5-2005

Polycyclic Aromatic Hydrocarbons from the Chattanooga Creek Flood Plain and Their Effects on Endothelial Cells via Group IVC Phospholipase A_2

Meghan Scott McNeilly
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by Meghan Scott McNeilly entitled "Polycyclic Aromatic Hydrocarbons from the Chattanooga Creek Flood Plain and Their Effects on Endothelial Cells via Group IVC Phospholipase A_2." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Comparative and Experimental Medicine.

Patricia K. Tithof, Major Professor

We have read this thesis and recommend its acceptance:

Gary S. Sayler, David Rotstein, Roger Carroll, Robert Donnell, John Sanseverino

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Meghan Scott McNeilly
May 2005

Acknowledgments

I wish to thank everyone who has helped me towards the completion of my Master of Science degree in Comparative and Experimental Medicine. I would especially like to thank my advisor, Dr. Tithof, for her unending patience and guidance in learning about the process of academic research. I would also like to thank Dr. Carroll, Dr. Donnell, Dr. Rotstein, Dr. Sanseverino, and Dr. Sayler for serving on my committee, and for all of their advice and interest in my project. Of course, I would like to thank Dr. Elgayyar and Wei Guan for teaching me both laboratory techniques and about life in general.

Finally, I would like to thank my family and friends, both two-legged and four-legged, whose encouragement and support has proven invaluable to me throughout my work.

Abstract

Exposure to environmental pollution can be a contributing factor to the development of cardiovascular disease (CVD). Due to contamination produced by long-term discharge of coal tar wastes into the creek, a 2.5-mile section of the Chattanooga Creek in south Chattanooga was designated as a Superfund site by the USEPA in 1994. In order to further investigate the potential health risks posed by creek contamination, 12 PAHs found in high levels in the sediment of the creek as compared to an uncontaminated control site were evaluated for their effects on the human coronary artery endothelial cell (HCAEC) phospholipase A_2 (PLA_2)/arachidonic acid (AA) cascade, a pathway known to play a significant role in endothelial cell inflammation, apoptosis, and atherosclerosis. Aortic tissue from feral mice trapped at the Superfund site exhibited an increase in markers of inflammation and apoptosis when compared to mouse tissue from the control site, suggesting that natural exposure to contaminants in the creek results in similar findings as in the *in vitro* studies. Six of the compounds studied (acenaphthylene, benz [e] acephenanthrylene, benzo [k] fluoranthene, fluoranthene, naphthalene, and phenanthrene) activated the AA cascade by targeting the isoform of PLA_2, Group IVC PLA_2. Upregulation of the enzyme was associated with an increase in apoptosis of HCAECs, as measured by ^{3}H-AA release and histone fragmentation, as well as Western blot analysis for PARP cleavage. Transfection with siRNA specific for Group IVC decreased the amount of histone fragmentation induced by the six compounds. Two compounds (anthracene and benz [a] anthracene) were inactive. Four compounds (benzo

[g,h,i] perylene, chrysene, indeno [1,2,3-c,d] pyrene, and pyrene) fluoresced independently of reagents used to measure histone fragmentation and were not able to be evaluated accurately in respect to this. However, these four compounds induced PARP cleavage and three of the four (chrysene, indeno [1,2,3-c,d] pyrene, and pyrene) produced significant ^{3}H-AA release from HCAECs. These data suggest that PAHs present in Chattanooga Creek have potential toxic effects on the cardiovascular system both *in vivo* and *in vitro*, and confirm the need for further studies.

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Nomenclature

AA	arachidonic acid
AP-1	activator protein-1
BSA	bovine serum albumin
CVD	cardiovascular disease
ER	endoplasmic reticulum
HCAEC	human coronary artery endothelial cells
HDL	high-density lipoprotein
LDL	low-density lipoprotein
NF-KB	nuclear factor-KB
PAH	polycyclic aromatic hydrocarbon
PARP	poly(ADP-ribose) polymerase
PLA_2	phospholipase A_2 enzyme

1. Background and Significance

Cardiovascular disease (CVD) is the number one cause of death for males and females in industrialized nations today (Bhatnagar). Greater than 1 million Americans die per year due to CVD, accounting for 40% of all deaths in the United States (Bhatnagar). Atherosclerosis is a particular kind of CVD, with its basis in the inflammatory response (Ross). The routes by which it develops are complex and insidious and can be related to a number of variables including an individual's genetic makeup, physical activity, diet, blood ratio of high-density lipoprotein (HDL) to low-density lipoprotein (LDL), alcohol intake and cigarette smoking (Bhatnagar, Ross).

Atherosclerotic lesions occur primarily in comparatively large and muscular arteries of the body. Rupture of lesions can result in embolism of the plaque to smaller arteries and cause complete blockage resulting in ischemia of organs such as the brain and the heart (Ross). When this occurs in the coronary circulation, it is known as acute myocardial infarction. The earliest stage of a lesion, a fatty streak, can occur prenatally, and is recognized as an increased intimal presence of migrating macrophages, hallmarks of the inflammatory response, and foam cells, which are macrophages already containing a cargo of lipid (Napoli, Stary, Ross). Genetic or diet-based changes in the LDL or blood pressure, in-take of free radicals via cigarette smoke, or any of the numerous aggravating sources will continue the processes causing endothelial cell dysfunction and simultaneous atherosclerotic lesion progression (Ross). The final lesion stage before possible infarction is the advanced lesion, a mass of

macrophages and activated mast cells surrounding a necrotic core and covered by a tenuous fibrous cap (Ross, Kovanen). (Figure 1) Plaques that contain high numbers of inflammatory cells express proteolytic enzymes (Ross). The continuing action of these enzymes thins the fibrous cap and plaque instability occurs (Ross). Unstable plaques are more prone to rupture, creating an embolism (Ross). (Figure 2)

Atherosclerosis and Environmental Pollution

Lately, interest has arisen as to the possible link between atherosclerosis and exposure to environmental pollution. Episodic increases in air pollution are associated with an increase in deaths due to acute myocardial infarction (Donaldson, Dockery). Brook, *et al.,* found that even short-term exposure to particulate matter less than 2.5 micrometers in diameter ($PM_{2.5}$), levels equivalent to levels occurring regularly in urban environments, results in vasoconstriction of major arteries. In general, it is also known that short-term air pollution exposure is an excellent prognosticator of increased admissions to hospitals and emergency rooms for cardiopulmonary disease (Wilson). Moreover, particulate air pollution (PM_{10} in particular) is associated with a system-wide inflammatory response including progression of previously existing atherosclerotic lesions in the hearts of rabbits (Suwa).

Polycyclic Aromatic Hydrocarbons, Inflammation and Atherosclerosis

Interestingly, air pollution is made up, among other things, of chemicals called polycyclic (or polynuclear) aromatic hydrocarbons (PAHs)

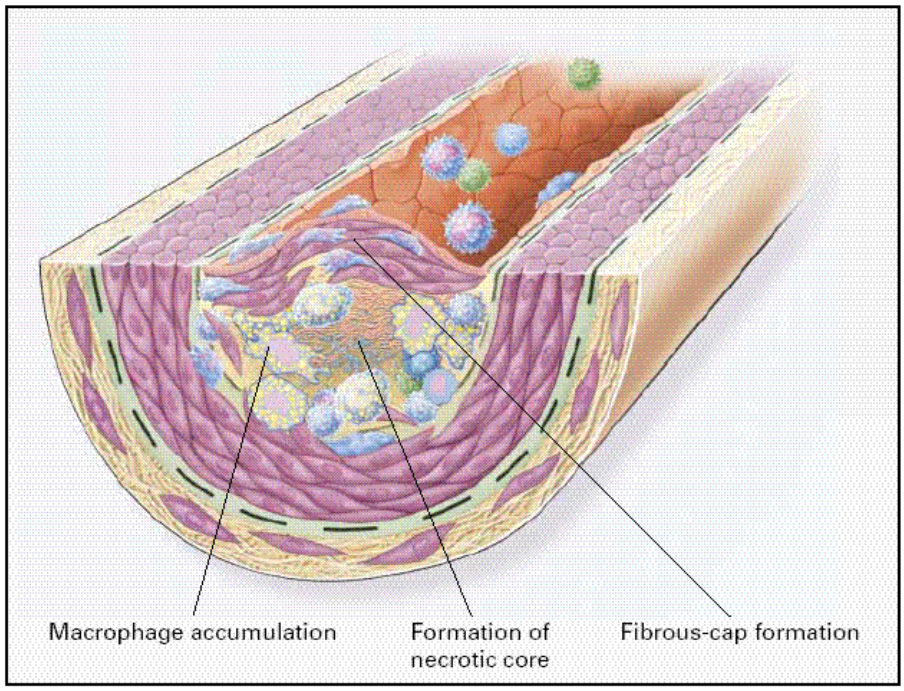


Fig. 1: Advanced Atherosclerotic Lesion. From Ross, 1999. Copyright © 1999, Massachusetts Medical Society.

