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**EFFECTS OF ENVIRONMENTAL POLLUTION ON ENDOTHELIAL
PROGENITOR CELLS AND VASCULAR REGENERATION**

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A dissertation
Submitted to the Faculty of the
School of Medicine the University of Louisville
in Partial Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy

Department of Physiology and Biophysics
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Louisville, Kentucky

December 2011

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ACKNOWLEDGMENTS

This dissertation is dedicated to my loving husband Mervin and family. My husband has been my rock I could always lean on and is the true love of my life. My family is and always will be the primary inspiration, joy and the reason I have always and will always work so hard to accomplish my goals. No matter how long my hours were or how far away I was, my husband and family always stood by me and my decisions and gave me strength through the hard times. They helped me never forget what I stood for and my morals that makes' me the person that I am. I would also like to thank Dr. Kenneth Schickler for keeping me alive for the past 25 years which wasn't easy. I wasn't always the best patient but he was always the best doctor, thanks to you I have lived to see my educational goals accomplished. I would also like to thank my co-authors and co-workers for contributing to this thesis and my educational training; Jason Hellmann, Petra Haberzettl, Timothy O'Toole, Daniel Conklin, James McCracken, and Mathew Burtke without their hard work and dedication none of this would have been possible. I would also like to acknowledge those who guided my scientific journey along the way, I would like to thank my mentor Dr. Aruni Bhatnagar, his

dedication and pride in his work and lab is truly inspiring and motivational. Also, to Dr. William B. Wead for continued support throughout the years, to those I am truly grateful.

ABTRACT

ACROLEIN SUPPRESSES MOBILIZATION OF ENDOTHELIAL PROGENITOR CELLS

Laura Anne Wheat Nissley

December 16th 2011

Acrolein is a common pollutant present in ambient air, automobile exhaust, and tobacco smoke. Previous studies show that exposure to acrolein increases cardiovascular disease risk. To determine whether acrolein affect cardiovascular regeneration, we investigated the cardiovascular effects of inhaled acrolein. Adult male C57BL/6 mice were exposed to room air or acrolein (0.5, 1 or 5 ppm) for and the endothelial progenitor cells (EPC) level in peripheral blood (PB) was measured by flow cytometry. To examine the proliferative capacity of EPCs, bone marrow cells (BMCs) were cultured on fibronectin-coated slides. Brief exposure to acrolein (5 ppm, 6h) or 4 day (1 ppm) exposure led to a 42% decrease in PB Flk⁺/Sca⁺ cells. BMCs isolated from mice exposed to acrolein for 1d (5 ppm) or 4d (1 ppm) formed more colonies than BMCs of air-exposed mice. Combined VEGF-A (100 µg/kg, ip, 4d) and AMD3100 (5 mg/kg; ip, last day only) treatment increased PB EPCs cells in air-exposed mice but not in acrolein-exposed mice (1 ppm, 4d). These data indicate that circulating EPCs

are a sensitive target of airborne pollutants and that acrolein at concentrations encountered in tobacco smoke suppresses EPC levels in the blood but it increases the proliferative capacity of EPCs in the bone marrow. My thesis is that EPCs are sensitive targets for environmental insults, such as acrolein, thus increasing the risk of developing cardiovascular disease and endothelial dysfunction. Studies here show that the effects of acrolein on EPC after exposure by inhalation (Aim 1), the environmental pollutant acrolein, prevents mobilization of EPCs by cytokine stimulation (Aim 2), evaluated perfusion recovery of hindlimb ischemia after exposure to acrolein inhalation (Aim 3).

The results of these studies support the hypothesis that exposure to environmental pollutant acrolein prevents EPC mobilization.

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CHAPTER I
GENERAL INTRODUCTION
ENVIRONMENTAL POLLUTION AND CIGARETTE SMOKE

The United States and other industrialized countries face an increasing amount of air pollution and related health concerns. The World Health Organization (WHO) data base has estimated that 1.5 billion people in the world are exposed to dangerously high levels of pollution on a daily basis¹. WHO also estimates 3 million deaths are attributed to outdoor air pollution and 1.6 million deaths are attributed to indoor-air pollution². The earth's atmosphere itself is a complex mixture of gases, changing in composition with each atmospheric layer. However, when defining environmental pollution the two layers of concern are the troposphere and stratosphere. The troposphere is the lowest portion of the earth's atmosphere, it contains approximately 80% of the total atmosphere mass and 99% of its water vapor and aerosols and the ozone layer is located within the stratosphere.

When defining pollution Webster dictionary simply states, "*pollution is the introduction of contaminants into a natural environment that causes instability, disorder, harm or discomfort to the ecosystem i.e. physical systems*

or living organisms". The EPA in conjunction with the Commission for Environmental Cooperation estimates each year the United States alone spews 3 million tons of toxic chemicals into the air, water and land ³. There are also naturally occurring sources of pollution such as volcanic eruptions, pollen, mold, sulfur release from oceans and forest fires ⁴⁻⁸. For the most part, pollution is man-made, has many sources and has escalated since the dawn of the industrial revolution. However, pollution isn't just a plague of modern society even in prehistoric time man was exposed to pollution. Scientists have found signs of indoor pollution on cave walls in the form of soot that resulted from building fires for heating and cooking with no or little ventilation ⁹. Modern pollution can still be attributed to wood burning but also the combustion of any petroleum product or byproduct, smelting, agriculture, cooking, coal burning, production of plastic, foam, energy, and nuclear power; the list goes on ¹⁰⁻¹⁴. Historically, pollution has been associated with severe or even life threatening health conditions. Cigarette smoke and second hand cigarette smoke exposure is a well publicized and published pollutant that is directly linked to the development of cancer; but can cause asthma, emphysema, decreased birth weight, increased risk for heart disease, stroke, coronary heart disease, and overall increased morbidity ¹⁵⁻¹⁹.

The EPA has classified 5 major pollutants; ozone (O₃), carbon monoxide (CO), nitrogen oxides (NO), particulate matter (PM), sulfur dioxide (SO₂), and lead. Each pollutant is produced from a number of sources and can have varying health effects. Ozone is a colorless gas that constitutes photochemical smog at the earth's surface. Ozone is formed in the lower troposphere as a result of chemical reactions between oxygen, volatile organic compounds and nitrogen oxides in the presence of

sunlight²⁰. Ozone has been associated with significant health problems including impaired lung function, chest pain, cough and lung inflammation^{21, 22}. Carbon monoxide is an odorless colorless gas emitted from the exhaust of vehicles and other kinds of engines where there is incomplete fossil fuel combustion²³. Carbon monoxide reduces the ability of blood to deliver oxygen to vital tissues, effecting primarily the cardiovascular and nervous systems²⁴⁻²⁶. Nitrogen dioxide is a light brown gas that results from the burning of fossil fuels in utilities, cars, and trucks²³. Nitrogen dioxide is the major cause of smog, acid rain and at high levels can be harmful to not only humans but vegetation as well^{27, 28}. Particulate matter (PM) can be solid or liquid droplets from smoke, dust, fly ash and condensed vapors. Particulate matter is produced from industrial processes, smelters, automobiles, burning industrial fuels, wood smoke, and dust from unpaved roads²⁹. These microscopic particles can affect breathing, respiratory tract health, increased respiratory disease, lung damage and linked to increased morbidity^{30, 31}. Sulfur dioxide is a colorless odorless gas emitted largely from industrial, institutional, utility, and apartment-house furnaces and boilers, as well as smelters, paper mills, petroleum refineries, and chemical plants^{32, 33}. Sulfur dioxide is one of the major pollutants that causes smog and can affect susceptible individuals like asthmatics^{23, 34}. Lead and lead compounds can adversely affect human health by either ingestion or direct inhalation of contaminated soil, dust, paint³⁵. Exposure to lead can affect mental development, kidney function, and blood chemistry^{36, 37}.

History has shown that exposure to increased amounts of pollution can be devastating. There have been several acute incidences displaying the effect that

pollution has on human health, and mortality. Several cities and towns have encountered these events first hand. Notable pollution related deaths have occurred in the past due to weather inversions in conjunction with increased industrial activity in low lying areas. One of the earliest recorded cases was in the Meuse Valley in Belgium, December 1930, killing 60 and sickening another 6,000 people. Deaths were attributed to “episodes of severe pulmonary attacks”. Investigators pointed to the probability that sulfur dioxide (SO_2) or oxidation products of that compound from factory smoke along with unusual weather patterns were the cause ³⁸. In 1948 over a span of 2 days the small town of Donora, Pennsylvania also suffered severe casualties. Thick smog trapped from an iron smelting plant combined with weather inversion, was responsible for killing 20 people and sickening 6,000 more ³⁹. Years later in 1952, 12,000 people died and 100,000 were sickened by pollution and stalled weather patterns in greater downtown London, England. Thousands of tons of black soot, tar particles, and sulfur dioxide had accumulated in the air due to the heavy coal combustion from factories along the river Thames. Estimates of PM_{10} concentrations during December, 1952, range between 3,000 and 14,000 $\mu\text{g}/\text{m}^3$ with the high range being approximately 50 times higher than normal levels ^{40, 41}. After this incident it became evident the Donora disaster was just the beginning and led the United States Congress to pass the first air pollution policy in 1955, the Air Pollution Clean Act. This policy was later followed by the formation and implementation of the EPA in 1970 by President Richard Nixon ^{42, 43}.

Depending on the geographic location, population density, and level of industrialization; pollution can vary in concentration and composition. Hong Kong,

China has a population of 7.2 million and a population density of 16,576 people/mile². Hong Kong is highly industrialized with little emission control policies enforced on industry. Therefore, it is not surprising that Hong Kong is one of the most polluted cities in the world ⁴⁴. The major 5 recognized pollutants vary in concentration; SO₂ 350 µg/m³, PM 180 µg/m³, NO 150 µg/m³, CO 10,000 µg/m³, O₃ 24 µg/m³ ^{45, 46}. Compared to Hong Kong, Los Angeles, California has drastically different levels of pollution. Los Angeles has a population of 3.8 million and density of 7,544 people/mile², California boasts extremely strict emission control laws and has a different industrial base, which accounts for the vast difference when compared to Hong Kong. The major 5 pollutants in ambient Los Angeles air; SO₂ 0.43 µg/m³, PM 39 µg/m³, NO 14.3 µg/m³, CO 304 µg/m³, O₃ 13 µg/m³ ^{47, 48}. When levels of these pollutants reach high enough levels, they can be hazardous or even deadly upon exposure and can cause serious health effects with long term repeated exposure. The Occupational Safety and Health Administration (OSHA) has compiled permissible exposure limits (PELs) for hundreds of pollutants. Studies have found there are no real “safe” or acceptable exposure concentrations of troposphere or ground-level ozone exposure. Overtime, exposure to ozone will have negative health related effects, but OSHA ozone PELs is 0.075ppm/8hr day. Sulfur dioxide PELs are not to exceed 140ppb in a 24hr period. Particulate matter PELs are not to exceed (PM_{2.5}) 35µg/m³ per 24 hour period, (PM₁₀)150µg/m³ per 24 hour period. Acceptable nitric oxide exposure levels have become more stringent, individuals are not to exceed 100ppb/hr of exposure. Carbon dioxide standards allow individual exposures to reach 9ppm/8hr day. OSHA has found these safety standard exposure levels to have no long-term health effects. However, data now show for every

10µg/ m³ of city specific particulate matter_{2.5} the mortality ratio rate (RR) is 1.13, demonstrating an inverse correlation between pollution levels and mortality^{49, 50}.

Environmental tobacco smoke (ETS) is a pollutant associated with severe health consequences. Smoke from cigarettes is a complex mixture of 4,000 different chemicals; 50 known carcinogens; 200 known poisons. ETS is made up of a combination of side stream and mainstream cigarette smoke. Side stream cigarette smoke is smoke coming off the end of a smoldering cigarette. Mainstream smoke is considered active smoking, the combination of inhaling and exhaling after taking a puff on a lit cigarette. The frequency of puffs, duration and volume all contribute to the make-up of mainstream smoke^{51, 52}. Each of the chemicals in cigarettes varies depending on brand and country of origin (**Table 1**)⁵³⁻⁵⁷. Studies have shown that exposure levels increase significantly from mainstream smoke to side stream smoke, particulate matter significantly increases by 5 fold like⁵⁸. Even though combustible tobacco products are full of numerous chemical aldehydes are highly reactant and abundant within cigarette smoke (**Table 2**). Even though acrolein is not necessarily the most prevalent of the aldehydes it is one of the most reactive.

Table 1: Cigarette chemical content (one burned cigarette) ⁵³⁻⁵⁷

Brand	Acrolein	CO	Nicotine	Tar	Country or origin
Merit	63 µg	9	0.8 mg	10 mg	USA
Marlboro	155 µg	10	1.8 mg	12 mg	USA
Camel	149 µg	16	1.1 mg	15 mg	USA
Sakura	350 µg	18	2.0 mg	26 mg	Japan

Table 2: Aldehyde content cigarettes (US Dept of Health and Service)

Aldehyde	Amount per cigarette
acetalehyde	1.4mg
crotonaldehyde	0.2µg
formaldehyde	1.6µg

Exposure to ETS whether it is from actively smoking or from second hand exposure is a major cardiovascular risk factor^{59, 60}. The WHO estimate over 26.5 % of males and 22.1 % of females in the world actively smoke; while 43 % of the world's children are exposed to second hand smoke⁶¹. Every year 5 million active smokers die, while 600,000 people exposed to second hand smoke also succumb to smoke related illnesses⁶². Cigarette smoke is associated with endothelial dysfunction, decreased angiogenesis, increased arteriosclerosis and various other cardiovascular diseases^{18, 63, 64}. Nevertheless, the specific chemicals in the tobacco smoke that cause toxicity have not been identified and the mechanisms underlying the cardiovascular toxicity of tobacco smoke remains unclear. The main questions remain what chemical(s) constituents, mechanism(s) and susceptibility factors associated with ETS and environmental pollution can account for increased mortality and decreased cardiovascular health.

Acrolein

Acrolein is an unsaturated aldehyde also known as acrylaldehyde, acrylic aldehyde, allyl aldehyde, ethylene aldehyde, 2-propenal, and prop-2-en-1-al.

Acrolein at room temperature is a colorless or yellow combustible liquid.

