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The bradykinin B_2 receptor antagonist Icatibant (HOE 140) corrects avid Na^+ retention in rats with CCl_4-induced liver cirrhosis: possible role of enhanced microvascular leakage

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Abstract

Avid Na^+ retention is a hallmark of liver cirrhosis. The aim of this study was to investigate whether and how bradykinin is involved in Na^+ retention in rats with CCl_4-induced liver cirrhosis. To this end the bradykinin B_2 receptor antagonist Icatibant (HOE 140) was used. On one hand, bradykinin has a renal natriuretic action. On the other hand, bradykinin is a potent mediator of both vasodilation and microvascular leakage. Both vascular mechanisms, which are reported for cirrhosis, could cause vascular underfilling and Na^+ retention by activating the renin–angiotensin–aldosterone system. Icatibant normalised Na^+ retention and reduced the hyperactivity of the renin–angiotensin–aldosterone system, suggesting a bradykinin-induced vascular disturbance. Icatibant had no significant effect on the mild hypotension which developed with CCl_4 treatment. However, there was indirect evidence for enhanced microvascular leakage that was strongly inhibited by Icatibant. Our experimental results demonstrate that bradykinin is a key mediator of Na^+ retention in liver cirrhosis and suggest that a bradykinin-induced increase in microvascular leakage is mainly responsible. © 1997 Elsevier Science B.V.

Keywords: CCl_4-induced liver cirrhosis; Hepatorenal syndrome; Na^+ retention; Microvascular leakage; Bradykinin B_2 receptor antagonist; HOE 140 (Icatibant)

1. Introduction

Na^+ retention is an early disturbance in liver cirrhosis. The mechanisms involved are not fully understood. Liver cirrhosis is not only associated with portal hypertension, which concerns the venous system, but also leads to excessive peripheral vasodilation, particularly in the mesenteric arterial bed, for which the mechanism is still enigmatic (Schrier et al., 1988). The ensuing underfilling of the vasculature is presumed to activate Na^+-retaining neuroendocrine systems, particularly the renin–angiotensin–aldosterone and the sympathetic nervous system. These neuroendocrine systems not only mediate Na^+ and water retention but also cause renal vasoconstriction, which finally leads to renal failure (the so-called hepatorenal syndrome, a mostly lethal pre-renal failure). This concept, referred to as the 'peripheral arterial vasodilation hypothesis' by Schrier et al. (1988), explains the early occurrence of Na^+ retention, decompensation with edema and ascites and the (pre)renal failure, the hepatorenal syndrome, as a continuum arising from the same vascular disturbance (Niederberger and Schrier, 1992). Interestingly enough, there is evidence for a further vascular disturbance: Enhanced microvascular leakage has been shown by several authors (Parving et al., 1977; Wallaert et al., 1992; Ohara et al., 1993) in patients and animals with liver cirrhosis, even in extra-splanchnic tissues. Enhanced filtration could directly lead to edema formation and contribute to ascites, although according to the 'peripheral vasodilation hypothesis' edema formation is considered a pure secondary event arising from Na^+ and water retention. Enhanced filtration would also cause vascular underfilling, activate Na^+-retaining hormonal systems and augment or even potentiate the effect of enhanced filtration on edema formation. Therefore, enhanced microvascular leakage may constitute an alternative explanation for Na^+ and water

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retention and renal failure in liver cirrhosis or may act in concert with peripheral vasodilation.

The vasoactive and inflammatory nonapeptide bradykinin is a potent mediator of both microvascular leakage and vasodilation (for review see Bhoola et al., 1992). Although these vascular actions of bradykinin would be expected to induce Na^+ retention, bradykinin can exert natriuretic effects by inhibiting the tubular reabsorption of Na^+ (Scicli and Carretero, 1986; Katori and Majima, 1996). Thus an increase as well as a decrease or no change in Na^+ excretion might result from an inhibition of bradykinin, should bradykinin be generated during the disease. Bradykinin is released from inactive plasma precursors, the kininogens, via kallikrein after activation by Hageman factor. Hageman factor is activated via diverse stimuli such as tissue damage, ischemia, heat, cold, low pH, non-physiological surfaces, collagen, urate, bacterial enzymes and lipopolysaccharides. Evidence has accumulated that the kallikrein–kinin system is activated in liver cirrhosis (Colman and Wong, 1977; Hayes et al., 1992; Cugno et al., 1995). Moreover, aprotinin, an inhibitor of kallikrein, has already shown a clear-cut natriuretic effect in patients with liver cirrhosis (MacGilchrist et al., 1994). Since aprotinin is a relatively non-specific serine protease inhibitor, its effect could not be attributed with sufficient certainty to an inhibition of the generation of bradykinin. The purpose of the current study was to investigate whether bradykinin plays a role in the Na^+ retention associated with CCl_4-induced liver cirrhosis in rats and to find out by which mechanisms bradykinin participates in the handling of Na^+ in liver cirrhosis. The selective and potent bradykinin B_2 receptor antagonist Icatibant (Hoe 140), which has already been shown to inhibit microvascular leakage in numerous disease models (Wirth et al., 1995), was used to block the action of bradykinin at the receptor level.

