Disrupted Nitric Oxide Metabolism from Type II Diabetes and Acute Exposure to Particulate Air Pollution

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Abstract

Type II diabetes is an established cause of vascular impairment. Particulate air pollution is known to exacerbate cardiovascular and respiratory conditions, particularly in susceptible populations. This study set out to determine the impact of exposure to traffic pollution, with and without particle filtration, on vascular endothelial function in Type II diabetes. Endothelial production of nitric oxide (NO) has previously been linked to vascular health. Reactive hyperemia induces a significant increase in plasma nitrite, the proximal metabolite of NO, in healthy subjects, while diabetics have a lower and more variable level of response. Twenty type II diabetics and 20 controls (ages 46–70 years) were taken on a 1.5hr roadway traffic air pollution exposure as passengers. We analyzed plasma nitrite, as a measure of vascular function, using forearm ischemia to elicit a reactive hyperemic response before and after exposure to one ride with and one without filtration of the particle components of pollution. Control subjects displayed a significant increase in plasma nitrite levels during reactive hyperemia. This response was no longer present following exposure to traffic air pollution, but did not vary with whether or not the particle phase was filtered out. Diabetics did not display an increase in nitrite levels following reactive hyperemia. This response was not altered following pollution exposure. These data suggest that components of acute traffic pollution exposure diminish vascular reactivity in non-diabetic individuals. It also confirms that type II diabetics have a preexisting diminished ability to appropriately respond to a vascular challenge, and that traffic pollution exposure does not cause a further measureable acute change in plasma nitrite levels in Type II diabetics.

Introduction

Epidemiological and toxicological studies have amply demonstrated associations between acute and chronic exposure to traffic-related air pollutants (TRAPs) and cardiorespiratory health effects [1]. TRAPs are a dynamic mixture of particles and gaseous compounds that arise from multiple sources. For many individuals exposure to substantial amounts of air pollutants likely occurs during daily commuting or at home when individuals live in close proximity to roads with high traffic volume [2–5]. A case- crossover study found that short term exposures to traffic pollution increased the risk of non-fatal myocardial infarction by 2 fold within a few hours of exposure [6]. Generally, mechanisms for health effects resulting from relatively brief exposures to TRAPs are not well understood. Diesel exhaust, an important contributor to TRAPs, has been found in human studies to cause inflammatory responses, adverse changes in cardiovascular biomarkers, and reversible health effects in response to controlled, acute exposures [7–12]. A small number of studies have investigated real world, acute exposures to TRAPs near roadways. These studies have shown effects on heart rate, respiratory inflammatory responses, adverse effects on lung function and alterations in oxidative stress [13–17].

The relative importance of the various components of the complex TRAPs mixture, as well as the biological pathways by which different TRAPs components may cause adverse health effects, are not well-understood. Exposure to TRAPs, diesel exhaust and particles increases inflammatory responses in animal and human respiratory tracts, in addition to cell systems. Oxidative stress is considered to be a key mechanism in these adverse responses [7, 17–22].

In previous studies, we found that nitrite and nitrate, relatively stable oxidation products of nitric oxide metabolism, were increased in exhaled breath condensate (EBC) among asthmatics after controlled exposure to diesel exhaust [9] and sub acutely following exposures to high levels of ambient pollutants, predominantly from traffic [17]. More recently, we demonstrated a similar effect in exhaled breath condensate immediately following a 1.5-hour highway exposure to TRAP [23]. The effects of TRAPs exposure on vascular oxidative stress have been established, but its impact on the vascular endothelium has yet to be examined.

Type 2 diabetes mellitus (T2DM) is associated with increased risk for acute and chronic cardiovascular disease [24–28]. Studies suggest that a major underlying factor in these pathologies is vascular endothelial dysfunction, recognized as an independent risk factor for cardiovascular events [29, 30].

The metabolically active vascular endothelium regulates blood flow through modulation of vascular smooth muscle tone via production of and response to molecules such as nitric oxide (NO) [31–33]. NO mediates numerous processes including vasodilation, vascular smooth muscle proliferation, and vascular inflammation. Reduced production and bioavailability of NO is a hallmark of endothelial dysfunction [24, 25, 34–36].

An increase in tissue metabolic demands due to physiological or chemical stressors, particularly oxidative stress, normally results in hypoxic vasodilation mediated by NO. NO has a short half-life and is rapidly oxidized into nitrite (NO_2^-) and subsequently into nitrate (NO_3^-) in the vasculature. Nitrite, although shorter-lived, is considered a more meaningful reflection of NO production, as it reflects NO produced by the endothelial cell lining of blood vessels, while nitrates in circulation may originate from alternate sources such as food constituents and preservatives [37].

The ability of the vasculature to respond appropriately to stress can be measured through the response to a brief local ischemic challenge known as reactive hyperemia. Typically termed brachial artery flow-mediated dilation (BAFMD) when ultrasound measurements of arterial diameter are made, other investigators have directly measured NO metabolites in blood as a

relevant endpoint [24, 37]. In healthy vascular tissue the response to occlusion is characterized by a rapid increase in both vessel size and blood flow mediated by metabolic alterations including a rapid increase in production of NO [24, 38]. Diabetics and those with cardiovascular disease (CVD) typically show a decreased ability to appropriately respond to ischemic and other stressors, termed endothelial dysfunction [39].

Previous studies have shown that adults with T2DM have reduced flow-mediated dilation [38, 40] on days with higher ambient pollution, and also have acutely decreased high frequency heart rate variability from roadway pollution [16]. Healthy adults had altered brachial artery diameter [41, 42] and endothelial function [43] after experimental exposures to concentrated ambient particulate matter (PM) or diesel exhaust.

We hypothesized that 1.5-hr rides in a passenger vehicle in morning rush-hour traffic on a major highway would cause acute increases in oxidative stress in the peripheral vasculature manifested by impaired generation of NO, observed as its metabolite nitrite, in response to a standard ischemic stimulus, and that the PM component of TRAP is responsible for these effects. We further hypothesized that the impairment in generation of NO would be greater in subjects with T2DM compared to healthy control subjects.

Materials and Methods

Study Population and Ethics Statement

Twenty subjects with T2DM aged 46 to 70 years and 20 non-diabetics were recruited from the community and from endocrinology clinics at RWJMS. All participants were medically cleared for the study with the following exclusions: pregnant and breast feeding women, unstable kidney/liver disease, unstable coronary artery disease or arrhythmia, peripheral arterial disease, uncontrolled hypertension, asthma, other pulmonary disease, cigarette smoking, or the inability to ride comfortably in a car for 2 hours. Prior to participation all subjects signed an informed consent approved by the UMDNJ (now Rutgers University) Internal Review Board.

Exposure Protocol

Treating physicians adjusted diabetes medications prior to each study visit to account for the lack of breakfast on study mornings (fasting after midnight) and blood glucose was monitored before and after the ride to avoid hypoglycemia. Subjects began pre exposure testing at approximately 7am with all study testing completed within a 4-hr window. Subjects lived within 20 km of the Environmental and Occupational Health Sciences Institute (EOHSI) and were encouraged to avoid highways when driving to reach the testing facility on days of their exposures. Exposure to weekday rush-hour traffic pollution occurred in a Ford Taurus on the New Jersey Turnpike (NJTPK). Each subject rode in the rear seat of the passenger vehicle, which was driven by professional drivers. The car rides were approximately 1.5 hours in length and approximately 79 miles round trip primarily in the diesel-enriched NJTPK truck/bus lanes (6 miles on local roads, 18 miles on Interstate 287, and 55 miles on the NJTPK). Windows were maintained closed but external air intakes were open (non-recirculation mode) with the fan on medium setting. Each subject participated in two randomly ordered exposure sessions at least one week apart. A powered air purifying respirator (PAPR) was utilized for both car rides. Exposures were either filtered (PAPR with HEPA filter in the respirator housing) or unfiltered (PAPR without HEPA filter). Subjects and technicians were blinded to presence or absence of the filter, which was randomized between rides.

