Increased Circulating Calcitonin in Cirrhosis. Relation to Severity of Disease and Calcitonin Gene–Related Peptide

Jens H. Henriksen, Søren Schifter, Søren Møller, and Flemming Bendtsen

Increased circulating levels of the neuropeptide calcitonin gene–related peptide (CGRP) have recently been described in cirrhosis. CGRP is formed by alternative transcription of the calcitonin/α-CGRP gene, which also gives rise to calcitonin (CT). This study was undertaken to determine circulating plasma concentrations of CT in patients with cirrhosis in relation to the severity of disease and the plasma level of CGRP. Moreover, the kinetics of CT was evaluated for different organ systems by determination of arteriovenous extraction. Thirty-nine patients with cirrhosis (Child-Turcotte classes A/B/C, n = 10/22/7) were studied under a hemodynamic investigation and compared with 13 control subjects without liver disease. CT and CGRP in arterial and organ venous plasma were determined by radioimmunoassays. In patients with cirrhosis, circulating CT was significantly increased versus control (12.1 *v* 6.9 pmol/L, $P < .001$) and a direct relation to the Child-Turcotte score was found ($P < .005$). The increased circulating CT was directly correlated with increased CGRP ($r = .29$, $P < .05$). No significant arteriovenous extraction of circulating CT was observed in the kidneys, hepatosplanchnic system, lower extremities, or peripheral circulation, but there was a substantial rate of pulmonary disposal and clearance ($P < .005$). It is concluded that in addition to thyroid production, increased circulating CT in cirrhosis is most likely due to overexpression of the calcitonin/α-CGRP gene, with relation to the severity of disease and possibly to an accompanying pulmonary dysfunction.

A NUMBER OF CIRCULATORY and neuroendocrine abnormalities have been described in cirrhosis.[1] Thus, such patients exhibit a hyperdynamic circulation with increased cardiac output, an activated renin-angiotensin-aldosterone system, and sympathetic nervous overactivity.[2,3] Increased circulating levels of calcitonin gene–related peptide (CGRP) have recently been described in patients with cirrhosis.[4-6] CGRP is a highly potent vasodilator[7] and is widely distributed in nerve fibers with relation to vascular structures.[8,9] The physiological importance of CGRP is not clarified, but it may include cardiovascular derangement owing to vascular hyporeactivity and low systemic hemodynamic resistance.[10] The calcitonin/α-CGRP gene gives rise to both calcitonin (CT) and α-CGRP.[8,11] A gene duplication gives rise to β-CGRP, which only differs from α-CGRP in 3 amino acids.[8-12].

Little information exists on CT in patients with cirrhosis.[13,14] As circulating CGRP is elevated in this condition, we found it of interest to clarify whether this is due to specific overexpression of CGRP or whether circulating concentrations of CT are also affected in patients with cirrhosis, thereby indicating a nonspecific overexpression of the calcitonin/α-CGRP gene. Moreover, vascular areas of possible extraction of CT were identified during a multiorgan venous catheterization.

SUBJECTS AND METHODS

Study Population

Thirty-nine patients (6 women and 33 men) with cirrhosis were referred for hemodynamic investigation to diagnose and quantify the degree of portal hypertension. Cirrhosis was biopsy-verified in 35 patients and established on accepted clinical, biochemical, and ultrasonographic criteria in the remaining patients.[15] The etiology was alcoholic in 35 patients, and no etiology was established in 4 patients. The age range was 33 to 74 years, with a mean of 53 years. None of the patients experienced recent gastrointestinal bleeding or had encephalopathy above grade I. All patients abstained from alcohol and had no withdrawal symptoms at the time of the study. The patients were divided into 3 groups, A, B, and C, according to the modified Child-Turcotte criteria.[16] Clinical and biochemical characteristics and CGRP results have been described recently for some of the patients in the present study.[6] Ten patients were Child-Turcotte class A, 22 class B, and 7 class C. Besides diuretic therapy (n = 16, furosemide 40 to 80 mg/d and spirolactone 100 mg/d), none of the patients received medication known to influence the circulatory or endocrine systems. The subgroup of patients with pulmonary catheterization (n = 16) were not different from the total study population with respect to severity of disease, diuretic treatment, and sex. Clinical and biochemical characteristics are summarized in Table 1.

