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Cerium and yttrium oxide nanoparticles are neuroprotective

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Abstract

The responses of cells exposed to nanoparticles have been studied with regard to toxicity, but very little attention has been paid to the possibility that some types of particles can protect cells from various forms of lethal stress. It is shown here that nanoparticles composed of cerium oxide or yttrium oxide protect nerve cells from oxidative stress and that the neuroprotection is independent of particle size. The ceria and yttria nanoparticles act as direct antioxidants to limit the amount of reactive oxygen species required to kill the cells. It follows that this group of nanoparticles could be used to modulate oxidative stress in biological systems.

Keywords: Antioxidant; Nanoparticle; Ceria; Yttria; Neuroprotection; Oxytosis

There has been a great deal of interest in the toxicity of particulate matter in the context of respiratory health [1]. While air born microscopic particles are a ubiquitous result of industrialization, the emerging development of nanotechnology has lead to concern related to the manufacturing and use of large quantities of nanoparticles [2–4]. There have, however, been very few studies that examine the biological consequences of the exposure of cells or animals to synthetic nanoparticles. Two studies in rodents showed that single-wall carbon nanotubes are cytotoxic [5,6], and more recently, Soto et al. [7] examined the cytotoxicity of a well-characterized group of nanoparticles on an established cell line of murine macrophages and showed that a variety of nanoparticles are cytotoxic. In addition, it has been shown that water soluble fullerenes are able to directly generate superoxide anions that are also cytotoxic [8].

The lung must deal with stresses that result in the generation of reactive oxygen species (ROS). Enhanced ROS production and oxidative stress in the lung is caused by breathing atmospheric particulates [9], as well as other conditions such as allergies [10], infection [11], and smoking [12]. Perhaps the most understood clonal cell system for analyzing oxidative stress is the HT22 cell line [13]. HT22 cells are derived from the rodent nervous system and are readily killed by conditions that lead to the endogenous production of ROS. The best studied natural pro-oxidant for HT22 cells is the amino acid glutamate. Exogenous glutamate competes with cystine for entry into the cell. Cystine is an essential amino acid that must be supplied by the culture medium and it is also a component of the major antioxidant in cells, the tripeptide glutathione (GSH). In the presence of glutamate, cystine uptake is blocked, glutathione levels are depleted, and the cells initiate a well-characterized cell death program called oxytosis [13]. The oxytosis cell death program requires ROS production by mitochondria and ultimately the influx of calcium. Since some nanoparticles have been shown to stimulate ROS production [7,8] and cause oxidative damage in animals [14], it was asked if nanoparticles alter the glutamate-induced cell death pathway in HT22 cells.

The particles examined in this study varied in size and composition. Cerium oxide (CeO_2, ceria) was chosen because it may act as a free radical scavenger [15]. This metal oxide possesses a cubic fluorite structure, and when prepared as described below, is characterized by fairly monodisperse particles that are single crystals with few

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twin boundaries or stacking faults [16] and with an expanded lattice parameter relative its bulk counterpart [17]. Moreover, ceria tends to be a nonstoichiometric compound with the cerium atom characterized by both +4 and +3 oxidation states. Recent research using X-ray photoelectron spectroscopy and X-ray absorption near edge spectroscopy suggests that the concentration of Ce^{3+} relative to Ce^{4+} increases as particle size decreases, with a conservative [Ce^{3+}] minimum of 6% in 6 nm nanoparticles and 1% in 10 nm particles [18]. This dual oxidation state means that these nanoparticles have oxygen vacancies or defects [19]. The loss of oxygen and the reduction of Ce^{4+} to Ce^{3+}, shown below, is accompanied by creation of an oxygen vacancy. This property is responsible for the interesting redox chemistry exhibited by ceria nanoparticles and makes them attractive for catalytic applications.

$$CeO_2 \underset{\text{oxidizing}}{\overset{\text{reducing}}{\rightleftharpoons}} CeO_{2-y} + \frac{y}{2}O_2$$

Nanoparticles made of other metal oxides were also considered to determine if this potential scavenger behavior is unique to ceria. These included particles of α- and γ-aluminum oxide (Al_2O_3, alumina), which only differ in crystal structure, and yttrium oxide (Y_2O_3, yttria). Alumina is thermodynamically stable at all temperatures and has a corundum structure with oxygen atoms adopting hexagonal close-packing and Al^{3+} ions filling 2/3 of the octahedral sites in the lattice [20]. The latter has a cubic structure [20].

