Mild increases in portal pressure upregulate vascular endothelial growth factor and endothelial nitric oxide synthase in the intestinal microcirculatory bed, leading to a hyperdynamic state

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The partial portal vein ligation (PVL) model has been shown to reproduce the systemic and hemodynamic abnormalities in PHT (37). Furthermore, its highly predictable chronobiology has allowed the elucidation of the sequences of events leading to hyperdynamic circulation (8, 33). Thus this model also offers the opportunity to sequentially evaluate the molecular mechanisms leading to the hemodynamic abnormalities observed in hyperdynamic circulation. In that regard, a recent study from our laboratory suggested that, in the commonly used PVL model, in which portal vein stenosis is calibrated over a 20-gauge needle (37), the myogenic response that occurs in the superior mesenteric artery (SMA) in response to acute and severe PHT triggers NO hyperproduction (36). In contrast, milder increases in portal pressure (PP) (calibrating PVL over the wider 18-gauge needle) did not result in reflex SMA vasoconstriction, and eNOS was not upregulated at the SMA (36). However, it is unknown whether, in this latter situation, which is more similar to what occurs initially in chronic liver disease, other signals located at more sensitive areas (i.e., the SMA microcirculation in the intestine) might activate NO production and lead to the hyperdynamic circulatory state.

The present study was designed 1) to explore whether rats with mild PHT (as in the early stages of chronic liver disease), which does not induce myogenic reflex vasoconstriction (as in acute and severe PHT) in SMA beds, still develop the hyperdynamic circulatory state, indicating that other signals are able to activate NO production in this syndrome; and 2) if this is the case, to elucidate which molecular signals might initiate the cascade of events leading to the hyperdynamic circulation in mild PHT. For these purposes, we developed a surgical model of stepwise, fixed increases in PP that was achieved by performing PVL over needles of different diameters.

METHODS

All experiments were performed in male Sprague-Dawley rats (Harlan Sprague-Dawley Laboratories; Indianapolis, IN) weighing 325–350 g. All procedures in this study were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the local Committee for Animal Research.
Surgical Procedures

Partial PVL and sham operation. PHT was induced surgically in aseptic conditions. Briefly, the portal vein was freed from the surrounding tissue after a midline abdominal incision was made. A ligature (silk gut 3-0) was placed around a 16-gauge (PVL-16G), 18-gauge (PVL-18G), or 20-gauge (PVL-20G) blunt-tipped needle lying along the portal vein. Subsequent removal of the needle yielded a calibrated stenosis of the portal vein. The diameters of the needles, and thus those of the stenosis, were as follows: 16-gauge, 1.651 mm; 18-gauge, 1.270 mm; and 20-gauge, 0.889 mm. In sham-operated rats, the same operation was performed, except that after the portal vein was isolated, no ligature was placed.

Hemodynamic measurements. Experiments were performed while the rats were kept under ketamine (100 mg/kg im) and diazepam (10 mg/kg im) anesthesia in a nonfasting state. Mean arterial pressure (MAP) and PP were measured by catheterization of the right femoral artery and ileocolic vein, respectively. Pressure was transmitted through a Hewlett-Packard transducer and recorded continuously. Mean arterial pressure (MAP) and PP were measured by catheterization of the right femoral

| Table 1. Baseline characteristics and systemic and splanchnic hemodynamics 4 days after sham or PVL surgery |
|---------------------------------------------------------|--------------------------------------------------|-------------------|-------------------|-------------------|-------------------|
| Weight, g                                               | 337±8                                           | 330±5             | 342±9             | 334±7             | NS                |
| Spleen/body wt                                          | 2.05±0.06                                       | 2.36±0.09*        | 2.61±0.15*        | 2.63±0.09*        | P = 0.0006        |
| Portal pressure, mmHg                                    | 9.29±0.14                                       | 10.93±0.33*       | 13.52±0.85*       | 15.25±0.86*       | P < 0.000001     |
| Portosystemic shunting, %                                | Below sensitivity                               | 10±4*             | 47±17*            | 69±15*            | P = 0.000003     |
| MAP, mmHg                                                | 145±1                                           | 129±4*            | 121±4*            | 123±5*            | P = 0.00001      |
| CI, ml/min⁻¹·100 g body wt⁻¹                              | 27.90±1.27                                      | 32.40±1.07*       | 36.43±2.14*       | 42.21±2.61*       | P = 0.000001     |
| SVR, mmHg·mmHg⁻¹·100 g body wt⁻¹                         | 5.34±0.27                                       | 4.06±0.19*        | 3.40±0.28*        | 2.95±0.17*        | P < 0.000001     |

Values are means ± SE; n, no. of animals. PVL, portal vein ligation; PVL-16G, PVL-18G, and PVL-20G, PVL with a 16-, 18-, or 20-gauge needle, respectively; MAP, mean arterial pressure; CI, cardiac index; SVR, systemic vascular resistance; NS, not significant. *P < 0.05 vs. sham-operated animals.