2. Materials and methods

2.1. Induction of liver cirrhosis by chronic administration of CCl_4

Female and male Wistar rats (breed Hattersheim, Hoechst, Frankfurt am Main, Germany) were used and liver cirrhosis was induced by oral administration of CCl_4 as described previously (Bickel et al., 1996).

Briefly, animals received CCl_4, 1 ml/kg p.o., dissolved in olive oil 1:1 (v/v) twice weekly on the basis of their individual body weight on the day of application of CCl_4. Animals were housed under standard conditions as described previously and were allowed standard rat chow Altromin 1321® and water ad libitum. Liver cirrhosis was verified by determining the collagen content of the liver, measured as hydroxyproline and by measuring cirrhosis-related serum variables (bilirubin, albumin and bile acids).

Ethical approval for the animal experiments was given by the Department of Veterinary Affairs of the Regierungspräsidium Darmstadt (11.06.1996).

2.2. Salidiuresis experiments: Experiment 1 and 2

2.2.1. Design of experiment 1: Liver cirrhosis

Five male (initial b.w. 171 ± 11 g) and five female rats (initial b.w. 154 ± 4 g) received 12 doses of CCl_4 within 6 consecutive weeks. Saluresis experiments were performed 10 days after the last CCl_4 administration. This procedure produces cirrhosis with no acute hepatocellular injury.

2.2.2. Design of experiment 2: Liver cirrhosis with superimposed acute hepatocellular damage

Ten female rats (initial b.w. 160 ± 7 g) received 22 doses of CCl_4 within 11 consecutive weeks. CCl_4 applications were maintained during the time of the salidiuresis experiments. In contrast to experiment 1, with this protocol acute hepatocellular injury is superimposed on an already existing cirrhosis (unpublished data).

Salidiuresis test in experiments 1–2: Rats were fasted for 16 h prior to the salidiuresis experiments but had free access to water till the onset of the salidiuresis experiment. Animals were placed in individual diuresis cages for 24 h and loaded with water (20 ml tap water per kg b.w. orally) at time point 0 h. Food and water were withdrawn.

Five (experiment 1) or seven days (experiment 2) after the control experiments with vehicle treatment the salidiuresis experiment was performed with the same animals treated with Icatibant. The bradykinin receptor antagonist was administered subcutaneously at time point 0 and 6 h at a dose of 0.3 mg/kg in a volume of 5 ml saline per kg b.w. A similar daily dose of 0.5 mg/kg given by minipumps has been shown to inhibit endogenous kinins for a period of 24 h (Wirth et al., 1995). The excretion of urine and electrolytes was measured in two collection periods, 0–5 and 6–24 h, after administration of the compound. The urine volumes of each rat during each observation period were collected separately. Na^+, K^+ and Cl^- were determined to assess the saluretic activity of the compound. An identical salidiuresis test with vehicle and Icatibant was performed for comparison in ten healthy age- and sex-related control rats.

In experiment 2, blood was taken at the end of each 24 h salidiuresis experiment for the determination of plasma aldosterone, plasma renin activity and angiotensin I levels. Thereafter the animals were killed to determine the degree of liver cirrhosis by measuring hydroxyproline.

2.3. Influence of Icatibant on systolic blood pressure, renal function and determination of the distribution volume of Iohexol (experiment 3)

36 male rats (initial b.w. 171 ± 11 g) were used. Six animals served as controls. 30 animals were treated with CCl_4. A total of 34 doses within 19 weeks was applied.

2.3.1. Systolic blood pressure

Systolic blood pressure was measured weekly by tail plethysmography under light ether anesthesia. In week 15 (day 102 after starting the experiment), when animals already had a lowered systolic pressure, Icatibant was given s.c. at a dose of 0.3 mg/kg three hours before the measurement of systolic blood pressure. Blood pressure measured at the same time the day before after injection of vehicle served as the control value.