Yttrium oxide is noteworthy because the free energy of oxide formation from elemental yttrium is among the highest known [21]. It is characterized by only small deviations from stoichiometry under normal conditions of temperature and pressure [22] and by absorption of water and carbon dioxide from the atmosphere [23]. The particular polymorph used in this experiment was the monoclinic B form. The structure is closely related to the A form which has hexagonal close-packing (hcp), but unlike the A form which has only sevenfold coordination, it has sixfold as well [22]. The B form structure is slightly less dense than the A form variant and has the yttrium cations in nonequivalent sites in the crystal [24].

The following paragraphs show that this group of nanoparticles are relatively nontoxic to HT22 cells and macrophages, and that CeO_2 and Y_2O_3 particles protect cells from death due to oxidative stress. It is also shown that this protection is due to the direct antioxidant properties of the nanoparticles.

Results and discussion

CeO_2, Y_2O_3, and Al_2O_3 nanoparticles are relatively nontoxic to HT22 cells

Since some types of nanoparticles are toxic to a mouse macrophage cell line [7], it was initially asked if particles composed of the oxides of Ce, Y, and Al are toxic to the nerve cell line HT22. Increasing amounts of particles were added to HT22 cells, and viability assayed 20 h later. Figs. 1A and B show that, with the exception of the 1 μ ceria nanoparticles, there was very little toxicity toward these cells. Significant toxicity was observed with 1 μ ceria at concentrations higher than 20 μg/mL. There was a large amount of scatter in the data, something that has been observed previously [7] and could be a reflection of the difficulty of applying suspension of particles uniformly to cells. Since most previous studies of nanoparticle toxicity have been done on macrophage cell lines, the mouse macrophage RAW164 cell line was exposed to increasing concentrations of Y_2O_3 and CeO_2 nanoparticles. Fig. 1C shows that the toxicity of these particles was about the same as with HT22 cells. There was no toxicity of the aluminum particles up to 80 μg/mL (data not presented).

