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The Effects of Benzo(A)Pyrene and Dimethylbenzanthracene on Cultured Porcine Aortic Endothelial Function

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THE EFFECTS OF BENZO(A)PYRENE AND DIMETHYLBENZANTHRACENE ON CULTURED PORCINE AORTIC ENDOTHELIAL FUNCTION

by

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Polycyclic aromatic hydrocarbons are environmental contaminants which have been shown to initiate and promote atherosclerosis and carcinogenesis in various animal species. These findings have been based primarily on the increase in incidence and size of smooth muscle cell tumors in these animals when exposed to benzo(a)pyrene (BaP) or dimethylbenzanthracene (DMBA). The endothelium has also been identified as an important mediator in vascular disease and atherogenesis, although the endothelial response to these PAHs has not been investigated. Thus, the focus of this study was on characterization of the endothelial response to BaP and DMBA. Concentration/response relationships for these chemicals were examined, in addition to evaluation of cellular toxicity and functional changes induced by BaP and DMBA which may suggest a role in the atherogenic process.

In this study, the effects of BaP and DMBA on endothelial morphology, cell proliferation, protein synthesis, monolayer permeability, LDL uptake, and monocyte adherence were characterized. Results indicate that BaP and DMBA are capable of inducing morphological changes as well as changes in cell proliferation and protein content that are time and concentration dependent. Results from functional studies indicate that although neither BaP or DMBA appear to have an effect on monolayer permeability, both chemicals are capable of inducing an increase in LDL uptake and monocyte adherence. Therefore, these results suggest that BaP and DMBA can induce an endothelial response, and may play a role in the atherogenesis.

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INTRODUCTION

Endothelial Cell Biology

The endothelium, which comprises the interface between the blood and the underlying vessel wall (Figure 1), is a powerful mediator of signals and blood components between the circulation and the tissues supplied by these vessels. Existing as a monolayer, the vascular endothelium was previously thought to be nothing more than a metabolically inert, selectively permeable barrier between the blood and tissue spaces (Pearson, 1991; Fajardo, 1989; van Hinsbergh, 1992). In recent years, however, it has been discovered that the endothelium is a critical factor in the regulation of vascular hemostasis, vascular tone, angiogenesis, macromolecular transport, response to mechanical stresses, and disease processes.

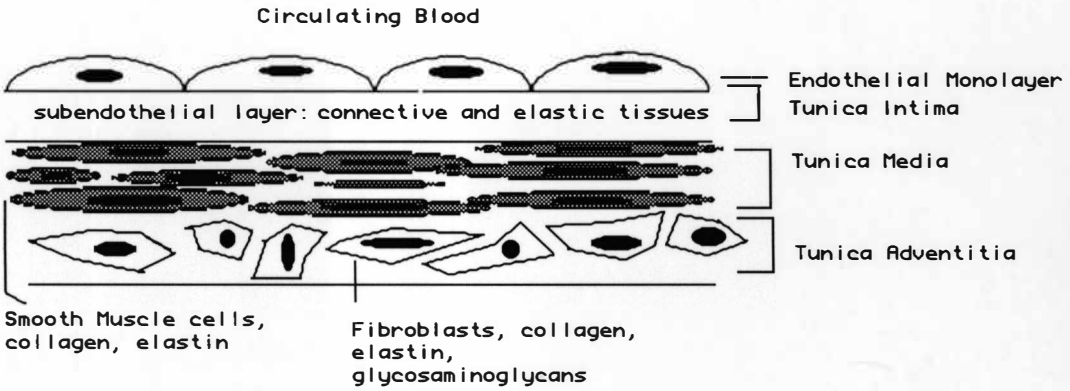

Figure 1. Cellular Composition of the Vascular Wall of Medium and Large Sized Blood Vessels.

Structure of the Endothelial Monolayer

The endothelium consists of a single layer of polygonal, flattened cells which line the luminal surface of the blood vessel wall. These cells typically display a cobblestone-like morphology, and retain this appearance in culture (Figure 2). The luminal surface of the endothelium is covered with a carbohydrate-abundant glycocalyx while the abluminal surface is anchored to the subendothelial matrix via binding proteins known as integrins, which are also responsible for intercellular adhesion of endothelial cells (Alberts et al., 1994). The subendothelial matrix is synthesized by the endothelium and consists of collagen, elastin, laminin, and fibronectin. Each of these matrix components have specific influences upon cell migration and proliferation as does the cytoskeleton (Alberts *et al.*, 1994). *In vivo* and *in vitro*, under simulated conditions of blood flow and shear stress (Davies and Tripathi, 1993), endothelial monolayers align themselves longitudinally in the direction of blood flow. It is believed that this is accomplished by transducing physical forces into biochemical signals which then act upon the proteins of the extracellular matrix and with the microfilaments of the cytoskeleton (Davies and Tripathi, 1993).

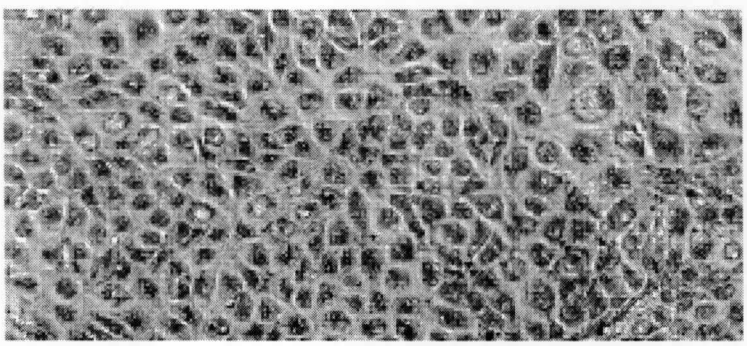

Figure 2. Porcine Aortic Endothelium *in Vitro*. 40 x magnification. Stained with 0.5% crystal violet.

Endothelial monolayers are heterogeneous; individual cell size ranges from 10 μ to 50 μ in diameter and from 0.1 μ to 0.5 μ in thickness. Depending upon location, endothelial monolayers may be continuous (without pores; CNS, lung, muscle, skin), sinusoidal (irregular, large gaps between cells; liver, bone marrow, spleen), or fenestrated (with pores/fenestrae; intestinal mucosa, endocrine glands, and renal glomerulus)(Leeson *et al.*, 1988; Fajardo, 1989). In addition, intercellular junctions vary with cell location; occludens, adherens, and gap junctions are found in some but not all endothelial monolayers (e.g., gap junctions are not found in the capillaries) (Leeson *et al.*, 1988).

Regulatory Functions of the Endothelium

The endothelium is now known to play a role in the regulation of vascular permeability, hemostasis, vascular tone, vascular cell growth, the expression of various receptors and binding sites, the secretion of specific proteins, the response to inflammatory stimuli, and angiogenesis (van Hinsbergh, 1992). Precise regulation of these processes is necessary for vascular homeostasis; alterations in this regulatory balance may lead to vascular disease and dysfunction.

Regulation of Permeability

The selectively permeable barrier provided by the endothelial monolayer consists of several pathways through which fluids, solutes, and macromolecules can pass from the lumen of the vessel into the subendothelial space. Small molecules such as oxygen are able to diffuse across the plasma membrane, while small lipids are able to pass into the subendothelial space via lateral movement across the membrane at intercellular junctions and transendothelial channels (van Hinsbergh,

1992). Solute exchange occurs passively through the cell membrane, through vesicles, through small and large pores of the cell, and via passage through intercellular junctions. Macromolecules rely upon carrier mediated exchange and receptor mediated transport (van Hinsbergh, 1992), while cellular components and many toxic substances are prevented from crossing the endothelial barrier.

Injury to the endothelium may cause a loss of cells from the monolayer, resulting in increased permeability and interstitial edema. In non-denuding injury due to reactive oxygen species or chemicals, changes in the cytoskeleton may occur, leading to enhanced permeability between cells (Coomber and Gotlieb, 1990). The endothelium must attempt to correct an increase in permeability by increasing cell migration and proliferation such that intercelluar junctions may be reestablished.

Regulation of Hemostasis

The endothelium is also involved in maintaining balance between a procoagulant and an anticoagulant state. The intact endothelium maintains a shift towards the anticoagulant state via the production of proteins which inhibit coagulation, platelet aggregation, and vasoconstriction, and which trigger the fibrinolysis pathway (van Hinsbergh, 1992). Endothelial injury may result in a shift towards the procoagulant state and is accompanied by platelet aggregation. However, a spontaneous shift towards this state can cause thrombus formation, resulting in tissue ischemia due to obstruction of blood flow (van Hinsbergh, 1992). Figure 3 illustrates some of the specific factors involved in endothelial regulated hemostasis.

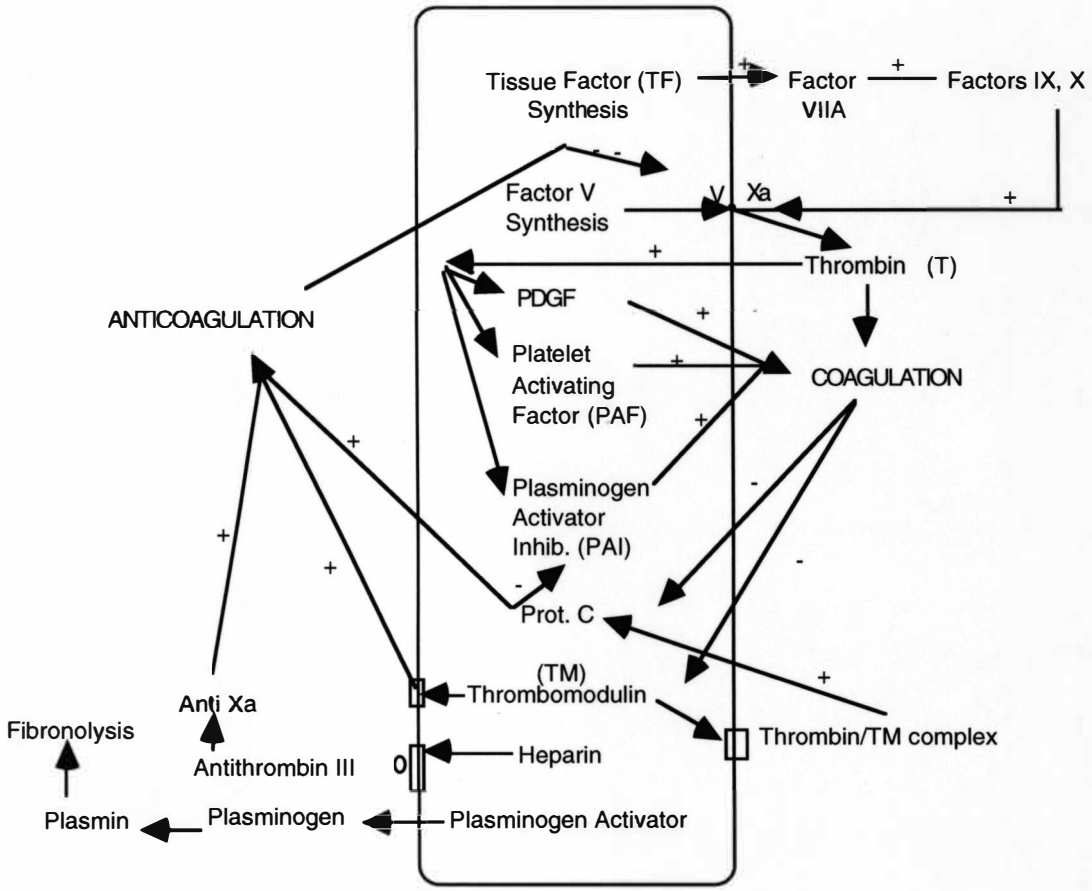

Figure 3. Endothelial Regulated Hemostasis. '+' indicates activation of pathway. '-' indicates inactivation of pathway. Rectangle represents an endothelial cell. (Nachman and Hajjar,1991).

Regulation of Vascular Tone and Vascular Cell Growth

Endothelial cells contribute to the regulation of smooth muscle contraction in the vessel wall via the release, catabolism, and conversion of vasoconstrictor and vasorelaxing substances. Examples of chemical conversion to vasoactive substances include the catabolism of ATP and ADP into AMP and adenosine, a potent vasodilator (van Hinsbergh, 1992); angiotensin I is converted by angiotensin converting

enzyme (ACE) into angiotensin II, a potent vasoconstrictor (van Hinsbergh, 1992). EDRF (endothelial derived relaxing factor) production can be induced by acetylcholine, histamine, bradykinin, substance P, and ATP. EDRF (also known as nitric oxide) then activates guanylate cyclase leading to cGMP production in vascular smooth muscle cells (VSMC), thus inducing relaxation (van Hinsbergh, 1992; Fajardo, 1989). Prostacyclin and prostaglandins are induced by the same mediators, but induce relaxation through activation of adenylate cyclase leading to cAMP production. It has also been suggested that endothelial mechanoreceptors responding to shear/mechanical stress induce the generation of vasoactive mediators within the endothelium (Davies and Tripathi, 1993).