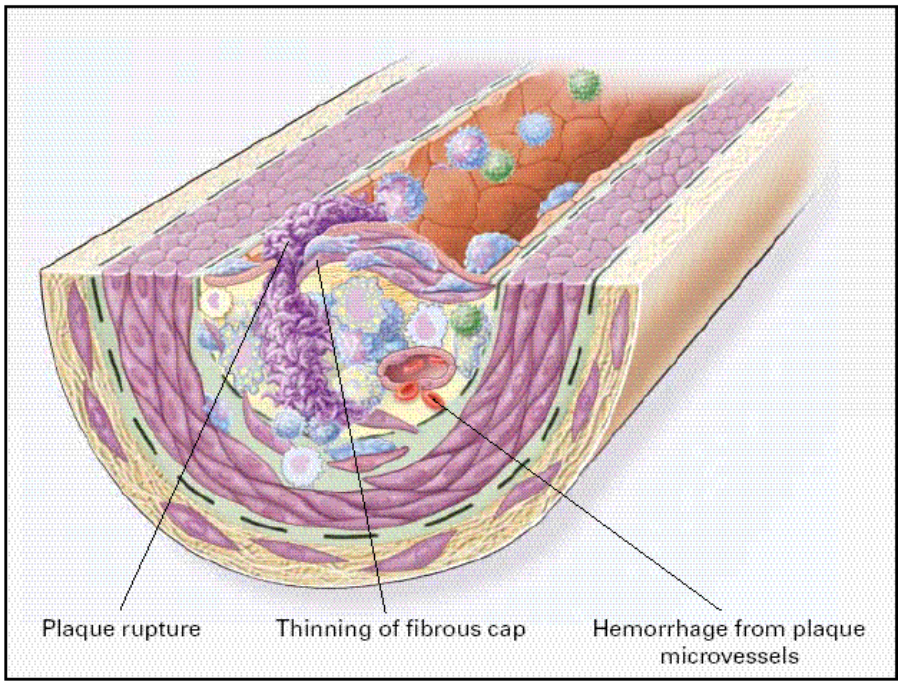
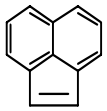


Fig. 2: Atherosclerotic Plaque Rupture. From Ross, 1999. Copyright © 1999, Massachusetts Medical Society.

(Bhatnagar). PAHs are a structurally related family of fat-soluble compounds with two or more benzene rings. (Figure 3) Sources of PAHs include incomplete combustion from industrial processes, automobile exhaust, burning of agricultural debris, cigarette smoke, and grilling and frying foods (Chiang, Kakareka). Early exposure to PAHs causes a myriad of toxic effects in embryonic fish, including hemorrhaging, pericardial edema, disruption of cardiac function, and death (Barron). PAHs also increase mortality in chicken, turkey, duck, and eider embryos (Brunstrom). Also, Penn and Snyder demonstrated that PAH administration to young roosters caused previously existing atherosclerotic plaques to grow larger, and more quickly, when compared to plaques present in control animals.

More recently, it was again shown that plaque size can be augmented by treatment with PAHs, in particular benzo [a] pyrene, by upregulation of the inflammatory response (Curfs). Using apoprotein-E (Apo-E)-deficient mice that spontaneously develop atherosclerosis when fed a normal chow diet, Curfs, *et al.*, evaluated the effects of benzo [a] pyrene. Treatment failed to influence the number or location of aortic lesions. Moreover, there was no accumulation of p53 nuclear protein or proliferation of cells within the plaques of treated animals, lack of which contributes to the evidence against the effect of benzo [a] pyrene on plaque initiation (Curfs). But, when compared with control mice, the treated mice did show significant increase in lesion size and lipid content, and, more importantly, an increase in the influx of inflammatory cells to the vascular wall. These data indicate that the PAH, benzo [a] pyrene, accelerates progression of

Acenaphthene



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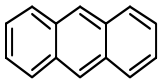
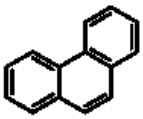
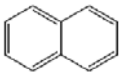


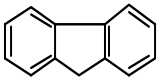
Anthracene



Phenanthrene

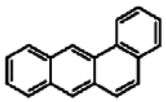


Naphthalene

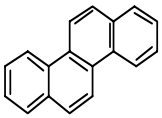


Fluorene

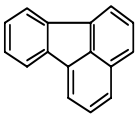
2- and 3-ring PAHs



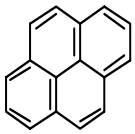
Benz (a) Anthracene



Chrysene

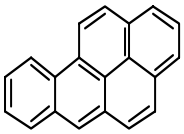


Fluoranthene

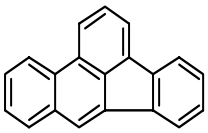


Pyrene

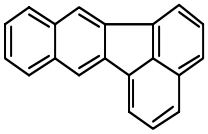
Four-ring PAHs



Benzo (a) Pyrene

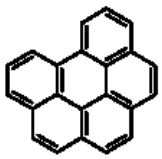


Benz (e) Acephenanthrylene

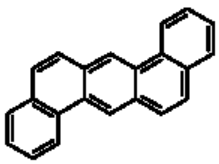


Benzo (k) Fluoranthene

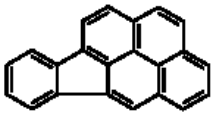
Five-ring PAHs



Benzo (g,h,i) Perylene



Dibenz (a,h) Anthracene



Indeno (1,2,3-c,d) Pyrene

Six-ring PAHs

Fig. 3: 16 EPA Priority PAH Chemical Structures. Those indicated in red are included in this study.

atherosclerotic lesions by promoting a local inflammatory response, rather than by initiating new plaque development (Curfs).

Apoptotic Cell Death and Atherosclerosis Progression

Numerous studies provide evidence that endothelial cell apoptosis is an early and critical event in the pathogenesis of CVD (Bennett, Isner, Kockx). Recent studies indicate that exposure to cigarette smoke and components of urban pollution can induce apoptosis (Smith). Apoptosis is the gene-directed death of a cell, characterized by a distinct set of features such as membrane shrinkage, chromatin condensation, membrane blebbing, formation of apoptotic bodies, and phagocytosis of the bodies by adjacent cells (Kockx). Apoptosis is a natural occurrence in the healthy individual and is, among other things, utilized to balance cell development, growth and senescence. However, exposure to toxicants can increase the incidence of apoptosis such that it exceeds the rate of cell proliferation. This effect of toxicants can lead to excessive cell loss (Kockx). Exposure to PAHs can cause cellular injury of human coronary artery endothelial cells (HCAECs) through apoptosis (Tithof, 2002). Enhanced endothelial cell loss causes intimal exposure, a condition that is central to leukocyte adherence and subsequent inflammatory change in the vessel wall that is the hallmark of atherosclerosis (Ross). The mechanism by which PAHs induce apoptosis of HCAECs involves the activation of the phospholipase A_2 (PLA_2)/arachidonic acid (AA) cascade, a pathway intimately linked to inflammation (Tithof, 1998). Several isoforms of PLA_2 have been linked to apoptosis.

PLA_2 Enzymes and Apoptosis

Phospholipase A_2 is a superfamily of enzymes that, in general, functions to hydrolyze membrane phospholipids at their *sn*-2 position, yielding a lysophospholipid and a free fatty acid (Balsinde, Capper). (Figure 4) The superfamily consists of at least 11 groups and several subgroups differing in subcellular location, substrate specificity, structure, and Ca^{++}-dependence (Balsinde, Murakami, Stewart). Functions of the PLA_2 isoforms are varied and include the liberation of energy stores (in the form of free fatty acids), provision of agents for signal transduction, generation of lipid mediators, and membrane remodeling (Six).

The Group IV A, B, and C PLA_2 are cytosolic or membrane-bound PLA_2s that hydrolyze phospholipids containing arachidonic acid (AA) at the *sn*-2 position, however the subgroups differ in substrate specificity (Six, Capper, Stewart). Important to our research is arachidonic acid, a fatty acid that is known to not only induce apoptosis itself but also to serve as the precursor for production of numerous eicosanoids (Six, Capper, Vosseler). (Figure 5) Eicosanoids, specifically prostaglandins and leukotrienes, are autocoids that mediate inflammation in the body and can also induce apoptosis. Group IVA PLA_2, also known as $cPLA_2\alpha$, is most selective for hydrolyzing membrane phospholipids containing AA at the *sn*-2 position, and has been the most studied (Six, Capper). Due to its marked specificity for phospholipids containing AA, group IVA PLA_2 is likely the major PLA_2 responsible for generating free AA for

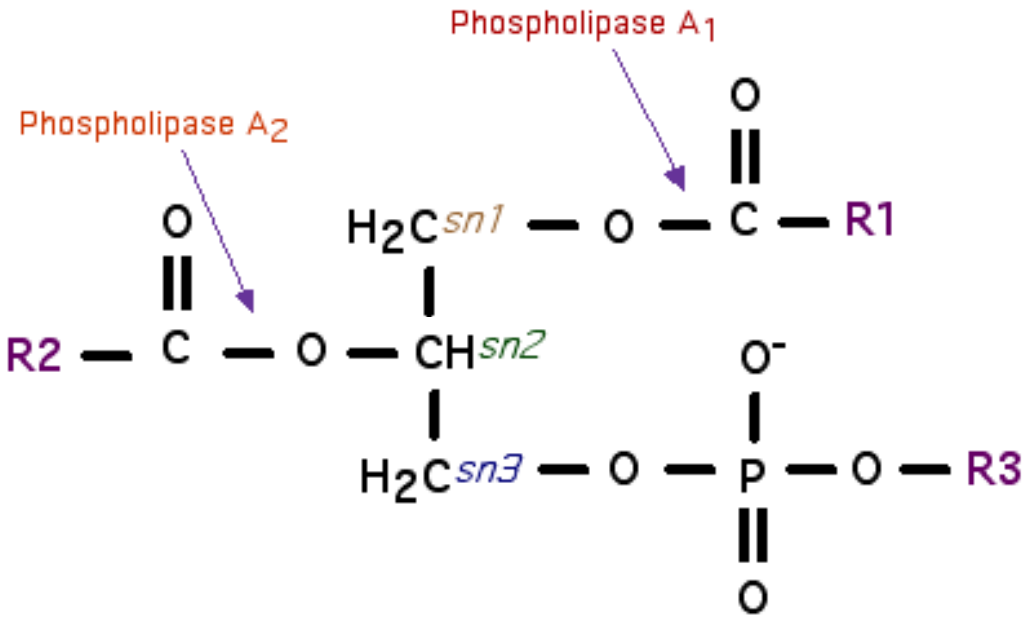


Fig. 4: Phospholipase Action Sites. Adapted from Capper, 2001.

