Endocannabinoids acting at vascular CB_1 receptors mediate the vasodilated state in advanced liver cirrhosis

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Advanced cirrhosis is associated with generalized vasodilation of unknown origin, which contributes to mortality. Cirrhotic patients are endotoxemic, and activation of vascular cannabinoid CB1 receptors has been implicated in endotoxin-induced hypotension. Here we show that rats with biliary cirrhosis have low blood pressure, which is elevated by the CB1 receptor antagonist SR141716A. The low blood pressure of rats with CCl_4-induced cirrhosis was similarly reversed by SR141716A, which also reduced the elevated mesenteric blood flow and portal pressure. Monocytes from cirrhotic but not control patients or rats elicited SR141716A-sensitive hypotension in normal recipient rats and showed significantly elevated levels of anandamide. Compared with non-cirrhotic controls, in cirrhotic human livers there was a three-fold increase in CB1 receptors on isolated vascular endothelial cells. These results implicate anandamide and vascular CB1 receptors in the vasodilated state in advanced cirrhosis and indicate a novel approach for its management.

Cirrhosis of the liver, which usually develops as a long-term consequence of chronic alcohol abuse or viral hepatitis, is a major cause of morbidity and mortality worldwide. The principal pathophysiologic feature of cirrhosis is an increase in portal pressure initiated by an increase in outflow resistance in the portal circulation, which is caused by extensive scarring of the liver. However, advanced cirrhosis is also associated with mesenteric arteriolar vasodilation[1], which contributes to portal hypertension and variceal hemorrhage by increasing portal inflow. Vasodilation also occurs in the systemic circulation, resulting in relatively low blood pressure[2,3], a decrease in effective circulating blood volume, sodium and water retention and, ultimately, ascites. Because the probability of survival is reduced after the appearance of ascites[4] and variceal hemorrhage is a life-threatening complication, the vasodilated state in cirrhosis contributes to mortality. Nitrates and β-blockers used to reduce portal hypertension owe their effectiveness, at least in part, to countering excessive splanchnic vasodilation[5]. However, the cirrhotic vasculature is highly resistant to conventional vasopressors[5,6], and attempts to correct the hyperdynamic circulation in cirrhosis by antagonism of putative endogenous vasodilator mediators have been unsuccessful so far[7].

It is well established that in cirrhosis, the bacterial endotoxin lipopolysaccharide (LPS) generated by normal intestinal flora gains access to the systemic circulation as a result of its impaired hepatic elimination and the increase in porto-systemic shunting[3,8]. Endotoxin is known to elicit hemodynamic changes similar to those present in cirrhosis, and there is a correlation between plasma endotoxin levels and the severity of hemodynamic changes in cirrhotic individuals[9,10]. Endotoxin might contribute to the vasodilated state in cirrhosis by increasing the production of nitric oxide by upregulating inducible nitric-oxide synthase (iNOS) activity[11]. However, there is evidence both for[12,13] and against[14,15] an increase in iNOS activity in cirrhosis, and there are similar conflicting reports on changes in endothelial cell (ec)NOS activity[16–18]. We have shown that in a rat model of endotoxic shock, the hypotension induced by LPS treatment is prevented by pretreatment with an antagonist of cannabinoid CB1 receptors[19]. Moreover, rat circulating macrophages and platelets exposed to LPS elicit CB1 receptor-mediated hypotension when injected into normal recipient rats, and these cells were found to contain the endogenous cannabinoids anandamide and 2-arachidonoyl glycerol[19]. These findings raise the possibility that endogenous cannabinoids and their receptors might be involved in mediating the vasodilation and low blood pressure of advanced liver cirrhosis. Here we tested this hypothesis using two different animal models of liver cirrhosis and circulating blood cells as well as vascular endothelial cells from cirrhotic patients. The results support the hypothesis that activation of vascular cannabinoid CB1 receptors by endogenous cannabinoids is involved in the vasodilated state associated with cirrhosis.