In addition, some of these factors have been found to have an effect upon VSMC growth. Recent reports suggest that vasoconstrictors such as endothelin-1 promote VSMC growth, while vasorelaxant substances such as nitric oxide (endothelial derived relaxing factor; EDRF) or prostacyclin tend to inhibit VSMC growth (Peiro, *et al.*, 1995). VSMC growth is also regulated by the quiescent endothelium and its matrix. Therefore, poor regulation of vasoactivity may lead to chronic vasoconstriction and hypertension, in addition to excessive VSMC division, resulting in a pathological condition.

Other Regulatory Processes

In addition to the regulation of permeability, hemostasis, vasoactivity and vascular cell growth, the endothelium is also actively involved in the response to inflammatory stimuli and angiogenesis. Inflammatory mediators in the circulation (interleukin-1, tumor necrosis factor, interferon gamma, bacterial lipopolysaccharide) are capable of activating protein synthesis within the

endothelium, although this ability decreases with age and senescence of the endothelium *in vitro* (Klein, *et al.*, 1995). Among the induced proteins are leukocyte adhesion molecules ELAM-1, VCAM-1, and ICAM-1, in addition to plasminogen activator inhibitor-1, tissue factor, MHC class I antigens, NO synthase, and cyclooxygenase. The effect of an acute inflammatory response by the endothelium can lead to increased intercellular permeability (via histamine and bradykinin binding to endothelial receptors (Pearson, 1991)) and edema; increased leukocyte adherence can result in extravasation of monocytes in to the subendothelium and subsequent formation of lipid laden foam cells. Thus, the induction of these proteins in response to inflammation has been found to contribute to the acceleration of atherosclerosis (van Hinsbergh, 1992).

Angiogenesis, the process of new vessel development, is also attributed to the endothelium. Under normal conditions, endothelial cell turnover in an intact monolayer is slow (due to the presence of inhibitory signals from neighboring pericytes), but in conditions of vascular disease or in wound healing the endothelium responds to angiogenic stimuli such as fibroblast growth factor, epidermal growth factor, and prostaglandins (Pearson, 1991). The angiogenic response induces the secretion of proteolytic enzymes from the endothelium (Pearson, 1991) which degrade the endothelial matrix components. The endothelial cells then migrate in response to chemotactic angiogenic factors where they form neovessels through replication and remodeling (Fajardo, 1989).

Thus, the endothelium is a critical factor in the maintenance of vascular homeostasis. Any defect in the endothelial regulation of vascular permeability, hemostasis, vascular tone, cell growth, angiogenesis, or the inflammatory response may result in vascular dysfunction and/or vascular disease. Elucidating the role of the endothelium in vascular disease processes such as atherosclerosis is therefore

essential to the understanding of the disease process as well as for the development of preventive measures and therapeutic targets.

Atherosclerosis

Atherosclerosis is a progressive disease initially affecting the intimal region of the medium and large vessels of the cardiovascular system, and ultimately inducing plaque and thrombus formation, occlusion of the vessel lumen, and focal degeneration of the vessel wall. Atherosclerosis contributes to virtually every form of cardiovascular disease, making it one of the leading causes of death in the United States. Once considered to be a consequence of aging, atherosclerosis is now understood to be a condition of chronic focal inflammation which may be induced or enhanced by various environmental, physiological, and chemical factors (Ross, 1993; Davies and Woolf, 1993). Such factors include elevated cholesterol and blood pressure, cigarette smoke, sex, weight, and heredity.

Fatty Streak Formation

The first stage in atherogenesis is the development of the fatty streak within the intima of the vessel wall (Figure 4). Once thought to be an inactive barrier between the blood and vessel wall, the endothelium is now understood to be actively involved in this disease process, as well as in vascular homeostasis. Lesion development is thought to begin with an alteration in normal endothelial function generally due to endothelial injury (i.e., in response to toxins, viruses, mechanical injury, or oxidized low density lipoprotein (oxLDL)). The result is an 'activated' endothelial cell which produces leukocyte adhesion molecules, monocytic activators, oxygen free radicals, mitogens and chemoattractants for smooth muscle cells, and

generally displays an increase in monolayer permeability (DiCorleto and Chisolm, 1986; Gimbrone, 1995; Ross, 1995; Nawroth *et al.*, 1993).

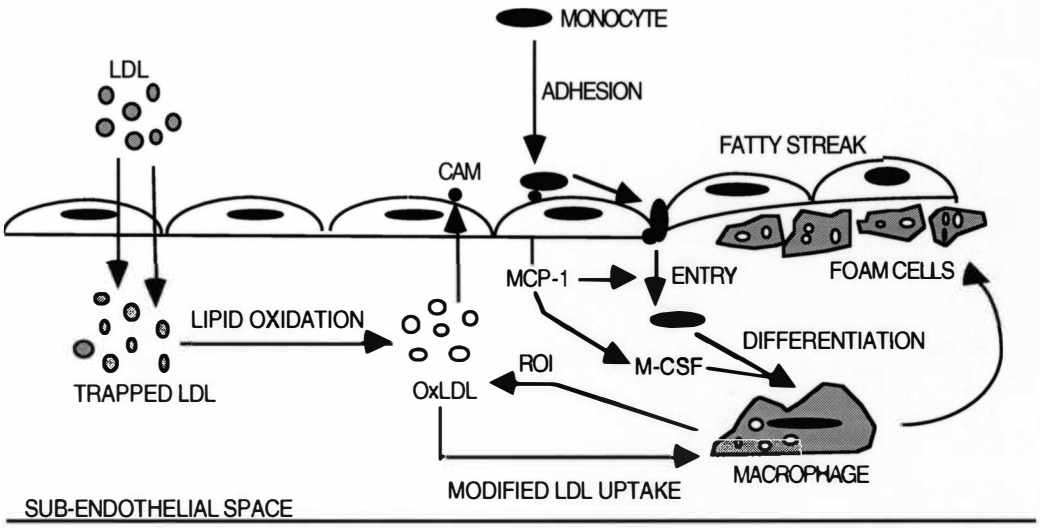

Figure 4. Fatty Streak Development. ROI: reactive oxygen intermediates. CAM: Cellular adhesion molecules. Adapted from Berliner *et al.*, 1995.

Under normal conditions, the endothelium internalizes LDL via receptor mediated endocytosis on coated pits, such that LDL can be utilized for membrane synthesis. Under conditions of endothelial activation, internalized LDL may be oxidatively modified within the cell where it may exert cytotoxic effects (Berliner *et al,* 1995; Ross 1993). Alternatively, the increased permeability of an activated endothelial monolayer allows movement of LDL from the circulation into the subendothelial space where it becomes trapped as a result of intimate association with the subendothelial matrix (Berliner *et al,* 1995). Here, the LDL is susceptible to oxidative modification by reactive oxygen intermediates secreted from various pathways within the cells of the arterial wall (Ross 1993). OxLDL in the subendothelial space induces the expression of adhesive glycoproteins (ICAM-1,

VCAM-1) on the luminal side of the endothelium and endothelial secretion of chemotactic molecules, resulting in the migration and adherence of monocytes to the endothelial cell surface. The chemotactic effects are mediated in part by lysophosphatidylcholine, which is formed during the oxidation of native LDL (Penn and Chisolm, 1994). The induction of endothelial monocyte chemotactic protein-1 (MCP-1) by oxLDL induces transendothelial migration of the monocytes at the intercellular junctions into the subendothelial space. Here, monocytes differentiate into macrophages in response to oxLDL-induced endothelial production of macrophage colony stimulating factor (M-CSF). Oxidized lipid in the subendothelial space is avidly ingested by subendothelial macrophages via scavenger receptors which are not down regulated by intracellular lipid (Davies and Woolf, 1993) such that the macrophages become engorged with lipid and take on a 'foam cell' appearance. These cells constitute the bulk of the fatty streak within the vessel intima, and are commonly found at sites of turbulent flow such as the bifurcations and curves.

The Intermediate or Fibrofatty Lesion

The lesion known as the fatty streak may regress, or under appropriate conditions may progress to a fibrofatty lesion. Following the formation of the fatty streak, lipid laden foam cells secrete smooth muscle growth factors and chemotactic molecules resulting in smooth muscle cell proliferation and infiltration into the intima. Further progression results in the formation of a necrotic core within the lesion; this core consists of extracellular lipid that has been released from dying foam cells (Davies and Woolf, 1993). Simultaneous production of excessive extracellular matrix components (collagen, elastin, and proteoglycans) occurs, contributing to the size of the lesion such that it begins pushing towards the

adventitia until it can no longer expand (Davies and Woolf, 1993).

The Fibrous Plaque

The fibrous plaque is the most advanced lesion in the atherosclerotic process. At this stage connective tissue has infiltrated the lesion, forming a fibrous cap which provides stability to the lesion. The cap covers the necrotic core which by this time contains macrophages, T lymphocytes, and smooth muscle cells in addition to lipid (Ross, 1995). The size of the plaque prevents further expansion into the intima and thus begins to protrude into the vessel lumen. Calcification of the plaque occurs and predisposes the plaque to rupture; reduced strength of the tissue as a result of the lesion may also play a role in plaque rupture (Davis and Woolf, 1993). As the plaque tears, blood is able to enter from the lumen and comes into contact with collagen and tissue factor (expressed in the adventitia) and thrombus formation ensues (Berliner *et al.*, 1995). Thrombus formation may be mural or occluding in nature; mural thrombi exist within the torn plaque, consist primarily of platelets, and do not protrude further into the vessel lumen although they contribute to stenosis (Davis and Woolf, 1993). Occluding thrombi exist both within the plaque and the lumen, consist of both platelets and fibrin, and may obstruct blood flow thus leading to a clinical event (Davis and Woolf, 1993).

Risk Factors and Protective Factors

High-density lipoprotein (HDL) has been found to play a protective role in preventing fatty streak formation. HDL has been found to inhibit the oxidation of LDL by metal ions both *in vivo* and *in vitro* as a result of the HDL associated enzymes platelet activating factor acetylhydrolase and paraoxonase (Navab *et al.*, 1995).

Thus HDL is capable of preventing some of the secondary effects of LDL oxidation, such as the induction of MCP-1 (monocyte chemotactic protein-1) and the subsequent infiltration of monocytes into the subendothelial space. In contrast, there are several risk factors associated with the progression of the atherosclerotic process including elevated blood cholesterol levels, high blood pressure, and cigarette smoking. Cigarette smoke, for example, has been found to enhance vasoconstriction, increase the incidence of oxidized lipid, and generate thrombin formation, all of which have been shown to contribute to the development of vascular disease (Kimura *et al*, 1994). Each stage of lesion formation is reversible should the damaging factors be removed and protective factors intervene to inhibit the fibroproliferative and inflammatory processes.

Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are environmental contaminants present in tar, coal, soot, and other fossil fuels, although they are also found in charred foods and cigarette smoke where many individuals may encounter them. Although these contaminants are relatively inert in their original form, biotransformation in humans and many animal species renders them potent carcinogens and putative atherogens (Parkinson, 1996). Benzo(a)pyrene (BaP) and dimethylbenzanthracene (DMBA) are two of the most toxic and well studied PAHs and will therefore be used in describing the nature of PAH metabolism and toxicity.

PAH Metabolism

PAHs are structurally related by an anthracene backbone (Figure 5). The degree of toxicity of each PAH depends upon the modifications to the anthracene ring;

DMBA is the most potent of the PAHs (Pitot and Dragan, 1996). Although these compounds are chemically inert in their original form, they are capable of accumulating within cells and perturbing cell function (Pitot and Dragan, 1996). Once metabolized, BaP and DMBA are immunosuppressants, carcinogens, and putative atherogens.

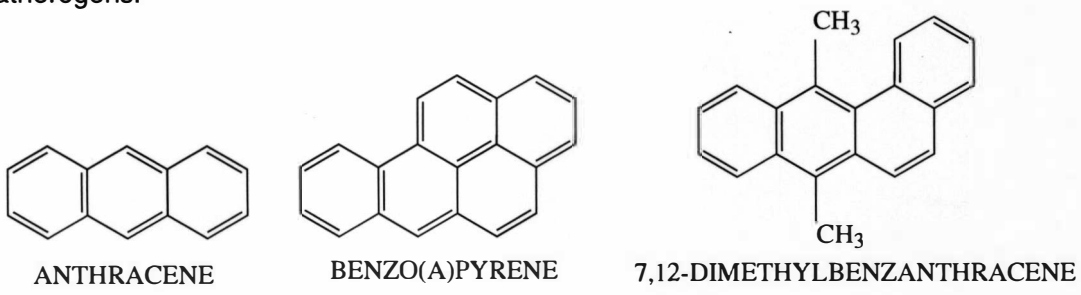

Figure 5. Chemcial Structures of Anthracene, Benzo(a)pyrene, and 7,12-Dimethylbenzanthracene.