2.3.2. Determination of the distribution volume and serum clearance of Iohexol

Under normal circumstances Iohexol, a non-ionic radiocontrast medium with a molecular weight of 821.14 g/mol, is appropriate to investigate extracellular water space and renal function simultaneously as its distribution volume corresponds to the extracellular water space in humans and other animals and excretion occurs nearly exclusively by glomerular filtration, at least in humans. Therefore, the serum clearance of Iohexol has been validated as an alternative method for the determination of glomerular filtration rate in humans (Krutzen et al., 1984). Our original intention was to determine extracellular water space and renal function in CCl_4-treated rats simultaneously with Iohexol because an expansion of the extracellular water space and a decrease in renal function could be present in CCl_4-induced liver cirrhosis. Binding to erythrocytes (Krutzen et al., 1990) or proteins (Krutzen et al., 1984) has been excluded. In humans the distribution phase of Iohexol (Krutzen et al., 1984) lasts for about 1 h.

Rats had free access to feed and water. 17 h before i.v. administration of Iohexol the first dose of Icatibant was given (0.3 mg/kg s.c. in a volume of 1 ml/kg). Injection of Icatibant was repeated 5 min before the i.v. injection of Iohexol. Blood (50 μl) was taken retroorbitally for the determination of Iohexol in serum at four time points between 60 and 240 min after i.v injection of Iohexol, which was given at a dose of 0.3 ml/kg, corresponding to 194 mg/kg b.w. Monitoring the elimination phase delivers the concentration at time zero (C_0), which allows calculation of the distribution volume (D_{vol}) by using the equation $D_{vol} = \text{Dose}/C_0$, and serum clearance by using the formula $S_{clear} = k_e(\text{elimination rate constant}) \times D_{vol}$.

2.3.3. Determination of creatinine clearance and salidiuresis

Creatinine clearance measurement after vehicle and Icatibant was performed in the 19th week on two consecutive days.

17 h after an administration of vehicle or Icatibant (0.3 mg/kg s.c. in a volume of 1 ml/kg) animals were given a 20 ml/kg oral (tap) water load and urine was collected for five hours for the determination of creatinine and electrolytes. 10 min before the oral water load, the injection of Icatibant was repeated. At the end of the 5 h collection period, a blood sample was taken for the determination of creatinine in serum. In contrast to experiment 1 and 2 animals had free access to feed and water before the collection period but feed and water were withdrawn during the collection period. Because of a possible influence of fasting on creatinine clearance this part of experiment 3 was performed in non-fasted animals. Autopsy was performed at the beginning of the 20th week.

2.4. Analytical methods

Na^+ and K^+ were measured by flame photometry (Flame photometer, Eppendorf, Hamburg) and Cl^- argentometrically by potentiometrical end point determination (Chloridmeter Eppendorf, Hamburg). The analytical results were used to calculate the excretion of urine (ml/kg b.w.) and electrolytes (mmol/kg b.w.). Creatinine in plasma and urine and renin, angiotensin I and aldosterone in plasma were measured as described previously (Hropot et al., 1994). The hydroxyproline content of the liver was determined according to Palmerini et al. (1985). Bilirubin, albumin and bile acids were measured as described earlier (Bickel et al., 1991).

Table 1
Effect of Icatibant on urinary Na^+ and electrolyte excretion in rats with CCl_4-induced liver cirrhosis (experiment 1)

	Collection period 0–5 h				Collection period 6–24 h			
	Vehicle		Icatibant		Vehicle		Icatibant	
	Healthy rats	CCl_4-treated rats	Healthy rats	CCl_4-treated rats	Healthy rats	CCl_4-treated rats	Healthy rats	CCl_4-treated rats
Urine (ml/kg)	32.35 ± 5.92	21.71 ± 3.42 [f]	29.17 ± 5.19	25.38 ± 4.70	28.95 ± 5.22	28.58 ± 8.07	23.78 ± 7.05	29.58 ± 8.53
Na^+ (mmol/kg)	0.66 ± 0.32	0.35 ± 0.20 [d]	1.02 ± 0.50	0.53 ± 0.22 [d]	3.13 ± 1.08	1.69 ± 0.70 [e]	2.73 ± 0.77	3.81 ± 1.07 [c,d]
K^+ (mmol/kg)	0.75 ± 0.24	0.66 ± 0.32	0.79 ± 0.33	0.51 ± 0.27	3.31 ± 0.65	3.30 ± 0.75	3.04 ± 1.00	2.20 ± 0.67 [b,d]
Cl^- (mmol/kg)	0.87 ± 0.34	0.48 ± 0.33 [d]	1.19 ± 0.55	0.39 ± 0.21 [f]	3.32 ± 0.84	1.57 ± 1.11 [f]	3.01 ± 0.41	3.01 ± 0.86 [a]

Healthy rats ($n = 10$), CCl_4-treated rats ($n = 10$).
[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$; comparison of vehicle with Icatibant in CCl_4-treated rats.
[d] $P < 0.05$.
[e] $P < 0.01$.
[f] $P < 0.001$ comparison of healthy rats with CCl_4-treated rats for either treatment with the vehicle or Icatibant.

