

Proinflammatory Liver and Antiinflammatory Intestinal Mediators Involved in Portal Hypertensive Rats

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Proinflammatory (TNF- α , IL-1 β , and NO) and antiinflammatory (IL-10, CO) levels were assayed in serum, liver, and small bowel in order to verify a hypothetic inflammatory etiopathogeny of portal hypertension that could be the cause of its evolutive heterogeneity. Male Wistar rats were divided into one control group ($n = 11$) and one group with a triple stenosing ligation of the portal vein ($n = 23$) after 28 days of evolution. In one subgroup of portal hypertensive rats, portal pressure, collateral venous circulation, mesenteric vasculopathy, and liver and spleen weights were determined. In the remaining rats with portal hypertension TNF- α , IL-1 β , and IL-10 were quantified in liver and ileum by enzyme-linked immunosorbent assay. NO synthase activity was studied in liver and ileum. CO and NO were measured in portal and systemic blood by spectrophotometry and Griess reaction, respectively. Portal hypertensive rats with mayor spleen weight show hepatomegaly and mayor development of collateral circulation. Ileum release of IL-10 (0.30 ± 0.12 versus 0.14 ± 0.02 pmol/mg protein; $P < .01$) is associated with a liver production of both proinflammatory mediators (TNF- α : 2 ± 0.21 versus 1.32 ± 0.60 pmol/mg protein; $P < .05$, IL-1 β : 19.17 ± 2.87 versus 5.96 ± 1.84 pmol/mg protein; $P = .005$, and NO: 132.10 ± 34.72 versus 61.05 ± 8.30 nmol/mL; $P = .005$) and an antiinflammatory mediator (CO: 6.49 ± 2.99 versus 3.03 ± 1.59 pmol/mL; $P = .005$). In short-term prehepatic portal hypertension a gut-liver inflammatory loop, which could be fundamental in the regulation both of the portal pressure and of its complications, could be proposed.

INTRODUCTION

Portal hypertension (PHI) is a clinical syndrome which is usually secondary to intrahepatic or extrahepatic obstruction of portal flow. It is characterized by a pathological increase in portal pressure, associated with splenomegaly, and by the development of portosystemic collateral circulation which diverts portal flow to the systemic circulation bypassing the liver [1, 2, 3]. Moreover, PHT is the main complication of cirrhosis and is responsible for most of its common complications: variceal hemorrhage, ascites, and portosystemic encephalopathy [4]. Making an effort to create new PHT models or to improve previously existing ones is justified by the need to study the physiopathological mechanisms of this frequent and severe clinical syndrome for which several different types of medical [5, 6] and surgical [7] treatments have been proposed.

Partial portal vein ligation (PVL) in the rat is the most frequently used experimental model to study in the short term the pathophysiology of prehepatic PHT [8, 9]. Constriction of the portal vein (PV) is immediately followed by increased portal resistance and portal pressure as well as decreased portal venous inflow [10]. Partial development of portosystemic collaterals is found after 4 days of PVL and, after two weeks of evolution most (95%) of the increased portal blood is diverted from the liver by an extensive portocollateral vascular bed [9, 10]. Then, portal resistance decreases to control values and the splanchnic blood flow, secondary to a decrease in the splanchnic arteriolar resistance, increases, all of which contributes to the maintenance of increased portal pressure [10, 11].

Therefore, until now PHT in the rat has always been considered to be a hemodynamic impairment with much more homogeneous alterations than those described in human PHT because of a narrow range of PHT, grade of portosystemic shunts, and hepatic atrophy [12]. However, this evolutive uniformity could not be verified from our previous studies using a modified technique of PV calibrated stenosis in the rat since the degree of hepatic

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atrophy, splenomegaly, and portosystemic collateral circulation that developed were variable [13]. A possible inflammatory etiopathogeny of PHT could be one cause of this evolutive heterogeneity. Thus, an increased infiltration of the intestinal mucosa and submucosa by mast cells has been described in PHT rats [14]. These inflammatory cells could be considered to be primed to a noxious stimulus and this mechanism could be responsible for the increased susceptibility of the PHT mucosa. If so, the anaphylactic degranulation of the mast cells may cause inflammatory episodes that vary in number and duration in each individual. This difference could explain the great variability observed in prehepatic PHT in the rat.

In order to verify a hypothetic inflammatory etiopathogeny of PHT we have studied some mediators involved in an inflammatory response. These included tumor necrosis factor- α (TNF- α), Interleukin-1 β (IL-1 β), interleukin-10 (IL-10), nitric oxide synthase (NOS) isoforms in tissue (liver and/or intestine), and nitric oxide (NO) and carbon monoxide (CO) in serum in rats with short-term prehepatic PHT.

MATERIAL AND METHODS

Animals

Male Wistar rats from the Complutense University Vivarium in Madrid with body weights from 230 to 250 g were used. The experimental procedures employed in this study are in accordance with the *Guidelines for the care and use of Laboratory Animals* (1986) published in Spain (Royal Decree 223/1988).

