipid metabolism and osmotic regulation in glial cells (6), and NAA is a normal neuronal marker (4). Finally, peak areas for each subject were corrected for head-coil loading and volume-of-interest position within the head coil. The resulting institutional units enabled intersubject comparisons.

Blood Sampling and Analysis

A peripheral venous blood sample was taken for analysis of ammonia and plasma aa profile at the beginning of each study.
immediately before the administration of the aa solution and was repeated 4 h after administration.

Ammonia. Plasma was obtained by centrifugation, deproteinized with trichloroacetic acid, and stored at −80 °C for spectrophotometric determination of ammonia (CobasMiraS; Hoffman-LaRoche, Switzerland) at a later date.

Amino acids. Plasma was obtained by centrifugation and deproteinized with sulphosalicylic acid for determination of aa by high-performance liquid chromatography (Pharmacia, Woerden, The Netherlands).

Statistics

All data are expressed as median and range. The neuropsychological data were compared before and after the administration of the aa solution using a Wilcoxon signed rank test. In the post hoc analysis, the neuropsychological deteriorators and nondeteriorators were compared by using the Mann-Whitney U-test. (Prism software version 3.0; GraphPad, San Diego, CA). A P value of <0.05 was considered significant. Prism software was also used to calculate linear regression of correlation between two variables (r value) with 95% confidence intervals. A P value of <0.05 was considered significant.

RESULTS

Patients

Sixteen patients were recruited; of these, 14 completed the study and are the subject of this report. One patient was unable to tolerate 1H-MRS due to claustrophobia, and we were unable to gather sufficient data for one other patient due to excessive movement inside the magnet. None of the patients who entered the study showed any evidence of altered mental state or overt HE after the administration of the aa solution.

Neuropsychological Function

There were no significant differences in the scores for the Trails B Test (P = 0.06), DSST (P = 0.06), and Reaction Time Test (P = 0.72) before and 4 h after receiving the aa solution. Significant differences were, however, apparent in the Immediate Story Recall Subtest of the Randt Memory Test (P = 0.001). In view of this apparent difference in the continuous data of the Immediate Story Recall Subtest, we decided to perform a post hoc analysis to identify whether there were any differences among the 14 patients that might determine whether they would deteriorate in the memory test in response to hyperammonemia. When the total deterioration in the score of 20 was calculated, 8 of the 14 subjects demonstrated deterioration in the score of 1 standard deviation (2.74) (mean 7) or more, regardless of the degree of induced hyperammonemia. We therefore went on to compare the group that deteriorated (n = 8) with the group that did not (n = 6).

Ammonia

Overall, the median fasting basal venous ammonia concentration and the degree of induced hyperammonemia were similar within the whole group. When the group that had a deterioration in memory score was compared with the group that did not, there was no significant difference in the fasting basal venous ammonia concentration [50.5 (42–76) vs. 68 (41–112) μM] (P = 0.42). Furthermore, the ammonia levels generated in response to the aa solution did not differ between the deteriorator and nondeteriorator groups at 4 h [90 (73–140) vs. 95 (46–221) μM] (P = 0.84).

Amino Acids

There were no significant differences in plasma aa profiles within the group as a whole. When the deteriorator and nondeteriorator groups were compared, the reduction in the concentration of isoleucine at 4 h after the aa solution was greater in the deteriorator group [26 (16.7–31) vs. 31 (28–41) μM] (P = 0.03). The remaining aa profiles were otherwise the same in both groups (Table 2). The Fischer Ratio (33) (branched-chain aa/aromatic aa) was not significantly different between the deteriorator and nondeteriorator groups.

Table 2. Levels of ammonia and/or amino acids in pre- and post-amino acid solution

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Pre-amino acid solution (t = 0 h)</th>
<th>Post-amino acid solution (t = 4 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia</td>
<td>53 (41–112)</td>
<td>89 (46–221)**</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>71.5 (42–94)</td>
<td>28.5 (16.7–41)***</td>
</tr>
<tr>
<td>Leucine</td>
<td>77.5 (36–140)</td>
<td>404.5 (158–880)***</td>
</tr>
<tr>
<td>Valine</td>
<td>129 (60–220.6)</td>
<td>657 (401–1359)***</td>
</tr>
<tr>
<td>Glutamine</td>
<td>614.5 (461–890)</td>
<td>818.5 (645–1300)**</td>
</tr>
<tr>
<td>Total essential amino acids</td>
<td>640.1 (470–918.1)</td>
<td>2014 (1176.5–2683.5)***</td>
</tr>
<tr>
<td>Total nonessential amino acids</td>
<td>972 (775–1367.3)</td>
<td>1905.95 (1435–4344)***</td>
</tr>
<tr>
<td>Branched-chain amino acids</td>
<td>264 (105.5–381.6)</td>
<td>1077 (452–1718.3)***</td>
</tr>
<tr>
<td>Total amino acids</td>
<td>1329 (980–1865.5)</td>
<td>2739.95 (2274–6044)***</td>
</tr>
</tbody>
</table>