Table 2
Effect of Icatibant on water and Na^+ retention (6–24 h collection period) and activity of the renin–angiotensin–aldosterone system in rats with CCl_4-induced liver cirrhosis and superimposed acute hepatocellular injury (experiment 2)

	Vehicle		Icatibant	
	Healthy rats	CCl_4-treated rats	Healthy rats	CCl_4-treated rats
Urine (ml/kg)	28.95 ± 5.22	15.78 ± 6.39 [e]	23.78 ± 7.05	27.78 ± 15.11 [b]
Na^+ (mmol/kg)	3.13 ± 1.08	2.03 ± 0.47 [c]	2.73 ± 0.77	3.25 ± 1.40 [a]
K^+ (mmol/kg)	3.31 ± 0.65	2.37 ± 1.12 [c]	3.04 ± 1.00	2.76 ± 1.24
Cl^- (mmol/kg)	3.32 ± 0.84	1.77 ± 0.34 [e]	3.01 ± 0.41	2.71 ± 1.46
Serum aldosterone (pg/ml)	252.10 ± 65.00	482.04 ± 117.6 [e]	210.90 ± 62.90	320.73 ± 114.93 [a]
Angiotensin I (ng/ml)	0.55 ± 0.13	0.94 ± 0.34 [b]	0.51 ± 0.15	0.65 ± 0.13 [a]
Plasma renin activity (ng Ang.I/ml per 10 min)	0.96 ± 0.43	2.09 ± 1.40	0.72 ± 0.42	1.02 ± 0.64 [a]

Healthy rats ($n = 10$), CCl_4-treated rats ($n = 8–9$).
[a] $P < 0.05$.
[b] $P < 0.01$. Wilcoxon signed rank test. Comparison of vehicle with Icatibant in CCl_4-treated rats.
[c] $P < 0.05$.
[d] $P < 0.01$.
[e] $P < 0.001$. Comparison of healthy rats with CCl_4-treated rats for either the vehicle or treatment with Icatibant.

Iohexol in serum was determined by high-performance liquid chromatography (HPLC) as described previously (Geisen et al., 1994).

2.5. Compounds

Iohexol (Omnipaque®) was purchased from Schering, Germany. Icatibant (HOE 140) was made by the Department of Pharma Synthesis at Hoechst AG.

2.6. Statistical analysis

Results given in Tables 1–6 are means ± standard deviations (S.D.). Testing for significant differences was performed by non-parametric Mann–Whitney rank sum test, if not otherwise mentioned. The respective level of significance for each parameter is indicated in the results.

Serum concentration/time-courses (Iohexol kinetics) were analyzed by simple analysis of linear regression after log-transformation of the individual serum concentrations. C_0 for t_0, distribution volume D_{vol} and $t_{1/2}$, serum half-life, were calculated from the corresponding regression lines.

3. Results

Rats with CCl_4-induced liver cirrhosis showed a significantly lower Na^+ and Cl^- excretion when compared with healthy controls in each of the three experiments. Treatment with Icatibant of rats with liver cirrhosis led to a highly significant natriuretic effect (Tables 1, 2 and 5), particularly in the second (6–24 h) collection period (Table 1) while it had no effect on salidiuresis in healthy animals. The saluretic effect of Icatibant was present in male and female rats of experiments 1–3 with no apparent gender difference. When healthy animals were loaded with 20 ml/kg of saline instead of water, Icatibant did not alter salidiuresis either (data not shown). Thus, Icatibant led to a total reversal of the pathological Na^+ retention. Cl^- excretion also rose significantly ($P < 0.05$) while K^+ excretion fell significantly ($P < 0.01$) and urine excretion was unchanged (Table 1). With a cumulated urine excretion of 20 to 30 ml in the first 5 h-collection period the animals had excreted the volume they were loaded with at time zero (20 ml), before water and food was withdrawn. The data for excretion during the control periods of healthy rats corresponded to our historical control values obtained over many years.

Diminution of water excretion was only found in experiment 2 (Table 2) where CCl_4 treatment was given as close as 4 days before each salidiuresis study period. This procedure leads to necrosis of remaining, functionally intact hepatocytes and constitutes cirrhosis on which acute hepatocellular injury is superimposed. Both water and Na^+ excretion became normal after treatment with Icatibant (Table 2).

