Research Article

JOURNAL OF HEPATOLOGY

Mouse hepatocytes and LSEC proteome reveal novel mechanisms of ischemia/reperfusion damage and protection by A2aR stimulation

Giorgia Mandili[1,2], Elisa Alchera[3,†], Simone Merlin[3,†], Chiara Imarisio[3,†], Bangalore R. Chandrashekar[3], Chiara Riganti[4], Alberto Bianchi[3], Francesco Novelli[1,2,†], Antonia Follenzi[3,†], Rita Carini[3,†,*]

[1]*Centre for Experimental and Clinical Studies CERMS, Azienda Universitaria Ospedaliera Città della Salute e della Scienza Città di Torino, Torino, Italy;* [2]*Department of Molecular Biotechnology and Heath Sciences, University of Torino, Torino, Italy;* [3]*Department of Health Science, University of Piemonte Orientale, Novara, Italy;* [4]*Department of Oncology, University of Torino, Torino, Italy*

Background & Aims: Ischemia-reperfusion (IR) of liver results in hepatocytes (HP) and sinusoidal endothelial cells (LSEC) irreversible damage. Ischemic preconditioning protects IR damage upon adenosine A2a receptor (A2aR) stimulation. Understanding the phenotypic changes that underlie hepatocellular damage and protection is critical to optimize strategies against IR.

Methods: The proteome of HP and LSEC, isolated from sham or IR exposed mice, receiving or not the A2aR agonist CGS21680 (0.5 mg/kg b.w.), was analyzed by 2-D DIGE/MALDI-TOF.

Results: We identified 64 proteins involved in cytoprotection, regeneration, energy metabolism and response to oxidative stress; among them, 34 were associated with IR injury and A2aR protection. The main pathways, downregulated by IR and upregulated by CGS21680 in HP and LSEC, were related to carbohydrate, protein and lipid supply and metabolism. In LSEC, IR reduced stress response enzymes that were instead upregulated by CGS21680 treatment. Functional validation experiments confirmed the metabolic involvement and showed that inhibition of pyruvate kinase, 3-chetoacylCoA thiolase, and arginase reduced the protection by CGS21680 of *in vitro* hypoxia-reoxygenation injury, whereas their metabolic products induced liver cell protection. Moreover, LSEC, but not HP, were sensitive to H_2O_2-induced oxidative damage and CGS21680 protected against this effect.

Conclusions: IR and A2aR stimulation produces pathological and protected liver cell phenotypes, respectively characterized by down- and upregulation of proteins involved in the response to O_2 and nutrients deprivation during ischemia, oxidative stress, and reactivation of aerobic energy synthesis at reperfusion. This provides novel insights into IR hepatocellular damage and protection, and suggests additional therapeutic options.

Introduction

Inflow occlusion during liver surgery, with consequent reperfusion, causes liver ischemia-reperfusion (IR) injury. IR causes up to 10% early graft dysfunction or failure during liver transplantation [1]. IR injury is the result of a complex series of alterations that mainly involve hepatocytes (HP) and sinusoidal endothelial cells (LSEC) [2]. Several events contribute to liver damage by IR. The lack of oxygen during the ischemic period is associated with mitochondrial de-energization, ATP depletion that impairs Ca^{2+}, H^+, and Na^+ homeostasis, with alteration of the volume regulatory mechanisms, and eventually necrosis. Upon oxygen readmission, the uncoupled mitochondria generate reactive oxygen species (ROS) with oxidative stress, mitochondrial permeability transition, and decreased capacity to synthesize ATP. These events, along with caspase activation, lead to cell death by both necrosis and apoptosis. Concomitantly, activation of the inflammatory reactions is also associated with the onset of IR [3,4]. Minimizing the adverse effects of IR could significantly increase the number of transplantable organs and improve the outcome of the grafts [5].

Preconditioning is a powerful protective phenomenon able to activate endogenous systems that make tissues resistant to a subsequent lethal stress [6]. Liver ischemic preconditioning, defined as brief periods of ischemia and reperfusion before sustained hepatic ischemia, can preserve energy loss, reduce transaminases release, inhibit inflammatory reactions, and promote liver regeneration after IR injury [4,7]. The surgical application of ischemic preconditioning represents a promising approach to protect against hepatic IR in humans. However, its use has the main disadvantage of inducing trauma to major vessels and stress to the target organ [8]; clinical studies have given conflicting results

Keywords: Preconditioning; Hepatoprotection; Antioxidants; Catabolic pathways; Liver damage; Energetic substrates.

