Plasma met-enkephalin, β-endorphin and leu-enkephalin levels in human hepatic encephalopathy

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مستويات الميت–إنكيفالين، والبيتا–إندورفين، واللو–إنكيفالين في البلازما في حالات اعتلال الدماغ الكبدي البشري

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الخلاصة: قام الباحثون بقياس الميت–إنكيفالين، والبيتا — إندورفين، واللو– إنكيفالين، لدى المرضى الذين يعانون من مستويات مختلفة من اعتلال الدماغ الكبدي المنشأ بالمقارنة مع المجموعات الشاهدة، ومرضى التليُّف (تشمُّع الكبد) لدراسة دور منظومة الأفيونيات في إحداث هذا المرض. فوجدوا أن مستويات الميت–إنكيفالين في البلازما كانت مرتفعة إحصائياً لدى المرضى الذين يعانون من التليُّف (التشمُّع) وكل درجات اعتلال الدماغ الكبدي، منها لدى المجموعات الشاهدة. أما مستويات البيتا–إندورفين فكانت متشابهة لدى المجموعات الثلاث. أما مستويات اللو–إنكيفالين في البلازما فكانت أعلى لدى مرضى المستوى الثاني والثالث والرابع من اعتلال الدماغ الكبدي منها لدى المجموعة الشاهدة ومرضى التليُّف، ومرضى المستوى الأول من اعتلال الدماغ الكبدي. وهذه النتائج تدعم المعطيات الخاصة بمسؤولية الميت–إنكيفالين واللو–إنكيفالين عن إمراض اعتلال الدماغ الكبدي، مما يسوِّغ منطقياً استخدام مضادات المستقبلات الأفيونية المفعول في معالجة هذا المرض الكبدي.

ABSTRACT To address the role of the opioid system in the pathogenesis of hepatic encephalopathy (HE) we measured plasma met-enkephalin, β-endorphin and leu-enkephalin in patients with different grades of HE compared to control subjects and patients with cirrhosis. Plasma met-enkephalin levels were significantly higher in patients with cirrhosis and all grades of HE than controls. Plasma β-endorphin levels were similar in the 3 groups. Plasma leu-enkephalin levels were significantly higher in HE grades II, III and IV than in controls, patients with cirrhosis and HE grade I patients. Our results support data on the involvement of met-enkephalin and leu-enkephalin in the pathogenesis of HE and provide a rationale for the use of opioid receptor antagonists in the treatment of HE.

Concentrations plasmatiques de met-enképhaline, de β-endorphine et de leu-enképhaline dans l'encéphalopathie hépatique chez l'homme

RÉSUMÉ Afin d'évaluer le rôle du système opioïde endogène dans la pathogénie de l'encéphalopathie hépatique (EH), nous avons comparé les concentrations plasmatiques de met-enképhaline, de β-endorphine et de leu-enképhaline mesurées chez des patients présentant différents degrés d'EH, des témoins et des patients souffrant de cirrhose. Les taux plasmatiques de met-enképhaline sont apparus significativement supérieurs en présence d'une cirrhose et d'une EH, quel qu'en soit le degré de sévérité, par rapport aux témoins. La β-endorphine plasmatique était comparable dans les 3 groupes, tandis que la leu-enképhaline atteignait une concentration significativement plus élevée dans les cas d'EH de grades II, III et IV que chez les témoins, les patients cirrhotiques et ceux présentant une EH de grade I. Les résultats que nous avons obtenus confirment l'implication de la met-enképhaline et de la leu-enképhaline dans la pathogénie de l'EH et justifient le recours aux antagonistes des récepteurs opioïdes dans le traitement de l'EH.

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Introduction

Hepatic encephalopathy (HE) continues to be a major clinical problem. In patients with cirrhosis, the child classification recognizes the prognostic significance of HE [*1*]. Alterations in the opioid system have been reported in patients with liver disease. Plasma met-enkephalin and leu-enkephalin levels have been found to be higher in patients with cirrhosis [*2*], and met-enkephalin to be elevated in plasma in acute liver disease [*3*]. Changes in the opioid system in the central nervous system have been reported in 2 animal models of fulminant hepatic failure [*4,5*]. Opioid peptides may contribute to some of the manifestations of chronic liver disease, such as fatigue [*2*], pruritis [*6,7*], ascites [*8*] and HE [*4*].