Vascular Reactivity

A five minute arm occlusion consisted of inflation of a blood pressure cuff to 50mmHg above systolic blood pressure. The cuff was placed on the biceps approximately 2–3 cm superior to the olecranon process allowing access to the median cubital vein for phlebotomy.

Blood Collection

Blood draws were taken prior to and immediately following TRAP exposure from the median cubital vein in the test arm. Samples were collected into a BD plastic whole blood tube with spray-coated K_2EDTA and immediately placed on ice. Draws were completed at baseline and 60 seconds following release of the upper arm occlusion. Samples were fractionated by centrifugation (13000 rpm for 20 minutes). The plasma was drawn off and aliquoted into three 1.5mL plastic eppendorf tubes. One plasma aliquot was placed on ice and analyzed for nitrite within 4 hours of sampling. The remaining plasma was frozen and stored at -80°C for future biochemical analyses.

Measurement of plasma nitrite

The NO metabolite, nitrite (NO_2^-), was analyzed using an ozone-based chemiluminescent technique with a Sievers Nitric Oxide Analyzer 280. NO_2^- analysis was completed at room temperature utilizing potassium iodide and acetic acid as reductants and argon as an inert carrier gas. This reaction specifically reduces NO_2^- into NO, but cannot reduce higher oxides of nitrogen (like nitrate (NO_3^-)) to NO. Standards for the assays were prepared by serial dilutions of sodium nitrite (Sigma Aldrich) with ultrapure water. Volumes of 10 μL of standards and 40 μL of sample were injected into the reaction vessel to give adequate reaction signal. Integrations of resulting peaks were calculated in the Liquid program to produce areas under the curve (AUCs) from standards and samples. Sample concentrations were calculated utilizing a formulated regression line from known standard concentrations.

Exposure Measurements

We made the following measurements with inlets at the subject's breathing zone inside the respirator (PAPR) face piece and separately near the center of the vehicle cabin: mass concentrations of PM with median cut-point of 2.5 μm ($PM_{2.5}$) at 1-min intervals using TSI SidePak model AM510 aerosol monitors (TSI, Inc, Shoreview, MN) with the calibration factor set at 0.32 (based on collocated gravimetric analysis of local ambient particles); number concentrations of particles with aerodynamic diameter from 0.01 to 1.0 μm at 1-min intervals using a condensation particle counter, TSI model 3007 (TSI, Inc.). Pollutants measured in the vehicle cabin only were: back carbon using an AE-51 microaethalometer (Aethlabs, San Francisco, CA) at flow rate of 100 ml/min and 1-min time base; nitrogen dioxide (NO_2) collected on triethanolamine-coated Sep-Pak cartridges (Waters, Corp, Millford, MA), analyzed using HPLC-UV as previously reported [44]; carbon monoxide (CO) and air temperature were measured continuously with a Langan T15v monitor (Langan, Inc, San Francisco, CA); and humidity with a HOBO 8 Pro Series monitor (Onset, Bourne, MA)

Statistical Analysis

Demographic characteristics between diabetics and controls and pollutant concentrations within the PAPR were examined with t-tests or chi-square tests, as appropriate. Histograms were examined for normality and outliers. Geometric means and 95% confidence intervals were calculated for the raw nitrite values as well as the change due to cuff at each time point.

Linear models with random effects to account for repeated measures were used to assess the main effects of diabetic status and exposure as well as the interaction of diabetic status and exposure on the nitrite measures. Log transformations were taken of nitrite values in order to stabilize the variance and ensure that the response variables were normally distributed. When examining exposure, the pre-exposure measure was included as a covariate (accounting for regression to the mean) and a random intercept for subject controlled for correlation within subject due to repeated exposure sessions. Ratios were calculated of changes due to the filtered versus unfiltered conditions, for example, as $(\exp(\beta)-1) \times 100\%$ where β was the regression coefficient associated with the dummy variable representing exposure ($= 1$ when unfiltered and $= 0$ for filtered). Confidence intervals were calculated similarly using the endpoints of the 95% confidence intervals for β.

Results

Subject Characteristics

Subject characteristics are summarized in Table 1. The control group had significantly lower systolic blood pressure (SBP), body mass index and weight than the diabetic group. Age, racial groupings, and diastolic blood pressure (DBP) did not differ between the diabetic and control groups. Diabetic subjects had Hb_{A1c} levels ranging from 7.0 to 11.2. Diabetics were prescribed a greater number of medications than controls for health issues beyond glycemic control, although 13 of the 20 controls had treated medical conditions such as hypertension or lipid abnormalities. The control group was comprised of a female majority (55%), while the diabetic cohort consisted of a male majority (75%). No subject had a blood glucose level below 90 mg/dl before or following a ride.

Exposure Characteristics

The pollutant measurements show no significant differences inside the vehicle cabin between filtered and unfiltered rides as the filter only affected the air directly inhaled by the subject through the PAPR (Table 2). However, inside the PAPR face piece mean $PM_{2.5}$ concentration was 83.9% lower ($p < 0.0001$) and mean particle number concentration was 99.9% lower ($p < 0.0001$) during HEPA-filtered rides compared to unfiltered rides.

Car ride effect on resting vascular nitrite concentrations

We examined whether car ride pollutant exposures would reduce vascular NO immediately after a ride, either at rest or post-ischemia (hyperemia). The control group's resting (pre-ischemia) plasma nitrite concentrations were found to be lower following unfiltered rides than filtered rides (18.3%, $p = 0.058$) (Fig 1A and Table 3). Diabetics showed no appreciable change from pre-ride values in pre-cuff nitrites following the ride (Fig 1B and Table 3).

Vascular nitrite response to ischemic challenge

Prior to the ride there was greater variability in resting nitrite levels within the diabetic group, as well as in their response to ischemic challenge, when compared to controls (Fig 2A vs. 2B). Geometric mean resting plasma nitrite levels were 26% higher in diabetic subjects than control subjects (Table 3).

Consistent with a healthy vascular response, controls showed a statistically significant 15.9% increase in plasma nitrite levels following ischemia ($p = 0.017$) prior to the car rides (Fig 2A and Table 3). In contrast, consistent with chronic endothelial dysfunction, nitrite concentrations following ischemia within the diabetic group did not show a significant increase (Fig 2B and Table 3).

Table 1. Subject characteristics (n = 40).

	Mean±SD	
	Controls	Diabetics
Age(years)	57.4±7.0	61.0±7.9
SBP(mmHg)[a]	125.7 ±13.5	137.5 ±12.9
DBP(mmHg)	76.1±9.6	76.7±9.2
BMI(kg/m^2)[a]	27.1±6.2	33.2±6.3
Hb$_{A1c}$(%)	N/A	8.6±1.3
Weight(kg)[a]	76.0±18.5	93.8±19.9
	Number of subjects	
SexWomen Men	119	515
Race		
Caucasian	15	16
Black	3	3
Other	2	1
Medications		
Diabetics medication		
Insulin	0	12
Metformin	0	12
Sulfonylureas	0	12
Dipeptidyl peptidase-4	0	2
Acarbose	0	1
Glitazone	0	1
Other medication		
Lipid lowering(statins)	4	18
Other(nonstatins)	5	4
Platelet aggregation inhibitors	3	14
Angiotensin-converting enzyme (ACE)inhibitors	4	10
Calcium channel blockers	1	6
Angiotensin II receptor blockers	2	6
Beta blockersHormone replacementMen (testosterone)Women (estrogen)	211	610

Systolic (SBP) and diastolic blood pressures (DBP) were reported as a mean of triplicate measurements following the rest period. Body mass index (BMI) was calculated from weight and height(not reported) after measurement at the facility. Medications and hemoglobin A1c (Hb$_{A1c}$) levels in diabetics were reported from medical records Hb$_{A1c}$ was not measured in control subjects and medications were self-reported.
[a]$p \leq 0.05$ Controls were significantly lower when compared to diabetics.