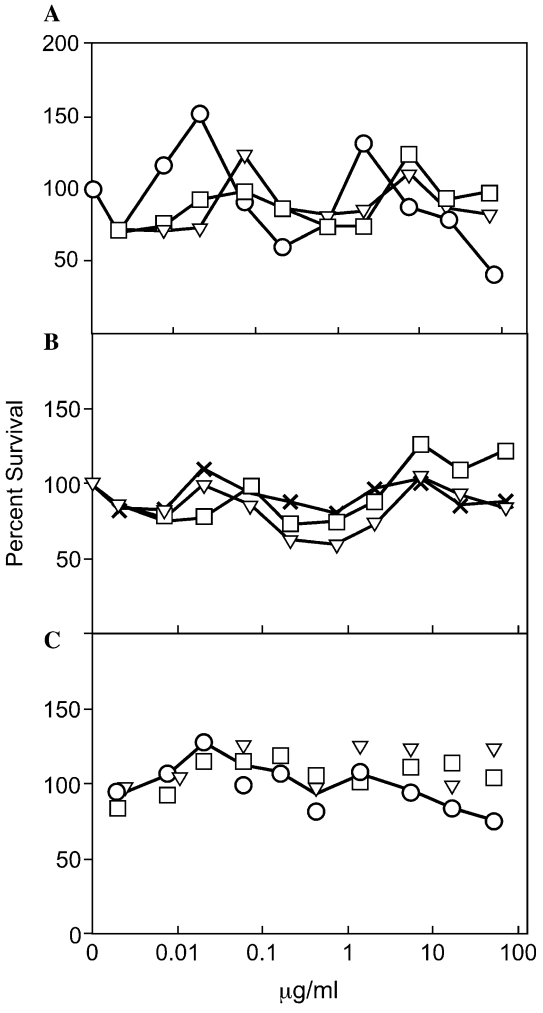

Fig. 1. Toxicity of nanoparticles. Increasing amounts of the different particles were added to HT22 or macrophage cells and 20 h later cell viability determined. The data are expressed as percent cell survival relative to controls exposed to vehicle alone. (A) HT22, ○–○ 1 μ CeO_2; ▽–▽ 6 nm CeO_2; □–□ 12 nm CeO_2. (B) HT22, ×–× 12 nm α-Al_2O_3; ▽–▽ 12 nm γ-Al_2O_3; □–□ Y_2O_3. (C) Macrophage, ○–○ 1 μ CeO_2; ▽–▽ 6 nm CeO_2; □–□ Y_2O_3.

Nanoparticles increase resistance to oxidative stress

Because of the redox chemistry of some nanoparticles, it is possible that they are biologically active as antioxidants. To determine if these nanoparticles can be protective in conditions of oxidative stress, HT22 cells were exposed to increasing concentrations of 6 nm, 12 nm and micron-sized CeO_2, 12 nm Y_2O_3, or 50 nm γ- or 300 nm α-Al_2O_3 nanoparticles, and 30 min later a toxic amount of glutamate was added to the cells. Fig. 2A shows that Y_2O_3 particles increased the viability of HT22 cells in a concentration dependent manner from 2 ng/mL to 20 μg/mL. Six and twelve nanometer CeO_2 particles were also protective, as were micron-sized CeO_2 particles, but there was no reproducible significant difference between the three sizes of CeO_2 particles in terms of protection from this stress condition. Two additional types of nanoparticles were also assayed, those of 300 nm α- and 50 nm γ-Al_2O_3. Fig. 2B shows that neither of these materials protected against oxidative stress, nor did nonparticle CeO_2. Equivalent concentrations of the nonparticle oxides and salts of these and related compounds, including silicon oxide, cerium nitrate, cerium chloride, yttrium oxide, and aluminum oxide had no protective activity at concentrations between 10 ng/mL and 50 μg/mL (data not shown). Therefore the protective effect is related to the structure of the nanoparticles, not simply their elemental composition. In addition, there is no significant size dependency on protection.

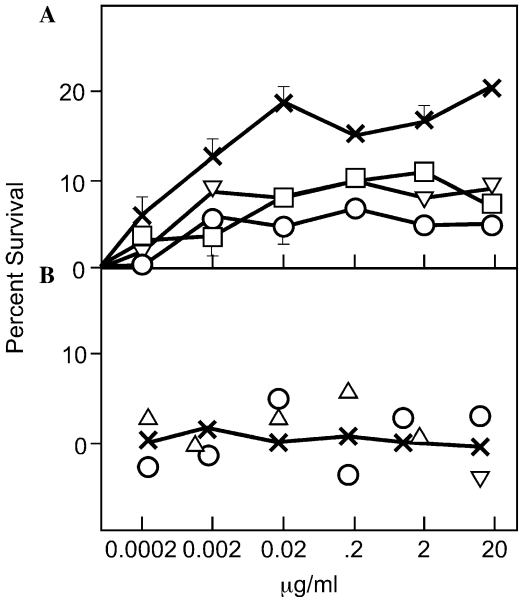

Fig. 2. Nanoparticles protect from oxidative stress. HT22 cells were exposed to the indicated concentrations of particles and 30 min later 5 mM glutamate was added. Cell viability was determined 20 h later and is expressed as percent of untreated controls. (A) ×–× Y_2O_3; ○–○ 1 μ CeO_2; □–□ 12 nm CeO_2; ▽–▽ 6 nm CeO_2. (B) ×–×, 300 nm α-aluminum oxide; △–△, 50 nm γ-aluminum oxide; ○–○, nonparticle cerium oxide. Using a Wilcoxon signed ranks test for two relative samples, P is less than 0.01 for Y_2O_3 and the cerium particles. There is no significant difference from controls with aluminum oxide particles. These experiments were repeated at least six times.