Opioid peptides are neuroactive substances that are found in the central nervous system and in peripheral tissues [*9*]. They coexist in the central nervous system with various neurotransmitters and may modulate their effects [*10,11*]. Opioid peptides interact in the central nervous system with a wide variety of neurotransmitter systems, including the gamma-aminobutyric acidergic, serotoninergic, dopaminergic and nor-adrenergic systems, all of which have been implicated in the multifactorial pathogenesis of HE [*12*]. HE is a metabolic encephalopathy characterized by neural inhibition [*13*] and there is evidence that opioid peptides may function as inhibitory neuromodulators [*14,15*]. As it is well known that patients with liver cirrhosis are hypersensitive to the neuroinhibitory effects of morphine [*16,17*], it is possible that opioid peptides in the central nervous system are implicated in some of the manifestations of HE. A pathophysiological link between the opioid system and HE is further supported by a demonstration that the opioid receptor antagonist naloxone induces amelioration of human HE [*18,19*] and improvement of HE in a rat model with fulminant hepatic failure [*4*].

This study, therefore, addressed the pathophysiological importance of the opioid system in human HE. Three representative opioid ligands were measured in plasma of patients with various grades of HE and compared to control subjects and cirrhotic patients.

Methods

A total of 73 subjects attending the Theodor Bilharz Research Institute between October 2003 and November 2004 were enrolled in the study, and were divided into 3 groups. Group I consisted of 10 "healthy" controls, age- and sex-matched with the liver cirrhosis and HE patients to control for these variables. The control group was limited to 10 because of cost considerations. The controls were recruited from the outpatient clinic where they were attending for check-up. Group II consisted of 14 patients with liver cirrhosis without HE: 7 were classified as Child–Pugh class B and 7 as Child–Pugh class C. The etiology of cirrhosis was hepatitis C in 10 of these patients, hepatitis B in 2 patients, mixed hepatitis B and C in 1 patient and autoimmune in 1 patient. Group III consisted of 49 patients with various grades of HE: 12 patients had HE grade I, 10 had HE grade II, 13 had HE grade III and 14 patients had HE grade IV. The predisposing factors for HE were identified as haematemesis in 12 patients, infection including spontaneous bacterial peritonitis in 17 patients, electrolyte disturbance either caused by the use of diuretics or by vomiting and diarrhoea in 10 patients and paracentesis in 7 patients. No predisposing factor was identified in 3 of these patients. The etiology of HE was hepatitis B in 12 of these patients, hepatitis

C in 27 patients, mixed hepatitis B and C in 2 patients, autoimmune in 2 patients and unknown etiology in 6 patients.

The diagnosis of HE was based on clinical criteria, and its severity was assessed by the West Haven Criteria for grading of mental status [*20*], which is based on changes of consciousness, intellectual function, and behaviour.

All patients underwent the following: full history taking, general abdominal examination, abdominal ultrasonography, upper endoscopy, and laboratory investigations. Liver biopsy was performed where feasible for cirrhosis and HE patients and histopathological examination carried out.

Venous blood samples were collected using plain tubes for routine tests and chilled polyethylene tubes containing aprotinin and/or ethylenediaminetetra-acetic acid (EDTA). Tubes were put on ice and blood samples were spun within 30 minutes at 1600 × *g* for 15 minutes at 0 ºC. Ammonia assay was carried out immediately. The remaining plasma was stored at –60 ºC until further processing.

Liver function tests including serum aminotransferases, alkaline phosphatase, albumin, bilirubin and prothrombin time concentration were measured by conventional methods. Seromarkers of hepatitis B [hepatitis B surface antigen (HBsAg) and hepatitis B surface antibody (HBsAb)] were assayed by enzyme-linked immunosorbent assay (ELISA) (Boehringer Mannheim, Germany) and those of hepatitis C by hepatitis C virus (HCV) antibodies (Murex Diagnostics, France, Version III ELISA) and HCV mRNA by polymerase chain reaction. For determination of plasma ammonia levels we used an enzymatic UV-assay (Randox, United Kingdom) [*21*].