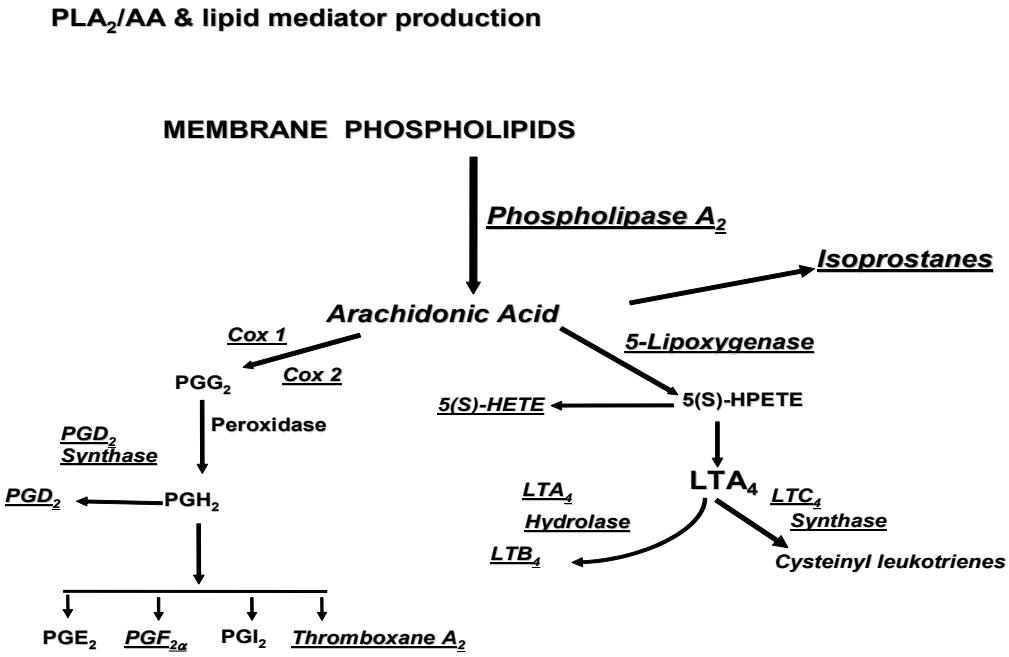


Fig. 5: Arachidonic Acid Cascade.

the production of eicosanoids (Capper). Group IVB (cPLA$_2$β), is markedly less selective for AA-containing phospholipids, and exhibits both PLA$_1$ and PLA$_2$ activity (Song). (Figure 4) Further, both cPLA$_2$α and -β require calcium for activity (Six, Capper).

Group IVC PLA$_2$ (cPLA$_2$γ), on the other hand, does not require calcium and is comparatively AA-nonselective in hydrolyzation when compared to Group IVA (Underwood, Stewart). Group IVC, like IVB, also exhibits PLA$_1$ activity (Capper). Asai, *et al.*, found that Group IVC PLA$_2$ is constitutively expressed in the endoplasmic reticulum (ER), supporting its role as a key player in membrane remodeling. Further, the membranes of the ER are important sites of phospholipid and eicosanoid biosynthesis (Underwood, Asai). Recent studies also suggest that Group IVC may function as a signaling PLA$_2$, linked to production of eicosanoids (Murakami). As apoptosis, a possible cellular outcome of the arachidonic acid cascade, can be a contributor to ongoing vascular inflammation as discussed above, the possible link between PLA$_2$s and atherosclerosis is of interest.

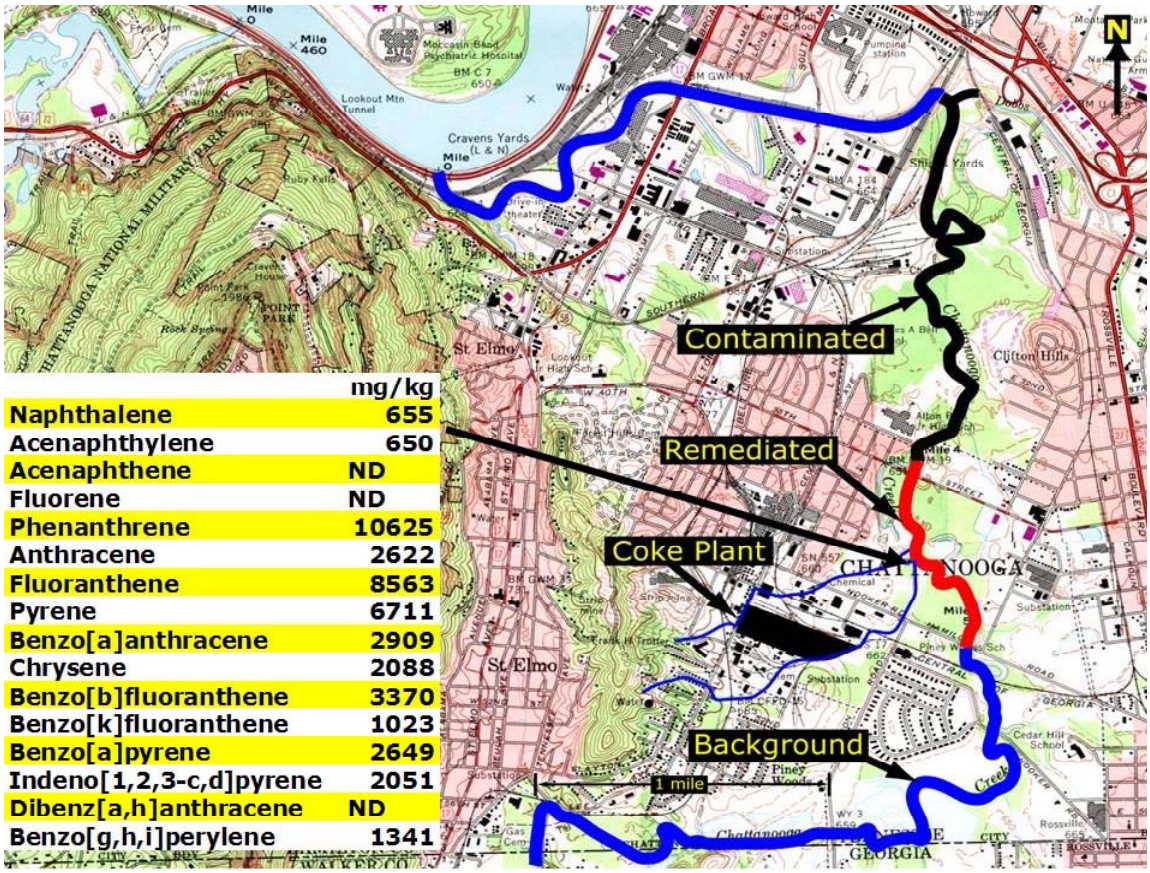
Of the many isoforms of Group IV PLA$_2$, we have selected Group IVC on which to focus our investigations. In previous studies, our lab has found that when ^{14}C-AA-PC is used as a substrate, the predominant PLA$_2$ activity in the cell is calcium-independent (Tithof, unpublished data). Groups IVA and B PLA$_2$ both require calcium for activity. Further, it has also been found that Group IVC is exogenously expressed in HCAECs (Tithof, 2002). Taken together, previous

data suggest that Group IVC is involved in PAH-induced AA release and apoptosis of HCAECs, enabling us to focus our studies on this isoform.

The Chattanooga Superfund Site and CVD

Like many metropolitan cities in America, Chattanooga, Tennessee, currently has high levels of pollution due to years of industrial development of the area. More than 100 years of coke furnace operation, chemical production facilities, foundries, tanning, and textile mills have resulted in significant pollution of South Chattanooga (Kennedy). Governmental housing projects were erected on inexpensive transects of land found in South Chattanooga in the 1950s and 1960s, and today greater than 6500 residents, 98% of them African-American, still reside there.

Running through the chemical quagmire of South Chattanooga is Chattanooga Creek, which also connects three of the government-planned neighborhoods: Alton Park, Clifton Hills, and Piney Woods. (Figure 6) The creek served as a wastewater disposal site for the numerous industries previously located in the area, and consequently it has experienced extreme contamination. It was determined that most of the Environment Protection Agency's (EPA) sixteen priority pollutant PAHs are found throughout the region at playgrounds, schools, housing projects, and retirement homes (Wells). (Figure 3) Although previous remediation steps, including coal tar dredging in 1997, have been taken by the government to clean this federally-designated Tennessee Products Superfund site, concern remains surrounding the movement of toxicants from the



	mg/kg
Naphthalene	655
Acenaphthylene	650
Acenaphthene	ND
Fluorene	ND
Phenanthrene	10625
Anthracene	2622
Fluoranthene	8563
Pyrene	6711
Benzo[a]anthracene	2909
Chrysene	2088
Benzo[b]fluoranthene	3370
Benzo[k]fluoranthene	1023
Benzo[a]pyrene	2649
Indeno[1,2,3-c,d]pyrene	2051
Dibenz[a,h]anthracene	ND
Benzo[g,h,i]perylene	1341

Fig. 6: Map of Chattanooga Creek Area. ND signifies a compound not detected in the area.

creek bed to the surrounding areas upon the regular flooding events that occur throughout the Chattanooga Creek area (USACE).

The purpose of this investigation was to test the hypothesis that the PAHs found throughout the Chattanooga Creek floodplain can induce apoptosis of human coronary artery endothelial cells, an event known to be important in the pathogenesis of atherosclerosis. We tested cells treated with PAHs for both PLA_2 expression and for markers of apoptosis. Additionally, we analyzed tissue samples from mice trapped both in the Chattanooga Creek flood plain and in a distant control site for markers of apoptosis. Sediment of the control site, upstream from the Chattanooga Creek site, was found to be free of PAH contamination.

