



Effect of increased hepatic platelet activating factor and its receptor portal hypertension in CCl₄-induced liver cirrhosis

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Abstract

AIM: To evaluate the changes in hepatic platelet activating factor (PAF) and its receptors and their effect on portal pressure of cirrhotic rats induced by CCl₄.

METHODS: A model of liver cirrhosis was replicated in rats by intra-peritoneal injection of CCl₄ for 8 wk. We determined the effect of hepatic PAF and its receptor level on portal and arterial pressure by EIA, saturation binding and RT-PCR technique.

RESULTS: Compared to control rats, cirrhotic rats had higher hepatic PAF levels and output as well as higher plasma PAF levels ($P < 0.01$, $P < 0.01$, $P < 0.05$, respectively). Both hepatic PAF receptor mRNA levels and PAF binding were nearly 3-fold greater in cirrhotic rats ($P < 0.01$). Portal injection of PAF (1 g/kg WT) increased the portal pressure by 22% and 33% in control and cirrhotic rats, respectively. In contrast, the arterial pressure was decreased in the both groups (54% in control rats and 42% in cirrhotic rats). Injection of the PAF antagonist BN52021 (5 mg/kg WT) decreased the portal pressure by 16% in cirrhotic rats but had no effect in the control rats.

CONCLUSION: The upregulation of the PAF system contributes to hepatic hemodynamic and metabolic abnormalities in cirrhosis, and the increased release of PAF into the circulation has impacts on the systemic hemodynamics.

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Key words: Platelet activating factor; PAF receptors; Endothelin; Portal hypertension; Cirrhosis

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INTRODUCTION

Platelet activating factor (PAF: 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a phospholipid exhibiting diverse biological actions. PAF acts both as a multifunctional, soluble, proinflammatory agent and as a specific membrane-bound adhesion molecule. PAF receptors have been identified in smooth muscle cells, cardiomyocytes, endothelial cells, neutrophils, monocyte-macrophages, Kupffer cells and eosinophils^[1]. The physiological actions of PAF play a role in platelet secretion and aggregation, bronchoconstriction, vascular permeability and systemic arterial hypotension^[1-4]. Exogenous PAF administration via the portal vein increases portal venous pressure and activates glycogenolysis both in perfused organs^[5] and *in vivo*^[6]. In contrast, systemic infusion of PAF reduces arterial blood pressure^[7,8]. Portal hypertension and hyperdynamic systemic circulation are two prominent clinical features of human and animal experimental liver cirrhosis^[9-11]. Circulatory responses to intravenous infusion of PAF resemble the hemodynamic change in advanced liver cirrhosis. Excessive deposition of extracellular matrix is a cause of the increased resistance to hepatic blood flow and consequent portal hypertension in cirrhosis, but studies in animals indicate that increased vascular tone is also a contributing factor^[12].

The PAF content in intact liver is elevated by various types of injury, including ischemia-reperfusion^[13], obstructive jaundice^[14], and endotoxin exposure *in vivo*. Based on these observations, we hypothesized that increased hepatic synthesis of PAF in cirrhosis could lead to increased plasma levels, and that PAF might play a role in portal hypertension. Our results have confirmed that hepatic PAF levels are elevated and PAF receptors are upregulated in cirrhosis. The reactivity of the hepatic vasculature to PAF is consequently increased, while that of the systemic vasculature is attenuated.

MATERIALS AND METHODS

Induction of cirrhosis

Cirrhosis was induced in male Sprague-Dawley rats

weighing 230-250 g as described previously^[10,15,16]. Briefly, intraperitoneal injection of CCl₄ (0.15 mL/kg WT, twice a week for 8 wk) was combined with drinking water containing phenobarbital (0.4 g/L). Control rats received injection of the vehicle (peanut oil) and phenobarbital water.