Structure:

Empirical formula: C₃H₄O

Molecular weight: 56.06 g/mol

Vapor pressure: 274 mmHg

Conversion factor: 1 ppm = 2.3 mg/ m³; 1mg/ m³ = 0.44 ppm

Acrolein is used in industry for the production of plastics, rubber and as an intermediate in the production of acrylic acid. Acrolein is also used as an herbicide, aquacide, slimicide; on farms, in drainage ditches and in paper production. Acrolein is also present in varying concentrations as a result of combustion of organic material (**Table 3**)⁶⁵⁻⁶⁷. Acrolein is highly water soluble and possesses low vaporizing pressure making acrolein a highly mobile substance. In ambient air acrolein exist solely in a vapor-phase, and exposure occurs primarily through atmospheric contact. For long-term outdoor pollution studies, government agencies use TO-11A method (active sampling with 2-4 dinitrophenylhydrazine [DNPH] coated solid sorbents). This particular method

samples air downstream of KI oxidant scrubbers which then pass on to silica gel cartridges coated with DNPH, DNHP derivatives are analyzed by liquid chromatography (LC) with detection by diode-array, UV-visible spectroscopy. The DNHP method is unstable with humidity, temperature and ozone level fluctuation. This method is used primarily on the west coast and mid-west regions. The most recent and highly stable system for collecting airborne aldehyde and ketone data is the modified passive aldehyde and ketone sampler (PAKs). PAKs method for collection efficiency is ~100% for most saturated carbonyls, and $99\pm5\%$ for acrolein even with humidity fluctuations from 30-90%. **(Table 4).**

Table 3: Sources and level of acrolein emissions

Source	Amount
Gas engine exhaust	0.05-27.7 mg/m ³
Diesel engine exhaust	0.12-0.21 mg/m ³
Smoky indoor environment	2.3-275 µg/m ³
Residential wood stoves	0.7-6.0 µg/m ³
Main stream cigarette smoke (exhaled smoke)	10-140 µg/m ³
Side stream cigarette smoke (smoldering ash)	100-1700 µg/m ³
Total industrial air emissions	208,108 lbs
Fires (Boston, USA)	6.9 mg/m ³

Table 4: Methods for acrolein sampling and testing.^{a,b}

Sample matrix	Sample preparation	Assay procedure	Limit of detection	Reference
Air	Adsorb on sorbent coated with 2-(hydroxymethyl)piperidine on XAD-2; desorb with toluene; analyse for oxazolidine derivative	GC/NSD	2 µg/sample (6.1 µg/m ³)	US OSHA, 1989; Eller, 1994
	Draw air through midget impinger containing acidified DNPH and isooctane; extract DNPH derivative with hexane:dichloromethane (70:30) solution; evaporate to dryness; dissolve in methanol	Reversed-phase HPLC/UV	NR	US EPA, 1988
	Draw air through bubblers in series containing 4-hexylresorcinol in an alcoholic trichloroacetic acid solvent medium with mercuric chloride	Colorimetry	22.9 µg/m ³ (10 ppb) ^c	Feldstein et al., 1989a
	Draw air through midget impinger containing 1% sodium bisulfite; react with 4-hexylresorcinol in an alcoholic trichloroacetic acid solvent medium with mercuric chloride	Colorimetry	22.9 µg/m ³ (10 ppb)	Feldstein et al., 1989b
Moist air	Collect in DNPH-impregnated adsorbent tubes (with calcium chloride tubes); extract with acetonitrile	HPLC/UV	0.3 µg/sample (0.01 mg/m ³)	Vainiotalo & Matveinen, 1992
Exhaust gas	Derivatize with O-benzylhydroxylamine to O-benzylloxime; brominate with sulfuric acid, potassium bromate, and potassium bromide; reduce with sodium thiosulfate; extract with diethyl ether	GC/ECD	NR	Nishikawa et al., 1987a
Aqueous solution	Derivatize with O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine	MIMS/EIMS	10 µg/litre (10 ppb)	Choudhury et al., 1992
Rainwater	Derivatize with O-methoxylamine to O-methyloxime; brominate with sulfuric acid, potassium bromate, and potassium bromide; reduce with sodium thiosulfate; elute with diethyl ether	GC/ECD	0.4 µg/litre	Nishikawa et al., 1987b
Liquid and solid wastes	Purge (inert gas); trap in suitable adsorbent material; desorb as vapour onto packed gas chromatographic column	GC/FID	0.7 µg/litre ^d	US EPA, 1986

Biological samples	Derivatize with DNPH; extract with chloroform, hydrochloric acid; dry with nitrogen; dissolve in methanol	HPLC/UV	1 ng	Boor & Ansari, 1986
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- ^a From IARC (1995).
- ^b Abbreviations used: DNPH = 2,4-dinitrophenylhydrazine; ECD = electron capture detection; FID = flame ionization detection; GC = gas chromatography; HPLC/UV = high-performance liquid chromatography/ultraviolet detection; MIMS/EIMS = membrane introduction mass spectrometry/electron impact mass spectrometry; MS = mass spectrometry; NR = not reported; NSD = nitrogen selective detection.
- ^c Note that 1 ppb = 1×10^{-9} .
- ^d Practical quantification limits for other matrices: 7 µg/litre for groundwater; 7 µg/kg for low-level soil samples; 350 µg/litre for water-miscible liquid waste samples; 875 µg/kg for high-level soil and sludge samples; 875 µg/litre for non-water-miscible waste samples.

The EPA reported mean ambient concentrations of acrolein to be $14.3\mu\text{g}/\text{m}^3$ (6 ppb) (**Table 3**)⁶⁵⁻⁶⁷.

Not only can we breathe in acrolein we can also ingest acrolein, through heating of carbohydrate-containing foods which results in the formation of reactive carbohydrate intermediates that can undergo carbon-carbon cleavage and ultimately produce acrolein⁶⁸. Acrolein is a highly reactive electrophile due to the polarization of the double bond by the aldehyde group. In the body acrolein reacts readily with protein nucleophiles, non-protein sulfhydryl groups, or primary and secondary amines found in nucleic acids. Acrolein is endogenously produced as a by-product of lipid peroxidation and possible mediator in several human diseases, as reviewed by Uchida *et al.*,^{69, 70} Acrolein conjugates readily and rapidly with sulfhydryl groups and is essentially irreversible. This reaction leads to the formation of thiazolidine derivatives and a decrease in glutathione (GSH) stores without an increase in oxidized GSH (GSSG). This pathway results in an acrolein-GSH adduct which is further metabolized by both mitochondrial and cytosolic aldehyde and alcohol dehydrogenase. Ultimately acrolein is excreted in urine as mercapturic acid derivatives, or remains as protein-acrolein adducts within the body (**Fig. 1**)^{67, 71}

Figure 1

Figure 1 metabolism of acrolein Intermediary metabolism can form Acrylic acid or Glycidaldehyde from acrolein, acrolein can also be excreted in the urine as mercapturic acid derivatives goes through several

It has been previously shown that administration of radioactive acrolein (0.82 mg/kg) to lactating goats, incorporation of the radioactivity appeared to follow incorporation of metabolites into normal biosynthetic pathways of both mother and nursing kid ⁷².

Acute exposure to acrolein at levels as low as 0.99 ppm for 5 min causes severe eye and nose irritation in humans ^{73, 74}. Lyons *et al.*, ⁷⁵ showed in a multi-species study containing fifteen Sprague-Dawley rats (male), seven to eight Princeton or Hartley guinea pigs (male), two pure bred beagle dogs (male); and nine squirrel monkeys (male). All species were exposed to acrolein, 8 hr/day, 5 days/week, for 6 weeks at concentrations of either 0.7ppm or 3.7ppm. No deaths were reported in the 0.7ppm group, histopathology of the lungs showed significantly increased inflammation in all species, however inflammation was found to be significantly more chronic in the lungs of more complex organisms; beagles and monkeys. In the 3.7ppm group, two monkeys died within two weeks after onset of exposure. All species exhibited extensive mucosal gland irritation within the first week which continued throughout the remainder of the study. In histological sections non-specific inflammatory changes were noted in the lungs, liver, and kidney of all species inflammatory changes within the lung were seen in the bronchi rather than the bronchioles. All study groups were found to be significantly different from that of control groups. Data supports acroleins ability to cause extreme tissue inflammation and damage not only in the lungs but can travel to various organs throughout the body and inflammation severity varies

from species to species. The more complex organisms seemed to be more susceptible to inflammation caused by acrolein inhalations.

Acrolein is classified as an environmental toxicant, the defined OSHA permissible exposure limit is 0.1ppm per 8 h/ d (time weighted average;TWA) or 0.3ppm for 15 min. short term exposure (TWA). Both are near or above acrolein's odor threshold of 0.2ppm. Acrolein can cause disruptions in sulfahydryl groups that are critical for chemical reactions within living cells, leading to disruption of intermediate metabolism, inhibition of cell growth or division, and cell death ⁷⁶. Acrolein is a known constituent of environmental tobacco smoke (ETS); ETS promotes arteriosclerosis and endothelial dysfunction by inducing proinflammatory stimuli. The link between cigarette smoke and cardiovascular disease is poorly understood, however due to the abundance, reactivity, and proinflammatory nature, acrolein is a likely candidate ⁷⁷. There is a need to further understand acrolein's possible role in cardiovascular disease and the link between cigarette smoke. The CDC reports people that smokers are 2-4 times more likely to develop coronary heart disease compared to non-smokers.

Endothelial Progenitor Cells

Prior to 1997, angiogenesis was thought to occur only in a neonatal state, as mesodermal cells differentiated into angioblasts, ultimately giving rise to endothelial cells, the process of neovascularization. However, in 1997 Asahara and colleagues revolutionized this dogma with the discovery of CD34⁺ cell subset

that possessed an endothelial phenotype. The endothelial like cells were sorted from human blood by magnetic beads and autologous transplants showed these cells had the ability to incorporated into sites of active angiogenesis and were beneficial in wound healing, they coined these cells Endothelial Progenitor Cells (EPCs) ⁷⁸.

Definition:

EPCs originate from the bone marrow (BM) and are present in either a quiescent state in the non-vascular niche of the BM or a proliferative state in the vascular niche from which they are mobilized^{79, 80}. EPCs can be defined by numerous markers in varying combinations, the most accepted is a combination of a stem cell marker along with an endothelial lineage marker. In the initial paper Asahara *et. al.*, human blood was drawn, sorted by magnetic bead for CD34⁺ and Flk-1⁺ cells, then stained for CD45, CD34, CD31, Flk-1, TIE-2 and E-Selectin⁷⁸. There are several markers commonly used to discern EPCs from other cells present within the body (**Table 5**) ^{81, 82}.

Table 5: Commonly used EPC markers and definition

Marker	Definition
Vascular Endothelial Growth Factor Receptor 2 ⁺ (VEGFR2, KDR, Flk-1)	Endothelial marker, surface receptor for VEGF (mouse)
von Willibrand factor (vWf) ⁺	Endothelial marker, cell surface marker bind to Factor VIII, important for platelet adhesion to wound sites (mouse)
CD34 ⁺	Hematopoietic stem cell lineage marker, important for cell-cell adhesion (human)
Lin ⁻	Cocktail of antibodies used to determine cellular lineage (human)
CD45 ^{-dim}	Surface marker on hematopoietic cells, protein tyrosine phosphatase (mouse)
CD133 ⁺	Also known as Prominin, cell surface marker of unknown function (mouse)
Lectin ⁺	Cell surface sugar binding protein (mouse)
Sca-1 ⁺	Stem cells marker-1, glycosyl phosphatidylinositol-anchored cell surface protein (mouse)
C-kit ⁺	Cytokine receptor, binds to stem cell factor
Tie-2 ⁺	Endothelial specific, identified as Angiopoietin I receptor (mouse)
CXCR4 ⁺	CXC chemokine receptor, bind SDF-1(mouse)

EPCs are evaluated by surface markers and angiogenic ability when seeded on Mitrigel®. Angiogenic ability of these cells allow for increased repair of damaged vasculature and improves overall vascular health. Other endothelial properties have been evaluated on EPCs as well such as, NO production which is very important for vasodilation and further EPC homing to sites of injury. It has been shown circulating endothelial progenitor cells account for only 0.01 % of circulating cells. However, three hours after hypoxic injury, circulating EPCs are visibly increased, and by 24 hours they account for 12 % of circulating cells⁸³.

Mobilization:

Found within red bone marrow is a heterogeneous population of cells that make up the stem cell niche, consisting of fibroblasts, osteoclasts, hematopoietic and endothelial cells. The bone marrow stromal cells that line the inside microenvironment of the bone marrow are responsible for providing extrinsic signals that maintain the stem cell niche and regulate the repopulation of stem cells^{81, 84}. While attached to the stromal cells, stem cells are maintained in the G₀ phase of the cell cycle, which is the quiescent non-proliferative phase.

Endothelial progenitor cell mobilization from the bone marrow occurs when cytokines interact between the bone marrow stromal cells and stem cells. This allows stem cells to disengage the bone marrow and pass into the blood stream. Respectively, during hypoxic injury, the Hypoxia Inducible Factor 1- α (HIF1- α) protein levels increase and cause up regulation of VEGF levels. Bone marrow stromal cells can bind to VEGF and increase production of nitric oxide (NO), NO

can then increase active-MMP-9 levels. MMP-9 is responsible for cleaving membrane bound kit ligand (mKitL), and thus, increasing the bioavailability of soluble kit ligand (sKitL). After levels of sKitL increase attached endothelial progenitor cells bind sKit and are pushed from a quiescent state into a proliferative state. Studies have concluded EPCs follow SDF-1/CXCR4 axis and gradients within the circulation (**Scheme 1**)^{81, 84-87}.

Scheme 1: Hypoxic injury and mobilization of EPCs from bone marrow

Recruitment/homing:

Stromal Derived Factor 1 (SDF-1) protein is crucial for homing and recruitment of circulating EPC to sites of injury. SDF-1 has chemotactic activity that controls adult stem cell trafficking and is the ligand for G-protein coupled receptor CXCR4⁸⁸. EPCs possess CXCR4 receptors and it has been shown local administration of SDF-1 at the sites of injury increases therapeutic neovascularization⁸⁹. With injury to the endothelium VEGF levels steadily increase along with numerous other cytokines and chemokines. Various stimuli promote mobilization and recruitment of EPCs to sites of injury⁹⁰. Once circulating EPCs come into contact with the appropriate recruitment stimulus, surface adhesion factors are thought to be upregulated and help with cell recruitment and cell adhesion (**Table 6**)⁹⁰⁻⁹⁷.

Table 6: EPC homing factors

Chemokine/ receptor and integrins	Cell type and markers	Animal model (species)
SDF-1/ CXCR4	EPC, CD34 ⁺	Hind limb ischemia (Murine)
IL-8 /CXCR2 or CXCR1	CD34 ⁺	Myocardial infarction (Murine)
HMGB-1	EPC	Myocardial infarction (Murine)
β 2-integrin	EPC	Myocardial infarction, Hind limb ischemia (Murine)
E- and P- Selectin	EPC	Tumor, Hind limb ischemia (Murine)

Transmigration:

EPC transmigration is similar to leukocyte rolling and transmigration. *In Vitro* studies have lead to evidence suggesting once the EPC has made contact with or near the injured tissue, low affinity contacts are made between selectins present on the cell surface and the vascular wall, much like the process seen with leukocyte rolling⁹⁸. The integrin, E-selectin, is important to not only homing but actually regulating the bone marrow-derived EPC recruitment to sites of injury. After cell rolling, further adhesion occurs through β 2-integrins^{99, 100}. For firm adhesion α 4 β 1 integrins and LFA-1 increase adhesiveness via their respective ligands, VCAM-1 and I-CAM-1. After rolling and firm adhesion, EPCs must transmigrate, however, little is known about the true mechanism behind this process. It is postulated that PECAM-1 and CD99 play a role in transmigration of EPCs (**Scheme 2**)¹⁰¹.

Scheme 2: EPC adhesion and transmigration

Differentiation:

After activation and mobilization into the circulation, EPCs start maturing and eventually lose stem cell markers and characteristics while simultaneously acquiring more mature endothelial cell characteristics¹⁰². In previous studies it has been shown phospho-Akt is induced in the ischemic muscle by cytokines such as VEGF and SDF-1, which are secreted by skeletal muscle cells, stromal cells, and endothelial cells in response to ischemia. In particular, the phosphorylation of Akt in endothelial cells leads to entrapment of systemically administered ex vivo-expanded EPCs into the ischemic muscle through various kinds of mechanisms, overexpression of ICAM-1 on endothelial cells (ECs) and the increased incorporation and transendothelial migration of EPCs¹⁰³. In order to study actual incorporation, EPCs were isolated and tagged with diacyl-LDL then injected into nude mice. Histology sections were taken of ischemic muscle and 56 ±4.7 % of the vessels had EPC incorporation out of the 16 section evaluated (10X field)¹⁰³. While still other studies using the same approach show only 0.01% of EPCs injected going to the site of injury and actively participate in angiogenesis¹⁰⁴. Various studies report different incorporation numbers for EPCs at the sites of injury, this could be accounted for by the methods used and the analysis of data. There are small amounts of EPCs in the circulation even after ischemic injury and so far histology and rudimentary counting has been the main published method, a lot could be missed or over counted by this approach just due to human error or bias.

When incorporated into the site of injury, EPCs act in a paracrine fashion releasing growth factors such as VEGF and stimulating release of NO, thus stimulating the release of growth factors from surrounding adult endothelial cells. The growth factors released recruit more EPC to the site of injury and other pro-angiogenic cells such as macrophages. The immature EPCs soon fully differentiate into mature endothelial cells, after exposure to growth factors such as VEGF, SDF-1, IGF-1, and HGF in the surrounding environment^{91, 105-108}.