Another way to approach the relationship between nanoparticle amount, size, and protection is to sample a reaction mixture in which nanoparticles are being formed, and to assay for their biological effect as a function of time. To this end $Ce(NO_3)_3{\cdot}6H_2O$ and hexamethylenetetramine were mixed and 10 μg/mL samples were periodically added to HT22 cells, followed immediately by 5 mM glutamate. The 10,000-fold dilution into serum containing medium most certainly stopped nanoparticle formation and growth. Fig. 3 shows that there was a protective effect of about 50% of maximal protection after about 10 min of particle formation. Neither of the starting materials, HMT nor $Ce(NO_3)_3{\cdot}6H_2O$, alone had any reproducible effect upon cell viability at the same dilution.

Mechanism of protection

The above experiments show that cerium and yttrium oxide nanoparticles are able to rescue cells from oxidative stress-induced cell death in a manner that appears to be dependent upon the structure of the particle but independent of its size within the 6–1000 nm range. There are three alternative explanations for the observation that the cerium oxide and yttrium oxide particles protect from oxidative stress. They may act as a direct antioxidants, they may block ROS production in HT22 cells by inhibiting a step in the programmed cell death pathway, or they may directly cause a low level of ROS production that rapidly induces a ROS defense system before the glutamate-induced cell death program is complete. The latter is a form of preconditioning that could be caused by the exposure of cells to particulate material known to induce low levels of ROS [9]. These alternatives were sequentially ruled out by published procedures [25].

To determine if yttrium oxide nanoparticles directly modified ROS metabolism, cells were exposed to 20 μg/mL

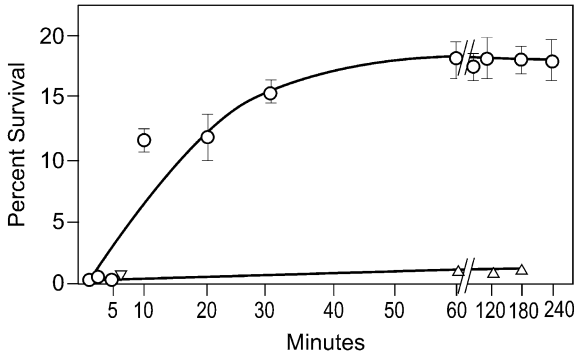

Fig. 3. Time course of CeO_2 nanoparticle generation. Solutions of $Ce(NO_3)_3{\cdot}6H_2O$ and hexamethylenetetramine were mixed and aliquots diluted at different times into the culture medium of HT22 cells. Five millimole glutamate was immediately added and cell viability determined 20 h later. The data are plotted as percent survival relative to untreated cultures as a function of time. They are presented as means plus or minus the standard error of the mean of triplicate determinations. ○–○, nanoparticles; △–△, $Ce(NO_3)_3{\cdot}6H_2O$ control. Hexamethylenetetramine had no effect.

of 12 nm Y_2O_3 particles and ROS measured by FACS analysis 10, 30, and 60 min following exposure of the cells. Fig. 4A shows that there was approximately 50% decrease in endogenous ROS during this time. Since several hours are required for the glutamate-induced cell death program to produce endogenous ROS [13], these data suggest that Y_2O_3 particles may have a direct antioxidant effect. To test this alternative, cells were exposed to glutamate for 8 h, allowing the accumulation of ROS. The nanoparticles were then added to the cells 15 min prior to harvesting for analysis and it was asked if they directly reduce the concentration of ROS. A lower level of ROS shows that the compound is acting as a direct antioxidant since the time frame is too short to induce any significant antioxidant response [25]. Fig. 4B shows that this is indeed the case, for 20 μg/mL of the Y_2O_3 particles reduced the accumulated ROS by about 50% and there was a tenfold reduction at 200 μg/mL. These data show that Y_2O_3 nanoparticles have a direct dose-dependent antioxidant activity. Figs. 4C and D show similar results for 12 nm ceria nanoparticles, although, in agreement with the protection data (Fig. 2), ceria was not as potent as Y_2O_3 particles. Neither α- nor γ-aluminum oxide nanoparticles had any effect on ROS accumulation (data not shown).