The controls were 13 patients (4 women and 9 men) with minor disorders (suspicion of intestinal ischemia, although not confirmed, irritable-bowel syndrome, postcholecystectomy pain, and fatty liver) who underwent a diagnostic catheterization. The age range was 31 to 69 years, with a mean of 54 years.

The study was approved by the Ethics Committee for Medical Research in Copenhagen. No complications or side effects were encountered during the study.

Catheterization

Patients and controls were studied in the morning after an overnight fast and at least 1 hour of resting supine. Catheterization of organ veins and the pulmonary artery was performed with a Swan-Ganz catheter (size 7F) via the femoral route under fluoroscopic control with the patients under local anesthesia as described elsewhere.[16-18] A small polyethylene catheter (size 5F) was placed in the femoral artery by the Seldinger technique. Pressures were measured with a capacitance transducer (Simonsen and Weel, Copenhagen, Denmark), the zero reference being the midaxillary level.

Cardiac output was determined by the indicator dilution technique after a bolus injection of 150 KBq ^{125}I-labeled human serum albumin into the right atrium as previously described.[18]

Arterial oxygen saturation was determined by an OSM-2 device (Radiometer, Copenhagen, Denmark).[17] Biochemical tests were performed by an autoanalyzer (SMAC, Terrytown, NY).

From the Departments of Clinical Physiology and Gastroenterology, Hvidovre Hospital, Hvidovre; and Department of Clinical Physiology, Glostrup Hospital, University of Copenhagen, Copenhagen, Denmark.

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Address reprint requests to Jens H. Henriksen, MD, Department of Clinical Physiology and Nuclear Medicine, 239, Hvidovre Hospital, DK-2650 Hvidovre, Denmark.

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Table 1. Clinical and Biochemical Data for 39 Patients With Cirrhosis

Variable	Reference Interval	Mean (range)
Age (yr)		53 (33-74)
Female/male ratio		6/33
Child-Turcotte class (A/B/C)		10/22/7
Hemoglobin (mmol/L)	F = 7.1-9.9, M = 8.1-10.9	8.3 (6.2-10.6)
Serum bilirubin (μmol/L)	2-17	28 (7-42)
Serum aspartate aminotransferase (U/L)	10-40	77 (29-278)
Serum alkaline phosphatase (U/L)	50-275	373 (172-1,341)
Serum albumin (μmol/L)	540-800	487 (313-688)
Coagulation factors 2, 7, and 10 (index)	0.70-1.30	0.58 (0.30-1.22)
Serum creatinine (μmol/L)	49-121	86 (50-126)
Serum sodium (μmol/L)	135-147	136 (125-145)
Hepatic venous pressure gradient (mm Hg)	<6	15 (8-26)
Cardiac output (L/min)	3.8-6.5	6.8 (4.5-9.4)
Arterial oxygen saturation (%)	93-99	95 (89-98)

Abbreviations: F, female; M, male.

Hormone Analysis

Blood samples were collected simultaneously from the following sites: femoral artery/hepatic vein (n = 33), femoral artery/renal vein (n = 32), femoral artery/inferior vena cava below the renal veins or iliac veins (n = 6), femoral artery/peripheral vein (n = 27), and pulmonary artery/femoral artery (n = 16). After clearing the catheter dead space, blood was collected in ice-chilled test tubes containing aprotinin-heparin (5,000 KIU/500 IU per 10-mL sample). The plasma was stored at −25°C until analysis.