The activity of the renin–angiotensin–aldosterone system was increased in CCl_4-treated rats compared with healthy controls and was reduced towards normal values

Table 3
Body weight and liver cirrhosis related variables at autopsy in female rats (experiment 2) after 22 CCl_4 applications

Groups	n	Body weight (g)	Liver weight (g)	Hydroxyproline (mg/g)	Bilirubin (μM)	Bile acids (μM)	Mortality (%)
Healthy rats	10	282 ± 9	9.46 ± 0.58	171 ± 16	1.97 ± 0.38	14.8 ± 7.71	0
CCl_4-treated rats	9	260 ± 15 [b]	9.48 ± 1.93	856 ± 356 [b]	3.61 ± 4.66	34.6 ± 38.43 [a]	10

[a] $P < 0.05$.
[b] $P < 0.01$. Comparison of CCl_4-treated rats with healthy rats.

Table 4
Distribution volume of Iohexol in CCl_4-treated rats after vehicle and Icatibant compared with healthy rats (experiment 3)

	Vehicle		Icatibant
	Healthy rats	CCl_4-treated rats	CCl_4-treated rats
C_0 (μg/ml)	909 ± 845	218 ± 95	413 ± 119 [b]
D_{vol} (ml/kg)	327 ± 168	1068 ± 527 [c]	514 ± 175 [b]
$T_{1/2}$ (min)	25.4 ± 1.27	31.9 ± 4.25	28.9 ± 5.12 [a]
S_{clear} (ml/min per kg)	8.84 ± 4.50	22.7 ± 8.87 [d]	12.5 ± 3.96 [b]

Healthy rats ($n = 5$), CCl_4-treated rats ($n = 20$). C_0 denotes Iohexol concentration at t_0, calculated from the serum concentration/time-course. $T_{1/2}$ is the half-life of Iohexol from the elimination phase. Coefficients of the regression curves for serum concentrations of Iohexol were 0.9814 for vehicle and 0.9982 for Icatibant. D_{vol} denotes the distribution volume and S_{clear}, serum clearance of Iohexol.
[a] $P < 0.05$.
[b] $P < 0.01$: Comparison of vehicle with Icatibant in CCl_4-treated rats.
[c] $P < 0.05$.
[d] $P < 0.01$: Comparison of healthy rats with CCl_4-treated rats.

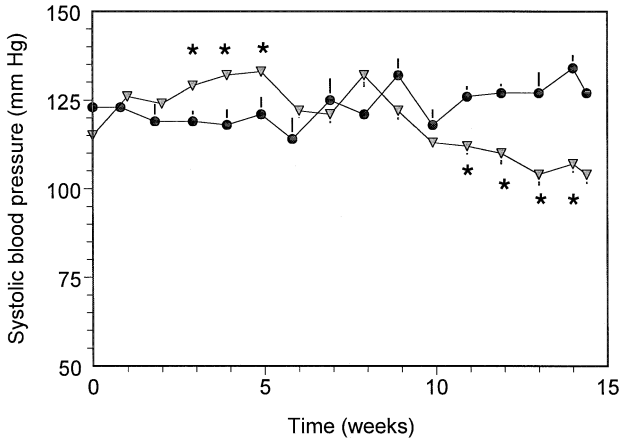

Fig. 1. Changes in systolic blood pressure during chronic treatment with CCl_4. CCl_4-treated rats (shaded triangles, $n = 20$–30). Healthy rats (closed circles, $n = 5$). Mean ± S.E.M. * $P < 0.01$ compared with healthy rats.

by Icatibant (Table 2) while Icatibant had no effect in healthy animals.

The saluretic effect of Icatibant occurred in fasted (experiment 1–2) and non-fasted (experiment 3) animals. It was also present under conditions of (acute) water loading (experiment 3) and thirst (in the 6– to 24-h collection period of experiment 1 and 2, where the water load had already been excreted before the beginning of this second collection period).

A highly significant correlation ($r = 0.779$, $P < 0.01$) was found between Icatibant-stimulated Na^+ excretion and the degree of liver cirrhosis (hydroxyproline). Cirrhosis was well documented in these animals (Table 3).

In experiment 3 mortality was 33% after 20 weeks of CCl_4 treatment with a total of 34 CCl_4 applications. Liver cirrhosis was documented at autopsy by determination of bilirubin, bile acid and albumin concentrations in blood and the hydroxyproline content of the liver (Table 6).

Systolic blood pressure showed a small and transient but statistically significant increase during week 4–7, then returned to the values of healthy animals and thereafter decreased moderately but statistically significantly after 10 weeks of CCl_4 treatment (Fig. 1). At day 102 Icatibant was administered. There was a small and nonsignificant increase in systolic blood pressure by 3 mmHg (109 ± 14 mmHg, mean ± S.D.) compared with that measured after vehicle injection the day before (106 ± 15 mmHg).

