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Carbamazepine suppresses calpain-mediated autophagy impairment after ischemia/reperfusion in mouse livers

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ABSTRACT

Onset of the mitochondrial permeability transition (MPT) plays a causative role in ischemia/reperfusion (I/R) injury. Current therapeutic strategies for reducing reperfusion injury remain disappointing. Autophagy is a lysosome-mediated, catabolic process that timely eliminates abnormal or damaged cellular constituents and organelles such as dysfunctional mitochondria. I/R induces calcium overloading and calpain activation, leading to degradation of key autophagy-related proteins (Atg). Carbamazepine (CBZ), an FDA-approved anticonvulsant drug, has recently been reported to increase autophagy. We investigated the effects of CBZ on hepatic I/R injury. Hepatocytes and livers from male C57BL/6 mice were subjected to simulated *in vitro*, as well as *in vivo* I/R, respectively. Cell death, intracellular calcium, calpain activity, changes in autophagy-related proteins (Atg), autophagic flux, MPT and mitochondrial membrane potential after I/R were analyzed in the presence and absence of 20 μM CBZ. CBZ significantly increased hepatocyte viability after reperfusion. Confocal microscopy revealed that CBZ prevented calcium overloading, the onset of the MPT and mitochondrial depolarization. Immunoblotting and fluorometric analysis showed that CBZ blocked calpain activation, depletion of Atg7 and Beclin-1 and loss of autophagic flux after reperfusion. Intravital multiphoton imaging of anesthetized mice demonstrated that CBZ substantially reversed autophagic defects and mitochondrial dysfunction after I/R *in vivo*. In conclusion, CBZ prevents calcium overloading and calpain activation, which, in turn, suppresses Atg7 and Beclin-1 depletion, defective autophagy, onset of the MPT and cell death after I/R.

Introduction

Liver ischemia during transplantation, hepatectomy, cardiac failure and hemorrhagic shock causes anoxia, depletion of glycolytic substrates, loss of ATP and acidosis. When blood flow returns to livers, the normal oxygen concentration and pH are restored, however, paradoxically, hepatic injury is prominent upon reperfusion (Kim et al., 2008). In the liver and other tissues, reperfusion causes onset of the mitochondrial permeability transition (MPT), leading to uncoupling of oxidative phosphorylation, mitochondrial dysfunction and cell death (Kim et al., 2008). Clinical outcomes from mitochondria-targeted therapies, however, remain disappointing largely due to the complex and integrated nature of mitochondrial dysfunction after I/R.

Macroautophagy (referred to as autophagy hereafter) is a lysosome-dependent cellular process that eliminates surplus or dysfunctional cytoplasmic proteins and organelles in a timely fashion. Autophagy is a primary catabolic process of hepatic proteins and confers cytoprotection against I/R liver injury (Kim et al., 2008; Wang et al., 2011). Mitophagy is a selective mitochondrial autophagy that targets and removes damaged or abnormal mitochondria (Kim et al., 2008; Wang et al., 2011). Hence, accelerating mitophagy may have a therapeutic potential for mitochondria-related diseases such as I/R injury. Indeed, overexpression of specific autophagy genes alleviates the MPT onset and mitochondrial dysfunction in both *in vitro* and *in vivo* I/R (Kim et al., 2008; Wang et al., 2011).

Despite a therapeutic potential, current strategies to accelerate autophagy, either genetically or pharmacologically, still face numerous obstacles. Major shortcomings surrounding gene therapy include issues on safety, delivery efficiency, immune response, mutagenesis and ethical concerns (Verma and Somia, 1997). Pharmacological enhancement of autophagy also presents a number of issues. Although rapamycin and its analogues are the most well-characterized autophagy inducers, they

Abbreviations: MPT, mitochondrial permeability transition; I/R, ischemia/reperfusion; CBZ, carbamazepine; FDA, Food & Drug Administration; Atg, autophagy-related proteins; KRH, Krebs–Ringer-N-2 hydroxyethylpiperazine-N-2 ethanesulfonic acid; PI, propidium iodide; TMRM, tetramethylrhodamine methylester; LC3, microtubule-associated protein 1 light chain 3; CQ, chloroquine; IP_3, inositol-1,4,5-triphosphate.

