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Exposure to Traffic-Generated Air Pollutants Mediates Alterations in Brain Microvascular Integrity in Wildtype Mice on a High-Fat Diet

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Abstract

Air pollution-exposure is associated with detrimental outcomes in the central nervous system (CNS) such as cerebrovascular disorders, including stroke, and neurodegenerative diseases. While the mechanisms of these CNS-related outcomes involved have not been fully elucidated, exposure to traffic-generated air pollutants has been associated with altered blood brain barrier (BBB) integrity and permeability. The current study investigated whether inhalation exposure to mixed vehicle emissions (MVE) alters cerebral microvascular integrity in healthy 3 mo old C57BL/6 mice, as well as whether exposure-mediated effects were exacerbated by a high-fat (HF) vs. low-fat (LF) diet. Mice on each diet were randomly assigned to be exposed to either filtered air (FA) or MVE [100 PM/m³ vehicle emissions mixture: 30 µg PM/m³ gasoline engine + 70 µg PM/m³ diesel engine emissions; median size ~60 nm; particle mass size distribution median of ~1 µm (range: <0.5 – 20µm)] for 6 hr/d, 7d/wk, for 30d. Using sodium fluorescein as a tracer, we observed a significant increase in BBB permeability in both HF+MVE exposed and HF+FA animals, compared to LF+FA controls. Exposure to HF+MVE also led to a significant increase plasma ox-LDL and ox-LDL scavenger receptors (LOX-1 and CD-36) expression in the cerebral vasculature. Histological analysis revealed decreased expression of TJ protein, claudin-5, associated with increased matrix metalloproteinase (MMP)-9 activity and oxidative stress in the cerebral vasculature of HF+MVE mice, compared to LF+MVE. Such findings indicate that inhalation exposure to traffic-generated pollutants, coupled with a HF diet, results in altered BBB integrity and increased ox-LDL signaling in the cerebral vasculature in a wildtype animal model.

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Conflict of Interests.

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Keywords

Air pollution; ox-LDL; LOX-1; BBB; Oxidative stress

Introduction

Findings from multiple epidemiological studies over the past two decades have provided a clear correlation between environmental air pollution-exposure and cardiovascular diseases, including stroke and ischemic heart disease (Crichton et al., 2016; Du et al., 2016). More recently, air pollution-exposure has been reported to be a major health risk that is associated with neuropathologies such as stroke, Alzheimer's disease (AD), Parkinson's diseases, as well as dementia-related neurotoxicity (Chen et al., 2015; Calderón-Garcidueñas et al., 2016a; Calderón-Garcidueñas et al., 2016b; Tian et al., 2017). The consequences of inhalation exposure to traffic-generated air pollution on the brain are complex, but is known to result in neuroinflammation, generation of reactive oxygen species (ROS), microglial activation, and alterations in blood brain barrier (BBB) permeability (Levesque et al., 2013; Oppenheim et al., 2013). The BBB is a dynamic structure, comprised of microvascular endothelial cells, which are joined by tight junction (TJ) proteins such as occludin and claudin-5, astrocytes, and pericytes that provide a physical and chemical barrier that regulates transport into and out of the brain parenchyma. Importantly, air pollution-exposure mediated alterations in TJ protein expression, in both animal models and human exposures, have been reported to result in decreased BBB integrity and increased BBB permeability that is associated with CNS-related disorders including stroke and AD (Alimonhamadi et al., 2016; Calderón-Garcidueñas et al., 2016; Oppenheim et al., 2013). The mechanisms involved in air pollution-exposure mediated alterations in the neurovasculature have not yet been fully elucidated; however, inflammation, ROS, and increased matrix metalloproteinase (MMP) activity have all been associated with exposure-mediated alterations in BBB integrity (Calderón-Garcidueñas et al., 2016).

Several studies have shown that the incidence of ischemic stroke is correlated with increased blood cholesterol levels (Kurth et al., 2007). Specifically, oxidized low-density lipoprotein (ox-LDL) is known to mediate the initiation and/or progression of atherosclerotic plaque growth and microvascular inflammation. The role of ox-LDL in atherogenesis has been reported to occur primarily via signaling through its receptors, including the low-density lipoprotein ox-LDL receptor (LOX-1), the primary ox-LDL receptor in the microvascular endothelium (Chen et al., 2007). Additionally, increased plasma ox-LDL is associated with an increased risk of acute cerebral infarction and/or may also predict the size of the ischemic lesion (Uno et al., 2005). In agreement with this premise, anti-ox-LDL antibody concentrations are significantly higher in patients who have suffered from an atherogenic ischemic stroke, compared to that in healthy individuals (Marta et al., 2014). Increased signaling through another scavenger receptor for ox-LDL, the CD36 receptor, is also associated with endothelial dysfunction and stroke (Cho, 2012). Oxidative lipids such as 4-hydroxy-trans-2-nonenal (4-HNE), which is a major product of lipid peroxidation, have also been found to be elevated in atherosclerosis (Chapple et al., 2013). As plasma ox-LDL typically increased with the consumption of a high-fat (HF) diet (Silaste et al., 2014),

understanding the interaction between diet and environmental exposures in mediating the detrimental effects on the microvascular system is critical. This becomes even more imperative when considering the staggering statistics that report more than 1/3 of children and adolescents in U.S. are overweight or obese, often associated with consumption of a high-fat diet, which in itself is a factor for risk of future cardiovascular disease (Freedman et al., 2007).