Values are median and range (in parentheses) of micromoles of ammonia and/or amino acids. **P < 0.01; ***P < 0.0001 (Wilcoxon signed-rank test)
P = 0.05 (Fig. 2). Four hours after administration of the aa solution, the Glx/Cr ratio was significantly increased in the patients that showed no deterioration but not in the patients that showed deterioration in the immediate memory test (basal ganglia, \( P < 0.003 \); temporal lobes, \( P < 0.01 \)). The correlation between the degree of deterioration in memory score and Glx/Cr in the basal ganglia was even greater at 4 h after administration of the aa solution (\( r = 0.69; P < 0.007 \)) than before its administration.

**Cho/Cr and NAA/Cr**

No significant changes were seen between the groups at \( t = 0 \) and 4 h after the administration of the aa solution (Table 3).

### DISCUSSION

Results of this study show that the deterioration in the immediate memory test with induction of hyperammonemia

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**Table 3. \(^1\)H-MRS data for basal ganglia and temporal lobe**

<table>
<thead>
<tr>
<th></th>
<th>Deteriorators</th>
<th>Nondeteriorators</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal Ganglia</td>
<td>Temporal Lobe</td>
</tr>
<tr>
<td></td>
<td>( r = 0 )</td>
<td>( r = 4 )</td>
</tr>
<tr>
<td>ml/Cr</td>
<td>0.42 (0.19–0.58)</td>
<td>0.3 (0.1–0.43)</td>
</tr>
<tr>
<td>Glx/Cr</td>
<td>0.99 (0.74–1.27)</td>
<td>1.26* (0.92–1.5)</td>
</tr>
<tr>
<td>Cho/Cr</td>
<td>0.71 (0.57–1.53)</td>
<td>1.3 (0.4–2.1)</td>
</tr>
<tr>
<td>NAA/Cr</td>
<td>1.84 (1.47–4.58)</td>
<td>1.85 (1.18–4.24)</td>
</tr>
</tbody>
</table>

Values are median and range in parentheses of \(^1\)H-MRS data obtained before \(( t = 0 )\) and 4 h \(( t = 4 )\) after administration of amino acid solution for the group that showed deterioration in memory score and the group that did not. ml, myo-inositol; Cr, creatine; Glx, glutamate/glutamine; Cho, choline; NAA, N-acetyl aspartate. Significant differences between \( t = 0 \) and 4 h are *\( P < 0.05 \), **\( P < 0.01 \).
was more likely to occur in the patients with low levels of mI/Cr measured by using 1H-MRS. This observation suggests that the effect of hyperammonemia is likely to be determined by the ability of the astrocytes to maintain osmotic equilibrium by losing osmolytes like mI in response to the ammonia-induced increase in glutamine.

Neuropsychological response to induced hyperammonemia is variable (24). Unlike the study by Riggio et al. (32), who found no significant change in mental state after an aa load, the investigators from the Newcastle group (28, 31) demonstrated significant changes in patient mental state after administration of glutamine, raising questions about neuropsychological factors that may determine the response to hyperammonemia. As we have recently shown, other factors such as systemic inflammatory response may exacerbate the neuropsychological effect of induced hyperammonemia (35). In the present study, we recruited a fairly homogenous group of patients with cirrhosis, who were metabolically stable without any evidence of infection or inflammation. It is therefore not surprising that there were no significant differences in the clinical and biochemical profile of the patients in whom a deterioration in the memory test was observed compared with those in whom no such deterioration was observed. In addition, the magnitude of change in ammonia and aa in the two groups after administration of the aa solution were similar.

Results of the spectroscopy provide support for the hypothesis that the buffering capacity of the brain measured by the relative concentrations of mI and Glx may be important in determining the effect of hyperammonemia. Accordingly, a state of cellular osmotic equilibrium may be maintained even with high brain glutamine levels, which are compensated for by very low levels of mI (or other unmeasured organic solutes). Therefore, in such a patient, a small increase in ammonia may cause a marked increase in osmotic stress by further increasing the accumulation of glutamine and therefore deterioration in the neuropsychological state. On the other hand, in the very well-compensated patient, the proportion of Glx to mI may be low, making this patient resistant to the effects of induced hyperammonemia. One way to gauge this potential buffering capacity is to calculate the mI-to-mI + Glx ratio (mI/mI + Glx), which accounts for the total measured osmolar capacity. The rationale for having mI as the denominator was to try to express the role of mI in relationship to the total measurable osmolality (using 1H-MRS). Therefore, conceptually, buffering capacity would be the buffer (mI in this case) divided by the total osmolytes. However, this is hypothetical and will have to be looked at prospectively in future studies. This ratio was significantly lower in the group that had deterioration in their Immediate Story Recall Test at \( t = 0 \) (\( P = 0.0007 \)) after administration of aa solution in the basal ganglia. This was less pronounced in the temporal lobe but still highly significant (\( P = 0.001 \)). Furthermore, the degree of deterioration in memory score correlated with mI/mI + Glx, such that the deterioration in memory score was greater when the basal \( t = 0 \) mI/mI + Glx was lower (basal ganglia \( r = -0.77, P = 0.003 \); temporal lobe \( r = -0.66, P = 0.01 \)) (Fig. 3).

