Endogenous benzodiazepine-like compounds and diazepam binding inhibitor in serum of patients with liver cirrhosis with and without overt encephalopathy

R Avallone, M L Zeneroli, I Venturini, L Corsi, P Schreier, M Kleinschnitz, C Ferrarese, F Farina, C Baraldi, N Pecora, M Frigo, M Baraldi

Abstract

Background/Aim—Despite some controversy, it has been suggested that endogenous benzodiazepine plays a role in the pathogenesis of hepatic encephalopathy. The aim of the present study was to evaluate the concentrations of endogenous benzodiazepines and the peptide, diazepam binding inhibitor, in the blood of patients with liver cirrhosis with and without overt encephalopathy, and to compare these levels with those of consumers of commercial benzodiazepines.

Subjects—Normal subjects (90), benzodiazepine consumers (14), and cirrhotic patients (113) were studied.

Methods—Endogenous benzodiazepines were measured by the radioligand binding technique after high performance liquid chromatography (HPLC) purification. The presence of diazepam and N-desmethyl-diazepam was assayed by HPLC-electrospray tandem mass spectrometry. Diazepam binding inhibitor was studied in serum by radioimmunoassay.

Results—Endogenous benzodiazepines were below the limit of detection in 7% of patients with encephalopathy. When detectable, their levels were at least comparable with those of benzodiazepine consumers and correlated with the liver dysfunction but not the stage of encephalopathy. Serum levels of diazepam binding inhibitor tended to decrease when endogenous benzodiazepines levels increased.

Conclusions—Endogenous benzodiazepines may accumulate in patients with liver cirrhosis during the course of the disease, and the phenomenon appears to be independent of the presence or absence of encephalopathy.

Keywords: benzodiazepine consumers; diazepam binding inhibitor; endogenous benzodiazepines; liver cirrhosis; overt hepatic encephalopathy

Hepatic encephalopathy is one of the major complications of liver cirrhosis, and it is a component of fulminant hepatic failure characterised by impairment of the central nervous system, which is believed to develop from increased tone of the inhibitory γ-aminobutyric acid (GABA$_\gamma$) receptor system (for reviews, $^1$ $^2$). The involvement of this receptor system in overt hepatic encephalopathy (OHE), discovered in the 1980s during studies on GABA$_\gamma$ receptors in the brain of animals with OHE, was considered likely when specific benzodiazepine receptor antagonists were shown to revert the symptoms of encephalopathy in animal models $^1$ $^6$ and in patients. $^5$ $^6$ Later, the observation of an increased presence of endogenous benzodiazepine receptor ligands (BZDs) in animals and patients with OHE $^7$ $^8$ suggested that this phenomenon may contribute to the enhancement of GABAergic neurotransmission. $^9$ $^10$ We cannot exclude, however, the possibility that compounds such as ammonia $^1$ $^3$ $^5$ or neurosteroids $^16$ contribute to the above mentioned increased functional activity of the GABA$_\gamma$ receptor system. BZD-like compounds and ammonia may potentiate inhibitory GABAergic neurotransmission by acting synergistically. $^17$

The endogenous receptor ligands found in blood and brain during OHE $^9$ were called BZD-like substances since they are a mixture of the halogenated 1,4-benzodiazepines (such as diazepam) and non-halogenated BZDs (called “endozepines”). Although the chemical structure of the endozepines is not yet fully characterised, it is fair to surmise that they contribute to OHE.

Halogenated BZDs are naturally present in several plants and vegetables, $^18$ in brain tissues of different animal species and in man. $^19$ Their sources have not yet been clarified, but the observation that they are present in human brain samples stored since 1940 $^20$ indicates that they do not derive from environmental pollution with synthetic BZDs, which have been produced commercially since 1959. These compounds and their precursors are components of our diet. $^21$ An exogenous biosynthetic pathway for the production of such compounds cannot, however, be excluded since we recently showed that a reduction in the intestinal bacterial flora caused by a non-absorbable antibiotic partially decreases the levels of these compounds in the blood. $^21$

Other endogenous BZDs such as the neuropeptide called diazepam binding inhibitor (DBI) and its metabolite, the octadecaneuropeptide, which decreases GABA$_\gamma$ neurotransmission, $^16$ have been found to be increased in the cerebrospinal fluid of patients with OHE $^7$ and in brain regions of rats with porta-caval anastomosis. $^{24}$
A–C, functional status of the liver classified according to Child-Pugh.

