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REVIEW

Intrahepatic vascular changes in non-alcoholic fatty liver disease: Potential role of insulin-resistance and endothelial dysfunction

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Abstract

Metabolic syndrome is a cluster of several clinical conditions characterized by insulin-resistance and high cardiovascular risk. Non-alcoholic fatty liver disease is the liver expression of the metabolic syndrome, and insulin resistance can be a frequent comorbidity in several chronic liver diseases, in particular hepatitis C virus infection and/or cirrhosis. Several studies have demonstrated that insulin action is not only relevant for glucose control, but also for vascular homeostasis. Insulin regulates nitric oxide production, which mediates to a large degree the vasodilating, anti-inflammatory and antithrombotic properties of a healthy endothelium, guaranteeing organ perfusion. The effects of insulin on the liver microvasculature and the effects of IR on sinusoidal endothelial cells have been studied in animal models of non-alcoholic fatty liver disease. The hypotheses derived from these studies and the potential translation of these results into humans are critically discussed in this review.

Key words: Non-alcoholic fatty liver disease; Endothelial dysfunction; Insulin resistance; Metabolic syndrome

Core tip: Insulin-resistance participates in the development of endothelial dysfunction and interferes with vascular homeostasis in patients with metabolic syndrome. This has been demonstrated in large conductance vessels, promoting atherosclerosis, but also occurs at a microcirculation level, suggesting an important role for Insulin in controlling vascular resistance and, finally, organ perfusion. We offer an overview of those pre-clinical and clinical studies exploring the liver microcirculation, and discuss the importance of early vascular changes induced by insulin-resistance in non-alcoholic fatty liver disease and in the most common chronic hepatitis in which Insulin-Resistance is a comorbidity.

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INTRODUCTION

Metabolic syndrome (MS) is a cluster of cardiovascular risk factors including glucose intolerance, hypertension, dyslipidemia, and visceral obesity, and has a prevalence of up to 25% in adults over 40 years old[1]. Non-alcoholic fatty liver disease (NAFLD) is the liver expression of MS, and constitutes a chronic disease associated with high cardio-vascular risk, with potential for progression to cirrhosis and hepatocellular carcinoma[2]. In other chronic liver conditions such as hepatitis C virus-related chronic hepatitis[3-5] and cirrhosis[6], MS can be an important comorbidity that potentially worsens liver histology[7] and increases the risk of decompensation[8]. Insulin-resistance (IR) is a common early finding in patients with MS and is the main pathogenic substrate for the development of NAFLD. IR is associated with endothelial dysfunction (ED)[9,10], a major pathogenic factor in arterial hypertension, coronary artery disease and atherosclerosis[11].

Several studies in animal models and humans have demonstrated that insulin action couples vascular and glucose homeostasis[12]. Indeed, in physiologic conditions, Insulin can influence the production of nitric oxide (NO) that mediates to a large part the vasodilating, anti-inflammatory and antithrombotic properties of a healthy endothelium. In patients with MS, the degree of IR parallels the severity of ED with important repercussion on structural and functional changes of the macro- and micro-circulation that may lead to impaired organ perfusion[11]. In the last few years, intrahepatic ED has been demonstrated in several models of liver disease including cirrhosis[13], ischemia-reperfusion[14], endotoxemia[15] and fatty liver[16,17]. As a consequence, vascular changes induced by IR, acting through the development of ED, are of potential therapeutic interest in the context of the most prevalent liver diseases. The present review offers an overview of the molecular mechanisms linking IR with ED in the control of vascular homeostasis, and reports the main biological and clinical findings on this topic in the context of the most common liver diseases.

INSULIN AND ENDOTHELIUM: FROM PHYSIOLOGY TO PATHOPHYSIOLOGY

Biological activity of NO is highly regulated in healthy conditions

NO is the main biochemical mediator of endothelium-dependent vasodilation in blood vessels. In physiologic conditions, it is constitutively synthesized by endothelial nitric oxide synthase (eNOS), whose activation results in a cascade of molecular events leading to smooth muscle relaxation[12]. The vasodilatory actions of NO play a key role in the renal control of extracellular fluid and is essential for the regulation of blood flow and blood pressure[18].