2. Materials and Methods

Materials

Naphthalene, acenaphthylene, phenanthrene, anthracene, 1-methylanthracene (1-MA; activates Group IVC PLA_2-- included as a positive control), fluoranthene, chrysene, benzo (b) fluoranthene (also known as benz [e] acephenanthrylene), benzo (k) fluoranthene, indeno (1,2,3-c,d) pyrene, and benzo (g,h,i) perylene were obtained from Sigma-Aldrich (St. Louis, MO). Pyrene and benz (a) anthracene were obtained from Fluka (Buchs SG, Switzerland). 1-palmitoyl-2-[arachidonoyl-1-14C] phosphatidylcholine, [5,6,8,9,11,12,14,15-3H], and 3H-arachidonic acid were purchased from American Radiolabeled Chemicals (St. Louis, MO). Anti-caspase-3 antibody was purchased from Cell Signaling (Beverly, MA). Anti-rabbit secondary antibody was purchased from Amersham Biosciences (Uppsala, Sweden). The Cell Death Detection ELISA^{PLUS} Kit was obtained from Roche (Indianapolis, IN).

Methods

Cell Culture: Human coronary artery endothelial cells (HCAECs) were purchased from Cambrex (Walkersville, MD) and maintained in microvascular endothelial growth media (Cambrex) containing 5% fetal bovine serum (FBS) at 37 degrees celsius in an atmosphere of 5% CO_2/95% O_2.

Arachidonic Acid Release: HCAECs were seeded into 6-well plates (105 cells/well) in growth medium. At 75% confluence, the cells were prelabeled with 0.25 µCi/mL of ^{3}H-arachidonic acid (^{3}H-AA) for a period of 24 hours. At the time of the experiments, cells were washed twice with Hanks' Balanced Salt Solution

(HBSS, Sigma-Aldrich) and equilibrated for 45 minutes in HBSS with 0.1% bovine serum albumin (BSA). The radiolabeled cells were then exposed to vehicle or various concentrations of the PAHs found in the polluted section of Chattanooga Creek for a period of 60 minutes. Scintillation counting was used to measure the cumulative release of ^{3}H-AA into the medium, and the data were expressed as absolute disintegrations per minute (DPM).

Animal Studies: We trapped feral mice (*Peromyscus gossypinus*) along the floodplain of the Chattanooga Superfund site and in a control site two miles upstream from the contaminated section of creek (the same sites where soil samples were taken). All animal procedures were in compliance with National Institute of Health guidelines on animal use and were approved by the University of Tennessee Institutional Animal Use and Care Committee. Traps were set each evening and checked each morning. Trapped animals were transported to the laboratory and anesthetized with CO_2. After the chest cavities were opened, the mice were exsanguinated by cardiac puncture and the distal aorta, heart and lungs perfused with phosphate buffered saline (PBS) to remove clotted blood. We removed the aortas and froze them in liquid nitrogen for Western blot analysis.

Western analysis for apoptosis: Poly(ADP-ribose) polymerase (PARP) is a 116 kD enzyme. During apoptosis, PARP is cleaved, producing the 89 kD cleavage product measured here, as well as a 25 kD product. Caspase-3 (32 kD) is a protease activated during early stages of apoptosis, and when active, forms a heterodimer of 17 kD and 12 kD subunits. The production of PARP cleavage

products (89 kD) and the caspase-3 active form (~17 kD) are hallmarks of apoptosis. After treatment with PAHs for 6 hours, PARP and caspase-3 cleavage was evaluated by preparing and suspending crude extracts of protein in ice-cold sample buffer (1 X RIPA buffer) containing protease inhibitors. Cells were lysed on ice by sonication, and the lysates subjected to 14% SDS-polyacrylamide gel electrophoresis. Following protein assay of the lysates, 40 µg of protein were loaded per lane. The proteins were then transferred to nitrocellulose membranes via a semi-dry blotting apparatus, the membrane blocked with casein in TBST (0.5 g per 100 mL), and incubated at 4 degrees C, overnight, with an anti-caspase 3 antibody or a PARP antibody. Enhanced chemiluminescence (ECL, Amersham Biosciences) was used to visualize the proteins.

Histone fragmentation assay: A Cell death detection ELISA^{plus} kit was used to quantify the relative apoptotic effect and to determine the kinetics of PAH-induced apoptosis. We conducted preliminary lysate-free ELISAs using the appropriate concentration of PAH as well as ingredients normally used in the assay to determine if any of the PAHs interfered with the assay. Because of fluorescence properties of benzo (g,h,i) perylene, chrysene, indeno (1,2,3-c,d) pyrene, and pyrene, they could not be included in the histone fragmentation optical density assays. Otherwise, HCAECs were treated at 80-90% confluency with 10 µM PAHs or vehicle for various times (30, 60 minutes, and 2 hours). Cells were then washed with warm PBS and lysed, and the lysates were incubated with antihistone-biotin and antiDNA-POD for two hours at room

temperature. We then washed with incubation buffer and 100 µL of substrate solution was added to the cells for color development. A positive control of DNA/histone complex (kit-supplied), a positive control of 1-methylanthracene, and a negative control of buffer without cells (to determine background absorbance) were included in each assay. A plate reader was used to take optical density measurements at 405 nm. Optical density increases directly with histone fragmentation as quantified in this assay by plate reading.

siRNA: Human coronary artery cells were seeded into 6-well plates and maintained until approximately 75% confluency. The cells were then transfected with siRNA corresponding to the sequence coding for Group IVC PLA_2 using the SuperFect Transfection Reagent and protocol (QIAGEN, Valencia, CA). The siRNA transfected into the cells (QIAGEN) had a target sequence of:

5' AAGATAATGAGCAGCCGGAAG 3'

and with sense:

5' GAUAAUGAGCAGCCGGAAGdTdT 3'

and antisense:

3' dTdTCUAUUACUCGUCGGCCUUC 5'

Following transfection, the cells containing siRNA, as well as control cells, transfected with vector alone, were subjected to PAH treatment and histone fragmentation assay as explained above. Silencing of the protein was confirmed by Western blot analysis.

Statistical analysis: Data is expressed as the mean +/- SEM. Analysis of variance (ANOVA) was used to analyze the data. For all studies, $p< 0.05$ was

used as the criterion for statistical significance. Additionally, post hoc tests of the Tukey method (significance indicated by $p < .05$), and additionally Bonferroni corrections for histone fragmentation data were utilized (significance indicated by $p < .05/8$ [the number of compounds tested] = $p < .006$ for initial histone fragmentation time course tests, and $p < .05/6 = p < .008$ for siRNA percent inhibition of histone fragmentation). Arcsine square root transformations were performed on percent data (not shown) prior to statistical analyses. Number of tests (n) was a minimum of 3, with usually 6 to 9 tests run.

3. Results

PAH-induced ^{3}H-AA release from HCAECs

Little ^{3}H-AA release was released from cells exposed to anthracene or vehicle, while acenaphthylene and phenanthrene produced significant ^{3}H-AA release at all concentrations (Figure 7). In contrast, naphthalene produced significant ^{3}H-AA release only at the highest concentration of 30 μM (Figure 7). As can be seen in Figure 8, response to benz (a) anthracene was not different from vehicle-treated control; however, the other 4-ringed compounds, chrysene, fluoranthene, and pyrene, induced significant concentration-dependent ^{3}H-AA release. The five-ringed compounds benz (e) acephenanthrylene and benzo (k) fluoranthene also induced significant release of ^{3}H-AA at all concentrations (Figure 9). While treatment with the 6-ringed compound benzo (g,h,i) perylene produced a response that was not different from vehicle-treated control, indeno (1,2,3-c,d) pyrene caused significant ^{3}H-AA release (Figure 10).

PAH-induced apoptosis

Western analysis for the cleavage product of PARP (poly [ADP-polymerase] ribose) was evaluated for all compounds. All compounds, with the exception of control and anthracene, caused cleavage of PARP (Figure 11).

Compounds that caused ^{3}H-AA release also induced significant apoptosis as determined by histone fragmentation (acenaphthylene, benz (e) acephenanthrylene, benzo (k) fluoranthene, fluoranthene, phenanthrene, collectively $p < .006$, and naphthalene, $p < .05$) (Figure 12 and Table 1). Further,