Radioimmunoassay for CT and CGRP

Human CT was measured with a radioimmunoassay.[19] Standards (Peninsula Laboratories Europe, St. Helens, UK) were prepared in 0.1 mol/L phosphate buffer, pH 7.5, containing 0.1% bovine albumin, 0.01% sodium azide, and aprotinin (Trasylol; Bayer, Leverkusen, Germany) 20 KIU/mL. Tracer was prepared by the Iodo-Gen method (Pierce, Rockford, IL). The total molecule was used as substrate. Purification was performed by high-performance liquid chromatography (HPLC). Total separation was obtained between mono- and di-iodinated CT. The mono-iodinated molecule was used as tracer. The antibody was obtained by immunization of Danish rabbits with a complex of human CT linked with glutaraldehyde to bovine serum albumin (Dako, Glostrup, Denmark). The antibody is specific for the midportion and C-terminal end of the CT molecule but is not dependent on C-terminal amidation. It was used in a final dilution of 1:400,000. Incubations were performed at 4°C as a nonequilibrium assay with incubation of calibrator/sample and antibody for 4 days followed by 2 days' incubation after addition of tracer. The nonspecific binding was 3% to 4%. The volume of sample was 200 μL, antibody 100 μL, and tracer 100 μL. Separation was performed with a solid-phase separation system (Tachisorb; Calbiochem-Novabiochem, Bad Soden, Germany). The specificity of the assay has been tested against salmon CT (<0.001%), human α-CGRP (<0.001%), and human β-CGRP (<0.001%), which showed no cross-reactivity. The recovery of synthetic CT added to serum samples varied from 87% to 118% in the range of 10 to 130 pmol/L. The detection limit of the assay is 0.4 pmol/L. Intraassay and interassay coefficients of variation were 5% and 10%, respectively. The normal range is 4 to 13 pmol/L (N = 222).

Chromatography

Heparin-aprotinin plasma (15 mL) containing heparin 50 IU/mL and aprotinin 500 IU/mL was mixed 1:2 with acetic ethanol (375 μL 37% HCl in 250 mL 95% ethanol). The sample was centrifuged at 10,000 × *g* for 20 minutes at 4°C, and the supernatant was vacuum-centrifuged to dryness. The extract was redissolved in 10 mL H_2O containing 8.8×10^{-3} mol/L (0.1%) triflouroacetic acid (TFA) and reextracted on Sep-Pack (Millipore, Waters Corp, MA) activated with 10 mL 100% methanol and 10 mL H_2O. The Sep-Pack was eluted with 3 mL 80% ethanol. The eluate was vacuum-centrifuged to a volume of 700 μL, mixed, and filtered through a 0.45-μm filter before HPLC analysis using a LiChrospher 100 RP-18, 5-μm, 4 × 250-mm reverse-phase column (Merck, Darmstadt, Germany) eluted at 50°C. The flow rate was 1 mL/min with a linear gradient over 30 minutes of 10% to 70% CH_3CN/H_2O containing 8.8×10^{-3} mol/L (0.1%) TFA. Fractions of 0.5 mL were collected and vacuum-dried before reconstitution in assay buffer.

CGRP was determined by a nonequilibrium radioimmunoassay with incubation for 4 + 2 days as previously described.[5] The assay does not discriminate between α-CGRP and β-CGRP. The recovery of synthetic CGRP added to serum samples varied from 94% to 102% in the range of 60 to 100 pmol/L. The detection limit of the assay is less than 1 pmol/L. Intraassay and interassay coefficients of variation were 4% and 7%, respectively. The normal range is 23 to 50 pmol/L (N = 232).

Calculations

The extraction ratio (E), plasma clearance (Cl), and disposal rate (J) were determined by the equations,[20] $E = (C_{AP} - C_A)/C_{AP}$, $Cl = CO (1 - Hct) \cdot E$, and $J = Cl \cdot C_{AP}$, where C_{AP} and C_A are the concentrations in the pulmonary artery and systemic (femoral) artery, respectively, CO is cardiac output, and Hct is the hematocrit.