Non-Bone Marrow, Endothelial Progenitor Cell Niches

EPC can also be found in resident tissue niches outside of the bone marrow. Stem cell niches provide a balance between quiescence and activity of the stem cell by protecting them from differentiation stimuli, apoptotic stimuli, and other stimuli that could challenge stem cell reserves. A stem cell niche is defined by functional dimensions and anatomical dimensions which specifically enable stem cells to asymmetrically reproduce and allow for self renewal¹⁰⁹⁻¹¹¹. Stem cells within a niche are embedded in a 3-D extra cellular matrix (ECM). The ECM can be divided into two groups, structural proteins and proteoglycans (PGs), including glycosaminoglycans (GAGs), which are primarily responsible for regulatory processes in disease and development. The ECM of the stem cell niche consists mainly of basement membrane components, such as collagens, laminins, fibronectin, and GAGs. Integrins connect the ECM niche to the internal cytoskeleton of the stem and progenitor cells and they transmit external signals directly to the stem cells. When mechanical force is applied to the ECM, this

influences the integrins and impacts stem cell fate by activation of various signaling pathways. Within each niche, some ECM proteins have specialized functions restricted to distinct stem cell niches¹¹². For example, β -1 integrins are predominately expressed within epidermal stem cell niches while tenascin c and osteopontin are specifically present in hematopoietic stem cell niches. Some investigators have suggested that bone marrow is the only source of stem/progenitor cells, however when the body depletes the bone marrow stem cells, non-bone marrow stem cell niches are called upon and play a vital role and help repair damaged tissues¹¹³. Non-bone marrow niches are found throughout the body some are better documented and studied than others. It is thought that niches are formed during embryogenesis and stem cell migrations during fetal tissue development and are actually maintained into adulthood^{114, 115}.

The spleen contains two distinct areas known as the red and white pulp, the red pulp contains macrophages that filter out debris and dead cells from the circulation. The white pulp or "lymphoid compartment" synthesizes antibodies against invading pathogens and releases platelets and neutrophils in response to bleeding and infections¹¹⁶⁻¹¹⁸. However, aside from these functions the spleen is the site of extramedullary hematopoiesis. In times of stress and disease when bone marrow cannot fulfill the demand for new cells, the spleen acts as a secondary hematopoietic center¹¹⁸. This is not surprising since the spleen is a site of hematopoiesis during gestation¹⁰⁴. Recent studies have shown cells with multi-lineage capability are found within the spleen and the spleen has been recognized not just as a secondary center for hematopoiesis but also a storage

site for multiple stem cell lineages. Many studies have linked this to a developmental origin called the aorta-gonad-mesonephros (AGM) ¹¹⁹. The AGM region is the first site for hematopoiesis, which then migrates to the liver and spleen, until ultimately the bone marrow takes over as the primary site of hematopoiesis ^{120, 121}. Recent studies have shown the AGM has a broader cell lineage potential than just producing CD45+ hematopoietic cells. When CD45- cells were harvested from the AGM region of mice expressing green fluorescence protein (GFP) and transplanted into the liver of neonatal mice ¹²²⁻¹²⁴, the transplanted cells were found not only in the bone marrow and spleen but also non-hematopoietic tissue as well- liver, kidney, lung, small intestine, vascular wall and uterus ¹²⁵⁻¹³¹. Literature suggests that stem cells with multi-lineage potential are found in the spleen as a result of early migration pattern from the AGM region [ref]. This leads to the idea of how niches could be formed not just within the spleen but various locations in the body due to early gestational development and stem cell migrations along developmental axis ^{122, 132}. It is currently believed that tissue-specific progenitor cells reside within niches proximal to the cells types they will regenerate.

Recent studies have led to the identification of several EPC niches in the spleen, liver, adipose tissue, adventitial tissue, and small intestine ¹²⁵⁻¹³¹. Using a parabiosis model coupled with a reverse bone marrow transplant and hindlimb ischemia, Aicher *et al.*, ⁹⁹ set out to discern the actually percentage of EPC that come from non-bone marrow niches. In this study $74 \pm 13\%$ of circulating progenitor cells are non-bone marrow derived and incorporated into hindlimb

ischemia (HLI) of rats. In addition as previously mentioned organs such as the liver and small intestine have a considerable number of resident progenitor cells, within progenitor cell niches. Sex-mismatched transplant of liver and small intestine of rats followed by the induction of HLI, showed that $4.7 \pm 3.7\%$ small intestine-derived endothelial progenitors incorporated in the ischemic tissue of HLI models and contributed to vasculogenesis, while the liver contributed $6.3 \pm 2.2\%$ ¹³³. Within the adipose tissue, there is the stromal vascular fraction (SVF). This fraction has angiogenic capacity equivalent to that of bone marrow-derived EPCs when injected after HLI ¹³⁴. Other studies have shown that the lung and skeletal muscle also possess endothelial progenitor cell niches. However, it is unclear whether these cells are truly tissue -resident or whether they are derived from the bone marrow ^{131, 135}. Vascular tissue itself has also been studied and a “vasculogenic zone” has been recently discovered. This zone lies between the adventitial and media of the vessel wall ¹²⁶. Cells within this vasculogenic zone have been identified as CD34+/Tie2+/KDR+/VE-Cadherin+, capable of differentiating into mature endothelial cells and form capillary like sprouts in Mitragel ¹²⁶.

Hill *et al.*, ¹³⁶ performed a series of experiments with male patients who had varying degrees of cardiovascular risk but had no history of cardiovascular disease. Peripheral blood was taken and EPC numbers were significantly reduced in patients with elevated cholesterol levels, hypertension and diabetics. They also found a significant inverse correlation between EPC numbers and Framingham scores of these patients. EPC depletion could be a by-product of

increased oxidative stress or other physiologic process within the patient. However, this decrease could also be due to continuous endothelial injury and EPC exhaustion compounding the risk for developing cardiovascular diseases. Previous studies have also shown that a decrease of circulating stem cells can be an important determinant of age-related conditions¹³⁶. Injection of EPC into the circulation of ischemic asymptomatic models shows a striking result, animals have increased limb salvage and increased angiogenesis of the ischemic leg¹³⁷.

By studying how pollutants affect EPCs we may be able to understand their mechanism of cardiovascular toxicity and to assess the cardiovascular disease risk due to pollutant exposure.

Goals of the project

The overall thesis of the work presented here is that exposure to environmental pollutants such as acrolein could deplete **EPC levels or interfere with EPC mobilization, and thereby increasing the risk of developing cardiovascular disease and endothelial dysfunction**. Although EPCs express high levels of manganese superoxide dismutase (MnSOD) and therefore are likely to be resistant to oxidative injury, recent studies have shown that exposure to tobacco smoke or the cardiovascular disease decreases EPC levels.

Nevertheless, the mechanisms by which EPC levels are decreased under these conditions remain completely unknown.. Hence, the aim of this project was to examine how inhalation exposure to a ubiquitous environmental pollutant such as

acrolein affects circulating EPC levels (Aim 1) and to understand the mechanism by which acrolein inhalation affects EPC mobilization (Aim 2) . To assess the functional significance of the effects of acrolein on EPCs, I also studied how exposure to this aldehyde affects EPC mobilization after hind-limb (Aim 3). The main purpose of these studies was to learn more about the mechanisms of cardiovascular toxicity due to acrolein exposure.

CHAPTER II:
ACROLEIN INHALATION DECREASES CIRCULATING LEVELS OF
ENDOTHELIAL PROGENITOR CELLS

Introduction

Several epidemiological studies show that exposure to combustion products increases the risk of developing cardiovascular disease. In many large population-based studies, long-term exposure to traffic-generated combustion products has been found to be associated with an increased risk for coronary heart disease¹³⁸, atherosclerosis¹³⁹ and fatal myocardial infarction¹⁴⁰. Likewise, exposure to products of wood or coal combustion is linked to increases in blood pressure and cardiovascular mortality^{141, 142}. Moreover, individuals such as bus drivers¹⁴³, chimney sweeps¹⁴⁴ and firefighters¹⁴⁵, who are repeatedly exposed to combustion products, have significantly higher rates of cardiovascular mortality than the general population. In addition, extensive data show that cardiovascular disease and mortality are increased by exposure to combustion products generated during smoking¹⁴⁶ or present in secondhand tobacco smoke¹⁴⁷. Experimental data with humans or animals exposed to automobile exhaust¹⁴⁸ or tobacco smoke^{146, 147} support these epidemiologic findings. These studies suggest that combustion products induce adverse cardiovascular effects.

Nonetheless, the chemicals that mediate the cardiovascular toxicity of combustion products are unidentified.

Combustion of organic material results in the generation of a complex chemical mixture. The composition of this mixture varies with the source; however, one of the chemicals common to all combustion sources is acrolein. It is produced in high amounts during combustion of organic material in any form (coal, wood, paper, cotton, gasoline, diesel or tobacco)¹⁴⁹. Recent estimates show that high levels of acrolein (between 6-8 ppm¹⁵⁰) are present in exhaust gases from petrol and diesel engine vehicles, and tobacco smoke (100-600 µg/cigarette)¹⁵¹ generates up to 50-70 ppm acrolein. Acrolein is a highly reactive and toxic chemical,^{76, 152} which could account, at least in part, for the cardiovascular toxicity induced by combustion products. Our previous studies showed that acrolein inhalation leads to endothelial dysfunction in susceptible mice¹⁵³. Endothelial injury is also an early and integral feature of the cardiovascular toxicity of automobile exhaust¹⁴⁸, tobacco^{146, 147} and wood smoke¹⁵⁴ exposures. However, mechanisms by which these exposures affect endothelial health remain unclear. Recent work suggests that endothelial repair and regeneration depends, in part, upon circulating cells with pro-angiogenic potential⁸⁶. These cells with pro-angiogenic potential have been coined Endothelial progenitor cells (EPCs) and are also known as Flk⁺/Sca⁺ cells, they possess both endothelial markers and stem cell markers. The blood level of Flk-1⁺/Sca-1⁺ cells is a sensitive index of endothelial health and is inversely correlated with cardiovascular disease risk⁸⁶. This current study was, therefore,

designed to test the hypothesis that acrolein exposure adversely affects the number and mobilization of circulating Flk-1⁺/Sca-1⁺ cells.

Methods

Acrolein Exposure

Acrolein atmospheres were generated from liquid acrolein (Sigma-Aldrich, St. Louis, MO, USA; $\geq 90\%$; diluted in dH₂O, 1:10). Acrolein exposure was performed using a custom vapor system (Teague Enterprises, Inc., Woodland, CA, USA) with a primary chamber as a constant source, and acrolein vapors diluted with HEPA-filtered room air in a secondary chamber. During exposure, acrolein concentration was continuously monitored using an in-line photo ionization detector (ppb RAE+, Rae Industries, Sunnyvale, CA, USA) upstream of the cage insert vapor delivery unit (Teague Enterprises, Inc.) mounted on a standard polycarbonate rat cage (41 cm x 34 cm x 21 cm; ≈ 31 L). Air or acrolein was distributed through a fine mesh screen at 3 lpm by delivery units with a cyclone-type top that distribute air within 10% of the mean concentration at six locations in the cage (**Scheme 3**). Exposure cages were placed partially over heating pads (≈ 22 °C) to allow mice to select preferable temperature. Mice were exposed to 5ppm acrolein for 2 or 6 h (5010 $\pm$ 49ppb of 2 different exposures) or to 1ppm acrolein for 4 days (1,053 $\pm$ 22ppb of 5 different exposures) (**Scheme 4**).

Scheme 3: acrolein inhalation setup

Scheme 4: 1ppm acrolein exposure level stability

Circulating endothelial progenitor cell mobilization

Acrolein (1ppm 6hr/4d) and Air (HEPA air 6hr/4d) exposed mice were injected with saline or VEGF₁₆₅ for 4 consecutive days. Immediately after the final exposure, VEGF-injected mice received the CXCR4 antagonist, AMD3100, and control mice received saline. Mice were euthanized 1hr after AMD3100 or saline injection (**Scheme 5**).

Scheme 5: VEGF/AMD3100 treatment regime

Peripheral blood mononuclear cells and flow cytometry

Whole blood (300–400 μ l) was lysed (4 ml; BD PharmLyse, BD BioSciences, San Jose, CA, USA; 10 min, RT) and after centrifugation (5 min, 400xg, RT), the supernatant was aspirated and the lysing/centrifugation/aspiration steps were repeated. The cell pellet was resuspended in 1% FBS/PBS and divided into 2 equal fractions. One fraction was fluorescently-labeled with anti-Sca-1 and anti-Flk-1 antibodies tagged with FITC (Fluorescein Isothiocyanate) and APC (Allophycocyanin), respectively. Following centrifugation (5 min, 400xg, RT), mononuclear cells were re-suspended in 1% FBS/PBS (20 μ l) with murine CD16/CD32 Fc Block to prevent non-specific binding to cell surface markers (0.5 μ g; BD Biosciences) and incubated for 10 min on ice. The FITC-Sca-1 (1 μ g; BD BioSciences) and APC-Flk-1 (1 μ g, BD BioSciences) antibodies or appropriate isotype controls (1 μ g; BD BioSciences) were added to cells (23 μ l) and incubated for 30 min on ice. Cells were then washed with 1% FBS/PBS (400 μ l) and centrifuged (5 min, 400xg, RT). Cells re-suspended in 1% FBS/PBS (400 μ l) were analyzed using a LSRII flow cytometer (BD BioSciences). Based on forward and side scatter, small non-debris events in a sub-lymphocyte population (3–5 μ m; sized using fluorescent beads, BD Biosciences) were gated electronically and displayed in a two-color dot plot. Data were subsequently analyzed using FACSDiva v6.0 software (BD Biosciences), and double positive events were normalized per 50,000 events or per μ l of assay volume. Preliminary flow cytometry data indicated that $\geq 98\%$ of circulating Flk-1⁺/Sca-1⁺ cells were also CD45⁺ (1 μ g, PerCP-CD45; BD Biosciences).

Endothelial progenitor cell characterization

For better characterization EPCs were mobilized. Mice were injected with saline or VEGF₁₆₅ saline (100 µg/kg/d; Peprotech, Inc., Rocky Hill, NJ, USA) (n=4,4) daily for 4 consecutive days, on the 5th day received CXCR4 antagonist, AMD3100, and control mice received saline (5mg/kg, i.p., 100 µl; Sigma-Aldrich) (n=4,4). Mice were euthanized 1h after AMD3100 or saline injection. Whole blood (1mL) was lysed (9 ml; BD PharmLyse, BD BioSciences, San Jose, CA, USA; 10 min, RT) and after centrifugation (5 min, 400xg, RT), the supernatant was aspirated and the lysing/centrifugation/aspiration steps were repeated. After washing cells were resuspended in 2% FBS/PBS with FcBlock for staining. All staining antibodies were purchased from eBioscience (San Diego). The following antibodies were used for analysis in three different groupings: **Stem cell marker panel 1:** Anti-Mouse CD150 FITC, Anti-Mouse Ly-6A/E (Sca-1) PE, Anti-Mouse CD115 (c-fms) PerCP-eFluor® 710, Anti-Mouse CD48 PE-Cy7, Anti-Mouse CD309 (FLK1) APC, Anti-Mouse CD11b APC-eFluor® 780, Anti-Mouse CD34 eFluor® 450, Anti-Mouse CD38 Biotin-Streptavidin 605 nanocrystal; **Stem cell marker panel 2:** Anti-Mouse CD133 (Prominin-1) Biotin 605, Anti-Mouse CD150 FITC, Anti-Mouse Ly-6A/E (Sca-1) PE, Anti-Mouse CD115 (c-fms) PerCP-eFluor® 710, Anti-Mouse CD48 PE-Cy7, Anti-Mouse CD309 (FLK1) APC, Anti-Mouse CD11b APC-eFluor® 780, Anti-Mouse CD34 eFluor® 450; **T and B-cell marker panel 3:** Anti-Mouse CD8a PerCP-Cy5.5, Anti-Mouse CD3 APC-eFluor® 780, Anti-Human/Mouse CD45R (B220) eFluor® 450, Anti-Mouse CD4 eFluor® 605NC, Anti-Mouse CD31 (PECAM-1) FITC, Anti-Mouse Ly-6A/E (Sca-1) PE,

Anti-Mouse CD309 (FLK1) APC, and Anti-Mouse CD19 PE-Cy7. Cells were analyzed by flow cytometry (BD LSRII, BioSciences) and data were analyzed using FlowJo (TreeStar Software; Ashland Oregon).