Fig. 1. Portal vein ligation (PVL) surgery performed over different needles of increasing caliber [16- (16G), 18- (18G), and 20-gauge (20G)] yielded rats with different degrees of portal pressure (PP) and portosystemic shunting (PSS). *P < 0.05 vs. sham.

Fig. 2. Systemic hemodynamics in rats with different degrees of stenosis of the portal vein. MAP, mean arterial pressure; CI, cardiac index; SVR, systemic vascular resistance; gbw, grams of body weight. *P < 0.05 vs. sham.
mistor was placed in the aortic arch through the left carotid artery, and the thermal indicator (100 μl of normal saline maintained at a temperature of 15°C or more below body temperature) was injected into the right atrium through a polyethylene-50 catheter placed into the right jugular vein. The aortic thermistor was connected to a CO computer (Columbus Instruments, Columbus, OH). Body temperature was maintained at 37.0 ± 0.2°C. At least three thermodilution curves were obtained for each CO measurement. Those curves with unusual morphology were discarded. A typical curve had a rapid upslope and a smooth decay. The final CO value was obtained from the arithmetic mean of at least three CO measurements. The cardiac index (CI) was calculated as CO per 100 g body wt. Systemic vascular resistance (SVR) was calculated from MAP divided by CI. Right atrial pressure was regarded as negligible. SMA blood flow (in ml/min) was measured using a nonconstrictive perivascular ultrasonic transit-time flow probe (1RB, 1-mm diameter; Transonic Systems, Ithaca, NY) placed around the vessel close to its aortic origin. The flow probe was connected to a small animal flowmeter (Transonic Systems). Resistance in the SMA (mmHg·ml⁻¹·min⁻¹·100 g body wt⁻¹) was calculated as (MAP – PP)/SMA blood flow. All hemodynamic readings were monitored and saved on a computer using the analog-to-digital PowerLab/MacLab system (ADInstruments, Milford, MA) with Chart 3.6 software (ADInstruments).

Portosystemic shunting. PSS was determined using the technique described by Chojkier and Groszmann (7) and substituting color for radioactive microspheres; 30,000 of 15-μm yellow microspheres (Dye Track; Triton Technology, San Diego, CA) were slowly injected into the spleen. The rats were euthanized, and the livers and lungs were dissected and placed into new polypropylene centrifuge tubes. The number of microspheres in each tissue was determined following the protocol provided by the manufacturer. In brief, 3,000 blue microspheres (Dye Track; Triton Technology) were added to each tube as an internal control. Tissue was digested overnight with 1 M KOH at 60°C and thoroughly sonicated. After centrifugation, the supernatant was removed, and the pellet was washed once with 10% Triton X-100 and twice with acidified ethanol. At the end of the process, a minimum pellet containing the microspheres was allowed to dry overnight. The color of the microspheres was diluted with 200 μl of acidified Cellosolve acetate (Spectrum Chemicals, Gardens, CA). The absorbance of the solution was read at 448-nm wavelength (yellow) and 670-nm wavelength (blue) in a spectrophotometer (Shimadzu, Columbia, MD), and the number of microspheres was calculated by com-

Fig. 3. Correlation between PP and hemodynamic parameters in rats with different degrees of stenosis of the portal vein (sham rats were excluded from the plot and analysis).

Fig. 4. Systemic (A) and splanchnic (B) hemodynamics 24 h after PVL-16G or sham surgery. SMABF, superior mesenteric artery (SMA) blood flow; SMAVR, SMA vascular resistance; ns, not significant.
parison with standards. Spillover between wavelengths was corrected with the matrix inversion technique. PSS was calculated as lung microspheres/(liver microspheres + lung microspheres). Assuming a worst-case scenario in which two-thirds of the microspheres remain trapped in the spleen, this technique can detect a minimum shunt of 3.5%. Studies using color microspheres have been shown to provide results similar to those using radioactive microspheres (19).

