Research Article

JOURNAL OF HEPATOLOGY

Cross-talk between autophagy and KLF2 determines endothelial cell phenotype and microvascular function in acute liver injury

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Background & Aims: The transcription factor Krüppel-like factor 2 (KLF2), inducible by simvastatin, confers endothelial vasoprotection. Considering recent data suggesting activation of autophagy by statins, we aimed to: 1) characterize the relationship between autophagy and KLF2 in the endothelium, 2) assess this relationship in acute liver injury (cold ischemia/reperfusion) and 3) study the effects of modulating KLF2-autophagy *in vitro* and *in vivo*.

Methods: Autophagic flux, the vasoprotective KLF2 pathway, cell viability and microvascular function were assessed in endothelial cells and in various pre-clinical models of acute liver injury (cold storage and warm reperfusion).

Results: Positive feedback between autophagy and KLF2 was observed in the endothelium: KLF2 inducers, pharmacological (statins, resveratrol, GGTI-298), biomechanical (shear stress) or genetic (adenovirus containing KLF2), caused endothelial KLF2 overexpression through a Rac1-rab7-autophagy dependent mechanism, both in the specialized liver sinusoidal endothelial cells (LSEC) and in human umbilical vein endothelial cells. In turn, KLF2 induction promoted further activation of autophagy.

Cold ischemia blunted autophagic flux. Upon reperfusion, LSEC stored in University of Wisconsin solution did not reactivate autophagy, which resulted in autophagosome accumulation probably due to impairment in autophagosome-lysosome fusion, ultimately leading to increased cell death and microvascular dysfunction.

Simvastatin pretreatment maintained autophagy (through the upregulation of rab7), resulting in increased KLF2, improved cell viability, and ameliorated hepatic damage and microvascular function.

Conclusions: We herein describe for the first time the complex autophagy-KLF2 relationship, modulating the phenotype and survival of the endothelium. These results help understanding the mechanisms of protection conferred by KLF2-inducers, such as simvastatin, in hepatic vascular disorders.

Keywords: Statins; Simvastatin; Ischemia/reperfusion; LSEC; HUVEC; Rab7; Rac1.
Received 21 December 2015; received in revised form 27 July 2016; accepted 30 July 2016; available online 18 August 2016

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Lay summary: Autophagy and the transcription factor KLF2 share a common activation pathway in the endothelium, being able to regulate each other.

Statins maintain microvascular function through the inhibition of Rac1, which consequently liberates Rab7, activates autophagy and increments the expression of KLF2.

Introduction

Autophagy is a constitutive process that maintains cellular homeostasis in a wide variety of cell types through the encapsulation of damaged proteins or organelles in double-membrane vesicles called autophagosomes, which fusing with lysosomes allow degradation of the cargo. As a result, cells obtain amino-acids, lipids and other components that will serve as a source of energy and new building blocks for synthesis. Regulation of autophagy is complex since it shares molecular signaling with cell proliferation and apoptosis [1,2]. Cells activate autophagy as a mechanism of cellular recycling and survival in response to cellular stresses like low nutrients, low ATP or hypoxia [3,4].

Autophagy plays a role in the pathophysiology of diverse liver diseases, including non-alcoholic steatohepatitis, viral hepatitis, fibrosis, and hepatocellular carcinoma [5–7]. However, there is no consensus about the role of autophagy in ischemia and reperfusion injury, where autophagy may be differentially regulated, and have opposite effects, depending on type of ischemia (warm or cold) or the preservation solution used [7–11]. Moreover, the possible role of autophagy in the maintenance of liver sinusoidal endothelial cells (LSEC) phenotype is completely unknown.

The vasoprotective transcription factor Krüppel-like factor 2 (KLF2) is expressed in the liver endothelium in response to blood flow-derived shear stress and plays a key role in the pathophysiology of hepatic ischemia and reperfusion injury [12,13]. In fact, when the liver is ischemic due to cold preservation or to surgical procedures, the endothelium rapidly loses its KLF2 expression leading to the dysregulation of its specialized phenotype (capillarization), development of hepatic microvascular dysfunction, and ultimately hepatic injury.

Pharmacologically, KLF2 can be efficiently induced by statins. Although these FDA/EMA approved drugs were designed to reduce cholesterol synthesis, several reports demonstrated that statins are potent KLF2 activators independently of their lipid lowering effects but dependent on the geranylgeranyl-pyrophosphate (GGPP) pathway [14,15]. In the field of acute liver injury, previous studies demonstrated that simvastatin protects healthy and steatotic rat livers through the maintenance of LSEC vasoprotective phenotype [13,16,17].

Considering this background and recent studies suggesting that KLF2 activators such as simvastatin and resveratrol might be able to increase autophagic flux in the endothelium [18–20], and that autophagy is required for the endothelium to respond to shear stress [21], the present study aimed at: 1) characterizing the possible cross-link between autophagy and KLF2 in the endothelium; 2) defining endothelial autophagy in the context of liver ischemia/reperfusion; and 3) modulating hepatic autophagy *in vitro* and *ex vivo* to revert the negative effects of ischemia/reperfusion.

Materials and methods

Animals

Male Wistar rats (Charles River) weighting 300–350 g were kept in environmentally controlled facilities at the IDIBAPS. All experiments 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 (European Economic Community (EEC) Directive 86/609).

Liver sinusoidal endothelial cells (LSEC) isolation

Rat LSEC were isolated as described in Supplementary methods. LSEC used in the present study showed average viability of 96% (by trypan blue exclusion) and 92% purity (by acetylated-low density lipoprotein (Ac-LDL) incorporation and Reca-1 staining) [22,23].