Endocannabinoid-mediated vasodilation in cirrhotic rats

Using an automated tail-cuff device, we monitored daily the systolic blood pressure of conscious CCl_4-treated rats and their controls. Within 10–12 weeks, CCl_4-treated rats became hypotensive

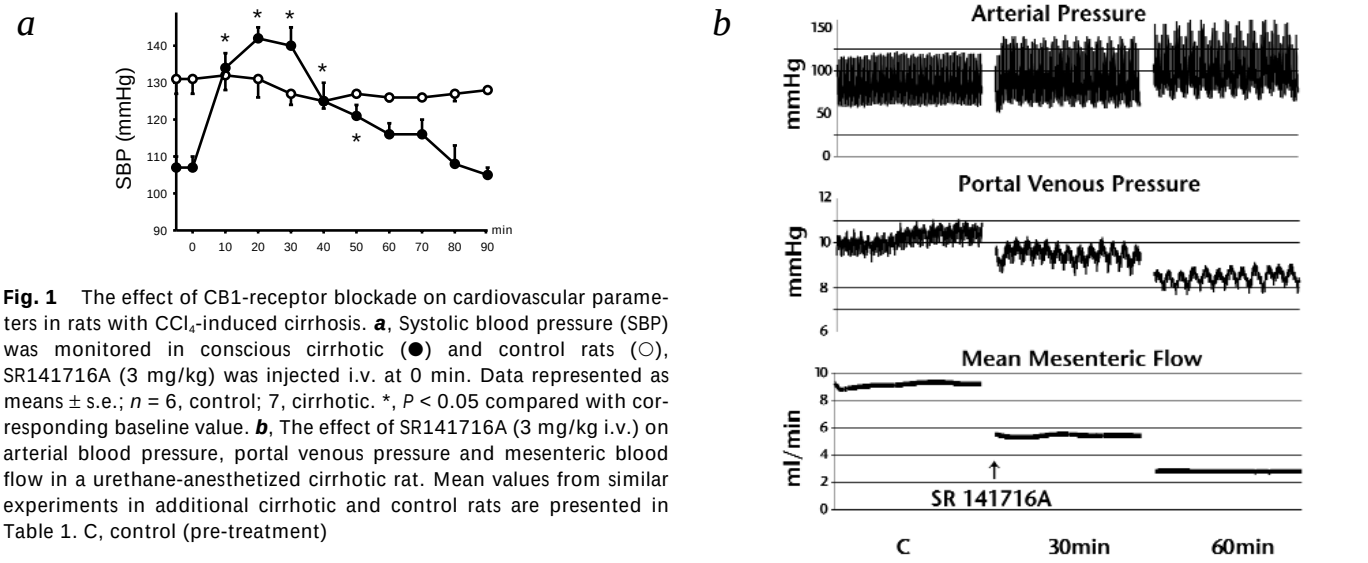

Fig. 1 The effect of CB1-receptor blockade on cardiovascular parameters in rats with CCl_4-induced cirrhosis. ***a***, Systolic blood pressure (SBP) was monitored in conscious cirrhotic (●) and control rats (○), SR141716A (3 mg/kg) was injected i.v. at 0 min. Data represented as means ± s.e.; $n = 6$, control; 7, cirrhotic. *, $P < 0.05$ compared with corresponding baseline value. ***b***, The effect of SR141716A (3 mg/kg i.v.) on arterial blood pressure, portal venous pressure and mesenteric blood flow in a urethane-anesthetized cirrhotic rat. Mean values from similar experiments in additional cirrhotic and control rats are presented in Table 1. C, control (pre-treatment)

relative to their controls, with a systolic blood pressure of 107 ± 3 mmHg versus 130 ± 7 mmHg in controls ($P < 0.005$). A single intravenous (i.v.) injection in conscious control rats with 3 mg/kg of SR141716A, a selective CB1 cannabinoid receptor antagonist[20], caused no significant change in systolic blood pressure (Fig. 1*a*). In contrast, the same dose of SR141716A in the CCl_4-treated rats caused a substantial increase in systolic blood pressure that lasted about an hour (Fig. 1*a*), and even a lower dose of SR141716 (0.5 mg/kg) caused a marked pressor response (from 107 ± 5 to 138 ± 1 mmHg; $P < 0.05$; $n = 4$; data not shown).

Within 6–10 days following the appearance of hypotension, ascites appeared in the CCl_4-treated animals, as detected by palpation and sudden weight gain, and its measured volume after laparotomy was 16 ± 5 ml. As illustrated by tracings from a typical experiment, i.v. injection of 3 mg/kg SR141716A in these anesthetized animals caused a gradually developing, long-lasting pressor response associated with a decrease in mesenteric arterial blood flow and portal venous pressure (Fig. 1*b*). These changes were statistically significant in a group of six cirrhotic animals (Table 1). In five pair-fed control animals, basal blood pressure was higher and basal mesenteric blood flow and portal venous pressure were lower than in the cirrhotic rats, and SR141716A did not significantly alter any of the three parameters (Table 1). Post-mortem microscopic examination of the liver by trichrome staining confirmed the presence of micronodular cirrhosis in all of the CCl_4-treated rats.