PAHs can be metabolized by phase I or phase II metabolism. Phase I metabolism occurs on the endoplasmic reticulum via the cytochrome P450 mixed function oxidase system, ultimately making the compound more polar by introducing oxygen. Phase II metabolism occurs in the cytoplasm through biosynthetic reactions involving conjugation by methylation, glucuronidation, sulfation, etc. (Parkinson, 1996). PAHs (as well as cigarette smoke) induce cytochrome P450-1A1 which biotransforms and metabolically activates these compounds into toxic epoxides capable of binding DNA or generating reactive oxygen intermediates. These reactive epoxides may also be formed during prostaglandin synthesis within the cell (Parkinson, 1996). Some epoxides are receptive to epoxide hydrolase, an enzyme found within the microsomes of most animal tissues; this enzyme detoxifies the epoxide by adding a water moiety to the epoxide bond (Parkinson, 1996). Phase II metabolism may detoxify these chemicals by glucuronidation or glutathione reduction of the epoxide.

The process by which PAHs are bioactivated through cytochrome P450 metabolism has been elucidated by Stegeman *et al.*, (1995) and is described below. PAHs induce transcriptional activation and message stabilization of the *CYP1A1* gene. Induction of the cyp1A1 protein requires transcriptional activation and derepression of the cytosolic arylhydrocarbon receptor. The arylhydrocarbon receptor exists in the cytoplasm bound to two hsp 90 proteins. Binding of the PAH ligand dissociates the heat shock proteins from the receptor, allowing the receptor to be phosphorylated via a tyrosine kinase which activates the receptor. The active arylhydrocarbon receptor is then translocated to the nucleus by complexing with an arylhydrocarbon receptor nuclear translocator (AhRcnt). Once in the nucleus, this complex binds the *CYP1A1* regulatory sequence, thereby inducing transcription of *CYP1A1*, such that the cyp1A1 protein will be translated and biotransformation of the PAH can occur. This metabolism can be carried out by a variety of cell types, including vascular endothleium (Stegeman *et al.*, 1995).

PAH Toxicity

Once metabolically activated, PAHs are capable of forming DNA adducts and inducing point mutations, making them potent genotoxins. Studies indicate that inhaled cigarette smoke induces more than a twofold increase in lung DNA adducts in rats (Bond *et al.*, 1989). Cell proliferation following mutation may fix DNA damage, or DNA damage may inhibit the cell cycle and induce apoptosis if the arrest is prolonged (Rogers and Kavlock, 1996). Unlike many xenobiotics, PAHs exert their toxicity (once metabolized) at the first site of contact (Pitot and Dragan, 1996), explaining the high incidence of skin carcinomas induced by these chemicals.

PAHs are also capable of inducing vascular damage which may be related to

the development of atherosclerosis. In studies using chickens and pigeons, PAHs were capable of inducing smooth muscle tumors in the vascular wall when given in an initiation-promotion sequence (Albert *et al.*, 1977; Bond, *et al.*, 1981; Majesky et al., 1985; Revis et al., 1984). The putative role that PAHs may play in the atherosclerotic process has been attributed primarily to effects on vascular smooth muscle. These chemicals are capable of deregulating vascular smooth muscle cell growth through interaction and inactivation of protein kinase C, while enhancing the transcription of growth related genes by binding to cytosolic receptors that act as ligand activated transcription factors, and ultimately alter gene expression (Ramos *et al.,* 1996).

REVIEW OF RELATED LITERATURE

Role of the Endothelium in LDL Uptake

Lipid accumulation in the vessel intima is characteristic of fatty streak development in the early stages of atherogenesis. Because these lesions occur in the absence of endothelial denudation, it has been suggested that subtle alterations in endothelial metabolism or ultrastructure allow the movement of lipids into the subendothelial space (Alexander *et al.*, 1991; Guretzki, *et al.*, 1994). Lipids in the circulation are subject to oxidative modification by reactive oxygen intermediates and by interaction with cellular components of the vessel; lipid may also be oxidized within the endothelium (Alexander *et al.*, 1991). Oxidized LDL is capable of exerting cytotoxic effects on the endothelium (Holland *et al.*, 1992), resulting in altered permeability such that more lipid is allowed to pass into the subendothelial space of the vessel. Additionally, Holland and coworkers (1992) have shown that the endothelium increases endocytosis of LDL when exposed to elevated circulating lipid levels. Intracellular oxidation of LDL alters endothelial permeability through an increase in intracellular calcium which induces changes in the cytoskeleton of the cell (Hamilton *et al.*, 1994). The increased permeability allows additional lipid to accumulate in the vessel wall. Enhanced permeability has also been identified in cells undergoing turnover. Weinbaum and Chien (1993) have shown that cells involved in turnover exhibit temporarily leaky intercellular junctions, also allowing the possibility of lipid uptake into the subendothelial space.

Increased endothelial permeability has also been attributed to the trapping of LDL within the subendothelial space. The cytoskeletal changes within the endothelial

cells allow accumulating lipid to contact the basement membrane which consists of collagen, laminin, and a heparin sulfate proteoglycan (HPSG) (Guretzki, *et al.*, 1994). The core protein of HPSG resembles the LDL receptor (Guretzki, *et al.*, 1994), such that LDL is able to adhere to the basement membrane and thus becomes trapped as a result of the increase in endothelial permeability to LDL.

Endocytosis of LDL occurs via one of two processes. There is a high affinity, saturable receptor which is active at LDL concentrations below 50 μg/ml protein, and there is a low affinity, non-saturable process which is important when LDL concentrations are elevated (Poumay and Ronveaux-Dupal, 1989). While the low affinity process has not been well characterized, the high affinity receptor mediated process is understood in more detail. Poumay and Ronveaux-Dupal (1989) have shown that binding of LDL to specific receptors occurs on the endothelial membrane, followed by invagination into the cell on coated pits, where the complexes are identified as endosomes. The LDL is then uncoupled from the receptor so that it may be recycled and the ligand is transferred to juxtanuclear endosomes where it is degraded and used for membrane synthesis (Poumay and Ronveaux-Dupal, 1989). In addition, there are several endothelial receptors which recognize and internalize LDL depending upon the extent to which it has been oxidized; the B/E LDL receptor recognizes native or minimally oxidized LDL while the scavenger receptor recognizes oxidized LDL (Penn and Chisolm, 1994).

Role of the Endothelium in Monocyte Adhesion

Focal monocyte adherence to the endothelium and subsequent migration into the subendothelial space are observed in the first stages of atherosclerosis. Monocyte adhesion occurs when endothelial cells become 'activated', such that they express adhesion molecules as well as stimulators and chemotactic molecules for

leukocytes. Endothelial cells may be activated by cytokines, chemical injury, inflammation, and oxidized LDL. There are three steps involved in the adhesion and transmigration process: the first stage involves the rolling of monocytes along the endothelial surface (mediated by selectin molecules), the second involves attachment to the endothelium (mediated by integrins and immunoglobulin-like molecules), and the third involves the transmigratory process of the monocytes into the subendothelium (Takahashi *et al.*,1994). Farqi and DiCorleto (1993) have identified inhibitors of endothelial-monocyte adhesion which act by inhibiting cytokine-stimulated expression of endothelial adhesion proteins through the inhibition of protein kinase C signaling mechanisms.

Selectins

Selectins (also known as LECCAMs) are calcium dependent adhesion molecules which mediate low strength adhesions (tethering) and rolling of leukocytes to the endothelium (McEver, 1993). The selectins belong to a family of glycoproteins which are not present on unstimulated endothelial cell surfaces, but appear on the surface of the endothelium after activation (Faruqi and DiCorleto, 1993). E-selectin (also known as ELAM-1; endothelial-leukocyte adhesion molecule-1) and P-selectin (also known as PADGEM or GMP-140; platelet activation dependent granule to external membrane protein or granule membrane protein-140, respectively) are the two selectins found on activated endothelium (Faruqi and DiCorleto, 1993).

Endothelial E-selectin expression is stimulated by IL-1 or TNF-α within 2-6 hours following stimulation (protein synthesis is necessary for its expression), while P-selectin is stimulated by histamine or thrombin and can be found both in

endothelium and in platelets within 5-30 minutes following stimulation (Beekhuizen and van Furth, 1993). P-selectin is stored in the Weibel-Palade bodies of the endothelium (α-granules in platelets) until cellular activation when it is translocated to the plasma membrane by exocytosis (Beekhuizen and van Furth, 1993). The selectins recognize carbohydrate ligands on the monocytes which are identified as sialyl Lewis X (sLeX) (Faruqi and DiCorleto, 1993). Monocytes loosely tethered to endothelial cells by selectins are exposed to activators which are capable of inducing tight endothelial-monocyte adhesion.

Immunoglobulin-like Molecules

Members of the immunoglobulin (Ig) superfamily are mediators of the immune response. Some Ig-like molecules have been identified which act as adhesion receptors in cell-cell interactions (Dianzani and Malavasi, 1995). ICAM-1 (intercellular adhesion molecule-1), ICAM-2, and VCAM-1 (vascular cell adhesion molecule-1) are Ig-like receptors present constitutively at low levels on the endothelial surface that are involved in endothelial-monocyte adhesion (Beekhuizen and van Furth, 1993). These receptors are upregulated within 12-48 hours following endothelial activation and bind with specific ligands on various leukoctyes (Beekhuizen and van Furth, 1993).

Integrins

Integrins are adhesion molecules which play critical roles in immune function, platelet aggregation, and wound healing (Dianzani and Malavasi, 1995). Certain specific integrins on monocyte cell surfaces mediate endothelial cell adhesion by binding to Ig-like counterreceptors (discussed previously) on the endothelial

surface (McEver, 1993). Activation of tethered monocytes results in rapid increases in the avidity of the integrin-counterreceptor interaction, although the mechanism behind this process remains to be elucidated (McEver, 1993). The integrin LFA-1 (lymphocyte function related antigen-1) is the counterreceptor to ICAM-1 and ICAM-2, and the integrin VLA-4 (very late antigen-4) is the counterreceptor to VCAM-1(Faruqi and DiCorleto, 1993). LFA-1 is found on lymphocytes, monocytes, and polymorphonuclear cells, while VLA-4 is present on lymphocytes, monocytes, and eosinophils, but not on neutrophils (Faruqi and DiCorleto, 1993). Integrins have also been found to interact with cytoskeletal and extracellular matrix components, making them important in the transmigratory process of monocytes into the subendothelium.

Role of the Endothelium in Monolayer Permeability

The integrity of the endothelial monolayer is critical in regulating the passage of plasma proteins and blood components between the circulation and the vessel wall. Alterations in monolayer permeability, especially to lipoproteins, have been found to be important to cardiovascular disease processes. Permeability across the endothelial monolayer is thought to be regulated by two pathways: a transcellular path via vescicle transcytosis and junctional transport via dynamic opening and closing of the intercellular junctions (Caveda *et al.*, 1995; Chen *et al*, 1995; Schnittler *et al.*, 1990). The transcytotic pathway shuttles macromolecules between the luminal and abluminal surfaces (Schnittler *et al.*, 1990), while the junctional pathway carries smaller molecules through a pore system; larger molecules such as LDL are able to cross via the junctional pathway of mitotic or dying cells where the junctions are leaky (Chen *et al*, 1995).

Platelets, plasma proteins, norepinephrine, and the vasodilator prostacyclin

(which is produced in response to bradykinin and thrombin) have been found to reduce vascular permeability (McDonagh, 1986; Casnocha *et al.*, 1989; Siflinger-Birnboim *et al.*, 1988). On the other hand, bradykinin, histamine, and thrombin have been found to increase permeability (Casnocha *et al.*, 1989; Siflinger-Birnboim *et al.*, 1988). Several studies suggest that these effects are mediated through cytoskeletal alterations, which induce changes in endothelial cell shape. In cases of acute inflammation, large gaps in the intercellular junctions are produced by contractile mechanisms of the cytoskeleton (Shasby *et al.*, 1985; Schnittler *et al.*, 1990). Schnittler and coworkers (1990) have discovered that endothelial cells contain actin and myosin which allow ATP-dependent contraction and shortening of the cell.

The increase in permeability is a result of gap formation within the intercellular junctions, thus enhancing paracellular transport. Gap formation is induced as a result of cytoskeletal changes. Schnittler *et al.* (1990) has suggested that this change in the contractile state of the cytoskeleton is due to increased intracellular calcium. The phospholipase C-mediated increase in calcium results in the formation of calcium-calmodulin complexes which activate the myosin light chain kinase (MCLK) (Schnittler *et al.*, 1990). MCLK becomes phosphorylated and induces contraction through myosin and actin interactions. In contrast, permeability is decreased by inducing relaxation. This is accomplished by increasing cAMP levels through the induction of specific G proteins and adenylate cyclase, although the precise mechanism has yet to be elucidated (Schnittler *et al.*,1990).

