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EXPRESSION OF ENDOTHELIN-1, AND ENDOTHELIN A AND B RECEPTORS IN PORTAL HYPERTENSIVE ESOPHAGUS OF RATS

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Nitric oxide synthase is overexpressed in the portal hypertensive (PHT) esophagus, suggesting that expression of other vasoactive mediators could also be affected. Therefore, in the present study we determined the expression of endothelin-1 (ET-1) and endothelin receptors, which could contribute to the regulation of the vascular tone in PHT esophagus. In esophageal specimens of PHT and sham operated rats, expression of ET-1 and its receptors A and B (ET_AR and ET_BR) mRNAs was studied by reverse transcription-polymerase chain reactions. ET-1 protein expression was assessed by immunostaining and enzyme immunoassay. In PHT esophagus, expression of ET-1, ET_AR and ET_BR mRNAs was significantly increased by 2.2-, 2.5- and 1.5-fold, respectively, compared with sham operated. The ET-1 protein was significantly increased by 2.2-fold vs. controls as measured by enzyme immunoassay. ET-1 protein was predominantly localized to endothelia of submucosal veins. Thus, portal hypertension induces over-expression of ET-1 in endothelia of esophageal submucosal vessels. Since ET-1 and its receptors could promote vascular proliferation and induce mucosal damage, the overexpressed ET-1 may play an important role in the development and rupture of esophageal varices in portal hypertension.

Key words: *portal hypertension, esophageal varices, endothelin-1, endothelin receptor.*

INTRODUCTION

In portal hypertension, a hyperdynamic splanchnic circulation is associated with collateralization of the portal system, leading to development of varices at various locations (1). The lower esophagus is a site at which varices are particularly prone to develop and rupture, but the mechanisms responsible for these processes have not been delineated.

Vascular and microvascular endothelium plays an important role in the regulation of microcirculatory hemodynamic. Endothelial cells synthesize and

release potent vasoactive substances such as vasodilators — prostacyclin and nitric oxide (NO) — and vasoconstrictors — endothelin-I (ET-1) and angiotensin-II (2). Recent studies suggested that increased synthesis and release of NO could account for the hyperdynamic circulation in portal hypertension (3, 4). Recently, we demonstrated overexpression of NO synthase in endothelia of submucosal veins in the portal hypertensive (PHT) rat esophagus (5). The overproduced NO, a potent vasodilator, could — at least in part — be responsible for the development of esophageal varices. Therefore, we postulated that vasoactive substances other than NO, e.g. ET-1, could also be overproduced. As a result, the interaction of these overproduced mediators may be the basis for development of esophageal varices in portal hypertension.

Endothelin-1 is a 21-amino acid peptide, initially noted for its powerful vasoconstrictor properties, but more recently recognized to have pleiotropic biological effects depending on its receptors (6). Specific endothelin receptors are divided into at least two types; endothelin A receptor (ET_AR) and endothelin B receptor (ET_BR). The ET_AR is localized in vascular smooth muscle and mediates vasoconstriction (7). The ET_BR is expressed predominantly in endothelial cells and mediates production of NO and prostacyclin (8–10). The ET-1 induced proliferation in cultured vascular smooth muscle cells is mediated by either ET_AR or ET_BR, depending on the smooth muscle phenotype (11, 12). Endothelin has been reported to be present in the rat gastrointestinal tract (13). However, the expression of ET-1 and its receptors in the PHT esophagus has not been explored. Therefore, the aim of this study was to determine the expression of ET-1 mRNA, ET receptor mRNA and ET-1 protein in the PHT esophageal mucosa.

MATERIALS AND METHODS

Animals

This study was approved by the Subcommittee on Animal Studies of the Veterans Administration Medical Center, Long Beach. Ninety Sprague-Dawley rats (250–300g) were used for the experiments. They were kept individually in wire bottom cages with free access to standard rat chow (Rodem Diet No. 8504; Harlan Teklad, Madison, WI, USA) and water. The animal room was illuminated on a 12 hours light-dark cycle. The room temperature was kept at 18–22°C and the humidity at 60–70%.

