Research Article

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Simvastatin maintains function and viability of steatotic rat livers procured for transplantation

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Background & Aims: Liver grafts obtained from healthy rat donors develop acute microcirculatory dysfunction due to cold-storage and warm-reperfusion injuries. These detrimental effects are avoided adding simvastatin to the cold-storage solution. Considering the importance of increasing organ donor pool for transplantation, we characterized whether simvastatin pretreatment can protect steatotic grafts from cold-storage and warm-reperfusion injuries.

Methods: Rats fed with high-fat diet received a single dose of simvastatin, or its vehicle, 30 min before liver procurement. Grafts were then cold stored for 0 h (control group) or 16 h and warm reperfused. At the end of the reperfusion period, hepatic vascular resistance, endothelial function, nitric oxide pathway, cell death, oxidative stress, autophagy, and liver injury were evaluated. Hepatic vascular resistance and endothelial function were determined in a group of simvastatin-treated livers in the presence of the nitric oxide synthase inhibitor L-NNA.

Results: Cold-stored rat steatotic livers exhibit increased hepatic vascular resistance and marked endothelial dysfunction, together with liver damage, oxidative stress, and low nitric oxide. Simvastatin markedly improved liver injury and prevented hepatic endothelial dysfunction. The beneficial effects of simvastatin were associated with cell death diminution, autophagy induction, and nitric oxide release. Statin-derived liver microcirculation protection was not observed when nitric oxide production was blunted.

Conclusions: Pretreatment of steatotic liver donors with simvastatin shortly before procurement of the liver graft strongly protects both parenchymal and endothelial components of the liver after warm reperfusion. Our data reinforce the use of statins to protect liver grafts undergoing transplantation.

Keywords: Endothelial dysfunction; Endothelium; Statins; Ischemia; Reperfusion.
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Abbreviations: I/R, ischemia reperfusion; O_2^-, superoxide; cGMP, cyclic guanosine monophosphate; NO, nitric oxide; Ach, acetylcholine.

Introduction

Liver transplantation is the only life-saving therapy for most types of end-stage liver disease. Despite the advancement in surgical techniques, postoperative care and immunosuppressive therapies, approximately 20% of liver transplants are associated with serious clinical problems [1]. Moreover, liver transplantation rates are limited by the shortage of adequate organs for clinical use, which have led to the use of steatotic liver grafts from extended-criteria donors. Unfortunately, these livers are much susceptible to ischemia/reperfusion (I/R) injury and, when used, have poorer outcome than non-steatotic livers [2,3]. Indeed, the use of steatotic livers for transplantation is associated with increased risk of primary graft dysfunction or failure after transplantation [4,5].

The process of hepatic I/R injury is a sequence of events involving many interconnected factors occurring in a variety of cell types. Liver endothelial cells are particularly vulnerable to I/R injury and develop severe alterations during cold storage and warm reperfusion, including cell activation and apoptosis, which are magnified in steatotic grafts [6,7]. Different mechanisms for endothelial damage during cold storage and/or warm reperfusion have been described [8–10]. Our group has unravelled that lack of hemodynamic stimulation occurring during cold storage conditions is a major detrimental effect of organ preservation for transplantation on the endothelial phenotype [11,12]. In fact, flow cessation *per se* results in a significant reduction in endothelial vasoprotective pathways leading to graft function deterioration and development of acute endothelial dysfunction. These negative effects of cold storage conditions, observed in

liver grafts from healthy rat donors, can be prevented adding simvastatin, a vasoprotective compound, to the cold preservation solution [12].

Considering that microcirculatory derangements are regarded as potentially responsible for the vulnerability of steatotic livers to I/R injury, and that simvastatin confers strong hepatic and vascular protection during cold storage [12], the present study aimed at investigating the potential use of this vasoprotective compound to ameliorate the hepatic and microcirculatory status of steatotic livers procured for transplantation. We report here our findings using two experimental models of liver steatosis, where we characterized the effects of cold storage on the hepatic endothelial vasoprotective phenotype, and investigated the effects of acute pretreatment of the donor with simvastatin on the hepatic I/R injury observed upon reperfusion.

Materials and methods

Animals and treatment

Male rats from Charles River laboratories, SA (Barcelona, Spain), weighting 275–300 grams, were used. Liver steatosis was induced feeding the animals with a safflower oil-based high-fat diet (28% carbohydrates, 58% fat, 14% protein; #5ALX, TestDiet, London, UK), as previously described by our group and others [13,14]. This high-fat diet was administered during 3 days to Wistar rats (as model of moderate steatosis with 20–40% of hepatocyte vesicular fatty infiltration) or 7 days to Sprague Dawley rats (advanced steatosis with over 75% of hepatocytes exhibiting macrovesicular fat) [13,14]. Age-matched control rats received standard chow. The animals were kept in environmentally controlled animal facilities at the August Pi i Sunyer Institute for Biomedical Research. 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).

Rats were anesthetized with ketamine hydrochloride (100 mg/kg intraperitoneally; Merial Laboratories, Barcelona, Spain) plus midazolam (5 mg/kg intraperitoneally; Laboratorios Reig Jofré, Barcelona, Spain). Simvastatin (1 mg/kg) or its vehicle (0.01% DMSO; n = 7 per group) was intravenously administered through the femoral vein. Thirty min later, liver vascular studies and liver cold storage were performed as described below.