Table 1 Cardiovascular effects of SR141716A in cirrhotic and control rats.

	Control		Cirrhotic	
	Basal	Δ due to SR	Basal	Δ due to SR
Mean arterial pressure (mmHg)	109.5 ± 8.0	–7.2 ± 5.6	83.1 ± 4.5[a]	+20.1 ± 3.0[d]
Mesenteric blood flow (ml/min)	4.9 ± 0.8	0 ± 0.4	12.3 ± 2.2[b]	–3.7 ± 1.2[c]
Portal venous pressure (mmHg)	8.5 ± 1.1	+0.3 ± 0.4	12.1 ± 1.1[a]	–1.5 ± 0.4[c]

Cardiovascular parameters were monitored in 6 urethane-anesthetized control rats and 6 rats with CCl_4-induced cirrhosis, as described in Methods. Δ values represent the maximal SR141716A-induced changes, measured 30–60 min following the i.v. injection of 3 mg/kg SR141716A. [a], $P < 0.05$; [b], $P < 0.005$; indicating significant difference from corresponding basal values in control rats. [c], $P < 0.05$; [d], $P < 0.005$; indicating significant difference from 0.

Ligation and transection of the biliary duct in another group of rats led to the development of biliary cirrhosis within 3–4 weeks. At this time, the animals were visibly icteric, with serum bilirubin values of 5.4 ± 0.8 mg/dL versus 0.9 ± 0.3 mg/dL in sham-operated controls. At the time of the experiment in the anesthetized animals, the presence of 10–25 ml of ascites fluid and significant hepato-splenomegaly were verified by laparotomy. Also similar to severe human cirrhosis, renal blood flow was reduced, as tested by ultrasonic volume flow measurements in six cirrhotic rats (1.0 ± 0.3 ml/min) and four sham-operated controls (2.6 ± 0.6 ml/min; $P < 0.05$).

Similar to CCl_4-induced cirrhosis, biliary cirrhosis was associated with significant hypotension reversible by SR141716A treatment. The basal mean blood pressure was 131 ± 4 mmHg in six control rats, and 93 ± 6 mmHg in seven rats with biliary cirrhosis ($P < 0.005$). In the cirrhotic rats, a single i.v. injection of 3 mg/kg SR141716A caused a gradual and sustained increase in blood pressure that reached 112 ± 3 mmHg at 1 hour ($P < 0.01$). In the controls, however, SR141716A caused a small but significant decrease in blood pressure to 110 ± 5 mmHg ($P < 0.01$). Thus the blood pressure difference between the two groups was abolished by SR141716A treatment.

We also tested the cardiovascular effect of the endogenous cannabinoid anandamide. In sham-operated control rats, the i.v. injection of four mg/kg anandamide caused a triphasic blood pressure response (Fig. 2*a*), of which the prolonged depressor phase is known to be CB1 receptor-mediated[21,22]. In the rats with biliary cirrhosis, the first two non-specific phases of the effect of anandamide were present but the subsequent hypotensive component was absent (Fig. 2*b*), and similar observations were made in rats with CCl_4-induced cirrhosis (data not shown).

When rats were treated with 20 mg/kg of nitro-L-arginine methyl ester (L-NAME), a non-selective inhibitor of NOS (ref. 23), a sustained increase in blood pressure was observed in both the biliary cirrhotic rats (Fig. 2*d*) and the sham-operated animals (Fig. 2*c*), and the increase was greater in the former than in the latter group. Despite the higher level of blood pressure following L-NAME treatment, anan-