In vitro cold ischemia and warm reperfusion (I/R)

Freshly isolated LSEC were washed twice with warm phosphate balanced saline (PBS) and cultured at 4 °C in cold Celsior or University of Wisconsin (UWS) solutions. Using an oxygen microsensor (Unisense OX-NP), O_2 availability was measured demonstrating that cold storage solutions have a 35% reduction in O_2 saturation in comparison to standard culture conditions, therefore ensuring an appropriate hypoxic environment.

After ischemia time, cells were washed twice with cold PBS and *in vitro* reperfusion was mimicked incubating LSEC in complete media during 2 h at 37 °C in normoxic humid atmosphere.

Endothelial cells treatments

Primary rat LSEC or human umbilical vein endothelial cells (HUVEC) (Lonza) were treated with 5 μM simvastatin (Calbiochem), 5 μM mevastatin (Calbiochem), 10 μM GGPP (Sigma), 5 μM GGTI-298 (Sigma), 20 μM chloroquine (CQ; Sigma), 2 μM rapamycin (Santa Cruz), 1 μM resveratrol (Sigma), 50 μM NSC23766 (Sigma) or 50 nM bafilomycin (Baf; Sigma) when appropriate. Drugs concentrations derive from previously published reports [14,19,23,24] or from preliminary studies performed by our team.

For each experiment, culture medium was aliquoted and complemented with the corresponding drug or vehicle and added to the corresponding wells. Results from endothelial cells derive from at least n = 3 independent experiments (different isolations for LSEC; different commercial batches for HUVEC always below passage 6) with n = 2–3 replicates for each experimental condition.

Adenoviral overexpression of KLF2

Cells were seeded at a 60% confluence in complete medium. At the time of infection, plates were washed twice with PBS and were incubated with 10 MOI of adenovirus containing KLF2 (Ad*KLF2*) or adenovirus containing green fluorescent protein (AdGFP) (kindly provided by Prof Garcia-Cardena) for 2 h in culture medium containing 2% FBS. After infection, the medium containing the adenovirus was removed and cells were incubated for 24 h [25].

Endothelial shear stress

HUVEC were seeded in gelatin-coated μSlide flow chambers (IBIDI) at a confluence of 100,000 cells/chamber and maintained at the incubator for 12 h. Afterwards, culture media (Medium 199 with 20% FBS, 2 mM L-glutamine, 1% penicillin/streptomycin, 0.1 mg/ml heparin, 0.05 mg/ml endothelial mitogen and 2% dextran) was added and cells were cultured for 24 h in static or dynamic conditions (12 dyn/cm^2) [23].

Endothelial viability

Cell death was analyzed by double staining with acridine orange and propidium iodide (AO-PI) and by trypan blue exclusion assay, as previously described [25]. Briefly, for AO-PI staining, endothelial cells were incubated with 800 ng/ml acridine orange and 5 μg/ml propidium iodide for 10 min. Plates were washed twice with PBS and complete Roswell Park Memorial Institute (RPMI) without phenol red media was added. Cells were observed with a fluorescence microscope (Olympus BX51 with a U-LH100HG light source). Four fields were randomly selected per well and pictures of visible light, green light (488 nm emission) and red light (555 nm emission) were taken at 200x magnification. Images were merged using ImageJ software (NIH). Cells stained in plain green were counted as viable, cells with bright and green dotted nuclei were counted as apoptotic, and cells stained in red were counted as necrotic.

The trypan blue exclusion assay was performed on plate. Trypan blue (Fluka) was added to the medium at a final concentration of 0.04 mg/ml. Cells were incubated at 37 °C for 10 min and pictures were taken for further analysis.

Two independent researchers (SG-M and FM) performed the analysis of all pictures blindly.

Immunofluorescence

Endothelial cells were seeded onto 12 mm confocal coverglasses (Electron Microscopy Sciences). At the end of treatments, cells were fixed with 4% paraformaldehyde for 10 min, rinsed with PBS and permeabilized with 0.1% triton X-100 (Sigma) for 5 min. Cells were blocked for 30 min with 1% BSA in PBS and subsequently incubated with primary antibodies against LC3B (1:200, cell signaling) and Lamp2 (1:50, Santa Cruz) overnight at 4 °C. Incubation with secondary antibodies conjugated with Alexa Fluor 488/555 (1:300, Invitrogen) was performed at room temperature for 1 h along with DAPI (3 ng/ml, Invitrogen). Preparations were then mounted using Fluoromount-G (Bionovacientífica) and dried overnight. Six images per preparation and channel (visible; green, 488 nm; red, 555 nm) were obtained with a spectral confocal microscope (Leica TCS-SP5). Images were then analyzed with the ImageJ software (NIH).

Ex vivo model of ischemia/reperfusion

Male Wistar rats (250–300 g; n = 8 per experimental condition) were pretreated with the autophagy inhibitor CQ (60 mg/kg, i.p., t = −24 h and −2 h) [26], or its vehicle, followed by the KLF2 inducer simvastatin (1 mg/kg, i.v., t = −1 h) or vehicle. Afterwards, rats were anesthetized with ketamine (80 mg/kg i.p; Merial Laboratories, Barcelona, Spain) + midazolam (5 mg/kg i.p; Laboratorios Reig Jofré, Barcelona, Spain). Livers were exsanguinated with Krebs buffer, flushed through the portal vein with 10 ml of ice-cold UWS and explanted. Grafts were kept submerged in this solution for 16 h and reperfused for 2 h with warm Krebs buffer [13,16]. Then, liver microvascular function was evaluated analyzing endothelium-dependent vasorelaxation to incremental doses of acetylcholine (10^{-7}–10^{-5} M) after pre-constriction with methoxamine (10^{-4} M) [27].

Samples of perfusion buffer and liver tissue were stored for molecular analyses.