Experimental design

The animals were divided for their study into three groups: one control group (I), in which the animals did not undergo any intervention, and two-subgroups (IIa and IIb), in which prehepatic PHT by triple stenosing ligation of the portal vein (TSLP) was carried out. All the animals were sacrificed by ether overdose after 28 days of evolution. In Group IIa portal pressure, collateral venous circulation, mesenteric vasculopathy, and hepatic and spleen weights were studied. In Group IIb TNF- α , IL-1 β , and IL-10 levels were assayed in ileum, endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) activity were measured in liver and in intestine (duodenum, jejunum, and ileum). Finally, NO and CO concentrations were quantified in PV, suprahepatic inferior vena cava (SH-IVC), and infrahepatic inferior vena cava (IH-IVC) blood.

Portal vein stenosis technique

The surgical technique used to produce PHT by TSLP has been described previously [15]. In brief, while rats were under ketamine hydrochloride (80 mg/kg) and Xylacin (12 mg/kg) im anesthesia, the PV was isolated and three stenosing ligations were performed in its superior, medial, and inferior portions. The stenoses were cali-

brated by a simultaneous ligation (4-0 silk) around the PV and a 20-gauge blunt-tipped needle. The midline incision was closed on two layers with catgut and 3-0 silk.

Portal vein pressure measurement

The portal pressure is measured by an indirect technique of intrasplenic puncture [16], inserting a 20 G needle in the splenic parenchyma which is, in turn, connected to a PE-50 tube. After verifying that free back blood flow was obtained, the catheter was then connected to a pressure recorder (PowerLab 200 ML 201) and to a transducer (Sensoror SN-844) with a Chart V 4.0 computer program (ADI Instruments). The system is calibrated before each experiment. The zero reference point was established at 1 cm above the operating table. Previous studies have demonstrated an excellent correlation between the indirect measurement of the portal pressure by intrasplenic puncture and the direct measurement by cannulation of the superior mesenteric vein [17].

Portosystemic collateral circulation study method

Portal hypertension was confirmed by the presence of splenomegaly and portosystemic collateral circulation. First, a midline abdominal incision with a large bilateral subcostal extension was made and then the areas in which the collateral venous circulation was developed, that is, the splenorenal (SR), gastroesophageal (paraesophageal collaterals (PE)), colorectal (hemorrhoidal or pararectal collateral (PR)) and hepatic hilum (portoportal (PP) and accessory hepatic vein (AHV)) [4], were carefully studied for the presence of increased collateral veins. The latter (AHV) reached the hepatic hilum following a pathway between the left lateral and caudate hepatic lobes [18].

Gross superior mesenteric vein study

Existence of dilation and tortuosity of the superior mesenteric vein branches has been named mesenteric venous vasculopathy (MVV). Three grades of MMV were considered: (i) Grade 0: normal appearance of the superior mesenteric vein branches; (ii) Grade I: dilation and tortuosity of the mentioned branches secondary to the Pringle maneuver, and (iii) Grade II in which the dilation and tortuosity of the superior mesenteric vein branches were spontaneous.

Blood extraction method

Blood samples (1 mL) were drawn by puncture of the IH-IVC and SH-IVC. After 15 minutes of centrifugation at 1500 g they were separated and transferred to sterile polypropylene tubes. The serum was then frozen at -80°C until NO and CO were assayed.

Preparation of organ homogenates

Liver and intestine (duodenum, jejunum, ileum) were quickly dissected and frozen in dry ice. Frozen organ samples were weighed on a Mettler balance (model AE 100; Mettler Instrument Corp, Hightstown, NJ) and

transferred to 50 mL polypropylene tubes (Falcon; Becton Dickinson, Lincoln Park, NJ) containing lysis buffer (4°C) at a ratio of 10 mL buffer/1 g of wet tissue. Lysis buffer consisted of 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma Chemical Company) and 1 µg/mL pepstatin A (Sigma Chemical Company), aprotinin (Sigma Chemical Company), antipain (Sigma Chemical Company), and leupeptin (Sigma Chemical Company) in 1x phosphate-buffered saline solution of pH 7.2 (Biofluids, Rockville, Md) containing 0.05% sodium azide (Sigma Chemical Company). Samples were homogenized for 30 seconds with an electrical homogenizer (Polytron; Brinkmann Instruments, Westminister, NY) at maximum speed, and the tubes were immediately frozen in liquid nitrogen. The samples were homogenized three times for optimal processing. The supernatants were frozen at -80°C to allow the formation of macromolecular aggregates. After thawing at 4°C, the aggregates were pelleted at 3000 g (4°C), and the final organ homogenate volume was measured with a graduated pipette [19]. The homogenates were stored at -80°C until assayed for the quantitative presence of rat TNF-α, IL-1β, and IL-10 with a rat enzyme-linked immunosorbent assay (ELISA).

Ilial and liver TNF-α, IL-1β, and IL-10 ELISA

Cytokine levels were measured using commercially available (ELISA) specific kits (BioNOVA Cientifica Ltd, Madrid, Spain). The minimum detectable level of TNF-α was 0.5 pg/mL ($n = 10$). The intraassay variation ranged from 3.1% (lower part of standard curve) to 4.2% (upper) and the interassay variation oscillated between 5.2% and 5.6%. The minimum detectable level of IL-1β was 1.5 pg/mL ($n = 10$). The intraassay variation ranged from 3.5% (lower part of standard curve) to 4.3% (upper) and the interassay variation osciled between 6.1% and 7.4%. The minimum detectable level of IL-10 was 2.5 pg/mL ($n = 10$). The intraassay variation ranged from 4.2% (lower part of standard curve) to 7.5% (upper) and the interassay variation osciled between 5.8% and 9.3%.