Objectives of the Study

Polycyclic aromatic hydrocarbons have been shown to initiate and to promote atherosclerosis and carcinogenesis in quail, chicken, and rabbit species (Ou and

Ramos, 1992; Majesky *et al.*, 1985; Bond *et al.*, 1981; Albert *et al.*, 1977). This has been demonstrated primarily by the increase in incidence and in size of vascular smooth muscle cell tumors in these animals when exposed to benzo(a)pyrene or dimethylbenzanthracene. The endothelium has also been identified as an important mediator in vascular disease and in the atherogenic process, although the endothelial effects induced by these chemicals have not been investigated. Therefore, the first objective of this study was to elucidate the concentrations at which benzo(a)pyrene or dimethylbenzanthracene might induce an endothelial response without causing overt cytotoxicity. Secondly, our objective was to assess the toxicity of these chemicals at these relevant concentrations, and thirdly, to determine what functional changes (changes in monolayer permeability, monocyte adherence, and LDL uptake) benzo(a)pyrene and dimethylbenzanthracene might induce in the endothelium which could contribute to the atherogenic process.

Significance of the Study

Atherosclerosis is now considered a leading cause of death in the United States, as it is an underlying component in most cases of cardiovascular disease. Elevated cholesterol levels, hypertension, and cigarette smoke are known risk factors which have been strongly correlated with the development and enhancement of atherosclerosis in humans. However, it has yet to be determined which of the greater than 4,000 chemicals in cigarette smoke may be responsible for atherogenic effects. Atherosclerotic lesions have been described as benign smooth muscle cell tumors within the vessel wall (Benditt and Benditt, 1973). Thus, a number of studies have been conducted to assess the ability of the carcinogenic polycyclic aromatic hydrocarbons found in cigarette smoke to induce and enhance the development and progression of atherosclerosis in various species.

Investigators have now shown the endothelium to be an important mediator in the onset and progression of the atherogenic process, although the effects of these hydrocarbons on the endothelium have not been determined. Elucidation of the general toxicity and of the functional changes induced in endothelium by these PAHs may further contribute to our understanding of the process by which cigarette smoke induces vascular damage.

Developing a preliminary profile of the effects of benzo(a)pyrene and dimethylbenzanthracene in cultured endothelium is inexpensive, yet provides a descriptive assessment of PAH-induced endothelial injury. The porcine aortic endothelium used in this study was chosen because of the tendency of this species to spontaneously develop atheromas similar in composition and in location to those found in the human aorta (McCauley and Bull, 1980). In addition, porcine aortic endothelial cells contain arylhydrocarbon hydroxylase (AHH), the cytochrome P450 1A1-dependent enzyme necessary for bioactivation of PAH's (Manchester, *et al.*, 1984; Parkinson, 1996).

MATERIALS AND METHODS

Cell Culture and Chemical Administration

Cell Culture

Porcine aortic endothelial cells (PECs) were isolated according to a modified method of Reindel *et al.* (1991). Sections of aorta (approximately 1.5 inches in length) were aseptically removed from freshly slaughtered hogs (obtained from Pease Meat Packing Co.), rinsed in sterile Hank's balanced salt solution (HBSS) (Sigma Chemical Company, St. Louis, MO) containing 3% antibiotic/antimycotic (Ab/Am) (300 U/ml penicillin, 300 μg/ml streptomycin, 0.75 μg/ml Fungizone; Gibco, Grand Island, NY), and then placed into fresh HBSS containing 3% Ab/Am on ice for transport to the laboratory. In a laminar flow hood, the aortic sections were prepared for cell isolation with careful dissection of adipose and connective tissue from the surrounding adventitia. A section of artery was then cut longitudinally, and placed lumen side down into a 100mm tissue culture plate containing a thin film of collagenase (Type 1A, 0.1%; Sigma). After 2-4 minutes in the collagenase, the vessel was carefully removed and the luminal side lightly scraped with a single brush of a sterile rubber policeman. The cells on the policeman were gently resuspended in a clean 100mm tissue culture plate containing HBSS. Using a micropipette, small groups of the resuspended cells were collected into 12-well tissue culture plates (Becton Dickinson, Lincoln Park, NJ) containing 0.5 ml Opti-Mem (Gibco) with 1% Ab/Am and 5% fetal bovine serum (FBS) (Intergen, Purchase, NY) under an inverted phase microscope (BioStar; Leica, Inc. (American

Optical), DePew, NY).

Cells were incubated for 14 days at 37°C with 5% CO_2/95% air, with fresh media given on day 7. On day 14, colonies of endothelial cells with cobblestone morphology, free of spindle cells resembling fibroblasts and/or smooth muscle, were passed into 6-well tissue culture plates with 0.25% trypsin/0.01M EDTA (Gibco) and allowed to reach confluence, with fresh media given every other day. After the 3rd passage, cells were plated and maintained in 100mm tissue culture plates containing 10 ml Opti-Mem with 1% Ab/Am and 3%FBS. Cells were passed at a 1:3 ratio every 5-7 days, and only cells between passages 4 and 10 were used for experimental procedures.

Chemical Preparation and Administration

Benzo(a)pyrene (BaP) (3,4-benzpyrene; Sigma) and 7,12-dimethylbenzanthracene (DMBA) (9,10-dimethyl-1,2-benzanthracene; Sigma) were dissolved in dimethylsulfoxide (DMSO) (Sigma) at a stock concentration of 25 mg/ml, and stored at 4°C. The stocks were serially diluted in DMSO such that administration of 5 µl of chemical to wells containing 2 ml of Opti-Mem or of 25 µl of chemical to plates containing 10 ml of Opti-Mem resulted in concentrations of 0.001, 0.01, 0.1, 1, or 10 µM BaP or DMBA. Administration of an equal volume of DMSO vehicle was used to treat control wells.

General Toxicity Assays

Morphology

Endothelial cells (ECs) were plated in 12-well tissue culture plates, allowed to reach confluence, and treated with 0.01, 0.1, or 1 µM BaP, DMBA, or vehicle.

Fresh medium containing the chemical of interest was administered every other day, providing continuous exposure throughout the experimental period. Cells were then examined microscopically for visible changes in morphology throughout the 240 hour experimental period. Examinations were performed at 0, 6, 12, 24, 48, 96, 168, and 240 hours. After two days of treatment, various wells were scraped with a single stroke of a rubber policeman to remove cells in one area of the well. These wells were then monitored throughout the remainder of the experimental period for the ability of the remaining cells (treated and controls) to migrate and divide so as to cover the scraped area of the well. Phase contrast photographs were then taken of live cultures and of cultures fixed with 10% formalin in HBSS and stained with 0.5% crystal violet (Sigma). The viability of detached cells in the wells was determined by trypan blue exclusion (Sigma).

Cell Number

Cells were plated and treated as described above. At each timepoint, triplicate wells from each treatment group were rinsed twice with calcium free HBSS, then adherent cells were removed by the addition of 1 ml trypsin/EDTA and diluted in 9 ml of isotonic diluent (USA Scientific; Ocala, FL). Cells were quantitated using a Coulter Counter (Model F; Coulter Electronics, Luton, England); each well was counted three times.

Protein Content

Cells were plated and treated as described above. At each timepoint, cells were washed twice with HBSS and then lysed in 1 ml of 0.1M NaOH. Cellular protein content was determined by a modification of the Bradford method (1976), using

Bovine serum albumin (Bio-Rad laboratories, Cambridge, MA) as a standard. Samples were analyzed in duplicate at 595nm using a microplate reader spectrophotometer (Model 450; Bio-Rad Laboratories). Results are expressed as mean protein content per cell.

Lactate Dehydrogenase Release

Release of lactate dehydrogenase (LDH) activity into the medium by ECs was determined by modification of the method of Bergmeyer and Bernt (1974). Medium was removed from triplicate wells in each treatment group for analysis, and each sample was analyzed in duplicate. Cell monolayers were then rinsed twice in HBSS, and lysed in 2 ml of 10% triton X-100 in HBSS. Lysate was sonicated and centrifuged and the supernatant was analyzed for LDH activity. Results are expressed as the percent of total LDH released into the medium.

Cell Proliferation/ DNA Synthesis

Cells were plated as described above. At confluence, EC monolayers were growth arrested for 72 hours in M199 medium (Gibco) containing 1% Ab/Am and 0.2% bovine serum albumin (BSA) (Sigma). At 72 hours, cells were given fresh Opti-Mem containing 1% Ab/Am and 3% FBS and were treated with either BaP or DMBA as described above. At each timepoint, thymidine incorporation was determined according to the method of Hoorn and Roth (1992). Cells were pulse-labeled with 2 μCi/ml of 5-methyl-^{3}H thymidine (40-60 Ci/mmole; ICN; Cleveland, OH) for 2 hours under the incubation conditions described above. Cells were then placed on ice for 30 minutes and washed three times in cold phosphate-buffered saline (PBS) to remove unincorporated tracer. 0.5 ml of ice cold 10%

trichloroacetic acid (TCA) was added to cells on ice, and after 10 minutes, wells were washed with 75% ethanol, and then with 95% ethanol. 0.5 ml of 1N NaOH was added to each well, and wells were incubated overnight. Finally, 0.5 ml of 1N HCl was added to each well, and 75 μl aliquots were added to 10 ml of liquid scintillant; each well was analyzed in duplicate. Radioactivity was determined in a liquid scintillation counter, and results reported as disintegration's per minute (dpm) per cell.

Colony Formation

Cells were plated into 100 mm tissue culture plates at a density of 1000 cells/plate and given 2 hours to adhere. At this time, plates were treated with vehicle, 0.001, 0.01, 0.1, 1, or 10 μM BaP or DMBA and were then incubated for 1 week. (Each treatment group for each chemical consisted of 6 plates). After 1 week, fresh medium was given to all plates, but only three of the six plates in each treatment group were retreated with BaP or DMBA. Plates were incubated for one more week, after which cells were fixed with 10% formalin in HBSS and stained with 0.5% crystal violet. Colonies consisting of greater than 50 cells were counted, and results were expressed as a percent of control. Results from plates given a single administration of chemical are reported separately from those which were retreated.

Functional Studies

Low-density Lipoprotein (LDL) Uptake

Cells were plated in 8-well tissue culture plates (Becton-Dickinson) on sterile glass coverslips (Sigma), and treated as described above. At each timepoint,

fresh Opti-Mem containing 1% Ab/Am, 3% FBS, and 10 mg/ml acetylated low density lipoprotein labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI-Ac-LDL) (Biomedical Technologies, Inc.; Stoughton, MA) was added for 4 hours of incubation. Medium was then removed and cells were washed twice in unlabeled Opti-Mem, and twice in HBSS. Coverslips were gently removed from the wells and placed into 3% formaldehyde in PBS for 20 minutes at room temperature. Coverslips were then rinsed for 5 seconds in distilled water, allowed to dry, and inverted on a microscope slide (Sigma) with a drop of 90% glycerol in PBS. LDL uptake was then quantified using the image analysis system, Image 1 (Universal Imaging Corp., West Chester, PA) with standard rhodamine excitation: emission filters. Cell number was determined visually under the microscope for each field. A minimum of 10 fields at 400X magnification were examined for each treatment group and results expressed as fluorescence intensity per cell.

Monolayer Permeability

Endothelial monolayer permeability to albumin was determined according to modified methods of Allen and Coleman (1995) and Gudgeon and Martin (1989). Cells were plated into 24-well plates (Becton-Dickinson) containing 0.4 μ cell culture inserts (Becton Dickinson) according to manufacturer's instructions. Cells were incubated for 5 days to ensure confluence, and then treated with either vehicle or 1μM BaP or DMBA. (Media and chemical were always placed both in the luminal chamber of the insert and in the abluminal chamber, or well, of the plate). Media changes were performed according to the instructions given with the cell culture inserts. At each timepoint, treated inserts were rinsed carefully with Krebs-

Heinslet buffer (Sigma) and placed into a fresh 24-well tissue culture plate containing 600 µl of Kreb's buffer. 100 µl of 40g/L albumin/trypan blue complex (albumin and trypan blue: Sigma) was added to the luminal chamber of each insert and incubated for 1 hour under standard incubation conditions. 200 µl from each abluminal chamber was then extracted (in duplicate) without removing the insert so that the permeability of the monolayer to the albumin/trypan complex could be assessed. Samples were analyzed using a microplate reader spectrophotometer (Model 450; Bio-Rad Laboratories) at 595 nm. Standards were prepared using a 5 g/L solution of the albumin-trypan blue complex. Results are expressed as a percent of equilibrium concentration, where 100% equilibrium concentration is equal to : (concentration of albumin/trypan complex; 40 g/L)(amount of complex added to luminal chamber; 100 µl)/(total volume; 700 µl)= 5.714 g/L.