Statistics

The results are expressed as the mean ± SEM. Statistical analysis was performed by 1-way ANOVA with Tukey's correction for multiple comparisons, or by the Kruskal-Wallis test with Dunn's test for multiple

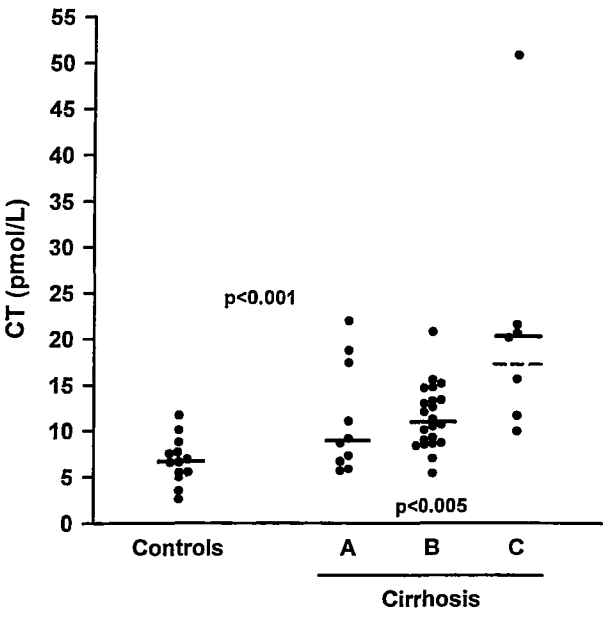

Fig 1. Arterial plasma CT concentration (pmol/L) in patients with cirrhosis (Child-Turcotte classes A, B, and C) and control subjects. Bars indicate mean values. (---) Mean after exclusion of 1 outlying high value.

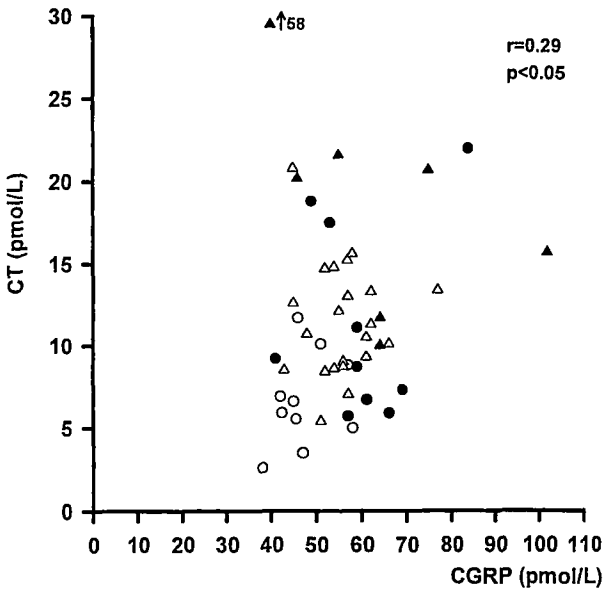

Fig 2. Relation between plasma CGRP and CT in patients with cirrhosis (Child-Turcotte classes A, B, and C, ●, △, and ▲) and controls (○).

comparisons. Comparisons of bivariate paired and unpaired data were performed by Student's paired and unpaired *t* test or the Wilcoxon and Mann-Whitney tests, respectively. Correlations were tested by Pearson or Spearman regression analysis. A *P* level less than .05 was considered significant.

RESULTS

In patients with cirrhosis, the circulating level of CT (mean, 12.1 ± 0.7 pmol/L) was significantly increased as compared with the control subjects (6.9 ± 0.9 pmol/L, $P < .001$) and the normal average (8 pmol/L, $P < .001$). Moreover, there was a significant relation to the severity of disease, as increasing levels of CT were observed through the Child-Turcotte classes A, B, and C ($P < .005$; Fig 1). Circulating CGRP was significantly increased in patients with cirrhosis ($P < .01$), and a significant direct correlation was found between CT and CGRP ($r = .29$, $P < .05$ and $r = .35$, $P < .05$ with exclusion of the outlying value; Fig 2). The arterial CT/CGRP concentration ratio was significantly higher in the patients versus the controls (0.24 *v* 0.14, $P < .05$), and it increased with the Child-Turcotte class ($P < .05$). HPLC analysis of plasma extracts shows heterogenous CT immunoreactivity with 2 major peaks (Fig 3). One of them elutes in fraction 40 with the same retention time as synthetic human CT and is therefore most likely identical to intact human CT. One other major peak elutes in fraction 36. The chromatograms do not show distinct differences between controls and cirrhotic patients.