The above data show that nanoparticles composed of the oxides of cerium and yttrium are relatively nontoxic to cultured cells and are able to protect HT22 nerve cells from oxidative stress caused by exogenous glutamic acid. Since an increase in ROS levels is necessary to complete the cell death program in these cells [13], it was asked if the particles can act as direct antioxidants. The ability to sequester accumulated ROS is perhaps the best way to assay for direct antioxidant activity in intact cells [25]. Fig. 4 shows that both Y_2O_3 and CeO_2 nanoparticles are able to rapidly reduce pools of preformed ROS. Because the nonnanoparticle oxides or salts of cerium and yttrium are not protective, it follows that there is a structural determinate for the antioxidant properties of the nanoparticles. Since other nanoparticles of similar size, such as those made of aluminum oxides, are not protective, it is unlikely that the cells are mounting some sort of a stress response to pinocytosed particulates that acts as a form of preconditioning to more severe stress. The time frame of ROS reduction by nanoparticles (10 min, Fig. 4) is also much too short for this to occur. What is the likely mechanism of the antioxidant activity?

Nanoparticles often exhibit properties different than their bulk counterparts. For example, cadmium selenide

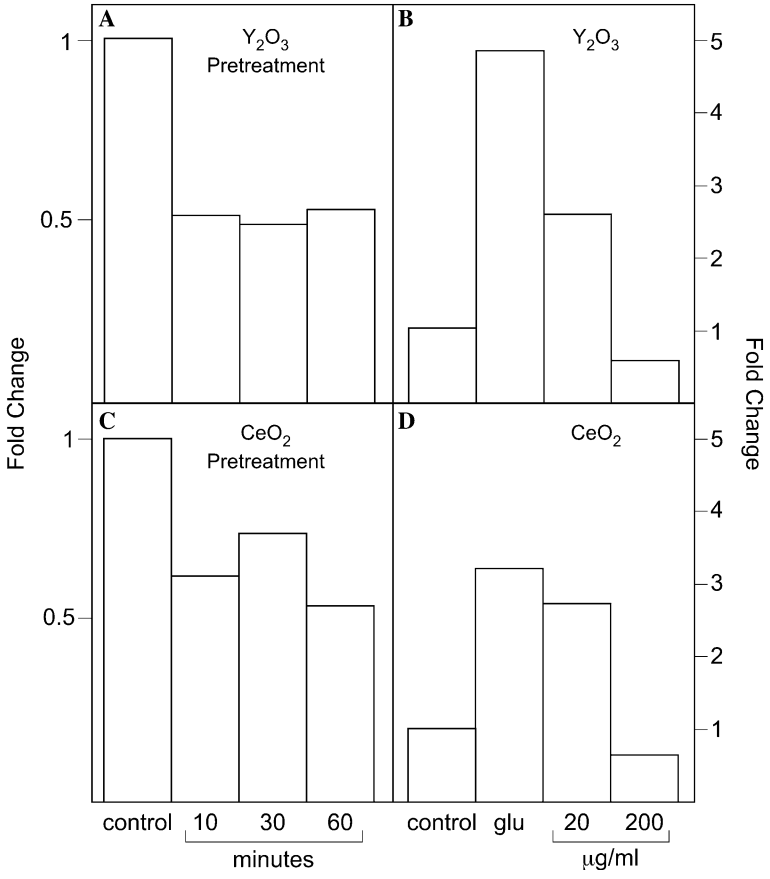

Fig. 4. Y_2O_3 and CeO_2 nanoparticles are antioxidants. Cells were either exposed directly to 20 μg/mL of 12 nm Y_2O_3 (A) or 12 nm CeO_2 (C) and ROS determined 10, 30, and 60 min later, or cells were exposed to glutamate for 8 h to generate endogenous ROS and then either 20 or 200 μg/mL of the nanoparticles added (B,D). The data are expressed as fold change in endogenous ROS in each experiment. The endogenous level of ROS was 14.35 (arbitrary units) in untreated cells and 67.66 units in cells treated with glutamate for 8 h. Each bar graph is the mean of 10,000 independent measurements, with an error of less than 2% between repeated measurements.