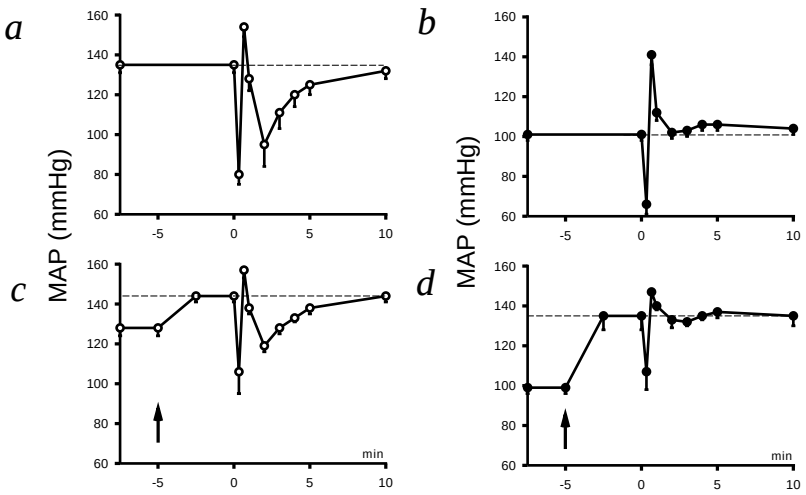

Fig. 2 The hypotensive effect of anandamide is absent in cirrhotic rats. ***a***–***d***, The effect of anandamide on mean arteriae pressure in sham-operated (○; $n = 6$) and biliary cirrhotic (●; $n = 8$) rats in the absence (a and b) and in the presence of L-NAME (c and d). Anandamide (4 mg/kg) was injected i.v. at 0 min. Arrows indicate the i.v. injection of L-NAME (20 mg/kg). Points and bars represent means ± s.e. The horizontal dashed line indicates basal mean blood pressure.

damide remained ineffective in lowering blood pressure in cirrhotic rats (Fig. 2*d*). In sham-operated animals, L-NAME treatment resulted in a 52 ± 12% reduction in the hypotensive response to anandamide ($P < 0.01$), as measured by the area under the curve (Fig. 2*a* vs 2*c*).

Cirrhotic monocytes cause hypotension via CB1 receptors

We next tested the effect of the monocyte fraction isolated from rat blood on the blood pressure of normal recipient rats pretreated with vehicle or 3 mg/kg of SR141716A. Intravenous injection of the monocyte fraction from biliary cirrhotic rats caused a gradually developing, prolonged hypotensive response in vehicle-pretreated recipient rats, whereas aliquots of the same cells caused no significant decrease in blood pressure in recipients pretreated with 3 mg/kg SR141716A (Fig. 3*a*). Monocytes isolated from sham-operated rats caused no significant changes in the blood pressure in recipient rats (Fig. 3*a*). A similar pattern of responses occurred when recipient rats were given an i.v. injection of monocytes isolated from cirrhotic patients or healthy volunteers (Fig. 3*b*). Cells from the 10 cirrhotic patients elicited long lasting hypotension, which could be prevented by pretreatment of the recipient rat with 3 mg/kg SR141716A. Again, cells from healthy individuals caused no change in the blood pressure of recipient rats.

Cirrhotic monocytes contain elevated levels of anandamide

To test whether the monocyte fraction contained endogenous anandamide, which might be responsible for the effects observed in the recipient animals, we isolated monocytes from 20 ml of pooled blood from cirrhotic or sham-operated rats. We subjected a control and a cirrhotic sample to in-line high-performance liquid chromatography/mass spectrometry (Fig. 4). Anandamide could be detected in both samples, but the amount detected was 2–3-fold greater in the cirrhotic than in the control sample. In 3 independent control samples the amount of anandamide detected in cells originating from 1 ml of blood was 90 ± 25 fmol; in 3 cirrhotic samples containing the same number of cells the amount of anandamide was 230 ± 10 fmol ($P < 0.005$). Anandamide could also be detected in human monocytes. Cells originating from 1 ml of blood from 5 healthy volunteers contained 15 ± 6 fmol of anandamide, whereas similar samples from 6 cirrhotic patients contained 234 ± 106 fmol of anandamide ($P < 0.05$).

Upregulation of endothelial CB1 receptors in cirrhosis

In addition to a possible increase in the production of endogenous cannabinoids by cells in the monocyte fraction, increased density of vascular cannabinoid receptors might also contribute to the appearance of the SR141716A-sensitive hypotension in cirrhosis. To test this possibility, we identified CB1-receptor mRNA by RT-PCR and quantified CB1-receptor binding sites by radioligand binding in hepatic arterial endothelial cells isolated from non-cirrhotic and cirrhotic human livers. There was a substantial increase in CB1-receptor mRNA relative to β-actin mRNA in cultured endothelial cells from a cirrhotic liver as compared with cells from a non-cirrhotic liver (Fig. 5*a*). A similar difference was observed in two additional pairs of preparations, resulting in a significant, 2.6 ± 0.5-fold increase ($P < 0.01$) in the optical density of the CB1 amplicon. This increase in mRNA was associated with a significant, three-fold increase in the cellular density of CB1 receptor binding sites in cirrhotic as compared with non-cirrhotic endothelial cells (Fig. 5*b*.)

