Metformin reduces hepatic resistance and portal pressure in cirrhotic rats

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ABSTRACT

Objective: Increased hepatic vascular resistance is the primary factor in the development of portal hypertension (PH). Metformin ameliorates vascular cells function in several vascular beds. Our study was aimed at evaluating the effects, and the underlying mechanisms, of metformin on hepatic and systemic hemodynamics in cirrhotic rats and its possible interaction with the effects of propranolol (Prop), the current standard treatment for PH. Methods: CCl$_4$-cirrhotic rats received by gavage metformin 300mg/kg or its vehicle once a day for 1 week, before measuring Mean Arterial Pressure (MAP), Portal Pressure (PP), Portal Blood Flow (PBF), Hepatic Vascular Resistance (HVR), and putative molecular/cellular mechanisms. In a subgroup of cirrhotic rats, the hemodynamic response to acute Prop (5mg/kg i.v.) was assessed. Effects of metformin±Prop on PP and MAP were validated in common bile duct ligated (CBDL)-cirrhotic rats.

Results: Metformin-treated CCl$_4$-cirrhotic rats had lower PP and HVR than vehicle-treated rats, without significant changes in MAP or PBF. Metformin caused a significant reduction in liver fibrosis (Sirius red), HSC-activation (alpha-SMA, PDGF-RB, TGFβR1 and RhoK), hepatic inflammation (CD68 and CD163), superoxide (dihydroethidium staining) and NO-scavenging (protein nitrotyrosination). Propranolol, by decreasing PBF, further reduced PP. Similar findings were observed in CBDL-cirrhotic rats. Conclusion: Metformin administration reduces PP by decreasing the structural and functional components of the elevated hepatic resistance of cirrhosis. This effect is additive to that of propranolol. The potential impact of this pharmacological combination, otherwise commonly used in patients with cirrhosis and diabetes, needs clinical evaluation.

Keywords: fibrosis; liver; portal hypertension; LSEC; cirrhosis.
INTRODUCTION

Portal hypertension is a serious consequence of liver cirrhosis that results in life-threatening complications with elevated morbidity and mortality. The initial factor for its development is the presence of an increased vascular resistance in the cirrhotic liver, due to hepatic architectural distortion and increased vascular tone (5). Histologically, cirrhosis is mainly characterized by increased deposition and altered composition of the extracellular matrix, and by the development of regenerative nodules. In the fibrotic liver, hepatic stellate cells (HSC) become activated and their phenotype change to be myofibroblasts-like, with contractile properties and elevated production of collagen (13; 38). The increased hepatic vascular tone in cirrhosis is considered to be mainly due to endothelial dysfunction, as a consequence of an insufficient nitric oxide (NO) bioavailability in sinusoidal endothelial cells (15). Low NO bioavailability has been related both to a decreased eNOS activity, which occurs despite normal eNOS protein expression, and to increased NO scavenging by superoxide radicals (19; 20).

The biguanide drug metformin, besides to have metabolic effects as insulin sensitizer in type 2 diabetes mellitus, also confers vascular protection improving the endothelial dysfunction in several cardiovascular diseases (7; 8; 10; 25; 31; 45). Indeed, previous studies in experimental models of arterial hypertension, diabetes or fatty liver disease proposed that metformin-derived vasoprotection may be due to an increase in NO bioavailability resulting from enhanced eNOS activity (42) and reduced oxidative stress (33; 34), as well as by an improvement in the phenotype of vascular smooth muscle cells (2; 24).

Then, it is reasonable to propose that metformin treatment may have a potential role reducing intrahepatic resistance and therefore portal hypertension in
cirrhosis. Interestingly, metformin has also been suggested to have an effect inhibiting the alpha adrenergic tone, which was shown to be synergistic with the effect of propranolol controlling arterial hypertension (35; 37). Therefore, the primary purpose of the present study was to evaluate the hemodynamic and molecular effects of metformin administration to rats with liver cirrhosis. In addition, we also tested whether metformin influences the hemodynamic response to non-selective beta-blockade.
MATERIALS & METHODS

The study was carried out in male Wistar and Sprague-Dawley rats (Charles River Laboratories, Barcelona, Spain). All procedures were approved by the Laboratory Animal Care and Use Committee of the University of Barcelona and were conducted in accordance with the European Community guidelines for the protection of animals used for experimental and other scientific purposes (EEC Directive 86/609). The personnel who prepared and administered treatments and those that performed the experimental studies were different. Treatments' codes were not open for interpretation of the results until the inclusion of all animals.