When investigating air pollution-mediated toxicity it is important to also study mixtures of environmental pollutants, including those from traffic-generated sources, as they are a significant component of ambient air pollution. The effects of exposure to components and/or mixtures of vehicle exhaust have emerged from studies reporting detrimental outcomes in the pulmonary, cardiovascular, and nervous system, as well as others. For example, exposure to diesel exhaust particulate matter (PM) was reported to generate the oxidation of LDL (e.g. increase ox-LDL) (Ikeda et al., 1995), higher levels of which are associated with progression of cardiovascular disease, atherosclerotic plaque growth, as well as poorer prognostic outcome in stroke (Ishigaki et al., 2009). Additionally, our laboratory has previously reported that inhalation exposure to a mixture of gasoline and diesel engine exhaust (MVE) resulted in increased circulating ox-LDL in atherosclerotic Apolipoprotein (Apo)E^{-/-} mice, which was associated with increased expression of microvascular LOX-1 and ROS (Lund et al., 2011; Lucero et al., 2017). Furthermore, exposure to air pollution has also been associated with altered BBB integrity and permeability in both laboratory studies and human exposures (Oppenheim et al., 2013; Calderón-Garcidueñas et al., 2016). Exposure to MVE, specifically, has been reported to result in a disruption of the BBB, associated with increased MMP-9 expression and activity and decreased TJ protein (occludin and claudin-5) expression, in the cerebral vasculature of ApoE^{-/-} mice (Oppenheim et al., 2015; Lucero et al., 2017). Importantly, alterations in the integrity of the BBB can lead to exacerbated pathology and/or hemorrhagic transformation during an ischemic stroke (Turner and Sharp, 2016).

We have previously reported the effects of traffic-generated air pollutants (MVE) in mediating microvascular toxicity and BBB disruption, associated with LOX-1 signaling, in atherosclerotic ApoE^{-/-} mice (Oppenheim et al., 2013; Lucero et al., 2017). However, to date, there is very little information on whether inhaled traffic-generated pollutants promote similar detrimental effects on the cerebral vasculature in a wildtype animal model and/or whether a HF diet may exacerbate any of the observed exposure-mediated outcomes. As such, we are investigating the hypothesis that exposure to traffic-generated air pollution (MVE) results in alterations in BBB integrity, related to ox-LDL and LOX-1 signaling pathways, in healthy wildtype (C57BL/6) mice, which is exacerbated by concurrent consumption of a HF diet.

Materials and Methods

Animals and Inhalation Exposure Protocol

Three mo-old male C57BL/6 mice were placed on either normal mouse chow or a HF diet (TD88137 Custom Research Diet, Harlan Teklad, Madison, WI; 21.2% fat content by weight, 1.5g/kg cholesterol content) beginning 30 days prior to initiation of exposure. Mice

were randomly grouped to be exposed by whole-body inhalation to a mixture of whole gasoline engine exhaust and diesel engine exhaust (MVE: 30 $\mu\text{g PM}/\text{m}^3$ gasoline engine emissions + 70 $\mu\text{g PM}/\text{m}^3$ diesel engine) or filtered-air (controls) for 6 h/d, 7 d/wk, for a period of 30 d. MVE was created by combining exhaust from a 1996 GM gasoline engine and a Yanmar diesel generator system, and exposures chemistries and PM characterized, as previously reported (McDonald et al., 2004; McDonald et al., 2008; Lund et al., 2011; Oppenheim et al., 2013; Mumaw et al., 2016; Lucero et al., 2017.). Particle size distribution was measured with a Fast Mobility Particle Sizer (FMPS, TSI, St. Paul, MN) for the ~10–500 nm size range and an Aerodynamic Particle Sizer (TSI, St Paul, MN) to measure the 0.5–20 μm size range. Particle mass concentration by gravimetric analysis of Teflon membrane filters at the inlet of the chamber and inside the exposure chamber was conducted once/wk throughout the duration of the exposure protocol. The particle number size distribution for this exposure had a median size of approximately 60 nm; particle mass size distribution had a median of ~1 μm (range: <0.5 –20 μm), with total particle mass for the mixture measured at $102.5 \pm 20.9 \mu\text{g}/\text{m}^3$ over the 30 d study. Particle composition was approximately: 60% elemental carbon, 15% organic carbon, 2% nitrates, 5% ammonium, 9% sulfates, 9% elements (metals) (Vedal et al., 2013). Mice were housed in standard shoebox cages within an AAALAC International-approved rodent housing facility (2m³ exposure chambers) for the entirety of the study, which maintained a constant temperature (20–24°C) and humidity (30–60% relative humidity). Mice had access to chow and water *ad libitum* throughout the study period, except during daily exposures when chow was removed. All procedures were approved by the Lovelace Respiratory Research Institute's Animal Care and Use Committee and conform to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

Tissue Collection

Animals were weighed (Table 1) and sacrificed within 14–16 hours after their last exposure. Mice were anesthetized with Euthasol (0.1 ml per 30 g mouse) and euthanized by exsanguination. The brain tissue was carefully dissected, meninges gently removed, and were either (1) embedded in tissue freezing medium (TFM; VWR Scientific) (n=3–4 per group; midbrain region) and frozen on dry ice or (2) immediately snap frozen in liquid nitrogen. Whole blood was also collected via cardiac-stick, using a heparinized syringe, centrifuged at 3000xg for 10 min, and plasma was separated, aliquoted, and stored at –80C.

BBB permeability

Changes in BBB permeability were assessed (in a subset of mice, n=6 per group) using the fluorescent tracer, sodium fluorescein (Na-F). Mice were injected intraperitoneally with 100 μl of 2% Na-F-PBS 30 min prior to the end of their exposure on day 30, as previously described (Oppenheim et al., 2013). At sacrifice, blood was collected via cardiac puncture, and then animals were perfused with PBS until perfusate ran clear. After complete perfusion, brains were removed, the meninges, cerebellum, and brain stem were gently dissected away. The cerebrum was homogenized in 10x vol of 50% trichloroacetic acid (TCA) to precipitate proteins and remove potential background fluorescence. The homogenate was then centrifuged at 13,000xg for 10 min at RT and the supernatant neutralized with 5 mol/L

NaOH (1:0.8). The serum was collected from blood samples and mixed 1:1 (vol) with 10% TCA and centrifuged at 13,000xg for 10 min at RT. Supernatant was neutralized as described for the cerebral tissue. Na-F fluorescence was measured in the supernatants of serum and cerebral tissue at ex/em wavelengths of 440/525 nm on a fluorometer and fluorescent dye content was calculated using external standards (10 to 200 ng/ml). Data were calculated as the amount (ng) of Na-F per mg of tissue / amount of Na-F per μ l of serum at the time of sacrifice/brain collection and expressed as fold-difference in the amount of Na-F uptake across diet/exposures groups compared to LF+FA controls, as previously described (Davidson et al., 2012; Amenta et al., 2014).