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have serious adverse effects, including immunosuppression and hyperlipidemia (Brattstrom et al., 1998; Hartford and Ratain, 2007). Recently, carbamazepine (CBZ), an FDA approved anticonvulsant and mood stabilizing drug, has been reported to stimulate autophagy by decreasing intracellular levels of inositol (Williams et al., 2008). How CBZ affects I/R-mediated autophagy defects in livers is currently unknown.

In the present study, we demonstrate that, in both *in vitro* and *in vivo* models of I/R, CBZ alleviates lethal reperfusion injury by preventing a temporal sequence of calcium overloading, calpain activation, Atg7 and Beclin-1 depletion, defective autophagy, onset of the MPT and cell death.

Material and methods

Reagents. Fluo-4/AM, xRhod-1/AM, tetramethylrhodamine methylester and calcein/AM were purchased from Life Technologies (Grand Island, NY). Embedding agents for transmission electron microscopy were purchased from Electron Microscopy Sciences (Hatfield, PA). All other chemicals were purchased from Sigma Aldrich (St. Louise, MO) except if noted otherwise.

Hepatocyte isolation and culture. Animals received humane care according to protocols approved by the Institutional Care and Use Committee of the University of Florida. 3-month-old male C57BL/6 mice were housed in a 12-hour light, 12-hour dark cycle, and temperature-controlled room. Mice were fed a standard chow with free access to water. Hepatocytes were isolated by the collagenase perfusion method and cultured overnight in Waymouth's medium, as previously described (Kim et al., 2003a). Cell viability after isolation, as determined by trypan blue exclusion, was consistently greater than 90%. For the cell death assay, aliquots (1 ml) of 1.7×10^5 cells were plated onto 24-well microtiter plates (Falcon, Lincoln Park, NJ). For immunoblotting analysis of LC3, Atg and calpains, hepatocytes were plated on 35 mm culture dishes at a concentration of 10^6 cells. For confocal microscopic studies, 4.0×10^5 cells were cultured on 42 mm round glass coverslips in 60 mm culture dishes. All plates, dishes and coverslips were coated with 0.1% Type 1 rat tail collagen. Hepatocytes were used after overnight culture in humidified 5% CO_2 and 95% air at 37 °C.

Simulated in vitro I/R in cultured mouse hepatocytes. Anoxia in the anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) was maintained under an atmosphere of 90% N_2–10% H_2 in the presence of a heated palladium catalyst to convert residual oxygen to water vapor. The anoxic buffer was prepared by equilibrating Krebs–Ringer-N-2 hydroxyethylpiperazine-N-2 ethanesulfonic acid buffer (KRH) containing 15 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$ and 25 mM HEPES (pH 6.2) overnight inside the anaerobic chamber prior to the experiments (Kim et al., 2008; Wang et al., 2011). Oxygen tension in the chamber and anoxic buffer was monitored by a gas analyzer (Model 10; Coy Laboratory Products) and was less than 0.001 Torr. To simulate the anoxia, substrate depletion and tissue acidosis of ischemia, hepatocytes were incubated in KRH at pH 6.2 in the anaerobic chamber for 4 h. To simulate reperfusion and the return to physiological pH, anaerobic KRH at pH 6.2 was replaced with aerobic KRH at pH 7.4. Some hepatocytes were treated with 20 μM CBZ overnight before ischemia and continuously during ischemia.

Cell death assay. Necrosis was assessed by propidium iodide (PI) fluorometry using a multi-well fluorescence scanner (SpectraMax M2; Molecular device, Sunnyvale, CA) (Kim et al., 2003a; Nieminen et al., 1992). To prevent oxygen back diffusion during ischemia, plates were sealed with vacuum tape (3 M, Minneapolis, MN) inside the anaerobic chamber.