Monocyte Adherence

Monocyte adherence to EC monolayers was determined by a modification of methods from Clerck *et al.* (1994) and Akeson and Woods (1993). Cells were plated in 24-well tissue culture plates and treated as described above. THP-1 cells, (ATCC; Rockville, MD) a human acute monocytic leukemia cell line, were maintained in standard incubation conditions with RPMI 1640 (Gibco) containing 1% Ab/Am, 10% FBS, and 5 x 10^{-5} M 2-mercaptoethanol. Before monocyte labeling, THP-1's were washed in HBSS and concentrated to 1 x10^5 cells/ml. Aliquots of 1x10^5 cells/ml were centrifuged at 400 x g for 10 minutes at room temperature and resuspended in 1 ml of 5 µM calcein-AM in HBSS (Molecular Probes; Eugene, OR), then incubated for 15 minutes under standard conditions. Labeling was stopped by washing twice with HBSS and centrifugation. For the adhesion assay, ECs were

washed twice with HBSS, and Calcein-AM labeled THP-1's (1 x10^5 cells/ml) were added to each well and co-incubated for 30 minutes under standard conditions. Following incubation, each well was carefully washed 3 times with PBS to remove non-adherent monocytes, followed by inversion of the plate and blotting of excess liquid on to absorbent paper. ECs and adherent THP-1's were maintained in PBS for microscopic examination. Adherent THP-1's were counted visually using standard fluorescein excitation: emission filters. One well of endothelial cells from each treatment group was used for cell number determination (procedure described in materials and methods under cell number). Results are expressed as a ratio of the total number of adherent monocytes (THP-1's) to EC number ± SEM.

Statistical Analysis

Data are reported as means ± SEM. [Alternatively the SED for the data set may be reported]. Data were analyzed using a random analysis of variance, and individual comparisons were made with Tukey's HSD test. Percentage data was transformed prior to statistical analysis using $\sqrt{\arcsin}$. The criterion for significance was $p<0.05$.

RESULTS

General Toxicity Assays

Morphology

Confluent monolayers treated with 0.01, 0.1, or 1 μM BaP or DMBA or with an equal volume of DMSO vehicle were examined throughout the 240 hour experimental period for visible changes in morphology. At 48 hours post-treatment, vacuolization of cells treated with either 0.1 or 1 μM BaP or DMBA was evident, and the monolayers became increasingly vacuolated at 96 hours, 168 hours, and 240 hours. Very little vacuolization was seen in vehicle and 0.01 μM BaP or DMBA treated cells throughout the 240 hour period. Cells treated with vehicle or 0.01 μM BaP or DMBA retained typical cobblestone morphology throughout the 240 hour experimental period, while cells treated with 0.1 or 1 μM BaP or DMBA were enlarged (in comparison to vehicle treated controls) and demonstrated an elongated, spindle-like morphology at 168 and 240 hours post-treatment (Figure 6). Increased cell detachment was also observed in the 0.1 and 1 μM BaP or DMBA treated monolayers at 168 and 240 hours post-treatment. Results from trypan blue exclusion indicated that the detached cells were viable.

In a separate experiment, the endothelial monolayer of both vehicle and 1 μM DMBA or BaP treated wells was partially removed with a single stroke of a rubber policeman at 2 days post-treatment. Cells were then examined throughout the remainder of the experimental period for their ability to migrate and divide so as to cover the scraped area of the well. Cells treated with vehicle were found to migrate

into the scraped area and divide; individual cells along the scraped region appeared to be elongated or stretching, so as to reach into the scraped area of the well. Cells treated with DMBA or BaP did not show the same extent of migration or division; individual cells along the scraped region became enlarged and rounded in appearance and were not elongating or stretching into the scraped area of the well.

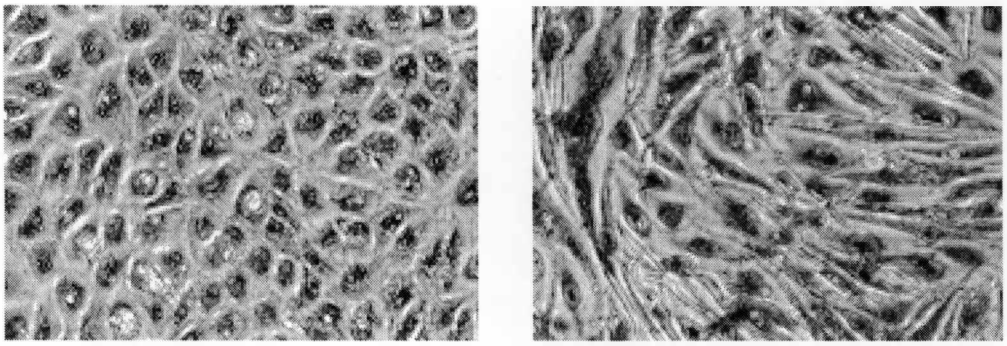

Figure 6. Treatment-Induced Morphological Changes in Cultured Porcine Aortic Endothelium. Cells were treated with DMSO vehicle (left) or 1 μM DMBA (right). Photographs were taken at 7 days post-treatment, 40 x magnification. Cells stained with 0.5% crystal violet.

Cell Number

The effect of continuous exposure to either BaP or DMBA on endothelial cell number was determined as described in Materials and Methods. Control PECs continued to divide and increase in number throughout the experimental period (Figures 7 and 8). Cells treated with 0.01 μM BaP or DMBA also increased in number throughout the experimental period. Monolayers treated with 0.1 μM BaP or DMBA showed increasing cellularity with time, although the cell number at 240 hours was significantly less in comparison to 240 hour controls for both BaP- and DMBA-treated monolayers. Cells treated with 1 μM BaP or DMBA showed only a

very slight increase in number with time. The number of cells in monolayers treated with 1 μM BaP or DMBA was significantly lower than controls at both 168 and 240 hours. While the cellularity of monolayers treated with 0.1 or 1 μM BaP or DMBA did not increase, monolayer cellularity at any point during the experiment was not lower than the cell number at the onset of the experiment.

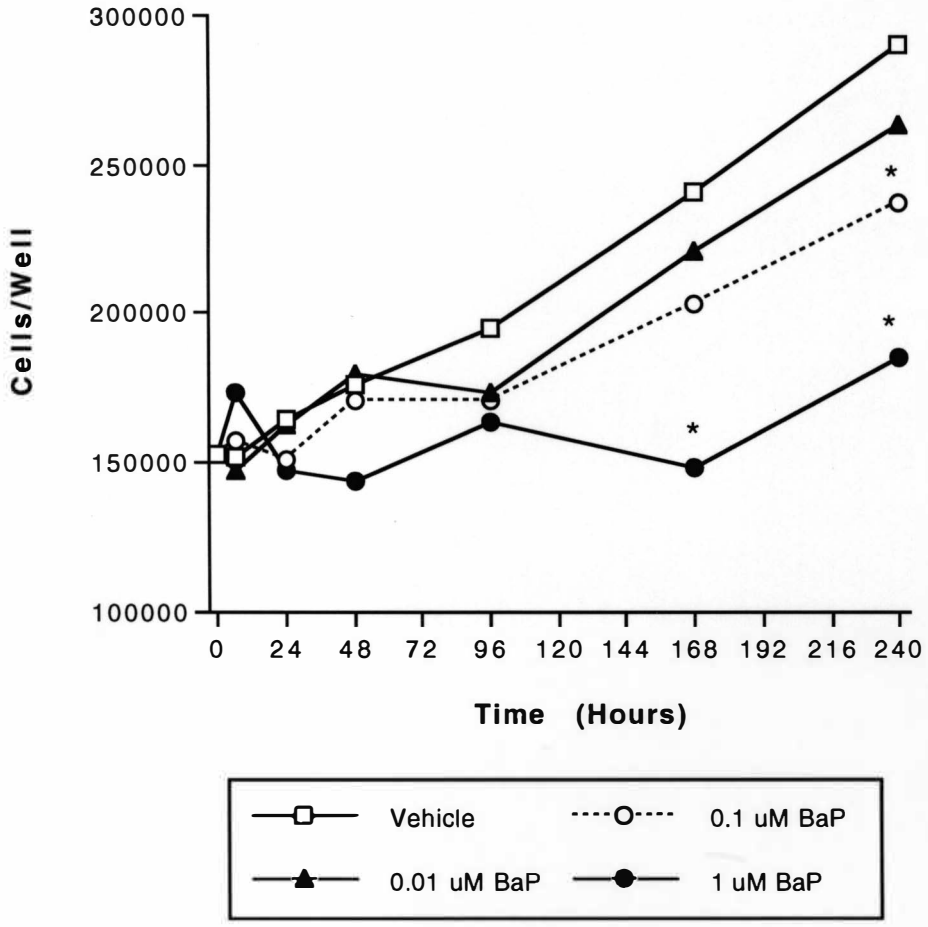

Figure 7. The Effects of BaP on Endothelial Cell Number. '*' denotes significance from vehicle controls. Standard Error of the Difference (SED) = 19,680. Values represent means of replicate studies (n=3).

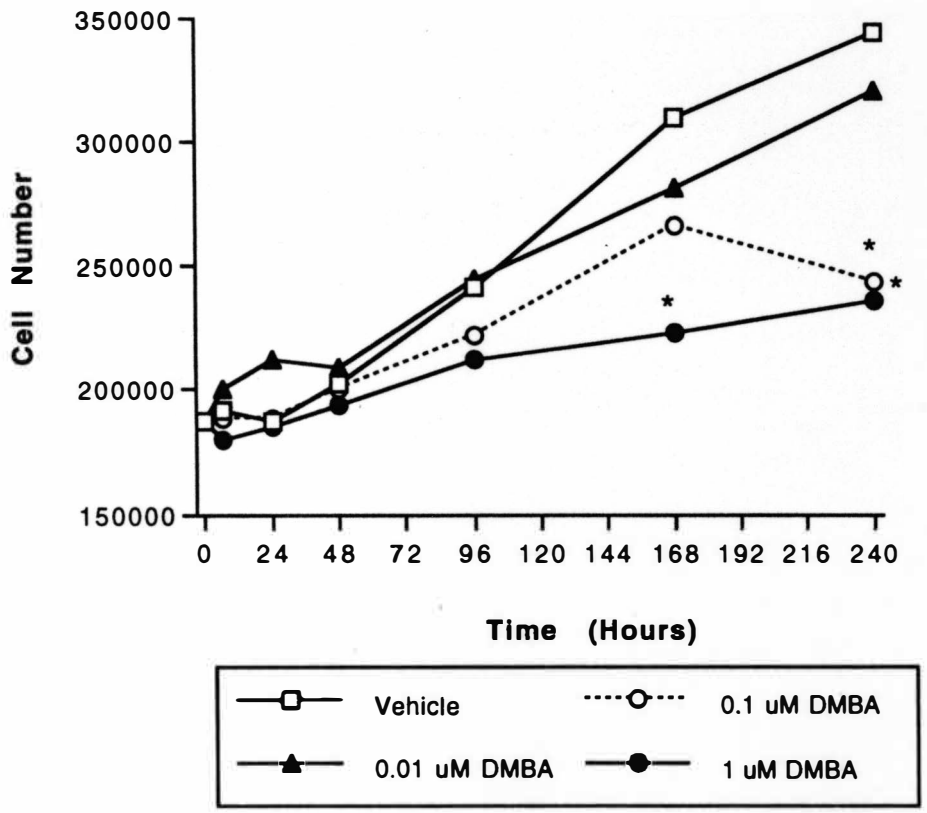

Figure 8. The Effects of DMBA on Endothelial Cell Number. '*' denotes significance from vehicle controls. Standard Error of the Difference (SED) = 17,755. Values represent means of replicate studies (n=3).

Protein Content

The effect of continuous exposure to either BaP or DMBA on endothelial cell protein content was determined as described in Materials and Methods. Total protein per well increased throughout the experimental period in cells treated with either BaP or DMBA. Wells treated with 0.1 μM or 1 μM BaP or DMBA showed a modest increase in total protein content in comparison to controls and 0.01 μM BaP- or DMBA-treated cells (data not shown). Figures 9 and 10 show changes in cellular

protein content for monolayers treated with BaP or DMBA, respectively. PEC monolayers treated with BaP showed no significant differences in cellular protein from vehicle controls at any time during the experimental period. Cellular protein decreased from 3.6 ng protein per cell to 2.1 ng protein per cell during the first 48 hours of the experiment, after which levels began to increase in all treatment groups. At 168 hours, cellular protein content had increased to 3.5-3.8 ng protein per cell, approximately equal to zero hour levels. At 240 hours, cellular protein had decreased to levels between 1.4 and 1.7 ng per cell, less than 50% of zero hour measurements.