No significant arteriovenous extraction of CT (or CGRP) was observed in the hepatosplanchnic bed, kidneys, lower extremities, or peripheral circulation (Table 2). However, a highly significant removal was identified in the lungs, as the systemic arterial concentration was significantly below that of the pulmonary artery ($P < .01$; Fig 4). The significant pulmonary extraction fraction, disposal rate, and clearance of CT were

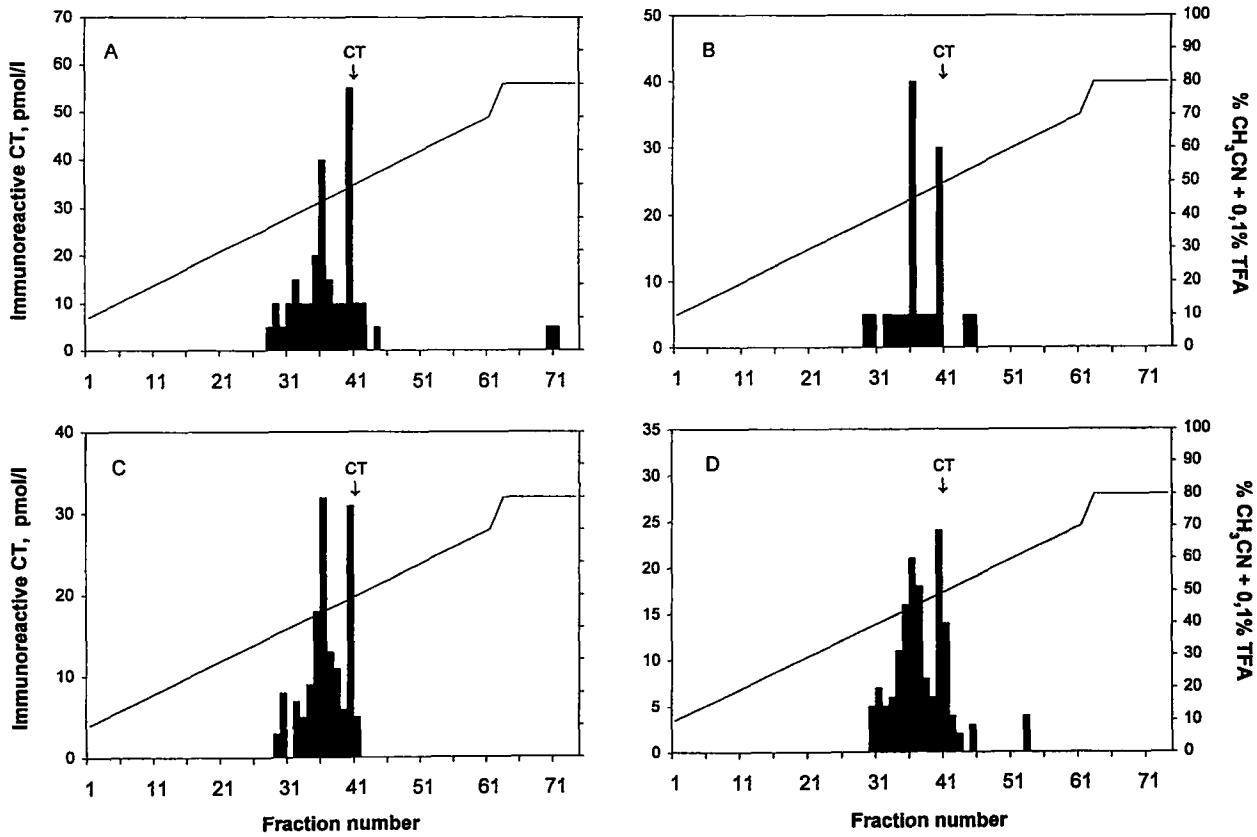

Fig 3. HPLC analyses for immunoreactive CT in plasma extracts. A and B, controls; C and D, patients with cirrhosis. The elution position of synthetic human CT is indicated by arrows.