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Car ride effect on vascular response to ischemic challenge

Following the car rides the significant nitrite increase from ischemia seen in controls prior to rides (15.9%, p = 0.017)(Fig 1A and Table 3) is no longer elicited in the unfiltered nor filtered condition, indicating a relative decrease in post-ischemia nitrite (and NO production) associated with the ride(5.0%, ns)(Fig 3A, Table 3, S1A and S1B Fig). There were no significant differences between filtered and unfiltered conditions within the diabetic group as they showed no significant change in response to ischemia following the ride (Fig 3B, S1C and S1D Fig).

Table 2. Characteristics of filtered and unfiltered car rides.

	Mean±SD	
	Filtered Ride(n = 40)	Unfiltered Ride(n = 40)
Particulate matter<2.5µm(µg/m³)CabinPAPR		
	15.4±4.9	15.0±5.4
	1.68±0.88[a]	10.4±5.64
Carbon monoxide	1.06±0.64	1.06±0.63
Nitrogen dioxide (ppb)	15.3±8.3	13.9±6.9
In vehicle temperature (°C)	23.8±2.4	24.1±3.0
Condensation particle count(#/cm³)CabinPAPR		
	$4.3 \times 10^4 \pm 1.6 \times 10^4$	$4.7 \times 10^5 \pm 2.2 \times 10^5$
	49.8±83.7[b]	$4.2 \times 10^4 \pm 2.0 \times 10^4$
Black carbon(µg/m³)	6.3±3.7	5.8±3.6
Relative humidity(%)	25.9±10.4	26.2±9.93

The mean of all rides and standard deviations (SD) are shown. Temperature and CO were monitored with a Langan T15v monitor, while relative humidity was measured with a HOBO 8 Pro Series monitor. Nitrogen dioxide was collected on calibrated triethanolamine-coated Sep-Pak cartridges with an SKC model 224-XR air pump and analyzed by high pressure liquid chromatography. Particulate matter concentrations (TSI SidePak model AM510 aerosol monitor) and condensation particle counts (condensation particle counter (CPC) TSI model 3007) were collected at 1-minute intervals, both within the vehicle cabin and within the PAPR. The levels were significantly lower in the PAPR during filtered rides due to HEPA filtration as indicated by [a] and [b].

[a]$p \leq 0.0001$ compared to unfiltered ride.

[b]$p \leq 0.0001$ compared to unfiltered ride.

Discussion

This study set out to determine the impact of both type II diabetes and an acute exposure to TRAP on vascular reactivity through nitric oxide and its proximal metabolite, plasma nitrite. The findings of this study included (i) higher resting nitrite levels in diabetics than controls; (ii) a borderline significant decrease among control subjects in resting nitrite following the
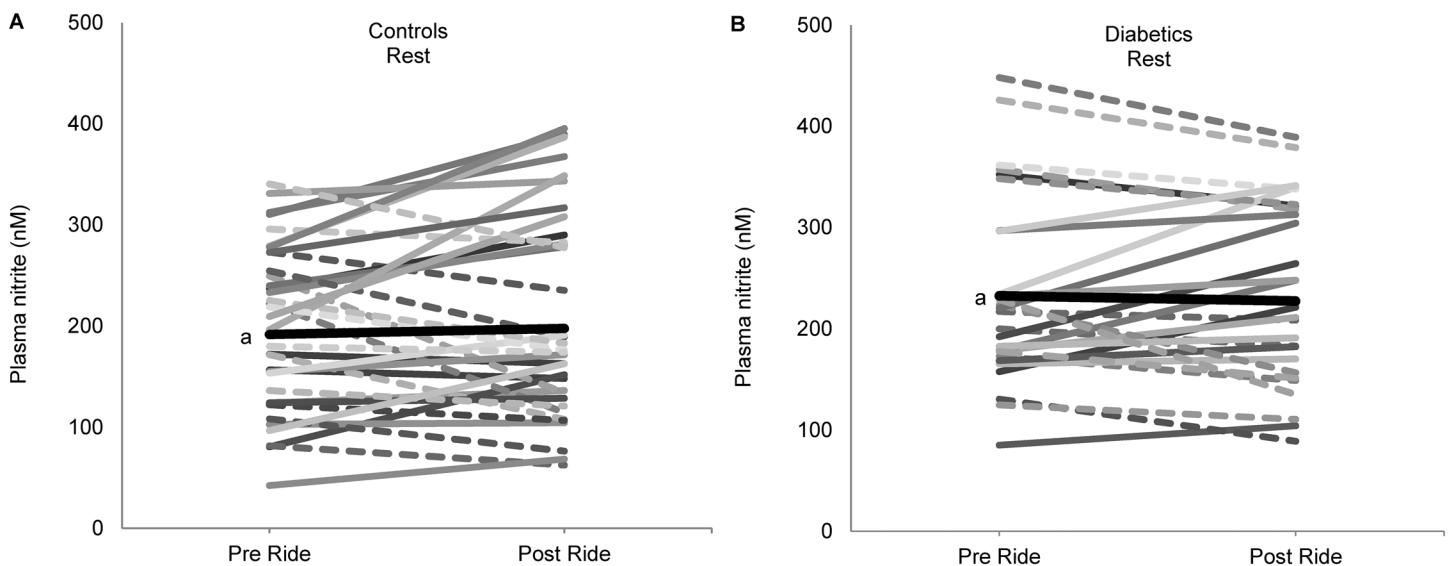

Fig 1. Acute ride (air pollution) effects on resting vascular nitrite levels (pre-ischemia). (A) Controls resting nitrite levels pre and post ride. (B) Diabetic resting nitrite levels pre and post ride. Dash lines indicate decreasing nitrite values, while solid lines indicate increasing values from pre to post ride. Unfiltered and filtered rides are combined. [a]denotes mean values (thick black line).

Table 3. Effect of ischemic challenge and air pollution on vascular nitrite concentrations.

	Diabetes		Control		Ratio of Diabetes versus Control	
Pre Car Ride[a]	*GeometricMean*	*95%CI(p-value)*	*GeometricMean*	*95%CI(p-value)*	*Ratio*[5]	*95%CI(p-value)****
Rest(nM)[1]	230.1	191.2,276.8	182.6	155.6,214.1	1.26	0.99,1.61(p = .0623)***
	Ratio	*95%CI*	*Ratio*	*95%CI*	*Ratio*	*95%CI*
Reactivehyperemia[2]	1.02	0.89,1.17(p = .7991)*	1.16	1.03,1.31(p = .0170)*	0.87	0.73,1.06(p = .1584)***
Car Ride Effect	*Ratio*	*95%CI*	*Ratio*	*95%CI*	*Ratio*	*95%CI*
Rest[3]	0.97	0.79,1.20(p = .7654)**	0.82	0.66,1.01(p = .0580)**	1.19	0.89,1.60(p = .2342)***
Reactivehyperemia[4]	1.03	0.87,1.22(p = .6800)	1.05	0.90,1.23(p = .5041)	0.99	0.81,1.22(p = .9398) ***

[1]Pre-ischemia (Rest) nitrite levels were higher in diabetics (n = 15) than control subjects(230.1 vs. 186.2nM).