Total cholesterol and ox-LDL in plasma

Total cholesterol levels in plasma were quantified as previously described (Lund et al., 2011). Values are reported as TBARS/ μ g of cholesterol.

Double Immunofluorescence

Frozen brain sections (10 μ m) of the cerebrum (between Bregma 0 through -2.5mm) were prepared for either LOX-1, CD-36, 4-HNE, occludin, claudin-5, MMP-9, and/or double immunofluorescence, using techniques previously described by our laboratory (Lund et al., 2011; Oppenheim et al., 2013) with the following primary antibodies: MMP-9 (1:1000; Abcam, Cambridge, MA; Cat. #38898), occludin (1:500; Abcam #168986), claudin-5 (1:500; Abcam #15106), LOX-1 (1:1000; Abcam #60178), CD-36 (1:1000; Abcam #64014), 4-HNE (1:1000; Abcam #46545), AT-1 (1:250; Abcam #18801) and von Willebrand factor (vWF) (1:1000; Abcam #11713) and anti-rabbit Alexa Fluor 555 (1:500) and anti-sheep Alexa Fluor 455 (1:500) secondary antibodies. Each endpoint was processed and analyzed as a single batch. Slides were imaged under fluorescent microscopy at 20x and 40x with the appropriate excitation/emission filter, digitally recorded, and analyzed using image densitometry with Image J software (NIH) by a blinded technician. Slides with no primary antibody were used as negative controls (data not shown). Only cerebral vessels 100 μ m in size were used for analysis; for most endpoints vessels were ~10–50 μ m in size. A minimum of 3–5 locations on each section (2 sections per slide), 3 slides and n=6 per group were used for analysis, as previously described by our laboratory (Lund et al., 2011).

In situ zymography

MMP-2 and -9 (gelatinase) activity was measured by *in situ* zymography, on 10 μ m thick cerebral sections, as previously described by our laboratory (Oppenheim et al., 2013; Lucero et al., 2017). Sections were analyzed using fluorescent microscopy and densitometry was calculated using white/black images and quantified using Image J software (NIH, Bethesda, MD; performed on 6 sections per sample, 3 regions per section, n=6 samples per group). Background fluorescence was subtracted from each section before statistical comparison between groups.

Statistical Analysis

Data are shown as mean $\pm$ SEM. A two-way ANOVA with posthoc Tukey's test was used to analyze statistical significance between diet/exposure permutations. Statistical analyses were

conducted using Sigma Plot 12.0 (Systat, San Jose, CA). A $p < 0.050$ was considered statistically significant for all endpoints. % change in expression of histological endpoints, compared to LF+FA control, provided in Supplemental Table I.

Results

Inhalation Exposure to MVE Alters Brain Microvascular Permeability and Integrity in C57BL/6 Wildtype Mice

To assess alterations in cerebral microvascular permeability and integrity, resulting from either diet and/or MVE-exposure, a subset of mice from each group on the study were injected with the molecular fluorescent tracer, sodium fluorescein (Na-F), on the final day of the study exposures. Na-F is a molecule with an MW of 376.3 Da, and as such should typically have limited transport from the blood across an intact cerebral vasculature under homeostatic conditions. Therefore, the increase of FITC fluorescence in the cerebral parenchyma indicates altered BBB integrity and increased permeability. Our results show that a HF diet, alone, increased cerebral microvascular permeability in 3 mo-old male C57BL/6 FA control mice, compared to filtered air FA+LF diet controls (Fig. 1, $p < 0.050$). When consumption of a HF diet is coupled with MVE-exposure, we observed an exacerbated increase in cerebral microvascular permeability, which is significantly higher than both LF+FA and HF+FA exposure groups (Fig. 1, $p < 0.050$ compared to each group). In addition to cerebral microvascular permeability, we also assessed diet- and or exposure-mediated alterations in integrity through analyzing expression of TJ proteins, occludin and claudin-5, in the cerebral vasculature. Consistent with those results observed in our permeability study, in comparison to LF+FA control mice (Fig 2A–C; Fig 3A–C) and LF +MVE-exposed mice (Fig 2D–F; Fig 3D–F), we observed only a trending decrease in TJ proteins occludin and claudin-5 in the HF+FA mice (Fig 2G–I; Fig 3G–I). However, there was a significant decrease (–15%) in claudin-5 expression in the vasculature of HF+MVE-exposed mice (Fig 3J–L, $p < 0.050$), compared to FA+LF controls, whereas occludin expression was not significantly altered (–9% decrease in expression) (Fig 2J–L).

MMP-9 Expression and Activity are increased with MVE-Exposure in C57BL/6 Wildtype Mice

We have previously reported that exposure to MVE results in increased MMP-9 expression and activity in both the systemic and cerebral vasculature of ApoE^{–/–} mice, which is associated with decreased TJ protein expression and increased BBB permeability (Lund et al., 2009; Oppenheim et al., 2015). Thus, we investigated whether MMP-9 expression and/or activity are altered in the cerebral vasculature of C57BL/6 wildtype mice, with either HF-diet and/or MVE-exposure. In comparison to LF+FA control mice (Fig 4A–C), we observed no significant change in MMP-9 expression in the cerebral vasculature of C57BL/6 mice exposed to MVE on an LF diet (Fig D–F). MMP-9 expression is, however, significantly upregulated (~19%) in HF+FA (Fig 4G–I, $p < 0.050$) and 23% in HF+MVE-exposed mice (Fig 4J–L, $p < 0.050$), when compared to LF+FA control animals. Interestingly, when assessing MMP-9 (and –2, both are classified as “gelatinases”) activity in the cerebral vasculature, via *in situ* zymography, compared to LF+FA and HF+FA animals (Fig 5A and 5C), MVE-exposure appears to be driving the increase in gelatinase activity in both MVE

+LF (Fig 5B, 51% increase) and MVE+HF exposure animals (Fig 5D; 90% increase). And while background fluorescence (and overall CNS fluorescence) was also increased in the MVE-exposed animals (Fig 5B, and 5D), once background was subtracted there was still a significant increase in MMP-9 (and -2) activity in the cerebral microvessels in groups exposed to MVE ($p<0.050$ for both LF+MVE and HF+MVE), compared to LF+FA controls.

