Brain suppression of AP-1 by inhaled diesel exhaust and reversal by cerium oxide nanoparticles

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Abstract
One of the uses of cerium oxide nanoparticles (nanoceria, CeO2) is as a diesel fuel additive to improve fuel efficiency. Gene/environment interactions are important determinants in the etiology of age-related disorders. Thus, it is possible that individuals on high-fat diet and genetic predisposition to vascular disease may be more vulnerable to the adverse health effects of particle exposure. The aim of this pilot study was to test the hypothesis that inhalation of diesel exhaust (DE) or diesel exhaust-containing cerium oxide nanoparticles (DCeE) induces stress in the brain of a susceptible animal model. Atherosclerotic prone, apolipoprotein E knockout (ApoE/C0/C0/C0) mice fed a high-fat diet, were exposed by inhalation to purified air (control), DE or DCeE. The stress-responsive transcription factor, activator protein-1 (AP-1), was significantly decreased in the cortical and subcortical fraction of the brain after DE exposure. The addition of nanoceria to the diesel fuel reversed this effect. The activation of another stress-related transcription factor (NF-κB) was not inhibited. AP-1 is composed of complexes of the Jun and/or Fos family of proteins. Exposure to DCeE caused c-Jun activation and this may be a mechanism by which addition of nanoceria to the fuel reversed the effect of DE exposure on AP-1 activation. This pilot study demonstrates that exposure to DE does impact the brain and addition of nanoceria may be protective. However, more extensive studies are necessary to determine how DE induced reduction of AP-1 activity and compensation by nanoceria impacts normal function of the brain.

Keywords
c-Jun, nanoceria, stress, transcription factors

Introduction
Diesel exhaust (DE), an important contributor to urban air pollution, consists of particulate matter (PM) and gases. While PM-induced adverse cardiopulmonary responses have been well documented (Donaldson et al., 2005; Frampton, 2001; Pope et al., 2002), the brain may also be another target (Block & Calderon-Garciduenas, 2009). Diesel exhaust particles (DEP) are directly toxic to neuronal cells (Block et al., 2004), and particles derived from a polluted site in California caused neurotoxicity (Morgan et al., 2011). Diesel exhaust particles (DEP) are directly toxic to neuronal cells (Block et al., 2004), and particles derived from a polluted site in California caused neurotoxicity (Morgan et al., 2011). Cerium oxide nanoparticles (nanoceria, CeO2) are used as a fuel additive to decrease particulate emissions while increasing fuel efficiency. A battery of in vitro screening of nanoceria showed no toxicity associated with exposure (Park et al., 2007). We have reported that at biologically relevant doses, the systemic toxicity of inhaled nanoceria is negligible. Furthermore, the addition of CeO2 to the fuel reduced particle numbers and reversed the atherosclerotic burden associated with DE exposure (Cassee et al., 2012). We did observe an increase in proinflammatory cytokine levels in the cerebellum and brainstem region of the central nervous system (CNS) after CeO2 exposure. This pilot study was designed to investigate if a general stress response underlies CNS changes after exposure to DE with or without nanoceria.

Apolipoprotein E knockout (ApoE-C0/C0/C0/C0) mice were exposed by inhalation to purified air (control), exhaust from an engine using standard diesel (DE) or diesel-containing nanoceria (DCeE) for 4 weeks. ApoE is a glycoprotein which functions in transport, uptake and redistribution of cholesterol. In the CNS, it plays an important role in maintaining neuronal integrity and repairing cellular damage (Duan et al., 2006). Furthermore, absence of ApoE increases proinflammatory gene expression after lipopolysaccharide challenge (Lynch et al., 2001) and its absence disrupts the integrity of the blood–brain barrier (Methia et al., 2001). Thus, the absence of ApoE can be considered a genetic susceptibility factor.