Isolation and culture of bone marrow-derived cells (BMDCs)

Bone marrow was aspirated from femur and tibia of both legs with 1mL HBSS (Clonetics/Lonza, Walkersville, MD, USA), and mononuclear cells were separated by Ficoll gradient centrifugation (Ficoll-Paque PREMIUM, GE Healthcare, Piscataway, NJ, USA; 400xg, 20 min, 4°C). Trypan blue-viable cells ($2-4 \times 10^6$ cells per mouse) were isolated and 8×10^5 cells were seeded on fibronectin-coated, 8-well chamber slides (10% human fibronectin, Sigma-Aldrich) in 500 μ L endothelial basal media (Clonetics/Lonza) supplemented with 20% FBS (Invitrogen, Carlsbad, CA, USA), human endothelial growth factor (hEGF), hydrocortisone, gentamycin/amphotericin B (GA) and bovine brain extract (BBE)(SingleQuot®, Clonetics/Lonza) under standard cell culture conditions (37°C, 5% CO₂)¹⁵⁵. On day 7, cells were incubated with Dil-acLDL (2.4 μ g/mL, Invitrogen) in media for 3 h. After media was removed, cells were washed three times with PBS, fixed in 4 % PFA/PBS (pH 7.4; RT) for 10 min, and then incubated with FITC-UE-lectin (50 μ g/ mL, Sigma-Aldrich) at 37°C for 30 min. For labeling of Flk-1 and Sca-1, cells were incubated with FITC-Sca-1 (1:25; BD BioSciences) and APC-Flk-1 (1:15, BD BioSciences) antibodies for 1 h at RT. Cells were washed three times with PBS before slides were stained with DAPI-

containing *Slow Fade*[®] Gold anti-fade reagent (Invitrogen). Dil-acLDL and FITC-UE-lectin double-positive cells were counted in 10 random fields.

Blood counts and plasma biochemistry

After select exposures, 100 µl of blood were used for complete blood count analysis (CBC; Hemavet 500, Coulter Counter, Oxford, CT). Plasma total, HDL and LDL cholesterol, triglycerides, total protein, albumin (Cholesterol CII Enzymatic Kit; L-Type TG-H Kit; Bradford reagent, bromocresol green, Wako, Richmond, VA, USA), ALT, AST (Infinity, ThermoElectron, Louisville, CO, USA), CK and LDH (Promega, Madison, WI, USA) levels were measured using commercially available assay reagents as indicated. Assays were performed using calibrated standards in 96-well plates or using a Cobas Mira Plus 5600 Autoanalyzer (Roche, Indianapolis, IN, USA). Plasma NO_x level (stable NO metabolites, nitrate and nitrite; 25 µl) was measured fluorimetrically according to manufacturer's instructions (NO_x kit; Calbiochem, Darmstadt, Germany).

Isolated aorta studies

Thoracic aorta were isolated and tested for intact VEGF signaling. Isolated aortas were incubated in autologous plasma and exposed to VEGF₁₆₅ for 15 min before freezing.

Vascular reactivity

Thoracic aortas were isolated and vascular reactivity was assayed as described previously ¹¹. Briefly, one 3-4-mm ring per mouse was hung on stainless steel hooks in 15-ml water-jacketed organ baths in physiological salt solution (PSS) bubbled with 95% O₂ and 5% CO₂ at 37°C. The composition of PSS was (in mM): NaCl, 130; KCl, 4.7; MgSO₄·7H₂O, 1.17; KH₂PO₄, 1.18; NaHCO₃, 14.9; CaCl₂, 2.0; glucose, 5.0; pH 7.4. Rings (≈1 g loading tension) were contracted with 100 mM potassium solution (2 times) followed by cumulative concentrations of phenylephrine (PE; 0.1 nM - 10 μM). PE-precontracted rings were relaxed with cumulative concentrations of acetylcholine (ACh; 0.1 nM - 10 μM) or of sodium nitroprusside (SNP; 0.1 nM -10 μM) to measure endothelium-dependent or –independent relaxation, respectively. Vessel contraction was quantified as mg tension, active stress (in mN/mm²) or normalized as percentage of the maximum PE contraction. Relaxation was calculated as percentage reduction of PE-induced tension. The effective concentration producing 50% response (EC₅₀) was assessed by normalizing cumulative concentration responses to 100%, plotting the response vs. the log [molar] agonist, and then interpolating the EC₅₀ value.

Western blot analyses

Bone marrow aspirates were centrifuged and pellets were suspended in lysis buffer (25 mM HEPES, pH 7.0; 1 mM EDTA; 1 mM EGTA; 1% Nonidet P40; 1% SDS) supplemented with 1:100 protease inhibitor cocktail (Sigma-Aldrich), 1:100

phosphatase inhibitor cocktail and 50mM N-ethylmaleimide (NEM; Pierce, Rockford, IL, USA). After sonication, samples were centrifuged (4,000xg, 15 min, 4°C) and supernatants were used for detection of protein-acrolein adducts by Western blotting. Pulverized aortas were lysed in RIPA buffer (50mM Tris•HCl, pH 7.4, 150mM NaCl, 1mM EDTA, 0.25% sodium desoxycholate, 1% NP-40; 1:100 protease inhibitor cocktail; 1:100 phosphatase inhibitor), sonicated and centrifuged (13,000xg, 15 min, 4°C). Heart was homogenized in membrane preparation lysis buffer (25mM Tris; 0.5mM EDTA; 0.5mM EGTA; protease inhibitor 1:100 dilution; phosphatase inhibitor, 1:100; pH 7.5) and centrifuged (14,000xg, 15 min, 4°C). Pellet was resuspended in lysis buffer supplemented with 1% NP-40 and incubated for 4h¹⁵⁶. After incubation and second centrifugation, the supernatant was collected as membrane fraction. Lungs were homogenized in ice cold lysis buffer (50mM Tris-HCL; 1mM EDTA; 1mM EGTA; protease inhibitor, 1:100; phosphatase inhibitor, 1:100 dilution; pH 7.4). Lysates were centrifuged (14,000xg, 15 min, 4°C) and supernatants used for Western blotting¹⁵⁷.

Total protein was measured using a commercially available kit (Bradford, Bio-Rad, Hercules, CA, USA). For Western blot analysis of protein-acrolein adducts, protein samples (150 µg) in 5x sample buffer (312.5 mM Tris base, pH 6.8 [Bio-Rad] or, 10% Glycerol, 11.5% SDS, 0.1% Bromphenol) supplemented with 50 mM NEM were separated under non-reducing conditions, whereas all other proteins (30-100 µg) were separated under reducing conditions (buffer supplemented with 50 mM DTT). Briefly, heat-denatured (5 min, 95°C) protein

samples were separated by SDS-PAGE and transferred to PVDF membranes (Bio-Rad). Membranes were processed by standard immunodetection techniques using a rabbit polyclonal antibody against KLH (Keyhole Limpet hemocyanin)-protein-acrolein adduct¹⁵⁸ or using a commercially available antibody against Phospho-Akt (Ser473), Akt, Phospho-eNOS (Ser1177), eNOS (BD Biosciences, 1:1000), Phospho-p42/44 (Thr202/Tyr204), p42/44 (1:1000; Cell Signaling) or VEGFR-2 (1:250; Santa Cruz) or MMP-9 (1:5000, Chemicon, Millipore, Temecula, CA, USA). Western blots were developed using ECL[®] plus reagent (Amersham Biosciences, Piscataway, NJ, USA) and detected with a Typhoon 9400 variable mode imager (Amersham Biosciences). Actin antibody (1:2000, Sigma-Aldrich) detection or amido-black staining was used as loading control. Quantification of band intensities was performed using Image Quant TL software (Amersham Biosciences).

Histology and immunohistochemistry

Pelvic bones were formalin-fixed (10 % neutral buffered formalin) for 24h, decalcified in Immunocal[®] (Decal Chemical Corp., Tallman, NY) for 6h, and washed in running tap water (0.5h). Paraffin-embedded sections (5 µm) of pelvic bones were stained with hematoxylin/eosin, anti-protein-acrolein adduct (rabbit polyclonal; 1:1,000), or normal rabbit or goat IgG (as negative controls)¹⁵⁸. A goat anti-rabbit secondary antibody and a Vector Elite staining kit with diaminobenzadine (Dako) as chromagen were used.

MoFlow cell sorting and ID1 staining

Whole blood (1000 µl) was lysed (10 ml; BD PharmLyse, BD BioSciences, San Jose, CA, USA; 10 min, RT) and after centrifugation (5 min, 400xg, RT), the supernatant was aspirated and the lysing/centrifugation/aspiration steps were repeated. The cell pellet was resuspended in 1 % FBS/PBS and divided into 2 equal fractions (i.e., isotype control or Flk-1/Sca-1 labeling). After centrifugation (5 min, 400xg, RT), mononuclear cells were re-suspended in 1% FBS/PBS with murine CD16/CD32 Fc Block (0.5 µg; BD Biosciences), incubated for 10 min on ice, followed by 30 min incubation on ice with anti-Sca-1 (FITC, 1 µg; BD BioSciences) and anti-Flk-1 (1 µg, BD BioSciences) antibodies or appropriate isotype control (1 µg; BD BioSciences). Cells were then washed with 1% FBS/PBS and centrifuged (5 min, 400xg, RT). Based on forward and side scatter, small non-debris events were gated electronically and 200 labeled Flk-1+/Sca-1+ cells were sorted into a 1.7 uL centrifuge tube containing 2 % FBS/PBS using MoFlo® (Beckman-Coulter). Cells were fixed with 1 % paraformaldehyde for 5 min. on ice. Triton-100X was then added to increase the permeability for 5 min. on ice. Cells were then washed using 0.1 % Triton-100X wash buffer, spun down at 3rpm for 5 min. then aspirated. Cells were then stained with murine anti-ID1 (1:50; Santa Cruze) for 30 min. in 0.1 % Triton on ice, then washed with 0.1% Triton wash buffer and aspirated. Cells were stained using secondary anti-rabbit PE fluorophore (1:25;). Cells were once again washed with 2 % PBS/FBS spun down and aspirated. Then 100uL of 2 % FBS/PBS was added and then cell spun onto a cytospin slide at 300Xg. Slides mounted in DAPI-containing *Slow Fade*®

Gold anti-fade reagent (Invitrogen), and viewed using a Zeiss confocal microscope.

Results

Flk⁺/Sca⁺ Cell characterization

To better understand the selective nature of acrolein inhibition, a more extensive screen of antigenic markers on Flk-1⁺/Sca-1⁺ cells (stem/progenitor and B-/T-cell markers) from saline and VEGF/AMD3100-treated mice was conducted by flow cytometry. VEGF/AMD3100 significantly increased Flk-1⁺/Sca-1⁺ cells expressing a variety of stem and B-/T-cell antigenic markers especially cells co-expressing CD31/CD45R(B220)/CD133 and CD31/CD45R(B220)/CD19 (**Fig. 2A-F**).

Effects of acrolein on circulating Flk-1⁺/ Sca-1⁺ cells

Analysis of the mononuclear cell population of peripheral blood showed that Flk-1⁺/Sca-1⁺ cells were a relatively small (3-5 μ m), sub-lymphocytic, non-debris cell population with further characterization showing stem and endothelial cell markers (**Figs. 3A-2E**).

Figure 2

□ saline ■ VEGF/AMD3100

C T and B cell markers

D

E

F

Figure 2: Hierarchical relationships of Flk⁺/Sca⁺ Cells. : A-B, Progenitor cell antigens, and, C-D, lymphocytic differentiation marker antigens expressed on Flk-1/Sca-1 cells. ,E, Progenitor cell antigens, and, F, lymphocytic differentiation marker antigens expressed on Flk-1/Sca-1 cells.

Figure 3

B.

C.

Figure 3: Characterization of Flk-1⁺/Sca-1⁺ progenitor cells by flow

cytometry and microscopy. Representative dot plots of **A**, forward- (FSC) and side-scatter (SSC) of circulating cells and size beads indicating an approximate size range of Flk-1⁺/Sca-1⁺ cells (3-5 μ m) Dot plots of SSC and PerCP-CD45 fluorescence of Flk-1⁺/Sca-1⁺ cells representing CD45 positive (CD45⁺, 98.5 $\pm$ 0.3 %) and CD45 negative cells (CD45⁻, 1.2 $\pm$ 0.3 %). Dot plots of FSC and PE-CXCR4 fluorescence of Flk-1⁺/Sca-1⁺ cells that are CXCR4 positive (97.2%). **B**, Brightfield and confocal images of Flk-1/Sca-1-double positive cells sorted by MoFlo for immunofluorescence detection of antigen co-localization. **C**, Confocal image of sorted Flk-1⁺/Sca-1⁺ cells stained with Id1 antibody. Nuclei were stained with DAPI.

Acrolein exposure induced a selective and dose-dependent decrease of circulating Flk-1⁺/Sca-1⁺ cells (**Fig. 4C**). The number of Flk-1⁺/Sca-1⁺ cells was significantly reduced by 77±10 % following exposure to 1 ppm acrolein for 4 consecutive days (6h/d; air: 413±109 cells per 50,000 events; acrolein: 97±19 cells per 50,000 events; n=8, 8; *p*<0.05). However, acrolein exposures (1ppm; 6h/d) for 1, 2, or 4 days did not affect the number of circulating Sca-1⁺ cells (**Fig. 4D**). Exposure of mice to a higher dose of acrolein (5 ppm; 6h), though, decreased Flk-1⁺/Sca-1⁺ cells significantly by 43±10 % compared to air-exposed mice (air: 4.3±0.7 cells/ μ L; acrolein: 2.4±0.4 cells/ μ L, n=8, 8; *p*<0.05; **Fig. 4E**). A brief acrolein exposure (5ppm; 2h), however, did not alter circulating Flk-1⁺/Sca-1⁺ cells (air: 6.3±2.4 cells/ μ L; acrolein: 7.7±1.4 cells/ μ L, n=4, 4; *p*<0.05) or Sca-1⁺ cell level. Acrolein exposure of 0.5 ppm (6h/d x 4d) had no effect on the number of circulating Flk-1⁺/Sca-1⁺ or Sca-1⁺ cells as compared with air controls indicating a narrow threshold range of acrolein action. Furthermore, mice allowed 7 days of recovery after a 4-day acrolein (1 ppm, 6h/d) exposure had similar numbers of circulating Flk-1⁺/Sca-1⁺ cells compared with air-exposed controls (**Fig. 4E**). Although 1 ppm acrolein (6h/d x 4d) did not alter plasma lipids (**Table 7**) or leukocyte blood cell counts, a 6h exposure to a higher acrolein level (5 ppm) significantly decreased leukocyte blood cell counts (**Table 8**). Acrolein exposure increased Annexin V/7AAD staining in circulating Flk-1/Sca-1 double positive cells (**Fig. 4G**).

Figure 4

B.

□ Air ■ Acrolein

□ Air ■ Acrolein

□ Air ■ Acrolein

Figure 3: Effects of acrolein on circulating progenitor cells. A, Flow cytometry analysis of forward- (FSC) and side-scatter (SSC) of peripheral blood mononuclear cells of mice breathing filtered air or acrolein (1ppm, 6h/day, 4 days). **B,** Representative two color (APC-Flk-1 and FITC-Sca-1) flow cytometry dot plots of circulating Flk-1⁺/Sca-1⁺ cells in peripheral blood of mice after 4 days of exposure to filtered air or acrolein. Levels of **C,** Flk-1⁺/Sca-1⁺ cells and **D,** Sca-1⁺ cells in peripheral blood after breathing filtered air or acrolein for indicated days (n=8-12). **E,** Flk-1⁺/Sca-1⁺ cells in blood obtained from mice breathing filtered air or 5 ppm acrolein for 2 or 6h. **F,** Flk-1⁺/Sca-1⁺ cells in blood of mice breathing air or 1 ppm acrolein (6h/d, 4d) and after 7 days recovery **G,** Representative dot plots of APC-Flk-1 and FITC-Sca-1 double positive cells stained for 7-AAD (necrosis marker) and Annexin V (apoptosis marker), and quantification of percentage Annexin V and 7-AAD stained cells from air and acrolein-exposed mice. (n=4; * $p < 0.05$).

