Roles of anandamide in the hepatic microcirculation in cirrhotic rats

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Am J Physiol Gastrointest Liver Physiol 290: G328–G334, 2006; doi:10.1152/ajpgi.00367.2005.—Cannabinoids have been reported to participate in the pathogenesis of peripheral vasodilatation in cirrhosis. However, their roles in increased intrahepatic resistance (IHR) in cirrhotic livers are unknown. We aimed to investigate the effects of cannabinoids in the hepatic microcirculation of cirrhotic rats produced by bile duct ligation. In isolated liver perfusion, portal perfusion pressure (PPP) and the production of eicosanoids in the perfusate were measured. In addition, various hepatic protein levels [cyclooxygenase (COX) isoform and 5-lipoxygenase (5-LOX)] were also determined. Finally, concentration-response curves for PPP and the corresponding production of eicosanoids in response to anandamide (1.44 \( \times \) 10^{-10}–1.44 \( \times \) 10^{-3} M) after indomethacin (COX inhibitor), piriprost (5-LOX inhibitor), or flurbiprofen (thromboxane A\(_2\) synthase inhibitor) preincubation were obtained. The study showed that cirrhotic livers had significantly higher levels of PPP, COX-2 and 5-LOX protein expression, and production of thromboxane B\(_2\) (TXB\(_2\)) and cysteinyl leukotrienes (Cys-LTs) than normal livers. Anandamide induced a dose-dependent increase in PPP in both normal and cirrhotic livers. The anandamide-induced increase in PPP was found concomitantly with a significant increase in TXB\(_2\) and Cys-LT production in the perfusate. In response to anandamide administration, cirrhotic livers exhibited a significantly greater increase in IHR and production of TXB\(_2\) and Cys-LTs than normal livers. Indomethacin and furegrelate, but not piriprost, significantly ameliorated the anandamide-induced increase in IHR in cirrhotic livers. In conclusion, anandamide plays, in part, an important role in increased IHR of cirrhotic livers. The anandamide-induced increase in IHR in cirrhotic livers may be mediated by increased COX-derived eicosanoid (mainly thromboxane A\(_2\)) production.

INCREASED INTRAHEPATIC RESISTANCE (IHR), an initial event in the development of portal hypertension, is a consequence of the distortion of liver vascular architecture caused by fibrosis, scarring, and nodule formation in cirrhosis (38, 39). Recent studies have demonstrated that there are contractile elements in cirrhotic livers that are able to constrict, in a reversible and graded manner, in response to several agonists, including endothelin 1, norepinephrine, thromboxanes, leukotrienes, etc. (13, 15, 36, 40). This dynamic component had found to be important in increased IHR, and its modulation may become a key therapeutic target in cirrhosis (2).

Cannabinoid receptors (CB\(_1\) and CB\(_2\)) and at least two endocannabinoids, anandamide and 2-arachidonoylglycerol, have been identified (5, 24). Anandamide, being the most extensively investigated, was present in peripheral tissues such as the spleen, heart, kidney, gut, cultured endothelial cells, and macrophage cell lines (4, 8, 25, 29). Elevation of circulating levels of anandamide and increased vascular CB\(_1\) receptor expression has been reported in cirrhotic humans and animals (1, 9, 37). Acute administration of a CB\(_1\) receptor antagonist reduced the elevated mesenteric blood flow and portal pressure, indicating that increased production of anandamide may play a role in splanchnic vasodilatation in CCL\(_4\)-induced cirrhotic rats (1). The mechanism underlying these vascular effects of anandamide has been proposed to involve in interactions with some vasoactive factors such as eicosanoids (10, 12, 27).

It has been suggested that an increase in the hepatic protein levels of cyclooxygenase (COX) and lipoxygenase (LOX) and the production of vasconstrictive eicosanoids play important roles in the increased IHR in the cirrhotic liver (13, 15). In fact, anandamide has been found to induce COX-2 expression and to release eicosanoids in several cell types (7, 18, 22, 32). However, the potential contribution of anandamide to the increased IHR in the cirrhotic liver has not been investigated. The aims of the present study was to evaluate the possible roles of anandamide in the intrahepatic microcirculation of cirrhotic livers and its interaction with the production of vasconstrictive eicosanoids.