quantum dots exhibit different fluorescent colors depending upon their particle sizes [26]. In some cases, differences between the particles and bulk chemicals are attributable to the higher ratio of surface area to volume found in nanoparticles, a property that makes nanotechnology useful for catalysis. As a catalyst in automobile catalytic converters, ceria (often doped with zirconium oxide) acts as an agent of oxygen storage and release with oxygen vacancies being generated and annihilated on the surface [27]. Since nanoparticles clearly have a higher surface to volume ratio than larger particles, they provide a greater surface area per unit volume for vacancy creation and annihilation. However, the above data show that there is little or no difference between the neuroprotective activity of 6 or 12 nm ceria particles and micron ceria. Therefore, the protective response must be dependent upon the physico-chemical properties of the nanoparticle that are relatively independent of its size and are most likely a property of the Redox properties of ceria described in the Introduction.

The similarities between the biological activity of Y_2O_3 and CeO_2 nanoparticles can be understood because yttrium behaves like the lanthanide elements that include cerium [28]. Although yttrium has no f-orbital electrons in its stable configuration, its cation has an inert gas configuration. Likewise, the cation radius of yttrium is in the same range as the lanthanides [29]. The radii values are dependent upon the coordination number (CN) of the cation, and cerium in ceria has a CN of 8 while yttrium in yttria has an average value of 6.5 [22]. Moreover, both ceria and yttria also exhibit some degree of nonstoichiometry [30], but the degree of nonstoichiometry in ceria is much more marked. Finally, the density of ceria is 1.4 times greater than yttria. These differences in the chemistry of Y_2O_3 and CeO_2 nanoparticles may not, however, explain the antioxidant properties of Y_2O_3 nanoparticles and the fact that they are more potent antioxidants than ceria.

The final group of nanoparticles that were studied is composed of aluminum oxides. Alumina is not noted for nonstoichiometry, and any defect concentration in the corundum phase would be small [22]. Therefore, unlike CeO_2 and Y_2O_3 nanoparticles, the aluminum oxide nanoparticles are not active as antioxidants.

It can be concluded that in contrast to the literature showing that nanoparticles are toxic, nanoparticles composed of cerium and yttrium oxides can have antioxidant properties that promote cell survival under conditions of oxidative stress. It follows that there is a potential for engineering this group of nanoparticles for therapeutic purposes.

Experimental procedures

Cell culture and toxicity studies. The HT22 hippocampal nerve cell line is a subclone of HT4 cells [31] which was selected for its sensitivity to glutamate toxicity. The cells do not possess active ionotropic glutamate receptors and are not subjected to excitotoxicity [32]. HT22 cells are propagated in Dulbecco's modified Eagle's medium (DMEM) [33] supplemented with 10% fetal bovine serum (FBS). The mouse macrophage cell line RAW164 was obtained from the American Type Culture Collection as grown in the same conditions as HT22. Cell survival was determined by the MTT (3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay as described [34]. In HT22 cells the MTT assay correlates with cell death as determined by trypan blue exclusion and a colony forming assay [32]. For the cytotoxicity assays, cells are dissociated with Pancreatin (Life Technologies, Gaithersburg, MD) and seeded onto 96-well microtiter plates in DMEM containing 10% FBS at a density of 2.5×10^3 cells per well in 100 μL medium. The next day cells are treated with various reagents according to the experimental design. Nanoparticles were freshly suspended in growth medium for each experiment. Twenty hours after the addition of glutamate, 10 μL of the MTT solution (2.5 μg/mL) is added to each well and the cells are incubated for 4 h at 37 °C. Solubilization solution (100 μL: 50% dimethylformamide, 20% SDS, pH 4.8) is then added to the wells and the next day the absorption values at 570 nm are measured. All experiments are done in triplicate and repeated at least three times. The results are expressed relative to the controls of glutamate alone which killed between 80% and 100% of the cells.