Determination of portal venous and systemic arterial pressure

Rats were anesthetized with 50 mg/kg (i.p.) pentobarbital, and placed on a heated water blanket maintained at 38°C. A PE-50 catheter (Thomas Scientific, Swedesboro, NJ, USA) was inserted into the femoral artery to monitor arterial blood pressure via a strain gauge pressure transducer connected to a 4-channel Grass polygraph (Model no. 79E, Quincy, MA, USA). The hepatic trigone was exposed via laparotomy, and the portal vein was skeletonized. The intestines and the abdominal cavity were covered with warm saline-soaked sponges, and a catheter was inserted into the portal vein and connected to a pressure transducer coupled to a 4-channel Grass polygraph to monitor portal pressure. After stable recordings of both portal and arterial blood pressure were obtained, 1 mL of blood was withdrawn from the femoral artery, portal vein and suprahepatic vena cava, respectively. Then, the liver was rapidly excised and washed in ice-cold phosphate buffered saline containing 0.1 mmol/L EDTA and 0.1 mmol/L EGTA. A portion of the liver tissue was stored in 10% buffered formalin, a portion in OCT, and the rest was snap-frozen in liquid nitrogen and stored at -80 °C.

Morphometric analysis

An established histological grading system was employed for the determination of pathological scores of liver injury^[17-19]. Paraffin-embedded liver sections of 4-μm thickness were stained with hematoxylin-eosin and Masson's trichrome. Steatosis, inflammation, necrosis and fibrosis were scored in at least three random fields of view in each tissue section and a score for each specific parameter was estimated. Steatosis was assessed by estimating the percentage of cells with micro- and macrovesicular fat as follows: 0 (absent), 1 (1-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%). Necrosis was scored as follows: 0 (absent), 1 (1-10 necrotic cells per view), 2 (11-20 necrotic cells per view), 3 (21-30 necrotic cells per view), 4 (31-40 necrotic cells per view), and 5 (41-50 necrotic cells per view). Inflammation was assessed as follows: 0 (absent), 1 (rare), 2 (scattered), 3 (scattered with localized foci), 4 (abundant with foci), and 5 (extensive). Architectural change, fibrosis and cirrhosis were estimated as follows: 0 (absent), 1 (rare), 2 (scattered deposition), 3 (scattered with localized deposition), 4 (abundant with minor bridging fibrosis), 5 (bridging fibrosis) and 6 (cirrhosis). A total pathology score was calculated by combining and summing the scores for the above pathological parameters, and the average score per animal/treatment group was calculated.

Determination of PAF in liver and blood

Lipids were extracted as previously described^[20]. Briefly, 100 mg of liver or 1 mL of blood was homogenized in 9.5 mL

of a mixture of methanol, chloroform and water (2:1:0.8, v:v:v). The homogenates were kept at room temperature for 1 h, followed by the addition of chloroform (2.5 mL) and water (2.5 mL). After being thoroughly mixed, the mixture was kept at room temperature for 1 h and centrifuged at 1200 r/min for 15 min. The chloroform layer was aspirated and dried under nitrogen at 35 °C. The residue was dissolved in 200 μL chloroform, and applied to the Bond Elut SI column (Amprep silica mini-columns; Amersham Pharmacia Biotech, Piscataway, NJ, USA). The column was washed with 3 mL of chloroform, 2 mL of chloroform-methanol (6:4; v/v) and 3 mL of chloroform-methanol-28% aqueous ammonia (70:85:7, v:v:v). PAF was eluted with 2 mL of chloroform-methanol-28% aqueous ammonia (50:50:7, v/v). The elute was evaporated to dryness under nitrogen, and the residue was dissolved in 200 μL of saline containing 0.01% Triton X-100. PAF concentration was determined by [³H]-PAF scintillation proximity assay (Amersham-Pharmacia Biotech).

Determination of hepatic PAF binding

Hepatic membranes were prepared as described previously^[10,16] and suspended in 50 mmol/L Tris-HCl (pH 8.0) containing 5 mmol/L MgCl₂, 125 mmol/L choline chloride, 0.1 mol/L PMSF, 0.1 μg/mL leupeptin and 1 μg/mL pepstatin. The membrane suspension was stored at -80 °C in aliquots after the protein concentration was adjusted to 10 mg protein/mL. Membranes (100 μg protein) were incubated in 50 mmol/L Tris-HCl (pH 7.2) containing 5 mmol/L MgCl₂, 125 mmol/L choline chloride, 0.25% BSA and 0.125-32 nmol/L 1-O-[³H]octadecyl-2-acetyl-sn-glycero-3-phosphocholine (151 Ci/mmol, 9.96 GBq/mg; Amersham) ± 10 μmol/L unlabeled PAF (Bachem Americas, King of Prussia, PA, USA) at 30 °C for 1 h. The reaction was terminated with the addition of 5 mL ice-cold assay buffer and filtration through Whatman GF/C filters (Whatman, Hillsboro, OR, USA) presoaked in assay buffer for 1 h. Filters were washed thrice with 4 mL of assay buffer, and the radioactivity was determined in a β-scintillation counter.