The endothelial NO production by eNOS is tightly regulated at the transcriptional and post-transcriptional level[19]. The expression of eNOS mRNA is largely restricted to the vascular endothelium[20]. Methylation is biologically associated with a marked impairment of promoter activity in mammalian cells and appears to play an important role in endothelial cell-specific expression of the human eNOS gene[21]. The eNOS promoter is heavily methylated in non-endothelial cells in comparison with endothelial cells. Kruppel-like factor 2 (KLF2) is a transcription factor that modulates the expression of multiple endothelial genes, including eNOS and thrombomodulin[22,23]. KLF2 is endothelial-specific and its expression, which is modulated by different flow patterns, confers endothelial protection against inflammation, thrombosis and excessive vasoconstriction[24,25]. Gracia-Sancho *et al*[26] have demonstrated that KLF2 can be activated by SIRT1 and that KLF2 overexpression activates vasoprotective genes in the vascular endothelium[27]. The same group demonstrated that simvastatin upregulates KLF2 expression in whole livers from cirrhotic rats[14] and in sinusoidal endothelial cells in culture[28]. This demonstrates that KLF2-mediated transcriptional regulation at the liver sinusoidal endothelial cells reproduces what occurs at peripheral endothelial cells.

The activity of eNOS is also thoroughly regulated

at the posttranslational level. These include protein-protein interactions, cofactors availability and protein phosphorylation[13]. The impact of these posttranslational modifications in liver endothelial eNOS has been thoroughly studied in animal models of liver disease[13]. Finally, the bioavailability of NO can be affected by the oxidative stress generated by several clinical conditions, and antioxidants have demonstrated the importance of redox environment to maintain microcirculation in conditions of compromised liver perfusion[29].

eNOS and iNOS: From physiology to pathophysiology

In addition to the constitutive form of eNOS, at least two other isoforms have been described: the inducible NOS (iNOS), and the neuronal NOS (nNOS). The potential pathogenic roles of eNOS and iNOS dysregulation have been assessed in several models of liver disease. Both isoforms produce NO, but their intracellular localization, activation, and concentration of NO produced are not the same, resulting in different biologic actions.

Physiological hepatic production of NO is derived from eNOS in response to stimuli such as shear stress and the presence of vasoconstrictors[30,31]. The classical activation of eNOS implies an increase of intracellular calcium (Ca^{2+}) and binding of Ca^{2+}/calmodulin to the enzyme. In addition, a Ca^{2+}-independent pathway regulating eNOS has been recently described. This pathway can be stimulated by several factors, among them shear stress and insulin[32,33]. Both shear stress and insulin increase endothelial NO production *via* activation of PI-3-kinase and protein kinase B (PKB/Akt), which activate eNOS by Ser1179-phosphorylation[34]. In addition, insulin upregulates the transcription of eNOS in endothelial cells[35].

In the liver, eNOS-derived NO targets hepatic stellate cells (HSC), promoting the synthesis of cyclic GMP (cGMP)[36]. The most important target of cGMP is protein kinase cGMP-dependent (PKG), which phosphorylates numerous proteins involved in the regulation of Ca^{2+} homeostasis, among them inositol 1,4,5 triphosphate-receptor. This leads to a decrease in the concentration of intracellular Ca^{2+} in HSC and produces relaxation with enduing decreased intrahepatic vascular resistance[37]. Thus, a physiological production of NO in the healthy liver offsets vasoconstrictor stimuli[38]. Since increased intrahepatic vascular tone is a major factor leading to portal hypertension in cirrhosis, different pharmacological strategies have been explored to increase liver NO availability[39-42].

iNOS was initially identified for its vital role in the immune system. When activated, it produces continuously large amounts of NO since, in contrast to eNOS, the substrate and cofactors are not limiting. iNOS is upregulated in metabolic tissues under different conditions of stress[43]. Although it is important for the immune system, iNOS activity can be harmful for other cell types, including pancreatic β cells[44] and vascular cells[45]. Recent studies have shown that iNOS-derived NO may play a role in the pathophysiology of obesity-induced metabolic dysfunction[43]. Among other mechanisms, it has been shown that iNOS is a critical modulator of PPAR-γ activation (a target of insulin sensitizing drugs)[46] and can decrease insulin sensitivity through S-nytrosilation of the insulin receptor[47]. Indeed, the inhibition of iNOS reduces hyperglycemia, hyperinsulinemia and improves liver insulin sensitivity[48]. Moreover, several studies with animal models have demonstrated that the induction of iNOS can cause ED through increased nitro-oxidative stress[49-51] and downregulation of eNOS[52]. Finally, the inhibition of iNOS in animal models that overexpress this enzyme restores a normal endothelial function[53-55].

