Research Article

Enoxaparin reduces hepatic vascular resistance and portal pressure in cirrhotic rats

Federica Cerini[1,†], Marina Vilaseca[1,3,5,†], Erica Lafoz[1,5], Oihane García-Irigoyen[2,3], Héctor García-Calderó[1,3,5], Dinesh M. Tripathi[1,5], Matias Avila[2,3], Juan Carlos Reverter[4,5], Jaime Bosch[1,3,5], Jordi Gracia-Sancho[1,3,5], Juan Carlos García-Pagán[1,3,5,*]

[1]*Barcelona Hepatic Hemodynamic Laboratory, Liver Unit, Hospital Clínic, Barcelona, Spain;* [2]*Centro de Investigación Médica Aplicada (CIMA), Division of Hepatology and Gene Therapy, Universidad de Navarra, Pamplona, Spain;* [3]*Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Spain;* [4]*Hemotherapy and Hemostasis Department, Hospital Clínic, Spain;* [5]*Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), University of Barcelona, Barcelona, Spain*

Background & Aims: Increased hepatic vascular resistance due to fibrosis and elevated hepatic vascular tone is the primary factor in the development of portal hypertension. Heparin may decrease fibrosis by inhibiting intrahepatic microthrombosis and thrombin-mediated hepatic stellate cell activation. In addition, heparin enhances eNOS activity, which may reduce hepatic vascular tone. Our study aimed at evaluating the effects of acute, short-, long-term and preventive enoxaparin administration on hepatic and systemic hemodynamics, liver fibrosis and nitric oxide availability in cirrhotic rats.

Methods: Enoxaparin (1.8 mg/kg subcutaneously), or its vehicle, was administered to CCl_4-cirrhotic rats 24 h and 1 h before the study (acute), daily for 1 week (short-term) or daily for 3 weeks (long-term) and to thioacetamide-cirrhotic rats daily for 3 weeks with/without thioacetamide (preventive/long-term, respectively). Mean arterial pressure, portal pressure, portal blood flow, hepatic vascular resistance and molecular/cellular mechanisms were evaluated.

Results: No significant changes in hemodynamic parameters were observed in acute administration. However, one-week, three-week and preventive treatments significantly decreased portal pressure mainly due to a decrease in hepatic vascular resistance without significant changes in mean arterial pressure. These findings were associated with significant reductions in liver fibrosis, hepatic stellate cell activation, and desmin expression. Moreover, a reduction in fibrin deposition was observed in enoxaparin-treated rats, suggesting reduced intrahepatic microthrombosis.

Conclusion: Enoxaparin reduces portal pressure in cirrhotic rats by improving the structural component of increased liver resistance. These findings describe the potentially beneficial effects of enoxaparin beyond the treatment/prevention of portal vein thrombosis in cirrhosis, which deserve further investigation.

Keywords: Portal hypertension; Anticoagulation; Low molecular weight heparin; Cirrhosis; Hepatic stellate cells.

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* Corresponding author. Address: Barcelona Hepatic Hemodynamic Lab – Hospital Clínic de Barcelona, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Villarroel 170, 08036 Barcelona, Spain. Tel.: +34 932275400x2824; fax: +34 932279856.

E-mail address: jcgarcia@clinic.cat (J.C. García-Pagán).

† These authors contributed equally as joint first authors.

Abbreviations: HSC, hepatic stellate cells; PAR, protease activated receptor; LMWH, low molecular weight heparin; PVT, portal vein thrombosis; NO, nitric oxide; CCl_4, carbon tetrachloride; MAP, mean arterial pressure; PP, portal pressure; PBF, portal blood flow; SMABF, superior mesenteric artery blood flow; HVR, hepatic vascular resistance; cGMP, cyclic guanosine monophosphate; O_2^-, superoxide; α-SMA, alpha-smooth muscle actin; PDGFRb, platelet derived growth factor receptor b; eNOS, endothelial NO synthase; $ONOO^-$, peroxynitrite.

