Cerium Oxide Nanoparticles Inhibits Oxidative Stress and Nuclear Factor- κ B Activation in H9c2 Cardiomyocytes Exposed to Cigarette Smoke Extract

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ABSTRACT

Cigarette smoke contains and generates a large amount of reactive oxygen species (ROS) that affect normal cellular function and have pathogenic consequences in the cardiovascular system. Increased oxidative stress and inflammation are considered to be an important mechanism of cardiovascular injury induced by cigarette smoke. Antioxidants may serve as effective therapeutic agents against smoke-related cardiovascular disease. Because of the presence of oxygen vacancies on its surface and self-regenerative cycle of its dual oxidation states, Ce^{3+} and Ce^{4+} , cerium oxide (CeO_2) nanoparticles offer a potential to quench ROS in biological systems. In this study, we determined the ability of CeO_2 nanoparticles to protect against cigarette smoke extract (CSE)-induced oxidative stress and inflammation in cultured rat H9c2 cardiomyocytes. CeO_2 nanoparticles pretreatment of H9c2 cells resulted in significant inhibition of CSE-induced ROS production and cell death. Pretreat-

ment of H9c2 cells with CeO_2 nanoparticles suppressed CSE-induced phosphorylation of $\text{I}\kappa\text{B}\alpha$, nuclear translocation of p65 subunit of nuclear factor- κ B (NF- κ B), and NF- κ B reporter activity in H9c2 cells. CeO_2 nanoparticles pretreatment also resulted in a significant down-regulation of NF- κ B-regulated inflammatory genes tumor necrosis factor- α , interleukin (IL)-1 β , IL-6, and inducible nitric-oxide synthase and further inhibited CSE-induced depletion of antioxidant enzymes, such as copper zinc superoxide dismutase, manganese superoxide dismutase, and intracellular glutathione content. These results indicate that CeO_2 nanoparticles can inhibit CSE-induced cell damage via inhibition of ROS generation, NF- κ B activation, inflammatory gene expression, and antioxidant depletion and may have a great potential for treatment of smoking-related diseases.

Introduction

Cigarette smoke is the most important risk factor for the development of atherosclerotic vascular diseases, including ischemic heart disease, stroke, and peripheral vascular disease (Law and Wald, 2003; Ambrose and Barua, 2004; Chelland Campbell et al., 2008). It is becoming increasingly evident that cigarette smoke exacerbates endothelium injury and the development of atherosclerosis. Several recent studies also demonstrated that chronic cigarette exposure can result in cardiomyopathy, characterized by the progressive

and irreversible deterioration of cardiac function associated with interstitial fibrosis, cardiac myocyte vacuolization, arteriolar hyalinosis, and immune reaction in the heart (Hartz et al., 1984; Leone et al., 2008; Armani et al., 2009; Minicucci et al., 2009). Although our understanding of the pathogenesis of smoke cardiomyopathy is still limited, several lines of evidence suggest that both oxidative stress and inflammatory responses play an essential role in the biological processes induced by smoke (Ambrose and Barua, 2004; Csiszar et al., 2009). Cigarette smoke can induce increased production of reactive oxygen metabolites and species. Elevated reactive oxygen species (ROS) may also activate nuclear factor- κ B (NF- κ B), a redox-sensitive transcription factor, and lead to increased production of inflammatory cytokines that are key players in the development and progres-

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ABBREVIATIONS: ROS, reactive oxygen species; CeO_2 , cerium oxide; NF- κ B, nuclear factor- κ B; $\text{I}\kappa\text{B}\alpha$, inhibitor of $\kappa\text{B}\alpha$; CSE, cigarette smoke extract; MCP-1, monocyte chemotactic protein-1; TNF- α , tumor necrosis factor- α ; IL-1 β , interleukin-1 β ; IL-6, interleukin-6; iNOS, inducible nitric-oxide synthase; SOD, superoxide dismutase; CuZnSOD, copper zinc SOD; MnSOD, manganese SOD; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate-buffered saline; DHR123, dihydrorhodamine 123; RT-PCR, reverse transcriptase-polymerase chain reaction; TUNEL, TdT-mediated dUTP nick-end-labeling; TMR, tetramethylrhodamine; Z-DEVD-R110, bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-aspartic acid amide

sion of cardiomyopathy (Ahn and Aggarwal, 2005; Hall et al., 2006).

Oxidant balance in the heart has a very important role in protecting the heart and in allowing normal cardiac contractile performance. An imbalance between ROS generation and antioxidant capacity favoring the former leads to oxidative stress and oxidative damage to the heart. It has been shown that smoking enhances oxidative stress not only through the production of ROS but also through weakening of the antioxidant defense system, such as antioxidant enzymes superoxide dismutase (SOD) and antioxidants such as GSH (Bernhard and Wang, 2007; Ramesh et al., 2008). Experimental studies have suggested a protective role for free-radical scavengers, such as vitamin E and carotenes, in modifying the major diseases related to cigarette smoke, including smoke-induced cardiomyopathy (Zornoff et al., 2006; Bernhard and Wang, 2007; Ramesh et al., 2008). However, many of these free-radical scavengers were found to be only partially successful, and the repeated dosing was needed to replace molecular species that were used in free radical reduction.