Determination of hepatic PAF receptor mRNA levels

The mRNA expression of PAF receptors was determined by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR). RNA was isolated from the livers using a RNA isolation kit (ToTALLY RNA™, Ambion, Austin, TX, USA). Two micrograms of total RNA was used for the preparation of cDNA by reverse transcription as described. cDNA equivalent of 5 ng original RNA was used in PCR. The reaction mixture (50 μL) contained 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.2 mmol/L dNTPs, 20 pmol of PCR primers and 2U platinum Taq DNA polymerase (GIBCO-Invitrogen, Carlsbad, CA, USA). Thirty-five cycles of reaction were carried out as follows: denaturation at 94 °C for 1 min, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. The PCR primers used for PAF cDNA were: 5'-GCCACAACAGAGGCTTGA-3'(forward) and 5'-TC CATTGCTCTGGGCAGGAA-3'(reverse) [product size, 121 bp^[21]]. For normalization, β-actin mRNA levels were measured as described previously^[15], using cDNA primers:

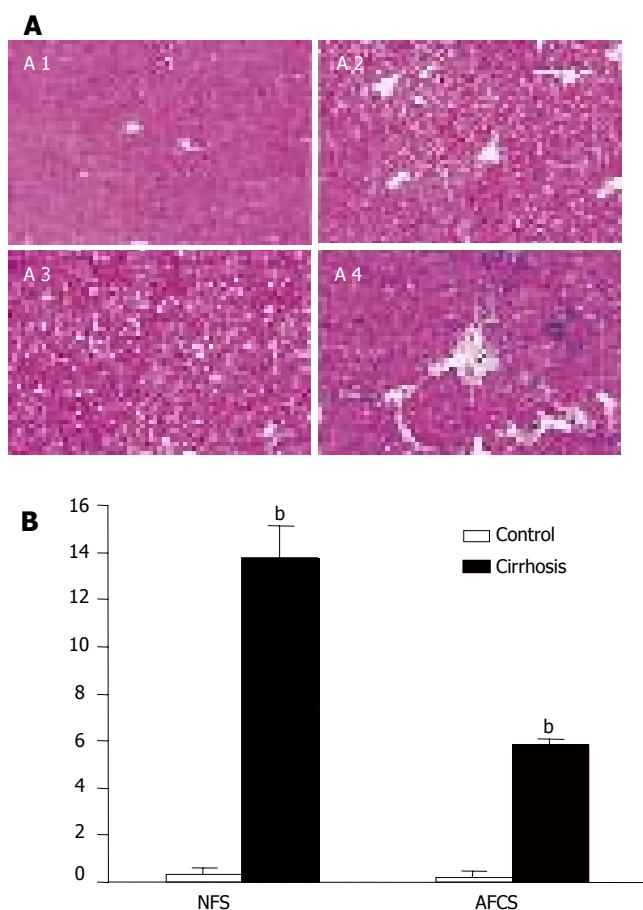


Figure 1 Morphometric analysis of cirrhotic liver 8 wk after CCl₄ or vehicle treatment (A) and scores for necroinflammatory NFS, architectural change, fibrosis and cirrhosis (AFCS) (B). ^b*P*<0.01 vs control.

Table 1 Characteristics of cirrhosis (mean ± SD)

	Control	Cirrhotic rats	<i>P</i>
Liver/body weight ratio (%)	4.80±0.25	4.90±0.44	NS
Weight of spleen (g)	0.73±0.06	1.36±0.15	0.001
Portal pressure (kPa)	0.71±0.08	1.63±0.09	0.001
MAP (kPa)	15.2±1.2	10.9±1.3	0.01
PAF (ng/g)	2.74±0.49	3.95±0.43	0.01

NS: not significant.