NOS activity

NOS activity was calculated as the rate of conversion of ¹⁴C-arginine in citrulline. Tissue was homogenized in 1 mL of a buffer containing 10⁻² M HEPES, 0.32 M sucrose, 10⁻⁴ M EDTA, 10⁻³ M dithiothreitol, soybean trypsin inhibitor, 10 µg/mL leupeptin, 2 µg/mL aprotinin, and 1 mg/mL phenylmethanesulfonyl fluoride and centrifuged for 20 minutes at 100 000 g at 4°C. An aliquot of the final supernatant was mixed with PBB containing calcium chloride, ¹⁴C-arginine, valine (to inhibit arginase activity) and NADPH, and incubated for 30 minutes at 37°C. After purification in a Dowex (50 W) column, radioactivity was measured. Constitutive NOS is calcium dependent while the inducible isoform is not. In contrast, the arginine analog N-methyl-L-arginine predominantly inhibits iNOS. To identify the activity of both NOS isoforms we

used either the calcium chelator EGTA (1.4 mM) to isolate the activity of the iNOS or N-methyl-L-arginine (140 µM) to isolate the activity of the constitutive NOS [20].

Serum NO assay

The serum NO content was measured by the Griess reaction as nitrite concentration after nitrate reduction to nitrite [21]. Briefly, the samples were deproteinized by the addition of sulfosalicylic acid. They were then incubated for 30 minutes at 4°C and subsequently centrifuged for 20 minutes at 12 000 g. After incubation of the supernatants with E colischerichial nitrate reductase (37°C, 30 minutes), 1 mL of Griess reagent (0.5% naphthylethylenediamine dihydrochloride, 5% sulfonilamide, 25% phosphoric acid) (Merck, Darmstad, Germany) was added. The reaction was performed at 22°C for 20 minutes and the absorbance was measured at 546 nm, using sodium nitrite solution as a standard. The minimum detectable level of NO was 0.1 nmol/mL ($n = 10$). The intraassay variation was 1.6% and the interassay variation 2.7%.

Serum CO assay

To quantify the amount of CO, hemoglobin was added to bind CO as carboxyhemoglobin and the proportion of carboxyhemoglobin was estimated [22]. For this purpose, hemoglobin (4 µM) was gently mixed with the sample and 1 minute was allowed to ensure maximum CO binding. Then, samples were diluted with phosphate buffer (0.01 M monobasic potassium phosphate/dibasic potassium phosphate, pH 6.85) containing sodium hydrosulfite, allowed to stand at room temperature for 10 minutes, and absorbance was read at 420 and 432 nm against a matched cuvette only containing buffer. The minimum detectable level of CO was 1 pmol/mL ($n = 10$). The intraassay variation was 2.9% and the interassay variation 6.8%.

Statistical analysis

The results are expressed as the mean ± the standard deviation. Analysis of variance (ANOVA), the Duncan test and Student *t* for unpaired data were used for the statistical comparison of the quantitative variables between the different groups. Chi-square test was used for comparison of the qualitative variables. The linear correlation between two mutually dependent variables was determined and expressed as the correlation coefficient (*r*). The results are considered as statistically significant if $P < .05$.

RESULTS

Splenomegaly is always present in portal hypertensive rats. Since in rats with portacaval anastomosis (PCA) spleen weights correlated with portal pressure and preservation of portal pressure after PCA provides metabolic and nutritional benefits [23], two subgroups were defined within the TSLP group: one group in which spleen/body weight ratio was less than 0.29 (IIa; $n = 9$) and one group with a spleen/body weight ratio higher than or equal to

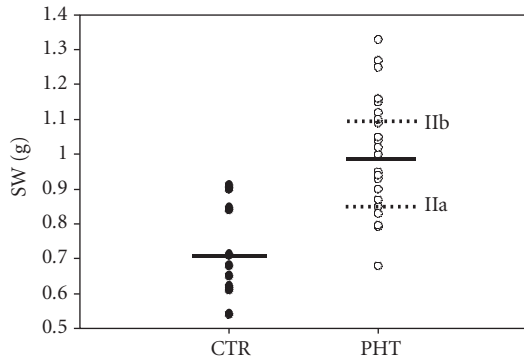


FIGURE 1. Residual plot showing the distribution of spleen weights in all control and TSLP rats. The solid lines represent the mean values for the overall group. The broken lines represent the mean value for the TSLP rats with lower spleen weights (IIa) and the TSLP rats with higher spleen weights (IIb).

0.30 (IIb; $n = 14$) (Figure 1). The spleen weight increase is significant ($P < .001$) in all TSLP rats, and this increase is greater ($P < .001$) in Group IIb compared to Group IIa (Table 1).

The body weight increase after 28 days of evolution was lower ($P < .001$) in all portal hypertensive rats (Table 1). The liver weight to body weight ratio does not change when all the animals with PHT are considered (Group II), but it is smaller in comparison to control animals in the subgroup of lower spleen weight (IIa) and increases in the subgroup with greater spleen weight (IIb) (Table 1). As shown in Table 2, portal pressure increases ($P < .001$) in rats with PHT, although we did not find differences between the two subgroups of animals with different spleen weights. Portal pressure correlates with body weight increase ($r = 0.71$; $P = .03$) in Group IIa, with lower spleen weight. In Group IIb, with higher spleen weight, portal pressure is correlated with body weight increase ($r = 0.56$; $P = .03$) and there is an inverse correlation between portal pressure and liver/body weight ratio ($r = -0.75$; $P = .002$) and between portal pressure and spleen/body weight ratio ($r = -0.68$; $P = .008$) (Table 3).