Experimental models of liver cirrhosis and metformin administration

Induction of cirrhosis by carbon tetrachloride (CCl₄)

Cirrhosis with intrahepatic portal hypertension was experimentally induced in Wistar rats by inhalation of CCl₄ three times a week, and phenobarbital (0.3 g/L) added to the drinking water (12). When cirrhotic rats developed ascites, after approximately 12–15 weeks of CCl₄ inhalation, administration of CCl₄ and phenobarbital was discontinued and treatments started one week later. A group of control rats, which only received phenobarbital, was included in the present study.

Induction of cirrhosis by common bile duct ligation (CBDL)

Secondary biliary cirrhosis with intrahepatic portal hypertension was experimentally induced in rats by common bile duct ligation (CBDL), as described (41). After 3 weeks of surgery, treatments started.

Metformin administration

Control and cirrhotic rats received by gavage, once a day, either metformin hydrochloride (300 mg/kg/day; Sigma-Aldrich; n=6 control, n=12 in CCl₄ and n=9
in CBDL) or vehicle (0.9% NaCl; n=6 control, n=12 in CCl₄ and n=9 in CBDL) during one week. Hemodynamic experiments were initiated 24h after the administration of the last dose of metformin, or vehicle (26; 43; 47). The dose of metformin has previously shown to improve vascular function in experimental models of arterial hypertension, diabetes and liver steatosis, among others (3; 9; 36; 43; 45).

**Experimental Studies**

**In vivo hemodynamic study**

All rats had free access to food and water until 12h before the study. Methods for the hemodynamic evaluation in portal hypertensive rat models have been extensively described in previous studies (1; 17; 41). Briefly, under anaesthesia and body temperature maintained at $37 \pm 0.5^\circ\text{C}$, portal pressure (PP; mmHg; ileocolic vein), mean arterial pressure (MAP, mmHg; femoral artery), portal blood flow (PBF; mL/min; portal vein as close as possible to the liver), and superior mesenteric artery blood flow (SMABF; mL/min; superior mesenteric artery) were measured using perivascular ultrasonic transit-time flow probes connected to a flow meter (Transonic Systems, Ithaca, NY), recorded using a PowerLab apparatus (4SP), and analyzed using the Chart v5.01 software (ADInstruments, Mountain View, LA). Hepatic vascular resistance (HVR, mmHg/mL-min·g⁻¹) was calculated as: 

$$\frac{PP}{PBF}.$$ 

In a sub-group of cirrhotic animals (n=9 in CCl₄ and n=6 in CBDL), after obtaining baseline hemodynamic data, rats received propranolol (5 mg/kg i.v.) for ten minutes through the femoral vein catheter (39), and MAP and PP were recorded again.
At the end the hemodynamic study, serum samples from cirrhotic rats were collected by cardiac puncture to subsequently evaluate alanine aminotransferase (ALT), aspartate aminotransferase (AST) and bilirubin, all by standard protocols.

**Evaluation of the hepatic endothelial phenotype**

- **Nitric oxide bioavailability**
  
  Cyclic guanosine monophosphate (cGMP), a marker of NO bioavailability, was determined in liver homogenates from cirrhotic rats treated with metformin or vehicle using an enzyme immunoassay (Cayman Chemical Company, Tallinn, Estonia) as previously described (1). In addition, real time levels of NO were determined using the DAF-FM-DA staining in LSEC freshly isolated from cirrhotic rats treated with metformin or vehicle, and in isolated LSEC from cirrhotic livers and treated in vitro with metformin (1mM; 24h) or its vehicle (PBS) (n=3 per condition) (18; 19).