Since few studies have been performed on endogenous circulating BZDs in patients with OHE due to fulminant hepatic failure\(^1\) or liver cirrhosis,\(^2\) the aim of the present study was to (a) evaluate the concentrations and nature of BZD-like compounds in the plasma of patients with liver cirrhosis with and without OHE, (b) compare the levels found in liver cirrhotic patients with those present in the plasma of consumers of commercial BZDs in order to estimate their pharmacological relevance, and (c) study the levels of DBI in both the patients and BZD consumers, bearing in mind that little is known about the mutual interaction of BZD compounds and DBI at the periphery.

**Methods**

SUBJECTS (TABLES 1 AND 2)

We studied 113 patients with liver cirrhosis and 90 normal subjects, who appeared to be free of commercial BZD medication for at least three months as verified by patient, family, and medication records. Moreover 14 normal subjects who were habitual consumers of commercial BZDs were included in the study. The diagnosis of liver cirrhosis was based on biochemical tests and liver biopsy. Fifty nine patients showed no evidence of OHE while the other 54 showed different stages of impaired mental status. The stage of OHE was evaluated on the basis of electroencephalographic pattern.\(^7\) This test allowed the classification of the cirrhotic patients into the following categories: 59 with stage 0, 22 with stage I, 19 with stage II, eight with stage III, and five with stage IV. The functional status of the liver was clinically classified according to the Child–Pugh classification.\(^2\) Table 1 gives the characteristics of the patients included in the study, and table 2 contains laboratory data.

The 14 regular consumers of BZDs, who used diazepam 2 mg per day or lorazepam 2.5 mg per day as sedatives, had normal liver and kidney function.

**Table 1 Patient characteristics**

<table>
<thead>
<tr>
<th>Child–Pugh class</th>
<th>Controls</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>BZD consumers</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>90</td>
<td>33</td>
<td>41</td>
<td>39</td>
<td>14</td>
</tr>
<tr>
<td>Men</td>
<td>46</td>
<td>18</td>
<td>28</td>
<td>30</td>
<td>3</td>
</tr>
<tr>
<td>Women</td>
<td>44</td>
<td>15</td>
<td>13</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>Age (y) (mean (SD))</td>
<td>63 (9)</td>
<td>59 (9)</td>
<td>57 (11)</td>
<td>50 (11)</td>
<td>65 (4)</td>
</tr>
<tr>
<td>Aetiology of cirrhosis</td>
<td>49 (54)</td>
<td>30 (30)</td>
<td>20 (20)</td>
<td>10 (10)</td>
<td>60 (60)</td>
</tr>
</tbody>
</table>

B–C, functional status of the liver classified according to Child-Pugh.

The serum obtained from all patients was stored at −80°C until used and individually processed for the assay of BZDs and DBI. The study was carried out with the approval of the local ethical committee.

**Quantification of endogenous BZD-like compounds**

As previously described,\(^2\) aliquots of all the serum samples (1 ml) were acidified with acetic acid (1 M), and centrifuged at 3000 g for ten minutes. The supernatant was passed through previously washed Sep-Pak C\(_18\) cartridges (Millipore, Medford, MA, USA). The material was eluted from Sep-Pak with acetone/0.1% trifluoroacetic acid (TFA) and then lyophilised. The lyophilised samples were reconstituted with 1 ml water, and aliquots (200 µl) were chromatographed in duplicate at 0.8 ml/min on a LiChrospher 100 RP-18 column (250 × 4.0 mm; 5 µm) equilibrated with 80% water/0.1% TFA and 20% acetonitrile. Absorbance was monitored at 230 nm. Samples were chromatographed using a water/0.1% TFA and acetonitrile gradient at 0.5% per minute from 20 to 58% acetonitrile. Seventy five fractions (one per minute) from each sample were collected, lyophilised, and reconstituted with water before radio-receptor assay. Known concentrations of diazepam, N-desmethyl-diazepam, oxazepam, lorazepam, delorazepam, and 2′-chlorodiazepam were run in parallel with the plasma samples.

Unless otherwise indicated, all reagents were obtained from Sigma Chemical Co. and were all high performance liquid chromatography (HPLC) grade. All the fractions were then tested for their ability to inhibit [\(^3\)H]flunitrazepam (1 nM; specific activity 87 Ci/mmoll; NEN, Boston, MA, USA) binding to rat cerebellar membrane preparations, which are a source of BZD receptor, and containing 180–200 µg protein/100 µl measured by Bradford’s method. Data were expressed as diazepam equivalents (DE) based on extrapolation from standard displacement curves generated using diazepam. The total concentration of BZD-like compounds present in each serum was calculated by determining the DE derived from the displacement activity of any single peak and then summing the values of all peaks.