[2]The reactive hyperemic response caused a statistically significant increase in plasma nitrite levels in controls before the car ride (n = 20) but not those with diabetes. The car ride (exposure) effect is represented by the ratio of geometric means/ratios of the outcomes post-car ride from unfiltered to filtered rides, adjusting for pre-exposure levels.

[3]Diabetes status (diabetics (n = 15) vs controls (n = 20)) does not modify effects of exposure (unfiltered vs filtered rides).

[4]Furthermore, diabetes status did not modify the effects of exposure on vascular nitrite response during hyperemia (controls (n = 19) and diabetics (n = 15)).

[5]In the last 2 columns ratios of the measures for diabetes versus controls along with percent Confidence Intervals (CI) and p-values are presented. A value of one indicates no difference between people with different disease status

[a] When examining the exposure effect, baseline values are included as covariates in the linear models rather than using raw differences between post- and pre-exposure levels as the outcome. By including the baseline value as a covariate, we allow for regression to the mean. This maintains reliability of the results in the case that there may be exposures en route to the session that influences the resting levels.

*p-value testing impact of reactive hyperemia on plasma nitrite levels

**p-value testing effect of exposure on either plasma nitrite levels or impact of reactive hyperemia

***p-value testing differences between diabetes and control.

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unfiltered car ride as compared to resting nitrites after the filtered ride; (iii) increased nitrites in controls but not diabetics during reactive hyperemia prior to exposure and abolition of this increase following acute TRAP exposure.

Resting plasma nitrite levels

Our findings showed baseline nitrite levels in diabetics to be substantially elevated compared to controls. This is consistent with a previous study that showed subjects with endothelial dysfunction to have increased baseline vascular nitrites [45]. This increase could indicate that diabetics may tonically produce more NO to compensate for varying levels of vascular dysfunction (i.e. damaged endothelial cells) or reduced bioavailability of NO due to accelerated consumption by increased levels of circulating ROS.

Subjects with diabetes showed a small decrease in baseline nitrite levels following the unfiltered rides as compared to filtered rides, while controls displayed a more substantial, borderline significant, decrease. It is possible that TRAP may have a greater impact on the baseline level of NO in healthy vascular systems than on those with disease-related endothelial dysfunction and consequently a pre-existing increase in NO production.

Plasma nitrite response to reactive hyperemia

Following reactive hyperemia but prior to the car ride, control subjects showed a significant increase in nitrite levels, while this response was blunted in diabetics. Allen showed a similar insufficient response in T2DM subjects following a graded exercise test (global reactive hyperemia inducer). It was anticipated that diabetics would have this limited ability to respond
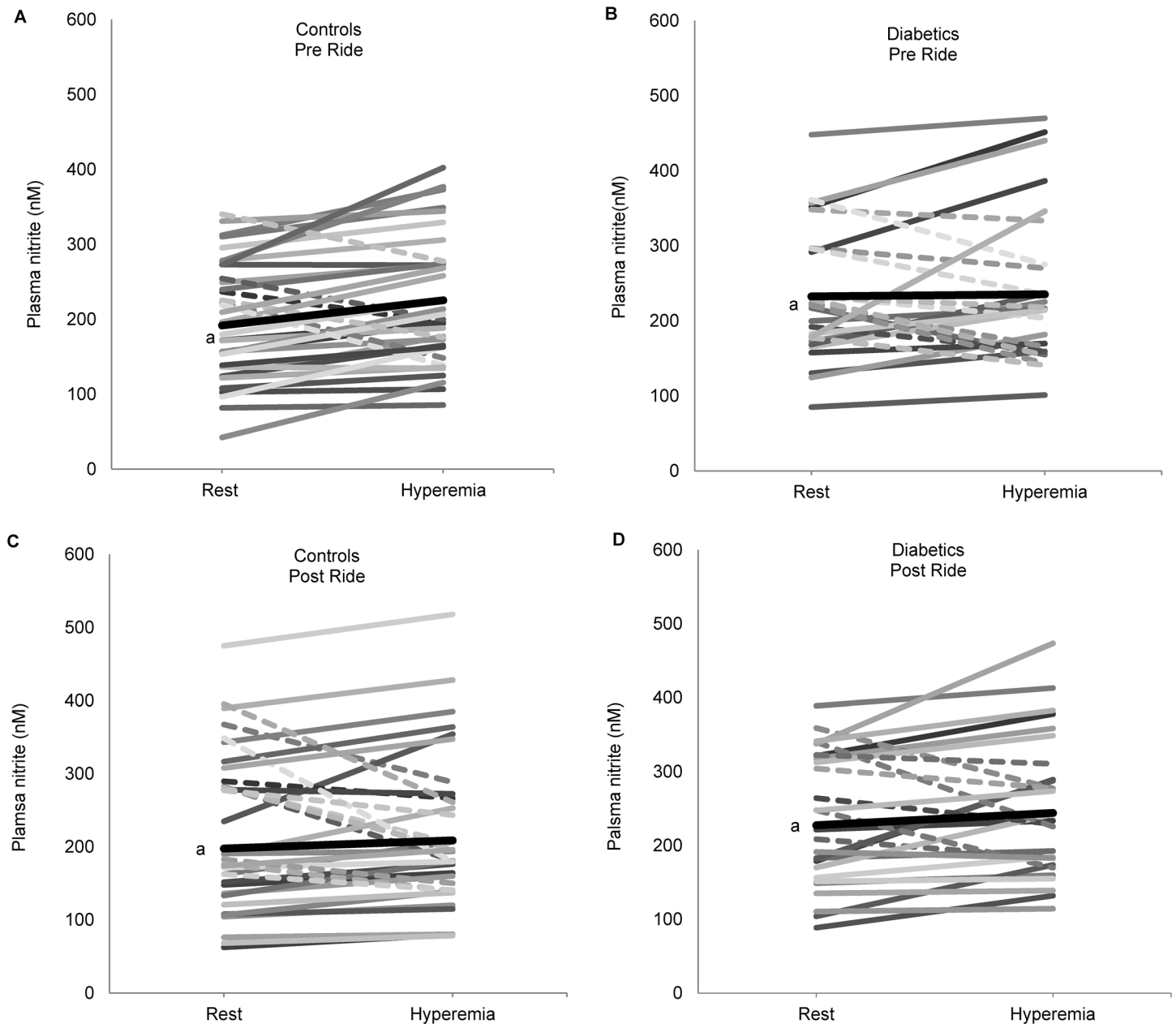
Fig 2. Vascular nitrite concentrations at rest and following ischemia in control and diabetic subjects. Blood draws were taken at rest and 60sec following release (hyperemia) of the five minute upper arm occlusion (50mmHg above SBP) before and following the ride. Dash lines indicate decreasing nitrite values, while solid lines indicate increasing values. Unfiltered and filtered rides are combined (A)Nitrite concentration (nM) changes in control subjects before the ride(n = 40); (B) Nitrite concentration (nM) changes in diabetic subjects before the ride(n = 30); (C) Nitrite concentration (nM) changes in control subjects following the ride(n = 30); (D) Nitrite concentration (nM) changes in diabetic subjects following the ride(n = 25). [a]denotes mean values (thick black line).