Forty-five rats were anesthetized with Nembutal 50 mg per kg intraperitoneally (i.p.) (Abbott Laboratories Ltd., North Chicago, IL, USA). Portal hypertension was produced by staged portal vein occlusion and splenic vein ligation as previously described (14). Forty-five sham operated rats (controls) underwent similar procedures without occluding the portal or splenic veins. After two weeks, rats were fasted for 24 hours and anesthetized with Nembutal 50 mg per kg i.p. Each rat underwent laparotomy and portal venous pressure was measured with a PE-50 catheter inserted through a peripheral mesenteric vein. The zero reference point was the inferior vena cava. Following pressure measurements, the lower esophagus (0.5 cm in length from esophago-gastric junction) was excised, and each specimen was divided longitudinally into two parts. One part was

immediately frozen in liquid nitrogen, then stored at -80°C for molecular biology and enzyme immunoassay, and another was fixed in 10% formalin or 4% paraformaldehyde solution for histochemical studies.

Quantitative histology

Esophageal specimens were fixed in 10% formalin, processed routinely and embedded in paraffin. Sections were stained with hematoxylin and eosin.

RNA isolation and reverse transcription polymerase chain reaction (RT/PCR) for ET-1 and endothelin receptors

Frozen esophageal specimens were homogenized with a Polytron homogenizer (Kinematica AG, Littau, Switzerland) in 4 mol/L guanidinium isothiocyanate, and total RNA was isolated using the guanidinium isothiocyanate-phenol-chloroform method (15). The total RNA concentration in each sample was determined from absorbance at 260nm, and the quality of each RNA preparation was determined by 1% agarose-formaldehyde gel electrophoresis and ethidium bromide staining.

Reverse transcription and polymerase chain reactions (RT/PCR) were carried out using a GeneAmp RNA polymerase chain reaction kit and a DNA thermal cycler (Perkin Elmer, Norwalk, CT, USA) according to standard techniques (16). Briefly, 0.3 μg of total RNA was used as the template to synthesize complementary DNA with 2.5 units of Moloney murine leukemia virus reverse transcriptase, in 10 μl of buffer containing 10 mmol/L Tris-HCl, pH 8.3; 50 mmol/L KCl; 5 mmol/L MgCl_2 ; 1 mmol/L each deoxyribonucleoside triphosphate; 2.5 mmol/L of random hexamer; and 1.4 unit/ μl of ribonuclease inhibitor. RT was performed at room temperature for 20 minutes, then at 42°C for 15 minutes, at 99°C for 5 minutes, and at 5°C for 5 minutes. The resulting complementary DNA was used as a template for subsequent PCR.

The PCR specific primer set used for rat ET-1 was

5'-CGTTGCTCCTGCTCCT-CCTTGATGG-3' (forward)

and

5'-AAGATCCCAGCCAGCATGGAGAGCG-3' (reverse) (17, 18).

The specific primer set used for rat ET_AR was

5'-TTCGTCATGGTA-CCCTTCGA-3' (forward)

and

5'-GATACTCGTTCCATTCATGG-3' (reverse) (19, 20).

The specific primer set used for rat ET_BR was

5'-TTCACCTCAGCAGGATTCTG-3' (forward)

and

5'-AGGTGTGGAAAGTTAGAACG-3' (reverse) (19, 21).

The PCR for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a positive control and an internal standard. The specific primer set for rat GAPDH (G3PDH Control Amplimer Set, Clontech Laboratories, Inc., Palo Alto, CA, USA) was

5'-ACCACAGTCCATGCCATCAC-3' (forward)

and

5'-TCCACCACCCTGTTGCT-GTA-3' (reverse) (22).

The PCR was performed in 50 μl of buffer containing 10 mmol/L Tris-HCl, pH 8.3; 2 mmol/L MgCl_2 ; 50 mmol/L KCl; 0.2 mmol/L each deoxyribonucleoside triphosphate; 0.25 μg of each primer; 2.5 μCi of [α - ^{32}P] dCTP; and 1.2 units of *Taq* DNA polymerase. In order to define the optimal amplification cycle, a series of pilot studies were performed using RT products from 0.3 μg