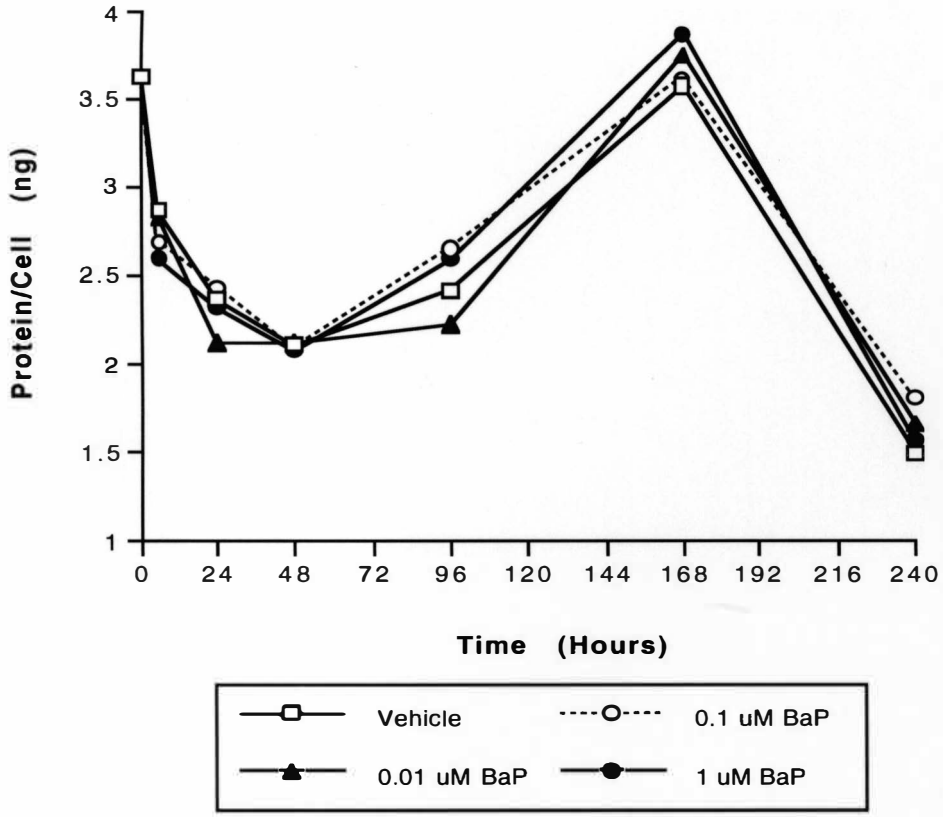

Figure 9. The Effects of BaP on Total EC Protein. '*' denotes significance from vehicle controls. Standard Error of the Difference (SED) = 0.2797. Values represent means of replicate studies (n=4).

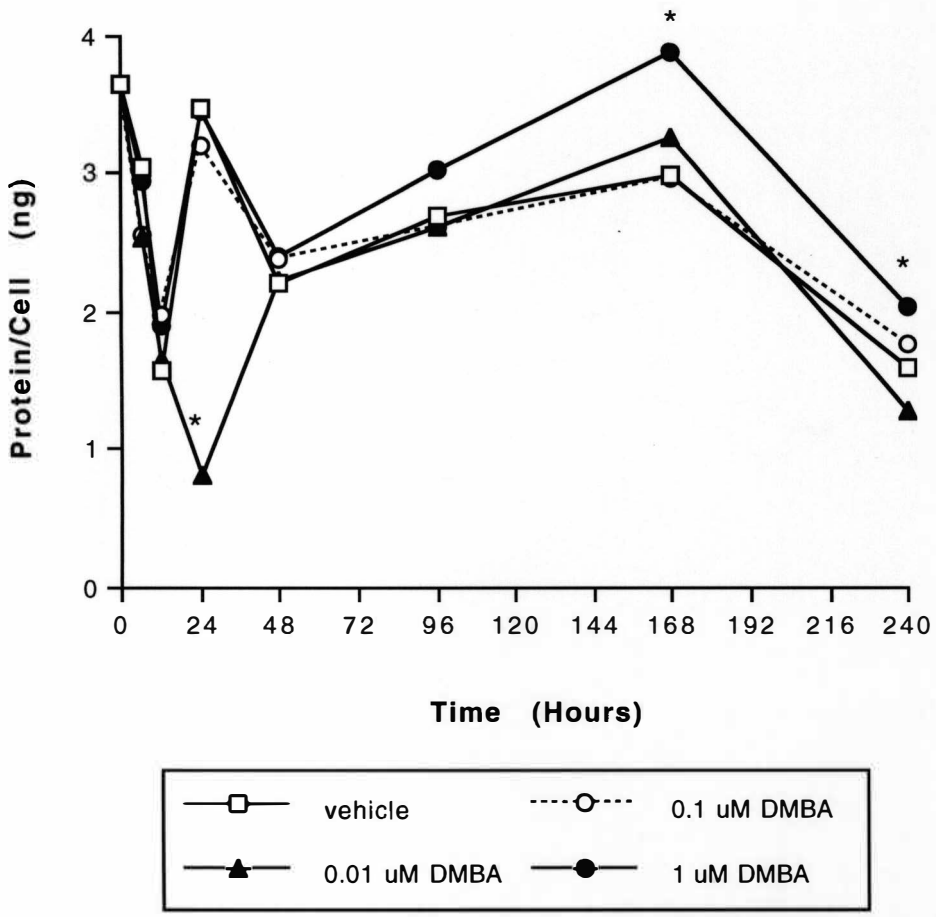

Figure 10. The Effects of DMBA on Total EC Protein. '*' denotes significance from vehicle controls. Standard Error of the Difference (SED) = 0.8165. Values represent means of replicate studies (n=4).

PEC monolayers treated with DMBA followed trends in protein content similar to BaP treated monolayers. Cellular protein decreased from 3.7 ng protein per cell to less than 2 ng protein per cell during the first 24 hours of the experiment in all treatment groups, after which levels increased (to approximately 3.4 ng protein per cell) in vehicle, 0.1, and 1 μM DMBA-treated cells. However, treated with 0.01 μM DMBA showed a significant decrease in cellular protein content

at 24 hours to less than 1ng protein per cell. At 48 hours, cellular protein content for all treatment groups was approximately 2.4 ng protein per cell. Cellular protein content increased in all treatment groups through 168 hours, at which time 1 μM DMBA treated monolayers had a significantly increased cellular protein content in comparison to vehicle treated controls. At 240 hours, cellular protein had decreased in all treatment groups, although 1 μM DMBA treated monolayers had a significantly increased cellular protein content in comparison to vehicle treated controls.

LDH Release

LDH release from treated monolayers was monitored in the culture medium as described in Materials and Methods as a measure of chemical-induced cytotoxicity. Significant LDH release was not evident in any of the BaP or DMBA treatment groups throughout the experimental period (data not shown).

DNA Synthesis

The effect of continuous exposure to either BaP or DMBA on endothelial cell DNA synthesis as indicated by incorporation of ^{3}H thymidine was determined as described in Materials and Methods. Figures 11 and 12 show cellular changes in DNA synthesis for monolayers treated with BaP or DMBA, respectively. Monolayers treated with BaP showed an increase in dpm per cell throughout the first 48 hours of the experimental period. Cells treated with 0.01 μM BaP showed a significant increase in thymidine incorporation at 24 hours (0.3376 dpm per cell) and 48 hours (0.4099 dpm per cell) in comparison to vehicle treated controls (0.2471 and 0.2769 dpm per cell), while monolayers treated with 1 μM BaP showed decreased

thymidine incorporation in comparison to vehicle treated controls at 24 hours (0.1408 dpm per cell) and 48 hours (0.2286 dpm per cell)(significant at 24 hours). By 96 hours, thymidine incorporation had returned to zero hour levels in all treatment groups, and showed little change at 168 hours. At 240 hours, dpm per cell was slightly lower than zero hour levels (0.1547 dpm per cell) for all treatment groups, with 1 μM BaP treated cells the lowest at 0.0389 dpm per cell.

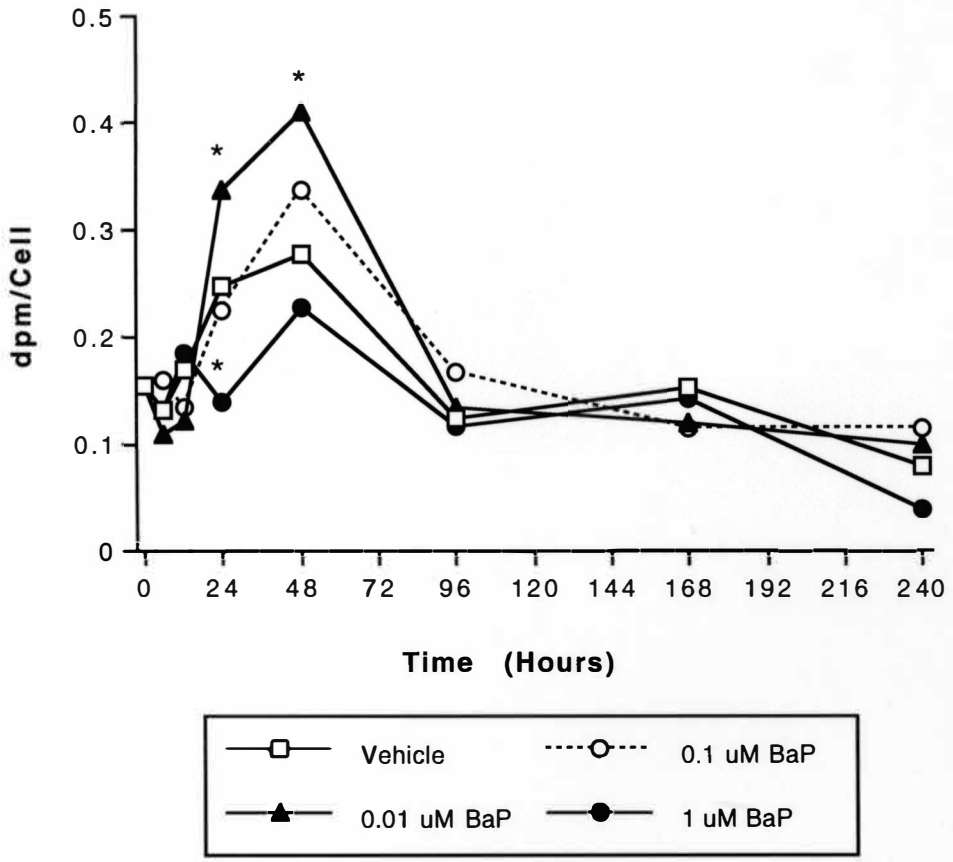

Figure 11. The Effects of BaP on DNA Synthesis. '*' denotes significance from vehicle controls. Standard Error of the Difference (SED) = 0.029. Values represent means of replicate studies (n=3).

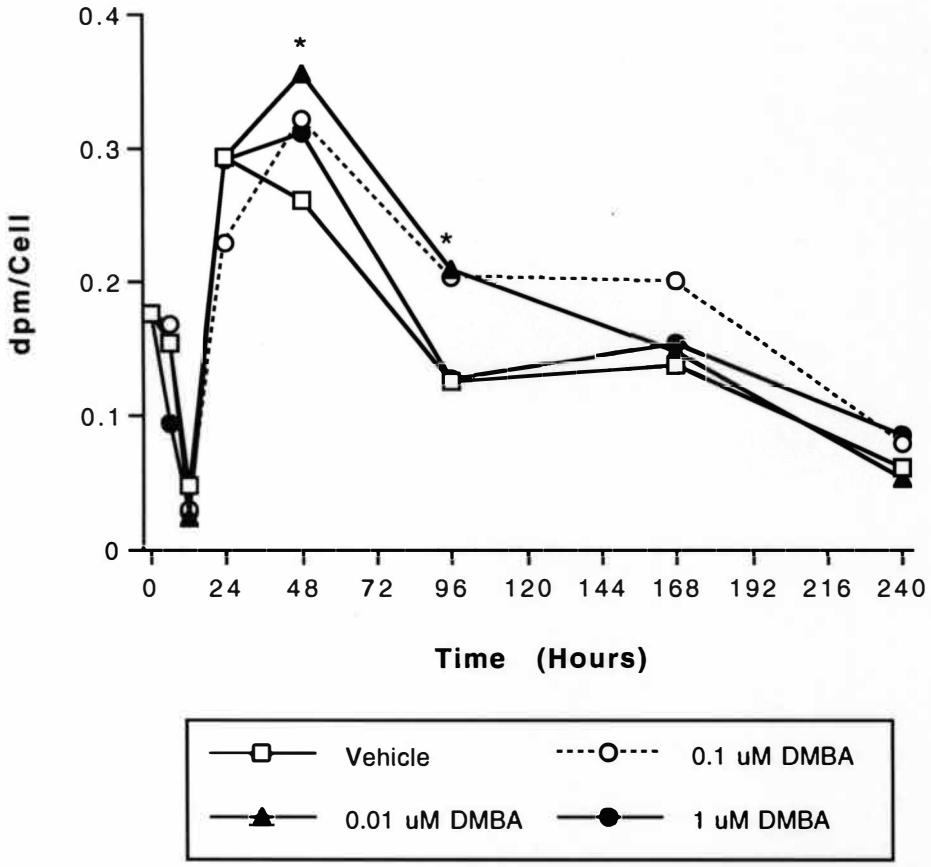

Figure 12. The Effects of DMBA on DNA Synthesis. '*' denotes significance from vehicle controls. Standard Error of the Difference (SED) = 0.025. Values represent means of replicate studies (n=4).