A decrease in the concentration of brain organic osmolytes, such as mI, indicates the activation of the process of regulatory volume decrease (37). Therefore, the release of mI from the astrocytes may be indicative of an adaptive change (40). In keeping with this, Cordoba et al. (12) showed that brain swelling induced by hyperammonemia is made significantly worse by hyponatremia, a condition known to reduce brain mI levels (5). The mechanism of why the mI levels were different in the two groups of patients (those that deteriorated and those that did not) has not been answered in the present study, because both groups of patients had similar severities of liver disease and ammonia concentrations at baseline. Although plasma sodium concentration was similar (within the normal range) at the time of the study, variations in its levels over the preceding weeks may contribute to lower mI levels. Furthermore, we have no data about other factors, such as the duration of hyperammonemia, cerebral blood flow, or the degree of activation of neurohormones that may be important, because they are known to alter the signaling pathways in the brain and, therefore, mI levels (3, 23, 36).

The simulated upper gastrointestinal bleed by oral administration of an aa solution is safe and well validated. Over 80 patients have been studied so far using this protocol in which we administered 75 g of the aa solution, which is not significantly different in protein content from the average daily protein intake of these patients. We were very careful in selecting patients for the study and excluded all those with any clinically detectable encephalopathy. Furthermore, the safety of administering aa solution to the cirrhotic patient has been extensively studied by our group (2, 21, 27) including four other published studies (15, 24, 28, 31) that have also evaluated

![Fig. 3. Correlation between the degree of deterioration in Immediate Story Recall Subtest score and the mI-to-mI + Glx ratio in the basal ganglia (A) and temporal lobes (B) at baseline (\( t = 0 \)). An improvement in memory score is represented by a negative value, and deterioration in memory score is represented by a positive value.](http://ajpgi.physiology.org/)
both glutamine challenge and aa challenge as safe methods to induce hyperammonemia in cirrhotic patients.

The observed deterioration in the memory function with induced hyperammonemia confirms the result of our previous study in which we administered an oral aa solution mimicking the hemoglobin molecule to examine neuropsychological changes. We showed that patients in the placebo group had a significant improvement in all of the neuropsychological function tests except the Immediate Story Recall Subtest. In contrast, patients who were administered the aa solution showed significant deterioration in the Immediate Story Recall Test but the results of the other three tests (Trails B Test, DSST, and Choice Reaction Time) did not change significantly (2). Other investigators (17, 38) have suggested that changes in memory tests are an important component of minimal HE and are commonly used as part of a standard test battery when characterizing minimal HE (1). The pathophysiological basis of the memory deficit in minimal HE is the likely result of the effect of hyperammonemia on neuronal pathways controlling attention, which is crucial for learning (39). Looking at this from a different pathophysiological perspective, the concentration of mI located mainly in glial cells may vary in different parts of the brain, which may also explain why there was a more significant difference between the deteriorators and nondeteriorators when looking at ml/ml + Glx in the basal ganglia than in the temporal lobe.

In conclusion, results of our study have shown that neuro-psychological response to induced hyperammonemia is determined by the underlying brain biochemistry and the ability of the brain to buffer the ammonia-induced increase in glutamine rather than the degree of induced hyperammonemia. Our data support the hypothesis that low brain ml levels may be used as a marker in patients with cirrhosis to predict patients that are at risk of developing HE and should be tested prospectively in suitably designed studies.

ACKNOWLEDGMENTS

Six of the patients in this study have also been reported in our previous paper (2).

GRANTS

This study was performed with grant support from the Wellcome Trust (London, UK) and a research grant from the National Health Service Research and Development Fund (Edinburgh, UK). 1H-MRS was performed at the SHEFC Brain Imaging Research Centre for Scotland, which was established with grants from the Scottish Higher Education Council and Medical Research Council, General Electric Medical Systems, Boehringer Ingelheim, Novartis, with grants from the Scottish Higher Education Council and Medical Research Council for Scotland, which was established.

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