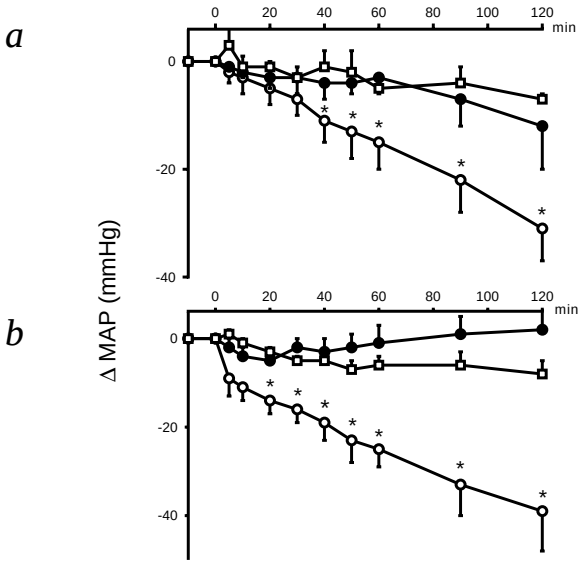

Fig. 3 CB1 receptor-mediated hypotensive response to monocytes from cirrhotic rats or patients. ***a*** and ***b***, The hypotensive response of normal recipient rats to the injection of monocytes isolated from rats with biliary cirrhosis (*a*) or from cirrhotic patients (*b*). Monocytes isolated from 5 ml of blood from control rats or subjects (□), or from cirrhotic patients or rats were injected at 0 min into recipient rats pretreated with 3 mg/kg vehicle (○) or SR141716A, (●). Cells were injected in 200 μL of saline at 0 min. Points and bars represent means ± s.e.; $n = 6$–10. *, significant difference from corresponding baseline (0 min) values.

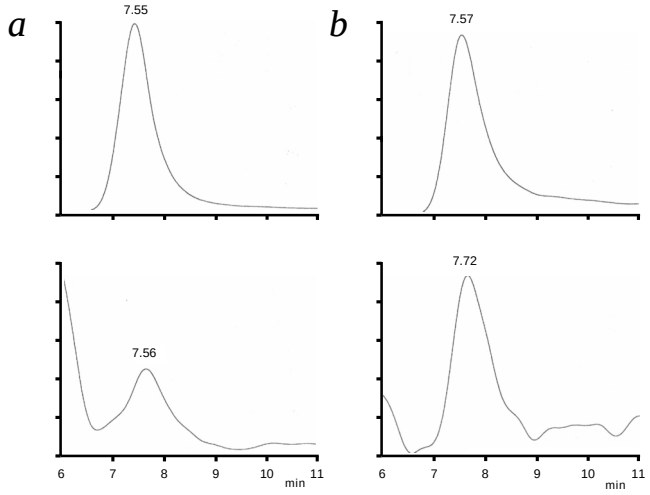

Fig. 4 Endogenous anandamide in monocytes from cirrhotic and control rats. ***a*** and ***b***, A representative LC/MS analysis of samples extracted from the monocyte fraction of 20 ml pooled blood of sham-operated (*a*) and cirrhotic (*b*) rats. Two selected ions were monitored simultaneously: m/z 356.2 for d_8-anandamide ions as internal standard (upper panels) and m/z 348.2 for anandamide ions (bottom panels). Numbers at the peaks indicate the Liquid chromatography (LC) retention time of the compounds in minutes.

Discussion

We present several lines of evidence to indicate that activation of vascular CB1 receptors by endogenous cannabinoids might be one of the mechanisms responsible for the vasodilated state in chronic liver cirrhosis that, in turn, has been implicated in the development of salt and water retention, ascites and decreased renal blood flow observed in the advanced stages of cirrhosis[24,25]. First, the decreased blood pressure associated with two different forms of cirrhosis in rats could be acutely reversed by a selective CB1 receptor antagonist, which also decreased the elevated mesenteric blood flow and portal pressure in these animals. Second, monocytes isolated from the blood of cirrhotic rats or patients, but not from normal controls, caused CB1-receptor–mediated hypotension when injected into normal rats. Third, monocytes from cirrhotic individuals or rats contain elevated levels of anandamide, as identified by liquid chromatography/mass spectrometry (LC/MS). And finally, hepatic vascular endothelial cells from cirrhotic patients contain elevated levels of CB1 receptors.