PEC monolayers treated with DMBA followed trends in thymidine incorporation similar to BaP treated monolayers. Monolayers treated with DMBA showed a decrease in thymidine incorporation throughout the first 12 hours of the experimental period, followed by an increase through 48 hours, evident in all treatment groups. At 48 hours, cells treated with 0.01, 0.1, or 1 μM DMBA showed an increase in thymidine incorporation in comparison to vehicle treated controls; the increase in 0.01 μM DMBA-treated cells (0.3555 dpm per cell) was significantly

greater than controls (0.261 dpm per cell). At 96 hours, thymidine incorporation had decreased in all treatment groups; levels in 0.01 and 0.1 μM DMBA-treated cells were significantly greater than vehicle treated controls. At 168 hours thymidine incorporation in vehicle, 0.1, and 1 μM BaP treated cells remained similar to 96 hour measurements, while 0.01 μM BaP treated cells showed a further decrease in thymidine incorporation. At 240 hours, dpm per cell had continued to decrease in all treatment groups.

Colony Formation

Cells were plated at low density and treated with either a single administration or a double administration of chemical as described in Materials and Methods. At the end of the experimental period, colonies consisting of at least 50 cells were counted and results reported as a percent of vehicle-treated controls. Results indicate that colony forming ability decreases with increasing concentration of BaP or DMBA (Figure 13). Cells treated with 0.001, 0.01, 0.1, 1, and 10 μM BaP were significantly inhibited in their ability to form colonies in comparison to controls. BaP reduced colony formation to 76%, 68%, 49%, 23%, and 16% of controls at concentrations of 0.001, 0.01, 0.1, 1, and 10 μM BaP, respectively. Cells treated with 0.01, 0.1, 1, and 10 μM DMBA were significantly inhibited in their ability to form colonies in comparison to controls. DMBA reduced colony formation to 90%, 65%, 44%, and 34% of controls at concentrations of 0.001, 0.01, 0.1, 1, and 10 μM DMBA, respectively. Colony formation in plates given a single administration of BaP or DMBA at time zero did not differ significantly from colony formation in plates which were given a second administration of chemical at 7 days post-treatment (data not shown).

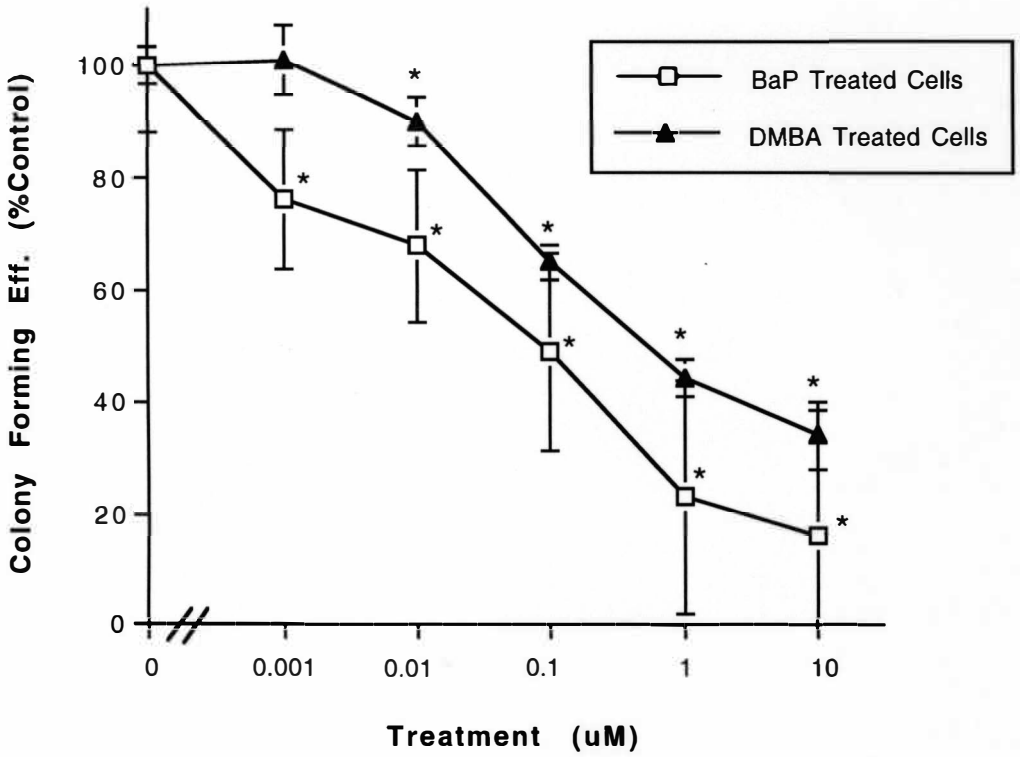

Figure 13. Colony Forming Efficiency of Cells Treated With BaP or DMBA. Graphed data represents cells treated with a single administration of chemical. '*' denotes significance from controls. Values represent means of replicate studies (n=3, BaP; n=4, DMBA). [100% represents 456 colonies per plate for BaP treated cells and 119 colonies per plate for DMBA treated cells].

Microscopic examination of colonies indicated that significant reductions in colony formation due to chemical treatment were not a result of cell death; individual EC cells remained adherent in the plates, although they did not divide to form colonies. These individual cells were enlarged and irregular in appearance. Colonies existing in plates treated with 1 or 10 μM BaP or DMBA consisted of fewer cells than colonies in plates treated with vehicle or 0.001 μM chemical.

Functional Studies

Low-density Lipoprotein Uptake

The effect of continuous exposure to either BaP or DMBA on endothelial cell LDL uptake was determined as described in Materials and Methods. Figures 14 and 15 show cellular changes in LDL uptake for monolayers treated with BaP or DMBA, respectively. Results from BaP experiments showed that there were no significant changes in LDL uptake of vehicle-treated cells over time. At 24 hours, 0.01 and 0.1 μM BaP treated cells showed an intensity per cell which was slightly less than vehicle treated controls, while 1 μM BaP-treated cells showed a slight increase. At 48 hours, 0.01, 0.1, and 1 μM BaP-treated cells showed a decreased intensity per cell in comparison to vehicle-treated controls; all treatment groups at 48 hours showed an increased intensity per cell in comparison to zero hour controls. At 96 hours 0.1 and 1 μM BaP treated cells showed an increased intensity per cell in comparison to vehicle controls and 0.01 μM BaP treated cells. At 168 hours all treatment groups showed elevated intensity per cell in comparison to vehicle treated controls, but only 1 μM BaP-treated cells showed a significant increase over controls.

Results from DMBA experiments showed that there were not significant changes in LDL uptake of vehicle-treated cells over time, except at 168 hours. At 24 hours all treatment groups from cells treated with DMBA showed an increase in the intensity per cell in comparison to vehicle treated controls. At 48 hours all DMBA treatment groups showed an increased intensity per cell in comparison to vehicle treated controls; cell intensity was significantly increased in 1 μM DMBA treated cells. At 96 hours 0.01, 0.1 and 1 μM DMBA treated cells showed a

significant increase in the intensity per cell in comparison to vehicle controls. At 168 hours 0.01 and 0.1 μM DMBA treated cells showed an increase in cell intensity in comparison to vehicle controls, while 1 μM DMBA treated cells showed a decrease in cell intensity.

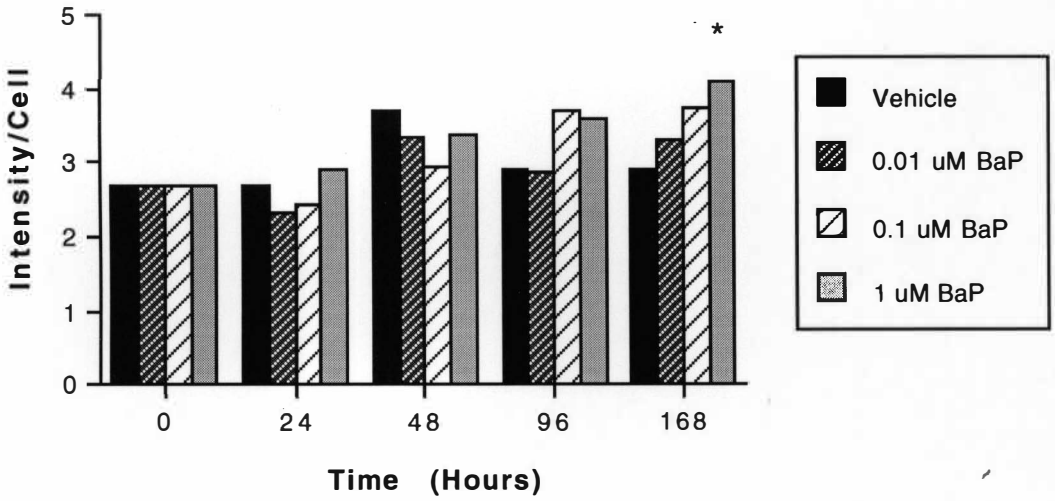

Figure 14. LDL Uptake of Cells Treated With BaP. '*' denotes significance from respective controls. SED = 0.643; n=3.

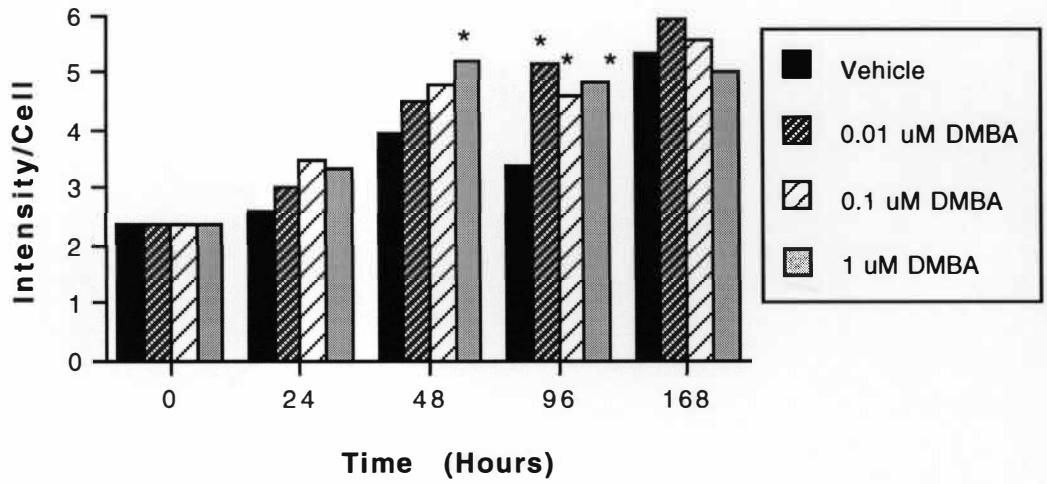

Figure 15. LDL Uptake of Cells Treated With DMBA. '*' denotes significance from respective controls. SED= 0.9949; n=3.

Monolayer Permeability

The effect of continuous exposure to either BaP or DMBA on endothelial monolayer permeability was determined as described in Materials and Methods. Control PECs showed a slight decrease in permeability at 24 and 48 hours, followed by an increase in permeability at 96 hours and 168 hours at which time the percent equilibrium returned to the level measured at the onset of the experiment (Figures 16 and 17). Cells treated with 1 μM BaP (Figure 16) showed a decrease in permeability at 24 and 48 hours, similar to the decrease in controls. At 96 hours, the permeability of 1 μM BaP treated cells appeared to increase in comparison to controls, while the permeability at 168 hours decreased to the level of controls.