Cerium oxide (CeO_2) nanoparticles, a rare-earth element of the lanthanide series, are widely used in ultraviolet absorbance (Tsunekawa et al., 1999), oxygen sensing (Das et al., 2007), and automotive catalytic converters (Yu et al., 2003). This biomaterial has both Ce^{3+} and Ce^{4+} oxidation states that could result in an autoregenerative redox cycle between Ce^{3+} and Ce^{4+} , shown in Fig. 1, accompanied by creation of oxygen defects on their surface, and offers many active sites for free radical scavenging (Yu et al., 2003; Hochella et al., 2008). A potential application of CeO_2 nanoparticles to quench ROS in biological systems is currently being investigated. For example, studies have demonstrated that CeO_2 nanoparticles are able to confer neuronal (Rzagalinski et al., 2006), ocular (Chen et al., 2006), and radioprotection (Tarnuzzer et al., 2005). We recently demonstrated that administration of CeO_2 nanoparticles protected heart from oxidative and inflammatory injury induced by cardiac-specific expression of monocyte chemotactic protein-1 (MCP-1) (Niu et al., 2007). In addition, it has been reported that cerium oxide can mimic the properties of SOD, an endogenous cellular defense against the free radicals (Korsvik et al., 2007; Heckert et al., 2008; Colon et al., 2010). Thus, cerium oxide nanoparticles may represent a novel agent to protect cells and tissues from damage by its regenerative free radical scavenging property. In this report, we investigated the ability of cerium oxide nanoparticles to protect against deleterious effects of cigarette smoke extracts (CSE), a specific in vitro model of cigarette smoke exposure, in cultured H9c2 cardiomyocytes by measuring the amount of ROS generation, NF- κ B activity, and the expression of antioxidant enzymes. Data presented here suggest that CeO_2 nanoparticle treatment protects the H9c2 cells from CSE-induced damage by

its free radical scavenging ability, the inhibition of NF- κ B activation, and the preservation of antioxidant activity and intracellular glutathione content.

Materials and Methods

Preparation of CeO_2 Nanoparticles. The CeO_2 nanoparticles were synthesized and characterized by the Nano Science Center at University of Central Florida as described previously (Korsvik et al., 2007; Heckert et al., 2008). The particles were resuspended in de-ionized water and diluted with Dulbecco's modified Eagle's medium (DMEM) to the final concentrations of 1, 10, or 100 nM.

Preparation of CSE. The CSE was prepared by a modification of the method described previously (Carnevali et al., 2003). In brief, two Kentucky 1R3F research-reference cigarettes (The Tobacco Research Institute, University of Kentucky, Lexington, KY) without filters were smoked using a modified syringe-driven apparatus and bubbled through 50 ml of serum-free DMEM. The resulting suspension was adjusted to pH 7.4 and then filtered through a 0.20- μm pore filter to remove bacteria and large particles, and this solution was regarded as 100% strength CSE. Dilutions of this extract were then made for experiments.

Cell Culture and Treatment. All cellular studies were conducted with H9c2 rat heart-derived embryonic myocytes (CRL-1446; American Type Culture Collection, Manassas, VA) incubated in culture using DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin G, 100 mg/ml streptomycin, and 2 mM L-glutamine. Cells were incubated at 37°C with 5% CO_2 and 95% air. For all experiments, cells were plated in six-well plates at 2.0×10^5 cells/ml. For CeO_2 nanoparticles pretreatment, cells were incubated with 1, 10, or 100 nM CeO_2 nanoparticles in a fresh cell culture medium for 24 h. For cell stimulation, old medium was removed and replaced with medium supplemented with 10% CSE. Cells were incubated for the indicated time points and then harvested for biochemical or molecular assays. All experiments were repeated at least three times to provide their reproducibility.

Cell Survival Analysis. Cell viability was determined by the CellTiter-Blue Cell Viability assay kit (Promega, Madison, WI) according to the manufacturer's instructions. In brief, cells were seeded in 96-well plates and treated with cerium oxide nanoparticles and 10% CSE as described above. The cells were washed carefully with Dulbecco's phosphate-buffered saline (DPBS), and CellTiter-Blue reagent was added to each well. After incubation for 1 h at 37°C to allow cells to convert resazurin to resorufin, the fluorescent signal was measured with a spectrophotometer at the excitation/emission wavelength pair of 560 $_{\text{Ex}}$ /590 $_{\text{Em}}$. Cell viability was expressed as the percentage of relative viability of treated cells to that of untreated cells.

Determination of Intracellular ROS. The presence of free radicals in the H9c2 cells after CSE stimulation was determined using a red fluorescent dye, dihydrorhodamine (DHR) 123 (Invitrogen, Carlsbad, CA), as described previously (Niu et al., 2009). In brief, the H9c2 cells were maintained in DMEM containing 10% FBS, 100 U/ml penicillin G, 100 mg/ml streptomycin, and 2 mM L-glutamine. After pretreatment of the H9c2 cells with 10 nM CeO_2 nanoparticles for 24 h, cells were stimulated with 10% CSE for indicated time points. Immediately after stimulation, cells were washed with DPBS and incubated in fresh culture medium containing DHR123 (10 μM) for 30 min at 37°C, during which DHR123 was oxidized directly to rhodamine 123. After three washes with DPBS, fluorescence images were obtained using a Nikon fluorescence microscope equipped with a rhodamine filter. Cells were then lysed with PBS containing 0.1% SDS; the fluorescence intensity values were analyzed by fluorescence spectrophotometer (Carry Eclipse, Mulgrave, VIC, Australia), and the average values from three different wells were presented.

Immunoblot Analysis. Cells were suspended with ice-cold lysis buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton

Fig. 1. Redox cycles between Ce^{3+} and Ce^{4+} oxidation states.