Under physiologic conditions, the only NOS expressed in the endothelium of the vessels is eNOS. During inflammation, blood vessels express iNOS and eNOS[56]. Overexpression of iNOS thus contributes to vascular dysfunction.

Insulin-resistance and eNOS activity

The binding of insulin to its receptors at the level of peripheral endothelial cells[57] activates the phosphorylation of the insulin-receptor substrate which initiates a phosphorylation of a series of down-stream substrates, among them the PI3K/Akt pathway[58,59], that finally activate eNOS[60,61]. The result of this set of reactions ultimately produces an increase in eNOS activity and increased production of nitric oxide (NO), leading to vasodilation (Figure 1).

In the presence of IR, the PI3K/Akt pathway (involved in metabolic functions) is impaired, while other pathways of insulin signalling remain unaffected, including the Ras/MAPK pathway (involved in the control of cell proliferation), resulting in an imbalance between insulin functions performed by the PI3K pathways and MAPK[62,63]. This imbalance leads to decreased activation of eNOS and thus lowered production of NO, resulting in ED.

INTRAHEPATIC VASCULAR CHANGES IN NAFLD

Microvascular abnormalities in models of fatty liver: Structural and functional increase of resistance

The isolated liver perfusion technique has been instrumental in the assessment of liver microvascular changes in fatty liver[16] (Figure 2). In several studies, substantial changes in vascular function and liver blood flow have been demonstrated in fatty liver disease, as reviewed elsewhere[64]. Studies in rabbits

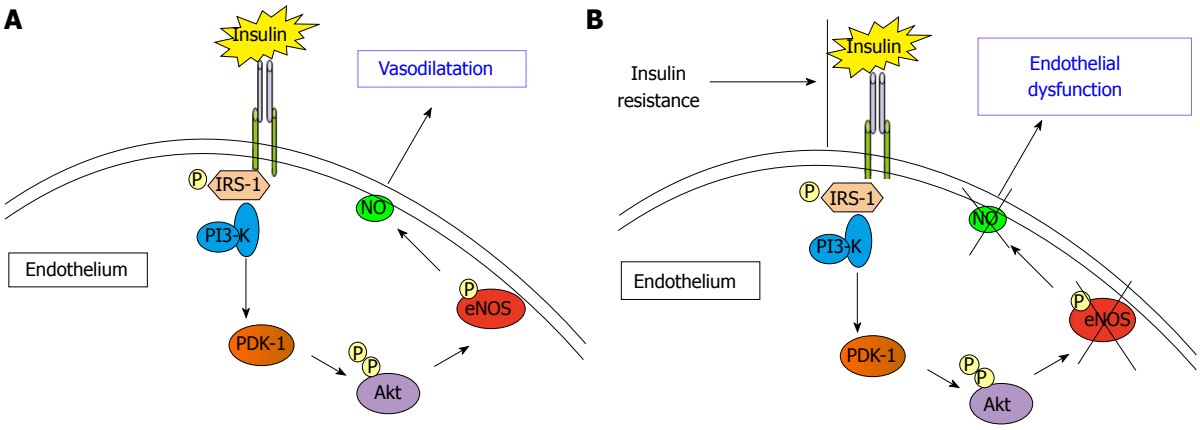

Figure 1 The binding of insulin to its receptor activates a series of phosphorylations of downstream receptors that finally activate nitric oxide-production by endothelial nitric oxide synthase. A: The release of nitric oxide (NO) causes endothelium dependent vasodilation; B: Insulin-resistance causes the reduction of Insulin-induced activation of endothelial nitric oxide synthase (eNOS). This is associated with reduction of NO bioavailability and, finally, endothelial dysfunction.