The levels of the opioid ligands met-enkephalin, β-endorphin and leu-enkephalin were measured in extracted plasma. Plasma extractions were performed as described previously [*22*], with some modifications. Briefly, isolation of opioid peptides from plasma was performed by adding 1% trifluoroacetic acid (TFA) (Merck, Darmstadt, Germany) to the plasma and then samples were centrifuged. The plasma solution was loaded on to pretreated C18 Sep-Pak columns (Waters, Milford, United States of America). The columns were washed twice with 1% TFA and then the peptide was eluted slowly with 60% acetonitrile in 1% TFA. The eluates were evaporated and the dried samples were reconstituted as required for the radioimmunoassay.

Plasma opioid peptides were determined by commercially available radioimmunoassay kits for met-enkephalin, β-endorphin and leu-enkephalin (Peninsula Laboratories Inc, Division of Bachem, United States of America) according to the instructions provided with each kit. The efficiency of the peptide extraction from plasma was approximately 80%, the reported data were corrected for extraction efficiency.

The sensitivities of the assays were 270 pg/tube for met-enkephalin, 13 ng/tube for β-endorphin and 9 pg/tube for leu-enkephalin. Intra-assay variance for all peptides was ≤ 5%.

Statistical analysis

Numerical data were expressed as mean and standard deviation (SD). Multiple intergroup comparisons were made by using one-way ANOVA. If a significant change was found in intergroup comparisons, post-hoc multiple comparison analysis with Tukey–Kramer multiple comparison test was performed. Correlation was computed using Spearman correlation coefficient. *P*-values < 0.05 were considered significant. *SPSS*, version 10 was used for data analysis.

Results

There was a significant increase in mean venous plasma ammonia levels in HE grade I patients (110.8 μmol/L, SD 12.6), grade II (164.4 μmol/L, SD 10.5), grade III (184.1 μmol/L, SD 14) and grade IV (279.5 μmol/L, SD 16.2) patients compared to both the control group (30.2 μmol/L, SD 10.1) and the cirrhosis group (43.2 μmol/L, SD 10.3) groups ($P < 0.01$) (Table 1, Figure 1A). In addition, there was a significant difference between HE grade II patients compared to grade I patients ($P < 0.01$). Plasma ammonia was significantly higher in HE grades III and VI patients compared to grade II patients ($P < 0.01$) and in HE grade IV patients compared to grade III patients ($P < 0.01$). Figure 2A shows that ammonia levels correlated with the severity of hepatic encephalopathy ($r = 0.944$, $P < 0.001$).

Plasma met-enkephalin levels were significantly higher ($P < 0.01$) in patients with cirrhosis (44 pg/mL, SD 11.1), HE grade I patients (63.7 pg/mL, SD 5.3), HE grade II patients (69.7 pg/mL, SD 8.1), HE grade III patients (90.6 pg/mL, SD 10.6), HE grade IV patients (133.2 pg/mL, SD 9.6) compared to controls (24.6 pg/mL, SD 3.2) (Table 1, Figure 1B).

Plasma met-enkephalin levels were significantly higher in HE grade III patients than both HE grade I and II patients ($P < 0.01$). Also, the differences between met-enkephalin levels in patients with HE grade IV and either HE grades I, II or III were significant ($P < 0.01$).

Met-enkephalin levels correlated with severity of hepatic encephalopathy ($r = 0.908$, $P < 0.001$) (Figure 2B).

Beta-endorphin levels did not show any statistically significance differences between the studied groups (Table 1, Figure 1C).

The mean elevation of plasma leu-enkephalin (pg/mL) was statistically signif-

Table 1 **Plasma levels of ammonia, met-enkephalin, β-endorphin and leu-enkephalin in the different studied groups**