X-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM Na_3VO_4 , and protease and phosphatase inhibitor cocktails (Thermo Fisher Scientific, Waltham, MA) for 15 min on ice and centrifuged at 14,000 rpm for 10 min. Cytosolic and nuclear extracts were prepared with NE-PER nuclear and cytoplasmic extraction kit using the manufacturer's protocol (Thermo Fisher Scientific). The protein concentration was determined by NanoDrop spectrophotometer assay (Thermo Fisher Scientific). Equal amounts of proteins were separated on 10 to 12% SDS-polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA) and transferred onto nitrocellulose membranes (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). The membranes were blocked with 3% (wt/vol) milk in 5 mM Tris-HCl, pH 7.4, containing 200 mM NaCl and 0.1% (v/v) Tween 20 for 1 h and then incubated overnight with specific antibodies against inducible nitric-oxide synthase (iNOS), NF- κ B-p65, MnSOD, CuZnSOD (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and phosphor-I κ B α (Cell Signaling Technology, Danvers, MA), respectively. The immune complexes were detected autoradiographically using appropriate peroxidase-labeled secondary antibodies (Santa Cruz Biotechnology, Inc.) and enhanced chemiluminescence detection reagent ECL (GE Healthcare). Anti- β -actin and antihistone antibodies served as loading controls. Specific bands were quantified by densitometry using analytic software (Alpha Innotech, San Leandro, CA).

Quantitative Real-Time RT-PCR. Total RNA was extracted from cell cultures using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Quality and concentration of RNA samples were detected using a Nanodrop spectrophotometer, and cDNA was generated using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time RT-PCR was performed with iCycler iQ real-time PCR detection system (Bio-Rad Laboratories) using the Quantitect SYBR Green RT-PCR kit (QIAGEN, Valencia, CA). Primer pairs used were as follows: TNF- α : forward, 5'-AAATGGGCTCCCTCTCATGTTTC-3' and reverse, 5'-TCTGCTTGGTGGTTTGCTACGAC-3'; IL-1 β : forward, 5'-CACCTCTCAAGCAGAGCAG-3' and reverse, 5'-GGGTTCCATGGTGAAGTCAAC-3'; IL-6: forward, 5'-TCCTACCCCAACTTCCAATGCTC-3' and reverse, 5'-TTGGATGGTCTTGGTCCTTAGCC-3'; and iNOS: forward, 5'-CAGCCCTCAGAGTACAACGAT-3' and reverse, 5'-CAGCAGGCACACGAATGAT-3'; β -actin: forward, 5'-AGGGAAATCGTGCGTGAC-3' and reverse, 5'-CGCTCATTGCCGATAGTG-3'. mRNA levels for TNF- α , IL-1 β , IL-6, and iNOS were determined relative to the housekeeping gene β -actin, and fold differences within each group were calculated.

NF- κ B Promoter Assay. The H9c2 cells were seeded at a concentration of 1.5×10^5 cells per well in six-well plates and cotransfected with 2 μ g of NF- κ B-driven luciferase reporter construct and 2 μ g of pSV40- β -galactosidase plasmid using the FuGENE 6 Transfection Reagent (Roche Applied Science, Indianapolis, IN) in FBS-free cell culture media. After 24-h exposure to the transfection mixture, the media on the transfected cells were removed and replaced with medium containing 10 nM CeO_2 nanoparticles for 24 h, and then the cells were exposed to 10% CSE for 24 h. After washing with DPBS, the cells were lysed with 1 $\times$ passive lysis buffer from Promega, and luciferase activity was measured by using the reporter luciferase assay system (Promega) according to the manufacturer's protocol and normalized with respect to β -galactosidase activity.

Immunocytochemistry. The H9c2 cells were seeded in Lab-Tek chamber glass slides (Nalge Nunc International, Rochester, NY) and treated with/without cerium oxide nanoparticles and CSE as described earlier. After treatment, cells were washed with DPBS, fixed, and permeabilized in 4% paraformaldehyde in PBS with 0.1% Triton X-100 for 10 min at room temperature. Cells were then washed with DPBS and blocked with 3% bovine serum albumin in PBS for 30 min and incubated with NF- κ B p65 antibody overnight at 4°C. After wash, cells were incubated with goat anti-rabbit Alexa Fluor 494-conjugated secondary antibody (Invitrogen) for 2 h at room temperature. Cells were viewed with a Nikon fluorescence microscopy, and

images were captured using an attached camera linked to a computer.

Glutathione Measurement. Intracellular glutathione level was determined with a GSH-Glo Glutathione Assay kit from Promega according to the manufacturer's instructions. In brief, cells were seeded in 96-well plates and treated with cerium oxide nanoparticles and 10% CSE as described earlier. The cells were washed carefully with DPBS, and the GSH-Glo reagent was added to each well. After a 30-min incubation at room temperature to allow cells to convert a luciferin derivative into luciferin, reconstituted Luciferin Detection Reagent was added to each well. After a 15-min incubation, the luminescent signal was recorded with a spectrophotometer microplate reader.

TdT-Mediated dUTP Nick-End-Labeling Assay. Apoptosis of the H9c2 cells was detected by TdT-mediated dUTP nick-end-labeling (TUNEL) assay using an in situ Cell Death Detection Kit, TMR Red (Roche Diagnostics) according to the manufacturer's instructions. In brief, cells were seeded in Lab-Tek chamber glass slides and treated with/without cerium oxide nanoparticles and CSE as described earlier. After treatment, cells were washed with DPBS and fixed in 4% paraformaldehyde in PBS for 10 min at room temperature, and then permeabilized with proteinase K and incubated with labeling buffer containing terminal deoxynucleotidyl transferase (TdT) and TMR Red-labeled dUTP at 37°C for 60 min. After wash, the slides were mounted, and the signals were visualized with TMR Red under a Nikon fluorescent microscope. The total TUNEL-positive cells were calculated in five randomly selected fields for each slide, and five slides were studied per group.