- **Nitric oxide molecular pathway**
  
  Protein expression of eNOS, phosphorylated eNOS, AMPK and phosphorylated AMPK, were assessed by Western blot in livers from cirrhotic rats treated with metformin or vehicle (17). Primary antibodies comprehended a mouse antibody recognizing eNOS (BD Transduction Laboratories, Lexington, KY), a rabbit anti-phosphorylated eNOS at Ser1176 (Cell Signaling Technology, Beverly, MA), a rabbit anti-AMPK (Cell Signalling Technology) or a rabbit P-AMPK at Thr172 (Cell Signaling Technology). Protein expression was determined by densitometric analysis using the Science Lab 2001, Image Gauge (Fuji Photo Film Gmbh, Düsseldorf). After stripping, blots were assayed for GAPDH content as standardization of sample loading. Quantitative densitometry values of eNOS and
AMPK were normalized to GAPDH. The degree of eNOS phosphorylation and AMPK phosphorylation were calculated as the ratio between the densitometry readings of P-eNOS/eNOS, and P-AMPK/AMPK.

- **Nitric oxide scavenging by superoxide (O$_2^-$)**

O$_2^-$ levels were quantified with the oxidative fluorescent dye dihydroethidium (DHE 10μM; Molecular Probes Inc., Eugene, OR) in liver slices and LSEC isolated from metformin- or vehicle-treated cirrhotic animals, as described (19; 21). Specificity of the assay was ensured using SOD (200 U/mL) as negative control. Nitrotyrosine content, as secondary marker of NO scavenging by O$_2^-$ to form peroxynitrite, was analyzed in liver sections (10 μM) through fluorohistochemistry (22), and in liver tissue through western blot (1:1000, Sigma) (19). Fluorescence images were obtained with a fluorescence microscope (Olympus BX51, Tokyo, Japan), and quantitative analysis of at least 10 images per slide was performed with Image J 1.44m software (National Institutes of Health, Bethesda, MD).

- **SOD activity**

Total SOD activity was measured in liver homogenates using a commercially available immunoassay (Sigma, Tres Cantos, Madrid), according to manufacturer’s instructions (22).

**Evaluation of hepatic fibrosis**

- **Quantification of hepatic fibrosis**

Semi-quantitative analysis of hepatic fibrosis was performed in liver tissue blocks fixed in 10% formalin, embedded in paraffin, sectioned and stained with 0.1% Sirius red. Eight fields from each slide were randomly selected, photographed and the red-stained area per total area was measured using AxioVision software (Carl
Zeiss MicroImaging, Germany) (41). Additionally, fibrosis was further quantified determining hepatic hydroxyproline content using a commercial kit (BioVision, Milpitas, CA) as previously described (4). Briefly, 40 mg of snap-frozen livers were hydrolysed in HCl (12 N) at 120°C for 3h and centrifuged to remove precipitates. Aliquots (10 μL) from each sample were evaporated to dryness and incubated with chloramine T (2.5 mM) for 5 min and Ehrlich's reagent (410 mM) for 90 min at 60°C. Absorption was measured at 560 nm and referred to a standard curve. Results are expressed as μg/mg liver tissue.

- HSC and Portal Myofibroblasts phenotypes

Hepatic protein expression of α-SMA (surrogate marker of HSC activation) and desmin (structural marker of HSC) were analyzed by immunohistochemistry. Immunostaining of paraffin-embedded liver sections was performed with a mouse anti-α-SMA antibody (1:1000, Sigma) and a mouse anti-desmin antibody (1:50, DAKO, Denmark) or, as a negative control, with phosphate-buffered saline. Bound antibodies were visualized using Dako Real Envision Detection System Peroxidase/DAB+, and slides were then counterstained with hematoxylin. α-SMA and desmin relative volume was determined by point-counting morphometry using a point grid to obtain the number of intercepts over α-SMA and desmin positive cells over the tissue. Twelve fields were counted in each liver. All measurements were performed by two blinded observers (12).

Fibrosis was further characterized in livers determining the expression of α-SMA, and PDGFRB, and the Rho kinase activity by Western blot in hepatic samples using a mouse antibody against α-SMA (1:1000, Sigma), a goat antibody against PDGFRB (1:500, Santa Cruz Biotechnology, California, USA), a mouse antibody recognizing moesin (1:200, Santa Cruz Biotechnology) and a mouse anti-phosphorylated
moesin at Thr558 antibody (1:200 Santa Cruz Biotechnology). Rho kinase activity was calculated as the ratio p-moesin/moesin (41). Taqman expression assays were used to determine hepatic mRNA expression of pro-collagen I, marker of fibrosis, collagen 15A1, marker of portal myofibroblasts (30), and metalloproteinases MMP-2, MMP-9 and MMP-13 and its inhibitors TIMP-1 and TIMP-2, as markers of fibrinolysis.