MVE-Exposure Increases plasma Ox-LDL and Ox-LDL Scavenger Receptor Expression in the Cerebral Vasculature of C57BL/6 Mice

In our previous *in vivo* studies involving MVE, we have consistently observed an exposure-mediated elevation in plasma ox-LDL associated with increased microvascular expression of the ox-LDL scavenger receptor, LOX-1, in an ApoE^{-/-} mouse model (Lund et al., 2011; Lucero et al., 2017). However, the effects of MVE-exposures on ox-LDL and scavenger receptor expression in the cerebral vasculature using a wildtype model have not yet been characterized. In agreement with previously published findings in healthy humans (Silaste et al., 2014), consumption of a HF diet resulted in increased plasma ox-LDL levels (Fig 6, $p<0.050$ compared to LF+FA controls), which was further increased when combined with the MVE-exposure in C57BL/6 mice (Fig 6, $p<0.050$ compared to LF+FA controls). Interestingly, compared to LF+FA controls (Fig 7A–C), we observed a 44% increase in cerebral microvascular LOX-1 expression in the LF+MVE (Fig 7D–F), a 24% increase in HF+FA (Fig 7G–I), and a 83% increase in HF+MVE (Fig 7J–L) exposure groups, which were all statistically increased compared to LF+FA controls ($p<0.050$ for each group). When analyzing the expression of the ox-LDL scavenger receptor CD36, compared to FA+LF controls (Fig 8A–C), only the HF+MVE exposure group (Fig 8J–L), exhibited an increase (46%) in expression in the cerebral vasculature of C57BL/6 mice, suggesting differential regulation of the receptors.

Induction of Lipid Peroxidation in the Cerebral Vasculature of C57BL/6 Mice Exposed to MVE

To assess whether inhalation exposure to MVE ± HF diet resulted in increased lipid peroxidation in the cerebral vasculature of C57BL/6 mice, we quantified 4-HNE expression. Compared to LF+FA controls (Fig 9A–C), we observed a ~13% increase in 4-HNE expression in LF+MVE exposed animals (Fig 9D–F), expression of which was further exacerbated (~17%) in the cerebral vasculature of the HF+MVE exposed animals (Fig 9J–L, $p<0.050$ compared to LF+FA and LF+MVE groups). Interestingly, there was no notable alteration in 4-HNE expression in the HF+FA group, suggesting the MVE-exposure mediated the increase in 4-HNE expression.

Discussion

While several recent studies have reported a strong correlation between exposure to environmental air pollutants and detrimental outcomes in the neurovasculature and CNS, very few studies have investigated these mechanisms and/or outcomes in a wildtype mouse model, findings from which are likely more translatable to pathophysiological outcomes in human exposure scenarios. Additionally, while consumption of a HF diet is a well-established link to both cardiovascular disease and BBB disruption in humans (Hsu and

Kanoski, 2014), little information exists on the combined effects of exposure to inhaled environmental air pollutants, concomitant with a HF diet, on the cerebral microvasculature. Thus, we investigated whether inhalation exposure to ubiquitous environmental air pollutants, namely a mixture of gasoline and diesel vehicle emissions, resulted in alterations in BBB integrity in a C57BL/6 wildtype mouse model, as well as whether consumption of a HF diet exacerbated these detrimental outcomes. Importantly, the concentrations and duration of exposure of MVE used in the current study are comparable to those previously reported in our ApoE^{-/-} mouse studies (Oppenheim et al., 2013; Lucero et al., 2017).

In agreement with our previous studies in ApoE^{-/-} mice (Oppenheim et al., 2013; Lucero et al., 2017), we observed alterations in cerebral microvascular integrity and permeability in our MVE-exposed C57BL/6 mice on a HF diet. This was evidenced by the increased Na-F transport from the blood into the parenchyma, as well as a through the observed significant decrease in expression of TJ protein claudin-5, when compared to LF+FA controls. We also observe increased BBB permeability in our HF diet-fed control (FA) animals, which concurs with previous studies that have reported that consumption of a HF diet, alone, alters BBB permeability and TJ protein expression in rats (Kanoski et al., 2010). As noted in Table 1, we do not observe a notable increase in body weight of C57BL/6 mice fed a high fat diet over the duration of the study; however, when combined with MVE-exposures we observe a significant increase in weight gain in C57BL/6 mice. This finding supports a growing body of literature that reports strong correlations between exposure to components of environmental air pollution and metabolic outcomes such as obesity, metabolic syndrome, and type 2 diabetes in humans (Krämer et al., 2010; Park et al., 2012; Rundle et al., 2012; Wei et al., 2016). As we did not assess food intake during this study period, future studies are necessary to determine the rationale for this significant weight gain that occurs in our MVE exposed animals concurrent with consumption of a high fat diet. Furthermore, our results suggest that concurrent consumption of a HF diet with environmental air pollution exposure leads to exacerbated disruption of the cerebral microvascular integrity, compared to exposure to either condition alone; however, we have not yet determined the pathways involved. As detrimental CNS-related outcomes, such as hemorrhagic stroke occurrence, multiple sclerosis, and/or Alzheimer's disease have been associated with air pollution exposure in humans (Han et al., 2016; Bergamaschi et al., 2017; González-Maciél et al., 2017), future in-depth mechanistic studies are needed to elucidate signaling pathways involved in these neurovascular and CNS-related pathologies, as well as how dietary fat and/or other components may be exacerbating these outcomes.

