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Cerium oxide nanoparticles accelerate the decay of peroxynitrite (ONOO⁻)

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Abstract

Cerium oxide nanoparticles (CeO₂ NPs) have been shown to possess a substantial oxygen storage capacity via the interchangeable surface reduction and oxidation of cerium atoms, cycling between the Ce⁴⁺ and Ce³⁺ redox states. It has been well established in many studies that depending on their reactivity and surface chemistry, CeO₂ NPs can effectively convert both reactive oxygen species (superoxide, O₂^{•-}, and hydrogen peroxide) into more inert species and scavenge reactive nitrogen species (RNS)(nitric oxide, •NO), both in vitro and in vivo. Since much of damage attributed to •NO and O₂^{•-} is actually the result of oxidation or nitration by peroxynitrite or its breakdown products and due to the multiple species that these nanoparticles target in vivo, it was logical to test their interaction with the highly reactive molecule peroxynitrite (ONOO⁻). Here, we report that CeO₂ NPs significantly accelerated the decay of ONOO⁻ by three independent methods. Additionally, our data suggest the ability of CeO₂ NPs to interact with ONOO⁻ is independent of the Ce³⁺/Ce⁴⁺ ratio on the surface of the CeO₂ NPs. The accelerated decay was not observed when reactions were carried out in an inert gas (argon), suggesting strongly that the decay of peroxynitrite is being accelerated due to a reaction of CeNPs with the carbonate radical anion. These results suggest that one of the protective effects of CeO₂ NPs during RNS is likely due to reduction in peroxynitrite or its reactive breakdown products.

Keywords

Antioxidant; Cerium oxide nanoparticles (CeO₂ NPs); Hydrogen peroxide; Nitric oxide; Peroxynitrite; Reactive oxygen species; Reactive nitrogen species; Superoxide

Biological cells survive in a constantly changing and challenging environment, responding to many factors including oxidative byproducts of normal metabolism. Progressive accumulation of damaged molecules or tissues is believed to be responsible for the ever-increasing susceptibility to disease and death [1]. Reactive oxygen species (ROS) and

reactive nitrogen species (RNS) cause damage to all types of biomolecules, including DNA, proteins, and lipids with the formation of toxic and mutagenic products. More recently, the role of RNS have been shown to have a direct role in cell signaling, vasodilation, and the immune response [2]. Nitrosative stress, defined by the excessive production of reactive nitrogen species, causes damage to macromolecules and can lead to degenerative diseases, contribute to metabolic diseases, and if in great excess, can lead to cell death through a variety of molecular mechanisms. The role of RNS in many age-related diseases, with the primary RNS species being peroxynitrite (ONOO^-), is now just being appreciated.

Cerium oxide nanoparticles (CeO_2 NPs) have been shown to possess a substantial oxygen storage capacity via the interchangeable surface reduction and oxidation of cerium atoms, cycling between the Ce^{4+} and Ce^{3+} redox states [3]. Due to this intrinsic capability, these materials have been employed for industrial use in three-way catalysts [4]. With the discovery that these nanoparticles can react effectively with biologically relevant radical species and oxidants, a new field has emerged studying these materials for use in biological systems. Biological uses of CeO_2 NPs have centered around their ability to scavenge free radicals under physiologically relevant conditions. This catalytic nature, which began with the discovery that water-based cerium oxide nanoparticles (with increased Ce^{3+} in their outer surface) could act as superoxide dismutase (SOD) mimetics [3, 5], has laid the foundation for their application in experimental and biomedical research. Subsequent studies have established cerium oxide nanoparticles (with decreased Ce^{3+} in their outer surface) have the ability to act as catalase mimetics [6] as well as scavenge nitric oxide [7]. Due to their structure–function relationship to other redox catalysts (superoxide dismutase [8] and catalase [9]), Ferrer-Sueta et al. demonstrated manganese porphyrins efficiently scavenge peroxynitrite and species derived from it [10]. Since CeO_2 NPs have catalytic activity towards $\text{O}_2^{\bullet-}$, H_2O_2 , and $\bullet\text{NO}$, we hypothesized that CeO_2 NPs would be able to interact with peroxynitrite. Additionally, much of damage attributed to $\bullet\text{NO}$ and $\text{O}_2^{\bullet-}$ is actually the result of oxidation or nitration by peroxynitrite or its breakdown products [11].

Peroxynitrite absorbs UV light at a wavelength of 302 nm, thus its decay can be measured kinetically using UV–spectroscopy [12]. Exploiting this property, we observed that two water-based preparations of CeO_2 NPs dramatically accelerated the normal rapid decay of peroxynitrite (Fig. 1). CeNP1 (with increased Ce^{3+} in their outer surface) (Fig. 1a) or CeNP2 (with decreased Ce^{3+} in their outer surface) (Fig. 2b) both accelerated the decay in manner similar to an established antioxidant, glutathione (GSH). We also tested the recognized peroxynitrite scavenger uric acid [13] and found similar results (Online Resource Fig. 1a). When we tested an unrelated metal oxide NP of similar size, SiO_2 (Online Resource Fig. 1a), there was no change in the rate of ONOO^- decay, suggesting that this was somewhat unique to CeO_2 NPs. A second addition of peroxynitrite after decay again was accelerated in the presence of CeNPs, suggesting strongly that these materials were acting as catalysts and not just reacting with peroxynitrite to generate a modified cerium oxide material (data not shown).