MATERIALS AND METHODS

**Animals**

Adult male Sprague-Dawley rats (250–350 g) were used in all experiments. Cirrhosis with portal hypertension was produced by common bile duct ligation (CBL) as previously described (21, 44). Sham-operated (sham) rats had their bile ducts exposed but not ligated. All rats were caged at 24°C, with a 12:12-h light-dark cycle, and allowed free access to food and water. Animal studies were approved by the Animal Experiment Committee of the university and conducted according to the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Science (Washington, DC).

**Study Protocol**

About 30 days after surgery, three sets of CBL and sham rats were included in the study. All rats were fasted for 18 h before experiments. In this study, COX, 5-LOX, and thromboxane A\(_2\) (TXA\(_2\)) synthase...
Experiment 1: Western blot analysis for COX-1, COX-2, and 5-LOX in livers. Liver sections obtained from the first set of animals were used to measure various protein expressions \((n = 7\) in each group). Liver microsomal fractions were prepared as previously described \((44)\). In brief, part of each liver was sliced off and homogenized in a solution containing 15 mM KCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 10 mM EDTA, and 50 mM Tris and protease inhibitors \((20 \mu M\) peptatin, 20 \(\mu M\) leupeptin, 1,000 U/ml aprotinin, and 1 mM PMSF) using a Potter-type Teflon glass homogenizer. Cells were solubilized by vigorous vortexing and sonication. The insoluble material in the homogenized solution was pelleted by centrifugation at 10,000 \(g\) for 15 min. The supernatant was then centrifuged at 105,000 \(g\) for 60 min. A pellet of microsomes was suspended in the homogenized solution and stored at \(-70^\circ C\) until used. Protein concentrations were determined by the Bradford method. The purified microsomal protein \((60 \mu g)\) was loaded into SDS-PAGE gels \((4\%\) stacking and 10\% resolving gels) and electrophoresed. After fractionation, proteins were electrophoretically transferred to nitrocellulose membranes and probed with primary \(\beta\)-anandamide-stimulated TXB2 productions were measured \((46)\). Thirty minutes later, concentration-response curves for PPP in the recirculating system were begun to estimate the basal production of eicosanoids. Thereafter, liver perfusates were obtained again after sequential doses of anandamide administration \((70\text{ min time point})\) to estimate anandamide-stimulated eicosanoid productions. The samples were stored at \(-80^\circ C\). To measure cysteinyl leukotrienes \((\text{Cys-LTs})\), methanol \((1.5 ml)\) was immediately added to the samples. Thromboxane \(B_2\) \((\text{TXB}_2)\), 6-keto-PGF\(_{1\alpha}\), and Cys-LT productions were quantified in duplicate using a commercially available EIA kit. Cys-LTs represent the sum of leukotriene \(C_4\), leukotriene \(D_4\), and leukotriene \(E_4\). TXB\(_2\) and 6-keto-PGF\(_{1\alpha}\), respectively, are the end metabolites of TXA\(_2\) and PGL\(_2\), respectively. The basal production of eicosanoids was expressed in picograms per milliliter per minute per liver weight. The extent of the anandamide-stimulated eicosanoid production was expressed as absolute changes \((\Delta)\) in picograms per milliliter per minute per liver weight from the basal level.

Experiment 3: effect of the TXA\(_2\)S inhibitor furegrelate in PPP concentration-response curves to anandamide and TXB\(_2\) production. The third set of CBL and sham rats \((n = 7\) in each group) was included in this experiment. The specific TXA\(_2\)S inhibitor furegrelate [5-(3-pyridinyl-methyl)bensofurancarboxylic acid, 1 mg/100 g body wt \((9\text{ M})\)] was added in the perfusate to inhibit TXA\(_2\) production, and cysteinyl leukotriene release in liver perfusates after preincubation with indomethacin or piriprost. Liver perfusates used in this experiment were obtained from the second set of livers \((n = 7\) in each group) in experiment 2.1. Three milliliters of the perfusate were obtained before \((0\text{ min time point})\) and 30 min \((30\text{ min time point})\) after the recirculating system was begun to estimate the basal production of eicosanoids. Thereafter, liver perfusates were obtained again after sequential doses of anandamide administration \((70\text{ min time point})\) to estimate anandamide-stimulated eicosanoid productions. The samples were stored at \(-80^\circ C\). To measure cysteinyl leukotrienes \((\text{Cys-LTs})\), methanol \((1.5 ml)\) was immediately added to the samples. Thromboxane \(B_2\) \((\text{TXB}_2)\), 6-keto-PGF\(_{1\alpha}\), and Cys-LT productions were quantified in duplicate using a commercially available EIA kit. Cys-LTs represent the sum of leukotriene \(C_4\), leukotriene \(D_4\), and leukotriene \(E_4\). TXB\(_2\) and 6-keto-PGF\(_{1\alpha}\), respectively, are the end metabolites of TXA\(_2\) and PGL\(_2\), respectively. The basal production of eicosanoids was expressed in picograms per milliliter per minute per liver weight. The extent of the anandamide-stimulated eicosanoid production was expressed as absolute changes \((\Delta)\) in picograms per milliliter per minute per liver weight from the basal level.