Variable	Control (*n* = 10)	Cirrhosis (*n* = 14)	HE I (*n* = 12)	HE II (*n* = 10)	HE III (*n* = 14)	HE IV (*n* = 13)
Ammonia (μmol/L)						
Mean (SD)	30.2 (10.1)	43.2 (10.3)	110.8 (12.6)[a, b]	164.4 (10.5)[a, b, c]	184.1 (14.0)[a, b, c, d]	279.5 (16.2)[a, b, c, d, e]
Range	18–24	29–65	93–128	150–187	160–206	256–310
Met-enkephalin (pg/mL)						
Mean (SD)	24.6 (3.2)	44.0 (11.1)[a]	63.7 (5.3)[a, b]	69.7 (8.1)[a, b]	90.6 (10.6)[a, b, c, d]	133.2 (9.6)[a, b, c, d, e]
Range	20–29	30–62	55–72	60–81	69–108	120–148
β-endorphin (ng/mL)						
Mean (SD)	9.1 (1.6)	12.1 (4.2)	10.5 (3.2)	11.9 (2.2)	11.5 (3.7)	12.1 (3.8)
Range	6.9–11.8	5.8–24	6.8–19	7.4–15	7.1–16.2	6–22.2
Leu-enkephalin (pg/mL)						
Mean (SD)	0.8 (0.1)	0.6 (0.2)	0.8 (0.4)	2.2 (0.7)[a, b, c]	2.9 (0.8)[a, b, c]	3.8 (1.1)[a, b, c, d, e]
Range	0.6–1	0.3–0.9	0.1–1.4	1.2–3.2	0.9–4.0	1.2–5.3

[a]P < *0.01 versus control group,* [b]P < *0.01 versus group with cirrhosis,* [c]P < *0.01 versus HE I,* [d]P < *0.01 versus HE II,* [e]P < *0.01 versus HE III.*
HE = hepatic encephalopathy.
SD = standard deviation.