Mesenteric vasculopathy, of either Grade I or II, is observed in all the animals with PV (Table 2). Portosystemic collateral circulation of superior and inferior SR, PE, and PR types is developed in rats with PHT. Two categories were established: one consisting in the development of 1 to 3 types of collateral circulation and another that consists in the development of 4 types of collateral circulation. When 3 or less types of collateral circulation are developed, superior and inferior SR types are the most frequent, and when four types exist PR and PE are the types associated.

Differences were observed between subgroups IIa and IIb in the development of collateral circulation. In the group with lower spleen weight (IIa) most rats (88.88%) had 3 or less types of collateral vessels, while in the group with greater spleen weight (IIb) 71.43% developed four types of collateral vessels, and this difference was statistically significant ($P = .005$) (Table 2). There is, therefore, a

significant correlation ($P = .003$) between splenic weight and the number of newly formed collateral vessels.

The concentrations of proinflammatory cytokines TNF- α and IL-1 β in ileum do not increase in rats with PHT (Figure 2). In contrast, IL-10 levels increase ($P < .01$) in these animals. TNF- α and IL-1 β levels increase in the liver ($P < .05$ and $P < .001$, resp) (Figure 3).

Liver and intestinal eNOS activity is similar in control and portal hypertensive-rats (Table 4). On the contrary, iNOS activity increases in liver ($P < .001$) and in jejunum ($P < .05$) in PHT rats (Table 5).

As shown in Figures 4 and 5, in rats with PHT NO and CO of hepatic source increase ($P = .005$) while NO decreases in IH-IVC ($P = .005$).

DISCUSSION

In rats with short-term prehepatic PHT, there is an ileum release of IL-10 which is associated with hepatic production of both proinflammatory mediators (TNF- α , IL-1 β , and NO) and an antiinflammatory mediator (CO).

IL-10 is a pleiotropic and regulator cytokine produced principally by both T cells and macrophages, which possesses both antiinflammatory and immunosuppressive properties [24]. In PHT, IL-10 of ileum origin can be produced by T and B lymphocytes of the gut-associated-lymphatic-tissue (GALT), monocytes/macrophages, and mast cells since all these cells are well-known sources of IL-10 [25]. The production of IL-10 is triggered by several stress factors and its concentrations in the blood and in tissue compartments often reflect the magnitude of the inflammatory stress [24]. Experimental studies in rodents and primates have revealed that the primary inducers of IL-10 synthesis are in fact more proximal proinflammatory cytokines, such as TNF- α and IL-1 [24]. Moreover, glucocorticoids, endotoxin, and reactive oxygen intermediates, all raised in PHT, have been shown to induce IL-10 release [15, 24, 26, 27].

In portal hypertensive rats it has been demonstrated that in the short term (1, 14, and 45 days postoperation) the serum concentrations of TNF- α , one of the factors to which the hyperdynamic syndrome is attributed, increase [28, 29]. Bacteria colonizing the gut represent a large reservoir of microbial products, such as lipopolysaccharides (LPS), endotoxins and other bacterial wall fragments capable of inducing inflammatory cytokines and lead to sustained NO production [30]. GALT has been shown to produce and release TNF in response to bacterial translocation, so the gut is a "cytokine-releasing" organ in PHT [30].

It can be hypothesized that immediately post PHT splanchnic hyperpressure could trigger an intestinal inflammatory reaction that would be highly tissue damaging and could require activation of NF- κ B with ensuing generation of chemokines and cytokines in a shorter developmental period than one month p.o. Then, intestinal

TABLE 1. Body weight increase (BWI), liver weight (LW), liver weight to body weight (BW), spleen weight (SW), and spleen weight to body weight in the control group (Group I) and in rats with triple stenosing ligation of portal vein (TSLP) (Group II) which, in turn, were divided into Groups IIa (SW/BW < 0.29) and IIb (SW/BW \geq 0.30). The results are expressed as mean \pm SD. * denotes that $P < .05$ in relation to control group, ** denotes that $P < .01$ in relation to control group, and *** denotes that $P < .001$ which is a statistically significant value in relation to the control group. ••• denotes that $P < .001$ which is a statistically significant value of Group IIb in relation to Group IIa.

Group	BWI (g)	LW (g)	LW/100 g BW	SW (g)	SW/100 g BW
I (control) <i>n</i> = 11	140 \pm 30	13.4 \pm 1.1	3.4 \pm 0.15	0.7 \pm 0.13	0.18 \pm 0.04
II (TSLP) <i>n</i> = 23	62 \pm 38***	11 \pm 2.5***	3.5 \pm 0.8	1.0 \pm 0.2***	0.3 \pm 0.06***
IIa (TSLP) <i>n</i> = 9	78 \pm 40***	10.1 \pm 1.5**	3.1 \pm 0.25	0.9 \pm 0.12*	0.3 \pm 0.02***
IIb (TSLP) <i>n</i> = 14	52 \pm 34***	11.1 \pm 2.9*	3.7 \pm 1	1.1 \pm 0.13***	0.4 \pm 0.04***,•••

TABLE 2. Portal pressure (PP), mesenteric vasculopathy (MV), and portosystemic collateral circulation (CC) in control rats (Group I) and in rats with triple stenosing ligation of portal vein (TSLP) (Group II) which, in turn, were divided into Group IIa (spleen weight/body weight < 0.29) and Group IIb (spleen weight/body weight \geq 0.30). The results are expressed as mean \pm SD. *** denotes that $P < .001$ which is a statistically significant value relative to the control group. •• denotes that $P = .005$ which is a statistically significant value of Group IIb relative to Group IIa.