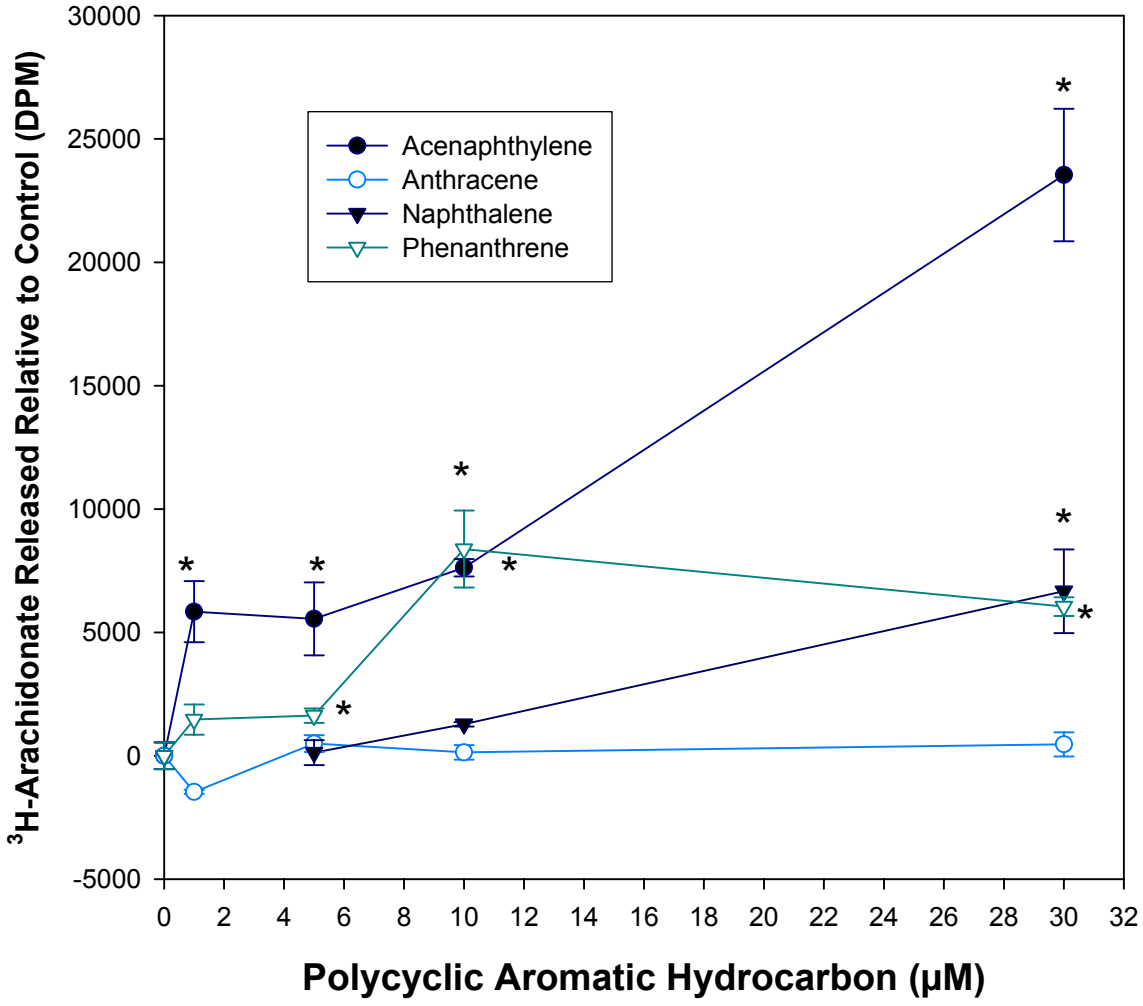


Fig. 7: Concentration-dependent release of ^{3}H-AA after exposure to 2- and 3-ring PAHs.
*** Significantly different from control; p < .05.**

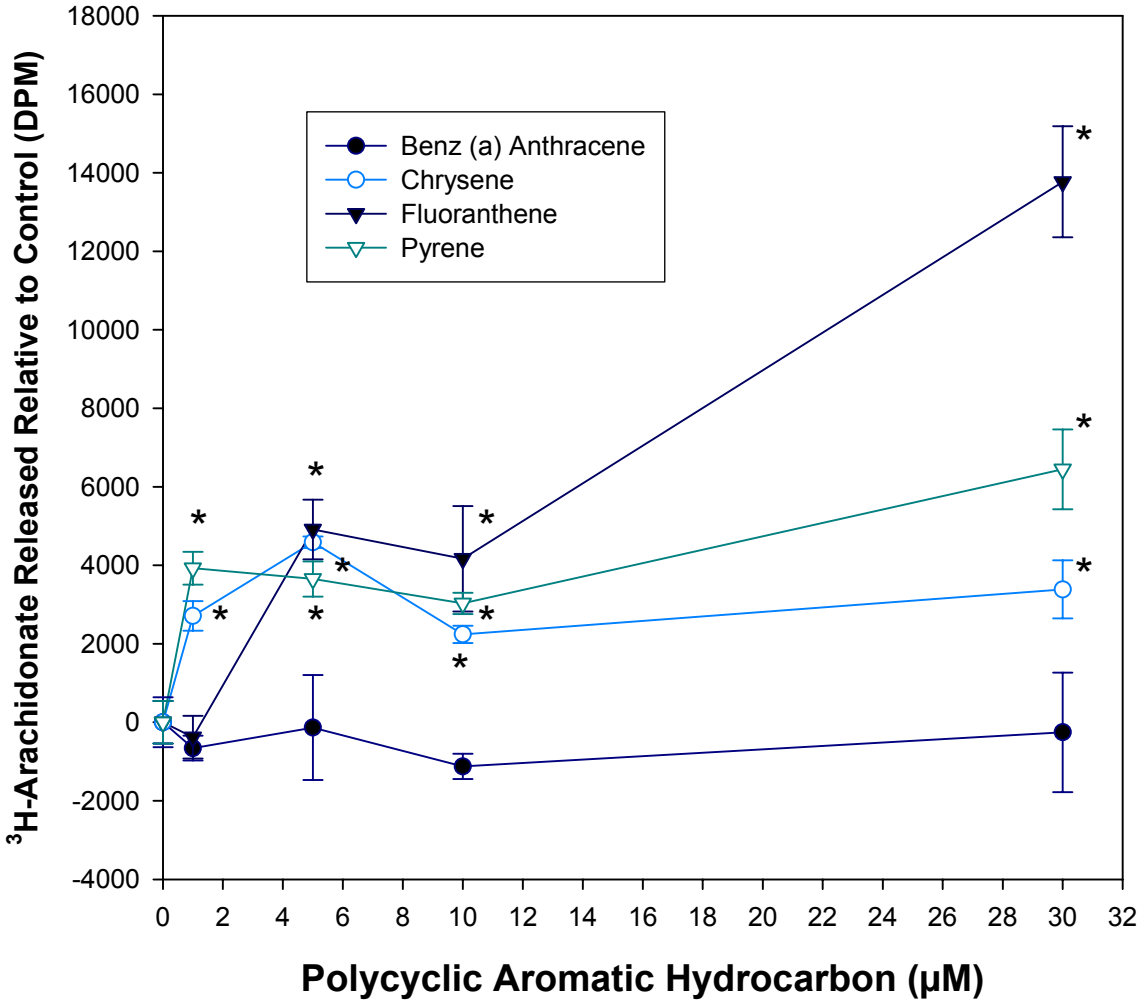


Fig. 8: Concentration-dependent release of ^{3}H-AA after exposure to 4-ring PAHs.
*** Significantly different from control; $p < .05$.**

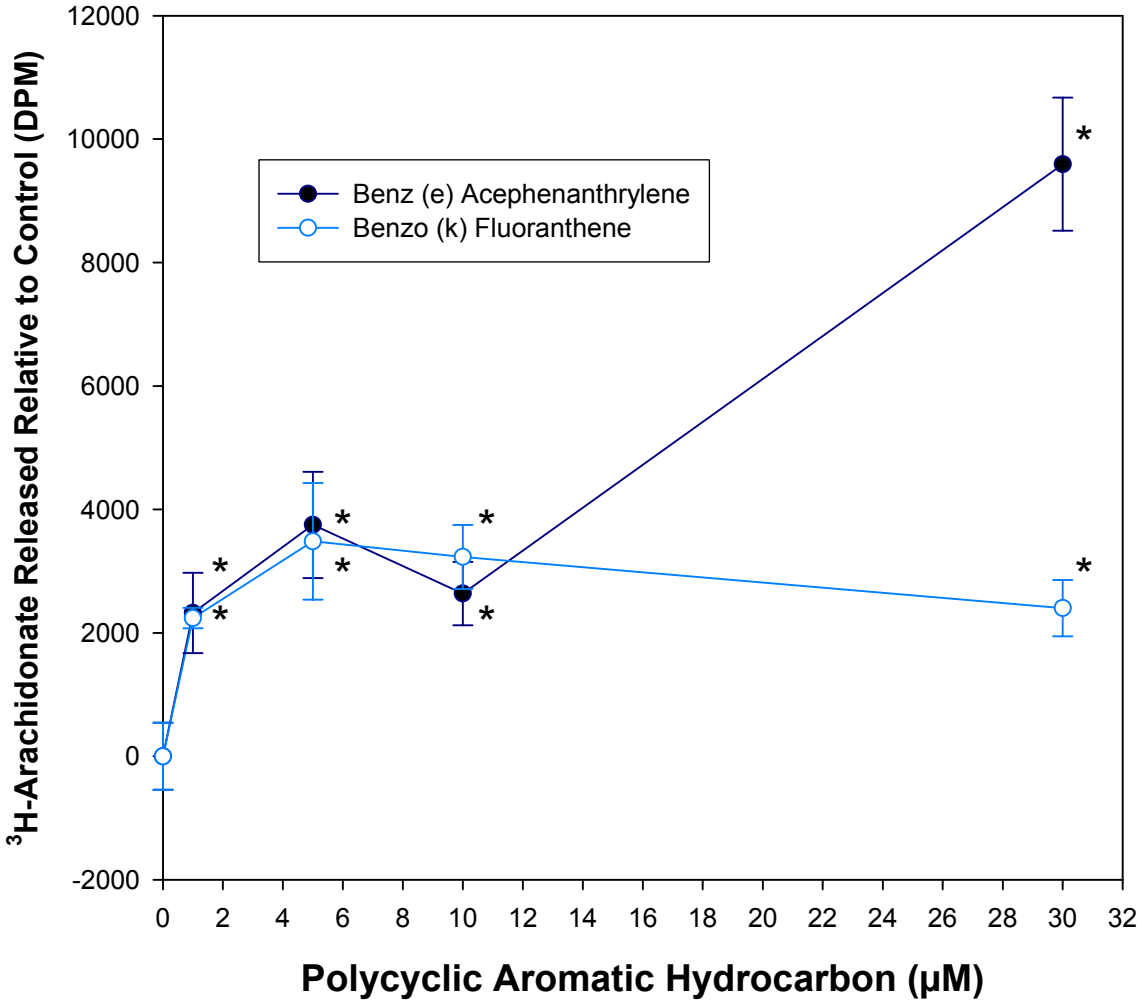


Fig. 9: Concentration-dependent release of ^{3}H-AA after exposure to 5-ring PAHs.
*** Significantly different from control; p < .05.**