At the doses used, SR141716A is a selective antagonist of CB1 receptors[20], which indicates that its pressor effect in the hypotensive, cirrhotic animals is due to the reversal of a CB1 receptor-mediated vasodilator tone. Equally important, SR141716A treatment was able to significantly reduce the markedly elevated mesenteric blood flow and portal venous pressure of the cirrhotic animals. These effects are opposite in direction to the recently reported effects of anandamide on mesenteric blood flow and portal pressure in control rats[26], which further indicates that the effects of SR141716A in the cirrhotic animals are due to reversal of an endocannabinoid 'tone'. In rats with CCl_4-induced cirrhosis, blood pressure at the peak of the SR141716A-induced pressor response briefly exceeded the blood pressure in normal controls (see Fig. 1*a*). This might have been due to unmasking the activity of neurohumoral vasoconstrictor systems, which is increased in cirrhosis[27]. The pressor effect of SR141716A could be of potential clinical significance, as decreasing inflow and thus pressure in the portal circulation is an important goal in the medical management of patients with advanced cirrhosis. Although the reduction of portal pressure by SR141716A was modest, this is in agreement with published information indicating that normalization of the elevated mesenteric inflow in cirrhosis reduces portal pressure by no more than 15–20%, as much of the increased pressure is due to increased resistance posed by the scar tissue[28,29].

The role of CB1 receptors in anandamide-induced hypotension is clearly indicated by the documented absence of this effect in CB1-receptor knockout mice[30,31], and the failure of exogenous anandamide to lower blood pressure in cirrhotic rats might be related to maximal activation of vascular CB1 receptors by an endogenous ligand, possibly anandamide. The alternative explanation that anandamide might not be able to further reduce blood pressure in already hypotensive animals is unlikely because anandamide remained ineffective after blood pressure was raised by L-NAME treatment of cirrhotic rats.

It has been proposed that the hyperdynamic circulation and vasodilated state in cirrhosis is mediated by NO (ref. 11), which might be derived from increased activity of iNOS, ecNOS or both. Indeed, treatment with the non-specific NOS inhibitor L-NAME caused a greater increase in blood pressure in rats with biliary cirrhosis than in their controls (Fig. 2), which was similar to observations by others[32]. However, L-NAME treatment also markedly reduced the hypotensive action of anandamide in normal rats (Fig. 2*a* and *c*), which indicates that some or most of the increase in NO production in cirrhotic rats might be mediated via the tonic activation of CB1 receptors. As shown here and elsewhere[33–35], CB1 receptors are present in vascular endothelial cells, and activation of endothelial CB1 receptors has been shown to lead to increased production of NO (refs. 33,36). It is unknown whether the activation of CB1 receptors can also lead to the well-documented upregulation of

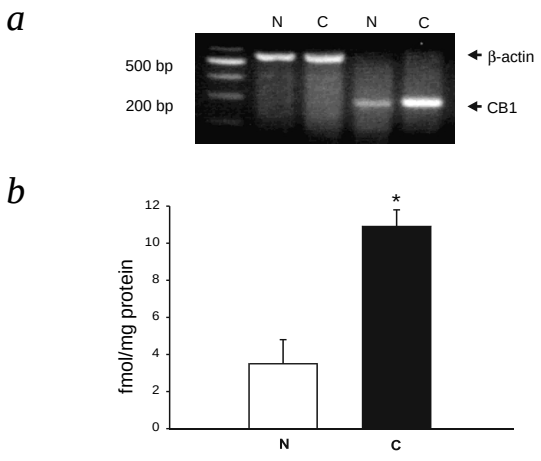

Fig. 5 CB1 receptors in vascular endothelium are upregulated in cirrhosis. ***a***, Identification of CB1-receptor mRNA by RT-PCR in hepatic arterial endothelial cells isolated from normal (N) and cirrhotic (C) human liver tissue. The β-actin gene was amplified as internal control. Left lane: DNA sizing ladder. ***b***, The density of CB1-receptor–binding sites in cultured endothelial cells isolated from cirrhotic (C) as compared with normal (N) human liver tissue. Columns and bars represent means ± s.e. from 3 normal and 3 cirrhotic preparations, *, difference from normal, $P < 0.01$.

ecNOS in cirrhosis[18], and additional mechanisms of increased NO production independent of CB1 receptor activation cannot be ruled out.

Cirrhotic patients are known to be endotoxemic[7–10], a finding confirmed here. Recent findings indicate that bacterial endotoxin (LPS) induces the production of endocannabinoids in circulating macrophages and platelets[19], which has led us to hypothesize that the cellular source of vasodilator endocannabinoids in cirrhosis might be circulating macrophages and platelets. Several of our findings further support this possibility. First, monocytes isolated from cirrhotic blood elicited hypotension susceptible to inhibition by the CB1-receptor antagonist, SR141716A. Although the monocyte fraction also contains a large number of lymphocytes, earlier studies with monocytes from rats with hemorrhagic shock have shown that pure lymphocytes, collected after allowing the macrophages and platelets to adhere to a plastic culture dish, are devoid of hypotensive activity[37]. Second, LC/MS analysis of lipid extracts of monocytes documented significantly higher amounts of anandamide in cells from cirrhotic patients and rats than in cells from the respective controls. It is well known that activated macrophages and platelets display increased adherence to the vascular endothelium *in vivo*[38], and anandamide might thus be delivered as a 'paracrine' mediator to its vascular site of action.