Caspase-3/7 Activity Assay. The activity of both caspases was determined using the APO-ONE Homogenous Caspase-3/7 Assay (Promega) as per instructions from the manufacturer. In brief, cells were seeded in six-well plates and treated with/without cerium oxide nanoparticles and CSE as described earlier. After treatment, cells were washed with ice-cold DPBS and lysed as described above. Crude cell lysate (100 μ l) was incubated with 100 μ l of Apo-ONE caspase-3/7 reagent containing bis-(*N*-CBZ-L-aspartyl-L-glutamyl-L-valyl-aspartic acid amide (Z-DEVD-R110), a caspase substrate known to be essentially cleaved by caspase-3 or caspase-7, respectively, for 1 h at room temperature. The fluorescence signal was recorded using a spectrofluorometer at the excitation/emission wavelength pair of 499_{Ex}/521_{Em}. Caspase activities were expressed as the percentage of relative fluorescence unit of treated cells to that of untreated cells.

Statistical Analysis. The results are presented as means $\pm$ S.E.M. Significant difference of values was calculated using the Student's *t* test. *P* < 0.05 was considered statistically significant.

Results

Cerium Oxide Nanoparticles Reduce ROS Levels and Attenuate CSE-Induced Cytotoxicity in H9c2 Cells. To investigate the ROS scavenging effects of CeO_2 nanoparticles because of its purported antioxidant properties, the H9c2 cells were pretreated with 1, 10, or 100 nM CeO_2 nanoparticles for 24 h and subsequently stimulated with 10% CSE in a fresh media for 24 h. ROS production was measured with a red fluorescent dye DHR123. Figure 2A shows representative examples of cells loaded with DHR123 in DMEM only or DMEM containing 10% CSE. The levels of intracellular ROS increased significantly in CSE-treated H9c2 cells. However, when cells were pretreated with CeO_2 nanoparticles for 24 h before CSE stimulation, the induction of intracellular ROS formation in CSE-stimulated cells was significantly inhibited by CeO_2 nanoparticles in a dose-dependent manner. We examined whether CeO_2 nanoparticles could protect cells from CSE-induced cell damage by its ROS scav-

Fig. 2. CeO₂ nanoparticles inhibited CSE-induced ROS production and cytotoxicity in H9c2 cells in dose-dependent manner. Cells were pretreated with 1, 10, or 100 nM CeO₂ nanoparticles (CeO₂ NP) for 24 h and followed by treatment with 10% CSE for 24 h. A, representative microphotographs of differential interference contrast (DIC) images and DHE123-loaded images at 24 h after CSE treatment. The red fluorescence indicates intracellular ROS formation (original magnification, 20×). B, cell viability was determined by the CellTiter-Blue cell viability assay kit (Promega), and the results are expressed as percentage of control cells for the indicated CeO₂ NP concentrations. *, $p < 0.05$ versus cells treated with CSE only; #, $p < 0.05$ versus 1 nM CeO₂ NP-pretreated cells, $n = 3$.

enging ability. Results show that the number of viable cells in culture was significantly decreased when cells were treated with DMEM containing 10% CSE compared with DMEM only (control) (Fig. 2B). However, cell viability was elevated when cells were pretreated with increasing concentrations of CeO₂ nanoparticles before CSE stimulation, indicating that CeO₂ nanoparticles interfered with CSE-induced cell death of H9c2 cardiomyocytes (Fig. 2B). There was no significant difference in ROS formation between the untreated control and the cells treated with CeO₂ nanoparticles only (data not shown). CSE-induced cell toxicity seemed to be time-dependent; we further examined the effects of CeO₂ nanoparticles on CSE-induced production of intracellular ROS and cell death at different time points. Cells were treated with 10 nM CeO₂ nanoparticles for 24 h and subsequently stimulated with 10% CSE in a fresh media for 6, 12,

and 24 h. Figure 3A shows representative examples of cells loaded with DHR123 in DMEM only (control) or DMEM containing 10% CSE for 6 h. Figure 2B shows the production of ROS, assayed by measurement of fluorescence intensity (directly proportional to ROS levels). The levels of intracellular ROS increased significantly in CSE-treated H9c2 cells, peaking at 6 h after addition of CSE, and maintained a high level at 24 h compared with that before treatment. However, this induction of intracellular ROS formation in CSE-stimulated cells was significantly inhibited at different time points when CeO₂ nanoparticles was added 24 h before stimulation (Fig. 3, A and B). Cell viability analysis showed that when cells were stimulated with 10% CSE for 6, 12, and 24 h, the number of viable cells in culture was significantly decreased compared with control after 12 and 24 h CSE exposure. However, when cells were pretreated with 10 nM CeO₂ nanoparticles for 24 h

Fig. 3. CeO₂ nanoparticles inhibited CSE-induced ROS production and cytotoxicity in H9c2 cells. Cells were pretreated with the 10 nM CeO₂ nanoparticles for 24 h and followed by treatment with 10% CSE for 6, 12, and 24 h. A, representative microphotographs of differential interference contrast (DIC) images and DHE123-loaded images at 6 h after CSE treatment. The red fluorescence indicates intracellular ROS formation (original magnification, 20×). B, ROS production is expressed as percentage of mean fluorescence intensity measured by a fluorescence spectrophotometer compared with untreated control cells for the indicated times. *, $p < 0.01$, #, $p < 0.05$ versus CSE-treated cells, $n = 4$. C, cell viability was determined by the CellTiter-Blue cell viability assay kit (Promega), and the results are expressed as percentage of control cells for the indicated times. *, $p < 0.05$ versus CSE-treated cells, $n = 4$.

before CSE exposure, the percentage of viable cell in culture was significantly increased, suggesting that CeO₂ nanoparticles protects cells from CSE cytotoxicity (Fig. 3C).