Introduction

Increased resistance to portal blood flow, derived from architectural alterations of the liver parenchyma and a dynamic increment in the hepatic vascular tone, is the primary factor in the development of portal hypertension [1,2]. Architectural distortion of the cirrhotic liver is partly due to excessive synthesis of extracellular matrix components performed by dys-regulated fibrogenic cells such as hepatic stellate cells (HSC) and portal myofibroblasts [3]. Several evidences suggest that liver fibrogenesis is positively influenced by inflammation and thrombosis [4]. Indeed, it has been demonstrated that different pro-coagulation factors, such as the serine protease thrombin, dysregulate HSC phenotype through the stimulation of their protease activated receptors (PARs) [5–9]. Moreover, PARs expression is highly upregulated in human livers undergoing acute and chronic injury [10], and their inhibition results in a significant amelioration in HSC phenotype and hepatic fibrosis progression in experimental models of mild liver damage [11,12]. Generation of thrombin, from its precursor prothrombin, is efficiently performed by the activated coagulation molecule factor Xa. It is nowadays recognized that either thrombin or factor Xa are agonists of PARs and PAR signaling represents one of the main pathways of HSC activation and collagen deposition [7,8,13–15].

In addition, thrombin-derived clot formation may promote flow disturbances and occlusion of small-sized intrahepatic veins and sinusoids, representing another triggering factor of liver tissue remodelling [16]. This mechanism, named parenchymal extinction, includes progressive vascular obliteration due to thrombosis or inflammation leading to both apoptosis and atrophy of the liver [17].

Heparins, through their interaction with antithrombin, which induces a conformational change in antithrombin molecule, greatly facilitates the interactions between antithrombin and serine protease targets such as thrombin, and also factor Xa, IXa, XIa and XIIa [18]. Potential beneficial effects of the low molecular weight heparin (LMWH)-induced inhibition of the coagulation cascade have been evaluated in liver fibrosis [19,20]. Previous translational studies, performed in experimental models of mild liver damage induced by CCl_4 and common bile duct ligation, have proposed that LMWH may improve hepatic regeneration, by the inhibition of HSC proliferation and/or by inducing a marked reduction in the hepatic cytonecrosis index respectively [19,21]. Whether the anti-fibrotic effects of the drug is directly due to its anticoagulative effect preventing thrombosis or to the inactivation of cell-mediated fibrogenic mechanisms remains unknown. In addition, it has not yet been evaluated whether these beneficial effects are also observed in more severe liver damage (ie. established cirrhosis). Indeed, a possible beneficial role of LMWH in the treatment of advanced liver disease was recently suggested by a randomised controlled trial investigating the effect of continuous use of the LMWH enoxaparin to prevent portal vein thrombosis (PVT) in patients with cirrhosis. In this study, enoxaparin reduced the incidence of PVT and, although the underlying mechanisms remain largely unknown, also reduced the incidence of liver decompensation and improved survival [22].

In addition, heparins may also have beneficial effects on the vasculature via endothelium-dependent mechanisms. Indeed, LMWH has been shown to upregulate nitric oxide (NO) levels by activation of its synthesis [23–25].

The present study aimed to characterize the effects, and underlying mechanisms, of acute, short, long-term and preventive administration of the LMWH enoxaparin on the hepatic and systemic hemodynamics and on liver fibrosis in two experimental models of cirrhosis (CCl_4 and thioacetamide).

Materials and methods

In vivo experiments

Induction of cirrhosis by CCl_4 and LMWH administration

Cirrhosis was induced in male Wistar rats (50–75 g) with CCl_4 inhalation three times a week. Phenobarbital (0.3 g/L) was added to drinking water as previously described [26]. When animals developed ascites, after approximately 12–15 weeks of CCl_4 inhalation, phenobarbital and CCl_4 administration was discontinued. Rats were then randomized to receive either enoxaparin (1.8 mg/kg body weight, subcutaneously; Clexane, Sanofi-Aventis), or its vehicle (saline 0.9%), according to three different protocols of study:

- Acute: Enoxaparin or vehicle (n = 10 per group) were administered 24 h and 1 h before the study.
- Short-term: Enoxaparin or vehicle (n = 13 per group) were given daily for 1 week.
- Long-term: Enoxaparin or vehicle (n = 8 per group) were administered daily for 3 weeks.