Chemicals

Anandamide, piriprost, furegrelate, COX isoform, and 5-LOX polyclonal antibodies and EIA kits for TXB\(_2\), 6-keto-PGF\(_{1\alpha}\), and Cys-LTs were purchased from Cayman Chemical (Ann Harbor, MI). Substances other than those described above were purchased from Sigma Chemical. Indomethacin was freshly prepared in a 100 mM sodium carbonate buffer (pH 11.4). Piriprost was freshly prepared in DMSO. Furegrelate was dissociated in saline. Control experiments indicated that all the solvent used in this study had no direct effect on PPP and eicosanoid productions at these concentrations.

Statistical Analysis

Data are given as means ± SE. Statistical significance in each group was tested using one-way ANOVA with individual means detected by the Student-Newman-Keuls test. When criteria for parametric testing were violated, the appropriate nonparametric test \((\text{Mann-Whitney U-test})\) was used. Concentration-response curves of PPP to anandamide were analyzed by repeated-measures ANOVA followed by a post hoc test \((\text{Games and Howell variant of the Turkey and t-test})\) to compare groups at each dose separately. Protein levels
between the two groups were compared using Student’s t-test. Significance was determined at $P < 0.05$.

RESULTS

All cirrhotic rats had portal hypertension, ascites, and splenomegaly by gross inspection. Body weight was not different between CBL (407 ± 13 g) and sham (378 ± 22 g) rats. Liver weight was greater in CBL (29.3 ± 2.2 g, $P < 0.01$) than sham (18.5 ± 0.9 g) rats.

Experiment 1: Western Blot Analysis for COX-1, COX-2, and 5-LOX in Livers

Hepatic protein levels of COX-2 and 5-LOX were higher in CBL than sham rats (Fig. 1, B and C). However, COX-1 protein expression was not different between normal and cirrhotic livers (Fig. 1A).

Experiment 2.1: PPP Concentration-Response Curves to Anandamide after Preincubation of Livers with Indomethacin or Piriprost

Overall, basal PPP and calculated IHR [PPP: 19.7 ± 1.8 (CBL) vs. 8.9 ± 0.7 (sham) mmHg, $P < 0.01$; IHR: 17.8 ± 4.7 (CBL) vs. 4.7 ± 0.7 (sham) mmHg·min⁻¹·g liver wet wt⁻¹, $P < 0.01$] were higher in cirrhotic livers than in normal livers. In addition, baseline PPP in the cirrhotic liver after preincubation with vehicle (20.1 ± 2.0 mmHg) was no different from the cirrhotic liver after preincubation with indomethacin (19.4 ± 1.3 mmHg), piriprost (19.8 ± 1.2 mmHg), or furegrelate (18.7 ± 3.2 mmHg). Similarly, baseline PPP in the normal liver after preincubation with vehicle (9.2 ± 2.5 mmHg) was no different from those after preincubation with indomethacin (8.9 ± 0.8 mmHg), piriprost (8.7 ± 0.5 mmHg), or furegrelate (9.3 ± 0.4 mmHg). In the vehicle-preincubated group, anandamide produced concentration-dependent increases in PPP in all livers. Magnitudes of the increases in PPP were significantly greater in cirrhotic livers than in normal livers with each concentration of anandamide (Fig. 2, A–C). Preincubation with indomethacin significantly attenuated the increased response of cirrhotic livers to anandamide (a rightward shift of the concentration-response curves of PPP, $P < 0.05$; Fig. 2A). However, preincubation with piriprost, a 5-LOX inhibitor, did not significantly change concentration-response curves of PPP to anandamide in cirrhotic livers (Fig. 2B). In normal livers, concentration-response curves of PPP to anandamide were not affected by preincubation with either indomethacin or piriprost (Fig. 2, A and B).