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effectively to stimuli, as their vascular systems may be compromised by chronically higher levels of oxidative stress and elevated NO scavenging. Additionally, a study by O'Neill showed diabetic subjects to have lower flow-mediated vascular reactivity than those subjects considered at risk for diabetes. This physiological response is consistent with the lack of nitrite response to reactive hyperemic stimulation among diabetics in our study. As nitrite represents a proximal
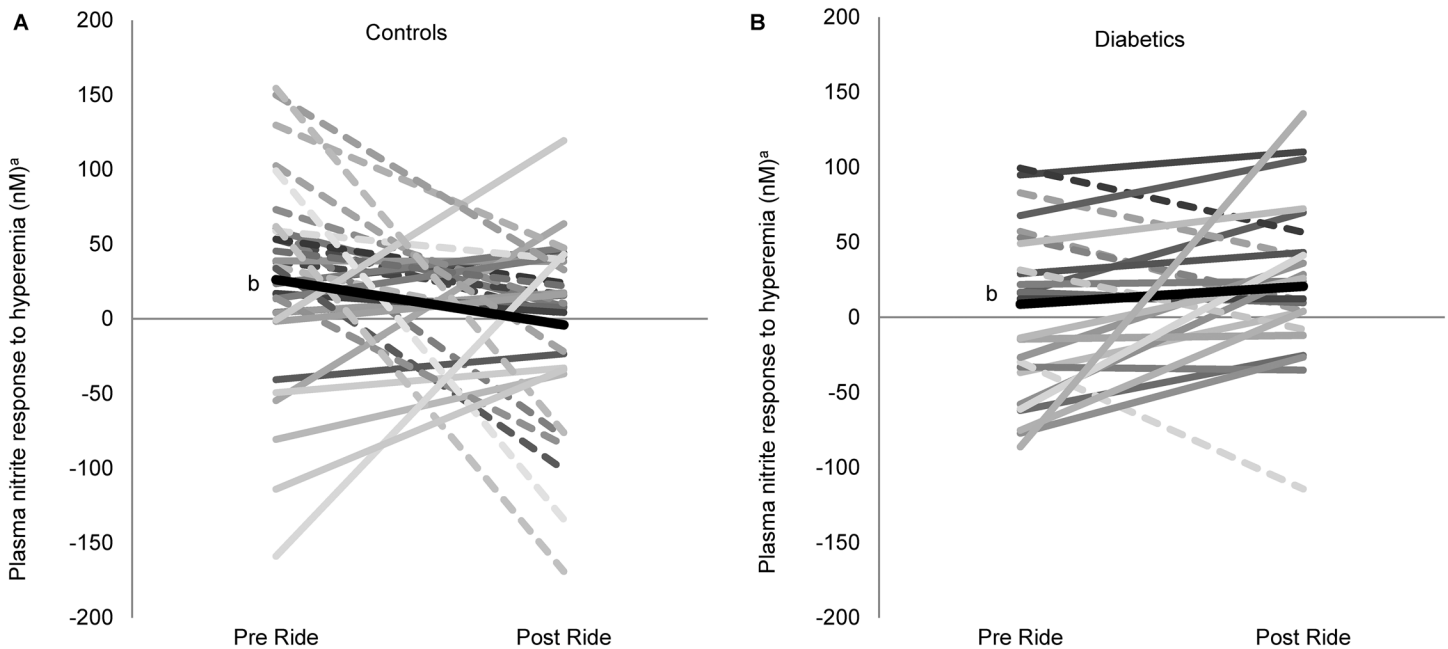
Fig 3. Air pollution exposure effects on vascular nitrite response to reactive hyperemia in controls and diabetics. A) Control subjects' change in plasma nitrite response to ischemia (resting nitrites subtracted from reactive hyperemia nitrites)before (pre ride) and following (post ride) exposure (n = 34). Mean changes in nitrite values (pre ride: 26.1nM; post ride:-4.1nM). B) Diabetic subjects' change in plasma nitrite response to ischemia before and following exposure (n = 25). Mean change in nitrite values (pre ride: 8.7nM; post ride: 20.6nM) Dash lines indicate decreasing nitrite values, while solid lines indicate increasing values. Unfiltered and filtered rides are combined. [a]denotes (pre ischemia (resting) nitrite level) subtracted from (post ischemia (reactive hyperemia)nitrite level). [b]denotes mean values (thick black line).

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measure of eNOS function these results are consistent with reduced eNOS function as the molecular mechanism of this physiological response.

Air pollution impact on reactive hyperemia

Our diabetic subjects displayed no change from their blunted pre-exposure reactive hyperemia response following the car ride. Their decreased responses during reactive hyperemia prior to exposure may indicate an already compromised vascular system, as discussed above. A recent study by Zanobetti (2014) showed no association between decreases in BAFMD and increases in ambient particulate pollution using a 5 day integrated sample in type II diabetics [46]. These data suggest that acute pollution exposure may not have a measureable impact on vascular response in those with pre-existing endothelial dysfunction.

The significant increase in plasma nitrite within the control group following reactive hyperemia is no longer present after exposure to TRAP (neither filtered nor unfiltered). This biochemical response is consistent with the observations of a study by Brook who found significant brachial artery vasoconstriction in a healthy population following acute exposure to a mixture of concentrated ambient fine particles and ozone [41]. The absence of an effect of particle filtration was not consistent with our recent studies of exhaled breath condensate nitrite [23] but is not inconsistent with human and animal studies of acute and chronic TRAP effects on vascular changes, where PM alone was not responsible for observed effects [47, 48]. These vascular responses are also in line with other previous animal studies, including a study by Ikeda (1995) that displayed increased vasoconstriction and inhibited dilation in rat aorta when exposed to diesel exhaust particles(DEP) or motorcycle exhaust [49]. The DEP response has been found to be related to NO-mediated dilators such as EDNO and NO donor drugs. These

reactions may be attenuated by treatment with antioxidants such as SOD, thereby connecting the response with generation of ROS [50]. PM exposures done on healthy animals, hypertensive and atherosclerotic models have shown similar results. Single bolus instillations and prolonged inhalation studies have shown that these responses persist from several hours up to 24 hours. DEP and urban PM have been found to significantly raise blood pressure in animal models of hypertension (induced and genetic) and atherosclerosis [51–56]

Location and duration of pollution exposure

In contrast to our findings, the diabetic subjects of the O'Neill study had altered vascular reactivity in association with multiple day increases in ambient air pollution, while the at-risk group displayed no change in response to air pollution. Since our diabetic subjects appeared to have severely comprised vascular NO responses prior to exposure, it is plausible that the impact of a relatively brief 2 hour car ride may not have been potent enough to further alter NO production in non-responsive systems. There is also a distinct possibility that physiological changes in brachial artery diameter occur even in the absence of significantly altered NO levels (i.e. due to nervous system alterations [57–59]).

The nature and length of exposure time may also play an important role in eliciting vascular changes. Our exposures were limited to 2 hours on highly trafficked roadways, while the O'Neill exposure metrics were average ambient urban pollution over 1–6 days in the Boston area. It is possible that a longer traffic exposure, repeated exposures over several days, or a later follow-up time may be necessary for impact on vascular reactivity. In addition, O'Neill also discussed sensitivity of diabetic subjects to pollution effects on vascular function as a possible reason for their findings; however, using the biochemical measure of nitrite, we found that diabetics appeared to be less sensitive than their non-diabetic counterparts to exposure to TRAP.

Health status impact of control group

Our control population responded with more modest nitrite increases during reactive hyperemia than those previously reported. This may be due to their more advanced age, elevated BMIs, and co-morbid conditions (other than diabetes) compared with subjects in previous reports [45]. The control subjects also had a significantly greater number of women than the diabetic group, which could account for a sex-related variation between the 2 groups. Additionally, medication differences between the two groups may alter vascular reactivity and NO production. For instance, consistent with their cardiovascular risk profile, 90% of the diabetic subjects were taking statins, in comparison to 20% of the controls. The marginal increase in diabetic resting plasma nitrite compared to controls could potentially be due to antioxidant effects of statins, in addition to potential compensatory mechanisms [9, 12, 60].