Experiments were performed 1 h after the last administration. The selected dose of enoxaparin was based on previous reports [19,27], and validated in our laboratory in preliminary studies in cirrhotic animals where prophylactic levels of anti-Xa in blood were measured 4 h after of its administration (0.2 ± 0.1 *vs.* 1.12 ± 0.2 UI/ml, vehicle-treated cirrhotic rats *vs.* enoxaparin-treated cirrhotic rats). Treatment was prepared by a third person not administering the drug/vehicle or performing the study.

Animals were kept in environmentally controlled animal facilities at the Institut d'Investigacions Biomediques August Pi i Sunyer (IDIBAPS). All experiments were approved by the Laboratory Animal Care and Use Committee of the University of Barcelona and were conducted in accordance with European Community guidelines for the protection of animals used for experimental or other scientific purposes (EEC Directive 86/609).

Induction of cirrhosis by thioacetamide (TAA) and LMWH administration

Cirrhosis was induced in male Sprague Dawley rats (150–200 g) with intraperitoneal injection of thioacetamide (TAA, Sigma-Aldrich) dissolved in saline, twice a week at a dose of 250 mg/kg for 12 weeks. In this model, 2 different treatment strategies were used:

- Long-term: Enoxaparin or vehicle (n = 5–7 per group) were administered daily for 3 weeks after completion of TAA intoxication period.
- Preventive: Enoxaparin or vehicle (n = 10–12 per group) were administered daily for 3 weeks since week 10 until week 12 of TAA administration.

In vivo hemodynamic study

In vivo measurement of hepatic and systemic hemodynamic parameters and assessment of liver microvascular function was performed as described in the Supplementary materials and methods. At the end of the study, blood samples and liver tissue were collected for biochemical, histological and molecular assays. Livers were washed with 20 ml of saline prior to collection. Aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels were measured in serum by standard techniques.

Measurement of cyclic guanosine monophosphate (cGMP) levels

cGMP, a marker of NO bioavailability, was determined in liver homogenates as previously described [28] using an enzyme immunoassay (Cayman Chemical Co.).

Measurement of superoxide (O_2^-) content in liver tissue

In situ O_2^- levels were evaluated with the oxidative fluorescent dye dihydroethidium (DHE; Molecular Probes) [29]. Fluorescence images were obtained with a laser scanning confocal microscope (TCS-SL DMIRE2, Leica), and quantitative analysis was performed with Image J 1.33u software (National Institutes of Health).

Quantification of hepatic fibrosis

Cirrhotic rat livers were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with 0.1% Sirius Red and Masson's Trichromic, photographed, and analyzed using a microscope equipped with a digital camera. The red-stained (Sirius Red) or the blue-stained (Masson's Trichromic) area was measured using Axiovision software [26]. Values are expressed as the mean of 8 fields per sample.

Western blot

Expression of α-SMA, Pdgfrβ, phosphorylated Moesin at Thr^{558} (p-Moesin; as a marker of Rho kinase activity), nitrotyrosinated proteins, fibrinogen, and TNF-α was determined by western blot in hepatic samples as described [30,31] using the following antibodies: α-SMA (Sigma-Aldrich), Pdgfrβ (Santa Cruz), p-Moesin (Santa Cruz), nitrotyrosine (Cayman Chemical Co.), fibrinogen (Nordic-MUbio), and TNF-α (Abcam).

Hepatic fibrin deposits were determined by western blot using an anti-fibrinogen antibody, which detects fibrinogen epitopes preserved in the insoluble fibrin deposits, as previously described [32,33]. Blots were stained with Ponceau or anti-GAPDH antibodies to show equal loading.

Immunohistochemistry

Immunostaining of paraffin-embedded liver sections was performed with a mouse anti- α-SMA antibody (Sigma), a mouse anti-desmin antibody (Dako), a rabbit anti-fibrinogen antibody (Dako) and a mouse anti-CD68 antibody (Biorad) [31,34]. Antibodies were visualized with Dako Real Envision detection system peroxidase/DAB+. Slides were counterstained with haematoxylin. For quantification, a point grid was placed over the slide-pictures and the number of positive