Since the chemical identities of all the components of the BZD-like material are not known, their extraction efficiencies could not be determined. The limit of detection of diazepam by [\(^3\)H]flunitrazepam binding was 2 nmol DE/l with a coefficient of variation of 0.52. Assays were performed in triplicate and variations from the mean were less than 15%.

**Determination of 1,4-benzodiazepines by HPLC-electrospray tandem mass spectrometry (HPLC–ESI–MS–MS)**

HPLC–ESI–MS–MS was carried out by the method of Kleinschmitz et al.\(^2\) The analysis was performed using a triple stage quadrupole TSQ 7000 LC–MS–MS system with electrospray interface (Finnigan MAT, Bremen, Germany). Data acquisition and mass spectrometric evaluation were conducted on a Personal
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ion [M+H]+ and the most abundant product ion pairs represent the protonated molecular and m/z 217/140 (−30 eV) for benzodiazepam. Selected ion pairs for the simultaneous experiments were (oxygenated) benzodiazepam and m/z 285/257 (−30 eV) for diazepam. The ions pairs represent the protonated molecular ion [M+H]+ and the most abundant product ion for each of the BZDs. The temperature of the heating capillary was 250°C. The electrospray capillary was set to 5 kV and the electron multiplier voltage was set to 2.3 kV. A sheath gas pressure of 73 Pa and an auxiliary gas flow of 5 litres/min were applied to support the droplet formation. Nitrogen served both as sheath and auxiliary gas. With this method a signal-to-noise ratio of 2:1 was reached.

STATISTICAL ANALYSIS

The Kruskal-Wallis test was used to determine whether a given variable differed significantly between groups. Comparisons between single groups were performed by means of the Mann-Whitney U test corrected as described by Bonferroni.

Results

BZD-LIKE COMPOUNDS

The extraction and purification of plasma samples from normal subjects and from patients with liver cirrhosis with and without OHE showed the presence of at least 12 different peaks, with a retention time ranging from 17 to 70 minutes under our chromatographic conditions. The number of peaks found in each patient ranged from one to four. The number of fractions containing BZD ligands was consistently lower in controls and in liver cirrhosis without OHE (one or two peaks) than in patients with OHE (three or four peaks).

Figure 1  Serum levels of benzodiazepine (BZD)-like compounds measured by the radioiodinated binding technique after HPLC purification in liver cirrhotic patients without encephalopathy (stage 0), with encephalopathy (OHE; stages I, II, III and IV) and in BZD consumers. The horizontal bar represents the mean value. Kruskal-Wallis one way analysis of variance shows a significant difference between groups (p<0.0001). The Mann-Whitney U test adjusted using the Bonferroni correction shows that patients with liver cirrhosis without OHE (stage 0) differed from all the patients with OHE (p<0.001). The levels found in patients with OHE stages I, II, III, and IV were not different from each other and not different from the levels found in BZD consumers.

DE Gesellschaft für Röntgenologische Diagnostik, (Finnigan MAT). For HPLC, an Applied Biosystem 140B Solvent Delivery System (Applied Biosystems, Foster City, CA, USA) equipped with two 10 ml syringes and a LiChrospher 60-RP select B column (100 × 2.0 mm internal diameter; 5 µm; Knauer, Berlin, Germany) was used. Aliquots of the lyophilised samples were redissolved in 100 µl methanol/water/acetonitrile (1:1:1, by vol.). Separations were performed using a linear gradient. Solvent A was 0.1% TFA in water, and solvent B was acetonitrile. The gradient program was: 0–10 min, 20–80% solvent B. The loop injection was 5 µl and the solvent flow was set to 200 µl/min. The mass spectrometer was operated in the selected reaction monitoring mode, with argon at a pressure of 0.27 Pa as collision gas. Selected ion pairs for the simultaneous experiment were (offset voltages in brackets): m/z 217/140 (−30 eV) for N-desmethyldiazepam and m/z 285/257 (−30 eV) for diazepam. The ions pairs represent the protonated molecular ion [M+H]+ and the most abundant product ion for each of the BZDs. The temperature of the heating capillary was 250°C. The electrospray capillary was set to 5 kV and the electron multiplier voltage was set to 2.3 kV. A sheath gas pressure of 73 Pa and an auxiliary gas flow of 5 litres/min were applied to support the droplet formation. Nitrogen served both as sheath and auxiliary gas. With this method a detection limit of 1.75 pmol/l for N-desmethyldiazepam and diazepam at a signal-to-noise ratio of 2:1 was reached.