Group	PP	MV		CC	
	mmHg	Grade I	Grade II	<i>n</i> \leq 3	<i>n</i> = 4
I (control) (<i>n</i> = 11)	7 \pm 0.6	—	—	—	—
II (TSLP) (<i>n</i> = 23)	13 \pm 3.5***	11 (48%)	12 (53%)	12 (53%)	11 (48%)
IIa (TSLP) <i>n</i> = 9	13 \pm 4***	5 (55.5%)	4 (44.4%)	8 (89%)	1 (11%)
IIb (TSLP) <i>n</i> = 14	13 \pm 3.4***	6 (43%)	8 (57%)	4** (28.6%)	10** (71.4%)

TABLE 3. Correlations between portal pressure and body weight increase and liver and spleen weights in rats with triple stenosing ligation of portal vein (TSLP) (Group II) which, in turn, were divided into Group IIa (spleen weight/body weight < 0.29) and Group IIb (spleen weight/body weight \geq 0.30).

Correlations	Correlation (<i>r</i>)		
	TSLP (IIa + IIb)	TSLP (IIa)	TSLP (IIb)
PP versus BWI	0.57 ($P = .004$)	0.71 ($P = .03$)	0.56 ($P = .03$)
PP versus LW/BW	-0.46 ($P = .02$)	—	-0.75 ($P = .002$)
PP versus SW/BW	—	—	-0.68 ($P = .008$)

release of IL-10, a cytokine that controls inflammatory processes by suppressing the production of proinflammatory cytokines that are transcriptionally regulated by NF- κ B, would occur. Moreover, IL-10 also inhibits the release of free oxygen radicals and NO [31]. More specifically, IL-10 is a pivotal cytokine in the control of intestinal inflammation and plays a central regulatory role in the immune responses of the intestine, limiting and ultimately terminating inflammatory responses [32]. It seems that its main function is to keep the inflammation under strict control by adjusting the intensity of the immune and inflammatory responses to the severity of the destruction

produced by a pathological condition or a pathogen and, thus, minimizing damage to the host tissues caused by either the pathogen or the immune system itself [33]. In this study, the intestinal production of IL-10, possibly acting in a paracrine/autocrine fashion, would effectively inhibit intestinal release of TNF- α , IL-1 β , and NO. The inhibitory effects of IL-10 on IL-1 and TNF production are crucial to its antiinflammatory activities, because these cytokines often have synergistic activities on inflammatory pathways and processes, and amplify these responses by inducing secondary mediators such as chemokines, prostaglandins, and PAF [34].

TABLE 4. Liver and intestinal endothelial nitric oxide synthase (eNOS) activity in control rats (Group I) and in rats with triple stenosing ligation of portal vein (TSLP) after 28 days of evolution. The results are represented as mean \pm SD.

Group	eNOS liver $\mu\text{mol/pg protein}$	eNOS duodenum $\mu\text{mol/pg protein}$	eNOS jejunum $\mu\text{mol/pg protein}$	eNOS ileum $\mu\text{mol/pg protein}$
I (control) (<i>n</i> = 8)	1.1 \pm 0.5	5.7 \pm 0.95	5 \pm 1	4 \pm 2.8
II (TSLP) (<i>n</i> = 8)	1.45 \pm 0.4	7 \pm 0.9	6.5 \pm 1	5 \pm 3.2

TABLE 5. Liver and intestinal inducible nitric oxide synthase (iNOS) activity in control rats (Group I) and in rats with triple stenosing ligation of portal vein (TSLP) (Group II) after 28 days of evolution. The results are represented as mean \pm SD. * denotes that $P < .05$ and *** $P < .001$ which is a statistically significant value in relation to the control group.

Group	iNOS liver $\mu\text{mol/pg protein}$	iNOS duodenum $\mu\text{mol/pg protein}$	iNOS jejunum $\mu\text{mol/pg protein}$	iNOS ileum $\mu\text{mol/pg protein}$
I (control) (<i>n</i> = 8)	1 \pm 0.4	6.0 \pm 0.7	5.7 \pm 0.9	5.7 \pm 2.3
II (TSLP) (<i>n</i> = 8)	3.8 \pm 0.8***	6.7 \pm 1.4	7.3 \pm 1.3*	6.5 \pm 1.5

However, the increase in IL-10 intestinal production could have another meaning because IL-10, as well as inhibiting inflammation,

- (1) has an important role in fetal wounds leading to reduced matrix deposition and scar-free healing [35],
- (2) is expressed at elevated levels in chronic venous ulcers and may be related to the failure of these wounds to progress to final wound healing [36],
- (3) has a modulatory effect on hepatic fibrogenesis, and on hepatocyte proliferation and limits liver necrosis [37].