Conclusion

This study demonstrated the adverse vascular effects of a two hour traffic exposure in an older non-diabetic population. The most pronounced change was seen in the decreased vascular reactivity response in non-diabetics following a car ride associated with increased TRAP inhalation. Before exposure, type II diabetic subjects demonstrated a decreased ability to respond appropriately to a vascular stressor and this was not altered by the TRAP exposure. Since the diabetic subjects show pre-existing compromise of their vascular systems, the extent to which a TRAP exposure affected them is unknown. The decrease of resting nitrite levels after TRAP pollution inhalation, and lowered vascular reactivity in non-diabetics, supports the proposition that real-world TRAP acutely increases endothelial dysfunction and alters NO metabolism

leading to inadequate vascular responses to hypoxia. These results further validate the previously observed detrimental effects of TRAP exposure on vascular reactivity in controlled diesel exposure studies, and provides a direct mechanism for the epidemiological observations of MI associated with acute traffic exposure [43, 61–63].

Supporting Information

S1 Fig. Air pollution exposure effects due to filtered and unfiltered ride on vascular nitrite response to reactive hyperemia in controls and diabetics. (A)Control subjects' change in plasma nitrite response to ischemia (resting nitrites subtracted from reactive hyperemia nitrites)before (pre ride) and following (post ride) exposure to filtered ride (n = 16). Mean changes in nitrite values (pre ride: 14.9nM; post ride: -2.9nM). (B) Control subjects' change in plasma nitrite response to ischemia before and following exposure to unfiltered ride (n = 17). Mean changes in nitrite values (pre ride: 36.7nM; post ride: 3.8nM). (C)Diabetic subjects' change in plasma nitrite response to ischemia before and following filtered ride (n = 14). Mean change in nitrite values (pre ride: 13.0nM; post ride: 30.3nM) (D)Diabetic subjects' change in plasma nitrite response to ischemia before and following unfiltered ride (n = 13). Mean change in nitrite values (pre ride: 20.8nM; post ride: 43.7nM). [a]denotes (pre ischemia (resting) nitrite level) subtracted from (post ischemia (reactive hyperemia)nitrite level). [b]denotes mean values (thick black line).
(TIF)

Acknowledgments

Author Contributions

Conceived and designed the experiments: HK AG RL. Performed the experiments: AP KKM CC LA SS SL. Analyzed the data: AP AG HK POS RL. Contributed reagents/materials/analysis tools: AG HK POS ZHF. Wrote the paper: AP HK AG RL. Recruited diabetic subjects and gave input on health related issues: LA SL SS.

References

1. Laumbach RJ, Kipen HM. Respiratory health effects of air pollution: update on biomass smoke and traffic pollution. The Journal of allergy and clinical immunology. 2012; 129(1):3–11; quiz 2–3. doi: 10.1016/j.jaci.2011.11.021 PMID: 22196520; PubMed Central PMCID: PMC3272333.

2. Zhu Y, Eiguren-Fernandez A, Hinds WC, Miguel AH. In-cabin commuter exposure to ultrafine particles on Los Angeles freeways. Environmental science & technology. 2007; 41(7):2138–45. Epub 2007/04/19. PMID: 17438754.

3. Zuurbier M, Hoek G, Oldenwening M, Lenters V, Meliefste K, van den Hazel P, et al. Commuters' exposure to particulate matter air pollution is affected by mode of transport, fuel type, and route. Environmental health perspectives. 2010; 118(6):783–9. doi: 10.1289/ehp.0901622 PMID: 20185385; PubMed Central PMCID: PMC2898854.

4. Zhu Y, Fung DC, Kennedy N, Hinds WC, Eiguren-Fernandez A. Measurements of ultrafine particles and other vehicular pollutants inside a mobile exposure system on Los Angeles freeways. Journal of the Air & Waste Management Association (1995). 2008; 58(3):424–34. Epub 2008/04/02. PMID: 18376645.

5. Hu S, Paulson SE, Fruin S, Kozawa K, Mara S, Winer AM. Observation of Elevated Air Pollutant Concentrations in a Residential Neighborhood of Los Angeles California Using a Mobile Platform. Atmospheric environment. 2012; 51:311–9. doi: 10.1016/j.atmosenv.2011.12.055 PMID: 23997642; PubMed Central PMCID: PMC3755476.

6. Peters A, von Klot S, Heier M, Trentinaglia I, Hormann A, Wichmann HE, et al. Exposure to traffic and the onset of myocardial infarction. The New England journal of medicine. 2004; 351(17):1721–30. doi: 10.1056/NEJMoa040203 PMID: 15496621.

7. Kipen HM, Gandhi S, Rich DQ, Ohman-Strickland P, Laumbach R, Fan ZH, et al. Acute decreases in proteasome pathway activity after inhalation of fresh diesel exhaust or secondary organic aerosol. Environmental health perspectives. 2011; 119(5):658–63. Epub 2010/12/18. doi: 10.1289/ehp.1002784 PMID: 21163722; PubMed Central PMCID: PMCPMC3094417.

8. Mills NL, Donaldson K, Hadoke PW, Boon NA, MacNee W, Cassee FR, et al. Adverse cardiovascular effects of air pollution. Nature clinical practice Cardiovascular medicine. 2009; 6(1):36–44. Epub 2008/11/26. doi: 10.1038/ncpcardio1399 PMID: 19029991.

9. Salvi SS, Nordenhall C, Blomberg A, Rudell B, Pourazar J, Kelly FJ, et al. Acute exposure to diesel exhaust increases IL-8 and GRO-alpha production in healthy human airways. American journal of respiratory and critical care medicine. 2000; 161(2 Pt 1):550–7. Epub 2000/02/15. doi: 10.1164/ajrccm.161.2.9905052 PMID: 10673199.

10. Lucking AJ, Lundback M, Mills NL, Faratian D, Barath SL, Pourazar J, et al. Diesel exhaust inhalation increases thrombus formation in man. European heart journal. 2008; 29(24):3043–51. Epub 2008/10/28. doi: 10.1093/eurheartj/ehn464 PMID: 18952612.

11. Tabit CE, Chung WB, Hamburg NM, Vita JA. Endothelial dysfunction in diabetes mellitus: molecular mechanisms and clinical implications. Reviews in endocrine & metabolic disorders. 2010; 11(1):61–74. Epub 2010/02/27. doi: 10.1007/s11154-010-9134-4 PMID: 20186491; PubMed Central PMCID: PMCPMC2882637.

12. Nordenhall C, Pourazar J, Blomberg A, Levin JO, Sandstrom T, Adelroth E. Airway inflammation following exposure to diesel exhaust: a study of time kinetics using induced sputum. The European respiratory journal. 2000; 15(6):1046–51. Epub 2000/07/08. PMID: 10885423.

13. McCreanor J, Cullinan P, Nieuwenhuijsen MJ, Stewart-Evans J, Malliarou E, Jarup L, et al. Respiratory effects of exposure to diesel traffic in persons with asthma. The New England journal of medicine. 2007; 357(23):2348–58. Epub 2007/12/07. doi: 10.1056/NEJMoa071535 PMID: 18057337.

14. Langrish JP, Mills NL, Chan JK, Leseman DL, Aitken RJ, Fokkens PH, et al. Beneficial cardiovascular effects of reducing exposure to particulate air pollution with a simple facemask. Particle and fibre toxicology. 2009; 6:8. Epub 2009/03/17. doi: 10.1186/1743-8977-6-8 PMID: 19284642; PubMed Central PMCID: PMCPMC2662779.