QUANTIFICATION OF DBI

The level of DBI-like immunoreactivity (DBI-LI) in serum was assayed in 16 controls, in 19 patients with liver cirrhosis without OHE, in 12 patients with liver cirrhosis with OHE, and in eight BZD consumers. To extract DBI and to precipitate plasma proteins, 1 ml plasma was diluted with 1 ml saline and 2 ml 2 M acetic acid, heated at 90°C for 10 min followed by the addition of 2 M NaOH (1 ml). After centrifugation at 20 000 g for 20 minutes, aliquots of supernatants were lyophilised in triplicate and used for DBI radioimmunoassay (DBI-RIA). The characterisation of DBI immunoreactivity detected in serum extracts by reverse phase HPLC and the DBI-RIA, performed using anti-serum produced in rabbits against human recombinant DBI, were determined by incubation of different aliquots of tissue extract that paralleled the standard curve and by use of reverse phase HPLC.

The specificity of the immunoreactive material detected was determined by incubation of different aliquots of tissue extract that paralleled the standard curve and by use of reverse phase HPLC.
The concentrations of BZDs in extracted and purified serum from normal subjects were below the limit of detection (less than 2 nmol DE/l) in 46 cases (51%). In the remaining 44, the total amount of BZD substances that displaced [3H]flunitrazepam binding ranged below the limit of detection (less than 2 nmol DE/l) in normalsubjectsand in 34 of 37 of the patients without OHE. In the remaining three patients, trace amounts of both compounds were found in two, and in one there was only N-desmethyldiazepam. In liver cirrhosis with OHE the two above compounds were below the detection limit in two patients with stage I OHE and were represented only in trace amounts of N-desmethyldiazepam in two patients with stage III and IV OHE.

Determination of 1,4-Benzodiazepines by HPLC-ESI-MS-MS (Fig 3)

Mass spectrometric studies utilising HPLC-ESI-MS-MS on the active fractions performed on 12 controls, 37 liver cirrhosis cases without OHE, and 16 liver cirrhosis cases with stages I–IV of OHE. Diazepam and N-desmethyldiazepam were below the detection limit in normal subjects and in 34 of 37 of the patients without OHE. In the remaining three patients, trace amounts of both compounds were found in two, and in one there was only N-desmethyldiazepam. In liver cirrhosis with OHE the two above compounds were below the detection limit in two patients with stage I OHE and were represented only in trace amounts of N-desmethyldiazepam in two patients with stage III and IV OHE.
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DIAZEPAM BINDING INHIBITOR (FIG 4)

patients with liver cirrhosis in correlation with worsening liver function, but not with the degree of OHE; (3) the measurable BZD-like compounds comprise both known BZDs such as diazepam and N-desmethyldiazepam and unknown BZD-like compounds, and these so-called "endozepines" seem to represent most of the displacing ligands in plasma; (4) when detectable, the BZD levels found in patients with OHE were comparable with those present in BZD consumers with normal states of consciousness; (5) DBI-LI levels were found to be decreased in cirrhotic patients independently of the presence or absence of OHE. In BZD consumers, in whom BZD levels were constantly elevated, we found a significant reduction of DBI-LI. These data indicate an inverse correlation with the levels of circulating BZDs.

The finding that encephalopathy may occur in liver cirrhotic patients with very low levels of circulating BZD-like compounds, if not below the detection limit, is in line with the results of previous studies on patients with OHE due to fulminating hepatic failure. In these studies, only 60% of patients showed increased levels of BZDs in serum and only 55% had increased concentrations in the brain. These findings are in line with the concept that BZDs in serum diffuse passively into the brain and are in equilibrium with BZDs in the brain.

The finding that, when detectable, circulating BZD-like compounds reach concentrations comparable with those found in BZD consumers, raises the question of what causes the difference in the response to BZDs by the brains of cirrhotic patients with OHE and those of BZD consumers.