Received 30 May 2014; received in revised form 11 September 2014; accepted 2 October 2014; available online 12 October 2014

* Corresponding author. Address: Department of Health Sciences, University of Piemonte Orientale 'A. Avogadro', via Solaroli, 17, Novara 28100, Italy. Tel.: +39 0321660685; fax: +39 0321620421.

E-mail address: carini@med.unipmn.it (R. Carini).

† These authors equally contributed to this work.

Abbreviations: HP, hepatocytes; LSEC, liver sinusoidal endothelial cells; A2aR, adenosine 2a receptor; IR, ischemia-reperfusion; CGS21680, 2p-(2-carboxyethyl)-phenyl-amino-50-N-ethylcarboxyamido-adenosine; ROS, reactive oxygen species; 2-DE, two-dimensional gel electrophoresis; DIGE, difference gel electrophoresis.

preventing the clinical use of ischemic preconditioning [4,8,9]. These observations show the need to explore alternative approaches to activate ischemic preconditioning in patients. To this respect, pharmacological induction of liver preconditioning could represent a more efficient and reliable technique. *In vitro* and *in vivo* studies have established a key role of the adenosine A2a receptor (A2aR) stimulation as an approach for pharmacological induction of liver preconditioning [4,10–12]. In fact, even short periods of hypoxia lead to the enhanced breakdown of adenine nucleotides to adenosine, because of the decreased production of ATP. Adenosine accumulation protects tissues from injury upon signalling through the adenosine receptor A2aR [4,12]. Expression of new synthesized proteins can also contribute to the production of the protected liver cell phenotypes [13]. The changes of protein expression of preconditioned as well as IR-injured HP and LSEC are presently poorly characterized.

With the aim of identifying new targets for the development of innovative therapeutic hepatoprotective approaches, the present work analysed the proteomic patterns of primary HP and LSEC isolated from mouse liver following IR, with or without pretreatment with the A2aR agonist CGS21680.

Materials and methods

Chemicals and reagents

Protease inhibitors, nuclease, ammonium persulfate (APS), bromophenol blue, glycerol, N,N,N9,N9-tetramethylethylene-diamine (TEMED), sodium dodecyl sulfate (SDS), TRIZMA, urea, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulphonate (CHAPS), dithiothreitol (DTT), iodoacetamide, Dulbecco's modified Eagle medium culture medium (DMEM), trypan blue, 2p-(2-carboxyethyl)-phenyl-amino-5-N-ethylcarboxya-mido-adenosine (CGS21680), palmitic acid, non-essential amino acid mixture (AA, 100X), suramine (SUR), norvaline (NRV), piruvate, trimetazidine (TMZ), 2,7-dichlorofluorescin diacetate (DCFH-DA), BCA kit, Enzymatic Assay of Pyruvate Kinase kit and ATP Bioluminescent Assay kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). DC Protein Assay kit, acrylamide, agarose, ready-made immobilized pH gradient (IPG) strip (17-cm IPG strips, pH 3-10NL) were purchased from Bio-Rad (Hercules, CA, USA). Ampholine pH 3.5–10, Western blot detection system, membranes for blotting, anti-rabbit and anti-mouse IgG horseradish-peroxidase-labeled antibodies were obtained from GE Healthcare (MI, Italy). Rabbit antibody against arginase 1 was purchased from Thermo Scientific (Illkirch Cedex, France), rabbit antibody against 3-ketoacyl-CoA thiolase from Aviva System Biology (San Diego, CA, USA). TaqMan gene expression master mix and TaqMan gene expression probes for mouse 3-ketoacyl-CoA thiolase, arginase 1, α-enolase and β-actin or 18S were purchased from Applied Biosystems Italia (Monza, Italy).

Animals

Male C57BL/6 mice used for this study were purchased from Harlan SRL, Italy. All experiments involving animals were approved by the Italian Ministry of Health and the ethical committee for animal care of the Università del Piemonte Orientale "A. Avogadro".

Ischemia-reperfusion injury

Mice were exposed to a non-lethal (~70% of the total liver volume) hepatic ischemia for 30 min, followed by 120 min reperfusion, as previously described [14]. Pharmacological A2aR activation was induced by i.p. injection of CGS21680 (0.5 mg/kg of body weight) 20 min before ischemia induction. Liver injury was assessed by measuring the ALT serum transaminase activity, with a commercial kit (Gesan Production, Italy), and the morphological alterations by histological observation. Details are provided in Supplementary Materials.