5'-TTCTACAATGAGCTGCGTGTG-3' (forward) and 5'-TTCATGGATGCCACAGGATTC-3' (reverse) [product size, 561 bp^[22]]. The PCR products were resolved in a 25 g/L agarose gel and stained with SYBR Green I (FMC Bioproduct, Rockland, ME, USA). The gels were scanned under blue fluorescent light using a phosphorimager and the band intensity was quantified using ImageQuaNT software (Molecular Dynamics, Sunnyvale, CA, USA).

Determination of PAF effect on portal venous and systemic arterial pressure

The experiment was essentially conducted for the measurement of baseline pressure. After a stable recording of portal and systemic mean arterial pressure (MAP) was

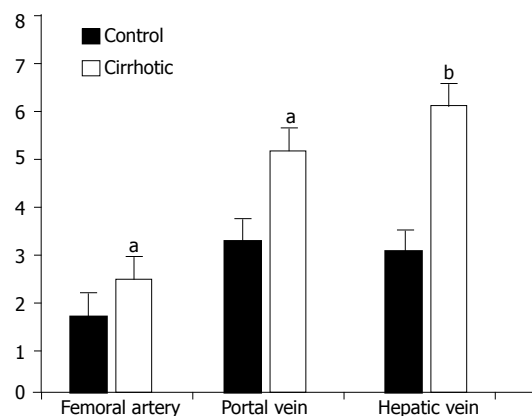


Figure 2 Effect of cirrhosis on circulating PAF levels. Concentrations of PAF in blood from femoral artery, portal and hepatic vein (suprahepatic vena cava) were determined. Values are mean±SD. ^a*P*<0.05 vs control; ^b*P*<0.01 vs control.

obtained, 1 mL of a solution containing PAF (1 µg/kg) was infused over 1 min from a 975 Harvard apparatus compact infusion pump into the portal vein via a 23 gauge needle/PE50 catheter. Portal venous pressure and MAP were monitored continuously for 15 min.

Statistical analysis

The values were presented as mean ± SD. Student's *t*-test was employed for statistical comparison between the groups. *P*<0.05 was considered statistically significant.

RESULTS

Characteristics of cirrhosis

There were no notable changes in liver histology throughout the 8 wk in control rats (Figure 1A1). In contrast, CCl₄-treated rats demonstrated extensive changes in liver morphology, including steatosis, inflammation, hepatocyte ballooning, and necrosis. Extensively distorted architecture due to excessive deposition of extracellular matrix was observed, which caused bridging of fibrosis, and infiltration of inflammatory cells in sinusoids and their accumulation around the islands of hepatocytes (Figure 1A3). Hepatic fibrosis was further validated histologically with Masson's trichrome staining (Figures 1A2 and A4).

The liver/body weight ratio was not significantly different between control and cirrhotic rats, whereas spleen weight of cirrhotic rats was nearly doubled (Table 1). MAP was significantly lower in cirrhotic rats (10.9 ± 1.3 kPa) than in control rats (15.2 ± 1.2 kPa). Portal venous pressure was higher in cirrhotic rats (1.63 ± 0.09 kPa) than in control rats (0.71 ± 0.08 kPa), indicating portal hypertension (Table 1).

Effect of cirrhosis on hepatic and circulating PAF and hepatic PAF receptor levels

Hepatic PAF concentrations were increased by 44% in cirrhotic rats (Table 1). Concentrations of PAF in the femoral arterial, portal venous, and hepatic arterial blood were significantly greater in cirrhotic rats than in control rats (Figure 2). Moreover, the concentration of PAF was significantly greater in hepatic venous blood than in