To test the reactivity of CeO_2 NPs with peroxynitrite by an alternative method, we followed the oxidation of 3'-(*p*-aminophenyl) fluorescein (APF) by fluorescence spectrometry in vitro. APF has no fluorescence at baseline, but when oxidized by peroxynitrite, it exhibits fluorescence [14]. Using this probe, we observed that CeNP1 and CeNP2 prevented the oxidation of APF in vitro, similar to GSH when challenged with peroxynitrite (Fig. 1c, d) [15]. Again, the same controls were tested in this assay with similar results as before with uric acid and SiO_2 NPs (Online Resource Fig. 1b). Collectively, these data illustrate a previously unknown catalytic property of CeO_2 NPs, namely their ability to accelerate the decay of peroxynitrite in vitro.

Protection against peroxynitrite-induced damage in vivo is provided by several antioxidants [16] as well as eliminated by many of the antioxidant enzymes including peroxiredoxins [17]. Modification of amino acid residues on the active site of key proteins may result in detrimental change in function of the proteins. Tyrosine nitration modification of proteins has become an important biomarker for inflammation and for nitrosative stress and has been detected in a number of diseases and pathological conditions [11]. Tyrosine nitration occurs with the incorporation of a nitro group ($-\text{NO}_2$) at position 3 of the aromatic ring of tyrosine [18].

To demonstrate the biological significance of CeO_2 NPs with ONOO^- , we tested whether CeO_2 NPs would reduce the level of peroxynitrite-induced protein tyrosine nitration of the protein bovine serum albumin (BSA) using a specific antibody for 3-nitrotyrosine. In Fig. 2, using quantitative densitometry analyses, we observed that bovine serum albumin (BSA), treated with $10\ \mu\text{M}$ peroxynitrite, exhibited high 3-nitrotyrosine immunoreactivity (Fig. 2, lane 1) as expected due to the production of nitrated tyrosines on the protein surface. By contrast, the same experiment carried out in the presence CeNP1 , CeNP2 , or GSH showed a significant decrease in tyrosine nitration (Fig. 2, lanes 2, 3, and 5, and inset). Addition of SiO_2 NPs had no effect (Fig. 2, lane 4 and inset). These results further demonstrate that CeO_2 NPs can interact with peroxynitrite or its break-down products, thus preventing the 3-NT modification of BSA in a manner similar to glutathione.

CeO_2 NPs have a mixed valence state of cerium containing both Ce^{3+} and Ce^{4+} . We have shown that upon incubation of CeO_2 NPs with hydrogen peroxide, CeO_2 NPs with a higher starting concentration of Ce^{3+} can convert to CeO_2 NPs containing increased Ce^{4+} on their surface [3]. Along with this change in oxidation state is the loss of their SOD mimetic ability. To determine whether this change in property also applies to ONOO^- interaction, we incubated CeNP1 with hydrogen peroxide and followed ONOO^- ability to oxidize APF (Fig. 3). Since CeNP2 had previously prevented the oxidation of APF (Fig. 1d), we were not surprised that H_2O_2 incubation did not affect CeNP1 ability to oxidize APF. Additionally, incubation with H_2O_2 did not affect zeta potential nor significantly affect their hydrodynamic radius (Online Resource Fig. 2). These results suggest that the interaction with ONOO^- does not specifically depend upon the Ce^{3+} sites.

The biochemistry of ONOO^- is vastly complicated due to the multiple reactions possible in the presence and absence of CO_2 , H^+ and metals during its decomposition [18]. The acceleration of peroxynitrite decay observed in the presence of cerium oxide nanoparticles represents compelling yet preliminary evidence that these nanomaterials readily react with peroxynitrite or one of the reactive oxidants and radicals that are a result of the non-enzymatic breakdown of peroxynitrite. The mechanism by which these materials can alter the catalytic decomposition of peroxynitrite has yet to be elucidated. Others have shown that thiols, metals, and carbon dioxide are the most likely targets of peroxynitrite in vivo [18, 19]. Assuming cerium oxide reacts directly with peroxynitrite (and not decay products such as carbonate radical), a putative scheme of one-electron oxidation and reduction reactions is shown that could explain the acceleration of peroxynitrite decay.

The proton-dependent decay of peroxynitrite results in production of hydroxyl radical and nitrogen dioxide radical. Each of these potent radicals, which are one-electron oxidants, could react with cerium oxide nanoparticles at the particle surface (in an oxygen vacancy) to oxidize Ce^{3+} to Ce^{4+} with the concomitant release of hydroxyl ion or nitrite. In our UV-visible measurements, an absorption peak near that of nitrite (229 nm) was observed that paralleled the decrease in peroxynitrite levels (data not shown).