Liver cells isolation and treatment

Liver cells were isolated by liver perfusion with collagenase digestion, from sham operated mice or mice exposed to IR, pretreated or not with CGS21680. HP were obtained by differential centrifugation at 50g for 5 min at 4 °C and LSEC by immunomagnetic separation, using a negative selection with a mouse anti-CD45, and a positive selection with anti-CD146 antibodies linked to immunomagnetic beads (Miltenyibiotec, Calderana di Reno, BO, Italy), as previously reported [15] and described in details in Supplementary Materials.

Isolated HP and LSEC for proteomic analysis were stored at −80 °C until solubilization.

For evaluation of hypoxia-reoxygenation injury, primary HP and LSEC were resuspended (10^6/ml cell density) in Viaspan solution (University of Wisconsin solution without additives), fluxed with 95% N_2/5% CO_2 and maintained at 4 °C for 16 hours in sealed flasks. For reoxygenation, cells were transferred to an oxygenated Krebs-Henseleit buffer containing 20 nmol/L N-(2-hydroxyethyl)-piperazine-N0-(2-ethanesulfonic acid) (pH 7.4 at 37 °C), and the incubation flasks were further fluxed with a 95% air/5% CO_2 gas mixture. When indicated, liver cells, suspended in the Viaspan solution, were preincubated 15 min at 37 °C before cold preservation with CGS21680 (5 μmol/L) and/or suramine (SUR, 20 μmol/L), norvaline (NRV, 50 μmol/L), trimetazidine (TMZ, 100 μmol/L), pyruvate (10 μmol/L), palmitic acid (PA, 2 μmol/L) or non-essential amino acid mixture (AA, 10%). To evaluate oxidative damage, HP or LSEC in Krebs-Henseleit buffer were treated with H_2O_2 (500 μmol/L) in the presence or absence of CGS21680 (5 μmol/L) and incubated for 30 min at 37 °C under a 95% air/5% CO_2 gas atmosphere.

Determination of cell viability

Cell viability was estimated by the determination of nuclear fluorescence staining with propidium iodide using a FACScan analyzer (Becton-Dickinson, San Jose, CA) and CellQuest software (Becton-Dickinson) [13].

Measurement of reactive oxygen species (ROS)

Intracellular ROS production was measured as reported in [14], by quantifying the DCFH-DA (2,7-dichlorofluorescin diacetate) fluorescence intensity with a Hitachi F-4500 fluorescence spectrophotometer. Details are provided in Supplementary Materials.

Data analysis

Statistical analysis was performed with InStat 3 statistical software (GraphPad Software, Inc., San Diego, CA) by 1-way analysis of variance, testing with Bonferroni correction for multiple comparisons when more than 2 groups were analyzed. The distribution normality of all groups was preliminarily verified with the Kolmogorov and Smirnov test. Significance was established at the 5% level.

Proteomic analysis

Two-dimensional gel electrophoresis (2-DE) on ready-made IPG strip (17-cm IPG strips, pH 3-10NL) was performed as described [16]. For 2-D DIGE analysis, fifty micrograms of each sample (control, CGS21680, IR or CGS21680+IR) was minimally labelled with CyDye DIGE Fluors following manufacturer's instructions (GE Healthcare). For 2DE Coomassie stained gel, 1 mg of total liver protein was loaded. Destaining and in-gel enzymatic digestion of G-stained spots were performed as previously described [16]. All digests were analyzed by MALDI-TOF (TofSpec SE, MicroMass). Details are provided in Supplementary Materials.

To verify the significance of protein expression variations, two-sided Student's *t* test was used. Experiments were performed in triplicate. Statistical significance was set at $p \leqslant 0.05$. Proteins were classified as differentially expressed if ratio in spot intensity was greater than 1.5-fold (protein overexpressed) or lower than 0.5-fold (protein underexpressed).

The protein and RNA levels of ketoacyl-CoA thiolase, arginase 1, and α-enolase were evaluated by Western blotting and RT-PCR as described in Supplementary Materials.

Enzymatic assays

Aldolase B activity was measured as described in [17], with minor modifications. α-enolase activity was measured accordingly to [18]. The activity of pyruvate kinase was detected with the Enzymatic Assay of Pyruvate Kinase kit, following manufacturer's instructions. Fatty acids β-oxidation was measured as previously reported [19], with minor modifications. The activity of carbamoyl phosphate synthase I was measured on mitochondrial extracts, isolated as previously reported [20]. Arginase activity was measured with a spectrophotometric method