Specific effects of metformin on HSC phenotype was investigated using the human activated HSC cell line LX-2, kindly provided by Dr Bataller. Cells were treated with 1mM metformin for 24h and the expression of α-SMA, PDGFRB, TGFβ, TGFβR1 and pro-collagen I was analyzed using western blot or qPCR as described above.

**Analysis of hepatic inflammation**

Hepatic macrophages M1 subtype (CD68+) and M2 subtype (CD163+) were evaluated by western blot in hepatic tissue samples using a mouse monoclonal antibody against CD68 (1:1000 BioRad) and CD163 (1:1000 BioRad).

**Statistics**

Statistical analysis was performed using the SPSS 19.0 statistical package (IBM Corp., Armonk, NY). Comparisons between groups were performed with U Mann-Whitney for continuous variables and Fisher test for categorical variables. Comparisons of results intra-group before and after propranolol administration were performed using paired t-test. All data are reported as mean ± standard deviation unless otherwise specified. Differences were considered significant at a $p$ value < 0.05.
RESULTS

Effects of metformin in CCl4 cirrhotic rats

Metformin lowers portal pressure in CCl4 cirrhotic animals

CCl4 cirrhotic animals exhibited portal hypertension when compared to control rats (supplementary table 1). CCl4 cirrhotic rats receiving metformin exhibited statistically significant lower portal pressure than rats receiving vehicle (10.2±2.8 vs. 13.9±2.6 mmHg; -27%; p= 0.003). This reduction was not associated with a significant change in PBF reflecting a fall in HVR (7.9±2.7 vs. 12.0±4.0 mmHg/mL·min·g^-1; -34%; p=0.007). MAP, SMABF and HR were not modified by metformin (Table 1).

Propranolol was given i.v. after obtaining baseline values. This produced a significant reduction in heart rate and PP in both groups. However, while the reduction in heart rate was similar (-30% in metformin pre-treated rats vs. -27% in the vehicle group), the reduction in PP was significantly greater in the metformin group (-32% vs. -17% in vehicle-rats; p=0.03). As a consequence, the final portal pressure in the metformin+propranolol group was markedly lower than that in the vehicle+propranolol group (6.6±2.2 vs. 10.9±1.8 mmHg; 40% difference; p=0.001).

No differences in biochemical parameters were found comparing both groups of CCl4-cirrhotic rats (Table 2).

Metformin increases liver endothelial NO bioavailability in CCl4 cirrhotic rats

No differences in cGMP content were found in liver homogenates from cirrhotic rats treated with metformin or vehicle (18.3±2.9 in metformin vs. 19.2±3.4 pmol/mL in vehicle; p=NS). However, LSEC isolated from metformin-treated
cirrhotic animals exhibited significantly higher NO levels than those from animals receiving vehicle (Figure 1A), as demonstrated by measuring specific endothelial NO bioavailability using DAF-FM staining. Such increased NO bioavailability was also observed in LSEC isolated from cirrhotic livers treated in vitro with metformin (Figure 1B).

**Metformin does not modify eNOS and AMPK pathways but exerts an antioxidant effect within the cirrhotic liver**

No effects of metformin administration were found analyzing eNOS, p-eNOS, AMPK or p-AMPK protein expression (data not shown). However, livers from cirrhotic rats treated with metformin exhibited markedly lower O$_2^-$ levels in comparison to the vehicle group (-76% in tissue, -71% in isolated LSEC; Figure 1C-D), which was associated with marked diminished levels of nitrotyrosinated proteins (-43% by fluorohistochemistry and -28% by western blot; Figure 1E-F), surrogate marker of NO scavenging by O$_2^-$. Liver SOD activity was significantly higher in metformin-treated cirrhotic rats, indicating greater liver antioxidant capacity than those treated with vehicle ($1.27 \pm 0.12$ vs. $2.06 \pm 0.13$ U/mL; +61%).