control RNA and 20–30 cycles of PCR amplification. Since the amplified products for all primer sets during 24–30 cycles were represented as bands on the autoradiography and densitometric intensity of these bands was linearly increased, the amplification of 28 cycles was used in this study. The temperature profile of amplification consisted of 94°C for 1 minute, 63°C (for ET-land GAPDH) or 54°C (for ET_AR, ET_BR and GAPDH) for 1 minute, and 72°C for 2 minutes. Ten-microliter aliquots of PCR-amplified mixture were electrophoresed using a 1.25% agarose gel. The gel was dried using a gel dryer (Model 543, BIO-RAD Laboratories, Hercules, CA) and subjected to autoradiography at room temperature for 1–3 hours. The identity of amplified cDNA products was confirmed by DNA sequence analysis (19). The intensity of bands on the x-ray film was measured by densitometric scanning (Ultrascan XL Laser Densitometer, Pharmacia LKB Biotechnology, Uppsala, Sweden). The signals of ET-1, ET_AR and ET_BR were standardized against GAPDH signal for each sample and results were expressed as ET-1 or endothelin receptor/GAPDH ratio.

Immunofluorescence staining

The esophageal specimens were fixed in 4% paraformaldehyde for 4 hours and subsequently transferred in 0.5 mol/L sucrose in phosphate-buffered saline for 24 hours. Then they were frozen at –80°C until cutting. Cryostat sections (10- μ m thick; Jung CRYOCUT 1800, Leica Inc., Deerfield, IL, USA) were treated with 0.05% saponin (Sigma Chemical Co., St. Louis, MO, USA) at room temperature for 30 minutes and incubated overnight with specific polyclonal antibodies against ET-1 (rabbit antiendothelin-1 IgG, Peninsula Laboratories, Fremont, CA, USA) diluted 1:100. Crossreactivity of this antiserum for ET-1, -2, -3, and big ET-1 was 100%, 7%, 7%, and 17%, respectively. For control studies, esophageal sections were also incubated overnight with phosphate-buffered saline instead of the primary antibody. After washing with phosphate-buffered saline, sections were incubated for 30 minutes with fluorescein-conjugated immunoglobulin (Sigma Chemical Co.) diluted 1:100. Immunofluorescence of coded esophageal sections was evaluated using a Nikon Optiphot epifluorescence microscope with B filter composition (Nikon Inc.). For the quantitative assessment of fluorescence intensity, a Nikon TMD Diaphot microscope connected to a video analysis system (Image-1/FL, Universal Imaging Corp., Westchester, PA, USA) was used (5, 23). The Image-1 system allows an image to be rapidly entered into computer memory in a fraction of second, eliminating the problem of fluorescence fading. This system distinguishes intensity of fluorescence on a scale of 0–255 units. All measurements of fluorescence intensity were made by two investigators unaware of the code. Fluorescence intensity in the endothelia of mucosal veins (collecting venules) and submucosal veins were measured in standardized rectangles under 400 \times magnification in 10 randomly selected fields of each section. Each measurement was standardized by subtracting the background intensity for each slide. All esophageal samples were processed and immunostained at the same time and fluorescence intensity was measured on coded sections during the same session and under the same conditions.

Enzyme immunoassay for ET-1

Esophageal specimens were homogenized with a Polytron (Kinematica AG) in 62.5mM EDTA, 50mM Tris, 0.4% deoxycholic acid and 1% NP40, and centrifuged at 15000rpm for 10 minutes at 4°C. The supernatants were collected for enzyme immunoassay for ET-1 and protein concentration assay.

ET-1 concentrations in the tissues were measured by a double-antibody sandwich technique (24), using the commercially available enzyme immunoassay kit (Endothelin-1 Enzyme Immunoassay Kit, Cayman Chemical Company, Ann Arbor, MI, USA). Briefly, 100 μ l of the supernatants or ET-1 standard and 100 μ l of acetylcholinesterase: endothelin Fab' conjugate were incubated overnight at 4°C in the plates pre-coated with a monoclonal antibody specific for ET-1 (endothelin

capture antibody). This allowed the two antibodies to form a 'sandwich' by binding on opposite sides of ET-1 molecule. Two hundred μ l of Ellman's reagent was added after the excess reagents were washed away. The plates were spectrophotometrically read at 412 nm, and ET-1 concentration in each sample was calculated from the standard curve. Intra-assay and interassay variations were 9% and 15%, respectively.

Protein concentrations were determined by the bicinchoninic acid protein assay (25), using the commercial kit (BCA Protein Assay Reagent, Pierce Chemical Company, Rockford, IL, USA).