with diet-induced steatosis of different severity confirmed that reduction in sinusoidal perfusion correlated with the severity of fat accumulation in parenchymal cells[65] and the severity of steatosis had a greater impact on microcirculation. These studies demonstrated that steatosis caused an increase in the mechanical component of intrahepatic vascular resistance to portal blood flow, independent of functional changes potentially induced by IR, a feature that could be observed also in patients with genetic susceptibility to NAFLD[66,67]. The potential impact of these hemodynamic changes on liver perfusion was subsequently explored in rats exposed to a high-cholesterol diet that developed steatosis. Compared to controls, steatotic rats had significantly reduced hepatic microcirculation and tissue oxygenation[68]. Interestingly, in this study, exposure to L-Arginine, the biochemical precursor of NO, improved tissue oxygenation, whereas L-NAME, a NOS inhibitor, further deteriorated hepatic microcirculation and hepatocyte oxygenation. These results allow for two major considerations. First, reduced oxygenation is an important issue if we consider the susceptibility of fatty livers to ischemic injury[69]. Second, all these results, from a biochemical point of view, suggest that NO, the marker of a healthy endothelium, is involved in the modulation of hepatic microcirculatory perfusion and oxygenation in rats with steatotic livers. Along these lines, we had previously demonstrated that intrahepatic ED in isolated and perfused livers from rats with several features of NAFLD and MS predated the development of fibrosis and inflammation[17], suggesting that liver ED contributes to increased intrahepatic resistance very early in the pathogenesis of NAFLD.

Sinusoidal endothelial dysfunction and fibrogenesis

Fibrogenesis is a complex biochemical process which represents the hallmark of any evolving chronic liver disease. Studies with animal models of fibrosis have demonstrated that fibrogenesis parallels neo-angiogenesis, confirming the importance of endothelial cells in this phenomenon[70]. In particular, the paracrine crosstalk of sinusoidal endothelial cells with hepatocytes, hepatic stellate cells and Kupffer cells is determinant in initiating and maintaining the fibrogenic reaction[71]. However, among all these cytotypes, there remains debate as to which cell initiates the process and when exactly these cells start changing their function towards a pathologic phenotype. In addition, the role of IR on this crosstalk and its impact on the pathogenesis and evolution of NAFLD has not been adequately addressed by *in vitro* and *in vivo* studies. Recently, Miyao *et al*[72], by using mice models of NAFLD/non-alcoholic steatohepatitis, confirmed that sinusoidal endothelial injury appeared in the steatotic phase, preceding the activation of Kupffer cells, hepatic stellate cells and, in turn, inflammation and fibrosis[72]. This suggests that sinusoidal endothelial cells may have a gatekeeper role in the progression from simple steatosis to steatohepatitis, but this would require confirmatory explorations. Interestingly, these functional changes in the endothelium parallel a replacement of regular sinusoidal anatomy into appearing disorganized and characterized by abnormal vascular interconnections[73]. Furthermore, the recent observation that fibrosis may be sustained by an abnormal activation of coagulation[74-76] suggests the theoretical scenario in which the loss of the anticoagulant properties of endothelium can play a mechanistic role in fibrogenesis even in NAFLD. Indeed, Kopec *et al*[77] demonstrated that steatosis due to 3 mo of high fat diet is associated with a pro-coagulant imbalance that has a cause-effect relation with the severity of liver damage. The real impact of all these microvascular abnormalities in the progression to