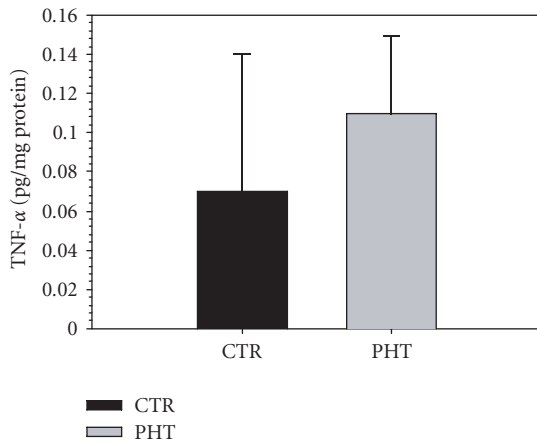
If induction of proinflammatory cytokines in liver is not counterbalanced by antiinflammatory cytokines, especially IL-10, there are inflammatory reactions leading to massive liver damage and fibrosis with pathologic progression to cirrhosis [38, 39]. Therefore, the intestinal rise in IL-10 in PHT would prevent two severe consequences of the proinflammatory response in a tissular area as large as the intestine: an early response that would combine necrosis and edema and a later one leading to intestinal fibrosis.

It could be hypothesized that in short-term portal hypertensive rats proinflammatory gut-derived mediators enter the liver directly through the PV or by an indirect route through intestinal lymphatics and/or systemic circulation. These gut-derived mediators would be a stimulus for hepatic synthesis of TNF- α and IL-1 β . Hypoxic stress has also been involved in cytokine synthesis by Kupffer cells [26] and in the liver Kupffer cells are major sources of proinflammatory cytokines that are produced in response to LPS [40]. Since PHT by PVL causes portal blood deprivation to the liver, it could be considered that this proinflammatory factor associated to LPS presence in

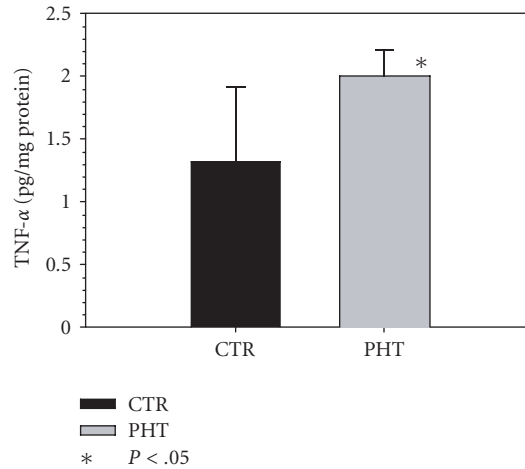
portal circulation [41] could stimulate the Kupffer cells to produce proinflammatory compounds, including TNF- α and IL-1 β .

In previous studies we have demonstrated that progressive liver steatosis is produced in rats with prehepatic PHT [42] and TNF- α and TNF-regulated cytokines are considered as effector molecules in nonalcoholic fatty liver disease (NAFLD) ranging from steatosis to cirrhosis [43]. It has been accepted that liver injury requires at least two "hits": one that increases exposure of the hepatocytes to TNF- α and another that interferes with the fat metabolism and renders the liver more vulnerable to a second injury, such as bacteria or LPS of intestinal origin, because the hepatocytes become sensitized to TNF-mediated cell death [43, 44]. TNF- α and other cytokines inhibit mitochondrial oxidative phosphorylation, with a decrease in free fatty acids (FFA) oxidation and oxidative stress [45]. TNF- α and TNF-related cytokines can contribute to the liver steatosis which occurs in PHT both by reducing the hepatic oxidation of fatty acids and by increasing lipid synthesis because IL-1 and, in particular TNF- α , stimulate hepatic lipogenesis in the rat and increase the plasma levels of FFA and triglycerides [46].

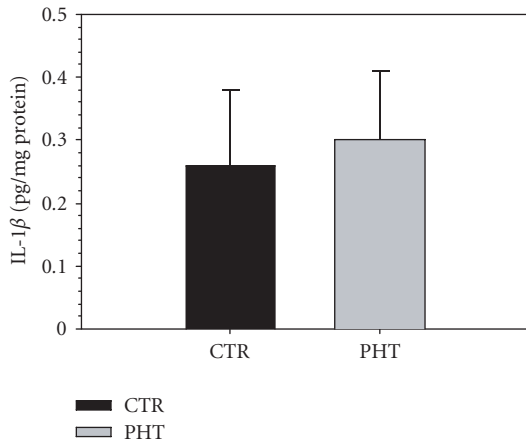
The direct biological response of TNF- α , which is ubiquitously expressed in response to stress, also referred to as an "alarm hormone" [47], is amplified by the secondary release of other cytokines and metabolic products like IL-1 [47] and NO [48]. Our results show an increased hepatic activity of iNOS 1 month after PVL. iNOS is synthesized de novo principally in macrophages, vascular smooth muscle cells, hepatic stellate cells, and hepatocytes, only after induction by LPS and inflammatory cytokines [49], both of which are increased in this experimental model. In 1991, Vallance and Moncada proposed