15. Adar SD, Gold DR, Coull BA, Schwartz J, Stone PH, Suh H. Focused exposures to airborne traffic particles and heart rate variability in the elderly. Epidemiology (Cambridge, Mass). 2007; 18(1):95–103. Epub 2006/12/07. doi: 10.1097/01.ede.0000249409.81050.46 PMID: 17149139.

16. Laumbach RJ, Rich DQ, Gandhi S, Amorosa L, Schneider S, Zhang J, et al. Acute changes in heart rate variability in subjects with diabetes following a highway traffic exposure. Journal of occupational and environmental medicine / American College of Occupational and Environmental Medicine. 2010; 52(3):324–31. Epub 2010/03/02. doi: 10.1097/JOM.0b013e3181d241fa PMID: 20190650.

17. Huang W, Wang G, Lu SE, Kipen H, Wang Y, Hu M, et al. Inflammatory and oxidative stress responses of healthy young adults to changes in air quality during the Beijing Olympics. American journal of respiratory and critical care medicine. 2012; 186(11):1150–9. doi: 10.1164/rccm.201205-0850OC PMID: 22936356; PubMed Central PMCID: PMC3530204.

18. Wan J, Diaz-Sanchez D. Antioxidant enzyme induction: a new protective approach against the adverse effects of diesel exhaust particles. Inhalation toxicology. 2007; 19 Suppl 1:177–82. Epub 2007/10/04. doi: 10.1080/08958370701496145 PMID: 17886065.

19. Banerjee A, Trueblood MB, Zhang X, Manda KR, Lobo P, Whitefield PD, et al. N-acetylcysteineamide (NACA) prevents inflammation and oxidative stress in animals exposed to diesel engine exhaust. Toxicology letters. 2009; 187(3):187–93. Epub 2009/05/12. doi: 10.1016/j.toxlet.2009.02.022 PMID: 19429263.

20. Zhao H, Ma JK, Barger MW, Mercer RR, Millecchia L, Schwegler-Berry D, et al. Reactive oxygen species- and nitric oxide-mediated lung inflammation and mitochondrial dysfunction in wild-type and iNOS-deficient mice exposed to diesel exhaust particles. Journal of toxicology and environmental health Part A. 2009; 72(8):560–70. Epub 2009/03/10. doi: 10.1080/15287390802706330 PMID: 19267316.

21. Pourazar J, Mudway IS, Samet JM, Helleday R, Blomberg A, Wilson SJ, et al. Diesel exhaust activates redox-sensitive transcription factors and kinases in human airways. American journal of physiology Lung cellular and molecular physiology. 2005; 289(5):L724–30. Epub 2005/03/08. doi: 10.1152/ajplung.00055.2005 PMID: 15749742.

22. Rich DQ, Kipen HM, Huang W, Wang G, Wang Y, Zhu P, et al. Association between changes in air pollution levels during the Beijing Olympics and biomarkers of inflammation and thrombosis in healthy young adults. JAMA: the journal of the American Medical Association. 2012; 307(19):2068–78. doi: 10.1001/jama.2012.3488 PMID: 22665106.

23. Laumbach RJ, Kipen HM, Ko S, Kelly-McNeil K, Cepeda C, Pettit A, et al. A controlled trial of acute effects of human exposure to traffic particles on pulmonary oxidative stress and heart rate variability. Particle and fibre toxicology. 2014; 11:45. doi: 10.1186/s12989-014-0045-5 PMID: 25361615; PubMed Central PMCID: PMC4236446.

24. Allen JD, Cobb FR, Gow AJ. Regional and whole-body markers of nitric oxide production following hyperemic stimuli. Free radical biology & medicine. 2005; 38(9):1164–9. doi: 10.1016/j.freeradbiomed.2004.12.018 PMID: 15808413.

25. Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proceedings of the National Academy of Sciences of the United States of America. 1987; 84(24):9265–9. PMID: 2827174; PubMed Central PMCID: PMC299734.

26. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Borden WB, et al. Heart disease and stroke statistics—2013 update: a report from the American Heart Association. Circulation. 2013; 127(1):e6–e245. doi: 10.1161/CIR.0b013e31828124ad PMID: 23239837.

27. Ness J, Nassimiha D, Feria MI, Aronow WS. Diabetes mellitus in older African-Americans, Hispanics, and whites in an academic hospital-based geriatrics practice. Coronary artery disease. 1999; 10 (5):343–6. PMID: 10421976.

28. Cooper ME, Bonnet F, Oldfield M, Jandeleit-Dahm K. Mechanisms of diabetic vasculopathy: an overview. American journal of hypertension. 2001; 14(5 Pt 1):475–86. PMID: 11368471.

29. De Meyer GR, Herman AG. Vascular endothelial dysfunction. Progress in cardiovascular diseases. 1997; 39(4):325–42. PMID: 9050818.

30. Haller H. Endothelial function. General considerations. Drugs. 1997; 53 Suppl 1:1–10. PMID: 9034750.

31. Mombouli JV. ACE inhibition, endothelial function and coronary artery lesions. Role of kinins and nitric oxide. Drugs. 1997; 54 Suppl 5:12–22. PMID: 9429841.

32. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature. 1980; 288(5789):373–6. PMID: 6253831.

33. Busse R, Fleming I, Hecker M. Signal transduction in endothelium-dependent vasodilatation. European heart journal. 1993; 14 Suppl I:2–9. PMID: 8293777.

34. Kadirvelu A, Han C.K., Lang C.C. Enothelial dysfuntion in cardiovascular diseases. Med Prog. 2002; (May):4–10.

35. Cines DB, Pollak ES, Buck CA, Loscalzo J, Zimmerman GA, McEver RP, et al. Endothelial cells in physiology and in the pathophysiology of vascular disorders. Blood. 1998; 91(10):3527–61. PMID: 9572988.

36. Verma S, Anderson TJ. The ten most commonly asked questions about endothelial function in cardiology. Cardiology in review. 2001; 9(5):250–2. PMID: 11569467.

37. Kelm M, Preik-Steinhoff H, Preik M, Strauer BE. Serum nitrite sensitively reflects endothelial NO formation in human forearm vasculature: evidence for biochemical assessment of the endothelial L-arginine-NO pathway. Cardiovascular research. 1999; 41(3):765–72. PMID: 10435049.

38. Allen JD, Miller EM, Schwark E, Robbins JL, Duscha BD, Annex BH. Plasma nitrite response and arterial reactivity differentiate vascular health and performance. Nitric oxide: biology and chemistry / official journal of the Nitric Oxide Society. 2009; 20(4):231–7. doi: 10.1016/j.niox.2009.01.002 PMID: 19371597; PubMed Central PMCID: PMC2676216.

39. James PE, Lang D, Tufnell-Barret T, Milsom AB, Frenneaux MP. Vasorelaxation by red blood cells and impairment in diabetes: reduced nitric oxide and oxygen delivery by glycated hemoglobin. Circulation research. 2004; 94(7):976–83. doi: 10.1161/01.RES.0000122044.21787.01 PMID: 14963010.

40. O'Neill MS, Veves A, Zanobetti A, Sarnat JA, Gold DR, Economides PA, et al. Diabetes enhances vulnerability to particulate air pollution-associated impairment in vascular reactivity and endothelial function. Circulation. 2005; 111(22):2913–20. doi: 10.1161/CIRCULATIONAHA.104.517110 PMID: 15927967.

41. Brook RD, Brook JR, Urch B, Vincent R, Rajagopalan S, Silverman F. Inhalation of fine particulate air pollution and ozone causes acute arterial vasoconstriction in healthy adults. Circulation. 2002; 105 (13):1534–6. PMID: 11927516.