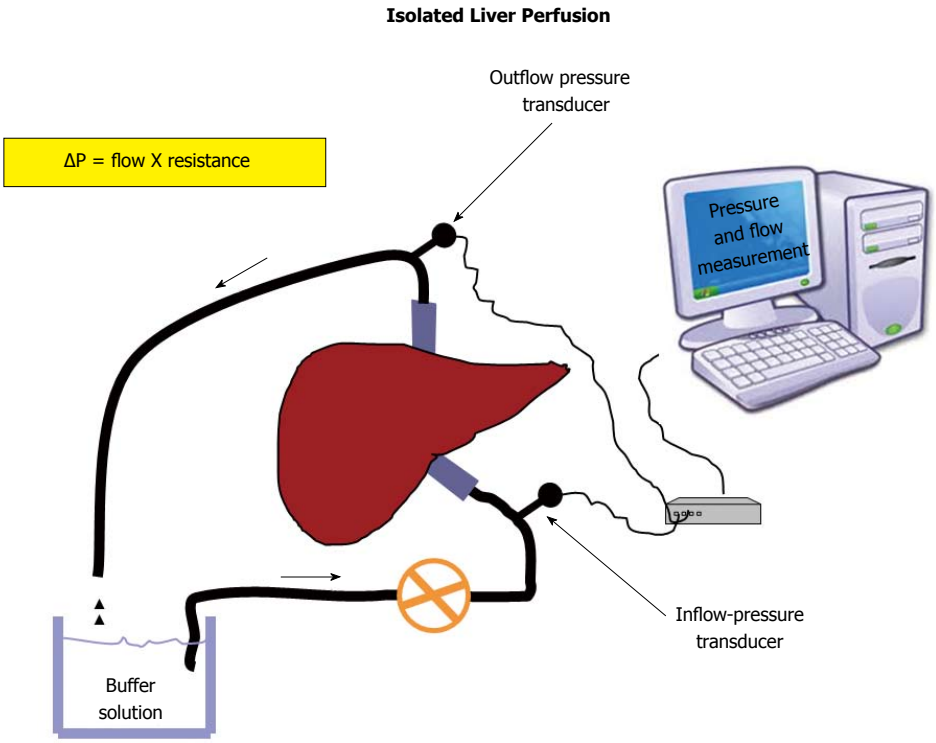

Figure 2 Livers are isolated and perfused at a constant velocity. Any change of pressure will be directly proportional to the resistance offered by sinusoids to the flow of a buffer solution according to Ohm's low applied to fluid-dynamic (ΔP = flow X resistance). This allows measuring the intrahepatic resistance offered by sinusoids.

steatohepatitis and cirrhosis is an intriguing question, and their role in the pathogenesis of NAFLD remains in need of further investigation.

Pathogenic link between intrahepatic microvascular abnormalities and IR

Many mechanisms observed in patients with IR, among them, lipotoxicity[78], oxidative stress[79,80], changes in local renin-angiotensin system[81], increased sensitivity to adrenergic stimuli of vascular smooth muscle cells[82], glucotoxicity (*via* oxidative stress, increased flow, activation of diacylglycerol, among others[83,84]) and inflammation[85,86], could explain the development of ED.

In a rat model of simple steatosis, we demonstrated the presence of insulin resistance in the liver sinusoidal endothelium that was mediated, at least in part, by the upregulation of iNOS[16]. As occurs in the peripheral circulation, in the normal liver, insulin results in liver vasodilation. In rats with fatty liver, insulin-dependent vasodilation in the liver vasculature was significantly impaired as compared to livers from control rats. This was partially restored in rats treated with the iNOS specific inhibitor 1400 W. Moreover, the insulin sensitizer metformin also restored hepatic vascular sensitivity to insulin while diminishing hepatic iNOS expression. Recently, the interaction between sinusoidal ED and iNOS has been explored in a rat model of endotoxemia (characterized by an overexpression of iNOS). These data further demonstrated that iNOS upregulation can induce, *per se*, sinusoidal ED[87]. All these findings support that in the hepatic vasculature, IR can be detected early in the course of the disease and may contribute to disease progression. A recent work by Gonzalez-Paredes *et al*[88]. confirmed the occurrence of intra-hepatic ED in rats with several features of MS and disclosed an important role of oxidative stress and cyclooxygenase end products in determining these functional abnormalities of the vasculature after 6 weeks of exposure to a high fat diet[88].