The chemical components of the MVE used in this study consists of elemental carbon, organic carbon, ammonium, sulfate, nitrate, metals, alkanes, carbonyl, polycyclic aromatic hydrocarbon (PAH), nitro-PAH, and volatile organic carbon (VOC) (McDonald et al., 2008; Mumaw et al., 2016). While multiple studies have been conducted to determine the toxicity of individual air pollutants (and/or sources), characterizing the toxicity of mixtures of environmental pollutants in the pulmonary, cardiovascular, and nervous system is critical to developing a more in-depth understanding of the resulting health effects. Exposure to diesel exhaust PM was reported to promote the oxidation of LDL (Ikeda et al., 1995), higher levels of which are associated with atherosclerotic plaque growth (Ishigaki et al., 2009). It has been previously reported that exposure to diesel exhaust PM results in BBB disruption and

increased neurovascular disease risk in a wildtype mouse model (Heidari Nejad et al., 2015); however, these exposures consisted of diesel exhaust PM at exposure levels of 20–30 mg/m³. Thus, the effects of whole mixtures of traffic-generated pollutants (gases + PM), at environmental and/or occupational exposure levels, and the mechanisms involved have not yet been investigated. Interestingly, the consumption of a HF or “Western” diet has also been linked with BBB degradation (Hsu and Kanoski, 2014). Our laboratory has previously reported that subacute exposure (6 hr/day for 7 days; 300 µg PM/m³) to MVE resulted in increased ox-LDL, plasma sLOX-1 levels, and microvascular expression of the LOX-1 receptor, in ApoE^{−/−} mice (Lund et al., 2011). This MVE-exposure mediated increase in circulating ox-LDL was associated with increased expression of factors involved in the progression of atherosclerosis, specifically endothelin (ET)-1 and MMP-9, downstream of LOX-1 receptor signaling. Furthermore, in humans, who were acutely exposed to diesel exhaust (2 hr; 100 µg PM/m³), plasma sLOX-1, ET-1, and MMP-9 were also reported to be upregulated, in comparison to FA (control) plasma measurements (Lund et al., 2011), owing to translational comparisons in these endpoints across animal model vs. human exposures.

Expression of factors, such as MMP-9, present in the cerebral vasculature has been reported to be deregulated during BBB disruption, as well as in animal models of ischemia and/or stroke (Shigemori et al., 2006). MMP-9, which is a proteolytic zymogen, is known to degrade many of the components of the extracellular matrix including gelatin and collagen. To determine MVE-exposure mediated MMP-9 activity, we utilized *in situ* zymography (Yan and Blomme, 2003) in the cerebral vasculature of our study mice. Interestingly, in HF-fed animals, we observed a 19% increase in MMP-9 protein expression in the cerebral vasculature, but not activity. However, when coupled with the MVE exposure, microvessels from animals on the HF diet showed a significant increased MMP-9 expression (~22% induction) and activity (~90% induction), while vessels from the LF+MVE group showed only significantly increased MMP-9 activity (~78% induction), compared to FA+ control animals, as determined by 2-way ANOVA. Such findings suggest that a component (or mixture of components) present in the MVE is directly mediating increased MMP-9 activity in the cerebral vasculature, independent of diet. Since the activity of MMP-9 is regulated through binding with tissue inhibitor of metalloproteinase (TIMP)-1, it is plausible MVE-exposure may be altering TIMP-1 expression; however, further studies are needed to determine this possible outcome. As increased MMP-9 expression and/or activity is associated with BBB disruption and decreased TJ protein expression, which can lead to increased risk of hemorrhagic transformation during an occurrence of stroke (Turner and Sharp, 2016), we assessed TJ protein levels in the cerebral vasculature of our study animals. Our data reveal that MVE-exposure mediated MMP-9 activity is associated with decreased expression of TJ protein, claudin-5, in animals on a HF diet, suggesting altered cerebral microvascular integrity when both “insults” are present. This premise is in agreement with previous studies that report increased MMP-9 activity results in decreased expression of TJ proteins in the BBB (Yang et al., 2007), expression of which was normalized through treatment with an MMP inhibitor (Reijerkerk et al., 2006). Thus, collectively, these results suggest that inhalation exposure to MVE increases MMP-9 expression and activity in the cerebral microvasculature of C57BL/6 wildtype mice. Interestingly, this increase in MMP-9 activity was only associated statistical alterations in integrity and permeability in cerebral

vasculature of the MVE+HF mice, suggesting there are additional pathways driving these alterations mediated by factors/physiological states derived from consumption of a HF diet.

Increased plasma ox-LDL is associated with increased risk of atherosclerosis, acute cerebral infarction, and may also predict the size of the resulting lesion during a stroke (Uno et al., 2003, 2005). In agreement with results presented in the current study, consumption of a HF diet is known to increase circulating ox-LDL levels (Silaste et al., 2004). We, as well as others, have previously reported that exposure to traffic-generated air pollutants results in increased plasma ox-LDL levels (Jacobs et al., 2011; Lund et al., 2011). While the medical community has historically associated elevated ox-LDL levels with “unhealthy” or “susceptible” (e.g. baseline cardiovascular disease) adults, we now observe elevated ox-LDL in our younger populations (e.g. over-weight/obese children) (Norris et al., 2011). As such, circulating ox-LDL levels may serve as a predictor for future risk of cardiovascular disease (Freedman et al., 2007) and thus it is critical to understand which factors may drive increased production of ox-LDL. As most previous studies investigating the deleterious effects of air pollution on the vasculature have been completed using “susceptible” animal models with baseline disease (e.g. ApoE^{-/-}, CD36^{-/-}, DIO, etc.), we chose to utilize a wildtype animal model for the current studies to gauge whether exposure to environmental air pollutants can have detrimental effects in the brain vasculature in the absence of other underlying disease states. The current study findings show that a HF diet statistically increases circulating ox-LDL levels in C57BL/6 mice (~1.6-fold), compared to animals fed an LF diet; however, plasma ox-LDL levels are increased more than 2.5-fold in MVE+HF exposed C57BL/6 mice. These findings suggest that inhalation exposure to MVE mediates increased oxidation of circulating LDL, compared to consumption of a HF diet, alone.