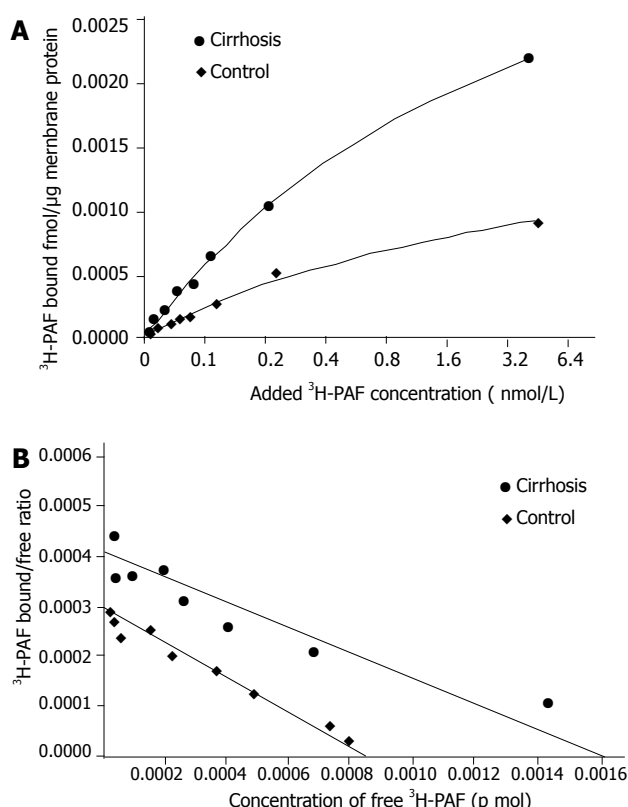


Figure 3 Effect of cirrhosis on hepatic PAF binding. **A:** Results of the saturation binding assay. ^3H -PAF was incubated with 100 μg membrane protein, in the presence or absence of 10 $\mu\text{mol/L}$ at 30 $^{\circ}\text{C}$ for 1 h; **B:** Scatchard plot analysis of ^3H -PAF binding to hepatic tissue of cirrhotic and control rats. Values are mean of separate experiments. Cirrhosis: $R=0.98$, $K_d=8.013$ nmol/L, $B_{\text{max}}=2.8\pm0.213$ fmol/ μg membrane protein; control: $R=0.99$, $K_d=5.794$ nmol/L, $B_{\text{max}}=0.9\pm0.061$ fmol/ μg membrane protein.

portal venous blood in cirrhotic rats (Figure 2). No such difference in hepatic venous and portal venous blood of control rats was observed.

Scatchard analysis of the saturation binding data revealed a 3-fold increase in PAF binding capacity in cirrhotic liver as compared to the control (B_{max} of 2.8 ± 0.21 vs 0.9 ± 0.06 fmol/ μg protein, $P<0.01$), whereas receptor affinity was unaltered (K_d : 8.01 ± 1.33 nmol/L for cirrhotic rats and 5.79 ± 0.96 nmol/L for control rats, $P<0.05$, Figure 3). Consistent with the increased receptor density, a similar increase in the PAF receptor mRNA transcript was observed in the cirrhotic liver as determined by RT-PCR (PAFR/ β -actin mRNA ratio of 0.51 ± 0.03 vs 0.16 ± 0.015 , $P<0.01$, Figure 4).

Effect of PAF on portal and arterial pressure in cirrhotic rats

A separate set of control and cirrhotic rats were used to determine the PAF-induced changes in portal and systemic blood pressure. Infusion of PAF (1 g/kg WT) via the portal vein after a short delay caused a greater increase ($P<0.01$) in portal pressure in cirrhotic rats (from 1.61 ± 0.08 to 2.13 ± 0.09 kPa; 33% increase) than in control rats (from 1.07 ± 0.10 to 1.30 ± 0.09 kPa; 22% increase) (Figures 5 and 6). Following the initial rise, the portal pressure decreased progressively with time and was lower than the basal value 9 min after the administration

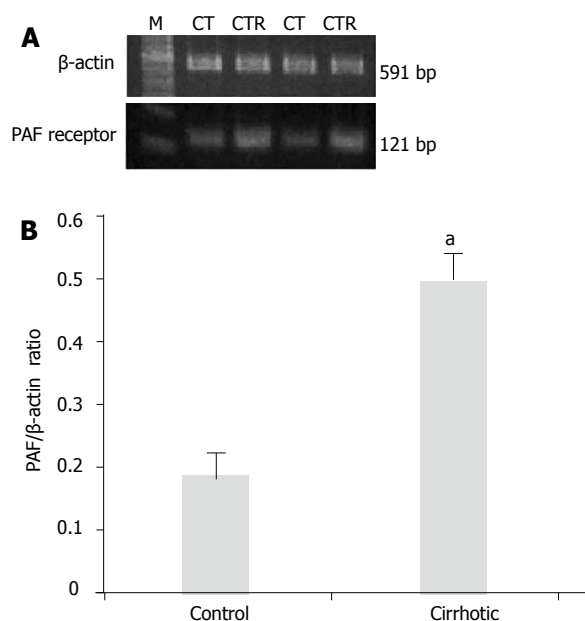


Figure 4 Effect of cirrhosis on mRNA expression of hepatic PAF receptor. **A:** PCR products of PAF and β -actin from control (CT) and cirrhotic (CR) rat livers; **B:** ratio of the PAF receptor and β -actin mRNA from six samples. ^a $P<0.01$ vs control.

of PAF.