Table 2. Plasma CT Concentration (pmol/L) in the Femoral Artery and Different Veins

Site	Hepatosplanchnic	Kidney	Peripheral/Iliac
Cirrhosis patients			
A	12.8 ± 0.9 (n = 33)	12.6 ± 0.6 (n = 32)	13.0 ± 0.6 (n = 27)
V	12.9 ± 0.7	12.08 ± 0.7	13.4 ± 0.6
P	NS	NS	NS
Controls			
A	6.3 ± 0.9 (n = 9)	7.1 ± 0.9 (n = 6)	7.1 ± 0.9 (n = 5)
V	5.9 ± 0.8	7.5 ± 0.8	7.0 ± 1.1
P	NS	NS	NS

NOTE. Results are the mean ± SEM.

Abbreviations: A, femoral artery; ns, not significant; V, hepatic, renal, and peripheral/iliac veins, respectively.

0.13 ± 0.03 ($P < .001$), 7.4 ± 2.4 pmol/min ($P < .01$), and 0.51 ± 0.12 L/min ($P < .001$), respectively, versus control values of 0.23, 6.9 pmol/min, and 0.87 L/min, respectively. The concentration of CGRP in the pulmonary and systemic artery was identical (61 ± 3 *v* 62 ± 4 pmol/L, NS).

The mean concentration of CT in mixed venous blood from vascular areas outside the head and neck was significantly below that of the pulmonary artery ($P < .005$), and this also applied to the CT/CGRP ratio ($P < .02$; Fig 5).

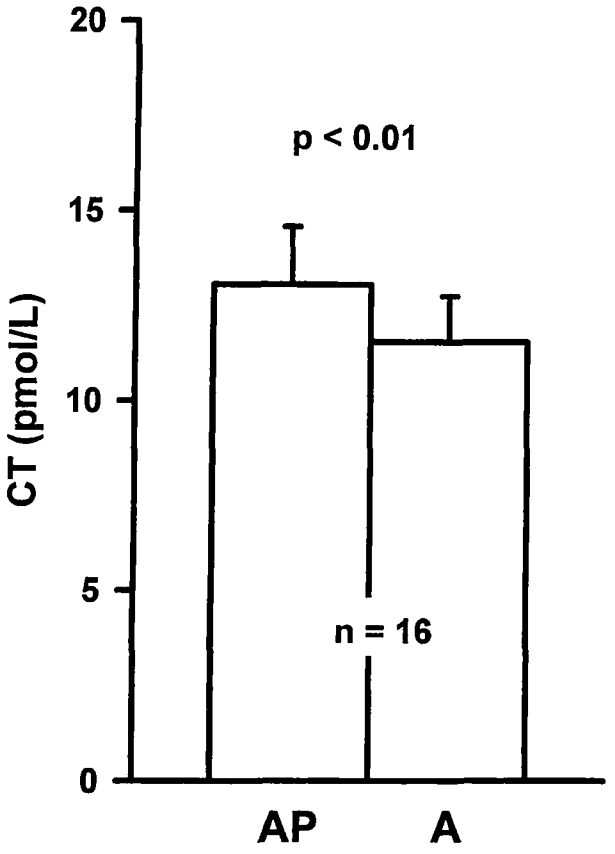

Fig 4. Significant difference in plasma concentration of CT in the pulmonary artery (AP) versus femoral artery (A) in 16 patients with cirrhosis.

Arterial oxygen saturation was decreased in patients with cirrhosis (95% ± 0.4% *v* 98% ± 0.6% in the controls, $P < .02$), but no correlation with circulating CT was present ($r = .03$, NS).