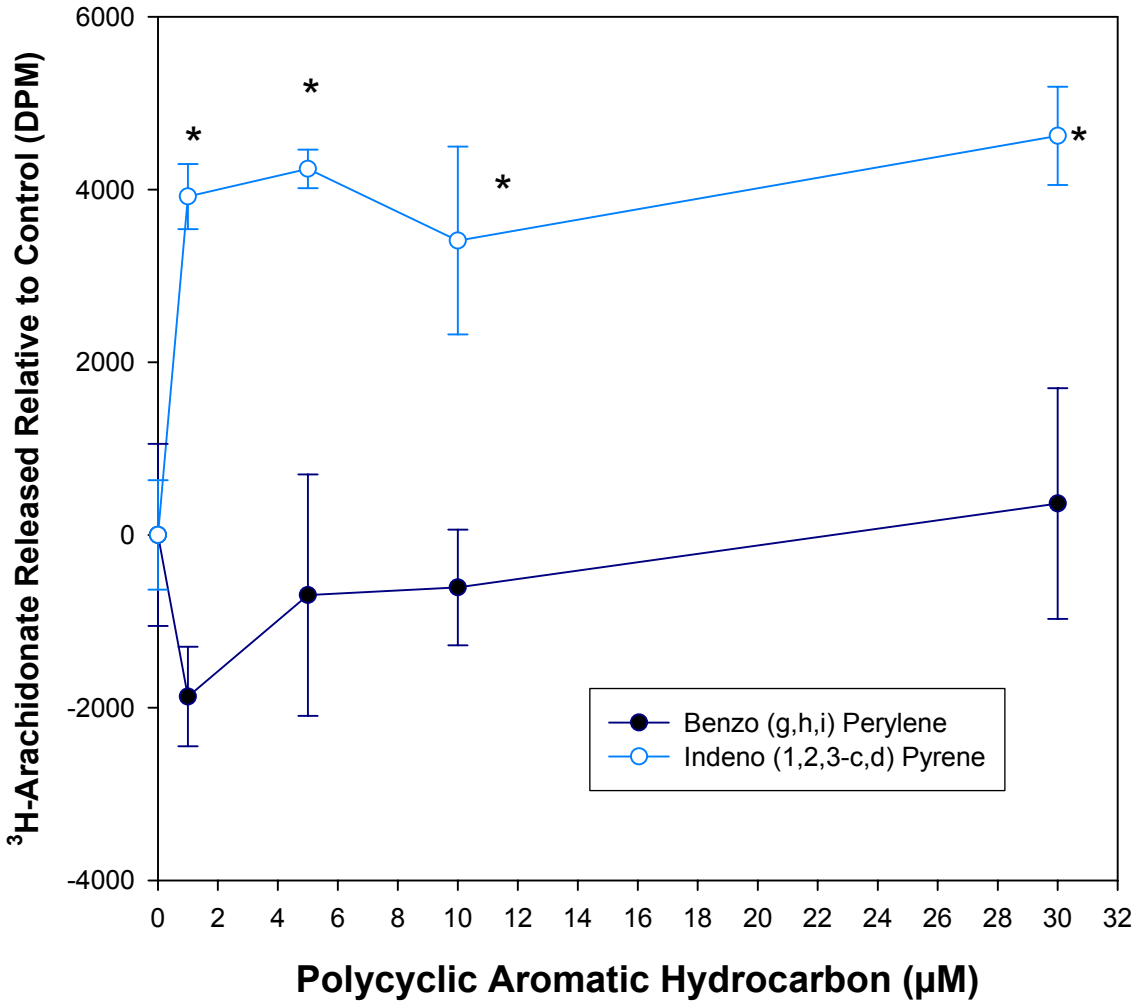
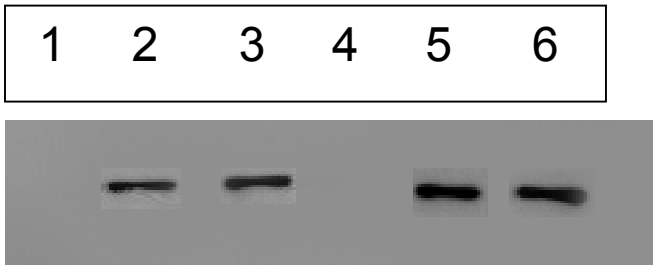


Fig. 10: Concentration-dependent release of ^{3}H-AA after exposure to 6-ring PAHs.
*** Significantly different from control; p < .05.**



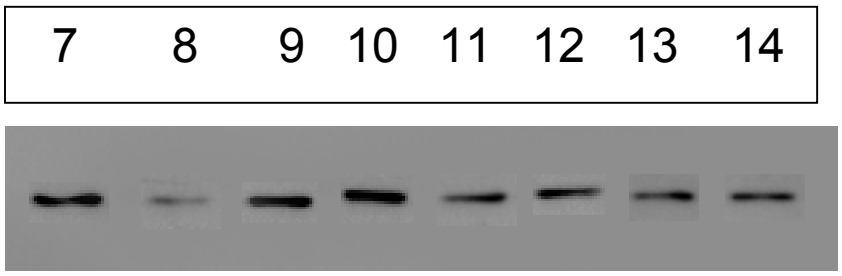


Fig. 11: 89 kD PARP cleavage product as expressed in HCAECs treated with PAHs.

1. DMSO
2. 1-Methylanthracene (positive control)

2- and 3-Ring Compounds:

3. Acenaphthylene
4. Anthracene
5. Naphthalene
6. Phenanthrene

4-Ring Compounds:

7. Benz (a) Anthracene
8. Chrysene
9. Fluoranthene
10. Pyrene

5-Ring Compounds:

11. Benz (e) Acephenanthrylene
12. Benzo (k) Fluoranthene

6-Ring Compounds:

13. Benzo (g,h,i) Perylene
14. Indeno (1,2,3-c,d) Pyrene

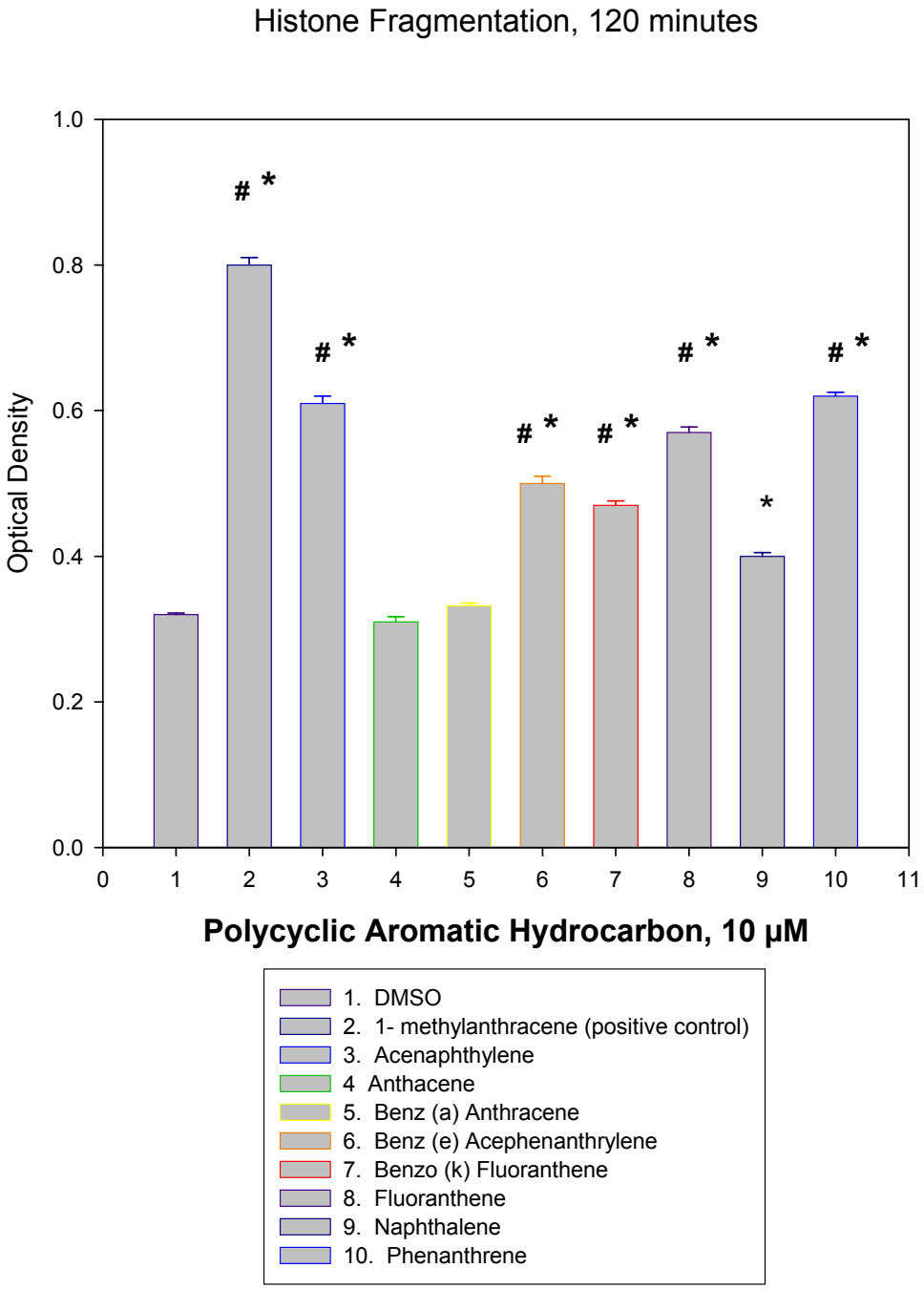


Fig. 12: Histone Fragmentation, 120 minutes. # indicates statistical significance when compared to control, $p < .006$. * indicates statistical significance when compared to control; $p < .05$.