Liver vascular studies

Liver vascular responses were assessed in the isolated, *in situ* liver perfusion system, as described previously [15]. Briefly, after cannulation of the bile duct, livers were perfused through the portal vein with oxygenated Krebs buffer in a recirculation fashion, at a constant flow rate of 30 ml/min with a total volume of 100 ml. An ultrasonic transit-time flow probe (model T201; Transonic Systems, Ithaca, NY) and a pressure transducer (Edwards Lifesciences, Irvine, CA) were placed on line, immediately ahead of the portal inlet cannula, to continuously monitor portal flow and perfusion pressure. Another pressure transducer was placed immediately after the thoracic vena cava outlet for measurement of outflow pressure. The flow probe and the two pressure transducers were connected to a PowerLab (4SP) linked to a computer using the Chart version 5.0.1 for Windows software (ADInstruments, Mountain View, LA). The portal flow, inflow and outflow pressures were continuously sampled and recorded. After 20 min of stabilization, the livers were flushed with cold Celsior Solution (CS; Genzyme Corporation, Cambridge, MA), harvested and then cold stored for 16 h in CS.

After cold storage, livers were exposed at room temperature (22 °C) for 20 min, to mimic warm ischemia period, and reperfused via the portal vein with oxygenated Krebs buffer (37 °C). During the first 5 min of warm reperfusion (initial stabilization period), portal flow was progressively increased up to 30 ml/min. The perfused preparations were continuously monitored during 60 min. Afterwards, liver endothelial 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) [16].

Control livers (not subject to cold storage) were perfused, flushed with CS, harvested and immediately warm reperfused *ex vivo*.

In an additional group of animals treated with simvastatin (n = 6), the nitric oxide synthase inhibitor L-NNA (10^{-3} M; Sigma, Tres Cantos, Madrid) was added to the perfusion buffer upon warm reperfusion and the intrahepatic circulation was assessed as described above. Hemodynamic data from all groups were blindly analyzed under code.

Aliquots of the perfusate were sampled for the measurement of transaminases and lactate dehydrogenase (LDH), using standard methods, at the Hospital Clinic de Barcelona's CORE lab. Bile output was evaluated during the study and results expressed as the difference in production comparing pre-explant and postreperfusion periods. Cholesterol levels were measured in hepatic samples homogenized in HEPES buffer (1:3 w:v), by standard methodology, at the Hospital Clinic's CORE lab.

Histological analysis

Liver samples were fixed in 10% formalin, embedded in paraffin, sectioned (thickness of 2 μm), and slides were stained with hematoxylin and eosin (H&E) [17]. Hepatic histology was analyzed by a third researcher under blind conditions. To detect neutral lipids, snap-frozen livers were fixed in a freezing medium (Jung, Leica Microsystems, Nussloch, Germany) and stained with Oil Red for 2 h at room temperature [18].

The samples were photographed using a microscope equipped with a digital camera and the assistance of Axiovision software (Zeiss, Jena, Germany).

Oxidative stress determination

Hepatic superoxide (O_2^-) levels were quantified using a commercially available assay (Sigma) with minor modifications. Briefly, livers were homogenized in buffer containing 20 mM HEPES, 1 mM EDTA, 210 mM mannitol and 70 mM sucrose. After centrifugation at 1500g for 5 min at 4 °C, the supernatant was collected and incubated during 20 min at 37 °C with WST-1, a tetrazolium salt that produces a formazan dye upon reaction with O_2^-. Absorbance at 440 nm was proportional to the amount of O_2^- radical. Positive (exogenous O_2^- generating enzyme) and negative (samples with high antioxidant capacity) internal controls were included.

Measurement of NO bioavailability

Levels of cGMP, a surrogate marker of NO bioavailability, were analyzed in liver homogenates using an enzyme immunoassay (Cayman Chemical Co., Ann Arbor, MI), as previously described [19].

Additionally, hepatic nitrites/nitrates (NOx) production was assessed in aliquots of perfusate using specific microelectrodes (Lazar Laboratories, Los Angeles, CA), according to manufacturer's instructions.

Western blotting

Liver samples were processed as previously described [15]. Aliquots from each sample containing equal amounts of protein (40–100 μg) were run on 8–15% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane. Equal loading was ensured by Ponceau staining. The blots were subsequently blocked for 1 h and probed overnight at 4 °C with antibodies recognizing eNOS (BD Transduction Laboratories, Lexington, KY), phosphorylated eNOS at Ser1176 (BD Transduction Laboratories), cleaved Caspase-3 (Cell Signaling Technology, Beverly, MA), LC3B (Cell Signaling), or KLF2 (Santa Cruz Biotech, Santa Cruz, CA), all 1:1000, followed by incubation with their corresponding HRP-conjugated secondary antibodies (1:10,000, Stressgen, Glandford Ave, Victoria, BC, Canada) for 1 h at room temperature. Blots were revealed by chemiluminescence. Protein expression was determined by densitometric analysis using the Science Lab 2001, Image Gauge (Fuji Photo Film Gmbh, Düsseldorf). Blots were assayed for GAPDH (Santa Cruz Biotech) content as standardization of sample loading.

Statistical analysis

Statistical analyses were performed with the SPSS 18.0 for Windows statistical package (IBM Corp., Armonk, NY). All results are expressed as mean ± standard error of the mean. Comparisons between groups were performed with analysis of variance followed by Tukey's test, or with Mann-Whitney test when adequate. Differences were considered significant at a p value less than 0.05.