Additionally, CO_2 plays a dramatic role in the decomposition of ONOO^- [20]. In tissues, where concentrations of CO_2 can reach 1–2 mM, the reaction with peroxynitrite is highly favored forming nitrosoperoxycarboxylate anion adduct (ONOOCO_2^-) that undergoes deleterious decay yielding carbonate radical ($\text{CO}_3^{\bullet-}$) and nitrogen dioxide radical ($\bullet\text{NO}_2$) [18]. To begin to understand whether the accelerated decay of ONOO^- in the presence of CeO_2 NPs was due to their interaction with the breakdown products of ONOO^- , we repeated the APF assays in an end-point format under inert conditions (argon atmosphere). Components were flushed with argon to remove as much normal atmosphere, including CO_2 , as possible as well the assays were performed in an argon flushed hood. With the removal of CO_2 , CeNP1 or CeNP2 were no longer able to accelerate the decay of ONOO^- (Online Resource Fig. 3). This suggests that CeO_2 NPs could be interacting with carbonate radical known to be formed during the decomposition of ONOO^- . Although somewhat speculative, it is well established that in air saturated buffer, the carbonate radical is the most reactive radical species present during the spontaneous decay of peroxynitrite, suggesting that it is the likely target of reaction with CeO_2 NPs.

It has been suggested in many studies that protection that has been observed in cell culture and animal studies is due to the presence of CeO_2 NPs and their ability to react with or scavenge the major deleterious ROS/RNS species [21–23]. Based on this study and our previous work on CeNPs it is clear that surface charge and oxygen vacancies can generate varied catalytic properties in CeNPs. Both ROS and RNS can be scavenged by various preparations of CeNPs in vitro, however what happens in vivo is still not well understood. In an attempt to correlate catalytic activity to surface oxidation, we have found that CeO_2 NPs that have higher levels of reduced cerium sites (3+) at the surface are more effective SOD mimetics [3, 5]. In contrast, CeO_2 NPs that have fewer reduced cerium sites exhibit better catalase mimetic [6] and $\bullet\text{NO}$ scavenging capabilities [7]. The results from this study suggest that in the presence of CeO_2 NPs, regardless of the oxidation state, peroxynitrite, or one of its breakdown products may react with the surface of CeO_2 NPs. The precise molecular mechanism behind each of these catalytic reactions is still not yet known but is the focus of our current research. What happens in the milieu of cells and tissues remains an enigma. However, many studies have shown protective effects when CeO_2 NPs are administered during oxidative or nitrosative stress. It is likely that the protection seen in many of these studies may be attributed to CeO_2 NPs interaction with ONOO^- and the nature of this reaction and its products are still actively under investigation by our group.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

CeNP1 and CeNP2 accelerate the decay of peroxynitrite in vitro. Relative absorbance of peroxynitrite (25 μ M) at 302 nm per unit time (seconds) either in the absence or presence of CeNPs or glutathione (GSH) (1 mM) using UV-visible spectrometry at pH9.5. **a** CeNP1 (100 μ M), **b** CeNP2 (100 μ M). Relative APF (10 μ M) fluorescence at 490 nm excitation and 515 nm emission wavelengths with either peroxynitrite (20 μ M) alone or with increasing concentrations of CeNP nanoparticles (100, 500, 1,000 μ M) or glutathione (1 mM) measured over a time period of 10 s at pH7.4. **c** CeNP1, **d** CeNP2. Statistics: Student's *t* test, *p* 0.05

Fig. 2.

CeNPs prevent 3-nitrotyrosine protein by peroxynitrite. Graphical representation of BSA western blots protected from nitration with dose-dependent addition of either CeNP1, CeNP2, GSH, or negative control SiO₂ NP. *Inset:* Representative slot-blots. All *lanes* contain 500 ng BSA treated with 10 μM peroxynitrite (ONOO⁻) in the absence and presence of 500 nM NPs or 1 mM GSH. Blots were probed with anti-3-nitrotyrosine antibody. Individual experiments were normalized to their individual BSA/ONOO⁻-treated lane. Data are representative of three or more independent experiments (see Online Resource—Methods and Materials). *p* 0.001. Statistics: Student's *t* test

Fig. 3. Peroxide-mediated changes in surface cerium oxidation state of CeNPs do not alter catalysis with peroxynitrite. Relative APF (10 μ M) fluorescence at 490 nm excitation and 515 nm emission wavelengths with either peroxynitrite (20 μ M) alone or in combination with CeNP nanoparticle CeNP1 treated with H₂O₂ (100 mM) or glutathione (1 mM) measured over a time period of 10 s at pH7.4. Representative traces of three or more experiments are shown (see Online Resource—Methods and Materials)