FROM BENCH TO BEDSIDE: POTENTIAL CLINICAL CONSEQUENCES

The effects of IR on hepatic vasculature could be of relevance in the pathogenesis and progression of NAFLD. The development of ED induced by IR may promote a pro-fibrogenic, pro-inflammatory and a pro-thrombotic environment, and impair regeneration after liver injury; aspects all related with the transition from steatosis to steatohepatitis and cirrhosis[72]. Unfortunately, in humans, the vascular abnormalities described in the liver of animal models with IR have been poorly investigated. Several authors have shown a correlation between IR and the severity of NAFLD[89], and have tested pharmacologic and non-pharmacologic strategies to improve IR in patients with NAFLD[90]. However, the association of these results with intra-

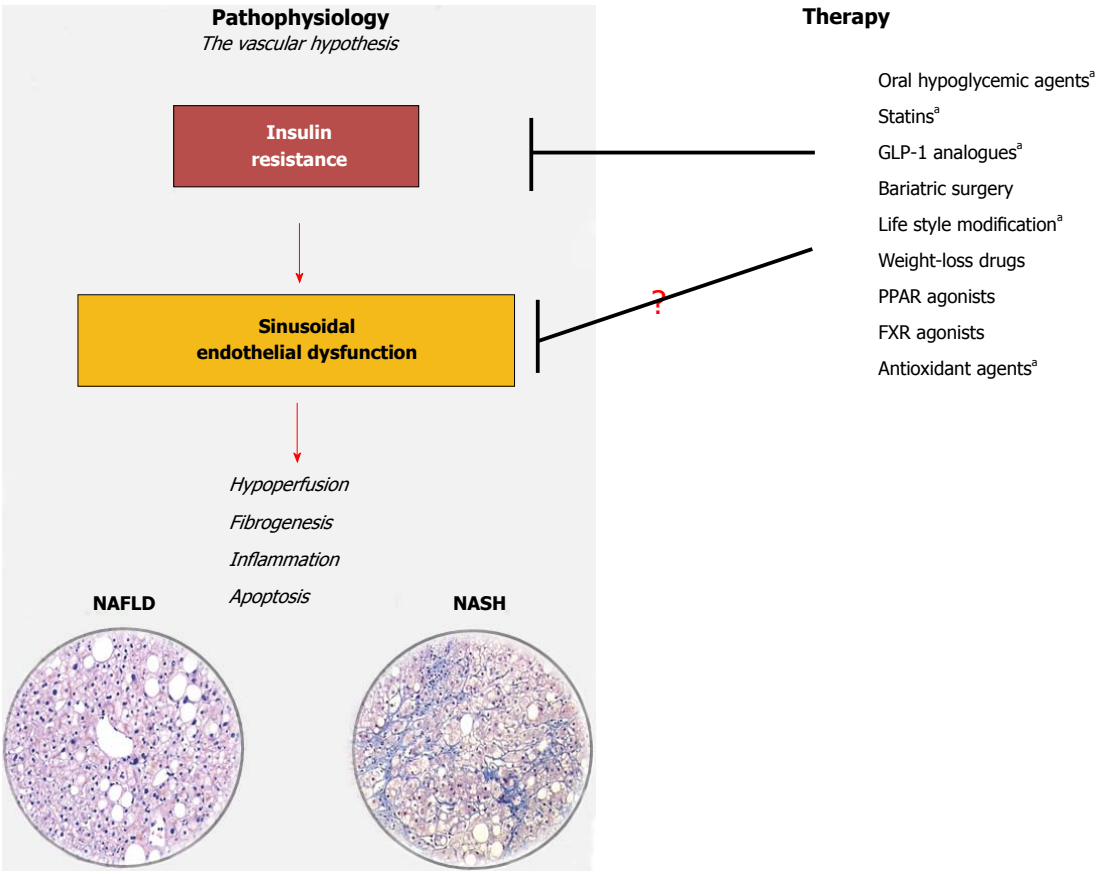

Figure 3 The vascular-hypothesis of liver damage in non-alcoholic fatty liver disease considers sinusoidal endothelial dysfunction due to insulin-resistance a key factor for the initiation and perpetuation of liver damage from simple steatosis to steatohepatitis. Any strategy of treatment ameliorating insulin-resistance may be efficacious in ameliorating sinusoidal endothelial dysfunction. Drugs marked with [a] are those with a proven efficacy on liver microcirculation (Ref. [16,42,95,103,104]). (Histological images are courtesy of Dr. Marco Maggioni, IRCCS Ca' Granda, Ospedale Maggiore Policlinico, Milan, Italy). NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis.

hepatic vascular abnormalities, although theoretically possible, has not been demonstrated so far.