The effect of PAF administered into the portal vein on arterial pressure was opposite to that observed on portal pressure (Figures 5 and 6). In contrast to the delay onset of the rise in portal venous pressure, MAP fell immediately after the administration of PAF. The decrease in MAP ($P<0.01$) was greater in control rats (54%) than in cirrhotic rats (42%). With the fall in MAP, pulse pressure fell markedly and did not recover, suggesting a reduction in cardiac contractility.

Effect of PAF antagonist BN52021 on portal and arterial pressure in cirrhotic rats

Administration of BN52021 (5 mg/kg WT) via the portal vein caused a 16% decrease in the portal pressure of cirrhotic rats (from 1.95 ± 0.21 to 1.64 ± 0.15 kPa, $P<0.01$). This effect occurred 2 to 3 min after the administration of BN52021. On the other hand, arterial pressure did not change upon treatment with BN52021 (from 11.0 ± 0.8 to 11.3 ± 0.67 kPa). In control rats, BN52021 had no effect on either portal or arterial pressure (Figure 7).

DISCUSSION

This investigation showed that increased production of PAF in the cirrhotic liver could lead to an increased circulating level of PAF, which is likely responsible for the arterial hypotension associated with the disease. In addition, hepatic PAF receptor mRNA expression was upregulated with a concomitant increase in PAF binding. Since PAF potentially increases portal pressure and hepatic vascular resistance^[6,7,23], it can be concluded that the up-regulated PAF system in the liver plays an important role in portal hypertension.

In the present study, the source of the increased hepatic PAF and the site of increased PAF receptors were not

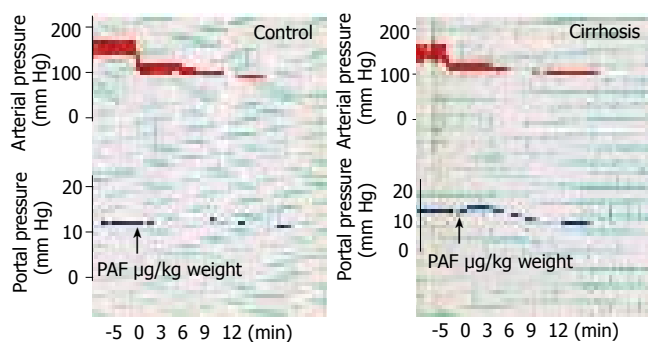


Figure 5 Tracings of the femoral arterial and portal venous pressure. After stabilization of arterial and portal venous pressure, PAF (1 µg/kg WT) was infused and the pressure was recorded continuously.