Statistical analysis

The results were expressed as the mean \pm SD. Unpaired two-tailed Student's t-test was used to determine the statistical significance between PHT and sham operated rats. One-way analysis of variance and Bonferroni correction (26) were used for multiple comparisons. For three-group comparisons, a p value of <0.0167 ($0.05/3$) was required for a significant departure from the null hypothesis at the level of 0.05.

RESULTS

Portal venous pressure and cross-sectional area of submucosal vein

In PHT rats, portal venous pressure was significantly higher than in sham operated controls (25.3 ± 1.7 vs. 15.8 ± 1.2 cm H₂O, respectively, $p < 0.01$).

Expression of ET-1, ET_AR and ET_BR mRNA in PHT esophagus

The RT/PCR demonstrated expression of ET-1, ET_AR and ET_BR mRNA in PHT and sham operated esophagus (Figs. 1 and 2). Densitometric analysis showed that ET-1 mRNA level in PHT esophagus was significantly increased by 2.2-fold compared with sham operated controls ($p < 0.01$, Fig. 3). Expression of ET_AR and ET_BR mRNA in PHT esophagus was also significantly increased by 2.5- and 1.5-fold, respectively, compared with controls ($p < 0.01$ and $p < 0.05$, Fig. 3).

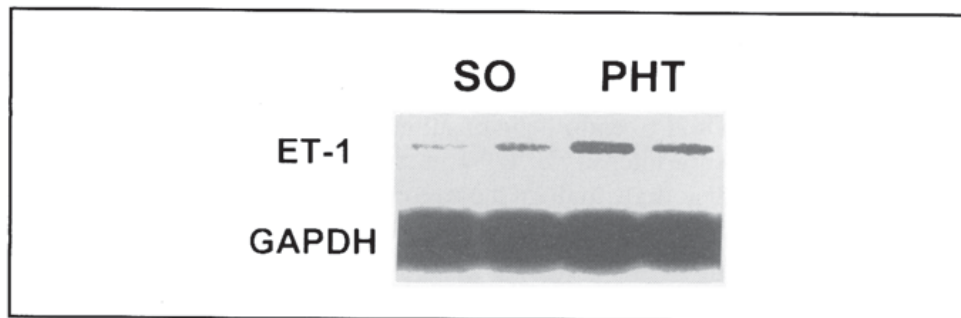


Fig. 1. Reverse transcription — polymerase chain reaction (RT/PCR) analysis of endothelin-1 (ET-1) mRNA in esophageal tissues of sham operated (SO) and portal hypertensive (PHT) rats. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) signal is an internal standard for each sample.

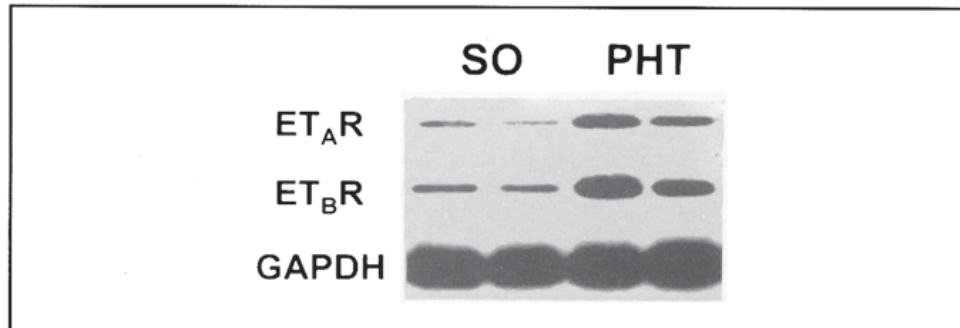


Fig. 2. Reverse transcription — polymerase chain reaction (RT/PCR) analysis of endothelin A receptor (ET_AR) and B receptor (ET_BR) mRNA in esophageal tissues of sham operated (SO) and portal hypertensive (PHT) rats. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) signal is an internal standard for each sample.

