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Therapeutic activity of superoxide dismutase-containing enzymosomes on rat liver ischaemia-reperfusion injury followed by magnetic resonance microscopy

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ABSTRACT

Liver ischaemia-reperfusion injury (IRI) may occur during hepatic surgery and is unavoidable in liver transplantation. Superoxide dismutase enzymosomes (SOD-enzymosomes), liposomes where SOD is at the liposomal surface expressing enzymatic activity in intact form without the need of liposomal disruption, were developed with the aim of having a better insight into its antioxidant therapeutic outcome in IRI. We also aimed at validating magnetic resonance microscopy (MRM) at 7 T as a tool to follow IRI. SOD-enzymosomes were characterized and tested in a rat ischaemia-reperfusion model and the therapeutic outcome was compared with conventional long circulating SOD liposomes and free SOD using biochemical liver injury biomarkers, histology and MRM. MRM results correlated with those obtained using classical biochemical biomarkers of liver injury and liver histology. Moreover, MRM images suggested that the therapeutic efficacy of both SOD liposomal formulations used was related to prevention of peripheral biliary ductular damage and disrupted vascular architecture. Therefore, MRM at 7 T is a useful technique to follow IRI. SOD-enzymosomes were more effective than conventional liposomes in reducing liver ischaemia-reperfusion injury and this may be due to a short therapeutic window.

1. Introduction

The interruption of blood flow to the liver is common during hepatic surgery and unavoidable in liver transplantation (LT). The lack of blood supply and consequent lack of oxygen and nutrients leads to cell damage through several mechanisms. This damage can be further exacerbated during liver revascularization (reperfusion), a clinical phenomenon known either as post-reperfusion syndrome or as ischaemia-reperfusion (IR) injury (IRI).

Liver injury due to ischaemia-reperfusion can be divided into two major categories. The first is warm IRI, which is initiated by hepatocyte damage and which occurs when the blood flow is temporarily interrupted during transplantation, trauma and shock and elective liver surgery. The second is cold IRI which is initiated by damage to hepatic sinusoidal endothelial cells and microcirculation disruption occurs when organs are preserved for transplantation and is usually coupled with warm IRI during LT (Klune and Tsung, 2010; Zhai et al., 2013).

The hallmark of IRI is the local inflammatory innate immune

Abbreviations: SOD, superoxide dismutase; MRM, magnetic resonance microscopy; MRI, Magnetic Resonance Imaging; LT, liver transplantation; IR, ischaemia-reperfusion; IRI, ischaemia-reperfusion injury; ROS, reactive oxygen species; RNS, reactive nitrogen species; SOD-enzymosomes, long circulating liposomes with superoxide dismutase covalently linked to the distal end of 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[maleimide (polyethylene glycol)-2000]; SOD-liposomes, superoxide dismutase long circulating liposomes

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activation response that is triggered. In liver IRI there is initially an ischemic injury, which includes a series of molecular events occurring during hypoxia which may lead to cell death after depletion of cellular ATP. This response is directed at organ preservation and repair, and is genetically determined, and thus can vary from patient to patient. In the reperfusion phase of IRI, two distinctive phases can be singled out: the first phase, occurring in the initial 1–2 h of reperfusion, is characterized by a significantly impaired microcirculation which prolongs the period of hypoxia, with areas of the liver remaining ischemic after the onset of reperfusion (Serracino-Inglott et al., 2001). This in turn is followed by activation of immunocompetent cells, like resident liver macrophages (Kupffer cells) and generation of reactive oxygen species (ROS), such as superoxide radical ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2), and reactive nitrogen (RNS) species, such as nitric oxide ($^{\cdot}NO$) and peroxynitrite ($ONOO^-$), leading to an initial stage of the inflammatory process and to oxidative stress. Activation of liver Kupffer cells is associated not only with increased rates of formation of ROS and RNS, but also with the release of proinflammatory cytokines (TNF-α, IL-1β, IFN-γ, and others), chemokines (MIP-2, MCP-1) and adhesion molecules, which mediate signalling pathways ensuing the recruitment of inflammatory cells, such as neutrophils, and liver infiltration by neutrophils (Diesen and Kuo, 2011; Jaeschke, 2011; Klune and Tsung, 2010). The second phase, occurring 6–48 h after reperfusion, involves accumulation of neutrophils and hepatocellular injury (Jaeschke and Farhood, 2002; Klune and Tsung, 2010; Lutz et al., 2010).

High levels and/or inadequate removal of $O_2^{\cdot -}$ and H_2O_2 by antioxidant enzymes results in molecular damage and disruption of redox signalling i.e., oxidative stress, which may cause oxidative stress-mediated diseases. $O_2^{\cdot -}$ main reactions in vivo are its dismutation, catalyzed by superoxide dismutases (SODs), to form H_2O_2 and oxygen and its reaction with nitric oxide to form peroxynitrite (Beckman and Koppenol, 1996). Superoxide dismutases started to be used as antioxidant therapy for inflammatory situations in the late 60s. Since then, SODs therapy has been shown to have a protective role, not just for inflammatory diseases, but also for other pathological situations where $O_2^{\cdot -}$ and endogenous SODs have a key role (Corvo et al., 2016). In fact, SODs have been used both in animal models and, also clinically, displaying positive therapeutic outcomes for rheumatoid arthritis, cancer, ischaemia-reperfusion injury, respiratory diseases, nervous tissue damage, infectious diseases and also for radiation side effects, such as fibrosis (Carillon et al., 2013).