Table 7

Blood and plasma variables in mice exposed to air or acrolein.				
	Air	Acrolein, 1ppm	Air	Acrolein, 5ppm
Cholesterol ^a	73.2±3.1	70.1±2.7	60.9±4.1	52.2±0.3
HDL ^a	54.6±3.2	45.6±3.6	45.8±3.2	50.2±0.3
LDL ^a	10.3±0.2	10.9±0.7	8.1±0.6	9.4±0.2
Triglycerides ^a	17.9±3.1	14.5±1.9	24.8±4.7	17.7±0.3
TP ^b	4.1±0.1	3.8±0.1	3.7±0.1	3.7±0.8
ALB ^b	2.7±0.1	2.7±0.1	2.5±0.1	2.5±0.9
LDH ^c	167.6±17.2	127.2±9.9	264.5±26.1	167.5±12.1 [*]
CK ^c	261.6±21.2	245.1±30.1	198±8.2	247±5.3
ALT ^c	56.5±7.8	48.2±6.4	25.7±3.6	25.8±0.9
AST ^c	42.2±7.3	25.9±3.9	62.8±8.7	69.3±10.7

Male, 12-14 week old mice were exposed to air or 1 ppm acrolein (6h/d, 4d, n=11 mice/group) or 5 ppm acrolein (6h/d, 1d, n=4 mice/group). Values are mean ± SEM. Abbr: HDL, high density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol; TP, total protein; ALB, albumin; LDH, lactate dehydrogenase; CK, creatine kinase; ALT, alanine aminotransferase; AST, aspartate aminotransferase. Units: ^a = [mg/dL], ^b = [g/dL], ^c = [U/L]; ^{*}, $p < 0.05$ vs. Air-matched group.

Table 8

Complete blood count in mice exposed to air or acrolein.

	Air	Acrolein, 1ppm	Air	Acrolein, 5 ppm
WBC ^a	2370±585	1775±293	2690±381	935±33*
NE ^a	362±64	355±51	472±61	138±16*
LY ^a	1942±518	1448±262	2135±325	752±21*
MO ^a	57±15	60±10	78±15	35±5*
RBC ^b	9.03±0.30	8.92±0.24	8.18±0.27	8.24±0.26
Hct (%)	42.2±1.2	41.4±1.3	37.4±1.1	36.9±1.2
Hb ^c	11.1±0.3	11.1±0.3	11.3±0.1	12.2±0.2
MCV ^d	46.9±0.3	46.3±0.6	45.7±0.3	44.8±0.4
MCH ^e	12.2±0.1	12.4±0.2	14.3±0.1	14.4±0.2
MCHC ^c	26.1±0.1	26.8±0.5	31.2±0.3	32.1±0.3
RDW (%)	17.3±0.5	17.5±1.7	17.3±0.3	16.7±0.1
PLT ^a	835±50	789±46	845±47	758±28
MPV ^d	3.8±0.1	3.8±0.1	4.1±0.1	3.9±0.2

Male, 12-14 week old mice were exposed to air or 1 ppm acrolein (6h/d, 4d, n=8 mice/group) or 5 ppm acrolein (6h/d, 1d, n=4 mice/group). Values are mean ± SEM. Abbr: WBC, white blood cells; NE, neutrophils; LY, lymphocytes; MO, monocytes; RBC, red blood cells; Hct, hematocrit; Hb, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; PLT, platelets; MPV, mean platelet volume. Units: ^a = [x10³/μL], ^b = [x10⁶/μL], ^c = [g/dL], ^d = [fL], ^e = [pg]; *, p<0.05 vs. air-matched group.

Acrolein exposure increased number of bone marrow-derived cells (BMDC)

To further evaluate the condition of the EPC within the bone marrow culture was performed. Acrolein exposure increased the number of Dil-acLDL/FITC-UE-lectin-double positive cells in culture on day 7 (**Fig. 5**). After 7 days of culture, outgrowths from bone marrow-derived Dil-acLDL/FITC-UE-lectin-double positive cells (**Fig. 5B**), had more cells from acrolein-exposed mice than air-exposed as indicated by DAPI staining (nuclear; blue fluorescence; **Fig. 5B**). Furthermore, the cells positive for Dil-acLDL (red fluorescence) and FITC-UE-lectin (green fluorescence) were increased by acrolein exposure compared with air-exposed controls (**Fig. 5B**). Image overlay showed co-localization of EPCs markers (Dil-acLDL, FITC-UE-lectin) indicating that acrolein enhanced the proliferative capacity of endothelial progenitor-like cells (orange) in culture. Compared with cells from air-exposed (control) mice, there was a 3- to 3.6-fold increase in the Dil-acLDL/ FITC-UE-lectin double positive cells isolated from mice exposed to acrolein (5 ppm, 6h; **Fig. 5C**; 1 ppm, 4d; **Fig. 5D**) after 7 days in culture. The identification of these proliferating cells as endothelial progenitor-like cells was also supported by immunocytochemical staining of cultured BMDC for APC-Flk-1 and FITC-Sca-1 (i.e., markers used in flow cytometry) on culture day 7. Thus, in comparison with air, acrolein exposure (1 ppm, 4d) increased Flk-1⁺/ Sca-1⁺ cells by 1.7-fold. Taken together, these data showed that acrolein exposure enhanced bone marrow-derived endothelial progenitor-like cell proliferation in culture.

Figure 5

A

B

C

Figure 5: Acrolein increases proliferation of bone marrow-derived cells.

Representative fluorescence images (upper panels) and the number of (lower panels) of Dil-acLDL⁺/FITC-UE-lectin⁺ cells isolated from bone marrow of mice exposed to air or **B**, 5 ppm acrolein for 6h **C**, or 1 ppm acrolein (6h/d, 4 days). Mononuclear cells were isolated from the bone marrow and grown in culture for 7 days. Cells were labeled with Dil-acLDL and FITC-UE-lectin and nuclei were stained with DAPI. Merged images show co-localization of both markers in orange (i.e., double-positive cells). **D**, Representative fluorescence images (upper panel) and analysis (lower panel) of Flk-1⁺/Sca-1⁺-cells from mice exposed to air or 1 ppm acrolein (6h/d, 4d) after day 7 of culture. Cells were labeled with FITC-Flk-1 and APC-Sca-1 and with DAPI for nuclear staining. Merged images show co-localization of the markers. Changes in the number of Dil-acLDL⁺/FITC-UE-lectin⁺ and Flk-1⁺/Sca-1⁺ cells are presented as fold increase in double positive cells compared with controls (n=4; * $p < 0.05$, air vs. acrolein exposure).

Protein acrolein adducts

To determine whether acrolein could directly reach the bone marrow cells, we examined the abundance of protein-acrolein adducts in plasma and bone marrow by Western blotting and immunohistochemistry (**Fig. 6**). Protein-acrolein adducts were detected by Western blotting of plasma (**Fig. 6A**) and bone marrow lysates (**Fig. 7B**) of air- and acrolein-exposed (1 ppm, 4d) mice. Abundant acrolein adducts were observed in proteins with molecular weights of ≈ 250 and ≈ 150 kDa in plasma and of ≈ 250 , ≈ 37 , ≈ 18 and ≈ 17 kDa in the bone marrow. Acrolein inhalation led to a significant increase in the intensity of several bands, including a 2.5-fold increase in a plasma protein band of ≈ 150 kDa and a 2-fold increase for a bone marrow protein band of ≈ 250 kDa, compared with controls (**Fig. 6**). Similarly, immunohistochemical staining of femur (proximal head) cross-sections with protein-acrolein adduct antibody was substantially stronger in acrolein-treated (1 ppm, 4d) bone compared with controls (**Fig. 6C**). Adduct distribution was quite heterogeneous in both acrolein- and air-exposed mice, however, distinct, intense positive staining of protein-acrolein adducts was observed in blood vessels within the connective tissue as well as in the ostea of the bone trabeculae (**Fig. 6C**, see black arrows). More diffuse staining was present in the bone marrow matrix, i.e., hematopoietic tissue (**Fig. 6C**, yellow arrows), where some focal, intense staining was localized to the largest bone marrow cells within the endosteum at the border between the bone marrow cavity and compact bone (**Fig. 6C**, yellow arrow).

Figure 6

C. Air

Acrolein

IgG controls:

Air

Acrolein

Figure 6: Formation of protein-acrolein adducts. Western blot analyses of protein-acrolein adducts in **A**, plasma or **B**, bone marrow extracts obtained from mice breathing air or 1 ppm acrolein for 4 days (6h/d). Amido-black stain (plasma) and actin detection (bone marrow) were used as loading controls. **C**, Bone marrow immunohistochemistry of acrolein antibody staining, air v. acrolein inhalation. Data are presented as fold increase of protein-acrolein adducts after acrolein exposure compared with air-exposed mice (* $p < 0.05$; $n = 4-5$).

Endothelial dysfunction and NO signaling

The number of circulating Flk-1⁺/Sca-1⁺ cells is in continuous flux due to the competing processes of mobilization (e.g., bone marrow/spleen) and recruitment to sites of injury (e.g., endothelium). To determine if the acrolein-induced decrease in circulating Flk-1⁺/Sca-1⁺ cells was due to increased recruitment to vascular sites of injury, endothelium function was measured in isolated aorta following exposures. No evidence of frank vascular smooth muscle or endothelial cell dysfunction was found after 1 ppm acrolein exposure (6h/d x 4d; **Table 9**), which is consistent with a previously published study using 1 ppm acrolein exposure in C57BL/6 mice ¹⁵³. In contrast, brief exposure of mice to 5 ppm acrolein (6h) induced a modest, yet significant, rightward shift in the acetylcholine-mediated vasorelaxation in phenylephrine-precontracted aorta indicating endothelial dysfunction (**Fig. 7A; Table 9**). There was no change in total percentage of NO-donor-mediated (sodium nitroprusside, SNP) relaxation, although there was a significant leftward shift in aortic sensitivity to SNP (EC₅₀: air, 16±3 nM; acrolein, 9±1 nM, n=8, 8, *p*<0.05; **Table 9**) – further indicating an endothelium-specific dysfunction.

Table 9

Vascular effects of air or acrolein exposure in mice.				
	Air	Acrolein, 1ppm	Air	Acrolein, 5 ppm
Phenylephrine (PE)				
Tension (mg)	738±74	659±62	1037±156	723±83
Active Stress (mN/mm ²)	8.9±0.9	8.1±1.2	na	na
EC ₅₀ (nM)	149±15	149±15	151±33	160±26
pD ₂	6.84±0.05	6.84±0.05	6.88±0.09	6.83±0.07
Acetylcholine (ACh)				
Relaxation (% PE)	-63±5	-64±7	-66±8	-66±3
EC ₅₀ (nM)	136±21	195±82	113±20	180±24*
pD ₂	6.92±0.09	6.92±0.15	7.00±0.10	6.77±0.06†
Sodium Nitroprusside (SNP)				
Relaxation (% PE)	-121±4	-121±6	-103±3	-104±4
EC ₅₀ (nM)	15±3	18±3	16±3	9±1*
pD ₂	7.91±0.10	7.80±0.07	7.84±0.09	8.08±0.06*

Male, 12-14 week old C57BL/6 mice were exposed to air or acrolein (1ppm, 6h/d, 4d or 5ppm, 6h/d, 1d). Values are mean ± SEM. Abbr: HI K⁺, 100 mM potassium buffer; na, not available; EC₅₀ = effective concentration producing 50% response; pD₂, -log[EC₅₀]; * p≤0.05 vs. matched air control (n=7-8 mice/group); † 0.10>p>0.05 vs. matched-air control.

To examine whether acrolein-induced endothelial dysfunction was due to changes in nitric oxide (NO) bioavailability, we examined changes in plasma of the stable end products (nitrate and nitrite) of the L-arginine/nitric oxide biosynthetic pathway otherwise known as NO_x, total eNOS, and the phosphorylation status of eNOS and Akt, which are critical mediators of endothelial signaling and function (**Fig. 8**). Acrolein exposure at 1 ppm (6h/d x 4d) or 5ppm (6h) significantly decreased plasma NO_x levels by 15 % (air: 100±8 %; acrolein: 85±4 %; n=8, 8; *p*<0.05) or 22 % (air: 100±8 %, acrolein; 78±4 %, n=8, 8; *p*<0.05), respectively (**Fig. 7C**). The acrolein-induced suppression of plasma NO_x level after 4-day acrolein exposure was reversed by 7 days of recovery in acrolein-free air (**Fig. 7A**). Similarly, the recovery of plasma NO_x level coincided with a return of circulating Flk-1⁺/Sca-1⁺ cell numbers in acrolein-exposed mice to the air control level (**Fig. 4F**).

Because acrolein exposure decreased both plasma NO_x and Flk-1⁺/Sca-1⁺ cell levels, eNOS protein content was measured in lysates of lung, heart and aorta (**Fig. 7C**) by Western blotting. Additionally, VEGFR-2 abundance was measured by Western blotting in bone marrow lysates. As shown in **Figure 7C**, acrolein exposure (1 ppm; 6h/d x 4d) did not alter eNOS protein abundance in lung, heart or aorta, nor did it change VEGFR-2 protein level in the bone marrow.

Figure 7

Figure 7: Endothelial response to acrolein exposure. A, Endothelial function assay from aorta of mice exposed to 5ppm 6hr acrolein (n=4, * $p<0.05$) **B,** NO_x levels in plasma of mice breathing filtered air or acrolein (n=8, * $p<0.05$). **C,** Western blot analyses of eNOS or VEGFR2 in lysates of lung, heart, aorta or bone marrow of mice breathing filtered air or 1 ppm acrolein (6h/d, 4d). Group densitometry data are presented as mean $\pm$ SEM (n=4). * $p<0.05$.

Figure 8

Figure 8: Effects of acrolein on enzymatically important constituents of EPC mobilization. A, Representative Western blots of phospho-eNOS (Ser1177), phospho-Akt (Ser473), eNOS and Akt in lysates of aortas obtained from animals exposed to air or Acrolein (5 ppm/6h) **B,** Akt and **C,** MMP-9 in lysates of bone marrow obtained from animals exposed to air or acrolein (5 ppm/6h). Group data are presented as mean $\pm$ SEM, n=4.

VEGF+AMD3100-induced mobilization of Flk-1⁺/Sca-1⁺ cells

Because acrolein exposure decreased circulating Flk-1⁺/Sca-1⁺ cells in the absence of frank aortic endothelial dysfunction, we tested if acrolein perturbed progenitor cell mobilization. Acrolein alone (1 ppm, 4d) significantly decreased Flk-1⁺/Sca-1⁺ cells compared with air controls (air + saline: 1.5±0.3 cells/ μL; acrolein + saline: 0.7±0.1 cells/ μL; n=4, 4; *p*<0.05; **Fig. 9A**). As shown before, VEGF+AMD3100 treatment led to a 3-fold increase in circulating Flk-1⁺/Sca-1⁺ cells¹⁵⁹. Acrolein inhalation significantly and specifically impeded VEGF+AMD3100-induced Flk-1⁺/Sca-1⁺ mobilization (normalized to air control: air+VEGF+AMD3100: 3.0±0.4 cells/ μL; acrolein+VEGF+AMD3100: 0.4±0.1 cells/ μL, n=8, 8; *p*<0.05; **Fig. 9A**). Although VEGF+AMD3100 treatment significantly increased Sca-1⁺ cells (normalized to air control: air+VEGF+AMD3100: 2.8±0.4 cells/ μL; acrolein+VEGF+AMD3100: 2.9±0.4 cells/ μL, n=8, 8) and overall white blood cell counts, indicating general mobilization of both stem cells and leukocytes, these effects were unaltered by acrolein exposure (**Table 10**), indicating that acrolein specifically decreased the endothelial progenitor cell population.

Co-treatment with VEGF+AMD3100 is known to modify mobilization without changing bone marrow cell function¹⁵⁹. Treatment with VEGF+AMD3100 did not alter aortic eNOS or Akt protein levels as analyzed by Western blotting regardless of exposure (**Fig. 9B-C**).

Our results indicate that acrolein at 1 ppm inhibits mobilization of Flk-1⁺/Sca-1⁺ cells via diminished NO bioavailability and impaired VEGF/ CXCR4 signaling.