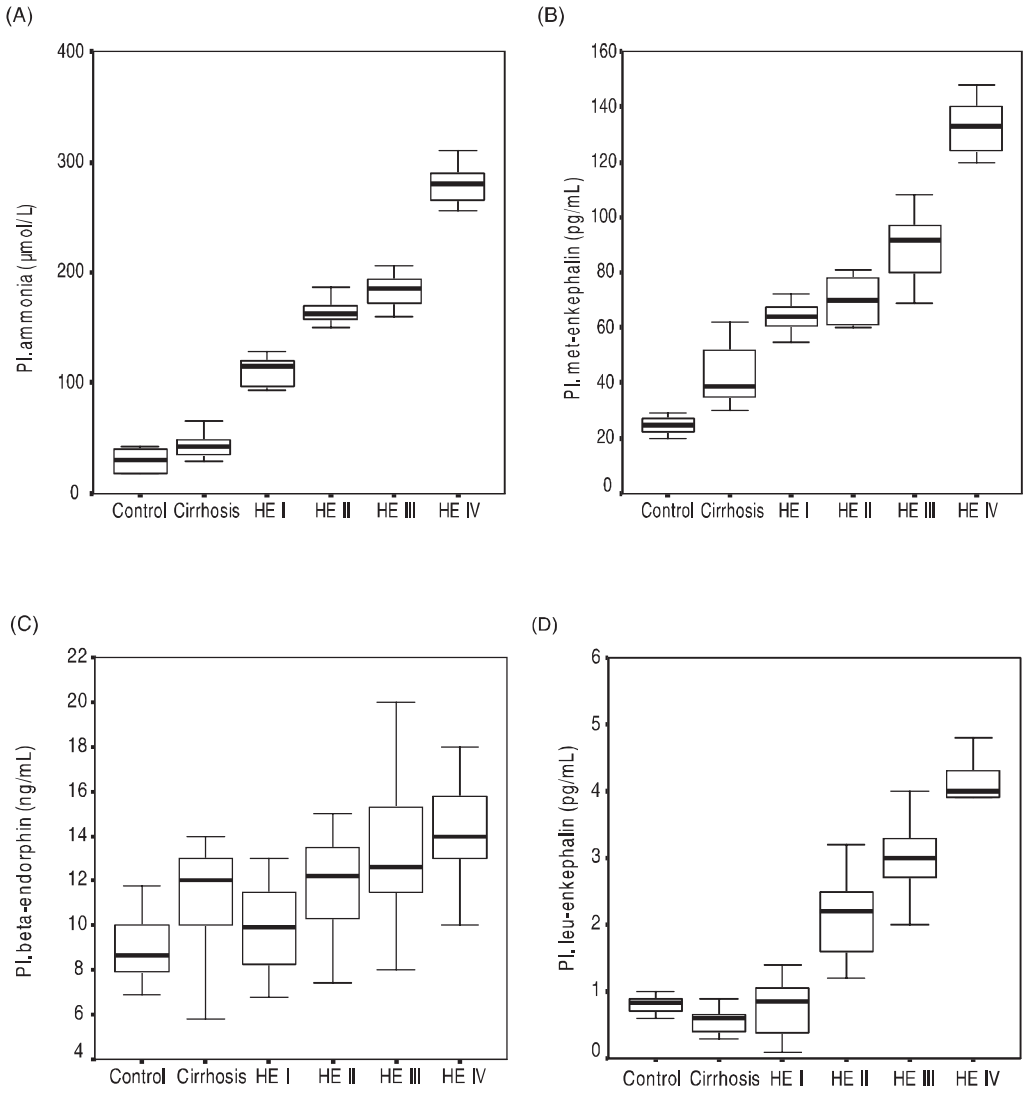

Figure 1 **Box plots showing plasma levels of (A) ammonia, (B) met-enkephalin, (C) β-endorphin and (D) leu-enkephalin in the studied groups. The top and bottom horizontal lines of the box indicate the 25th and 75th percentiles respectively. The lines within the box indicate the median values.**

icant ($P < 0.01$) in HE grade II (2.2 pg/mL, SD 0.7), grade III (2.9 pg/mL, SD 0.8) and grade IV (3.8 pg/mL, SD 1.1) patients as compared to controls (0.8 pg/mL, SD 0.1), patients with cirrhosis (0.6 pg/mL, SD 0.2) and HE grade I patients (0.8 pg/mL, SD

0.4) (Table 1, Figure 1D). Significant differences were found between HE grade IV patients and both HE grade II, III patients ($P < 0.01$).

Again, plasma leu-enkephalin levels correlated with severity of hepatic encephalopathy ($r = 0.823$, $P < 0.001$) (Figure 2C).

Discussion

In the present study, venous ammonia as well as 3 prototype opioid ligands, met-enkephalin, leu-enkephalin and β-endorphin, were measured in patients with various grades of HE as compared to control subjects and cirrhotic patients.

Our finding that ammonia levels correlated with the severity of hepatic encephalopathy ($r = 0.944$, $P < 0.001$) is in agreement with Ong et al., who also found that venous sampling was adequate for ammonia measurement and that there was no additional advantage of measuring the partial pressure of ammonia compared to total ammonia [*23*]. Conversely, other researchers have found that arterial ammonia levels are more accurate than venous values, but still correlate poorly with severity of HE and that scoring of ammonia levels is arbitrary [*24*]. Despite the significant correlation between partial pressure of ammonia and HE, Nicolao et al. suggested that neither partial pressure of ammonia nor arterial ammonia are, from the clinical point of view, more useful than venous ammonia; all 3 determinations being limited both for the diagnosis of HE and for the clinical management of patients [*25*].

The finding of elevated plasma met-enkephalin levels in patients with cirrhosis is in accordance with the data reported by Thornton and Losowsky [*2*]. They addressed met-enkephalin levels in patients with primary biliary cirrhosis, where the presence of cholestasis may be linked to elevated