The scavenger receptor, LOX-1, is the primary receptor for ox-LDL on microvascular endothelial cells (Chen et al., 2007), including those that comprise the BBB (Lin et al., 2010). LOX-1 expression is typically very low in healthy microvascular endothelial cells but is reported to be significantly upregulated during pathophysiological conditions (Mehta et al., 2006; Ogura et al., 2009). In agreement with our previous study in ApoE^{-/-} mice (Lucero et al., 2017), we observe that either a HF diet or MVE-exposure, independently, caused enhanced expression of LOX-1 receptor in the cerebral microvascular endothelium, with the highest expression observed in the MVE+HF group. Several previous studies have reported that LOX-1 receptor expression is correlated to BBB disruption. For example, early-onset preeclampsia is related with neurological complications that can lead to BBB disruption in the mother, which is associated with increased expression of the LOX-1 and plasma ox-LDL levels (Schreurs et al., 2013). Additionally, increased ox-LDL-LOX-1 signaling is also associated with increased microvascular MMP-9 expression and activity, which can also serve as a biomarker of BBB disruption (Shigemori et al., 2006; Lin et al., 2010; Lund et al., 2011; Oppenheim et al., 2013). We have previously reported that LOX-1 Ab-treatment attenuates MVE-mediated induction of LOX-1 expression and MMP-9 activity in both the systemic and cerebral vasculature of ApoE^{-/-} mice (Lund et al., 2011; Lucero et al., 2017). Thus, it is plausible that MVE-exposure induced ox-LDL-LOX-1 signaling may be driving MMP-9 activity in the vasculature of C57BL/6 mice as well.

The CD36 scavenger receptor is a second type of ox-LDL receptor that is expressed on microvascular endothelial cells, which also internalizes and/or mediates ox-LDL signaling (in addition to other ligands)(Swerlick et al., 1992). Increased CD36 expression and downstream signaling have been associated with the pathophysiology of both cardio-and cerebrovascular diseases. For example, ligand-CD36 signaling has been shown to induce inflammatory signaling and endothelial dysfunction (Cho, 2012). Exposure to diesel exhaust has been shown to increase oxidation of systemic lipids and also the expression of CD36 in the plaques of atherosclerotic ApoE^{-/-} mice (Bai et al., 2011). More recently, CD36 has also been implicated in mediating air pollution (ozone) induced microglial activation (Mumaw et al., 2016). Unlike LOX-1 expression, we did not see an increase in CD36 expression in our LF+MVE or HF+FA groups; however, we did observe a 46% increase in CD36 expression in the cerebral vasculature of C57BL/6 mice exposed to MVE+HF diet, suggesting the combined “insults” drive the statistical increase in CD36 receptor expression.

4-hydroxynonenal (4-HNE) is a secondary byproduct of lipid peroxidation and thus serves as a biomarker for MVE-induced lipid peroxidation in the current study. It has previously been reported that 4-HNE contributes to the pathogenesis of cardiovascular disease-states such as atherosclerosis. Ox-LDL has been shown to promote the accumulation of 4-HNE in endothelial cells, which is associated with increased endoplasmic reticulum stress (Chapple et al., 2013). Moreover, 4-HNE can bind to and decrease the activity of glutathione, which has also been associated with increased BBB permeability (Mertsch et al., 2001). We observe a significant increase (~17%) in 4-HNE expression in the cerebral microvasculature of the HF+MVE group, compared to FA+LF controls. Future studies with additional time points (acute vs. subchronic) for backend analysis of these endpoints, as well as additional ROS quantification assays, will be needed to clearly delineate the role of diet vs. exposure in the generation of oxidative stress in the CNS.

There is a growing body of literature highlighting the detrimental effects of traffic-generated air pollution on the CNS resulting in pathological outcomes in humans such as neuroinflammation, dementia-related diseases, and onset of stroke, which may be mediated by alterations in neurovascular structure and/or function. The mechanisms involved in pollution-mediated effects in the CNS have not yet been fully elucidated, and even less is understood about the interactions that may occur in the neurovasculature when multiple “insults”, such as concurrent consumption of a HF diet, are present in an individual. In the current study, we report that inhalation exposure to ubiquitous environmental air pollutants, namely vehicle emissions, results in increased BBB permeability associated with decreased TJ protein (claudin-5) expression in the cerebral vasculature of C57BL/6 wildtype mice fed a HF diet. Findings from our study suggest that either a HF diet or MVE-exposure, alone, can drive the alterations in BBB permeability; however, the alterations in TJ protein expression appear to be primarily mediated via MVE-exposures. These results are in agreement with our *in situ* zymography results, which show MMP-9 and (-2) activity are significantly increased in our MVE-exposure groups (regardless of diet). Finally, we also observe HF diet and MVE-mediated increase in plasma ox-LDL and ox-LDL scavenger receptor (LOX-1 and/or CD36) expression in the cerebral vasculature of C57BL/6 mice; however, these levels are the most pronounced in the HF+MVE group. Collectively, the findings from this study suggest that exposure to environmental-relevant levels of MVE can

alter BBB integrity and induce expression of factors associated with atherogenesis and/or occurrence of stroke, which is exacerbated when consuming a HF diet, in a wildtype animal model. Such findings highlight the necessity of: (1) understanding key components of environmental air pollution that drive the observed responses; (2) elucidating key mechanistic pathways, induced by exposure to environmental air pollutants that may contribute to the pathogenesis of cardiovascular and/or neurovascular disease states in a “healthy” individual; and (3) the importance of determining how diet, combined with environmental exposures, can exacerbate signaling through these mechanistic pathways in both “susceptible” and healthy” individuals in the human population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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All procedures were approved by the Lovelace Respiratory Research Institute’s Animal Care and Use Committee (Protocol # FY08-070) and conform to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Abbreviations