Table 1: Histone Fragmentation, 30 min., 60 min., and 120 min.			
Optical density: measured by plate reader (405 nm).			
PAH	**30 min.**	**60 min.**	**120 min.**
DMSO	0.20 ± 0.0040	0.24 ± 0.021	0.32 ± 0.0020
1- methylanthracene (+ control)	0.20 ± 0.0060	$0.41 \pm 0.0060_{AB}$	$0.80 \pm 0.010_{AB}$
Acenaphthylene	$0.30 \pm 0.017_{A}$	$0.41 \pm 0.0030_{AB}$	$0.61 \pm 0.010_{AB}$
Anthracene	0.20 ± 0.0070	$0.29 \pm 0.0090_{B}$	0.31 ± 0.0070
Benz (a) Anthracene	0.21 ± 0.0040	$0.26 \pm 0.0040_{B}$	$0.33 \pm 0.0040_{B}$
Benz (e) Acephenanthrylene	$0.37 \pm 0.011_{A}$	$0.41 \pm 0.0050_{A}$	$0.50 \pm 0.010_{AB}$
Benzo (k) Fluoranthene	$0.33 \pm 0.0090_{A}$	$0.38 \pm 0.010_{A}$	$0.47 \pm 0.0060_{AB}$
Fluoranthene	0.17 ± 0.023	0.24 ± 0.027	$0.57 \pm 0.0070_{AB}$
Naphthalene	$0.23 \pm 0.0090_{A}$	$0.31 \pm 0.0060_{B}$	$0.40 \pm 0.0050_{B}$
Phenanthrene	$0.23 \pm 0.014_{A}$	0.26 ± 0.021	$0.62 \pm 0.0050_{AB}$
** Subscript A indicates statistical difference when compared with control. Subscript B indicates statistical difference when compared with the previous time point of that PAH. ($p < .006$)			

anthracene, while failing to induce ^{3}H-AA release, also failed to induce significant apoptosis (Table 1).

Inhibition of apoptosis by siRNA

To ensure that our siRNA was effective in silencing the gene for Group IVC PLA_2, Western analysis was performed with cells treated with the siRNA or vector alone for expression of Group IVC PLA_2. Gene expression was markedly inhibited in siRNA-treated cells (Figure 13, lane 1).

To investigate whether PLA_2 activation is required for PAH-induced apoptosis, histone fragmentation was determined in the presence and absence of the siRNA complementary to the PLA_2 gene. Apoptosis induced by acenaphthylene, benz (e) acephenanthrylene, benzo (k) fluoranthene, and fluoranthene (collectively, $p < .008$) was significantly inhibited by treatment with siRNA (Figure 14).

PLA_2 expression and apoptosis of aortic tissue from feral mice trapped in the floodplain of the Chattanooga Superfund Site

Aortic and lung tissue from mice (*Peromyscus gossypinus*) from the contaminated floodplain showed an increase in markers of apoptosis including caspase-3 and PARP cleavage (Figure 15, lanes SA and SL, respectively) when compared to mice from the uncontaminated site (Figure 15, lanes CA and CL, respectively). Moreover, there was a significant increase in the aortic expression of Group IVC PLA_2 in mice from the superfund site (Figure 16, lane 1) when compared to control (Figure 16, lane 2).

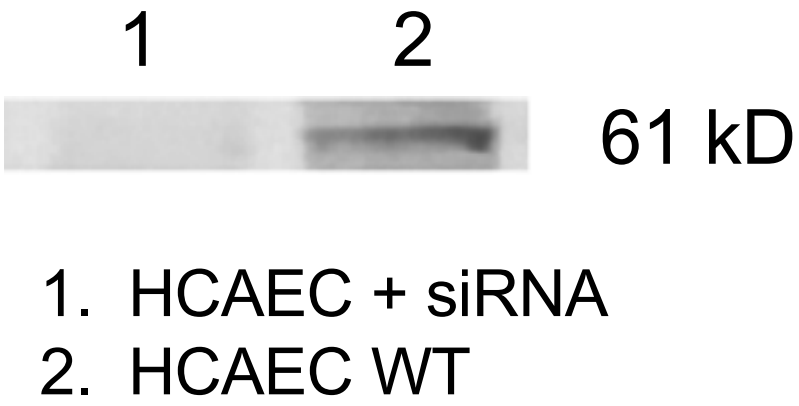


Fig. 13: Inhibition by siRNA of gene for Group IVC PLA_2.

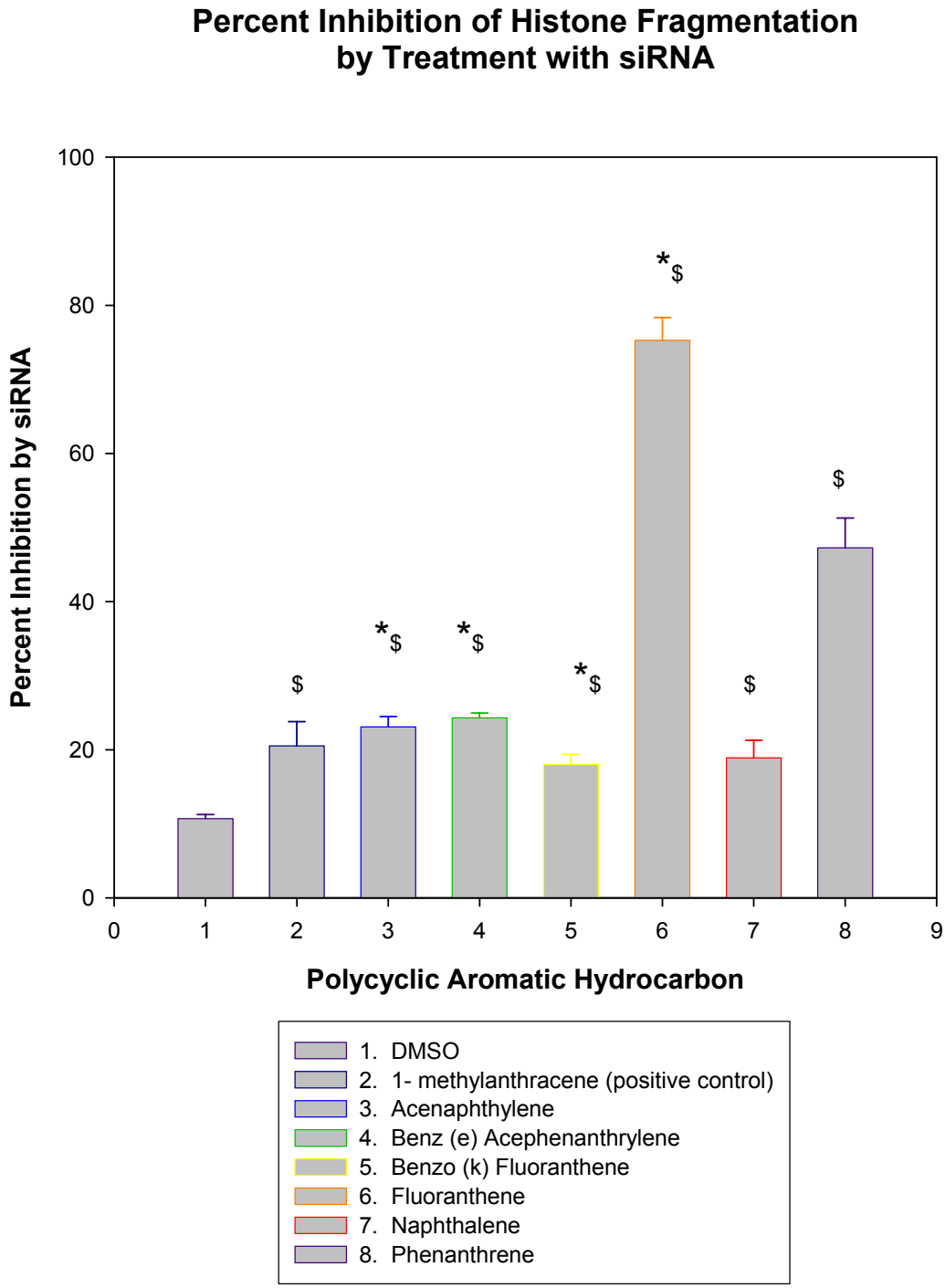


Fig. 14: Percent Inhibition of Histone Fragmentation by Treatment with siRNA. * indicates statistical difference, $p < .008$. $ indicates statistical difference, $p < .06$.

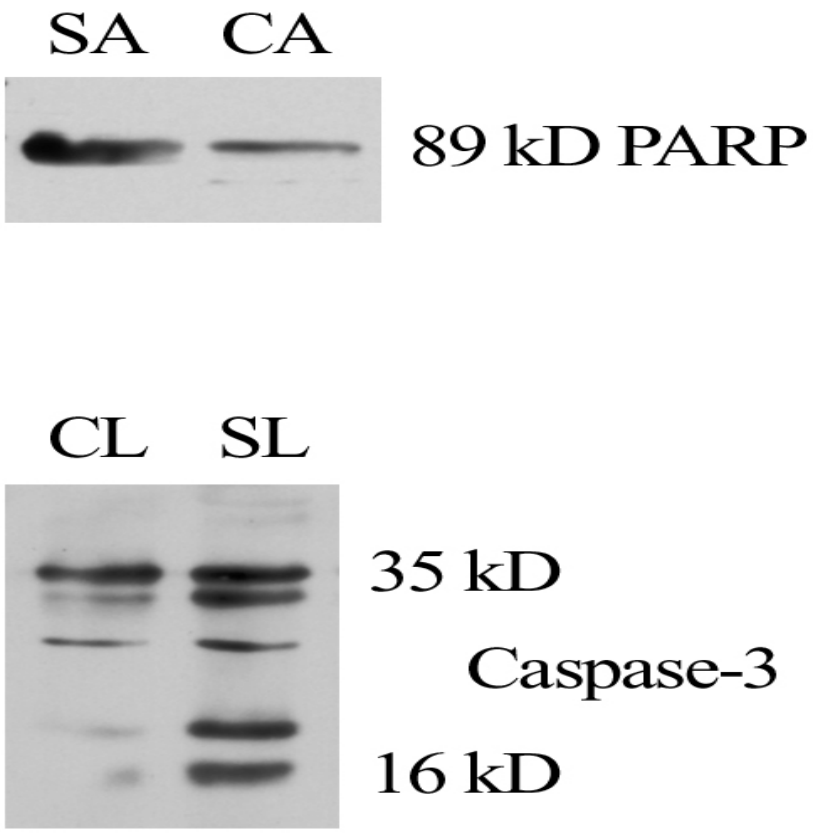


Fig. 15: Markers of Apoptosis (PARP and Caspase-3) as Found in Aortic Tissue and Lung Tissue of Mice from the Chattanooga Creek site (SA and SL, respectively) and from a Control Site (CA and CL, respectively).