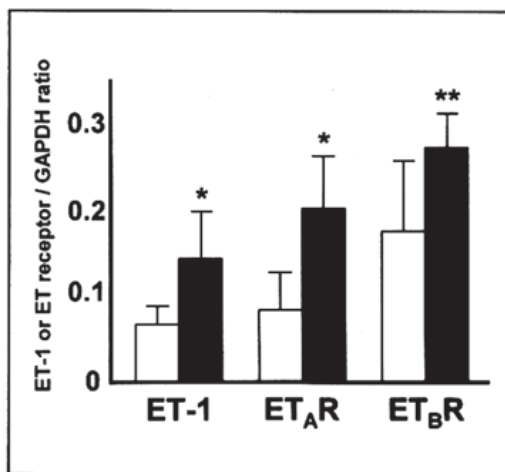


Fig. 3. Quantitative analysis of endothelin-1 (ET-1), and endothelin A receptor (ET_AR) and B receptor (ET_BR) mRNA in the esophageal tissues using densitometric scanning of amplified PCR products. Each ET-1 or endothelin receptor signal was standardized against the corresponding glyceraldehyde 3-phosphate dehydrogenase (GAPDH) signal and results were expressed as ET-1 or endothelin receptor/GAPDH ratio. The values are mean \pm SD (n = 7–8 animals per each group). * p < 0.01, ** p < 0.05 compared with sham operated controls. SO: sham operated rats, PHT: portal hypertensive rats.

Expression of ET-1 protein in PHT esophagus

The specific fluorescence signals for ET-1 were localized to the muscularis mucosae and propria and to endothelia of submucosal vessels in the esophagus (Fig. 4). The fluorescence intensity of ET-1 in endothelia of submucosal vessels was significantly higher than that in the muscularis mucosae and propria in each section (sham operated rats: endothelia of submucosal veins — 107.7 ± 21.1 vs muscularis mucosae — 56.0 ± 7.3 and muscularis propria — 67.7 ± 9.3 image units; p < 0.01. PHT rats: endothelia of submucosal veins — 202.7 ± 29.4 vs. muscularis mucosae — 63.1 ± 6.8 and muscularis propria — 62.4 ± 12.3 image units, p < 0.01). Signal intensity for ET-1 in endothelia of the esophageal submucosal veins in PHT rats was significantly stronger than in sham operated controls (p < 0.01).

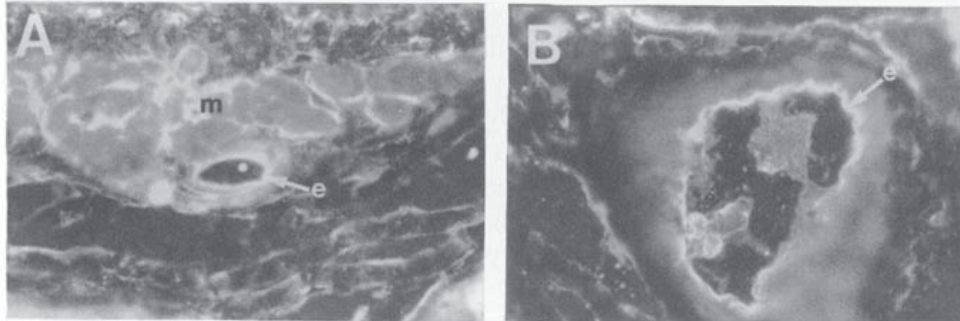


Fig. 4. Photomicrographs of immunofluorescence staining for endothelin-1 in the esophageal mucosa of sham operated (A) and portal hypertensive (B) rats. Staining is predominantly localized to endothelia of submucosal vessels. *m*: muscularis mucosae, *e*: endothelia of submucosal vein (magnification 400 \times).

Enzyme immunoassay demonstrated that ET-1 concentrations in PHT esophagus were significantly increased by 2.2-fold compared with sham operated controls ($p < 0.01$, *Fig. 5*).

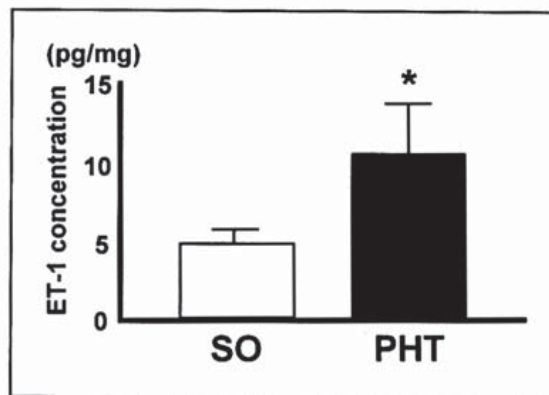


Fig. 5. Tissue concentration of endothelin-1 in sham operated (SO) and portal hypertensive (PHT) esophagus in the enzyme immunoassay. The values are mean \pm SD in pg/mg of total protein ($n = 6$ animals per each group). * $p < 0.01$ compared with sham operated controls.