Activator protein-1 (AP-1) is a DNA-binding transcription factor composed of homodimeric or heterodimeric complexes of the Jun and/or Fos family of proteins.
Physiological function of AP-1 is related to its role in proliferation and differentiation (Hess et al., 2004). Factors such as cellular stress, steroid hormones, and inflammatory cytokines modulate activation of this transcription factor (Pérez-Cadahía et al., 2011). In the brain, c-Jun is important in formation of the AP-1 transcription factor complex and has a dual role in mediating either neurodegeneration or neuronal repair (Herdegen & Waetzig, 2001; Raivich & Behrens, 2006). Phosphorylated c-Jun (p-c-Jun) is the activated protein. NF-kB is another transcription factor which has been associated with the stress response. Activation of this transcription factor underlies innate immune responses (Baueuerle & Henkel, 1994; Hatada et al., 2000). In the present study, AP-1, NF-kB, c-Jun and p-c-Jun levels were measured in the brain of control and exposed animals. We hypothesized that exposure to DE or DCE would activate markers of stress response in the brain of ApoE−/− mice. It is more informative to determine effects in more specific brain regions known to be affected in disease states. However, because of the size of the mouse brain and sample amount necessary for planned biochemical analysis, markers were evaluated in two segments of the brain [(i) cortical plus subcortical region and (ii) cerebellum plus brainstem region].

Methods and materials

The methods and materials are the same as that used in our previously published article (Cassee et al., 2012). Thus, the following is an overview. Please refer to the previous study for more detail.

Animal exposures

Apolipoprotein E-deficient (ApoE−/−) mice (B6.129P2-Apoelm1Unc N11) were fed a ‘Western’ diet high in fat (21% fat; AB Diets, the Netherlands) ad libitum for 7 weeks. The high-fat diet was initiated at the age of 11 weeks and continued till the end of the study. At 14 weeks of age, the animals were exposed (nose only) to filtered air or diluted exhaust originating from an engine fueled with standard diesel with or without 9 ppm nanosized CeO2 (Envirox; Oxonica, Haddenham Buckinghamshire, UK; 3 h/day and 5 days/week for 28 days). Animals were sacrificed 3 days after the last exposure. For this pilot study, the brains of five males from each of the three experimental groups (control, DE and DCE) were collected and analyzed.

Generation and characterization of DE and DCE

An Ingersoll Rand engine was run at 1500 rpm with a generator load of 35 kW fueled with low sulfur diesel (EN590). The DCE also contained 9 ppm nanoceria. The exhaust was collected and diluted with humidified compressed air to obtain a concentration of 1.7 mg/m3 of particles during exposures. Dilution was adjusted to ensure equal mass concentration for both DE and DCE. A TEOM 1400a (Rupprecht & Patashnick, Co., Albany, NY) was used for continuous mass recordings. Particle number was measured by a condensation nucleus particle number and surface area was measured using a nanoparticle surface area monitor (CPC 3022A; TSI Inc., St. Paul, MN). Cerium content was determined by high-resolution inductively coupled plasma mass spectroscopy (HR-ICPMS, Thermo Fisher Scientific, Bremen, Germany).

Sample preparation for western blot analysis and gel shift mobility assay

The brain was collected and divided into two regions: (i) cortical and subcortical region and (ii) cerebellum and brainstem. Cytoplasmic and nuclear protein fractions were prepared using a published method (Lahiri & Ge, 2000).

Electrophoretic mobility shift assay

A protocol developed by Promega Corporation (Madison, WI) was used per manufacturer’s instruction. Protein amount was determined with the BCA protein assay kit (Thermo Fisher Scientific). About 50 µg of each sample was incubated with 32P-labeled oligonucleotides containing either AP-1 or NF-κB consensus sequence. The samples were loaded on polyacrylamide gel and ran along with negative control (blank) and competitor reactions (specific and non-specific). The specific competitor contained unlabeled AP-1 or NF-κB consensus nucleotide while the non-specific competitor contained unlabeled SP-1 consensus oligonucleotide. The competitor reactions also contained HELA cell extract (positive control). X-ray films were developed manually. The mean intensity of each band was measured and quantitated using KODAK 1500 gel logic imaging system (Eastman Kodak Company, New Haven, CT).

Western blot

Proteins (25 µg) were separated on polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was blocked for 1 h and then incubated overnight with specific antibodies against c-Jun, p-c-Jun (Ser63) or p-c-Jun (Ser73) (Cell Signaling Technology, Danvers, MA). The membrane was then incubated with the respective horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) for 60 min at room temperature. Proteins were visualized by chemiluminescence detection reagent ECL (Thermo Scientific, Waltham, MA). An antibody against β-actin (Millipore, Billerca, MA) served as loading control. The mean intensity of each band was measured and quantitated using KODAK 1500 gel logic imaging system.

Statistical analysis

Differences among groups were examined by one-way analysis of variance (ANOVA). Tukey’s test was used in pair-wise comparisons.