Henceforth, we studied VEGF signaling in aortas isolated from air- or acrolein- (1 ppm, 6h/d x 4d) exposed mice. Aortas were incubated *ex vivo* with VEGF (20 ng/mL) for 15 min and phosphorylation status of Akt (**Fig. 9D**), eNOS (**Fig. 9E**) and ERK (**Fig. 9F**) proteins was analyzed. In control aortas, VEGF stimulated phosphorylation of Akt (2-fold) and eNOS (2-fold) – an effect absent in aortas of acrolein-exposed mice (**Figs. 9D and 9E**). Although VEGF-induced phosphorylation of ERK was not influenced by inhalation exposure, acrolein-exposed aortas had a significantly greater level of basal phospho-ERK (p42/44) (**Fig. 9F**). These findings indicated that acrolein exposure significantly impaired VEGF/ CXCR4 signaling – an effect that could account for the diminished levels of basal and VEGF+AMD3100-mobilized Flk-1⁺/ Sca-1⁺ progenitor cells, as well as, the decreased plasma NO_x level

Figure 9

A

B **D** **E**

F

Phospho-
(Thr 202/Tyr 204)

Figure 9: Effect of acrolein on the mobilization of Flk-1⁺/Sca-1⁺ cells. A,

Representative two color (Flk-1 and Sca-1) flow cytometry dot plots (upper panel) of circulating cells in the peripheral blood of mice breathing filtered air or 1 ppm acrolein (6h/d, 4 d) after combination of VEGF/AMD3100 treatment. Levels of Flk-1⁺/Sca-1⁺ cells per μ l blood (left y-axis) and Sca-1⁺ cells (right y-axis; fold-change relative to air control) of mice breathing filtered air or 1 ppm acrolein (6h/d, 4d) after treatment with VEGF/AMD3100 (* $p < 0.05$, $n = 8$). **B**, Representative Western blots and densitometric analysis of Akt in bone marrow lysates obtained from mice after saline or VEGF/AMD3100 treatment ($n = 4$). **C**, Representative Western blots and densitometric analysis of eNOS in lysates of aortas obtained from mice breathing air or acrolein (1 ppm, 6h/d, 4d) after saline or VEGF+AMD3100 treatment ($n = 4$). **D-F**, Analysis of VEGF signaling in aortas isolated from mice breathing air or acrolein (1 ppm, 6h/d, 4d) and treated with VEGF in autologous plasma for 15 min. Representative Western blots and analysis of membranes probed for **D**, phospho-Akt (Ser473)/total Akt; **E**, phospho-eNOS (Ser1177)/total eNOS; and **F**, phospho-ERK (Thr202/Tyr204) /total ERK ($n = 3-7$; * $p < 0.05$).

Table 10

Complete blood counts in mice exposed to air or acrolein.

	+Saline		+VEGF+AMD3100	
	Air	Acrolein, 1ppm	Air	Acrolein, 1ppm
WBC ^a	1365±210	2137±368 [*]	7630±810 [*]	8030±475 [†]
NE ^a	422±79	497±179 [*]	2345±362 [*]	2821±280 [†]
LY ^a	897±165	1600±192 [*]	5042±494 [*]	4981±285 [†]
MO ^a	35±11	61±32 [*]	232±17 [*]	220±16 [†]
RBC ^b	6.72±0.80	8.68±0.33	7.94±0.46	8.32±0.47
Hct (%)	32.2±2.3	39.2±1.5	34.9±1.9	37.1±2.2
Hb ^c	9.1±1.2	11.9±0.6	10.8±0.7	11.1±0.5
MCV ^d	44.7±0.7	45.1±0.2	44.5±0.3	44.2±0.4
MCH ^e	13.3±0.3	13.8±0.1	13.4±0.2	13.5±0.3
MCHC ^c	29.9±0.5	30.5±0.3	30.8±0.9	30.2±0.5
RDW (%)	16.5±0.3	17.3±0.2	17.8±0.2	17.3±0.3
PLT ^a	799±122	858±76	917±91	809±78
MPV ^d	4.4±0.1	4.2±0.1	4.4±0.1	4.2±0.1

Male, 12-14 week old mice were exposed to air or 1 ppm acrolein (6h/d, 4d, n=8 mice/group). Values are mean ± SEM. Abbr: WBC, white blood cells, NE, neutrophils, LY, lymphocytes, MO, monocytes, RBC, red blood cells, Hct, hematocrit, Hb, hemoglobin, MCV, mean corpuscular volume, MCH, mean corpuscular hemoglobin, MCHC, mean corpuscular hemoglobin concentration, RDW, red cell distribution width, PLT, platelets, MPV, mean platelet volume. Units: ^a = [x10³/μL], ^b = [x10⁶/μL], ^c = [g/dL], ^d = [fL], ^e = [pg]. ^{*}, p<0.05 vs air-matched group, [†], p<0.05 vs Air+VEGF/AMD3100 matched group.

Discussion

Results of the current study showed that inhalation of acrolein suppressed the level of Flk-1⁺/Sca-1⁺ cells in the peripheral blood and prevented their mobilization by VEGF/SDF-1. The effects of acrolein were specific because acrolein decreased Flk-1⁺/Sca-1⁺ cells without affecting the level of Sca-1⁺ cells, indicating a unique sensitivity of this heterogeneous cell population to acrolein. Effects of acrolein inhalation were, however, transient, and recovery in an acrolein-free environment for 7 days led to complete restoration of the level of these cells in the peripheral blood. Moreover, the decrease in Flk-1⁺/Sca-1⁺ cells was associated with decreased plasma NO_x level, prevention of VEGF/CXCR4-induced mobilization of Flk-1⁺/Sca-1⁺ cells and inhibition of VEGF-induced Akt and eNOS phosphorylation – a constellation of changes impinging on vascular function and repair.

Our results also indicated that the bone marrow could be a specific locus of acrolein action. We found that acrolein inhalation increases accumulation of protein-acrolein adducts in the plasma and the bone marrow, suggesting that despite its high reactivity and rapid metabolism, acrolein was delivered from the lung into the systemic circulation and to distal vascular sites. Our previous studies have shown that exposure to tobacco smoke or acrolein resulted in the formation of protein-acrolein adducts in non-pulmonary sites^{153, 160}. Thus, acrolein appeared to cross from the lungs and directly induce bone marrow toxicity.

Although acrolein inhalation decreased levels of circulating Flk-1⁺/Sca-1⁺ cells, it increased the number of bone marrow-derived Flk-1⁺/Sca-1⁺ cells that accumulated acLDL and were positive for *Ulex* lectin binding in culture. Because the number of cells that grow out of the bone marrow on fibronectin-coated dishes is indicative of their population in the bone marrow ¹⁵⁹, it appeared likely that exposure to acrolein increased proliferation of Flk-1⁺/Sca-1⁺ cells in the bone marrow, while preventing their egress into the circulation. Alternatively, decreased mobilization by acrolein could indirectly result in the expansion of the bone marrow population of these cells. Nevertheless, this increase in levels of Flk-1⁺/Sca-1⁺ cells in the bone marrow indicated that acute acrolein exposure did not permanently impair the growth or viability of these cells but that it prevented their mobilization from the bone marrow. We suggest that this defect could be due to alterations in VEGF/SDF-1 signaling, but it also may be related to other specific processes such as cell release from the bone marrow stroma by matrix metalloproteases (MMPs), which is a NO-dependent process. Indeed, our measurements showed that exposure to acrolein is associated with a decreased level of active MMP-9 (73 kDa) in bone marrow lysate (**Fig. 8C**).

Depletion of Flk-1⁺/Sca-1⁺ cells in the peripheral blood in acrolein-exposed mice could also be due induction of cell death. Our measurements showed that even brief exposure (2h) to acrolein resulted in a significant increase in the markers of cell death within the circulating Flk-1⁺/Sca-1⁺ but not the Sca-1⁺ cell population (**Fig. 4**). These observations reinforce the view that the Flk-1⁺/Sca-1⁺ population is uniquely sensitive to acrolein; however, persistent suppression of

the steady-state levels of circulating Flk-1⁺/Sca-1⁺ cells (**Fig. 4**) implies that the primary defect was in the bone marrow because the depletion of these cells, due to increased cell death or increased recruitment to sites of injury, was not adequately compensated via mobilization from the bone marrow.

Because acrolein is one of the most reactive and toxic components of tobacco smoke, it is not surprising that many effects we observed with acrolein inhalation are consistent with findings of tobacco smoke exposure. Our observation that acrolein induced endothelial injury was consistent with evidence showing that exposure to combustion products generated by automobile exhaust¹⁴⁸ or tobacco smoke^{146, 147} impairs endothelial function. Previous studies show that chronic smoking decreases the number of endothelial progenitor cells (EPCs) and that smoking cessation restores EPC levels in human subjects¹⁶¹. The reversible effects of smoking are similar to the reversible effects of acrolein inhalation (see **Fig. 4F**), as well as particulate matter (PM_{2.5}) exposure³¹. In contrast to chronic smoking, brief exposure to secondhand smoke increases EPC levels¹⁶². Although we studied low-dose (0.5ppm) and brief (2h) exposures, we did not observe an increase in Flk-1⁺/Sca-1⁺ cells in acrolein-exposed mice. We speculate that the increase in EPCs upon brief exposure to secondhand smoke may be related to other combustion products such as CO¹⁶³, which by inducing transient or pseudo-hypoxia could increase EPC mobilization. Yet, inhibition of VEGF signaling by secondhand smoke exposure¹⁶⁴ is similar to the effect of acrolein (**Fig. 9**) indicating that some of the pathological effects of secondhand smoke could be mediated by acrolein.

The levels of acrolein used in the current mouse exposure studies are relevant to those encountered by passive and active smokers as well as humans with occupations that include high level or chronic exposure to vehicle exhaust or smoke (e.g., bus drivers, bartenders, firefighters). Given the obvious lack of systemic toxicity (**Tables 5 and 7**) in mice, our observations suggested that acrolein at environmentally relevant levels could suppress circulating Flk-1⁺/Sca-1⁺ cell numbers without inducing overt toxicity. Moreover, it is important to point out that humans are exposed to acrolein not only from combustion, but also from foods and beverages, which contain high levels of acrolein¹⁶⁵. In addition, acrolein is generated endogenously during lipid peroxidation and via myeloperoxidase activity at sites of inflammation¹⁶⁶. Hence acrolein generated endogenously from distal inflamed tissues could also suppress Flk-1⁺/Sca-1⁺ cell mobilization from bone marrow (and other sites) and decrease the circulating levels of these cells. Nevertheless, the pathophysiological significance of acrolein-induced changes in Flk-1⁺/Sca-1⁺ cells remains unclear and deserves additional assessment. Previous studies show that the circulating levels of these cells are increased acutely in response to injury and that chronic suppression of similar progenitor cells is associated with an elevated CVD risk in humans¹⁷. In our analyses, we measured considerable antigenic diversity within the Flk-1⁺/Sca-1⁺ population, which has characteristics of monocytes, T- and B-cells. However, most of these cells were CXCR4⁺ (**Fig. 3C**) and they were mobilized by VEGF/AMD3100 which indicated that this population was likely to be recruited to the site of hypoxic or traumatic tissue injury. Moreover, the cells were also Id1⁺

(**Fig. 2E**). Recent work suggests that Id1 is a selective marker of EPCs because ablation of Id1 in bone marrow-derived cells reduces circulating EPC levels and induces significant defects in angiogenesis as Id1⁺ cells are incorporated in tumor neo-vessels¹⁶⁷. Thus, the Flk-1/Sca-1 population affected by acrolein could be important for wound healing and angiogenesis.

Wound healing is a complex process. It involves the clearance of cell debris, regeneration of injured tissue, and the growth of new blood vessels. This process requires the recruitment of a diverse set of progenitor cells containing both monocytic¹⁶⁸ and endothelial¹⁶⁷ characteristics, which ultimately turn into macrophages and vascular cells to secrete proteases, cytokines and growth factors that promote growth of tissue-resident cells. Therefore, we speculate that suppression of this recruitable and pro-angiogenic cell population by acrolein could lead to deficits in wound healing capacity. Future work is required to identify the effects of acrolein on specific cell populations and how these interfere with individual steps in the wound healing response.

CHAPTER III

EFFECTS OF ACROLEIN ON ANGIOGENESIS

Introduction

Angiogenesis and vasculogenesis are two processes responsible for vascular development in both adults and fetal gestation¹⁶⁹. The process of angiogenesis is the development of new vessels from preexisting vessels, while vasculogenesis refers to the development of a primitive vessel frame work by stem and progenitor cells¹⁷⁰. Angiogenesis is highly regulated and controlled by a balance of stimulators (e.g. vascular endothelial growth factor, angiopoetin-1, and erythropoietin) and inhibitors (e.g. angiostatin, platelet factor 4, interferon- β) of vascular formation¹⁷¹. In ischemic diseases, both hypoxia and inflammation are fundamental for the stimulation of angiogenesis¹⁷². Several disease states such as Alzheimer's¹⁷³, coronary artery disease¹⁷⁴, arthrosclerosis¹⁷⁵, and diabetes¹⁷⁶ have decreased angiogenesis and wound healing ability. Diabetics often suffer from a plethora of angiogenic-related health issues retinopathy¹⁷⁷, neuropathy¹⁷⁸, nephropathy¹⁷⁹, which can lead to ischemic ulcers and gangrene. Aside from diabetes, active and passive smokers are subject to increased cardiovascular health risks brought on by decreased angiogenesis¹⁸⁰. Studies have shown smokers do not respond to ischemic injury or stimuli as do healthy

non-smokers¹⁸¹. Cigarette smoke exposure has been shown to decrease angiogenesis, both *in vivo* and *in vitro*¹⁸². The chemical(s) found in cigarette-smoke that affect angiogenesis in active smokers and those exposed to second hand smoke are yet to be identified. Angiogenesis was thought to develop solely from the sprouting of local endothelial cells, however, the discovery of endothelial progenitor cells (EPCs) revolutionized this dogma⁷⁸. EPCs are vital for ongoing endothelial repair of damaged vessels¹³⁶. Circulating EPCs incorporate into the sites of neovascularization and injury, improving endothelial function¹⁸³. Previous studies have also shown active smokers have decreased numbers of circulating EPCs^{184, 185}, which can account for decreased angiogenesis and cardiovascular health seen in smokers¹⁸⁵. EPCs are positively correlated with wound healing and angiogenic ability¹⁸⁶. Studies from our lab, presented in chapter II of this thesis show EPCs (Flk-1⁺/Sca-1⁺) are decreased in a time and dose dependent manner when mice were exposed to acrolein, much like the phenomenon seen in chronic smokers¹⁸⁷.

In this study we hypothesized that exposure to acrolein could prevent angiogenesis by preventing ischemic injury-induced mobilization of EPCs.

Methods

All Experimental procedures and protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Louisville, and conform to the NIH "Guide for the Care and Use of Laboratory Animals".

Tube forming assay

Human Umbilical Vein Endothelial Cells (HUVECs) were cultured in endothelial basal media (Clonetics/Lonza) supplemented with 20 % FBS (Invitrogen), human endothelial growth factor (hEGF), hydrocortisone, gentamycin/amphotericin B (GA) and bovine brain extract (BBE; SingleQuot®, Clonetics/Lonza) under standard cell culture conditions (37°C, 5 % CO₂). Cells were grown in 10cm dishes until 80 % confluent. Cells were treated with 0, 2.5, or 5mM acrolein in 5mL of Hanks Buffering solution (HBSS⁺) supplemented with 5μM glucose for 30min (37°C, 5 % CO₂), while controls were incubated in 5mL of HBSS⁺ alone. Cells were washed with warm HBSS⁺ and allowed to recover for 4h in fresh media (37°C, 5 % CO₂). Cells were then trypsinized and seeded in a 96 well plate (1X10⁴ cell/well) coated with Matrigel® (BD Biosciences). Pictures were taken every 30min of tube formation and counted for 6h using Evos® microscope.