The serum kinetics of Iohexol were originally going to be used to determine the extracellular water space in CCl_4-treated rats. However, in CCl_4-treated rats, the distribution volume of Iohexol, calculated from C_0 and dose, was surprisingly increased (1068 ± 527 ml/kg b.w.), close to whole body volume, and was much higher than the distribution volume of 327 ml/kg of healthy rats (Table 4). The value found for healthy rats is in the range of values found with inulin (340 ml/kg), a marker for the extracellular water space (Sugimoto et al., 1993) and the radiocontrast agent Iodixanol (Heglund et al., 1995).

Icatibant reduced the distribution volume by 52% in CCl_4-treated rats. The inhibition of the increase in distribution volume amounted to about 75% of control.

The elimination half-life was slightly increased in CCl_4-treated rats compared with healthy controls and showed a small but statistically significant decrease by 9%

Table 5
Creatinine clearance and salidiuresis in rats with CCl_4-induced liver cirrhosis (experiment 3)

	Vehicle		Icatibant
	healthy rats	CCl_4-treated rats	CCl_4-treated rats
Urine (ml/kg per h)	3.15 ± 1.17 (100)	3.10 ± 0.96 (98)	2.65 ± 1.00 (84)
Na^+ (μmol/kg per h)	74.4 ± 41.8 (100)	52.3 ± 28.1 (70)	71.4 ± 26.2 [a] (96)
Cl^- (μmol/kg per h)	162 ± 95.5 (100)	82.9 ± 41.3 (51)	130 ± 51.3 [b] (80)
K^+ (μmol/kg per h)	258 ± 103	187 ± 69.9	192 ± 47.4
Serum creatinine (μmol/l)	42.8 ± 3.03	40.3 ± 7.64	42.4 ± 6.78
GFR (ml/min per kg)	4.87 ± 0.92 (100)	3.92 ± 1.35 (80)	4.41 ± 1.41 (91)

Healthy rats ($n = 5$), CCl_4-treated rats ($n = 20$). Values in parentheses (%) are absolute percentages of values compared to healthy vehicle-treated rats (= 100%).
[a] $P < 0.05$.
[b] $P < 0.01$ Comparison of vehicle with Icatibant in CCl_4-treated rats.

Table 6
Body weight, liver weight and liver cirrhosis related variables at autopsy after 34 CCl_4 applications (experiment 3)

Groups	*n*	Body weight (g)	Liver weight (g)	Hydroxyproline (mg/g)	Bilirubin (μM)	Bile acids (μM)	Albumin (g/l)	Mortality (%)
Healthy rats	5	476 ± 18	13.6 ± 0.71	233 ± 27	2.16 ± 0.33	12.4 ± 12.2	23.1 ± 0.79	0
CCl_4-treated rats	20	412 ± 46 [b]	14.2 ± 3.11	1223 ± 503 [b]	3.97 ± 3.57 [a]	68.5 ± 58.9 [b]	19.6 ± 3.92 [b]	33

[a] $P < 0.05$.
[b] $P < 0.01$. Comparison of CCl_4-treated rats with healthy rats.

after Icatibant (Table 4). The serum clearance of Iohexol, calculated from the product of the elimination rate constant k_e and distribution volume by the formula ($S_{clear} = k_e \times D_{vol}$), was 8.9 ml/min per kg, exactly as reported for healthy Wistar rats (Geisen et al., 1994) and was about twice that for creatinine (4.87 ml/min per kg). Obviously in rats another elimination mechanism for Iohexol apart from renal filtration must be involved, in contrast to the situation in humans.

The increase in the serum clearance of Iohexol in CCl_4-treated rats by 168% was entirely due to the large increase in its distribution volume and was strongly reduced by Icatibant, being only 38% higher than in healthy animals. The serum clearance, elimination half-life and distribution volume of Iohexol in healthy rats are in agreement with values found for the radiocontrast agent Iodixanol (Heglund et al., 1995).

Creatinine clearance showed no statistically significant changes between the groups (Table 5). Values for controls are in agreement with values for historical controls and with values reported in the literature (Hropot et al., 1994). It was only slightly decreased in CCl_4-treated rats compared with healthy animals. Creatinine clearance increased slightly by 13% (nonsignificant) with Icatibant in CCl_4-treated rats, in line with the statistically significant diminution of the half-life of Iohexol, which is mainly excreted by the kidney. Na^+ excretion became normal, reaching 96% of that of the healthy rats, and Cl^- excretion increased also significantly (Table 5). Finally, body weight, liver weight and liver cirrhosis related variables at autopsy after 34 CCl_4 applications (experiment 3) are shown in Table 6.