**Metformin stimulates reduction of fibrosis in CCl$_4$ cirrhotic rats**

As expected, CCl$_4$ cirrhotic rats exhibited marked distortion of hepatic parenchyma with abundant fibrosis, as evaluated by Sirius Red staining. As shown in figure 2, metformin produced a significant reduction in hepatic fibrosis in comparison to vehicle administration (-41% in Sirius Red; -17% in hydroxyproline; -34% in pro-collagen I), however it did not reach normal values. Importantly, fibrosis improvement was associated with significant diminutions in the expression of α-SMA (-74% by western blot; -61% by immunohistochemistry), desmin (-46%) and
PDGFRB (-39%), and in Rho kinase activity (-55% in p-moesin/total moesin ratio), altogether suggesting decreased activation and abundance of HSC. Fibrinolysis characterization revealed no changes in the mRNA expression of MMP2, MMP9 and TIMP-2 (data not shown). However, we observed a trend to reduction in MMP13 (-50%; p=0.3) and TIMP-1 (-30%; p=0.2).

In addition, livers from metformin-treated animals showed reduced levels of the recently proposed marker of portal myofibroblasts, collagen 15A1, although it did not reach statistical significance (Figure 2F).

As shown in Figure 3, cellular studies confirmed the effects of metformin improving HSC phenotype. Indeed, LX-2 cells treated with metformin showed decreased expression of pro-collagen I (-50%), \( \alpha \)-SMA (-48%) and PDGFRB (-41%) and TGF\( \beta \)R1 (-16%), without significant differences in TGF\( \beta \) expression (data not shown).

**Metformin reduces hepatic inflammation in CCl4 cirrhotic rats**

Analysis of metformin effects on hepatic inflammation showed that M1 subtype macrophages (CD68+) were decreased (-31%; p=0.04) in comparison to vehicle treated cirrhotic rats. No differences in the expression of M2 subtype (CD163+) macrophages were observed.

**Effects of metformin in CBDL cirrhotic rats**

**Metformin ameliorates portal hypertension in CBDL cirrhotic rats**

Animals with cirrhosis of the liver caused by CBDL were used as a validation group. In this experimental model, animals treated with metformin also exhibited significantly lower PP than the vehicle treated group (17.2±2.3 vs. 19.1±2.7 mmHg; -10%; p= 0.009), with no differences in MAP or HR (Table 3).
Similar to what observed in CCl₄-cirrhotic rats, i.v. propranolol produced a significant reduction in PP in CBDL rats treated with either metformin or vehicle. As a consequence, the final portal pressure in the metformin+propranolol group was markedly lower than that in the vehicle+propranolol group (14.8±1.7 vs. 17.5±1.4 mmHg; -15%; p=0.01). No differences in biochemical parameters were found comparing CBDL-cirrhotic rats receiving metformin or vehicle (Table 2).

*Metformin improves eNOS activity but does not affect liver fibrosis in CBDL-cirrhotic animals*

Metformin treatment enhanced the phosphorylation of eNOS, suggesting improvement in its enzymatic activity, without modifying eNOS, AMPK or p-AMPK (Figure 4A-B). Fibrosis evaluation using Sirius Red staining revealed no differences between CBDL cirrhotic rats receiving metformin or vehicle (Figure 4C). Quantification of portal myofibroblasts using collagen 15A1, although 18% lower in the metformin treated rats, the difference did not reach statistical significance between both groups (data not shown).
In liver cirrhosis, increased hepatic vascular resistance, due to an increased hepatic vascular tone and to architectural abnormalities of the liver parenchyma, is the main player in the development of portal hypertension. Different studies have evaluated the possibility of reducing hepatic vascular resistance by enhancing hepatic NO bioavailability and reducing hepatic vascular tone using several experimental strategies (14; 21; 29). Although these studies showed beneficial effects, novel therapeutic strategies based on EMA/FDA-approved drugs with no systemic adverse effects are required to improve treatments for patients with portal hypertension.