Acrolein inhalation

Acrolein atmospheres were generated from a liquid refillable humidification tube (LFH, Kin Tek Laboratories, La Marque, TX, USA). Acrolein emission rate of the permeability tube was controlled by temperature, 1ppm acrolein levels were produced by placing tube in a hot water bath at a set temperature of 50°C. Immersion of the permeability tube in the inert gas Nitrogen allowed for trace element of the acrolein to be emitted with high dosage accuracy. During exposure, acrolein concentration was continuously monitored using an in-line

photo ionization detector (ppb RAE+, Rae Industries, Sunnyvale, CA, USA) upstream of the cage insert vapor delivery unit (Teague Enterprises, Inc.) mounted on a standard polycarbonate rat cage (41 cm x 34 cm x 21 cm; ≈31L). Air or acrolein was distributed through a fine mesh screen at 3 lpm by delivery units with a cyclone-type top that distributed air within 10% of the mean concentration at six locations in the cage (**Scheme 6**). Exposure cages were placed partially over heating pads (≈22 °C) to allow mice to select preferable temperature. Mice were exposed to 1 ppm acrolein for 4 days 6h/d (995.7±1.4ppb of) prior to induction of hindlimb ischemia (HLI) or to 2 ppm acrolein for 4 days (2138±5.4 ppb) prior to HLI. Acrolein inhalation was continued the day after induction of HLI in all study groups and perfusion recovery was followed by laser Doppler imaging.

Scheme 6

Punch wound

Male C57/BL6 mice at 12 weeks of age (Jackson Laboratories) were anesthetized with Isoflurane 1-3 % in 100 % oxygen at 1 mL/ minute. Mice were shaved 24h before punch wounds were induced dorsally. A double punch wound was made on the back between the shoulder blades using a 5mm punch skin biopsy pin (Integra Miltex Inc, York, PA, USA). (**Fig. 10A**)

Figure 10

Figure. 10 punch wound model representative picture of 5mm punch wound location

Hind limb ischemia

Male C57/BL6 mice at 12 weeks of age (Jackson Laboratories) were anesthetized with isoflurane 1-3 % in 100 % oxygen at 1 mL/ minute. Mice were shaved and then sensitive skin Nair (Church & Dwight Co., Princeton, NJ, USA) applied to remove all hair on the lower limbs 24h before surgery (**Fig. 11A**). Mice were anesthetized with isoflurane 1-3 % in 100 % oxygen at 1 mL/ minute, the femoral artery was ligated distal of the femoral bifurcation and proximal to the ankle joint using 7-0 silk suture (Johnson & Johnson Co., Ethicon, Cornelia, GA, USA), approximately 1 cm of femoral artery was excised between the ligation points (**Fig.12B**). Skin was closed using 5-0 polyethyl (Ethicon) discontinuous suture. Mice were given 0.05 mg/kg of buprenorphine for pain management 1 hour before surgery. Mice were allowed to recover for 24h before starting acrolein inhalation. Mice were euthanized and changes in EPC levels were measured after surgery by flow cytometry (see Chapter 2). Perfusion recovery was measured on days 2, 4, 8, 12 or 18 after HLI surgery independent of inhalation exposures.

Figure 11

Figure 11. Representative pictures **A**, shows transverse abdominal line and area shaved then depilatory cream used for hair removal before HLI surgery, **B**, shows a representation of the HLI surgery and excision of the femoral artery and ligation points.

Laser Doppler perfusion imaging

Blood perfusion in the ischemic (right) and normal (left) hind limb was measured with laser Doppler perfusion imaging (LDPI) system (PIMII, Perimed Instruments, North Royalton, OH). Low or no blood perfusions were displayed in dark blue, and the highest perfusion intervals were displayed in red. When laser light penetrates the tissue under study it is scattered or partly absorbed. Some of the scattered light returns to the tissue surface, where it is registered by a photo detector inside the instrument head. Information sent back to the instrument head which is read by the machine and provides information about the microcirculatory blood flow. According to the Doppler principle, light particles which hit moving blood cells undergo a change in wavelength/frequency otherwise known as Doppler shift. The perfusion can be calculated because the magnitude and frequency distribution of the Doppler shifted light are directly related to the number and velocity of blood cells but unrelated to their direction of movement. Perfusion data was shown as mean perfusion in volts. Mice were placed at a depth of 12cm from the head of the PIM II on a black non-reflectant surface. The laser was allowed to warm up for 15-20min before use. The machine was set on high resolution, with a set threshold voltage of 6. Analysis of hind limb ischemia data compared ischemic to non-ischemic limbs and subtraction of average background voltage from each captured image. Data was presented as the perfusion ratio ischemic to non-ischemic limb.

Complete blood count

After select exposures, 100 μ l of blood were used for complete blood count analysis (CBC; Hemavet 500, Coulter Counter, Oxford, CT).

Histology

Animals were anesthetized (pentobarbital; 40mg/kg). Whole thigh muscle including adductor longus, gracilis, adductor magnus and biceps femoris of both legs were harvested (**Fig 12A-I**). The gastronemius muscles were also harvested from both ischemic and non-ischemic limbs and fixed in 10 % neutral buffered formalin (NBF) for 24h. Fixed tissue was paraffin-embedded and cut into 5 μ M thick sections and placed on slides. Sections were stained with UE-Lectin-FITC (1:50; Vector Laboratories, Burlingame, CA, USA) and anti- α -Smooth Muscle Actin-FITC (1:250; Sigma) antibody for 1h at 37 °C. Slides were washed and nuclei were stained with DAPI (Molecular Probes, Invitrogen, Carlsbad, CA, USA). Auto-fluorescence was quenched with 0.1 % Sudan black solution for 30 min at RT. Slides were again washed under running distilled water and cover slipped using *Slow Fade*[®] Gold anti-fade mounting reagent (Invitrogen). Capillary densities were calculated as the area of vessels positive for FITC-Lectin within each field. Arteriogenesis was calculated by counting the total number of α -SMA positive vessels within each field. Slides were examined by fluorescent microscopy (Evos microscope 20X) in four different random fields for capillary area calculations, while seven random fields were taken for α -SMA positive vessels. Pictures were analyzed using Nikon Elements software.

Figure 12

Figure 12. Representative pictures of **A-I**, removal of ischemic and non-ischemic muscle that is to be fixed, processed and stained for immunohistochemistry after HLI surgery.

Results

Tube forming assay

To examine the effects acrolein has on angiogenesis, we first studied its effects on the spontaneous tube formation by endothelial cells. For this, HUVECs were cultured to 80% confluency, then treated with HBSS⁺, 2.5 or 5 μ M of acrolein in HBSS⁺ for 30min. Acrolein decreased tube formation at both 2.5 and 5 μ M concentrations (**Fig.13A-B**). However, 2.5 μ M acrolein treated HUVECs recovered to that of control by the 6h time point, while the tube formation plateaued at 3h and never recovered after 5 μ M acrolein treatment (**Fig.13A-B**), showing that acrolein can affect the angiogenic ability of endothelial cells.

Figure 13

Figure 13. Model of *in vitro* angiogenesis. **A**, Representative images of *in vitro* tube formation after treatment with acrolein 0, 2.5, or 5 μ M for 30min. **B**, Quantification of tube formation following acrolein incubations. Data are mean $\pm$ SEM (n=3/group). * p<0.05 vs. control.

Ischemic injury recovery

Ischemic injury has been shown to mobilize EPCs and play an intricate part in wound healing and neovascularization of injured tissue¹³⁶. Our studies have shown that acrolein decreases circulating EPCs but increases proliferation within the bone marrow¹⁸⁷. Any changes in mobilization of EPCs due to acrolein inhalation will be more apparent when EPC levels peak within the circulation after induction of HLI. To determine peak EPC mobilization after ischemic injury, we performed a time course study. EPCs were measured in mice without HLI, and at 2, 4, 8, and 12 (n=4) days after the induction of HLI. EPC levels in circulation peaked 2 days after hindlimb ischemia, then returned baseline levels by day 12 (**Fig.14B**). To determine the effect acrolein has on mobilization of EPCs in response to ischemic injury, mice were exposed to 1ppm acrolein inhalation 4 days pre-surgery and continued acrolein inhalation 2 days post-surgery (**Scheme 7**). Peripheral blood was drawn and flow cytometry analysis performed to determine circulating EPC levels. EPCs were gated as previously described, Flk-1⁺/Sca-1⁺ cells were gated out and plotted for CXCR4 positivity (**Fig.14A**). Acrolein did not affect circulating Sca-1⁺/CXCR4⁺ cell number (**Fig. 14C**). However, acrolein did decrease Flk-1⁺/Sca-1⁺/CXCR4⁺ cells within the circulation (n=6,6; air 4.7±0.8 cells/μL; acrolein 2.9±1 cells/μL; p<0.05; n=6,6; **Fig. 14D**). To determine whether EPCs are in an increased proliferative state within the bone marrow, we examined cell cycle of Flk-1⁺/Sca-1⁺ cells and found no difference between cell cycle or total cell numbers (**Fig. 14E**). Stem, T-and B-cells that mobilized were measured to determine if any subset of Flk-1⁺/Sca-1⁺ EPCs were

affected. A subset within the Flk-1⁺/Sca-1⁺ cell population, CD31⁺/B220⁺/CD8⁻/CD19⁺/CD3⁺ cells were increased in acrolein-exposed mice compared with air (air 0.05%±0.005; acrolein 0.08%±0.005; p<0.05; n=6,6; **Fig. 14E-G**). The stem cell panel showed that two different subsets of Flk⁺/Sca⁺ cells were decreased in acrolein-exposed mice compared with air; subset 1 B220⁺/CD31⁺/CD11b⁻, CD115⁻/CD19⁺ (air 0.08±0.002%; acrolein 0.05±0.001%; n= 6,6; p<0.05; n= 6,6; **Fig. 14H-I**) subset 2 B220⁺/CD31⁺/CD11b⁻, CD115⁻/CD19⁻ (air 0.09±0.001; acrolein 0.04±0.001; n=6; p<0.05; n=6,6; **Fig. 14H-I**).

External wound healing and EPC response to ischemic injury is hampered in smokers but is reversible with cessation of smoking¹⁸⁸. To further elucidate if acrolein had any effect on superficial wound healing like what is seen in smokers, double 5mm punch wound technique was employed. A short 2 day punch model was used to test if acrolein inhalation affected mobilization of EPC in response to the injury (**Scheme 7**). Circulating EPC levels in the punch wound model was not significant between air-exposed and acrolein-exposed mice (**Fig.15A-D**).

Scheme 7 Two day hindlimb ischemia treatment protocol

Figure 14

A.

Air

Acrolein

F.

H.

Figure 14. Two day mouse model of hindlimb ischemia and characterization

and **A**, representative dot plots of gated FSC and PE-CXCR4 fluorescence of Flk-1⁺/Sca-1⁺ cells that are also CXCR4 positive from air- and acrolein- treated mice. **B**, Quantification of time course EPC levels per μ L after the induction of hindlimb ischemia, measurements taken from baseline, days 2, 4, 8 and 12 from whole blood. **C**, Quantification of CXCR4⁺/Sca-1⁺ cells from 2 day hindlimb ischemia after 1ppm acrolein inhalation from whole peripheral blood. **D**, Quantification of Flk-1⁺/Sca-1⁺/CXCR4⁺ cells 2 days after hindlimb ischemia and 1ppm acrolein inhalation from whole blood. **E**, Quantification of cell cycle from bone marrow Flk-1⁺/Sca-1⁺ cells of 2 day 1ppm acrolein hindlimb ischemia mice. **F**, representative dot plots of gated FSC and gated T-cell and B-cell markers. **G**, Characterization of Flk-1⁺/Sca-1⁺ cell using T and B-cell markers after 2 days of hindlimb ischemia and 1ppm acrolein exposure. **H**, representative dot plots of gated FSC and Stem cell markers **I**, Characterization of Flk-1⁺/Sca-1⁺ cell using stem cell markers after 2 days of hindlimb ischemia and 1ppm acrolein exposure. Data are mean $\pm$ SEM (n=4-6/group). * p<0.05 vs. control.

In order to determine if acrolein could affect wound closure we examined the progression of wound healing. Double 5mm punch wounds were induced dorsally and followed until 90% closure (**Scheme 8**). Wound closure of acrolein-exposed mice was not significantly different when compared to air controls, in both groups there was 90% wound closure within 10 days (**Fig.15E-G**).

To further determine acrolein's effect on ischemia induced EPC mobilization, a more severe model of hindlimb ischemia was employed. Mice were exposed to 1ppm acrolein for 4 days prior to HLI surgery. After surgery perfusion recovery was followed and laser Doppler perfusion images were taken of ischemic and non-ischemic limbs on days 0, 8, 12, and 18 (**Scheme 9**). There was no significance between perfusion recovery of acrolein-exposed mice when compared to air-exposed mice (**Fig. 16A-B**). Circulating EPC levels were compared on day 20 of HLI, Sca-1⁺/CXCR4⁺ cells and Flk-1⁺/Sca-1⁺/CXCR4⁺ cells were not significant when compared with air-exposed mice (**Fig. 16C-D**). For further confirmation and analysis of angiogenesis thigh and Gastrocnemius muscles of both ischemic and non-ischemic legs were harvested and stained for immunofluorescence. Tissue sections were stained for UE-Lectin to detect capillary density and α -smooth muscle actin (α -SMA) in order to detect arterioles (**Fig. 16E**). Quantification of capillary density and total number of α -SMA positive vessels showed there were no changes in angiogenesis after hindlimb ischemia between acrolein- and air-exposed mice (**Fig. 16F-G**).

Scheme 8 Two day punch wound model treatment protocol

Scheme 9 Ten day punch wound treatment protocol

Figure 15

Figure 15. Punch wound mouse model. **A**, Quantification of CXCR4⁺/Sca-1⁺ cells from 2 day punch wound model after 1ppm acrolein inhalation from whole peripheral blood. **B**, Quantification of Flk-1⁺/Sca-1⁺/CXCR4⁺ cells after 2 days punch wound model after 1ppm acrolein inhalation from whole blood. **C**, Quantification of CXCR4⁺/Sca-1⁺ cells from 10 day punch wound model after 1ppm acrolein inhalation from whole peripheral blood **D**, Quantification of CXCR4⁺/Sca-1⁺ cells from 10 day punch wound model after 1ppm acrolein inhalation from whole peripheral blood **E**, Representative images of 5mm punch wound on day 0 and 8 **F**, Quantification of punch wound healing every 2 days until day 8. Data are mean±SEM (n=6/group). * p<0.05 vs. control.

Scheme10 Twenty day hindlimb ischemia treatment protocol

Figure 16

B.

E.

F.

G.

Figure 16. Hindlimb ischemia perfusion recovery and acrolein 1ppm inhalation. **A**, Representative laser Doppler measurements at days 0, 8, 12 and 18. **B**, Quantification of laser Doppler perfusion ratios following hindlimb ischemia in control mice, or mice exposed to 1ppm acrolein inhalation. **C**, Quantification of Sca+ cells from 20 day hindlimb ischemia model after 1ppm acrolein inhalation from whole peripheral blood **D**, Quantification of Flk-1+/Sca-1+ cells from 20 days of hindlimb ischemia and 1ppm acrolein inhalation from whole peripheral blood. **E**, Representative photographs of thigh muscle from 20 day hindlimb ischemia mice and 1ppm acrolein exposure, stained for UE-Lectin and arterioles. **F**, Quantification of capillary and, **G**, total α -SMA positive vessels. Data are mean $\pm$ SEM (n=6/group). * p<0.05 vs. control.

HLI perfusion was followed for an extended time to determine the affect acrolein potentially has on angiogenesis *in vivo*. Higher concentrations of acrolein (2ppm) were used to determine if the effect is dose dependant (**Scheme 11**). Acrolein mice did not have hampered leg reperfusion, both air- and acrolein-exposed mice had 74-76% recovery of blood flow by day 10 post-surgery (**Fig.17 A-B**). Circulating Sca-1⁺/CXCR4⁺ cells remained insignificant at this time point as well. Acrolein-exposed mice had significantly decreased levels of EPCs (Flk-1⁺/Sca-1⁺/CXCR4⁺) compared with air-exposed mice (air 4.4±0.5; acrolein 1.4±0.3; n=6,6; p<0.05; **Fig. 17 C-D**). Complete blood counts of acrolein-exposed mice were decreased after induction of HLI in both 1ppm/2d acrolein inhalations and 2ppm/10d acrolein inhalations (**Table 11**).

Scheme 11 Ten day hindlimb ischemia treatment protocol

Figure 17

A.

B.

C.

D.