Increased target organ sensitivity might also contribute to the development of an endocannabinoid vasodilator tone in cirrhosis. This possibility is strongly supported by the upregulation of both CB1-receptor message and binding-site density in vascular endothelial cells from cirrhotic versus normal human livers. It remains to be determined whether CB1 receptors present in vascular smooth muscle cells[39] are similarly upregulated in cirrhosis. Thus, both increased production (and release) of the endogenous mediator and increased sensitivity of at least some of its vascular receptors might contribute to the cannabinoid-mediated vasodilator tone in liver cirrhosis. Here we provide evidence for such a mechanism in two different animal models of cirrhosis as well as in cells isolated from patients with cirrhosis of different etiologies. This indicates that the appearance of an endocannabinoid-mediated vasodilator tone might be a general pathogenic feature in different forms of cirrhosis.

The prevailing peripheral vasodilation theory[5,24] posits that an initial increase in vascular capacitance caused by as yet unidentified endogenous vasodilator(s) contributes to the maintenance of portal hypertension and the development of ascites. The present findings suggest that this elusive mediator might be an endocannabinoid acting at vascular CB1 receptors, and antagonists of these receptors might offer a therapeutic approach to the management of patients with advanced liver cirrhosis awaiting liver transplantation.

Methods

Rat model of micronodular cirrhosis. Male Sprague–Dawley rats (200 g) received phenobarbital (35 mg/dL) through drinking water and were gavaged weekly with CCl_4, 40–80 μg in corn oil, or with corn oil only (controls) as described[40]. Body weight was monitored daily and the dose of CCl_4 was adjusted individually. Systolic blood pressure (BP) was monitored daily using an automated tail-cuff procedure. After 10–12 wk of treatment, CCl_4-treated rats became hypotensive and 6–10 d later started to develop ascites. After hypotension set in, the effect of a single i.v. dose of SR141716A on systolic BP was tested in the conscious state. After the first signs of ascites several days later, rats were anesthetized with urethane (0.3 g/kg intraperitoneally (i.p.) + 0.7 g/kg i.v.), laparotomized and the ascites fluid was measured. The femoral artery and vein were cannulated for monitoring of BP and drug injections, respectively. A side branch of the portal vein was cannulated for measurement of portal venous pressure. Mesenteric or renal blood flow was monitored using a T206 small animal blood-flow meter (Transonic Systems, Ithaca, New York), which calculates the difference in integrated transit times detected by a probe placed around the artery, as a measure of volume flow rather than velocity. Cirrhosis was verified by post-mortem microscopic examination of trichrome-stained sections of the liver.

Rat model of biliary cirrhosis. Male Sprague–Dawley rats (250–300 g) were anesthetized and laparotomized, and the common biliary duct was ligated and transected. Weight gain in the surviving animals (~70% of those operated) was markedly reduced, and they developed icterus by 3–4 wk following surgery. Their stool became acholic, and postmortem examination confirmed enlarged liver and spleen, and the presence of ascites (10–25 ml). Sham-operated controls displayed normal post-operative recovery. 4 wk following surgery, the animals were re-anesthetized with urethane (0.3 g/kg + 0.7 g/kg i.v.) and instrumented for monitoring cardiovascular parameters, as described above.

Patients with chronic cirrhosis. In a total of 10 patients, cirrhosis was confirmed by liver biopsy or diagnosed by the clinical constellation of chronic liver disease, liver failure and portal hypertension in the absence of portal vein thrombosis. The average age of the 6 male and 4 female patients was 52.9 ± 4.1 y, and the etiology of the cirrhosis was chronic alcoholism (4), hepatitis C (4), graft versus host hepatitis (1) and chronic autoimmune hepatitis (1). All patients had signed an informed consent form, approved by the Institutional Ethics Committee. Blood cultures drawn at admission were sterile. Serum bilirubin was elevated (9.5 ± 3.9 mg/dL), serum albumin was reduced (2.4 ± 0.1 mg/dL), and there was moderate hyponatremia (131 ± 2 mmol/L). All patients had very advanced cirrhosis as evidenced by a mean Child–Pugh score of 11. The patients were hypotensive with a mean blood pressure of 67.4 ± 1.8 mmHg (systolic/diastolic: 91.8 ± 1.5/55.4 ± 2.4 mmHg), and heart rate was 88 ± 4 beats/min. Control blood samples were obtained from 10 healthy volunteers. Plasma endotoxin level was measured by the limulus amebocyte lysate endotoxin reaction[41] in 9 patients and 4 volunteers, and was 6.2 ± 2.0 pg/ml in the patients and below detectability (< 0.5 pg/ml) in all 4 volunteers.