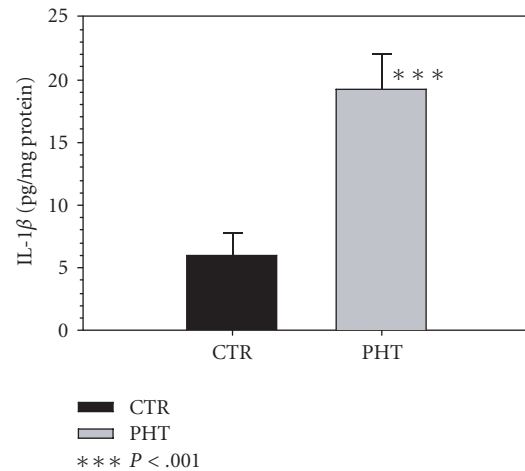
(a)



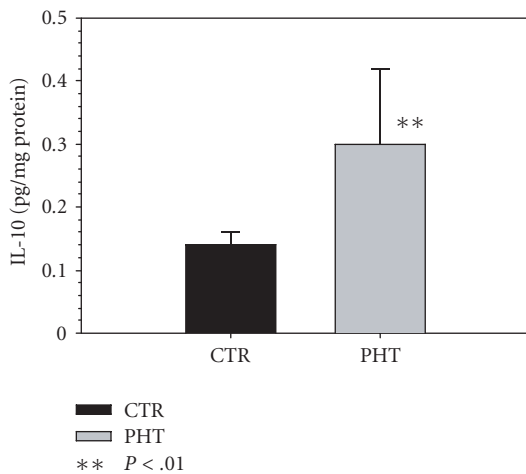
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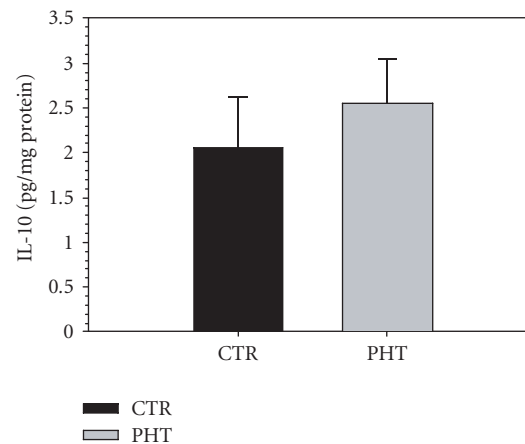
(b)



(b)



(c)



(c)

FIGURE 2. Concentrations of (a) TNF- α , (b) IL-1 β , and (c) IL-10 in the ileum of control rats (Group I) and of rats with triple stenosing ligation of portal vein (TSLP) (Group IIb) after 28 days of evolution.

FIGURE 3. Concentrations of (a) TNF- α , (b) IL-1 β , and (c) IL-10 in liver of control rats (Group I) and in rats with portal hypertension (PHT) by triple stenosing ligation of portal vein (TSLP) (Group IIb) after 28 days of evolution.

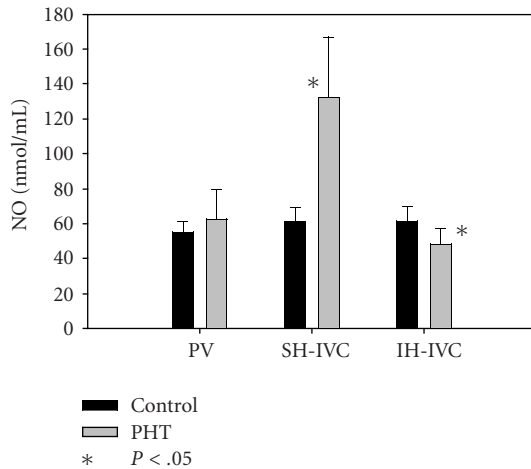


FIGURE 4. Nitric oxide (NO) in portal vein (PV), suprahepatic inferior vena cava (SH-IVC), and infrahepatic inferior vena cava (IH-IVC) in control rats (Group I) and in rats with triple stenosing ligation of portal vein (TSLP) (Group IIb) after 28 days of evolution.

that the increased synthesis and release of the vascular endothelium-derived vasodilator NO, induced by endotoxin directly or indirectly by cytokines, could account for the peripheral cardiovascular abnormalities of cirrhosis [50]. However, the main enzymatic source of the NO systemic and splanchnic overproduction which occurs in PHT [51] is eNOS, the constitutive isoform of NOS. eNOS protein expression increases, eNOS activity enhances and endothelial NO release is produced in response to flow and shear stress in mesenteric and systemic vessels of PHT animals [49]. Models of PHT developed by PV stenosis are associated with a chronic increase in splanchnic shear stress, related to high blood flow, which may contribute to NO overproduction by upregulation of eNOS [52]. This could represent an adaptive mechanism of the endothelium in response to chronic increases in flow-induced shear stress [51, 53].

However, activation of iNOS in vascular smooth wall has also been described in prehepatic PHT [54, 55] leading us to deduce that possibly a small part of NO derived from iNOS, upregulated in mesentery vasculature, may also contribute to hyperdynamic circulation in PHT [56]. The contribution of each isoform of NOS to the pathogenesis of the hyperdynamic syndrome probably depends on the etiology or severity of cirrhosis in human studies and in animal studies on the PHT model [57] perhaps on the time considered after PVL and especially on the tissue in which the enzyme is measured. This could cause the increased eNOS shown in splanchnic and systemic vascular beds [50, 51, 52] while we found an increased iNOS activity in liver. Thus, 1 month after PVL our results suggest that liver proinflammatory cytokines (TNF- α and IL-1 β) and endotoxin of intestinal origin could increase the hepatic synthesis of NO by iNOS upregulation.