Preparation of cerium oxide nanoparticles. Cerium oxide nanoparticles used in the experiments above were produced in the following manner. A 0.07 g/mL (0.5 M) solution of hexamethylenetetramine (HMT) and a 0.016 g/mL (0.038 M) solution of $Ce(NO_3)_3{\cdot}6H_2O$ were prepared and mixed separately for 30 min. The two solutions were then combined and mixed for a length of time dependent upon the desired particle size. Earlier studies have shown a correlation between mixing time and diameter [35]. They were then centrifuged for a length of time dependent on the expected particle size and density as well as the centrifuge rotor dimensions and revolutions per minute (rpm) selected [36]. For example, the 6 nm particles were prepared by mixing the combined reactants for 6 h followed by 30 min centrifugation at 3900 rpm in a Centra-CL2 equipped with a swinging bucket rotor. The 12 nm particles were prepared by mixing for 13 h followed by 15 min centrifugation in an Eppendorf 5804 equipped with a F-34-6-38 rotor and operating at 11,000 rpm. The micron ceria was prepared by mixing the reactants together for 36 h followed by centrifugation and 12 h annealing at 1200 °C.

For the cerium oxide nanoparticles time course, the initial procedure is similar to the preparation method described above, except that the particles are not harvested by centrifugation. $Ce(NO_3)_3{\cdot}6H_2O$ and hexamethylenetetramine were dissolved in water and equal volumes mixed and stirred gently. Aliquots were removed at the times indicated and the particles diluted 3 parts per 10,000 into the culture medium and 5 mM glutamate added immediately. Cell viability was determined 20 h later by the MTT assay. The final CeO_2 concentration was 10 μg/mL.

Reactive oxygen species levels. Intracellular accumulation of reactive oxygen species (ROS) was determined with dichlorofluorescein-di-acetate (H_2DCF-dA) [25]. This nonfluorescent compound accumulates within cells upon de-acetylation. H_2DCF then reacts with ROS to form fluorescent dichlorofluorescein (DCF). HT22 cells were dissociated from tissue culture dishes with pancreatin in DMEM in the presence of 10 μM H_2DCF-dA for 10 min at 37 °C, washed once with room temperature Hepes-DMEM without phenol red supplemented with 2% dialyzed FBS and resuspended in 750 μL of the same solution containing 2 μg/mL propidium iodide (PI). The use of pancreatin did not affect the outcome of flow cytometric experiments as confirmed by fluorescence microscopy. Flow cytometric analysis was performed using a FACScan instrument (Becton–Dickinson, San Jose, CA, USA) with the excitation wavelength (λ_{ex}) of 475 nm and the emission wavelength of (λ_{em}) of 525 nm. Data were collected in list mode on 10,000 cells after gating only for characteristic forward versus orthogonal light scatter and low PI fluorescence to exclude dead cells. Median fluorescence intensities of control and test samples were determined with CellQuest software (Becton–Dickinson). The counting error in the experiments described here was less than 2% for repeated measurements, therefore error bars are not presented in the FACS experiments.

Materials. The Y_2O_3 was obtained from Nanophase (Romeoville, IL). The SiO_2 was a 30% colloidal suspension in water produced by Sigma (St. Louis, MO). The $Ce(NO_3)_3{\cdot}6H_2O$ (99.5%) was obtained from Alfa Aesar (Ward Hill, MA). The HMT (99+%) was obtained from Lancaster Synthesis (Pelham, NH). The 300 nm α-alumina particles were obtained from

LECO (St. Joseph, MI). The 50 nm γ-alumina particles were from Buehler (Lake Bluff, IL). All other reagents were from Sigma (St. Louis, MO).

Acknowledgments

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