Results

Particle characterization

Table 1 is a summary of the particle number, mass, surface area and cerium content measured in the exhaust collected from burning diesel fuel or diesel fuel-containing nanoceria. Particle number and surface area was significantly decreased while levels of cerium were increased in DCE compared to DE. For a more detailed discussion refer to our previously published study (Cassee et al., 2012).
Transcription factor binding in the cortical and subcortical brain region

Exposure to DE caused a significant decrease in the amount of basal AP-1 binding. Addition of nanoceria to the diesel fuel reversed this effect and returned values to control levels (Figure 1). NF-κB activation was not significantly altered after exposure (Figure 2).

c-Jun and p-c-Jun levels in the cortical and subcortical brain region

c-Jun is one of the main proteins which form the AP-1 complex. Activated c-Jun is phosphorylated on Ser63 or Ser73 residues. The levels of c-Jun, p-c-Jun (Ser63) and p-c-Jun (Ser73) were assessed in cytoplasmic fractions and nuclear fractions. p-c-Jun (Ser63) was not detected. In the nuclear fractions, both c-Jun and p-c-Jun (Ser73) levels were increased after DCE exposure compared to DE exposure (Figures 3 and 4). In the cytoplasmic fraction, only the level of p-c-Jun was significantly increased after DCE exposure (Figure 4).

Discussion

The addition of CeO₂ may lessen the toxicity of DE by reducing the number and surface area of particles emitted by combustion of diesel fuel. However, the amount of cerium in the emitted particles is enhanced (Table 1; Cassee et al., 2012). This release into the environment may increase biological absorption. Cerium-containing particles in lung tissue do not appear to be easily degraded and are persistent in the human respiratory tract (Pairon et al., 1995). In cultured human lung epithelial cells, cerium oxide nanoparticles decrease viability by inducing oxidative stress (Park et al., 2008). Thus, in the lungs, nanoceria may cause adverse effects. In rats, 90 days after a single intravenous injection of citrate-stabilized ceria, cerium was detected in all organs including the brain (Yokel et al., 2012). This finding reflects the longevity of the element in biological systems. After inhalation, cerium was detected in the brain and levels appear to accumulate with repeated exposures (Geraets et al., 2012). Thus, it is reasonable to predict that prolonged inhalation of nanoceria may lead to bioaccumulation of the particles in brain tissue which may eventually alter normal cellular function and cause toxicity. In a related study, we reported that inhalation of nanoceria-containing DE increased

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**Table 1. Particle characteristics.**

| Particle                          | DE     | DCE
|-----------------------------------|--------|-----
| Mass (time integrated)            | 1741 ± 153 µg/m³ | 1740 ± 162 µg/m³
| Mass (continuous)                 | 1925 ± 79 µg/m³  | 1893 ± 136 µg/m³
| Mass median diameter              | 82.0 ± 1.8 nm     | 83.0 ± 1.8 nm
| Surface area (µm²/cm³)            | 4018 ± 605        | 3636 ± 517
| Number (#/cm³ x 10⁸)              | 5.3 ± 0.1         | 3.6 ± 0.5
| Cerium content (µg Ce/mg soot)    | 0.2 ± 0.1         | 5.4 ± 4.0

Note: Values are mean ± standard deviation. This data is derived from a previously published report (Cassee et al., 2012).

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**Figure 1.** AP-1 levels in nuclear fractions of mouse brain. (A) Sample gel showing the AP-1 shifted band in mouse brain nuclear fractions. B = blank; NC = non-specific competitor; SC = specific competitor. (B) The intensity of AP-1 shifted band is shown in the bar graph. Values given are mean ± standard error of mean; n = 5; *p ≤ 0.05 compared to control.

**Figure 2.** NF-κB levels in nuclear fractions of mouse brain. (A) Sample gel showing the NF-κB shifted band in mouse brain nuclear fractions. B = blank; NC = non-specific competitor; SC = specific competitor. (B) The intensity of the NF-κB shifted band is shown in the bar graph. Values given are mean ± standard error of mean; n = 5.