Chronic exposure to commercial BZDs produces tolerance represented by reduced GABA-BZD receptor function: this means that administration of increased doses of the drug is required to maintain the pharmacological effect. In contrast, in patients with liver cirrhosis, rather than tolerance, there is increased cerebral sensitivity to BZD administration. It has been shown that the reduction in BZD dose requirements in these patients is due to changes in the cerebral sensitivity more than to changes in drug disposition. Hence it seems fair to surmise that the enhanced GABAergic tone cannot be attributed to increased endogenous BZD-like compounds per se, but more to the presence of pre-existing brain dysfunction related, for example, to ammonia toxicity. In this situation, a concentration of circulating BZD-like compounds that would have no effect in a normal subject may facilitate sedation and worsen an episode of encephalopathy in a liver cirrhotic patient.

Finally, as regards the nature of the BZD-like compounds in serum, we found the presence of both diazepam and N-desmethyldiazepam by HPLC-ESI-MS-MS analysis. These halogenated compounds, however, represented less than 20% of the total BZD receptor ligands. This observation, which confirms the results of previous studies, indicates that most of these compounds are substances of unknown origin and nature called "endozepines." Both

DIAZEPAM BINDING INHIBITOR (FIG 4)

The DBI-LI levels ranged between 0.31 and 2.37 nmol/l (mean value 1.04 nmol/l) in control subjects, between 0.28 and 1.01 nmol/l (mean value 0.55 nmol/l) in liver cirrhosis without OHE, and between 0.13 and 0.57 nmol/l (mean value 0.34 nmol/l) in liver cirrhosis with OHE. Interestingly, the levels of DBI-LI in BZD consumers ranged between 0.15 and 0.57 nmol/l (mean value 0.33 nmol/l). Kruskal-Wallis one way analysis of variance shows a significant difference between groups (p<0.0001). The Mann-Whitney U test adjusted using the Bonferroni correction shows that the levels found in liver cirrhotic patients with or without OHE were statistically different from controls (p<0.05 and p<0.001 respectively). The DBI-LI in BZD consumers was different from controls (p<0.005) and practically equal to those found in cirrhotic patients with or without OHE.

Discussion

We have shown in this study, which includes a large number of fully characterised liver cirrhotic patients, that: (1) endogenous BZD-like compounds are, under our experimental conditions, below the detection limit (2 nmol DE/l) in 51% of normal subjects, in 16% of liver cirrhotic patients without OHE, and in 7% of those with OHE; (2) when detectable, BZD levels rise in the serum of cirrhotic
halogenated and non-halogenated BZDs were found inconsistently in patients with OHE and were sometimes not raised at all. It remains, however, unclear why BZDs accumulate in the blood of some liver cirrhotic patients and not in others with the same pathological condition. Retrospective control of the diet and therapy used in our patients as well as establishment of the aetiology of the liver cirrhosis did not show any substantial difference to explain this phenomenon.

As regards DBI, we found that the levels of this peptide are significantly decreased in those patients with liver cirrhosis and increased levels of BZDs independently of the presence or absence of OHE. The levels of DBI do not correlate with neuronal dysfunction or the severity of the liver disease. This finding would appear to exclude any direct effect of the liver dysfunction and the encephalopathy on the metabolism of this circulating peptide and suggests the presence in the periphery of a negative regulatory feedback mechanism exerted by BZDs on DBI. Accordingly, the same decrease is present in BZD consumers. The relation between DBI levels in plasma and those in the central nervous system is still poorly understood, as is also the regulation of its synthesis and metabolism in peripheral tissues. From these data we can surmise that the ratio between DBI and BZDs in the periphery is probably regulated by different mechanisms from those operating in the central nervous system. In liver cirrhotic patients with OHE, in fact, DBI was shown to be increased in cerebrospinal fluid in the presence of increased levels of BZDs, and the phenomenon was interpreted as an episode of compensatory reaction by DBI to an increased presence of BZDs. Whatever the regulatory mechanism in the periphery may be, the described decrease in DBI in the blood of the liver cirrhotic patients may be of relevance from the metabolic point of view, since this peptide, through stimulation of peripheral BZ receptors, regulates the intermediate metabolism and steroid biosynthesis. In conclusion, endogenous compounds with sedative action may accumulate in patients with liver cirrhosis during the course of the disease, and the phenomenon appears to be independent of the presence or absence of encephalopathy. The observation that circulating BZD-like compounds reach levels comparable with those found in BZD consumers with a normal state of consciousness reinforces the concept that these compounds may be more effective in those patients with pre-existing altered brain function.

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