Experiment 2.2: Anandamide-Stimulated Intrahepatic TXB₂, 6-Keto-PGF₁α, and Cys-LT Release in Liver Perfusates after Preincubation with Indomethacin or Piriprost

Basal productions of TXB₂, 6-keto-PGF₁α, and Cys-LTs were significantly higher in cirrhotic livers than in normal livers (Fig. 3, A–C). In the vehicle-preincubated group, cumulative doses of anandamide caused a significant increase in the production of TXB₂ and Cys-LTs in the perfusate (Fig. 4, A–C). The absolute increases in TXB₂ and Cys-LTs were significantly greater in cirrhotic livers than in normal livers (sham vs. CBL: 184 ± 84 vs. 2,469 ± 654 pg·ml⁻¹·min⁻¹·g liver wet wt⁻¹ for TXB₂ and 205 ± 82 vs. 441 ± 91 pg·ml⁻¹·min⁻¹·g liver wt⁻¹ for Cys-LTs, $P < 0.01$; Fig. 4, A and C). However, anandamide only induced a little increase of 6-keto-PGF₁α in both normal and cirrhotic livers (sham vs. CBL: 12.6 ± 7.5 vs. 14.0 ± 6.1 pg·ml⁻¹·min⁻¹·g liver wt⁻¹, Fig. 4B). Preincubation with indomethacin or piriprost significantly inhibited anandamide-related TXB₂ and Cys-LT productions, indicating that the doses used in this study were adequate to block the increased COX and 5-LOX activity in cirrhotic livers (Fig. 4, A and C).

Experiment 3: Effect of the TXA₂S Inhibitor Furegrelate in PPP Concentration-Response Curves to Anandamide and TXB₂ Production

Preincubation with furegrelate significantly ameliorated the anandamide-related increase in PPP in cirrhotic livers (Fig. 2C) as well as the reduction in anandamide-stimulated TXB₂ production (Fig. 4A).

DISCUSSION

It has been reported that hepatic protein levels of COX and 5-LOX and the corresponding production of TXA₂ and Cys-LTs were increased in cirrhotic livers (13, 15). Meanwhile,
increased intrahepatic production of TXA₂ and Cys-LTs has been demonstrated to play an important role in increased IHR in cirrhosis (13, 15, 46). In line with these observations, the present study shows that protein levels of COX-2 and 5-LOX in cirrhosis (13, 15, 46). In line with these observations, the present study shows that protein levels of COX-2 and 5-LOX were significantly higher in cirrhotic livers than in normal livers (Figs. 1 and 3). However, protein levels of COX-1 and 6-keto-PGF₁α production in the perfusate were similar between normal and cirrhotic livers. Previous studies have demonstrated that, although higher hepatic COX-2 mRNA expression was observed in cirrhotic livers than in normal livers, COX-1 plays a major role in increased IHR in cirrhotic livers and is the main source responsible for the increased hepatic production of TXA₂ (13, 14, 46).

It has been suggested that certain actions of the cannabinoids could be explained by the mechanism involving the synthesis of eicosanoids (7, 18, 22, 32). In bovine coronary arteries, cannabinoids induced vasorelaxation by the release of vasodilatory eicosanoids (32). An in vitro study (20) of smooth muscle from the guinea pig distal colon demonstrated a contractile response of cannabinoids after the release of eicosanoids. Eicosanoids are the major products of arachidonic acids, which can be divided into two groups according to their vasodilator or vasoconstrictor effects (38). To our knowledge, the effects of anandamide in increased IHR and intrahepatic eicosanoid productions in cirrhosis have not been established. Our data demonstrated that anandamide induced a significantly greater increase in IHR in cirrhotic livers than in normal livers (Fig. 2). In other words, the cirrhotic livers may exert a hyperresponse to anandamide. It has been shown that the plasma levels of anandamide drawn from a peripheral vein were higher in cirrhotic patients than in healthy subjects (9). Theoretically, the concentration of anandamide in the portal vein should also be increased in cirrhotic patients or animals, although there are no data reporting the concentration of anandamide in portal venous blood. In the present study, using the liver perfusion model, the cumulated increase in anandamide dosage in the perfusate may be similar to the elevated concentration of anandamide in portal venous blood in vivo. Accordingly, the present study suggested that anandamide plays, in part, a role in increased IHR in the cirrhotic liver.