Cerium Oxide Nanoparticles Inhibit CSE-Induced Apoptosis and Caspase-3 Activation in H9c2 Cells. To test whether the cell viability reflected apoptosis, TUNEL staining experiments were performed to examine whether CeO₂ nanoparticles improved cell survival rate by inhibiting CSE-induced apoptosis of the H9c2 cells. A representative double staining of cell nuclei with TMR Red/4'-6-diamidino-2-phenylindole from cells exposed to 10% CSE for 24 h is displayed in Fig. 4A. The proportion of TMR Red/4'-6-diamidino-2-phenylindole-staining cells was significantly lower in cells treated with CeO₂ nanoparticles plus 10% CSE than in cells exposed to 10% CSE alone (Fig. 4B), revealing that CeO₂ nanoparticles prevented CSE-induced cell death, including apoptosis in the H9c2 cells.

Activation of caspase cascade is critical in initiation of apoptosis, and caspase-3 constitutes the major pool of executor caspases (Kothakota et al., 1997). To address whether caspase-3 was involved in cytotoxicity of CSE exposure, caspase-3/7 activity was measured using the APO-ONE Homogenous Caspase-3/7 Assay kit (Promega). The results show that the levels of caspase 3/7 activity significantly increased in cells exposed to 10% CSE for 24 h compared with the untreated cells (control). However, when cells were treated with CeO₂ nanoparticles for 24 h before CSE exposure, the caspase 3/7 activity was significantly inhibited (Fig. 4C). Taken together, these findings indicate that CeO₂ nanoparticles enhanced the survival of CSE-exposed H9c2 cells by suppression of apoptosis.

Cerium Oxide Nanoparticles Inhibit CSE-Induced Activation of NF- κ B in H9c2 Cells. NF- κ B is one of the key transcription factor activated by cigarette smoke and has been implicated in the process of myocardial inflammation (Hall et al., 2006). NF- κ B activity is regulated by cytoplasmic degradation of its inhibitory unit, I κ B. In response to a variety of stimuli, I κ B is rapidly phosphorylated and degraded by proteasomes, resulting in p65 subunit of NF- κ B translocation to the nucleus and binding to DNA, leading to expression of target genes. As shown in Fig. 5A, a slight cytoplasmic immunostaining with anti-p65 antibody was observed in un-

treated cells, whereas cells treated with 10% CSE for 24 h showed an intense nuclear fluorescence, and cerium oxide nanoparticles pretreated cells exposed to CSE showed a significant diminution in nuclear p65 immunostaining, indicating an inhibitory effect of CeO₂ nanoparticles on the translocation of p65 subunits of NF- κ B into the nuclei. This observation was further confirmed by immunoblot analysis as shown in Fig. 5B. An increased NF- κ B/p65 level in the nucleus was seen in CSE-treated cells, and this increase in the nuclear levels of NF- κ B/p65 protein was suppressed by the pretreatment with CeO₂ nanoparticles, signifying that CeO₂ nanoparticles inhibit CSE-induced nuclear translocation of p65 subunit of NF- κ B in the H9c2 cells.

To determine whether the inhibitory action of CeO₂ nanoparticles on NF- κ B/p65 nuclear translocation was attributable to its effect on I κ B α degradation, the cytosolic levels of I κ B α was determined by immunoblot analysis after pretreatment of the H9c2 cells with CeO₂ nanoparticles followed by exposure to 10% CSE. The results showed that CSE exposure resulted in increased phosphorylation of I κ B α and that pretreatment of cells with CeO₂ nanoparticles suppressed this phosphorylation (Fig. 5C). These results indicate that CeO₂ nanoparticles treatment of the H9c2 cells resulted in inhibition of CSE-induced phosphorylation and degradation of I κ B α , and subsequent activation of NF- κ B.

CSE treatment seems to activate NF- κ B in H9c2 cells, which should result in the transcriptional activation of NF- κ B-inducible genes. We examined whether CeO₂ nanoparticles treatment could block CSE-induced increase in NF- κ B reporter gene activity. The H9c2 cells were transiently transfected with NF- κ B promoter-luciferase reporter plasmid and then exposed to 10% CSE in the presence and absence of CeO₂ nanoparticles. Treatment of cells with 10% CSE for 24 h resulted in a significant increase in luciferase reporter expression compared with the untreated control, whereas this increase in CSE-induced luciferase reporter expression was suppressed in the presence of CeO₂ nanoparticles (Fig. 5D), suggesting that CeO₂ nanoparticles inhibit CSE-induced NF- κ B promoter activity.

Cerium Oxide Nanoparticles Inhibit CSE-Induced Activation of Inflammatory Genes in H9c2 Cells. The ultimate consequence of NF- κ B signaling is the activation of

Fig. 4. CeO₂ nanoparticles inhibited CSE-induced apoptosis in H9c2 cells. Cells were pretreated with the 10 nM CeO₂ nanoparticles (CeO₂ NP) for 24 h and followed by treatment with 10% CSE for 24 h. A, representative microphotographs of double staining of TUNEL (TMR, red) and 4'-6-diamidino-2-phenylindole (DAPI, blue) at 24 h after CSE treatment (original magnification, 20 $\times$). Nuclei stained pink represent apoptotic nuclei. B, apoptotic cells are expressed as a percentage of total cells in five randomly selected fields for each slide and five slides were studied per group. *, $p < 0.05$ versus CSE-treated cells, $n = 4$. C, the activity of caspase-3/7 from cytosolic fraction was measured by determining the cleaved fluorogenic substrate, Z-DEVD-R110. The data represent the mean $\pm$ S.E.M. *, $p < 0.05$ versus CSE-treated cells, $n = 3$.