Recently we began studies to establish both the therapeutic effectiveness of different liposomal formulations of bovine CuZn-SOD on animal models of arthritis and IRI, as well as to test a new contrast agent for Magnetic Resonance Imaging (MRI) with high spatial resolution (magnetic resonance microscopy, MRM) (Corvo et al., 2015; Martins et al., 2014). MRM has been suggested as a promising technique for medical imaging not only to diagnose acute ischaemia but also to follow up the complex chain of events that follows an acute ischaemia (Cheung et al., 2011; Martins et al., 2014). MRM can be used on live animals and when used for imaging of fixed specimens has advantages over conventional microscopy because it is non-destructive, can be 3D, and is inherently digital (Driehuys et al., 2008). Moreover, since the fixed samples do not have to be dehydrated for MRM, as it happens in conventional histology, MRM images display the natural distribution of water in tissues and organs and provide a detailed functional and anatomical picture thus allowing to characterize lesions not possible to visualize in other conditions (Driehuys et al., 2008). We developed long circulating liposomes with SOD covalently linked to the distal end of DSPE-PEG (SOD-enzymosomes) which when tested in an animal model of arthritis showed an enhanced therapeutic result. An animal model of liver IRI was used to make a preliminary assessment of the outcome of treatment with SOD-enzymosomes and with SOD-long circulating liposomes (SOD-liposomes). We found that treatment with SOD-enzymosomes, unlike SOD-liposomes or free SOD, decreased significantly alanine aminotransferase (AST) activity in serum, a biomarker of liver cell damage due to IRI. As previously demonstrated, both these nanosystems have long circulating capabilities and enhanced retention at inflamed sites/pathologies (Corvo et al., 1999, 2015; Martins et al., 2014). At 24 h post-injection, both formulations have similar biodistribution profiles, namely in the liver (Corvo et al., 2015). However, SOD-enzymosomes have a unique capability of expressing enzymatic activity in intact form without the need of liposomal disruption, thus enabling a faster therapeutic outcome when compared to SOD-liposomes. Our previous results suggested that a therapeutic approach to IRI using SOD-enzymosomes would be beneficial, decreasing liver cell damage due to ischaemia-reperfusion. Here we aimed at further characterizing the therapeutic effect of the administration of two liposomal formulations of SOD (SOD-long circulating liposomes) and SOD-enzymosomes in the final stage of liver ischaemia induced in rats by evaluating their effect on IRI. We also aimed at validating MRM as a tool to follow IRI by using known liver injury biochemical biomarkers and histological analyses.

2. Materials and methods

2.1. Materials

1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[maleimide (polyethylene glycol)-2000] (ammonium salt), (maleimide-PEG-PE) was purchased from Avanti Polar Lipids. Egg-phosphatidylcholine (PC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy (polyethylene glycol)-2000] (PEG-PE) were obtained from Lipoid GmbH. Bovine Cu,Zn-superoxide dismutase (SOD), dimethylformamide (DMF), *N*-(2-hydroxyethyl) and Cholesterol (Chol), were obtained from Sigma. Ethylenediaminetetraacetic (EDTA) was from Merck. *N*-succinimidyl *S*-acethylthioacetate (SATA) was from Pierce. Imalgene 1000® (ketamine) was obtained from Merial Portuguesa Saúde Animal Lda, Rio de Mouro, Portugal and Domitor® (medetomidine) and Antisedam® (atipamezole) were obtained from Pfizer Animal Health, Pennsylvania, EUA.

2.2. Animals

For the therapeutic activity experiments male Wistar rats weighting 200–250 g were provided by Instituto Bento da Rocha Cabral, Lisbon, Portugal. Animals were fed with standard laboratory food and water ad libitum. All animal experiments were carried with the permission of the local animal ethical committee, and in accordance with the Declaration of Helsinki, EEC Directive (2010/63/UE) and Portuguese Law (DL 113/2013, Despacho n° 2880/2015), and all following legislations for the humane care of animals in research.

2.3. Methods

2.3.1. SOD formulations for therapeutic activity studies

2.3.1.1. SOD-enzymosomes. SOD-enzymosomes were prepared as described in (Corvo et al., 2015). Briefly, PC:Chol:Maleimide-PEG-PE:PEG-PE at the molar ratio of 68.25:30.5:0.75:0.5 and dispersed in 145 mM NaCl/10 mM citrate buffer, pH 6.0 (20 μmol lipid/mL), dimensioned through a final polycarbonate membrane pore size of 0.05 μm. The conjugation was performed with a SATA:SOD molar ratio of 4:1 and a SOD concentration of 1 mg/mL. The conjugated enzyme was separated from the enzymosome dispersion by ultracentrifugation, at 300,000 × *g* for 120 min at 4 °C in a Beckman LM-80 ultracentrifuge. Finally, liposomes were dispersed in 0.145 M NaCl/10 mM citrate buffer pH 5.6.

2.3.1.2. SOD-liposomes. SOD-liposomes were prepared as described before (Corvo et al., 2002). Briefly, liposomes were prepared by the dehydration-rehydration method, followed by sequential extrusion through polycarbonate filters up to a final pore size of 0.05 μm. The