4-HNE	4-hydroxynonenal
AD	Alzheimer’s disease
ApoE^{-/-} mice	Apolipoprotein E null mice
BBB	blood brain barrier
CD-36 receptor	cluster of differentiation 36 receptor
CNS	central nervous system
DIO	diet-induced obesity
FA	filtered air
HF	high-fat diet
LDL	low-density lipoprotein
LF	low-fat diet
LOX-1	lectin-like oxidized LDL receptor

MMP	matrix metalloproteinase
MVE	mixed gasoline and diesel vehicle exhaust
NAD(P)H oxidase	nicotinamide adenine dinucleotide phosphate-oxidase
Na-F	sodium fluorescein
ox-LDL	oxidized low-density lipoprotein
PM	particulate matter
ROS	reactive oxygen species
TIMP	tissue inhibitor of matrix metalloproteinases
TJ	tight junction proteins

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Highlights

- Exposure to vehicle emissions alters BBB permeability in high-fat-fed C57BL/6 mice.
- Vehicle emission exposures exacerbate oxLDL levels in animals on a high-fat diet.
- Alterations in neurovascular integrity are associated with increased oxLDL receptors.
- Exposures result in reduced TJ protein expression associated with MMP-2/9 activity.

Figure 1. Inhalation Exposure to Mixed Vehicle Emissions Alters Blood Brain Barrier Permeability in High Fat-fed C57BL/6 Mice

Na-F uptake levels in a subset of C57BL/6 mice (n=6 per group) exposed for 6hr/d, 7 d/wk, for 30 d to either filtered air (FA), or 100 $\mu\text{g}/\text{m}^3$ PM of mixed gasoline and diesel engine emissions mixed engine emissions (MVE), while being fed either a low-fat (LF), or high-fat (HF) diet. Mice were injected i.p. with 100 μl of 2% of the fluorescent tracer sodium fluorescein (Na-F)-PBS 30 min prior to the end of their final exposure on day 30. Total fluorescence in the cerebrum and plasma (at time of sacrifice) was measured by fluorometry. Data are expressed as fold-increase uptake compared to LF+FA controls. *p < 0.05 compared to LF+FA controls, # p < 0.05 compared to HF+FA.

Figure 2. Tight Junction Protein, Occludin, Expression in the Cerebral Vasculature of C57BL/6 Mice is not Altered through Exposure to Mixed Vehicle Emissions

Double immunofluorescence of occludin (red) and vWF (green) in cerebral microvessels from 4 mo old C57BL/6 mice on a low-fat (LF; A–C) or high-fat diet (HF; G–I) exposed filtered air (FA) or on a LF (D–F) or HF diet (J–L) and to exposed MVE at 100 $\mu\text{g}/\text{m}^3$ PM of mixed gasoline and diesel engine emissions for 6hr/d, 7 d/wk, for 30 d (n=3–4 per exposure group; 4 sections each, 3–5 vessels per section). Right panels (yellow; C, F, I, L) are merged figures of left (red; A, D, G, J) and center (green, E, H, K) panels for occludin and vWF. Blue fluorescence is Hoechst stained nuclei. Scale bar = 100 μm . Results shown are mean $\pm$ SEM.

Figure 3. Tight Junction Protein, Claudin-5, Expression is decreased in the Cerebral Vasculature of C57BL/6 Mice on a High-Fat Diet, Exposed to Mixed Vehicle Emissions

Double immunofluorescence of claudin-5 (red) and vWF (green) in cerebral microvessels from 4 mo old C57BL/6 mice on a low-fat (LF; A–C) or high-fat diet (HF; G–I) exposed filtered air (FA) or on a LF (D–F) or HF diet (J–L) and exposed to MVE at $100 \mu\text{g}/\text{m}^3$ PM of mixed gasoline and diesel engine emissions for 6hr/d, 7 d/wk, for 30 d ($n=3-4$ per exposure group; 4 sections each, 3–5 vessels per section). Right panels (yellow; C, F, I, L) are merged figures of left (red; A, D, G, J) and center (green; B, E, H, K) panels for claudin-5 and vWF. Blue fluorescence is Hoechst stained nuclei. Arrows indicate areas of increased endothelial-

specific claudin-5 expression (merged fluorescence). Scale bar = 100 μ m. Results shown are mean $\pm$ SEM. *p < 0.05 compared to LF+FA.

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Figure 4. Cerebral Microvascular Expression of MMP-9 is increased in C57BL/6 Mice on a High-Fat Diet and Exposed to Mixed Vehicle Emissions

Double immunofluorescence of MMP-9 (red) and vWF (green) in cerebral microvessels from 4 mo old C57BL/6 mice on a low-fat (LF; A–C) or high-fat diet (HF; G–I) exposed filtered air (FA) or on a LF (D–F) or HF diet (J–L) and exposed to MVE at 100 $\mu\text{g}/\text{m}^3$ PM of mixed gasoline and diesel engine emissions for 6hr/d, 7 d/wk, for 30 d (n=3–4 per exposure group; 4 sections each, 3–5 vessels per section). Right panels (yellow; C, F, I, L) are merged figures of left (red; A, D, G, J) and center (green; B, E, H, K) panels for MMP-9 and vWF. Blue fluorescence is Hoechst stained nuclei. Arrows indicate areas of increased MMP-9

expression (fluorescence). Scale bar = 100 μ m. Results shown are mean $\pm$ SEM. *p < 0.05 compared to LF+FA controls.