Fig. 5. CeO₂ nanoparticles prevented CSE-induced activation of NF-κB in H9c2 cells. Cells were pretreated with the 10 nM CeO₂ nanoparticles (CeO₂ NP) for 24 h and followed by treatment with 10% CSE for 24 h. A, representative photomicrographs of cells showing NF-κB p65 subunit nuclear translocation identified by fluorescence immunocytochemical staining with anti-p65 antibody. NF-κB p65 translocation was detected in the nuclei of cells exposed to CSE alone, whereas CeO₂ NP pretreatment effectively inhibited this translocation as determined by significant diminution in nuclear p65 immunostaining fluorescence intensity. B, CeO₂ NP inhibited CSE-induced nuclear translocation of NF-κB p65, as shown by a decrease in NF-κB p65 levels in nuclear extract compared with CSE treatment alone, assayed by immunoblot analysis. Equal protein loading was evaluated by histone. Densitometry measurements of the nuclear NF-κB p65 protein are normalized to histone and shown in the bar graph. *, $p < 0.01$ versus CSE-treated cells, $n = 3$. C, CeO₂ NP inhibited CSE-induced phosphorylation of IκBα, as shown by a decrease in IκBα levels in cytosolic extract compared with CSE treatment alone, assayed by immunoblot analysis. Equal protein loading was evaluated by β-actin. Densitometry measurements of the cytosolic IκBα protein are normalized to β-actin and shown in the bar graph. *, $p < 0.05$ versus CSE-treated cells, $n = 3$. D, CeO₂ NP inhibited NF-κB-dependent reporter gene activity induced by CSE. H9c2 cells were seeded at a concentration of 1.5×10^5 cells per well in six-well plates and cotransfected with NF-κB-driven luciferase reporter construct and pSV40-β-galactosidase plasmid. After 24 h, cells were treated with CeO₂ NP for 24 h followed by CSE treatment for 24 h, and luciferase activity was measured and normalized with respect to β-galactosidase activity. The data represent the mean $\pm$ S.E.M. *, $p < 0.05$ versus CSE-treated cells, $n = 3$.

inflammatory genes, including genes encoding the proinflammatory cytokines, TNF- α , IL-1 β , and IL-6 (Ahn and Aggarwal, 2005). Because CeO₂ nanoparticles seem to inhibit CSE-induced NF-κB activation, we examined whether expression of these NF-κB-dependent genes could be abrogated by CeO₂ nanoparticles. Real-time PCR analysis showed that CSE exposure to H9c2 cells for 24 h resulted in an increase in the mRNA expression of TNF- α , IL-1 β , and IL-6 compared with the untreated control, whereas CeO₂ nanoparticles treatment of cells led to a significant inhibition of the CSE-induced increase in the expression of these proinflammatory genes (Fig. 6), suggesting the anti-inflammatory potential of CeO₂ nanoparticles.

Cerium Oxide Nanoparticles Inhibit CSE-Induced Expression of iNOS in H9c2 Cells. Increased iNOS expression is a component of the immune response and has been demonstrated in cardiomyocytes in septic shock, myocarditis, transplant rejection, and dilated cardiomyopathy (Umar and van der Laarse, 2010). A pathological role of iNOS-derived excessive nitric oxide production has been considered as an essential factor of smoke injury (Ambrose and Barua, 2004; Csizsar et al., 2009). We tested the effect of CeO₂ nanoparticles on CSE-induced expression of iNOS, which is dependent on NF-κB activation (Xie et al., 1994). As shown in Fig. 7, CSE exposure to the H9c2 cells resulted in

increased expression of iNOS at both mRNA and protein levels as determined by real-time PCR and immunoblot, respectively. CeO₂ nanoparticles pretreatment of cells resulted in a significant suppression of the CSE-induced increase in the expression of iNOS at both its mRNA and protein levels, indicating that CeO₂ nanoparticles protect against CSE-induced iNOS production in H9c2 cells.

Cerium Oxide Nanoparticles Prevent CSE-Induced Antioxidant Depletion in H9c2 Cells. It has been shown that smoking can weaken the cellular antioxidant defense mechanisms (Ramesh et al., 2008). CSE-induced free radicals might consume cellular antioxidants, such as antioxidant enzymes and intracellular GSH. To test this possibility, we examined whether CeO₂ nanoparticles limit the CSE toxicity in the H9c2 cells by preservation of its antioxidant defense system. As shown in Fig. 7, CSE exposure resulted in decreased levels of CuZnSOD and MnSOD in H9c2 cells compared with untreated control, and CeO₂ nanoparticle pretreatment inhibited CSE-induced depletion of both CuZnSOD and MnSOD as determined by immunoblots (Fig. 8, A and B). We also observed that cells exposed to CSE showed the decreased levels of GSH compared with untreated control, and the diminished level of GSH was significantly restored by CeO₂ nanoparticle pretreatment compared with cells treated with CSE alone.

Fig. 6. CeO₂ nanoparticles inhibited CSE-induced production of inflammatory cytokines in H9c2 cells. Cells were pretreated with the 10 nM CeO₂ nanoparticles (CeO₂ NP) for 24 h and followed by treatment with 10% CSE for 24 h. Cells were harvested, and total RNA was prepared for real-time PCR analysis as detailed under *Materials and Methods*. CSE treatment of H9c2 cells showed a marked increase in TNF- α (A), IL-1 β (B), and IL-6 (C) mRNA levels, which was significantly down-regulated by CeO₂ NP pretreatment. The data represent the mean $\pm$ S.E.M. *, $p < 0.05$ versus CSE-treated cells, $n = 5$.