In this early phase of PHT we have also shown the increased hepatic synthesis of CO, which besides its va-

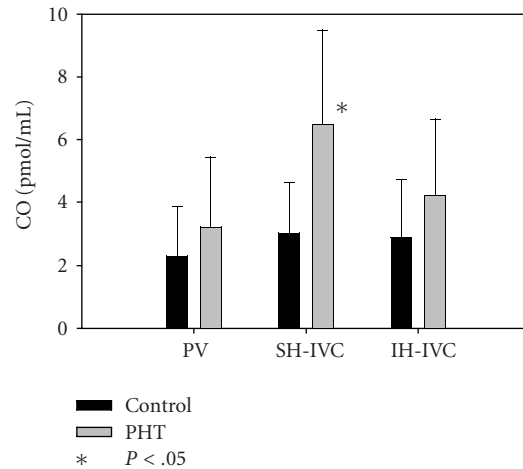


FIGURE 5. Carbon monoxide (CO) in portal vein (PV), suprahepatic inferior vena cava (SH-IVC), and infrahepatic inferior vena cava (IH-IVC) in control rats (Group I) and in rats with triple stenosing ligation of portal vein (TSLP) (Group IIb) after 28 days of evolution.

sodilator and antiapoptotic effects [58], is also an antiinflammatory molecule [59]. Both isoforms of heme oxygenase (HO), the enzyme involved in the generation of CO, the inducible and the constitutive ones, can be upregulated in PHT rats. Several physical or chemical factors that may be increased during PHT, including shear stress, hypoxia, NO, glucagon, as well as the proinflammatory agents endotoxin and cytokines can induce HO-1, the inducible isoform of HO [58]. HO-1 expression is upregulated in hepatocytes and splanchnic organs from PVL rats after 7 days of the operation [59]. In relation to HO-2, the constitutive isoform of HO, the only chemical inducers identified are adrenal glucocorticoids [60]. We have previously demonstrated that there is an increase in plasma levels of corticosterone in rats with short-term PVL [13] and therefore, HO-2 could be activated by glucocorticoids in this experimental model.

The increase in serum CO levels after 1 month in portal hypertensive rats could be related to their actions. Thus, CO acts as an endogenous regulator that is required for maintaining hepatic microvascular blood flow [61]. In addition, CO acts as a potent antiinflammatory molecule that selectively inhibits expression of the proinflammatory cytokines TNF- α , IL-1 β and macrophage inflammatory protein-1 β (MIP-1 β) [62].

In this early stage of prehepatic PHT in the rat, the hepatic hyperproduction of proinflammatory cytokines, that is, TNF- α and IL-1 β , as well as of NO and CO, could favor the development of mesenteric vasculopathy with splanchnic arteriolar vasodilation and systemic hyperdynamic circulation. The dilation and tortuosity of the mesenteric vein branches, which we have called mesenteric vasculopathy and that also have been described in association with a marked dilation in microcirculation [63], are signs that can be attributed to intestinal inflammation in this PHT experimental model.

In this early state of prehepatic PHT, a gut-liver inflammatory loop could be proposed. Proinflammatory gut-derived mediators produced initially in response to sharp and sudden PHT would be a stimulus for hepatic synthesis of TNF- α and IL-1 β , with iNOS activation. Liver TNF- α and IL-1 β , NO and CO, in turn, could leave the liver and enter the gut to complete the inflammatory loop via bile and/or the systemic recirculation.

It has been shown that in rats with portocaval anastomosis the spleen size reflects portal pressure, and preservation of portal pressure attenuates the shunt sequelae and provides metabolic and nutritional benefits [23]. However, in a group of rats with PHT, spleen weight is inversely correlated with portal pressure (Table 3). In these animals portal pressure has an inverse correlation with spleen/body weight and liver/body weight ratios. Moreover, portal pressure and spleen weight are related with a greater development of collateral circulation (Table 2). All the above mentioned findings suggest that this subgroup of animals represents one type of PHT which is characterized by hepatosplenomegaly and a great development of portosystemic collateral circulation.

PHT results from a pathological increase in either portal venous inflow ("forward hypothesis") or resistance ("backward hypothesis") and the maintenance of an elevated portal pressure depends, in part, on enhanced portal collateral resistance [2]. It could, therefore, be considered that in this group of animals the greater development of collateral circulation could be involved in regulating mesenteric flow and, subsequently, in controlling portal pressure.

Although PHT has been considered as a uniform experimental model, the results described previously could suggest an evolutive heterogeneity in this model. If we consider that in this subgroup of portal hypertensive rats, TNF- α and IL-1 β are involved in the production of hepatomegaly and that this increased liver size is secondary to steatosis, then the hyperproduction of IL-10 by the gut would be a mechanism, not only to decrease the inflammatory response, but also to attenuate one of its pathologic consequences, steatosis. Finally, the coexistence of greater spleen weight and greater development of collateral circulation could help to formulate a physiopathological definition of an evolutive type of PHT.

In summary, a hepatointestinal relationship seems to play an essential role in regulating both the portal pressure and its complications. This is only logical because the gut-liver axis is a portal system and its functions are distributed along this axis.

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