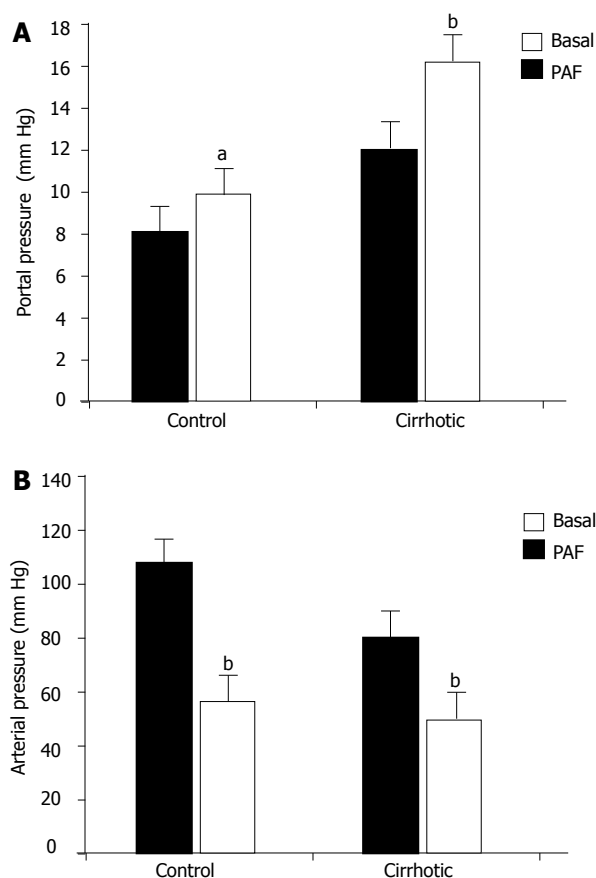


Figure 6 Effect of cirrhosis on PAF-induced changes in arterial and portal pressure. Arterial and portal venous pressure before and after portal administration of PAF were measured. Values are mean±SD of six determinations. ^a $P < 0.05$ vs basal value; ^b $P < 0.01$ vs basal value.

determined. Yang *et al.*^[10] reported that Kupffer cells are a major source of increased PAF in CCl₄-induced cirrhotic rat liver. Synthesis of PAF by Kupffer cells is stimulated by a number of inflammatory mediators and bacterial endotoxin (ET)^[2,24,25]. Increased circulating ET is a strong candidate contributor to the elevation of hepatic portal pressure in cirrhosis^[15,26]. We have previously reported that hepatic ET-1 and its receptors increase in rats 24 h after the administration of CCl₄^[15] and progressively thereafter during the development of cirrhosis. In the present study, hepatic PAF did not increase during the first

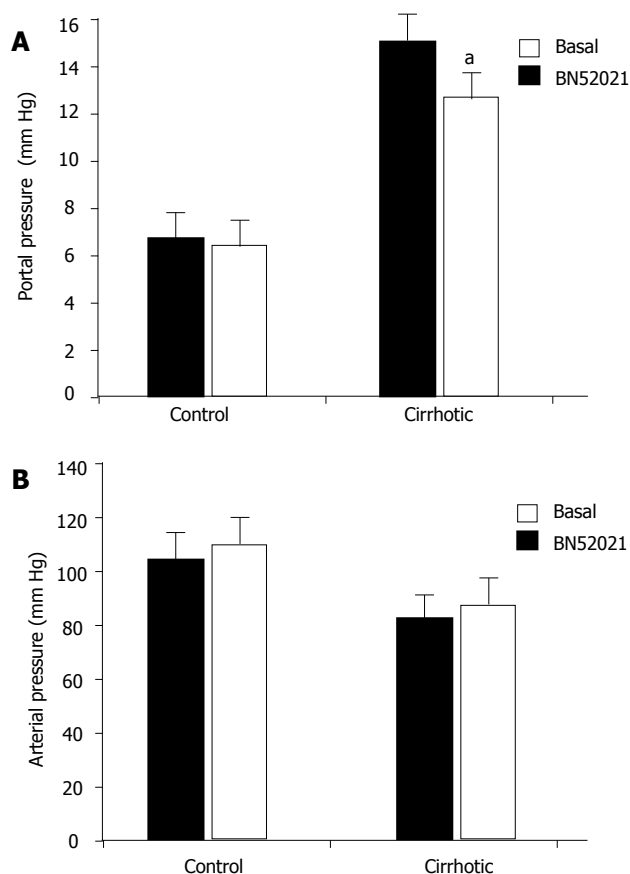


Figure 7 Effect of BN52021 on femoral arterial and portal venous pressure of cirrhotic rats 8 wk after vehicle or CCl₄ treatment. Values are mean±SD 5-6 min after introduction of BN52021. ^a $P < 0.05$ vs basal value.

week of CCl₄ treatment (2.70 ± 0.75 ng/g in control rats and 2.74 ± 0.46 ng/g in CCl₄-treated rats, $P > 0.05$) but increased after 2 wk of treatment. Since PAF synthesis by Kupffer cells is increased by ET-1^[24], the early elevation of ET-1 may contribute to the long-term increase in hepatic PAF. To further investigate the interaction between these two potent mediators during cirrhosis, we infused the powerful vasoconstrictor ET-1 into some experimental animals. After stabilization of the portal and arterial pressures following the administration of PAF, ET-1 (0.75 nmol) was infused as a bolus into the portal vein. The magnitude of ET-1 effect was much greater in control rats (200% increase) than in cirrhotic rats (80% increase, $P < 0.01$). We have previously reported that portal infusion of ET-1 alone causes a 150% increase in portal pressure in control rats and a 23% increase in cirrhotic rats^[15], suggesting that with or without prior infusion of PAF, the qualitative responses to ET-1 are similar between control and cirrhotic rats. Quantitatively, prior infusion of PAF appears to sensitize the hepatic vasculature to ET-1 moderately in control rats (200% vs 150%) and greatly in cirrhotic rats (80% vs 23%). The interaction between these two potent mediators during cirrhosis warrants further investigation.