Fig. 16: PLA_2 Expression in Aortic Tissue of Mice Trapped on both the Chattanooga Creek site (SA) and a Control Site (CA).

4. Discussion

This study confirms that several of the polycyclic aromatic hydrocarbons present in sediment samples from the Chattanooga Creek area induce apoptosis of endothelial cells, an important event in the pathogenesis of atherosclerosis (Table 2). Of the 12 tested, six compounds: acenaphthylene, benz [e] acephenanthrylene, benzo [k] fluoranthene, fluoranthene, naphthalene, and phenanthrene, induced ^{3}H-AA release in a concentration-dependent manner, and this event preceded the onset of apoptosis. Two compounds, anthracene and benz (a) anthracene, produced neither ^{3}H-AA release nor apoptosis, suggesting that these compounds are inactive. Chrysene, indeno (1,2,3-c,d) pyrene, and pyrene induced significant concentration-dependent ^{3}H-AA release and apoptosis as measured by PARP cleavage. Benzo (g,h,i) perylene, in contrast

Table 2: *In vitro* Results Summary.

PAH	Lowest Concentration Inducing ^{3}H-AA Release	Concentration at which Histone Fragmentation Seen	Histone Fragmentation inhibited by siRNA?	PARP Cleavage?
Acenaphthylene	1 μM	10 μM	yes (p < .008)	yes
Anthracene	not induced	not seen	not seen	no
Benz (a) Anthracene	not induced	not seen	not seen	yes
Benz (e) Acephenanthrylene	1 μM	10 μM	yes (p < .008)	yes
Benzo (g,h,i) Perylene	not induced	NA	NA	yes
Benzo (k) Fluoranthene	1 μM	10 μM	yes (p < .008)	yes
Chrysene	1 μM	NA	NA	yes
Fluoranthene	5 μM	10 μM	yes (p < .008)	yes
Indeno (1,2,3-c,d) Pyrene	1 μM	NA	NA	yes
Naphthalene	30 μM	10 μM	yes (p < .06)	yes
Phenanthrene	5 μM	10 μM	yes (p < .06)	yes
Pyrene	1 μM	NA	NA	yes

did not induce ^{3}H-AA release, but it did induce PARP cleavage. Inhibition of the Group IVC PLA_2 with siRNA significantly attenuated PAH-induced apoptosis in the same 6 compounds that induced both ^{3}H-AA release and apoptosis (see above). Because histone fragmentation effects of benzo (g,h,i) perylene, chrysene, indeno (1,2,3-c,d) pyrene, and pyrene could not be verified due to treatment-independent compound fluorescence, other methods of determining apoptosis should be pursued in future studies. Aortic and lung tissues taken from mice trapped along the flood plain of the Superfund site displayed marked increases in PARP and caspase-3 cleavage, both markers of apoptosis. In addition, upregulation of expression of Group IVC PLA_2 was also observed when compared to results obtained from a control site where no PAHs were detected. These data support the concept that PAHs cause endothelial cell death by a mechanism that involves Group IVC PLA_2-induced release of fatty acids from the *sn*-2 position of membrane phospholipids. Moreover, the data obtained in the feral mouse samples parallel the *in vitro* data, suggesting the relevancy of our studies to residents of the Chattanooga Creek.

In previous studies with human coronary artery endothelial cells, Tithof, *et al.*, found that Group IVC PLA_2 is constitutively expressed. Moreover, treatment with active PAHs increases the expression of Group IVC enzyme. In the current study significant silencing of the gene was accomplished by transfecting with siRNA specific to Group IVC PLA_2. Further, results of this study suggest that the Group IVC PLA_2 enzyme is responsible, at least in part for apoptosis caused by PAHs because siRNA specific to this isoform inhibited apoptosis in response to

PAH exposure. Although these data suggest that Group IVC PLA_2 mediates apoptosis of HCAECs, the data do not rule out the activity of other enzymes. Apoptosis can also be mediated by other PLA_2 isoforms, including Groups IVA PLA_2 and VI $iPLA_2$, as well as some secreted isoforms of PLA_2 (Hayakawa, Tithof, 2002). Isoforms other than Group IVC were not involved in our siRNA oligonucleotide transfection, and could still be actively participating in apoptosis. Determining the specific enzymes involved in PAH-induced endothelial cell apoptosis, and in turn, their functionality, is the subject of future investigations.

PAHs have been found to induce apoptosis by various mechanisms unrelated to PLA_2 activation. In this study, apoptosis induced by benzo (g,h,i) perylene is likely caused by mechanisms other than PLA_2 activation. Additionally, the fact that naphthalene induces ^{3}H-AA release at 30 μM but apoptosis at only 10 μM suggests that naphthalene may activate more than one mechanism of apoptosis, producing an additive effect at a lower concentration. DNA damage, especially such that the cell experiences a loss of RNA splicing capabilities, can induce apoptosis (Kockx). It is also well known that activations of various pathways, such as the pathway induced by binding of the cell surface beta-adrenergic receptors, result in cell death by apoptosis (Iwai-Kanai). The mechanism by which PAHs activate Group IVC PLA_2 is not known.

Beyond activity directly on PLA_2s, many PAHs are known to bind the aromatic hydrocarbon receptor (AHR), an action which promotes oxidative stress and, in turn, apoptosis (Chaloupka, Nebert). Further, reactive oxygen species are known to be produced during cellular metabolism of PAHs, and such oxygen

radicals can induce apoptosis of endothelial cells (Blaha, Dana). Oxidative stress also may activate the Group IVC enzyme (Asai). Asai, *et al*., found that cPLA$_2$γ may be activated by hydrogen peroxide via a tyrosine phosphorylation pathway, although the data are not repeated by other groups. The ability for PAHs to bind or otherwise interact with as yet unidentified receptors that are involved in the activation of PLA$_2$ should also be considered. PAHs have already been found to bind steroid hormone receptors and glycine N-methyltransferase, another known receptor, clearly indicating PAHs' ability to bind and potentially alter various cellular proteins (Chang, Bhat).

PAH mechanism of inducing apoptosis may also be related to lipophilicity. Because of their fat-solubility, PAHs may be able to enhance the interaction of PLA$_2$ enzymes with their respective substrates. Other lipophilic compounds, such as arachidonic acid, have already been shown to facilitate assembly of protein complexes. Arachidonic acid induces a conformational change in NADPH oxidase, enabling the proper assembly of the enzyme complex responsible for action of the superoxide anion (Tithof, 1999, Rodriguez, Goodnight). Determining whether PAHs cause apoptosis by binding cellular receptors, inducing oxidative stress, or by augmenting interactions between enzymes and phospholipids in a given situation will be pursued in future studies.

PAHs may also act on PLA$_2$ enzymes via phosphorylation of various signaling molecules. It has been found that PAH-metabolites can be responsible for both an increase in intracellular Ca^{++}, which can upregulate active protein kinase C (PKC), and for an increase in tyrosine kinase (PTK) (Mounho). In fact

Krieger, *et al*., found the effect of PAHs upon PTKs to be important to the rise of Ca^{++}. Further, it has been suggested that PKC has a large role in inducing $sPLA_2$-mediated neutrophil migration to a site of inflammation (Gambero).

The results of this study also suggest that PAHs have both immediate and long-term effects on Group IVC PLA_2. As elucidated in previous studies, PAHs induce release of ^{3}H-AA from endothelial cells within minutes of treatment (Tithof, 2002). More long-term exposure to PAHs induces an increase in expression of Group IVC PLA_2, suggesting that active PAHs may exert transcriptional control over the enzyme. It is also possible that PAHs act by increasing protein stability. Moreover, it is already known that arachidonic and other fatty acids activate transcription factors such as AP-1 and NF-KB, both of which are involved in the regulation of apoptotic cell death (Bécuwe).

Although less information is available concerning the means by which environmental pollution causes heart disease, the mechanism by which cigarette smoking induces heart disease is much better understood. Because cigarette smoke contains many of the PAHs and other toxicants commonly found in urban pollution, studies examining the pathophysiology of smoking-induced heart disease are relevant. The Honolulu Heart Program began in 1968 and provided a 20-year prospective study of 8006 Japanese-American men aged 45 to 65. It demonstrated that morbidity and mortality due to CVD in this group of men was significantly reduced (by 50%) in smokers who consumed diets high in omega-3 fatty acids, compounds known to inhibit the AA cascade at several levels (Rodriguez, Ridker). The Honolulu Heart Project, as well as other studies, went

on to suggest that components of cigarette smoke were the activators of the AA cascade (McCarty). This project is one of several that describe a distinct link between exposure to compounds found in cigarette smoke, PLA_2/AA activation, and CVD.

Clearly, as endothelial cell injury is known to be important to the process of CVD, induction of endothelial cell apoptosis by components of both cigarette smoke and environmental pollution may represent an important mechanism of augmentation of cardiovascular diseases. Further information about specific requirements for PLA_2 activation and subsequent apoptosis of endothelial cells, as well as a more complete understanding of how PAHs induce apoptosis, may aid in understanding potential toxicities of PAHs found in cigarette smoke as well as in the environment. This information could prove valuable in risk assessment and in development of both preventative and therapeutic techniques for patients at-risk for CVD, not unlike those in the Chattanooga Creek Area.

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