The study of intrahepatic ED in clinical practice is demanding and probably represents the Achilles' heel to translating experimental observations of vascular abnormalities from animal models to humans. Catheterization of the suprahepatic vein and measurement of the hepatic venous pressure gradient (HVPG) and hepatic blood flow with indicator-dilution techniques is the most reliable tool to measure hepatic resistance to portal blood flow[91]. These measurements can be more informative if performed dynamically, after stimulating the hepatic circulation by increased splanchnic blood flow, for example with a test meal. The rationale for this is that if ED occurs in the liver, the liver circulation is less efficient in accommodating an increase in blood flow, with a consequent abrupt increase in portal pressure[92]. Unfortunately, the use of invasive methods in patients with early disease is challenging. Notwithstanding, Francque *et al*[93] published a series of 50 patients with biopsy-proven NAFLD who underwent liver hemodynamic studies. They found that up to 28% of patients had a HVPG over 5 mmHg (the threshold indicating sinusoidal portal hypertension), even though only one patient had histological documentation of cirrhosis. In that series, HVPG significantly correlated with the degree of steatosis, suggesting that the ballooning of hepatocytes causes narrowing of sinusoidal spaces and consequently increases intrahepatic resistance to portal blood flow, in keeping with the experimental observations in animal models of steatosis. The investigation of intra-hepatic hemodynamic changes induced by IR has been addressed by studies including patients with cirrhosis and comorbidities related to MS. Cirrhosis is frequently associated with IR[6,94]. Berzigotti *et al*[8] have demonstrated that obesity is an independent risk factor for higher portal pressure and disease progression in patients with cirrhosis (independent of the etiology) and might suggest that clinical features of IR could worsen the degree of intrahepatic ED. Interestingly, the same authors demonstrated that a program of weight loss through diet and physical exercise reduced portal pressure in overweight/obese patients with cirrhosis with positive results[95].

Beyond HVPG measurement, some authors have separately explored less invasive methodologies or biomarkers of inflammation, coagulation, platelet

activation that may be linked to ED in NAFLD. In human studies, imaging techniques such as Doppler flowmetry[96], positron emission tomography[97] and magnetic resonance[98] have demonstrated the potential for investigating intravascular changes and hepatic perfusion within the steatotic liver. It has been much harder to identify biomarkers specific for intrahepatic ED. Selectins (such as P-Selectin, E-Selectin), von Willebrand Factor, Isoprostans, asymmetric dimethyl-arginine, endothelins and a series of molecules involved in the inflammatory and hemostatic activity of the endothelium have been used by cardiologists to describe and monitor ED[99]. However, unless these biomarkers are measured at the level of the hepatic veins or, alternatively, be discriminable by their organ-origin, the impairment of all these tests can only be interpreted as consequence of IR/MS but is not specifically liver-related. This underlines once again that NAFLD is the liver expression of a systemic disease. As matter of fact, several authors have demonstrated that the severity of NAFLD correlates with some key features of the systemic cardiovascular risk described in patients with MS/IR, among them, peripheral ED measured by vasodilatory response of the brachial artery to ischemia[10], mild chronic inflammation and low fibrinolytic activity[100,101]. Presently, it would be hard to recommend a pharmacologic strategy specifically targeting intrahepatic ED. However, any strategy of treatment recommended for NAFLD[102] which can modulate MS/IR has the potential to benefit sinusoidal endothelial cells in this clinical setting (Figure 3). Furthermore, the ongoing research on new drugs targeted against apoptosis, inflammation, fibrogenesis could offer in the next future an alternative/adjuvant therapy to contrast the downstream effects of the vascular changes induced by IR.

CONCLUSION

Several studies with animal models have demonstrated that IR is associated with narrow sinusoids and sinusoidal endothelial dysfunction, which cause both a mechanical and functional increase of hepatic vascular resistance to portal blood flow, even in the absence of cirrhosis. Due to the high prevalence of metabolic syndrome in patients with chronic liver diseases, the influence of these vascular changes on the natural history of NAFLD and of cirrhosis of other etiologies is highly plausible and should be explored by specifically designed human studies. This could certainly result in new strategies for the treatment of patients with chronic liver disease.

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