Potentially, PAF could be increased by a decrease in the activity of plasma PAF-acetylhydrolase, which is predominantly responsible for the hydrolysis of PAF to lyso-PAF^[27,28]. Plasma PAF-acetylhydrolase activity is similar

in normal individuals and patients with alcohol-induced liver cirrhosis^[29]. Patients with primary and secondary biliary cirrhosis have elevated levels of circulating PAF and serum PAF-acetylhydrolase activity^[30], suggesting that synthesis of PAF increased by plasma PAF-acetylhydrolase is the mechanism underlying the elevated circulating levels of PAF.

The augmented effect of PAF on the vasculature of chronically injured liver appears to be independent of the hemodynamic state of the liver (i.e. normotensive or portal hypertensive). The vasoconstrictor effect of PAF in the perfused livers is greater in rats with thioacetamide-induced hepatic fibrosis than in control rats, even though the fibrosis rats have no portal hypertension^[31]. In the present study, PAF-induced portal hypertension was of greater magnitude in the cirrhotic rats than in the control rats. These results suggest that the increased reactivity to PAF is associated with the pathology of hepatic cirrhosis or injury rather than the pre-existing presence or absence of portal hypertension. That is to say, the response to PAF is not dependent upon the already increased hepatic vascular tone.

The reduction of portal pressure in cirrhotic rats induced by the PAF antagonist BN52021 provides convincing evidence that endogenous PAF is involved in the development of portal hypertension. The mechanisms of PAF-induced hepatic vasoconstriction are very complex and remain to be defined. PAF receptors have been found in smooth muscle cells^[32]. PAF elevates cytosolic free calcium in smooth cells^[33,34], which is necessary for muscle contraction. Exogenous PAF causes contraction of smooth muscle from ileal and peripheral lung strips^[35,36] and contributes to endothelin-induced vascular constriction in rat mesentery^[37]. Therefore, the contractile effect of PAF on the hepatic vasculature may be elicited by its direct action on smooth muscle cells. PAF may also act by stimulating the synthesis of eicosanoids such as thromboxane, PGE₂ and PGD₂, which are known to cause portal vasoconstriction^[38-41].

The combination of portal hypertension and peripheral vasodilatation with systemic arterial hypotension is characteristic of liver cirrhosis and implicates PAF. The observation that PAF induces smooth muscle contraction of ileal^[35] and pulmonary strips^[36], and micro-vessels^[37] is apparently in contradiction with the observation that it induces hypotension when administered intravenously to rats^[42]. In fact, PAF produces hypotension in all animal species studied^[8]. Increased plasma levels of PAF in cirrhosis are associated with low peripheral vascular resistance that is reversed by PAF antagonist BN52021^[9,29,43]. Sakaguchi *et al*^[44] and Hines *et al*^[6] reported that intravenous administration of PAF (1.5 µg/kg WT) causes arterial hypotension. The mechanisms of PAF-induced systemic arterial hypotension are not clearly understood. PAF-induced hypotension is not mediated by the central nervous system, renin-angiotensin system, β-adrenergic and dopaminergic eicosanoids, as well as Ca²⁺ influx and thyrotropin releasing steroids^[45,46]. However, PAF-induced delay and persistent hypotension is inhibited by the nitric oxide synthase inhibitor, N^ω-nitro-L-arginine^[47] while nitric oxide plays a role in PAF-induced relaxation

of rat thoracic aorta^[48]. This can be partially explained by the observation that PAF released by the liver may regulate systemic hemodynamics or stimulate the release of very powerful hypotensive agents such as nitric oxide^[47,48]. The involvement of nitric oxide requires clarification.

In conclusion, PAF is an important mediator of hepatic pathology during chronic liver injury. Cirrhotic liver is a source of circulating PAF and a major contributor to the systemic hypotension associated with liver cirrhosis.

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