Western blot analysis SMA and superior mesentary veins (SMV) were harvested after freeing the vessels from the surrounding tissue at a length of 2–3 cm. A 5-cm portion of the jejunum was harvested from the area corresponding to the 15–20 cm cranial to the cecum. All tissues were immediately snap frozen in liquid nitrogen and kept at −70°C until analyzed. Vessels and jejunum samples were homogenized in a lysis buffer containing 50 mM Tris-HCl, 0.1 mM EGTA, 0.1 mM EDTA, 5 mM sodium fluoride, 1 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, protease inhibitor cocktail tablet (Roche Diagnostics; Mannheim, Germany), 1% (vol/vol) Nonidet P40, 0.1% SDS, and 0.1% deoxycholate; pH 7.5. Jejunum tissue was crushed to powder while frozen before it was added to the lysis buffer. The protein concentration in the supernatants was quantified using the Lowry assay, and equal amounts of protein from each sample were loaded by SDS-PAGE and electroblotted onto nitrocellulose membranes. Equal loading was ensured by Ponceau staining. Membranes were probed with an antibody that recognizes eNOS (Transduction Laboratories, Lexington, KY), phosphorylated eNOS at Ser1177 (Cell Signaling Technology, Beverly, MA), VEGF (Santa Cruz Biotechnology, Santa Cruz, CA), and β-actin (Sigma, St. Louis, MO). After being washed, membranes were probed with secondary antibodies conjugated with fluorescent markers, and quantification of bands was performed the Odyssey Infrared Imaging System (Li-Cor Biotechnology; Lincoln, NE). β-Actin expression was used for standardization.

Immunofluorescence. A small portion of the jejunum was removed, oriented in OCT (Ted Pella, Redding, CA) on a plastic mold, and frozen in dry ice. Ten-micrometers-thick cryosections were cut using a cryostat, mounted on slides, and fixed in −20°C cold acetone for 10 min. Tissue was blocked with 5% goat serum plus 1% BSA in PBS for 45 min at room temperature, followed by an overnight incubation with primary antibody (polyclonal anti-VEGF and monoclonal anti-PECAM) at 4°C. Alexa Fluor 488-labeled anti-rabbit (diluted 1:500) or anti-mouse (diluted 1:500) secondary antibodies were incubated for 45 min at room temperature. Tissue sections were counterstained with rhodamine-labeled phalloidin (1:100) and DAPI, an indicator for nucleus. Slides were observed using a Zeiss Axiovert 200 fluorescence microscope (Carl Zeiss MicroImaging; Thornwood, NY), and images were captured using Openlab3 software (Improvision, Lexington, MA).

Drug administration. SU5416 (Calbiochem, San Diego, CA) was dissolved in DMSO to a concentration of 0.285 mg/ml and administered as a single 5 mg/kg dose through the penile vein. This dose achieves therapeutic plasma levels in the rat (35), which result in prolonged inhibition of the VEGF receptor (27).

Statistical Analysis

Results are expressed as means ± SE. Statistical analysis was performed with SPSS 12.0 statistical software (SPSS, Chicago, IL). Results were assessed using one-way ANOVA followed by pre-

Fig. 5. Expressions of endothelial nitric oxide synthase (eNOS), phosphorylated (P)-eNOS, and VEGF are upregulated in the jejunal microcirculation but not in the SMA or superior mesenteric vein (SMV) 24 h after PVL-16G or sham surgery (n = 5 rats/group). AU, arbitrary units.
planned contrast analysis to compare each group of PVL rats with sham rats. Linear trends were using polynomial contrasts (3). Unpaired t-test or Mann-Whitney test was used in those experiments comparing two groups of rats. A two-tailed P value of <0.05 was considered statistically significant.

RESULTS

Hyperdynamic Circulation in Rats with Mild PHT

To evaluate whether rats with mild PHT develop a hyperdynamic circulatory state and PSS, 24 rats underwent portal vein ligation over a 16- (n = 12), 18- (n = 6), or 20-gauge needle (n = 6) or sham operation (n = 10). Four days after surgery, PVL performed over 16-, 18-, and 20-gauge needles yielded increasing degrees of PHT and PSS (Table 1 and Fig. 1). PSS was not detectable in any sham rats. All three groups of PVL rats showed features of the hyperdynamic circulatory state: CI was significantly higher and SVR and MAP were significantly lower in all three groups of PVL rats compared with sham rats (Table 1 and Fig. 2). The magnitude of these changes, indicators of hyperdynamic circulation, were proportional to the degree of PHT (all linear trends P < 0.00005). Furthermore, MAP, CI, and SVR significantly correlated with PP (Fig. 3). These results suggest that the development of hyperdynamic circulation is not an all-or-nothing phenomenon but occurs gradually from the initial stages of PHT.