Fig. 7. CeO₂ nanoparticles inhibited CSE-induced expression of iNOS in H9c2 cells. Cells were pretreated with the 10 nM CeO₂ nanoparticles (CeO₂ NP) for 24 h and followed by treatment with 10% CSE for 24 h. Total RNA and protein were prepared for real-time PCR and immunoblot analysis, respectively. A, CSE treatment of H9c2 cells showed a marked increase in iNOS mRNA levels, which was significantly down-regulated by CeO₂ NP pretreatment. The data represent the mean $\pm$ S.E.M. *, $p < 0.01$ versus CSE-treated cells, $n = 5$. B, CeO₂ NP inhibited CSE-induced expression of iNOS, as shown by a decrease in iNOS protein levels in cell lysates compared with CSE treatment alone, assayed by immunoblot analysis. Equal protein loading was evaluated by β -actin. Densitometry measurements of iNOS protein are normalized to β -actin and shown in the bar graph. *, $p < 0.05$ versus CSE-treated cells, $n = 3$.

Discussion

Cigarette smoke has been identified as a major risk factor for cardiovascular diseases, and increased oxidative stress and inflammation are considered to be an important mechanism of cardiovascular remodeling induced by smoking (Le-

one et al., 2008; Armani et al., 2009; Csiszar et al., 2009; Minicucci et al., 2009). Recent studies in rodent models of smoking have shown that acute CS exposure causes increased cardiac oxidative stress that is associated with depleted cellular antioxidant capacity (Bernhard and Wang, 2007; Ramesh et al., 2008). In the present study, we demonstrated that pretreatment of the H9c2 cardiomyocytes with CeO₂ nanoparticles prevented ROS generation, antioxidant depletion, NF- κ B activation, inflammatory cytokine production, and iNOS expression that occurred in H9c2 cardiomyocytes exposed to CSE. The present study provides the first evidence that CeO₂ nanoparticles act as antioxidants that prevent oxidative stress and suppress inflammatory response that occurs in cardiomyocytes after cigarette smoking.

Cellular response to ROS may depend on the reduction-oxidation state in the cell. If the amounts of oxidants do not exceed the reducing ability of the cell, ROS are involved in physiological functions such as signal transduction (Palmer and Paulson, 1997). However, when ROS levels exceed the antioxidative ability of the cell, the cell undergoes oxidative stress, finally leading to cell death. Oxidative stress in cardiomyocytes has been recognized as a key event in the initiation and/or progression of a wide array of cardiac pathologies and is also implicated in smoke-induced cardiomyopathy (Leone et al., 2008; Armani et al., 2009). Experimental studies have dealt with evaluation of the cardioprotective potential of antioxidative supplementation in cigarette smoke-induced cardiac remodeling. However, the direct deleterious effect of cigarette smoke to cardiomyocytes has not yet been well assessed. In the present study, the use of H9c2 cardiomyocyte cell line exposed to CSE, a specific in vitro model of cigarette smoke exposure, we found that CSE treatment can increase intracellular ROS level and cause the H9c2 cardiomyocyte death. The discrepancy between CSE-generated intracellular ROS and cell viability, shown in Fig. 3, was probably the result of the decreased living cell numbers from the longer CSE exposure-induced cell death because these dead cells were lost due to wash procedure to exclude the unloaded

Fig. 8. CeO₂ nanoparticles inhibited CSE-induced antioxidant depletion in H9c2 cells. Cells were pretreated with the 10 nM CeO₂ NP for 24 h and followed by treatment with 10% CSE for 24 h. A and B, expression pattern of antioxidant enzymes were analyzed by immunoblot with anti-CuZnSOD and anti-MnSOD antibodies. Equal protein loading was evaluated by β -actin. Densitometry analysis of showed that CuZnSOD or MnSOD levels is normalized to β -actin and shown in the bar graph. *, $p < 0.05$ versus CSE-treated cells, $n = 3$. C, cellular GSH levels were assayed by using GSH-Glo glutathione assay kit (Promega). The data represent the mean $\pm$ S.E.M. *, $p < 0.05$ versus CSE-treated cells, $n = 4$.

DHR123 for measurement of DHR123 fluorescence intensity. This explanation was also indicated by Fig. 3A that was taken at 6 h after CSE exposure and showed relatively higher living cells compared with cell exposure to CSE for 24 h as indicated in Fig. 2A and is consistent with cell viability assay as shown in Fig. 3C. CSE-induced cell damage seemed to be time-dependent, and cell viability was significantly reduced at 24 h after CSE exposure. Accordingly, at this time point (most of our data generated from this end point), we did find an increase in TUNEL-positive cells in the H9c2 cells exposed to CSE. However, CSE cytotoxicity was prevented when the induced ROS was scavenged by CeO₂ nanoparticles, thereby implicating a role of oxidative stress in the mechanism of cigarette smoke-induced cytotoxicity (Csiszar et al., 2009). Therefore, our results clearly demonstrate that CSE can induce H9c2 cardiomyocyte death by generating ROS. Moreover, CeO₂ nanoparticles may be effective in inhibiting H9c2 cell death by controlling ROS level in the cells.