The present study shows that one-week metformin administration decreases portal pressure in two different models of liver cirrhosis. In fact, in CCl₄-cirrhotic rats treated with metformin portal pressure was 27% lower than in those receiving vehicle. The decrease in portal pressure was not associated with modifications in portal blood flow, pointing to a decreased hepatic vascular resistance. Importantly, the beneficial effects of metformin reducing portal hypertension were confirmed, although of less magnitude, in CBDL-cirrhotic rats. Interestingly, metformin appears to ameliorate intrahepatic vascular resistance differently in the two cirrhotic models used. Indeed, in CCl₄-cirrhotic animals treated with metformin, a marked amelioration in fibrosis was observed, which was associated with an improvement in HSC phenotype, and reductions in the hepatic content of HSC and probably, although to a less extent, of portal myofibroblasts. This finding is in agreement with previous studies suggesting that long-term treatment with metformin ameliorates mild fibrosis in liver and heart (6; 40; 50), nevertheless our report describes for the first time the effects of
metformin reducing fibrosis in a pathology where exaggerated collagen deposition exists. Characterization of the underlying molecular mechanisms leading to fibrosis diminution in terms of fibrosis regression revealed a decrease in the expression of the metalloproteinase MMP13 and metalloproteinase inhibitor TIMP-1, although it did not achieve significance, with no differences in MMP2, MMP9 and TIMP-2 in comparison to vehicle-treated cirrhotic rats. Overall these data suggest that matrix degradation, at the dose and duration of treatment, may not play a major role in the reduction of fibrosis. Metformin mediated HSC de-activation may result from its capability to reduce oxidative stress, a well-known pro-fibrogenic stimulus, but also from the inhibition of the proliferative and pro-fibrogenic pathways PDGF, TGFβ and Rho Kinase (38; 47). Indeed, our study agrees with previous data demonstrating that inhibition of Rho kinase results in HSC phenotype amelioration and senescence (27). Metformin-derived inhibition of Rho kinase may occur through, at least, two different mechanisms: increment in NO bioavailability, and enhancement of the Rac1-cdc42 signalling pathway, both of them have been described as Rho kinase inhibitors (28; 32).

By contrast, we were unable to demonstrate an effect of metformin on liver fibrosis in the CBDL model. This discrepancy may be due to the different characteristics of fibrogenesis in the two models; the CBDL model is characterized by a very rapid progression of fibrosis with no possibility of spontaneous regression due to the persistence of the bile obstruction, while the CCl₄ is much slower and susceptible to regression upon stopping administration of the toxic. Importantly, discrepancies in the mechanisms explaining the beneficial effects of a certain drug when comparing different experimental models of cirrhosis are not new. In fact, previous works from our team and others using the thromboxane A2
receptor antagonist Terutroban or the FXR agonist Obeticholic Acid already demonstrated such phenomena (41; 44).

In addition, our data also show that metformin enhances liver NO bioavailability, which contributes to reduce hepatic vascular resistance and portal pressure. In the CCl4-cirrhotic model we did not detect any increase in eNOS phosphorylation, a marker of increased eNOS activity, but we observed an increased endothelial NO bioavailability that was mainly derived from an up-regulated SOD activity, thus reducing NO scavenging and formation of peroxynitrite. The use of antioxidants to increase liver NO was primarily described by our group and validated using different antioxidants (11; 12; 14; 19; 48; 49). So, our results agree with previous reports demonstrating the antioxidant effects of metformin in other vascular beds (33; 34). On the other hand, in the CBDL-cirrhosis model we found that metformin induces the activation of the NO-generating enzyme eNOS. Previous in vitro studies showed that metformin is able to increase eNOS-dependent NO production in endothelial cells [11-13]. The reason for such discrepancy between experimental models of cirrhosis is not clear. However, this has been previously reported by our group and others (16; 41; 44), emphasizing the need of extending studies to different experimental models to increase the chances of translating to human beings the findings observed at the bench-side. It is important to note that the reduction in portal pressure due to metformin was observed in both models of cirrhosis, although the magnitude of the reduction, probably because of the observed reduction in fibrosis, was greater in the CCl4 than in the CBDL model. Although NO certainly plays a central role modulating vascular tone within the cirrhotic liver (46), we cannot discard that other mechanisms that have been implicated in metformin-derived vasoprotection, such as inhibition of pro-
inflammatory responses (31; 40), suppression of vasoconstrictor prostanoids (34), or modulation of calcium flux within endothelial cells (23; 34), may also contribute to improve the intrahepatic vascular tone in cirrhosis.