DISCUSSION

The present study shows that circulating CT is increased in patients with cirrhosis, with a direct relation to the severity of disease and to increased circulating CGRP. No significant extraction of CT immunoreactivity could be established across the hepatosplanchnic bed, kidneys, or peripheral vascular bed. However, a highly significant pulmonary disposal was observed.

Fujiyama et al[13] found increased plasma CT in hepatocellular carcinoma and other liver diseases, and Capra et al[14] reported increased CT in a small group of patients with alcoholic and posthepatitis cirrhosis. Thus, the present findings confirm elevated circulating CT in patients with cirrhosis and establish a relation to the severity of liver disease.

Increased circulating CT is hardly related to abnormalities of ionized calcium, as no signs of hypercalcemia or hypocalcemia are present in most patients with cirrhosis.[21] Parathyroid hormone (PTH) was not determined in the present study. However, in another group of similar patients with cirrhosis (n = 15), we found that circulating PTH was not significantly different from that in controls (median, 48 (11 to 111) *v* 58 (23 to 155) mg/mL in controls, n = 20, NS). Nor was the serum calcium level different (2.25 (2.05 to 2.42) *v* 2.13 (2.00 to 2.35) mmol/L, NS). Hence, it is not likely that the highly increased levels of CT are caused by any major change in calcium levels, but we did not measure urine calcium. It seems more likely that in addition to thyroid parafollicular cell production, increased expression of the calcitonin/α-CGRP gene is related to the circulatory derangement that is present in cirrhosis.[6,7,17,21] The direct correlation with the increased circulating CGRP supports a common gene overexpression.

A significant plasma clearance of CT was established in the pulmonary circulation. No disposal over major circulatory beds in other organs could be detected (Table 2), but pulmonary extraction was highly significant. Moreover, when the venous concentration of CT from the integral part of the inferior vena cava and drainage from areas outside the head/neck was considered, a step-increase in the mixed venous plasma concentration of CT (absolute as well as relative to CGRP) could be detected (Fig 5). Quantitative consideration of the spillover into the circulation of CT suggests that the lungs may be a major site for CT disposal, and overall differences in the disposal of CT and CGRP may contribute to the different CT/CGRP plasma ratios. Furthermore, the magnitude of the CT clearance rate corresponds largely to the blood flow inthe head and neck region, which suggests that this region is a source of circulating immunoreactive CT. However, this investigation does not clarify the organ-specific origin of CT, but it is interesting to speculate that in addition to thyroid production, increased circulating CT in cirrhosis may be due to a widespread overexpression of the calcitonin/α-CGRP gene. Codetermination of β-CGRP seems to be of minor quantitative importance.[5,8,11]

CT clearance and degradation is not elucidated in detail. Tracer-labeled CT shows early uptake in various organs (kidney