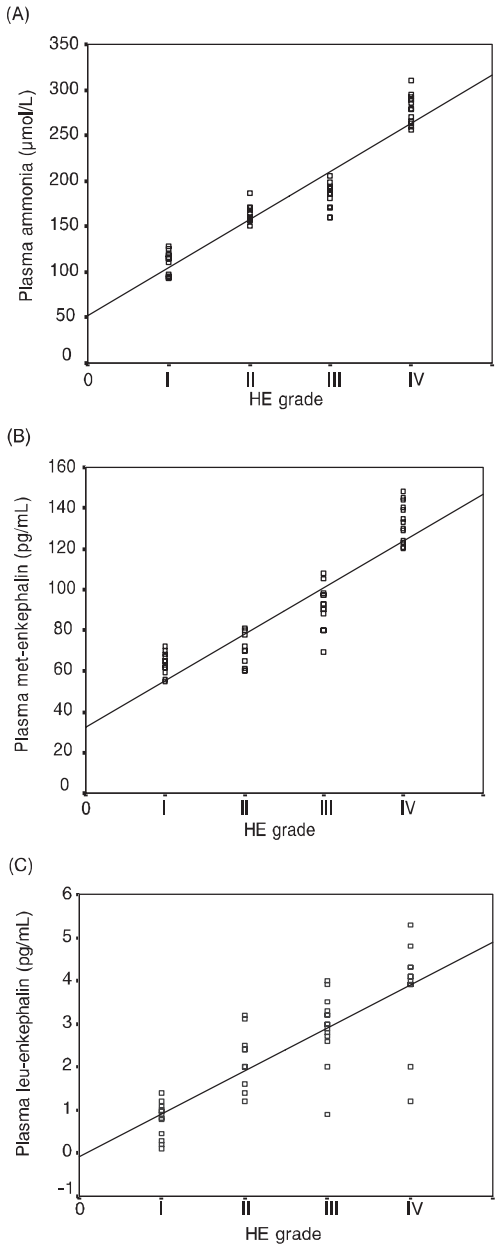

Figure 2 **Correlation of hepatic encephalopathy (HE) grades and: A. plasma ammonia level ($r = 0.944$, $P < 0.001$); B. plasma met-enkephalin level ($r = 0.908$, $P < 0.001$); C. plasma leu-enkephalin level ($r = 0.823$, $P < 0.001$).**

met-enkephalin levels [*4,7*]. Conversely, Yurdaydin et al. found met-enkephalin levels unchanged in patients with cirrhosis and they suggested that diminished hepatic clearance secondary to the liver damage of cirrhosis does not significantly contribute to the elevated levels of plasma met-enkephalin in HE [*26*].

Our findings also concur with other researchers who have found an elevation of plasma met-enkephalin levels in patients with HE as compared to controls; the increase was mainly observed in patients with HE grades III and IV, while in grades I and II plasma met-enkephalin overlapped with those of healthy controls [*26*]. The changes in plasma met-enkephalin levels in the Yurdaydin study were of the same magnitude as those reported in patients with acute liver disease [*3*]. Our data on met-enkephalin levels are also in line with data reported in an animal model of HE [*3*]. In our study met-enkephalin levels correlated with severity of HE which is in agreement with others [*4*].

We suggest that increased plasma met-enkephalin levels in patients with HE may be secondary to increased secretion or diminished hepatic clearance or both [*3*]. Potential sources of increased met-enkephalin secretion may be the adrenal glands [*27*], the gut [*28*], the sympathetic nerves [*29*] and the liver [*30*].

Beta-endorphin levels did not show any statistically significance differences between the studied groups, which supports previous findings in patients with cirrhosis [*26*]. However, these results are at variance with studies in different animal models [*4,5*], and the findings may be species or model dependent.

The mean elevation of plasma leu-enkephalin was statistically significant in HE grades II, III and grade IV patients as compared to controls, patients with cirrhosis and HE grade I patients. Significant differences were also found between HE grade IV patients and both HE grade II and III patients. This is in contrast to published data obtained from patients with cirrhosis without HE where the difference between studied groups was not significant [*2*]. Also, other researchers have found no statistically significant differences between plasma leu-enkephalin levels in patients with HE and healthy controls [*26*]. In addition, we found that plasma leu-enkephalin levels correlated with severity of hepatic encephalopathy.

Patients with liver disease have increased plasma concentrations of the endogenous opioid peptides met-enkephalin and leu-enkephalin [*2*]. Nalmefene, a specific opioid antagonist devoid of agonist activity, was given to patients with cirrhosis and clinical manifestations of the hepatic disease improved, indicating that blocking opioid receptors has an effect on some of the metabolic abnormalities of liver disease [*17,19*].

Overall, the results of our study suggest that changes may occur in the activity of the opioid system in HE and may be primarily linked to the enkephalin pentapeptides. This line of reasoning suggests that the finding of significantly elevated levels of met-enkephalin and leu-enkephalin in the plasma of humans with HE implies a pathophysiological role for opioids in HE, and this should provide a rationale for the study of opioid receptor antagonists in the treatment of HE.

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