Research Article

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Cerium oxide nanoparticles reduce steatosis, portal hypertension and display anti-inflammatory properties in rats with liver fibrosis

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Background & Aims: Cerium oxide nanoparticles (CeO_2NPs) have proven to behave as free radical scavengers and/or anti-inflammatory agents. The aim of the study was to determine whether CeO_2NPs display hepatoprotective properties in experimental chronic liver disease.

Methods: Systemic and hepatic effects of nanoparticles were assessed in CCl_4-treated rats receiving CeO_2NPs or vehicle twice weekly for two weeks and CCl_4 treatment was continued for 8 additional weeks. Thereafter, mean arterial pressure and portal pressure (PP) were assessed and serum samples obtained to measure standard hepatic and renal function tests. Organ and subcellular distribution of NPs were assessed using mass spectrometry (ICP-MS) and transmission electron microscopy. Liver samples were obtained to evaluate steatosis, α-SMA expression, macrophage infiltration, apoptosis and mRNA expression of oxidative stress, inflammatory or vasoactive related genes.

Results: Most CeO_2NPs were located in the liver and it reduced hepatic steatosis, ameliorated systemic inflammatory biomarkers and improved PP without affecting mean arterial pressure. In addition, a marked reduction in mRNA expression of inflammatory cytokines (*TNFα*, *IL1β*, *COX-2*, *iNOS*), ET-1 and messengers related to oxidative (*Epx*, *Ncf1*, *Ncf2*) or endoplasmic reticulum (*Atf3*, *Hspa5*) stress signaling pathways was observed in the liver of rats receiving CeO_2NPs. This was associated with reduced macrophage infiltration and reduced abundance of caspase-3, α-SMA and inflammatory cytokines.

Conclusions: CeO_2NPs administration to CCl_4-treated rats protects against chronic liver injury by reducing liver steatosis and portal hypertension and markedly attenuating the intensity of the inflammatory response, thereby suggesting that CeO_2NPs may be of therapeutic value in chronic liver disease.

Introduction

Deregulated inflammation is thought to be a common step of many pathological processes including vascular, metabolic and neurological diseases [1]. In this context, liver diseases are not an exception. In fact, regardless of whether its viral, metabolic or toxic etiology, acute inflammation is a common event that after evolving to chronic inflammation, leads to extracellular matrix remodeling, cirrhosis and eventually, liver failure [2]. Resolution of inflammatory response has classically been considered a passive process resulting from the progressive dilution of cell mediators involved in inflammatory response such as cytokines and chemokines [3]. However, during the last few years the concept that inflammatory resolution is an active response that can be modulated and facilitated by specialized pro-resolving mediators has gained increased attention [4]. These findings raised the possibility of using pro-resolving substances as a novel therapeutic strategy.

In the current investigation we explored the possibility that engineered ceria nanoparticles (CeO_2NPs) may behave as exogenous pro-resolving mediators in liver disease. Actually, such nanoparticles have already demonstrated their utility for local targeting and delivery, whereas most ceria applications are based on its redox activity, including its biomedical use [5,6]. In this regard, most therapeutic CeO_2NPs applications are proposed based on their ability to reduce *in vitro* the levels of reactive oxygen species (ROS) and consequently, most inflammatory mediators such as inducible nitric oxide synthase (iNOS), nuclear

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Abbreviations: α-SMA, α-smooth muscle actin; HSCs, hepatic stellate cells; NASH, non-alcoholic steatohepatitis; iNOS, inducible nitric oxide synthase; ROS, reactive oxygen species; NF-κβ, nuclear factor κ-β; TNF-α, tumor necrosis factor α; TMAOH, tetramethylammonium hydroxide; TEM, transmission electron microscopy; MRI, magnetic resonance imaging; AST, aspartate transaminase; GGT, gamma-glutamyl transferase; ALT, alanine transaminase; ER, endoplasmic reticulum; DCF, 2′,7′-dichlorofluorescein; SOD, superoxide dismutase.

factor κ-β (NF-κβ), tumor necrosis factor α (TNF-α) and interleukins [7]. Consequently, suggestions have been raised indicating that CeO_2NPs may be useful in the prevention and/or treatment of diabetic cardiomyopathy, lung disease, retinal degeneration, stroke and neurodegenerative disorders [8]. However, whether CeO_2NPs are of therapeutic value in liver disease is not known. We assessed the organ distribution, subcellular localization, systemic and hepatic effects of intravenous administration of CeO_2NPs to CCl_4-treated rats. The aim of the study was to determine whether CeO_2NPs display inflammatory pro-resolving activity and hepatoprotective properties in experimental chronic liver disease.