Hepatocellular loading of xRhod-1, Fluo-4, calcein, TMRM and propidium iodide. To monitor mitochondrial and extramitochondrial Ca^{2+}, hepatocytes on glass coverslips were co-loaded with 10 μM xRhod-1/AM and 10 μM Fluo-4/AM, respectively, in anoxic KRH at 37 °C for 30 min during ischemia (Gerencser AA and Adam-Vizi, 2001; Kim et al., 2006). To image temporal changes of mitochondrial membrane potential ($\Delta\Psi_m$), onset of the MPT and cell death, hepatocytes cultured on glass coverslips were co-loaded with 100 nM of tetramethylrhodamine methylester (TMRM), 1 μM calcein/AM and 3 μM propidium iodide (PI), respectively, for 30 min before reperfusion (Kim et al., 2008; Wang et al., 2011).

Autophagic flux measurement. For autophagic flux, hepatocytes were treated with 10 μM chloroquine or 100 nM bafilomycin for 3 h or 20 min prior to reperfusion, respectively. Changes in LC3 expression were monitored by immunoblotting.

Adenoviral labeling of GFP-LC3 and mCherry-GFP-LC3. Hepatocytes were infected with adenovirus encoding GFP-LC3 and mCherry-GFP-LC3 at the concentration of 10 MOI (multiplicity of infection) in hormonally defined medium, as previously described (Kim et al., 2008; Wang et al., 2011). Adenovirus harboring β-galactosidase (AdLacZ) was used for the viral control. For *in vivo* livers, mice were intraperitoneally injected with 10^{10} virus particles of adenovirus overnight.

Immunoblotting analysis. Hepatocyte and liver lysates were prepared and expression of Atg7, Beclin-1, LC3-I/II, calpain 2 and β-actin were detected on the same gel using primary polyclonal antibodies (Cell Signaling Technology, Danvers, MA) (Kim et al., 2008; Wang et al., 2011). Changes in protein expression were determined using IMAGE J software (NIH, Bethesda, MD).

Calpain activity assay. The activity of calpains was fluorometrically assessed using 20 μM succinyl-Leu-Leu-Val-Tyr-7-amino-4-methyl-coumarin (SLLVY-AMC, Sigma, St. Louis, MO), a membrane-permeable calpain substrate, in the presence and absence of CBZ (Kim et al., 2008).

Confocal laser scanning microscopy. Glass coverslips containing hepatocytes were mounted in a closed gas-tight cultivation chamber (Zeiss, Germany) inside the anoxic chamber. Confocal images of calcein, Fluo-4, TMRM, GFP-LC3, mCherry-GFP-LC3, PI and xRhod-1 were collected using an inverted Zeiss 510 laser scanning confocal microscope, as previously described (Kim et al., 2008; Wang et al., 2011).

Transmission electron microscopy. Hepatocytes were fixed with 2% paraformaldehyde/2% glutaraldehyde/125 mM cacodylate/2.2 mM $CaCl_2$ (pH 7.4) and stained with 1% OsO_4 (Electron Microscopy Sciences, Hatfield, PA) (Dunn, 1990). Images were collected with a JEOL 100CX transmission electron microscope (JEOL, Peabody, MA).

In vivo I/R. Hepatic ischemia in male C57BL/6 mice was induced by occluding the portal triad for 45 min, as previously described (Kim et al., 2008). Reperfusion was initiated by removing a microvascular clamp. Some mice were intraperitoneally injected with CBZ at the concentration of 25 mg/kg of body weight overnight prior to I/R. Liver biopsies from the left lateral lobe were collected during I/R and immediately frozen in liquid nitrogen for the analysis of autophagy proteins.

Intravital multiphoton microscopy. To visualize autophagosomes and autophagic flux, livers were labeled with adenoviral GFP-LC3 or mCherry-GFP-LC3 (Wang et al., 2011). After 15 min of reperfusion *in vivo*, a 24-gauge catheter was inserted into the portal vein. Rhodamine 123 (50 ml of 10 μM/animal), a $\Delta\Psi_m$-sensitive fluorophore, was infused for 10 min. The liver was gently withdrawn from the abdominal cavity