4. Discussion

Na^+ retention, a hallmark of liver cirrhosis in humans and other animals, was present in our rats with liver cirrhosis and was totally reversed by Icatibant. When an acute hepatocellular injury was superimposed on liver cirrhosis, water excretion was also diminished and normalised by Icatibant. Creatinine was only slightly decreased in CCl_4-treated rats compared with healthy animals, suggesting that Na^+ and water retention are due to an enhanced reabsorption rather than to a decrease in glomerular filtration rate. After Icatibant creatinine clearance improved slightly (not significant), in line with the significant diminution of the half-life of Iohexol, which is mainly excreted renally, suggesting an improvement of the renal function. In cirrhotic rats GFR is mostly found to be unchanged (Wensing et al., 1995), but decreases have also been reported (Brunkhorst et al., 1993). No changes in GFR have been reported for Icatibant in healthy rats (Madeddu et al., 1992).

The highly significant correlation between the natriuretic effect of Icatibant and the degree of liver damage suggests that the importance of bradykinin in Na^+ retention increases with the severity of the disease.

These data suggest that bradykinin is involved as a key mediator of Na^+ retention in liver cirrhosis, most likely via a vascular mechanism for the following reasons: First, the enhanced activity of the renin–angiotensin–aldosterone system in CCl_4-treated rats decreased after Icatibant while a rise would be expected for a salidiuretic effect as found with Icatibant. A direct stimulation of aldosterone secretion by bradykinin in rats has been excluded (Rudichenko et al., 1993). In experiment 1, where water excretion was unchanged, K^+ excretion decreased, suggesting a fading influence of aldosterone. Second, the effect was delayed relative to the time profile generally seen for salidiuretic agents, in line with the assumption that downregulation of hormonal systems takes time. Third, Icatibant has been shown to have anti-natriuretic effects in certain hypertension models (Madeddu et al., 1992; Majima et al., 1993), which is explained by the inhibition of the natriuretic effect of bradykinin on the distal tubules of the kidney (Scicli and Carretero, 1986; Katori and Majima, 1996). The decreased urinary kallikrein excretion found in patients with cirrhosis (Hattori et al., 1984) supports the idea that an impaired renal kallikrein–kinin system could contribute to an increased sensitivity towards the Na^+-retaining action of aldosterone.

As Icatibant is clearly natriuretic in cirrhotic rats, a possible anti-natriuretic effect of Icatibant in the renal tubules must have been largely overridden by an improvement of the vascular situation. Interestingly, the potent vasoconstrictor endothelin showed natriuretic effects when given to rats with CCl_4-induced liver cirrhosis (Claria et al., 1991), suggesting that hypotension and underfilling of the vasculature play an important role. Rats with CCl_4-induced liver cirrhosis show renal and hemodynamic fea-

tures that closely resemble those of human liver cirrhosis, including Na^+ retention and a decreased peripheral resistance (Fernandez-Munoz et al., 1985).

Both excessive vasodilation as well as an increase in microvascular leakage, which are well documented in liver cirrhosis, can lead to underfilling of the vasculature and activate Na^+-retaining hormonal systems. In our attempt to determine the contribution of the two possible vascular mechanisms, we measured systolic blood pressure over the whole experimental period. Systolic blood pressure was significantly lower in cirrhotic rats after 10 weeks of CCl_4 treatment, suggesting the presence of peripheral vasodilation, but did not significantly increase after Icatibant, thus excluding a major, acute role for bradykinin in the moderately hypotensive state. On the basis of these data we cannot fully exclude a partial reversal of a presumed excessive vasodilation because cardiac output is usually very much enhanced in rats with liver cirrhosis (Fernandez-Munoz et al., 1985) and might have been the first variable to benefit from a possible correction of excessive vasodilation.

While our data excluded a major contribution of bradykinin to the modest hypotension found in these cirrhotic animals, we were surprised by the marked increase in the distribution volume of Iohexol in CCl_4-treated rats. This increase could not be explained by an increased extracellular water space given the magnitude of the increase (close to whole body volume) and the strong inhibitory effect Icatibant exerted (minus 554 ml/kg) in only 24 h of treatment. A unifying explanation for the two observations, namely the marked increase in the distribution volume of Iohexol and Na^+ retention in liver cirrhosis, that takes into account the fact that both are mediated by bradykinin, as indicated by the strong effects of Icatibant and, additionally, the postulated vascular action of bradykinin, is an enhancement of microvascular leakage by bradykinin. Enhanced microvascular leakage could be shown by several authors in patients with liver cirrhosis and in animal models (André et al., 1976; Wallaert et al., 1992; Ohara et al., 1993) and an enhanced rate of escape of labelled albumin from blood has also been observed (Parving et al., 1977).