Isolation of circulating monocytes. 10 ml of heparinized blood was obtained through venipuncture from each of 6 healthy volunteers and 10 patients with chronic cirrhosis, and from sham-operated animals and rats with biliary cirrhosis. The monocyte fraction, which contained lymphocytes, circulating macrophages and contaminating platelets, was isolated by the Ficolpaque method[37]. The cells were washed 3 times and resuspended in PBS. Aliquots of cells (0.2 ml) originating from 5 ml of human or rat blood were injected i.v. into urethane-anesthetized normal rats instrumented for monitoring blood pressure and heart rate. For LC/MS analysis of endogenous anandamide, monocytes were isolated from 20-ml samples of pooled normal or cirrhotic rat blood or from 25-ml blood samples obtained from 5 healthy volunteers and 6 cirrhotic patients. 1 nmol of d_8-anandamide was added as external standard, and cells with medium were then extracted 3 times with 2 volumes of chloroform:methanol (2:1, v/v). The organic phase was dried under nitrogen and resuspended in 100 μL of methanol for analysis by LC/MS.

Analysis of endogenous anandamide by LC/MS. Monocyte extracts were fractionated by reversed-phase HPLC on an ODS column (Supelco, Bellefonte, Pennsylvania, 5 μm, 4.6 mm × 15 cm), using a mobile phase of methanol:water:acetic acid (85:15:0.03 v/v/v) at a flow rate of 1 ml/min on a Waters 2690 system. This was followed by in-line mass spectroscopic analysis on a Micromass Quattro II mass spectrometer equipped with an atmospheric pressure chemical ionization source. Anandamide and d_8-anandamide were monitored as positive ions at m/z 348.2 and 356.2, respectively. The limit of sensitivity was ~50 fmol (20 pg).

Isolation and culture of human hepatic artery endothelial cells. Normal human liver tissue was obtained from liver lobes resected for metastatic

colon cancer, which had a 2–3-cm rim of normal tissue around the tumor. Cirrhotic human livers were obtained at the time of liver transplant. Pieces of normal or cirrhotic liver tissue were minced and incubated in collagenase overnight. Vascular endothelial cells were isolated by incubating the dissociated cells with Ulex Europeus I (UEA)-coated Dynabead suspension as described[42]. The cells were maintained in endothelial growth medium #199 containing 10% fresh human serum, hydrocortisone, heparin epidermal growth factor and endothelial growth supplement (100 mg), under standard conditions. The endothelial nature of the cells was verified by their DiL-Ac-LDL uptake, a selective marker for vascular endothelial cells[35].

RT-PCR. Isolation of total cellular RNA from confluent cultures of human hepatic artery endothelial cells, reverse transcription of the RNA into single stranded cDNA, and PCR amplification using the cDNA template were as described[35]. Primers used to amplify a 276-bp segment of the human CB1 receptor gene and a 500-bp segment of the human β-actin gene as internal control have been reported[35].

CB1-receptor–binding assays. The density of CB1 receptor-binding sites on cultured hepatic artery endothelial cells was estimated by the specific binding of the antagonist radioligand [^{125}I]AM-251 (ref. 43), as described[35]. Due to the limited number of cells available, binding was tested at a single, near-saturating concentration of the radioligand (0.2 nM), in triplicate aliquots in the absence or presence of 10 μM SR141716A as unlabelled competitor.

Drugs. SR141716A (N-[piperidin-1-yl]-5-[chlorophenyl]-1-[2,4-dichlorophenyl]-4-methyl-1H-pyrazole-3-carboxamide HCl) was a gift from Sanofi Recherche (Montpellier, France). Anandamide and d_8-anandamide were from Deva Biotech (Hatboro, Pennsylvania). L-NAME (S[+]-N5-[imino{nitroamino}methyl]-L-ornithine dihydrochloride) was from RBI (Natick, Massachusetts).

Statistical analyses. The time-dependent effects of drugs on blood pressure were analyzed by two-way ANOVA followed by Tukey's post-hoc test. Differences between two groups of samples were analyzed by Student's unpaired *t*-test or the Mann–Whitney *U*-test, as appropriate. Differences with a P value < 0.05 were considered statistically significant.

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