It is worthy to note that no detrimental systemic effects, analyzed as changes in mean arterial pressure, superior mesenteric artery blood flow or in heart rate, were observed in cirrhotic animals receiving metformin. This observation is in agreement with a recently published study demonstrating no contraindications of metformin when administered to patients with liver cirrhosis (51). Such differential effects of metformin according to the vascular bed could be attributed to the known affinity of metformin for the damaged/dysfunctional endothelium, evidenced by previous studies demonstrating no effect of metformin on vascular function in normal rats (25; 33; 35; 45). In fact, our results support this hypothesis since no significant changes in portal pressure or in systemic hemodynamics were observed when metformin was administered to control rats (table 4).

Considering that beta-blockers are accepted therapeutic agents for primary and secondary prophylaxis in portal hypertensive patients, we further evaluated the possible synergistic effects of an acute administration of propranolol in animals under metformin treatment. Metformin-receiving animals exhibited a further decrease in portal pressure after propranolol administration, reaching much lower levels than in animals treated with placebo, with no differences in systemic hemodynamics, thus demonstrating that metformin may add a significant advantage to the established treatment with propranolol. The additive effects of both treatments reducing portal pressure may result from the capability of metformin to reduce the hepatic vascular resistance together with the reduction in portal blood inflow due to non-selective beta-blockade. These results are in
accordance with previous reports describing the combined effect of metformin and propranolol ameliorating arterial hypertension (35; 37).

In conclusion, the present study provides novel information showing that metformin administration to cirrhotic animals decreases portal pressure, thus ameliorating portal hypertension. Moreover, the metformin-derived improvement has a synergistic effect with beta-blockers reducing portal pressure, suggesting a new therapeutic approach to treat portal hypertensive patients.
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Disclosures: None

Authors’ contributions: DMT and EE designed the study, performed experiments, analyzed data and wrote the manuscript; EL and HG-C performed experiments and analyzed data; SKS critically reviewed the manuscript; JB conceived ideas, critically reviewed the manuscript and obtained funding; JG-S designed the study, conceived ideas, wrote the manuscript, obtained funding and co-directed the study; JCG-P designed the study, conceived ideas, wrote the manuscript, obtained funding and co-directed the study.


50. Zhang CX, Pan SN, Meng RS, Peng CQ, Xiong ZJ, Chen BL, Chen GQ, Yao FJ, Chen YL, Ma YD and Dong YG. Metformin attenuates ventricular

FIGURE CAPTIONS

Figure 1. Metformin improves endothelial nitric oxide bioavailability and oxidative stress in CCl₄-cirrhotic livers. In vivo real-time production of nitric oxide (NO) determined in LSEC freshly isolated from cirrhotic rats treated with metformin or vehicle (A), or in cirrhotic LSEC treated for 24h in vitro with metformin or vehicle (B). Intrahepatic superoxide (O₂⁻) determined in cirrhotic rats treated with metformin or vehicle (C), or in LSEC isolated from cirrhotic rats receiving metformin or vehicle (D). Hepatic levels of nitrotyrosinated proteins (E, fluorohistochemistry), and (F, western blot), determined in tissue samples from control rats (c) and cirrhotic rats treated with metformin (m) or vehicle (v). Depicted results derive from n=3 independent experiments for cellular studies, and from n=6 (control) and n=12 (cirrhotic) animals per group for tissular determinations. Values represent mean ± SEM. * p<0.05 vs. cirrhotic-vehicle, # p<0.05 vs. control. All images 20X.

Figure 2. Metformin promotes liver fibrosis regression in CCl₄-cirrhotic animals. A, Left: representative histological images of livers stained with Sirius red from control rats and cirrhotic rats treated for 1 week with vehicle or metformin, and quantification of fibrosis (Sirius red staining area per total area; n=6 (control) and n=12 (cirrhotic) per group); Right: hydroxyproline levels (top) and procollagen I mRNA expression (bottom) determined in livers described before. B, representative α-SMA immunohistochemistry and western blot with quantifications from livers described in A. C, desmin detection and quantification in livers described above. D, PDGFRB mRNA expression in livers described in A. E, rho kinase activity expressed as the ratio between p-moesin and moesin in livers described above. F, mRNA expression of collagen 15A1 determined in livers described above. Slides quantifications derive from 8 (Sirius Red) or 12 (immunohistochemistry) pictures per preparation. Values represent mean ± SEM, * p<0.05 vs. vehicle, # p<0.05 vs. control. All images 10X.