42. Peretz A, Sullivan JH, Leotta DF, Trenga CA, Sands FN, Allen J, et al. Diesel exhaust inhalation elicits acute vasoconstriction in vivo. Environmental health perspectives. 2008; 116(7):937–42. doi: 10.1289/ehp.11027 PMID: 18629317; PubMed Central PMCID: PMC2453163.

43. Barath S, Mills NL, Lundback M, Tornqvist H, Lucking AJ, Langrish JP, et al. Impaired vascular function after exposure to diesel exhaust generated at urban transient running conditions. Particle and fibre toxicology. 2010; 7:19. doi: 10.1186/1743-8977-7-19 PMID: 20653945; PubMed Central PMCID: PMC2918524.

44. McCreanor J, Cullinan P, Nieuwenhuijsen MJ, Stewart-Evans J, Malliarou E, Jarup L, et al. Respiratory effects of exposure to diesel traffic in persons with asthma. New England Journal of Medicine. 2007; 357(23):2348–58. PMID: 18057337.

45. Rassaf T, Heiss C, Hendgen-Cotta U, Balzer J, Matern S, Kleinbongard P, et al. Plasma nitrite reserve and endothelial function in the human forearm circulation. Free radical biology & medicine. 2006; 41 (2):295–301. doi: 10.1016/j.freeradbiomed.2006.04.006 PMID: 16814110.

46. Zanobetti A, Luttmann-Gibson H, Horton ES, Cohen A, Coull BA, Hoffmann B, et al. Brachial Artery Responses to Ambient Pollution, Temperature, and Humidity in People with Type 2 Diabetes: A Repeated-Measures Study. Environmental health perspectives. 2014. Epub 2014/01/09. doi: 10.1289/ehp.1206136 PMID: 24398072.

47. Hoffmann B, Moebus S, Mohlenkamp S, Stang A, Lehmann N, Dragano N, et al. Residential exposure to traffic is associated with coronary atherosclerosis. Circulation. 2007; 116(5):489–96. doi: 10.1161/CIRCULATIONAHA.107.693622 PMID: 17638927.

48. Campen M, Robertson S, Lund A, Lucero J, McDonald J. Engine exhaust particulate and gas phase contributions to vascular toxicity. Inhalation toxicology. 2014; 26(6):353–60. doi: 10.3109/08958378.2014.897776 PMID: 24730681; PubMed Central PMCID: PMC4137573.

49. Ikeda M, Suzuki M, Watarai K, Sagai M, Tomita T. Impairment of endothelium-dependent relaxation by diesel exhaust particles in rat thoracic aorta. Japanese journal of pharmacology. 1995; 68(2):183–9. PMID: 7563975.

50. Miller MR, Borthwick SJ, Shaw CA, McLean SG, McClure D, Mills NL, et al. Direct impairment of vascular function by diesel exhaust particulate through reduced bioavailability of endothelium-derived nitric oxide induced by superoxide free radicals. Environmental health perspectives. 2009; 117(4):611–6. doi: 10.1289/ehp.0800235 PMID: 19440501; PubMed Central PMCID: PMC2679606.

51. Miller MR, Shaw CA, Langrish JP. From particles to patients: oxidative stress and the cardiovascular effects of air pollution. Future cardiology. 2012; 8(4):577–602. doi: 10.2217/fca.12.43 PMID: 22871197.

52. Xu X, Yavar Z, Verdin M, Ying Z, Mihai G, Kampfrath T, et al. Effect of early particulate air pollution exposure on obesity in mice: role of p47phox. Arteriosclerosis, thrombosis, and vascular biology. 2010; 30(12):2518–27. doi: 10.1161/ATVBAHA.110.215350 PMID: 20864666; PubMed Central PMCID: PMC3065931.

53. Ying Z, Yue P, Xu X, Zhong M, Sun Q, Mikolaj M, et al. Air pollution and cardiac remodeling: a role for RhoA/Rho-kinase. American journal of physiology Heart and circulatory physiology. 2009; 296(5): H1540–50. doi: 10.1152/ajpheart.01270.2008 PMID: 19286943; PubMed Central PMCID: PMC2685340.

54. Cozzi E, Hazarika S, Stallings HW 3rd, Cascio WE, Devlin RB, Lust RM, et al. Ultrafine particulate matter exposure augments ischemia-reperfusion injury in mice. American journal of physiology Heart and circulatory physiology. 2006; 291(2):H894–903. doi: 10.1152/ajpheart.01362.2005 PMID: 16582015.

55. Vesterdal LK, Folkmann JK, Jacobsen NR, Sheykhzade M, Wallin H, Loft S, et al. Pulmonary exposure to carbon black nanoparticles and vascular effects. Particle and fibre toxicology. 2010; 7:33. doi: 10.1186/1743-8977-7-33 PMID: 21054825; PubMed Central PMCID: PMC2991279.

56. Ying Z, Kampfrath T, Thurston G, Farrar B, Lippmann M, Wang A, et al. Ambient particulates alter vascular function through induction of reactive oxygen and nitrogen species. Toxicological sciences: an official journal of the Society of Toxicology. 2009; 111(1):80–8. doi: 10.1093/toxsci/kfp004 PMID: 19182107; PubMed Central PMCID: PMC2726294.

57. Lefrandt JD, Smit AJ, Zeebregts CJ, Gans RO, Hoogenberg KH. Autonomic dysfunction in diabetes: a consequence of cardiovascular damage. Current diabetes reviews. 2010; 6(6):348–58. PMID: 20879972.

58. Manzella D, Paolisso G. Cardiac autonomic activity and Type II diabetes mellitus. Clinical science. 2005; 108(2):93–9. doi: 10.1042/CS20040223 PMID: 15476437.

59. Canani LH, Copstein E, Pecis M, Friedman R, Leitao CB, Azevedo MJ, et al. Cardiovascular autonomic neuropathy in type 2 diabetes mellitus patients with peripheral artery disease. Diabetology & metabolic syndrome. 2013; 5(1):54. doi: 10.1186/1758-5996-5-54 PMID: 24295032; PubMed Central PMCID: PMC3849595.

60. Peters A, von Klot S, Heier M, Trentinaglia I, Hormann A, Wichmann HE, et al. Exposure to traffic and the onset of myocardial infarction. The New England journal of medicine. 2004; 351(17):1721–30. Epub 2004/10/22. doi: 10.1056/NEJMoa040203 PMID: 15496621.

61. Langrish JP, Unosson J, Bosson J, Barath S, Muala A, Blackwell S, et al. Altered nitric oxide bioavailability contributes to diesel exhaust inhalation-induced cardiovascular dysfunction in man. Journal of the American Heart Association. 2013; 2(1):e004309. doi: 10.1161/JAHA.112.004309 PMID: 23525434; PubMed Central PMCID: PMC3603248.

62. Mills NL, Miller MR, Lucking AJ, Beveridge J, Flint L, Boere AJ, et al. Combustion-derived nanoparticulate induces the adverse vascular effects of diesel exhaust inhalation. European heart journal. 2011; 32 (21):2660–71. doi: 10.1093/eurheartj/ehr195 PMID: 21753226; PubMed Central PMCID: PMC3205591.

63. Mills NL, Tornqvist H, Robinson SD, Gonzalez M, Darnley K, MacNee W, et al. Diesel exhaust inhalation causes vascular dysfunction and impaired endogenous fibrinolysis. Circulation. 2005; 112 (25):3930–6. doi: 10.1161/CIRCULATIONAHA.105.588962 PMID: 16365212.