Oxidative stress could lead to activation of redox-sensitive transcription factors, especially NF- κ B that is activated by cigarette smoke and has been called the “smoke sensor” of the body (Ahn and Aggarwal, 2005). Activation of NF- κ B in the heart has been linked to myocardial inflammation, cardiac remodeling, and heart failure (Hall et al., 2006). Our data show that CSE treatment stimulated generation of ROS and activation of NF- κ B in the H9c2 cardiomyocytes. These findings are consistent with those of previous studies that have shown that NF- κ B is activated by cigarette smoke and suggest a mechanism by which the exposure of cardiovascular system to cigarette smoke may predispose the heart to inflammation and ultimately cardiomyopathy and failure given that activation of the NF- κ B pathway activates genes that produce inflammatory cytokines. Indeed, we did see a significant induction of TNF- α , IL- β , and IL-6 and proinflammatory enzymes such as iNOS in the H9c2 cells exposed to CSE. However, treatment of cells with CeO₂ nanoparticles resulted in the inhibition of CSE-induced activation of NF- κ B and production of inflammatory cytokines in addition to decreasing ROS. This is similar to our previous findings that CeO₂ nanoparticles attenuated myocardial inflammation and ventricular dysfunction in a murine model of cardiomyopathy induced by cardiac-specific expression of MCP-1 (Niu et al., 2007) and consistent with the recent report showing the anti-inflammatory properties of CeO₂ nanoparticles by inhibition of proinflammatory iNOS protein production in J774A.1 murine macrophages (Hirst et al., 2009). However, the current study is the first to show the anti-inflammatory mechanism of CeO₂ nanoparticles through inhibition of NF- κ B activation and suggests that CeO₂ nanoparticles can be useful in preventing the occurrence of an inflammatory environment apparently involved in the pathogenesis of cigarette smoke-induced cardiomyopathy.

Antioxidants in the cell constitute the primary defense system that limits the oxidative stress. Among the critical antioxidant enzymes that protect the cells against oxidative stress are superoxide dismutases CuZnSOD and MnSOD. GSH is the most abundant intracellular nonenzymic antioxidant, and it has been reported that the intracellular depletion of GSH enhances cytotoxicity and apoptosis (Jiang et al., 2000). The reduction of both SOD activity and intracellular GSH content has been reported in the myocardium of rats exposed to cigarette smoke (Ramesh et al., 2008). Consistent

with these findings, our results demonstrate a significant decrease in CuZnSOD and MnSOD activities and intracellular GSH content in the H9c2 cells exposed to CSE. The decreased activity of these antioxidant enzymes and GSH level may be due to its increased utilization during the burst of ROS production induced by CSE exposure. These data are in accordance with findings of other author that demonstrated an induction of MnSOD protein expression by CeO₂ nanoparticles in normal human colon cells (Colon et al., 2010). The preservation in the activity of these antioxidant enzymes and intracellular GSH content by CeO₂ nanoparticles may be explained by its scavenging effect on free radicals. Taken together, these data suggest that retention of the activity of these antioxidant enzymes and intracellular GSH content may be crucial for maintenance of cellular homeostasis under conditions of oxidative stress, and CeO₂ nanoparticles seemed to have a potential to be considered as a cardioprotective agent by its ROS-scavenging ability.

The antioxidative effect of CeO₂ nanoparticles is attributed to its direct exchange between ROS and the high ratio of electrons on the larger surface area of the nanoparticles (Yu et al., 2003; Hochella et al., 2008). CeO₂ nanoparticles have both 3⁺ and 4⁺ oxidation states that could result in an autoregenerative reaction cycle (Ce³⁺ $\rightarrow$ Ce⁴⁺ $\rightarrow$ Ce³⁺) continuing on their surface accompanied by creation of oxygen vacancies or defects (Yu et al., 2003; Hochella et al., 2008). Thus, the high reactivity of CeO₂ nanoparticles results in a high ability to scavenge ROS. Our findings provide the evidence that CeO₂ nanoparticles can decrease ROS production, inhibit inflammation, and preserve antioxidant enzymes and GSH content in a biological system. We show for the first time that CeO₂ nanoparticles can inhibit NF- κ B activation and expression of inflammatory genes. In addition, the results suggest that the cytoprotective effect of CeO₂ nanoparticles is associated with the regulation of activity of antioxidant enzymes and intracellular GSH level. However, the overall significance, safety, and bioavailability of CeO₂ nanoparticles in the in vivo setting and also clinical applications remain to be investigated. When we injected CeO₂ nanoparticles into experimental mice intravenously, we routinely harvested organs (liver, heart, lung, and kidney) and analyzed the levels of CeO₂ nanoparticles in various tissues and found highest levels of CeO₂ nanoparticles in the hearts (J. Niu and P. E. Kolattukudy, unpublished data). We did not observe any overt systemic toxicity, even with the CeO₂ nanoparticles solution used at a relative high concentration of 15 nM twice a week for 2 weeks, but beneficial effects were observed for CeO₂ nanoparticles treatment (Niu et al., 2007). A recent study reported that CeO₂ nanoparticles protected against hepatic oxidative damage in rat and that administration of CeO₂ nanoparticles showed no visible systemic toxicity (Amin et al., 2011), supporting the notion that CeO₂ nanoparticles may be safe in the in vivo setting that are currently under investigation.

In summary, we show that cigarette smoke extract can increase intracellular ROS production, activate the NF- κ B signaling pathway and inflammatory gene expression in cardiomyocytes, and cause cell damage, which can be eliminated by the application of CeO₂ nanoparticles. Therefore, there may be potential for applying CeO₂ nanoparticles as a novel agent to protect against the development of cardiovascular diseases related to cigarette smoke.

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Authorship Contributions

Participated in research design: Niu.

Conducted experiments: Wang and Niu.

Contributed new reagents or analytic tools:

Performed data analysis: Wang and Niu.

Wrote or contributed to the writing of the manuscript: Niu and Kolattukudy.

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