Materials and methods

Synthesis and characterization of CeO_2NPs

CeO_2NPs were synthesized by the chemical precipitation of cerium (III) nitrate hexahydrated (Sigma-Aldrich, St. Louis, MO, USA) in a basic aqueous solution [9]. Modifying the pH conditions, different sizes can be obtained. Here, we used a mixture of different sized nanoparticles (from 4 to 20 nm), at a concentration of 1 mg/ml. For 4 nm CeO_2NPs; in a first step, 10 mM of cerium (III) nitrate hexahydrate was dissolved in 100 ml of absolute ethanol at room temperature. The solution was left under stirring for about 30 min. To the 100 ml solution was added 1 ml of tetramethylammonium hydroxide solution (TMAOH, 1.0 ± 0.02 M in H_2O) at a final concentration of 10 mM, and the mixture was left under stirring. For 10 nm CeO_2NPs; in a first step, 10 mM of cerium (III) nitrate hexahydrate was dissolved in 90 ml of absolute ethanol at room temperature. To this solution 10 mL of hexamethylenetetramine (HMT, 1 M) was added at a final concentration of 100 mM, and the solution was left under stirring. For all samples, NPs were purified by centrifugation and resuspended in aqueous solution of 10 mM TMAOH, which acts as a stabilizer. CeO_2NPs were kept at 4 °C until administration to animals. The surface charge of the NPs was characterized in a Z-sizer (Malvern, Worcestershire, UK), while the crystal size was characterized by high-resolution (HR-TEM) in the Tecnai G2 F20 at 200 kV (FEI, Oregon, USA). The crystal structure was analyzed by HR-TEM (Tecnai 200 kV) and XRD (Xpert Pannalytical, MA, USA), and the light interaction by UV-VIS spectroscopy (Shimatzu, Kyoto, Japan). Size distribution was computer analyzed by ImageJ (National Institutes of Health, Bethesda, MD, USA).

Induction of hepatic fibrosis in rats

See Supplementary material and methods.

Organ distribution of Ce in CCl_4-treated rats

Organs were collected from 24 CCl_4-treated rats receiving CeO_2NPs. Once 5 min of CCl_4 inhalation was reached the animals received CeO_2NPs (0.1 mg/kg bw) twice weekly for two weeks and CCl_4 treatment was maintained thereafter. CeO_2-NPs were dispersed in saline solution and intravenously given as a bolus (500 μl) through the tail vein. Animals were euthanized at day 1, 21, 42 and 56 after the last administration of CeO_2NPs and organs dissected and kept at −80 °C for further analysis. Samples were diluted with an aqueous solution of HNO_3 2% w/w (Trace Metal Basis; Sigma-Aldrich) and analyzed for cerium concentration by inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7500; Agilent Technologies, California, USA). The quantification is done by interpolation in a standard curve obtained from a commercial 1000 ppm Ce standard (Sigma-Aldrich).

Subcellular location of CeO_2NPs in the liver of CCl_4-treated rats

Location of CeO_2NPs was assessed using transmission electron microscopy (TEM). Two rats were intravenously injected (500 μl) with CeO_2NPs (0.1 mg/kg bw) or vehicle (saline solution containing TMAOH ammonium salts 0.8 mM) twice a week for 2 weeks starting at 8 weeks of fibrosis induction as described. The rats were euthanized 90 min after the last administration and liver samples were fixed in 2% paraformaldehyde and 2.5% gluteraldehyde for further ultrastructural examination. Tissues were embedded in Spur's resin and thin sections (50–55 nm) were cut and placed on copper grids and then stained with uranyl acetate and lead citrate. After staining, the sections were examined at low electron power microscope to increase contrast in a JEOL-1010 TEM (JEOL, Tokyo, Japan) operated at 80 kV and equipped with a BioScan camera (Gatan, CA, USA) and digital photomicrographs were taken.

Systemic and hepatic effects of CeO_2NPs in CCl_4-treated rats

The hemodynamic and gene expression effects of CeO_2NPs were assessed in CCl_4-induced fibrosis rats receiving CeO_2NPs (0.1 mg/kg.bw, n = 10) or vehicle (TMAOH ammonium salts 0.8 mM, n = 15) as previously described. Following NPs administration the fibrosis induction protocol was maintained for 8 additional weeks. Thereafter, animals were instrumented as described below and a blood sample was obtained to measure standard liver and renal function tests in baseline conditions. A hemodynamic study was performed to measure mean arterial pressure, portal pressure (PP), heart rate and splanchnic perfusion pressure. The animals were sacrificed by isofluorane overdose. Liver specimens were obtained from each animal, immediately frozen in dry ice, and stored at −80 °C or fixed in 10% buffered formalin for further hematoxylin and eosin (H&E) and immunostaining analysis.

Additional materials and methods are provided in the Supplementary material section.

Results

Characterization of CeO_2NPs

HR-TEM analysis of CeO_2NPs revealed that the particles had a spherical morphology (Fig. 1A–C) and were predominantly in the size range of 4–20 nm. See Supplementary Results.

Liver and spleen are major targets for CeO_2NPs in CCl_4-treated rats

It is well known that after systemic distribution small inorganic NPs accumulate in the liver and spleen [10]. This was confirmed in our laboratory using MRI (Oró *et al.*, data not shown). NPs accumulation in these tissues was apparent as early as 30 min following intravenous injection. We further confirmed these findings by analyzing tissue Ce accumulation by ICP-MS following intravenous CeO_2NPs injection in fibrotic rats (Fig. 2A). We found that 90 min after the administration of NPs, 84% and 12% of the total dose of Ce collected was accumulated in the liver and spleen, respectively. Considerably less Ce accumulated in the lungs and kidneys (2.25% and 0.63%, respectively), with very little in the heart and brain (data not shown). Interestingly, Ce was detected in these organs for over 8 weeks.

Subcellular localization of CeO_2NPs

Under TEM, following CCl_4 administration, hepatocytes became swollen and the mitochondria appeared as broken crests. CeO_2NPs 90 min after injection did not induce noticeable alterations in cell morphology in comparison to CCl_4-treated rats receiving vehicle. CeO_2NPs were present in the form of agglomerates of different sizes in the intracellular space of the liver parenchyma (Fig. 2B). CeO_2NPs were also observed within intracellular single-membrane organelles. From the ultrastructural appearance, these organelles were identified as lysosomes. NPs agglomerates had sizes of around 30 nm, although some NPs agglomerates were considerably large (250–270 nm). CeO_2NPs were not observed in the other organelles of the different liver cell types.