Figure 17. Hindlimb ischemia perfusion recovery of acrolein 2ppm inhalation mice. **A**, Representative laser Doppler measurements at days 0, 1, 3, 6, 10. **B**, Quantification of laser Doppler perfusion ratios following hindlimb ischemia in control mice, or mice exposed to 2ppm acrolein inhalation. **C**, Quantification of Sca-1⁺ cells from 10 day hindlimb ischemia model after 2ppm acrolein inhalation from whole peripheral blood **D**, Quantification of Flk-1⁺/Sca-1⁺ cells from 10 days of hindlimb ischemia and 2ppm acrolein inhalation from whole peripheral blood.

Table 11

Blood parameters in C57BL/6 mice exposed to air or acrolein with HLI

	Control no HLI		2D HLI		20DHLI		10DHLI	
	Air		Air	Acrolein, 1ppm	Air	Acrolein, 1ppm	Air	Acrolein, 2ppm
WBC ^a	2370±585		3832±399	2173±178*	2100±320	1663±172	3363±376	1676±334*
NE ^a	362±64		744±93	400±34*	334±80	183±23	686±55	313±42*
LY ^a	1942±518		2098±391	1708±173*	1668±246	1397±146	2528±330	1286±283*
MO ^a	57±15		82±17	45±32*	74±17	78±11	141±24	73±17*
RBC ^b	9035±298		7796±283	7915±199	7750±274	7996±274	8716±227	8418±224
HCT (%)	42.2±1.2		33.9±1.3	34.3±0.9	34.8±0.4	35.9±1.1	37.38±1.0	36.5±1.1
Hb ^c	11.1±0.3		11.4±0.5	11.8±0.3	11.6±0.2	11.7±0.4	12.15±0.3	12.0±0.3
PLT ^a	835±50		579±35	509±25	565±17	569±21	783±53	652±32
MCV ^d	46.9±0.3		43.8±0.2	43.7±0.2	44.9±0.4	44.8±0.3	42.9±0.6	43.4±0.2
MCH ^a	12.2±0.1		14.7±0.2	14.9±0.2	14.9±0.2	14.6±0.1	13.9±0.2	14.2±0.1
MCHC ^c	26.1±0.1		33.4±0.3	34.1±0.4	33.2±0.2	32.6±0.3	32.5±0.3	32.8±0.3
RDW (%)	17.3±0.5		16.8±0.2	16.1±0.3	17.1±0.3	17.0±0.2	17.5±0.3	17.3±0.2
MPV ^d	3.8±0.1		4.1±0.03	4.1±0.03	4.0±0.03	4.0±0.03	4.3±0.06	4.1±0.1

Male, 12-14 week old mice were exposed to air or acrolein (1 or 6h/day) for 4 days, with or without HLI, n=6 per group. Mice were euthanized immediately after exposure. Units: ^a = [K/mL], ^b = [M/mL], ^c = [g/dL], ^d = [ng/ml]; Values are mean ± SEM; * p < 0.05 vs. air-matched group; n=8 mice/group. Abbr.: WBC, white blood cell; NE, neutrophil; LY, lymphocyte; MO, monocytes; RBC, red blood cell; HCT, hematocrit; Hb, hemoglobin; PLT, platelet; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; MPV, mean platelet volume. p<0.05, One-Way ANOVA; compared to there prospective air groups

Discussion

In the present study, we show that acrolein treatment decreases spontaneous tube formation of HUVECs *in vitro* and acrolein inhalation decreases mobilization of EPCs in response to ischemic injury.

Previous studies have shown that exposure to cigarette smoke decreases circulating EPCs and decreases angiogenic recovery after induction HLI¹⁸⁹.

Acrolein inhalations that were performed are pure exposures to acrolein, compared to the plethora of chemicals contained in cigarette smoke, so there cannot be a direct comparison between the results of these two studies. However, acrolein is a highly reactive component found within cigarette smoke and is a likely candidate for the development of vascular pathologies seen in smokers. Indeed, in this present study we report a decrease in tube formation by HUVECs treated with acrolein for 30min.

Angiogenesis and neovascularization are complex biologic responses and are important processes in ischemic injury and disease¹⁹⁰. In both post-natal physiological and pathological neovascularization bone marrow-EPCs play an essential role¹⁹¹. Studies have shown HLI causes mobilization of EPCs from bone marrow to the site of ischemic injury, mobilization of EPCs is essential for perfusion recovery and limb salvage¹⁹². Ischemic injury is a strong stimulus for increased release of VEGF, which is a potent EPC mobilizer¹⁹³. In an attempt to elucidate if acrolein affects mobilization of EPCs in response to ischemic injury, mice were exposed to 1ppm acrolein for 4 days before HLI surgery and euthanized at the peak of EPC mobilization 2 days post-surgery. Circulating

EPCs were significantly decreased in acrolein-exposed mice, confirming a blunted response to ischemic stimuli. Previous data have shown acrolein-exposed mice have a hampered mobilization to VEGF-injections. These results support the conclusion that EPCs are not responding to endogenous VEGF level increases that occur after ischemic injury and thus do not mobilize¹⁸⁷. However, the precise mechanism responsible for this blunted response and mobilization remains to be elucidated.

Circulating EPC levels have been positively correlated to cardiovascular health and increased angiogenesis with improved ischemic recovery¹³⁶. We further investigated the decrease of circulating EPC levels and if the decrease would affect ischemic recovery of HLI. Serial Doppler imaging showed perfusion recovery was not affected in any HLI model; at 1ppm 20 day post-HLI nor the 2ppm 10 day post-HLI model.

In conclusion, acrolein does affect EPC mobilization in response to ischemic injury. However, angiogenesis and reperfusion does not seem to be affected by acrolein inhalations. Despite the negative results reported here, acrolein could still affect angiogenic recovery after induction of HLI. The question remains open and further research and technique development is needed to determine the affect acrolein exposure has on angiogenesis and the mechanism(s) involved.

CHAPTER IV

CONCLUDING DISCUSSION

Studies presented here were designed to develop an understanding of how acrolein, a byproduct of environmental pollutants, affects vascular health by altering the recruitment and mobilization of endothelial progenitor cells (EPCs). **My thesis is that EPCs are sensitive targets for environmental insults, such as acrolein, thus increasing the risk of developing cardiovascular disease and endothelial dysfunction. Studies described here show that exposure to acrolein by inhalation (Aim 1), prevents mobilization of EPCs by cytokine stimulation (Aim 2). These studies also, evaluated perfusion recovery after hindlimb ischemia in mice exposed to acrolein inhalation (Aim 3).**

Results of the first set of experiments (Chapter II), show that inhaled acrolein preferentially suppresses circulating endothelial progenitor cells (EPCs). Acrolein specifically decreases this cell population without affecting the level of Sca-1⁺ cells, indicating a unique sensitivity of the EPC population to acrolein. The effects of acrolein on this population were, however, transient, and recovery in acrolein-free environment for 7 days led to a return of circulating EPCs to baseline levels.

The decrease in circulating levels of EPCs appears to be the result of a defect in mobilization. Acrolein selectively suppressed basal levels of EPCs but not the Sca-1⁺ cell pool indicating that it preferentially affects VEGFR-2-expressing cells. In support of this conclusion, we found that acrolein exposure *in vivo* blocked VEGF-induced phosphorylation of Akt and eNOS in isolated aorta *ex vivo*. The prevention of EPC mobilization was also associated with decreased plasma NO_x levels as well, suggesting that some of the effects of acrolein on EPC mobilization could be attributed in part to a decrease in NO production or bioavailability.

We also found that acrolein inhalation was associated with the accumulation of protein-acrolein adducts in the plasma and bone marrow. This observation suggests that despite its high reactivity, acrolein is delivered from the lung into the systemic circulation and to distal vascular sites. Thus, acrolein appears to cross from the lungs and directly stimulate bone marrow EPCs. Indeed, our studies showed that bone marrow derived cells (BMDC) from acrolein-exposed mice were more proliferative when cultured on fibronectin-coated plates. Enhanced BMDC proliferation was observed as early as 1-2 days during initial colony formation, which contain clusters that stained for Flk-1⁺/Sca-1⁺ as well as positive for acLDL uptake and *Ulex* lectin binding. The apparent increase in proliferation and a decrease in circulating EPC levels indicate that acrolein does not impair the growth or viability of these cells, but that it prevents their mobilization from the bone marrow. We suggest that this defect may be due to alterations in VEGF/ CXCR4 signaling but could also be related to other

processes such as cell release from the bone marrow stroma. The specific mechanism by which acrolein enhances BMDC proliferation but inhibits their mobilization remains unknown and needs further investigation.

Because acrolein is one of the most reactive and toxic components of tobacco smoke, it is not surprising that many of the effects we observed with acrolein inhalation are consistent with tobacco smoke exposure. Our observations that acute high level acrolein slightly injures the murine endothelium are similar to reported effects of tobacco smoke on endothelial cells¹⁹⁴. The acrolein-induced suppression of NO_x could be related to diminished levels of EPCs because both parameters returned to baseline levels following a 7-day recovery period. Moreover, we found that exposure to 1 ppm acrolein for 4 days did not induce frank endothelial dysfunction although the levels of EPCs were decreased, suggesting that decreases in the circulating EPC levels precede endothelial dysfunction.

Humans are exposed to acrolein not only from tobacco smoke and air pollution, but also to acrolein present in foods and beverages¹⁶⁵. In addition, acrolein is generated endogenously during lipid peroxidation and myeloperoxidase activity at sites of inflammation¹⁶⁶. Our studies suggest that acrolein generated endogenously within the bone marrow or transported to the bone marrow from distal inflammatory processes could also suppress EPC mobilization and decrease circulating levels of EPCs by triggering mechanisms similar to those seen in animals exposed to inhaled acrolein.

In this project, we characterized and counted EPCs independent of CD45⁺ status because the majority (>90 %) of EPCs express CD45 antigen. We reasoned that because of acrolein's propensity for targeting the endothelium, acrolein exposure could result in injury and mobilization of EPCs for repair. Chronic smoking is associated with a decrease in EPC levels in humans similar to the suppression of EPCs in blood that we have reported¹⁹⁵. Hence, these findings suggest a novel connection between a specific component of cigarette smoke, acrolein, and air pollution generated from the combustion of organic materials and provide new insights into the mechanisms by which exposure to these pollutants can increase the risk of developing cardiovascular disease.

We next wanted to determine the effects acrolein had on vascular regeneration using *in vivo* and *ex vivo* approaches. Our cell culture studies showed that acrolein treatment decreased spontaneous tube formation of HUVECs seeding on Matrigel. To further elucidate the effects acrolein on angiogenesis and perfusion recovery we studied two models of wound healing, i.e., the punch model and hindlimb ischemia. However, we found that the progression of wound healing in these models was not affected by acrolein exposure. We reasoned that the punch wound model is not a severe model of ischemia and does not elicit the hypoxic response necessary to significantly increase plasma VEGF and increase circulating EPC levels to the extent of HLI model¹⁹⁶. Studies have shown VEGF exerts dominant functions during angiogenesis and is necessary for EPC mobilization from the bone marrow. Hindlimb ischemia is also a potent stimulator of VEGF release and mobilizes

EPCs into the circulation almost 13 fold over baseline¹⁹⁷. We found that with the induction of hindlimb ischemia, circulating EPC levels peak on day 2 of ischemic injury. However, after day 2 of HLI mice exposed to 1ppm acrolein had significantly decreased circulating EPC levels. This data suggests exposure to acrolein prevents EPC mobilization due to hindlimb ischemia perhaps by preventing the stimulatory effects of endogenous VEGF on the mobilization of EPCs from the bone marrow in response to HLI. To develop these observations further, an extended 1ppm acrolein exposure was carried out to determine if decreased circulating EPCs affected angiogenesis and reperfusion recovery after HLI. Our results showed that acrolein exposure did not affect circulating EPC levels or the rate of recovery of tissue perfusion. Hence, no determination could be made if EPCs affected perfusion recovery because EPC levels did not differ between control and inhalation animals. To examine whether the effects of acrolein on angiogenic recovery might be a dose-dependent phenomena, acrolein exposure levels were increased to 2ppm. Our results with the higher dose showed a trend towards decreased perfusion recovery of 2ppm acrolein-exposed mice, however this did not reach statistical significance. Circulating EPCs were also not significantly different in HLI of 2ppm acrolein-exposed mice when compared with air-exposed mice. Both 1ppm and 2ppm HLI studies produced negative results, however, in chapter III EPC levels were shown to return to baseline by day 12 of HLI. EPCs may become desensitized to mobilization stimuli after a period of time or EPCs mobilization could become exhausted after prolonged ischemic stimulation. Further research is needed to

determine the possible detrimental effects that acrolein could have on angiogenesis, vascular repair and how this could potentially effect wound healing.

Overall, the data presented here support the global hypothesis that EPCs are a sensitive target for acrolein, thus increasing the risk for developing cardiovascular disease and endothelial dysfunction.

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CURRICULUM VITAE

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CITIZENSHIP:

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2011	Ph.D	University of Louisville	
	Physiology/Biophysics	School of Medicine	
		Louisville, KY	
2008	M.S.	University of Louisville	
	Physiology/Biophysics	School of Medicine	
		Louisville, KY	

2006	B.A.	Lindsey Wilson College Columbia, KY	Major Biology Minor Chemistry
2006	A.A.	Lindsey Wilson College Columbia, KY	Health Sciences

APPOINTMENTS

September 2002 - December 2003	Medical Assistant, Dr. John Kilgallin, Russell Springs, KY
August 2003 - May 2005	Student Tutor, Lindsey Wilson College, Columbia, KY
August 2004 – May 2005	Student Assistant Biology Department, Lindsey Wilson College, Columbia, KY

COMMUNITY SERVICE

2002-2006	Volunteer - Adanta Adult Day Care
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CHARATABLE SERVICE

2008-Present	Sponsorship Chair - Lupus foundation Walk for Lupus Now campaign
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AWARDS and HONORS

2001	Kentucky Govenor Scholar
2006	Integrated Programs in Biomedical Sciences (IPIBS) fellowship award

Part II: Research

My research at the University of Louisville was initiated as an attempt to understand the link between environmental pollution and increased mortality rates. Previous studies have shown a strong negative correlation between pollution and developing heart disease, diabetes, and decreased wound healing ability, however the physiologic reason behind this still remains a mystery. In my research I studied adult endothelial progenitor cells (EPCs), a small subset of cells that have stem cell and endothelial cells properties and heal damaged endothelium. There is also a strong negative correlation between the number of circulating EPC and the risk for developing heart disease, diabetes, and decreased wound healing. Little has been done with the characterization of EPC in mice models, so our lab has done an extensive set of cell markers by flow cytometry and developing the first heirarchy of mouse EPCs. My main field of study came to be EPC and how they were affected by acrolein. Acrolein is a

highly reactive unsaturated aldehyde present in all forms of pollution in high amounts. In my research I have found acrolein inhalation does indeed decrease circulating endothelial cells and hinders angiogenic assays in vitro, in a time and dose dependant manner. With this knowledge our lab can now began to unravel the mystery behind pollution and high mortality rates, thus decreasing or even preventing pollution related deaths.

Part III: Bibliography

Original Articles (refereed journals)

Wheat, L., Hellmann, J., Haberzettl, P., Baba, S., Bertke, M., Lee, J., McCracken, J., O'Toole, T., Bhatnagar, A., Conklin, D.J. Acrolein inhalation decreases circulating levels and VEGF-induced mobilization of Flk-1+/Sca-1+ cells. *ATVB* 2011. (Submitted).

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Dissertation

Wheat, L. Effects of Environmental Pollution on Endothelial Progenitor Cells and Vascular Regeneration [dissertation] Louisville (KY): University of Louisville; 2011

Abstracts

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Hellmann, J., Haberzettl, P., **Wheat, L.**, Conklin, D.J., O'Toole, T., Bhatnagar, A., Aldose Reductase-Dependant Effects on Endothelial Dysfunction and

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Louisville*

2009

Conklin, D.J., **Wheat, L.**, Hellmann, J., Haberzettl, P., O'Toole, T.,
Bhatnagar, A.

Inhaled Acrolein Decreases Circulating Endothelial Progenitor
Cells and Their Recruitment. *Society of Toxicology poster 2010*

Wheat, L., Hellmann, J., Haberzettl, P., Conklin, D.J., O'Toole, T.,
Bhatnagar, A.,

Acrolein Suppresses Mobilization of Flk-1+/Sca-1+ Progenitor
Cells.

FASEB 2010