DISCUSSION

In the present study, we demonstrated that portal hypertension activates ET-1, ET_AR and ET_BR genes in the esophagus. ET-1 protein was significantly overexpressed in the endothelia of submucosal veins in the PHT esophagus. In addition, as demonstrated by the immunostaining study, signal intensity of ET-1 fluorescence in endothelia of submucosal veins (which can reflect protein concentration in the endothelial cells) was increased by about 2-fold in portal hypertension compared with sham operation. Therefore, both ET-1 gene and

protein are overexpressed in the PHT esophagus. Our data are consistent with clinical studies, which demonstrated that ET-1 plasma levels are elevated in patients with liver cirrhosis and portal hypertension (27—29). These increased levels of ET-1 seem to be produced by greater net splanchnic release rather than by decreased extraction (30). In the liver tissue of cirrhotic rats with ascites, ET-1 tissue concentrations are significantly increased compared with those in controls, while there is no difference in the ET-1 tissue concentrations in lung and kidney in the two groups (31).

Hartleb, et al. reported that pressor responses to exogenous ET-1 in rats with cirrhosis are significantly lower than in normal rats (32). However, pressor responses to high doses of ET-1 did not differ between cirrhotic and normal rats after pretreatment with nitric oxide (NO) synthase inhibitors. Therefore, NO and ET-1 may interact and systemically regulate vascular tone in portal hypertension. Since ET-1 should be regarded more as a paracrine rather than as an endocrine hormone (33), ET-1 may play an important role for regulation of vascular tone in portal hypertension in regional circulation rather than in systemic circulation.

Since ET-1 works as a vasoconstrictor through ET_AR and promotes NO and prostacyclin synthesis through ET_BR (7—10), the role of overexpressed ET-1 in the PHT esophagus may be quite complex. However, both these endothelin receptors participate in vascular proliferation (11, 12), thus overexpressed ET-1 and its receptors are likely to contribute to the development of dilated submucosal veins (varices) in PHT esophagus. Since ET-1 induces mucosal injury (34, 35), the overproduction of ET-1 may well contribute to esophageal injury and rupture of the esophageal varices.

The mechanism(s) responsible for the overexpression of ET-1 in the PHT esophagus are not clear from this study. It is known from the literature that mechanical stimuli such as angioplasty, increased shear stress (19, 36), hypoxia (37, 38), and cytokines such as tumor necrosis factor- α (TNF- α) and interleukin-1 β (39, 40) are potential contributors to activation of this gene. Specifically, TNF- α may be one of the important regulators of ET-1 in portal hypertension. In PHT rats, the plasma level of TNF- α is significantly higher than in normal rats (41). Moreover, exogenous TNF- α elevates the plasma level of ET-1 resulting in increased systemic and pulmonary vascular resistance (42). Furthermore, TNF- α regulates expression of the ET-1 gene in endothelial cells *in vitro* (39). Recently, it was shown that TNF- α might be a major contributor to the hyperdynamic circulation in PHT rats via the NO pathway (43, 44). Our previous study demonstrated that TNF- α is a regulator of constitutive NO synthase in PHT gastric mucosa (45). Therefore, in portal hypertension, elevated TNF- α seems to activate not only the NO synthase gene, but also the ET-1 gene.

We have previously demonstrated that, in the PHT esophagus, expression of basic fibroblast growth factor and its receptor is significantly decreased in

the muscularis mucosae (46). Expression of fibronectin receptor is also reduced in this setting (47). These factors, in combination with the presence of thinner muscularis mucosae (46) and the overexpressed ET-1 leading to mucosal injury (35, 36), are likely to cause weakness of the PHT esophageal wall and predispose varices to the rupture.

In conclusion: Portal hypertension activates ET-1 and its receptor genes with overexpression of ET-1 protein in endothelia of esophageal submucosal veins. Since ET-1 and its receptors exert strong biological effects on the vasculature, overexpressed ET-1, combined with increased NO production, may play an important role in the development and rupture of the esophageal varices.

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