Early Molecular Vasodilatory and Angiogenic Signals Activated by Mild PHT

NO (41) and VEGF (12) are the main mediators described thus far to explain, respectively, the vasodilation and angiogenesis that occur in PHT. Because PVL-16G rats exhibited a mild degree of PHT, we investigated whether even mild increases in PP are able to activate these signals in this model, and, if that is the case, what is the molecular mechanism that activates these signals? For that

Fig. 6. Immunofluorescent staining of the jejunum in sham and PVL-16G rats 24 h after surgery. VEGF expression (green) was localized at the intestinal mucosa (A) in highly vascularized areas, as shown by PECAM staining (green; B). Tissue structure was oriented by F-actin staining (red) and nucleus (blue).
purpose, 10 rats underwent sham surgery (n = 5) or PVL-16G (n = 5). Twenty-four hours after surgery, PP was higher in PVL-16G rats than in sham rats. There was no difference in systemic hemodynamics between the two groups, and, in keeping with our previous study (36), there were no significant differences in SMA blood flow or SMA vascular resistance (Fig. 4). PVL-16G rats showed increased VEGF (P = 0.007) and eNOS (P = 0.02) jejunum expression compared with sham rats. Furthermore, eNOS phosphorylation, an active form of eNOS, was detectable only in PVL-16G rats. In contrast, there was no difference in VEGF, eNOS, or eNOS phosphorylation at the SMA trunk or at the SMV between sham and PVL-16 rats (Fig. 5). Immunofluorescence staining showed that VEGF was located predominantly at the mucosa in highly vascularized areas according to PECAM staining (Fig. 6). Taken together, these findings indicate that in mild PHT, there is activation of eNOS and VEGF selectively at the intestinal mucosa microvasculature, which precedes the development of vasodilation and the development of PSS.

Finally, because VEGF has been shown to increase eNOS expression (29), we aimed to determine whether initial eNOS upregulation in mild PHT is VEGF dependent. Twelve rats were treated with the VEGF receptor 2 inhibitor SU5416 (n = 6) or its vehicle (DMSO, n = 6) immediately after PVL-16G surgery. Four sham rats treated with DMSO were used as negative controls. Twenty-four hours after PVL, both groups of rats showed a similar increase in VEGF expression at the jejunal wall. However, in rats treated with SU5416, the increase in eNOS expression was markedly attenuated (P = 0.003; Fig. 7), but eNOS levels were still twice those of sham rats treated with DMSO (not significant). This indicates that VEGF upregulation in the intestinal microcirculation accounts, in large part, for the initial eNOS upregulation in mild PHT.

**DISCUSSION**

In this study, we demonstrated that even very mild increases in PP are enough to activate vasodilatory and angiogenic signals and, thus, to activate the cascade of events leading to hyperdynamic circulation and the development of PSS. Because these are the main features that account for the complications of PHT, the findings of this study are of importance not only to understand the pathophysiology of early PHT but also to rationally address the prevention of its complications. Additionally, we have shown that the intensity of the hemodynamic changes in the systemic circulation is proportional to the severity of PHT, further supporting the well-established concept (5) that PP is a meaningful target in the treatment of patients with chronic liver diseases even at the early stage.

Another novel and salient finding of this study is that, in mild PHT, eNOS is initially upregulated selectively at the intestinal mucosa microcirculation, an indicator that this vascular bed is the main site for the transduction of the increased PP signal into the molecular signals that account for vasodilation, the initial step in the development of hyperdynamic circulation (18, 31). Because mucosal arterioles account for 25% of the total mesenteric vascular resistance (16), NO activation at this level could have a marked impact in regional splanchnic hemodynamics.

We chose to study the expression of these molecules as early as 24 h after the induction of PHT because it is well established that, once hyperdynamic circulation is developed, other mechanisms such as shear stress increase eNOS expression (20), hindering the real initial mechanism that triggers initial NO overproduction. We also have demonstrated herein, confirming our previous data in severe PHT (43), that upregulation of eNOS precedes arterial vasodilation in the splanchnic circulation.

Recently, Fernández et al. (12) showed that VEGF has a major role in the development of portosystemic collateralization in a model of severe PHT, because inhibition of VEGF signaling decreased by >50% the development of PSS. In our study, we expanded these findings by showing that mild PHT is enough to activate VEGF at the intestinal mucosa and that this activation occurs before the development of portosystemic collateralization, because PSS was not detected 24 h after PVL (12, 33).