With regard to the unexpected distribution of Iohexol, it is important to mention that the reported leakage of albumin would inevitably lead to an increase in its distribution volume, although the magnitude of the changes would be much smaller than those for Iohexol, for which filtration would be much higher due to its small size. This would be expected to lead to a more complex pharmacokinetic pattern. Indeed, a second elimination phase with a long half-life of about 12.6 h has been reported for healthy men, indicating the existence of a further compartment (Edelson et al., 1984).

Based on our indirect evidence for an enhanced bradykinin-induced microvascular leakage the edema that occurs in liver cirrhosis can be regarded as a primary vascular process reinforced by secondary Na^+ and water retention, portal hypertension and hypoalbuminemia.

Our results showing that bradykinin is deeply involved in Na^+ retention in liver cirrhosis support the results of a clinical study with aprotinin in patients with liver cirrhosis (MacGilchrist et al., 1994). During infusion of relatively high doses of aprotinin, which are necessary to inhibit plasma kallikrein (aprotinin is a strong inhibitor of tissue kallikrein, but a less potent inhibitor of plasma kallikrein), patients showed a doubling of urinary Na^+ excretion and an increase in renal plasma flow and glomerular filtration rate. This was accompanied by a 10% increase in systemic vascular resistance, a decrease in renal vascular resistance by about 29% and a small rise in mean arterial pressure by 3 mmHg. The activity of the renin–angiotensin–aldosterone system strongly decreased, in line with our data. The improvement of renal function was explained by an improvement of peripheral vasodilation. Since there is an obvious discrepancy between the impressive renal improvement and the modest hemodynamic effect achieved with aprotinin, bradykinin-induced microvascular leakage may have been involved. However, since aprotinin inhibits a wide range of enzymes, the improvement could not be attributed with certainty to inhibition of bradykinin generation. These clinical data with aprotinin and our data with the selective bradykinin B_2 receptor antagonist are mutually confirmatory.

Activation of the kalllikrein–kinin system occurs in liver cirrhosis (Colman and Wong, 1977, Hayes et al., 1992; Cugno et al., 1995) by the Hageman factor which is activated by lipopolysaccharides (Pettinger and Young, 1970; Kaplan et al., 1977). Endotoxinemia has been found in liver cirrhosis (Fukui et al., 1991; Lin et al., 1995). It is assumed that lipopolysaccarides are absorbed from the gut, but are not sufficiently cleared when liver function is severely impaired. Moreover, a recent paper shows that the rat liver has a large capacity to degrade bradykinin, as does the lung (Griswold et al., 1996). Hence, insufficient clearance of both endotoxins and bradykinin by the diseased liver and a considerable collateral venous blood flow that by-passes the liver may lead to an enhanced generation and decreased metabolism of bradykinin.

Kinin receptors are subdivided into two different subtypes (Regoli et al., 1977). Most of the demonstrated physiological and pathophysiological actions of kinins are mediated by the bradykinin B_2 receptor, to which bradykinin binds as the preferred ligand. However, evidence is accumulating that B_1 receptors, which bind the bradykinin metabolite $DesArg^9$-bradykinin with high affinity, become more important following some types of tissue injury and after stimulation with lipopolysaccharides, which results in their selective upregulation (Marceau, 1995). B_1 receptors have similar actions as B_2 receptors, but most of the known actions of kinins are mediated by the B_2 receptor. The magnitude of the effect of the selective bradykinin B_2 receptor antagonist Icatibant on salidiuresis,

renin–angiotensin–aldosterone system activity and distribution volume of Iohexol suggests that most of these pathological changes are mediated by the bradykinin B_2 receptor, leaving little room for a major role of the B_1 receptor. It could be argued that a B_1 effect is responsible for the hypotension seen in our CCl_4-treated rats, which would explain the lack of a clear effect of Icatibant on systolic blood pressure. However, the hemodynamic improvement with aprotinin was also relatively weak, although aprotinin inhibits the generation of bradykinin, which is the precursor for Des-Arg^9-bradykinin, the active ligand at B_1 receptors. This excludes an important role for a B_1-receptor mediated effect, at least in patients with liver cirrhosis. Thus, while we demonstrated the involvement of bradykinin in Na^+ retention and presented indirect evidence that a bradykinin-induced enhancement of microvascular leakage is involved, the mediator(s) and mechanism(s) responsible for hypotension remain to be elucidated.

In conclusion, our experimental results obtained with the selective bradykinin B_2 receptor antagonist Icatibant in rats with CCl_4-induced liver cirrhosis show that bradykinin is a key mediator of the pathological Na^+ retention which we presume is the consequence of vascular underfilling resulting from a bradykinin-induced enhancement of microvascular leakage.

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