It is of interest to observe the existence of different hemodynamic characteristics in the peripheral and intrahepatic circulation in cirrhosis. The systemic and splanchnic vasculatures exhibit marked vasodilatation, but the intrahepatic microvasculature displays marked vasoconstriction in cirrhosis (3, 43). PGF₂α, TXA₂, and Cys-LTs are metabolites of arachidonic acid, which is released by chemical and mechanical stimuli (28). Under normal physiological conditions, normal vascular tone is maintained by the balance between the activity of vasodilators and vasoconstrictors. In cirrhosis with portal hypertension, anandamide acts as a vasodilator in the peripheral circulation, as previously reported (1, 7, 27), whereas it serves as a vasoconstrictor in the hepatic microcirculation, as shown in the present study.

A recent study (6) has reported that dilatation of rabbit cerebral arterioles by anandamide, possibly via the production of vasodilatory prostanooids, is blocked by indomethacin. In this...
study, preincubation with both indomethacin (a COX inhibitor) and piriprost (a 5-LOX inhibitor) attenuated the anandamide-related increase in IHR in cirrhotic and normal livers. However, the magnitude of changes reached statistical significance only in cirrhotic livers with indomethacin preincubation. In addition, preincubation of indomethacin decreased TXB₂ production, whereas 6-keto-PGF₁α production remained unchanged. Moreover, preincubation with furegrelate (a TXA₂S inhibitor) caused an attenuation of the anandamide-induced increase in IHR and a decrease in TXB₂ production, which is similar to that observed in the cirrhotic liver with indomethacin preincubation. Together, these results indicate that the increase in IHR by anandamide in cirrhotic livers was mainly mediated by the increase in COX activity with subsequent TXA₂ production.

A variety of receptor-mediated (CB₁ and CB₂ receptor) effects of cannabinoids have been reported (18). Anandamide can induce an EDHF-mediated relaxation response, via the vascular CB₁ receptor, in isolated rat mesenteric and coronary arteries (33, 35). Several investigations have recently reported that anandamide production can be stimulated during hemorrhagic shock or by the administration of bacterial lipopolysaccharide (41, 42). In addition, the increased anandamide production was mostly from monocytes, because separated mono-

Fig. 3. Production of thromoxane B₂ (TXB₂; A), 6-keto-PGF₁α (B), and cysteinyl leukotrienes (Cys-LTs; C) in perfusates of cirrhotic and normal livers. #P < 0.01 vs. sham.

Fig. 4. Anandamide-stimulated (1.44 × 10⁻¹⁰-1.44 × 10⁻³ M) absolute increases (Δ) in hepatic TXB₂ (A), 6-keto-PGF₁α (B), and Cys-LT (C) production. #P < 0.05 vs. S-V; *P < 0.05 vs. CBL-V.

cytes administered to a euvoletic recipient animal caused a marked hypotensive effect (41, 42). Moreover, the hypotensive effect induced by the administration of monocytes from cirrhotic patients or cirrhotic rats to normal rats can be prevented by pretreatment with specific CB₁ receptor antagonists (1). Recent studies (16, 19, 23) have demonstrated that CB₁ and CB₂ receptors are marked upregulated in stellate cells of cirrhotic livers. In contrast, several effects of cannabinoids are not mediated via cannabinoid receptors such as inhibition of L-type Ca²⁺ channels, stimulation of vanilloid VR₁ receptors, transient changes in intracellular Ca²⁺, and disruption of gap junction function (18). However, data on the effects of cannabinoids in cirrhotic livers are limited, and more studies are needed to elucidate the role of cannabinoids in cirrhotic livers (2, 31, 34).

In summary, this study suggests that anandamide contributes in part to increased IHR in cirrhotic livers. In addition, the anandamide-related increase in IHR in cirrhotic livers may be mediated by increased production of COX-derived eicosanoids (mainly TXA₂).
REFERENCES