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Figure 5. Inhalation Exposure to Mixed Vehicle Emissions Increases Gelatinase (MMP-2 and 9) Activity in the Cerebral Vasculature of C57BL/6 Mice

MMP-9 (and -2) activity, as shown by *in situ* zymography in cerebral microvessels from 4 mo old C57BL/6 mice on a low-fat (LF; A) or high-fat diet (HF; C) exposed filtered air (FA) or on a LF (B) or HF diet (D) and exposed to 100 µg/m³ PM of mixed gasoline and diesel engine emissions (MVE) for 6hr/d, 7 d/wk, for 30 d (n=3–4 per exposure group; 4 sections each, 3–5 vessels per section). Arrows indicate higher levels of fluorescence correlated to gelatinase activity. Scale bar = 100µm. Results shown are mean ± SEM. *p < 0.05 compared to LF+FA controls.

Figure 6. Plasma oxidized LDL is elevated in C57BL/6 Mice Exposed to Mixed Vehicle Emissions, which is Further Exacerbated with Consumption of a High-Fat Diet

Plasma ox-LDL levels in C57BL/6 mice exposed for 6hr/d, 7 d/wk, for 30 d to either filtered air (FA), 100 $\mu\text{g}/\text{m}^3$ PM of mixed gasoline and diesel engine emissions (MVE) and fed either a low-fat (LF) or high-fat (HF) diet. *p 0.05 compared to LF+FA controls; # p 0.05 compared to HF+FA controls.

Figure 7. Inhalation Exposure to Mixed Vehicle Emissions and Consumption of a High-Fat Diet each Promote an Increase in Cerebral Microvascular LOX-1 Receptor Expression in C57BL/6 Mice

Double immunofluorescence of LOX-1 (red) and vWF (green) in cerebral microvessels from 4 mo old C57BL/6 mice on a low-fat (LF; A–C) or high-fat diet (HF; G–I) exposed filtered air (FA) or on a LF (D–F) or HF diet (J–L) and exposed to MVE at 100 $\mu\text{g}/\text{m}^3$ PM of mixed gasoline and diesel engine emissions for 6hr/d, 7 d/wk, for 30 d (n=3–4 per exposure group; 4 sections each, 3–5 vessels per section). Right panels (yellow; C, F, I, L) are merged figures of left (red; A, D, G, J) and center (green; B, E, H, K) panels for LOX-1 and vWF. Arrows indicate areas of increased endothelial-specific LOX-1 expression (merged fluorescence).

Blue fluorescence is Hoechst stained nuclei. Scale bar = 100 μ m. Results shown are mean $\pm$ SEM. *p < 0.05 compared to LF+FA controls, # p < 0.05 compared to HF+FA.

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Figure 8. Cerebral Microvascular CD-36 Receptor Expression is increased with Mixed Vehicle Emission Exposure in C57BL/6 Mice

Double immunofluorescence of CD36 (green) and vWF (red) in cerebral microvessels from 4 mo old C57BL/6 mice on a low-fat (LF; A–C) or high-fat diet (HF; G–I) exposed filtered air (FA) or on a LF (D–F) or HF diet (J–L) and exposed to MVE at 100 $\mu\text{g}/\text{m}^3$ PM of mixed gasoline and diesel engine emissions for 6hr/d, 7 d/wk, for 30 d (n=3–4 per exposure group; 4 sections each, 3–5 vessels per section). Right panels (yellow; C, F, I, L) are merged figures of left (green; A, D, G, J) and center (red; B, E, H, K) panels for LOX-1 and vWF. Arrows indicate areas of increased endothelial-specific CD-36 expression (merged fluorescence).

Blue fluorescence is Hoechst stained nuclei. Scale bar = 100 μ m. Results shown are mean $\pm$ SEM. *p < 0.05 compared to LF+FA controls, # p < 0.05 compared to HF+FA.

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Figure 9. Inhalation Exposure to Mixed Vehicle Emissions Mediates an increase in Lipid Peroxidation in the Cerebral Vasculature of C57BL/6 Mice

Double immunofluorescence of 4-HNE (red) and vWF (green) in cerebral microvessels from 4 mo old C57BL/6 mice on a low-fat (LF; A–C) or high-fat diet (HF; G–I) exposed filtered air (FA) or on a LF (D–F) or HF diet (J–L) and exposed to MVE at 100 $\mu\text{g}/\text{m}^3$ PM of mixed gasoline and diesel engine emissions for 6hr/d, 7 d/wk, for 30 d (n=3–4 per exposure group; 4 sections each, 3–5 vessels per section). Right panels (yellow; C, F, I, L) are merged figures of left (red; A, D, G, J) and center (green; B, E, H, K) panels for 4-HNE and vWF. Arrows indicate areas of increased endothelial-specific 4-HNE expression (merged fluorescence).

Blue fluorescence is Hoechst stained nuclei. Scale bar = 100 μ m. Results shown are mean $\pm$ SEM. *p < 0.05 compared to LF+FA controls, # p < 0.05 compared to HF+FA.

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Table 1**Animal Body Weights**

Animals	Diet	Exposure	End of Study Weight (g)
C57Bl6 male mice	Low-fat diet	Filtered air	28.7±1.8
C57Bl6 male mice	Low-fat diet ^a	Mixed vehicle exhaust ^b	33.7±2.8
C57Bl6 male mice	High-fat diet	Filtered air	29.3±1.9
C57Bl6 male mice	High-fat diet	Mixed vehicle exhaust	37.2±2.8 *

^aHigh fat diet = TD88137 Custom Research Diet, Harlan Teklad, Madison, WI; 21.2% fat content by weight, 1.5g/kg cholesterol content, beginning 30 days prior to initiation of exposure.

^bMixed vehicle exhaust, 100 µg PM/m³ = mixture of whole gasoline engine exhaust and diesel engine exhaust (30 µg PM/m³ gasoline engine emissions + 70 µg PM/m³ diesel engine) for 6 h/d, 7 d/wk, for a period of 30 d.

* p<0.050 compared to Filtered Air + Low-fat diet controls.