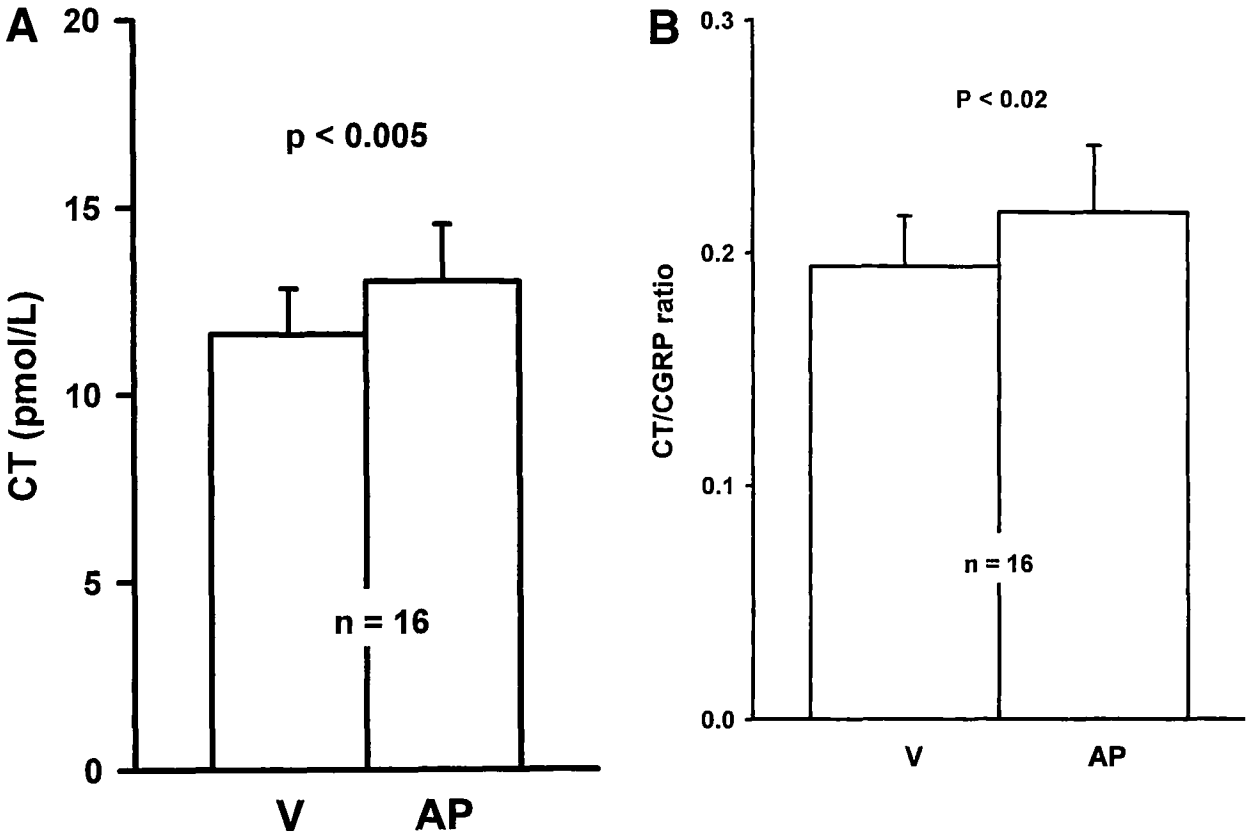

Fig 5. (A) Concentration of CT in mixed venous plasma (V) from the hepatic vein, renal vein, inferior vena cava/iliac vein, and peripheral vein (venous drainage excluding head and neck) and in plasma from the pulmonary artery (AP) in 16 patients with cirrhosis. (B) Concentration ratio of CT and CGRP in the same vessels as in A.

and gastrointestinal tract) but not in normal bone,[22] and there are indications of rapid breakdown in relation to tissue receptors.[22] Receptors for CT have been demonstrated in the lung tissue of humans,[23,24] pigs, and rats.[25,26] This may suggest that the pulmonary clearance is not solely due to nonspecific degradation, but may be caused by receptor-specific binding of CT in the pulmonary tissue. Unfortunately, since only a few controls in the present investigation had their pulmonary artery catheterized, a statistically significant difference versus normal pulmonary disposal cannot be evaluated from the present study. The present high clearance rate of CT is in keeping with previous reports in man.[27]

Chromatography did not show major differences between patients with cirrhosis and controls. A second peak (fraction 36) in both cirrhotics and controls has not been further identified, but may represent methionine-oxidized CT.

The consequences of increased circulating CT in patients with cirrhosis remain to be established. In 2 recent studies, parenteral CT was given to patients with primary biliary cirrhosis, 1 without beneficial effect[28] and the other with some beneficial effect on the associated metabolic bone disease, which, however, could be related to 1,25-dihydroxyvitamin D.[29] A pathophysiological role for CT in cirrhosis is further obscured by possible target-organ resistance after long-term exposure, at least in the skeleton.[30]

In conclusion, in addition to thyroid production, increased circulating CT in cirrhosis is most likely due to an overexpression of the calcitonin/α-CGRP gene, with relation to the severity of disease and speculatively to the accompanying pulmunary dysfunction. The pathophysiological and clinical consequences remain to be established.

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