In addition to angiogenesis, VEGF is a well-known activator of eNOS and causes NO-dependent vasodilation (21, 25, 44). VEGF upregulates eNOS both at the transcriptional and posttranslational levels (15, 29). Indeed, VEGF increases eNOS
expression in endothelial cells in culture (29), and this effect is mediated via VEGF receptor 2 (23, 32). Thus, in the present study, we tested whether VEGF plays a role in increased eNOS expression in the intestinal microcirculation in mild PHT. Our data clearly indicate that VEGF is involved in eNOS upregulation in the intestinal microcirculation in mild PHT, because the inhibition of VEGF receptor 2 signaling with SU5416 markedly attenuated eNOS expression. These findings suggest that VEGF upregulation accounts, for the most part, for initial eNOS upregulation in mild PHT and could have potential therapeutic implications, because a number of VEGF blockers are already available for human use (14). In that regard, Fernández et al. (11) have recently shown in PVL rats that treatment with SU5416, initiated immediately after PVL and maintained for 5 days, markedly attenuated the hyperdynamic circulation of PHT. Treated rats showed a 45% decrease in portal blood inflow and a 30% decrease in CO compared with untreated rats, indicating that VEGF plays a role not only in the development of PSS but also in the pathophysiology of hyperdynamic circulation.

The present study raises an important question regarding the stimulus for VEGF and eNOS upregulation in the intestinal microcirculation in PHT. It has been shown that one important stimulus for VEGF expression is tissue hypoxia (13). Thus a possible mechanism could be hypoxia in the intestinal microcirculation in PHT. Although it has been demonstrated that a mild increase in mesenteric venous pressure does not decrease mesenteric blood flow, it alters the distribution of blood flow within the bowel wall, decreasing mucosal blood flow and increasing muscularis blood flow (17). This was further confirmed in an intravital microscopic study (9) in the rat intestine that demonstrated constriction of mucosal arterioles and dilatation of arterioles in the muscularis layer when venous pressure was elevated. After venous pressure elevation, the redistribution of flow within the bowel from the mucosa to the muscularis (17) may cause a certain degree of hypoxia in the mucosa that is sufficient to stimulate VEGF production. Although intestinal blood flow must decrease to 60–70% to impair oxygen exchange (4), a less-compromised blood flow could result in hypoxia during nutrient absorption periods that markedly increase oxygen consumption at the mucosal level (6). To further address this question, we investigated whether hypoxia-inducible factor (HIF)-1α (a transcription factor induced by hypoxia that upregulates VEGF) was involved in the intestinal upregulation of VEGF in mild PHT. A small portion of the intestine was isolated 24 h after PVL-16G or sham surgery, when VEGF is already induced. HIF-1α expression was assessed using Western blot analysis in both whole lysates and nuclear lysates of the intestine, because HIF-1α is highly localized in the nucleus in the hypoxic condition (38). In both preparations, the levels of expression were similar between two groups (data not shown). This observation, however, does not rule out (although it makes unlikely) the possibility of the involvement of HIF-1α in the upregulation of VEGF expression in our model, because HIF-1α induction may occur immediately after the generation of PHT but might be transient. Detailed studies specifically addressing dynamical mucosal oxygenation and HIF-1α expression would be required to explore this possibility.

The most likely mechanism that could be implicated in both eNOS and VEGF upregulation is circumferential vascular stretch, which indeed occurs in the intestinal microcirculation after an increase in venous pressure in the mesenteric system (9) and previously has been shown to activate VEGF (10, 26, 28, 30, 34) and eNOS (24). Moreover, the preferential distribution of VEGF expression in the mucosal vasculature may be due to a greater percentage change in mesenteric venous pressure being transmitted into the mucosal layer than into the muscularis (9).

In conclusion, mild increases in PP, which may be similar to what occurs in early cirrhosis, trigger the production of VEGF and an increase in eNOS expression in the intestinal microcirculation. These phenomena might be the early molecular signals that induce the cascade of events leading to the hyperdynamic circulatory state and the development of PSS in mild PHT.

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REFERENCES

12. Fernández M, Vizzutti F, García-Pagan JC, Rodes J, and Bosch J. Anti-VEGF receptor-2 monoclonal antibody prevents portal-systemic col-


25. Li B, Ogasawara AK, Yang R, Wei W, He CW, ZioniCheck TF, Bunting S, de Vos AM, and Jin H. KDR (VEGF receptor 2) is the major mediator for the hypotensive effect of VEGF. *Hypertension* 39: 1095–1100, 2002.