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DE exposure decreased AP-1 binding in the cortical and subcortical region of the brain. We saw a similar trend in the cerebellum and brainstem, but the results did not reach statistical significance (data not shown). This finding is consistent with a previous report showing a decrease in brain AP-1 activation after exposure to ultrafine PM derived from an urban environment (Campbell et al., 2009). However, because the same animal model was used, the effect may be dependent on the ApoE/− phenotype. A recent study reported that exposure to vehicle emissions for 30 days increased the permeability of BBB in this animal model (Oppenheim et al., 2013). We were not able to discern a change in AP-1 activation in different brain regions of normal rats exposed to DE (Gerlofs-Nijland et al., 2010). It is plausible that DE-induced AP-1 suppression may depend on a combination of stress factors. A recent study reported that inhaled DE alters genetic expression in the olfactory bulb. Both upregulated and downregulated genes were mainly associated with immune responses. Enrichment of the rearing environment reversed the genetic changes (Yokota et al., 2013). Thus, decreasing environmental stress may alleviate brain response to DE exposure. In a genetic mouse model of obesity, the stress hormone corticosterone, caused AP-1 suppression and reduced expression of brain-derived neurotrophic factor (BDNF) (Wosiski-Kuhn et al., 2014). The promoter of the gene for BDNF is under the control of AP-1. It is interesting that after exposure to ambient particles, BDNF expression was decreased in the olfactory bulb of exposed mice (Bos et al., 2012). In the brain, AP-1 generally promotes immediate-early genes in response to different stimuli including cellular stress and cytokines (Pérez-Cadahía et al., 2011). AP-1 regulates diverse mechanisms such that both neurodegenerative and neuroprotective pathways have been attributed to the activation of the transcription factor (Herdegen & Waetzig et al., 2001; Raivich & Behrens, 2006). It has been observed that the c-Jun/AP-1 pathway plays a physiological role in learning and memory (Klusa et al., 2006). It has been observed that exposure to PM may contribute to cognitive impairments (Power et al., 2011; Suglia et al., 2008; Weuve et al., 2012). Since AP-1 has been shown to play a role in learning and memory, it is possible that DE-induced downregulation of AP-1 in the brain may provide a molecular basis for the cognitive changes reported. More extensive studies are needed to determine if DE-induced change in AP-1 is associated with cognitive impairment and if the addition of nanoceria to the fuel may have a protective effect.

The addition of nanoceria to diesel fuel reversed AP-1 inhibition. It is possible that nanoceria physically interacts with and binds components of DE which cause AP-1 suppression. This is a likely scenario because as noted above, CeO2 did reduce the number of particles emitted. Since cellular redox status influences AP-1 activity (Gius et al., 1999), another mechanism by which cerium caused the observed effect may be via alteration of redox activity. Free radical scavenging activity of nanoceria has been reported (Heckert et al., 2008). However, in the brain, injection of nanoceria showed pro-oxidant effects (Hardas et al., 2012). In the liver, injection of nanoceria caused an increase in pro-oxidant markers up to 30 days post-exposure, but after 90

proinflammatory cytokines in the cerebellum and brainstem region of the ApoE/− mouse brain (Cassee et al., 2012). NF-kB results in this brain region support our earlier finding (data not shown).
days, nanoceria behaved as an antioxidant (Yokel et al., 2012). Thus, there may be complex temporal patterns in regards to how nanoceria behaves in biological systems and more studies are warranted before concluding that the effect of nanoceria on AP-1 activation was mediated by modulation of cellular redox status.

Nanoceria may reverse DE-induced AP-1 suppression by an increase in upstream constituents of the transcription factor. After the addition of nanoceria to the fuel, phosphorylated levels of c-Jun increased in both the nuclear fraction and cytoplasmic fraction. Thus, it is plausible that nanoceria modulates AP-1 via activation of the components that comprise the AP-1 dimer. Activation of c-Jun may be due to a stress-dependent increase of the mitogen-activated protein kinase (MAPK) c-Jun N-terminal kinase (JNK). In the brain, JNK has been implicated in both beneficial and pathogenic pathways (Mehan et al., 2011). More specific studies are warranted to establish impact of nanoceria on this pathway and determine if changes in the brain are harmful or protective.

Conclusions

Many factors will influence brain responses to DE or DCeE. These include dose and duration of inhalation exposures, brain region analyzed, as well as species and genetic predisposition of the animal model used. Although oxidative, inflammatory and stress pathways may be interrelated, one time point analysis may not suffice to decipher the complex spatiotemporal relationship between these events. Thus, to better understand the CNS consequences of DE or DCeE exposure, future studies need to include multi-time point analysis of biologically relevant doses in both normal and genetically susceptible animals.

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