Figure 3. Metformin promotes HSC de-activation. Human HSC LX-2 were treated with metformin (m), or its vehicle (v), for 24h and the expression of procollagen I (A), α-SMA (B), PDGFRB (C), and TGFβR1 (D) were analyzed. Results
expressed as mean ± SEM derive from n=3 independent experiments. * p<0.05 vs. vehicle.

**Figure 4. Effects of metformin in CBDL-cirrhotic animals.** A, eNOS and p-eNOS expression determined in CBDL-cirrhotic animals treated for 1 week with vehicle (left) or metformin (right, n=9 per group). B, expression of AMPK and its phosphorylated form in livers described above. C, representative images of fibrosis detection using Sirius red staining (10X) with their quantification from livers described in A. Slides quantifications derive from 8 pictures per preparation. Values represent mean ± SEM, * p<0.05 vs. vehicle.
Figure 1
**Figure 2**

(A) Sirius Red (% total area)

(B) Pro-collagen I mRNA relative to control

(C) Desmin relative to vehicle

(D) α-SMA relative to vehicle

(E) p-Moesin relative to vehicle

(F) Collagen 15A1 mRNA relative to vehicle
Figure 3
Figure 4
**Tables**

**Table 1:** Effects of metformin on hepatic and systemic hemodynamics in CCl₄-cirrhotic rats.

<table>
<thead>
<tr>
<th>Parameter</th>
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<td>369±9</td>
<td>373±8</td>
<td>0.75</td>
</tr>
</tbody>
</table>

MAP, mean arterial pressure; PP, portal pressure; PBF, portal blood flow; SMABF, superior mesenteric artery blood flow; HVR, hepatic vascular resistance; HR, heart rate. Values are expressed as mean±SD.

**Table 2:** Effects of metformin on biochemical parameters in CCl₄-cirrhotic and CBDL-cirrhotic rats.

<table>
<thead>
<tr>
<th>CCl₄</th>
<th>Parameter</th>
<th>Vehicle (n=12)</th>
<th>Metformin (n=12)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (U/L)</td>
<td>269±120</td>
<td>264±65</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>148±41</td>
<td>103±37</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td>Bilirubin (mg/dL)</td>
<td>0.20±0.07</td>
<td>0.18±0.06</td>
<td>0.87</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CBDL</th>
<th>Parameter</th>
<th>Vehicle (n=9)</th>
<th>Metformin (n=9)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (U/L)</td>
<td>486±222</td>
<td>460±201</td>
<td>0.91</td>
<td></td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>106±19</td>
<td>67±36</td>
<td>0.075</td>
<td></td>
</tr>
<tr>
<td>Bilirubin (mg/dL)</td>
<td>0.9±0.2</td>
<td>0.7±0.2</td>
<td>0.11</td>
<td></td>
</tr>
</tbody>
</table>

AST, aspartate aminotransferase; ALT, alanine aminotransferase. Values are expressed as mean±SD.

**Table 3:** Effects of metformin on hepatic and systemic hemodynamics in CBDL-cirrhotic rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle (n=9)</th>
<th>Metformin (n=9)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP (mmHg)</td>
<td>98±11</td>
<td>100±19</td>
<td>1.00</td>
</tr>
<tr>
<td>PP (mmHg)</td>
<td>19.1±2.7</td>
<td>17.2±2.3</td>
<td>0.009</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>340±35</td>
<td>335±49</td>
<td>0.83</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>336±4</td>
<td>331±5</td>
<td>0.48</td>
</tr>
</tbody>
</table>

MAP, mean arterial pressure; PP, portal pressure; HR, heart rate. Values are expressed as mean±SD.
Table 4: Effects of metformin on hepatic and systemic hemodynamics in control rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle n=6</th>
<th>Metformin n=6</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP (mmHg)</td>
<td>124 ± 18</td>
<td>135 ± 17</td>
<td>0.23</td>
</tr>
<tr>
<td>PP (mmHg)</td>
<td>7.8 ± 1.1</td>
<td>7.9 ± 1.5</td>
<td>0.72</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>346 ± 42</td>
<td>337 ± 43</td>
<td>0.89</td>
</tr>
</tbody>
</table>

MAP, mean arterial pressure; PP, portal pressure; HR, heart rate. Values are expressed as mean±SD.