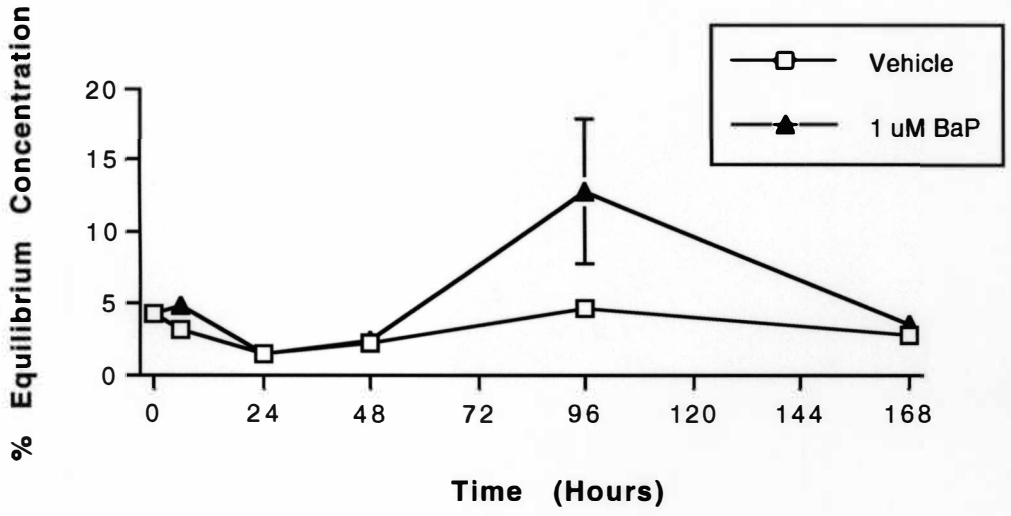

Figure 16. The Effects of BaP on Endothelial Cell Monolayer Permeability to Albumin. Values are represented as means ± SEM; n=2 (insufficient for statistical analysis).

Cells treated with 1 μM DMBA (Figure 17) showed a decrease in permeability at 24 hours, similar to the decrease in controls. Permeability to

albumin at 48 hours following DMBA treatment appears to increase, although the large variability in results suggests that these values are not different from controls. At 96 hours and 168 hours, the permeability of 1 μM DMBA-treated monolayers to albumin did not appear to change in comparison to controls. Preliminary results suggest that the permeability of PEC monolayers to albumin is not affected by treatment with either BaP or DMBA.

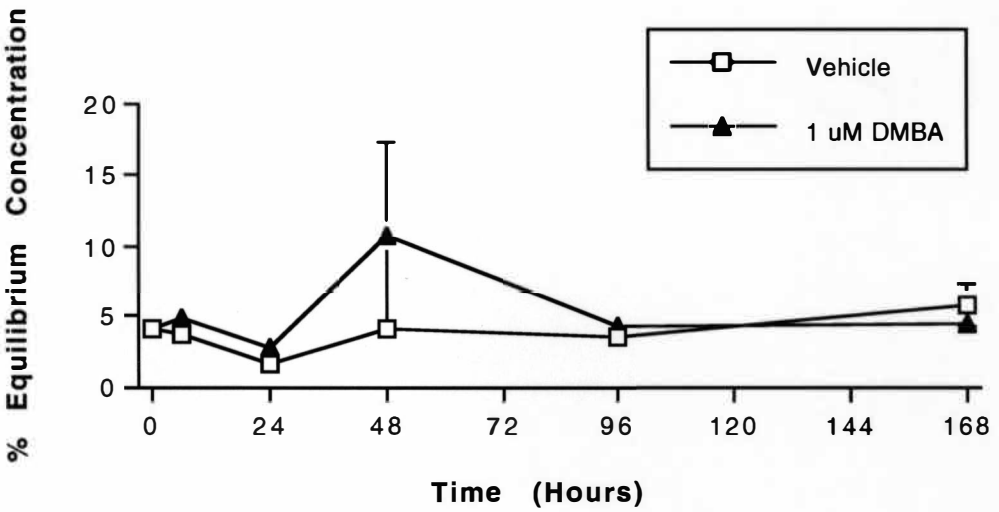

Figure 17. The Effects of DMBA on Endothelial Cell Monolayer Permeability to Albumin. Values are represented as means ± SEM; n=2 (insufficient for statistical analysis).

Monocyte Adherence

The effect of continuous exposure to either BaP or DMBA on monocyte adherence to endothelial cells was determined as described in Materials and Methods. Figures 18 and 19 show changes in monocyte adherence for monolayers treated with BaP or DMBA, respectively. Results from BaP experiments showed that there was an increase in monocyte adherence to vehicle-treated endothelial cells over time. BaP

treated cells show little change in monocyte adherence in any of the treatment groups in comparison to vehicle controls at 0 and 24 hours. At 48 hours, monocyte adherence in 0.01 and 0.1 μM BaP treated cells is increased in comparison to 48 hour controls; 1 μM BaP treated cells show a decrease in adherence in comparison to controls. At 96 hours monocyte adherence is decreased in all BaP treatment groups in comparison to vehicle controls. At 168 hours, monocyte adherence is elevated in all BaP treatment groups, with the greatest adherence in the 0.01 μM BaP treated cells.

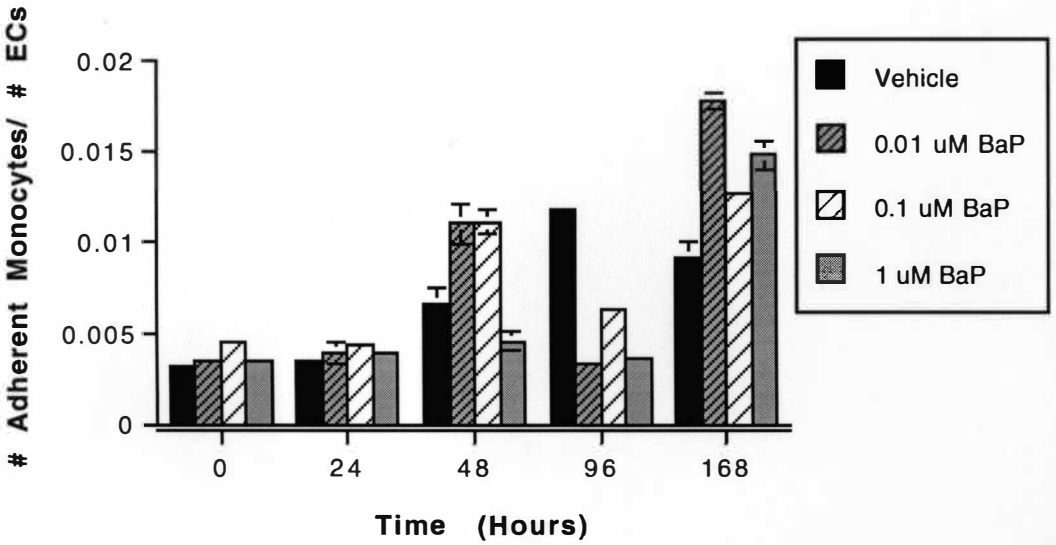

Figure 18. The Effect of BaP on Monocyte Adherence to Endothelial Cells. Values are represented as means ± SEM; n=2.

Changes in monocyte adherence to ECs with DMBA treatment are similar to the changes which occur in BaP treated cells. Results from DMBA experiments showed that there was an increase in monocyte adherence to vehicle-treated endothelial cells at 168 hours. At 0 and 24 hours there is little change in monocyte

adherence to ECs in any of the DMBA treatment groups. At 48 hours all DMBA treatment groups show an increase in monocyte adherence in comparison to vehicle controls; the greatest increase in adherence is evident in 0.1 μM DMBA treated cells. At 96 hours monocyte adherence is increased in all DMBA treatment groups, with the greatest increase in the 1 μM DMBA treated cells. At 168 hours monocyte adherence is increased in 0.01 and 0.1 μM DMBA treated cells, but is decreased in 1 μM DMBA treated cells in comparison to vehicle controls.

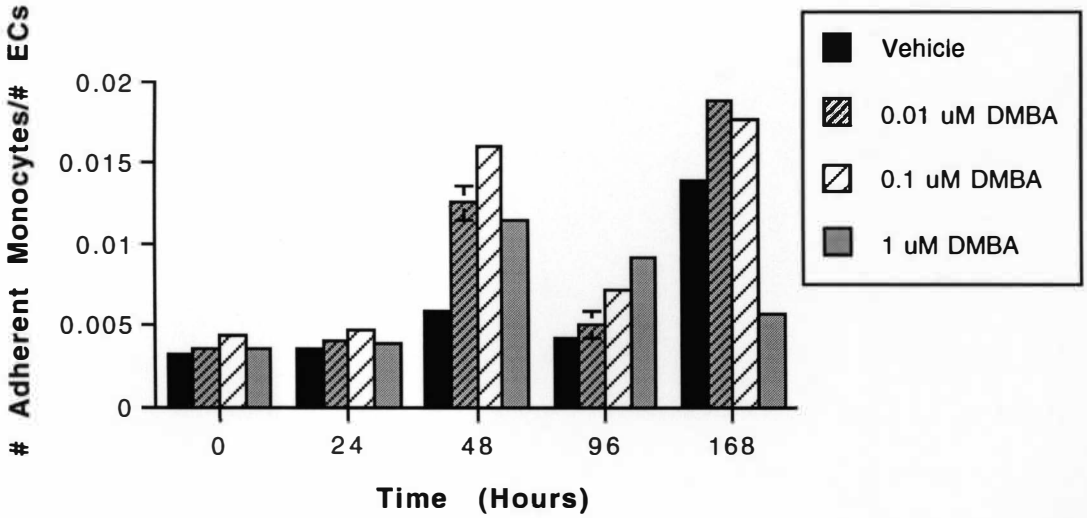

Figure 19. The Effect of DMBA on Monocyte Adherence to Endothelial Cells. Values are represented as means ± SEM; n=2.

DISCUSSION

Polycyclic aromatic hydrocarbons (PAHs) have been shown to initiate and to promote atherosclerosis and carcinogenesis in various animal species (Ou and Ramos, 1992; Majesky *et al.*, 1985; Bond *et al.*, 1981; Albert *et al.*, 1977). These findings have been demonstrated primarily by the increase in incidence and in size of smooth muscle cell tumors in these animals when exposed to BaP and DMBA, two of the most potent PAHs. The endothelium has been identified as a critical regulatory component of vascular homeostasis, as well as an important mediator in vascular dysfunction and disease (Nachman and Hajjar, 1991; Fajardo, 1989; Gimbrone *et al.*, 1995; van Hinsberg, 1992). However, the endothelial effects induced by BaP and DMBA have not been studied. Therefore, the first objective of this study was to elucidate the concentrations and the course of time for which BaP and DMBA induce an endothelial response without an acute cytotoxic effect. Secondly, our objective was to assess the toxicity of these chemicals at relevant concentrations, which may contribute to chronic vascular disease as a result of prolonged PAH exposure. Thirdly, we set out to determine what functional changes BaP and DMBA might induce in the endothelium which are indicative of proatherogenic changes.

The effect of BaP and DMBA on EC morphology was examined throughout the experimental period. There was an increased incidence of vacuolization and cell detachment with time and increasing concentration of chemical treatment, in addition to cell enlargement and shape change. Figure 6 depicts these changes in 1 μM DMBA treated cells in comparison to vehicle controls at 168 hours, suggesting that these PAHs are capable of inducing an endothelial response. The inability of ECs to migrate and spread into an area void of cells with increasing time and concentration of

chemical treatment additionally supports the hypothesis that prolonged PAH exposure at low concentrations induces endothelial injury. Coomber and Gottlieb (1990) suggested that cell migration plays a role in the propogation of endothelial cell replication following injury. Therefore, if PAHs are capable of inhibiting EC migration, they may also be capable of inhibiting the proliferative response to monolayer injury. Previous studies (Fyfe *et al.*, 1995; Coomber and Gottlieb, 1990) have shown that the proliferative response to endothelial monolayer injury is critical for preventing the development of vascular disease.

The hypothesis that BaP and DMBA affect EC proliferation was further investigated by evaluating changes in cell number throughout the experimental period. Results from replicate cell number studies show that cell number is significantly reduced in comparison to controls (Figures 7 and 8) at 168 hours when treated with 0.1 μM BaP or DMBA and at 168 and 240 hours when treated with 1 μM BaP or DMBA. The results also suggest a direct dose/response relationship with regard to PAH treatment and cell division; with increasing concentration of chemical treatment the smaller the cell number in comparison to vehicle controls (it is important to note that while treated monolayers did not increase in cell number to the same extent as controls, numbers did not decrease i.e., there was no overt cell death as a result of chemical treatment). These proliferative responses to PAH treatment become more pronounced with time, suggesting that prolonged exposure to BaP or DMBA produces substantial effects. The repair process following endothelial cell injury includes cell migration, spreading, and proliferation (Coomber and Gottlieb, 1990). The alteration of these cellular processes as a result of PAH treatment suggests an inability to repair injury which may result in prolonged endothelial dysfunction. This may profoundly affect the onset of vascular disease initiated by other risk factors, or may itself be a key factor in initiating

vascular dysfunction. Although atherosclerosis is typically associated with an increase in both EC and SMC proliferation, Fyfe *et al.* (1995) have shown that the immunosuppressant cyclosporin A inhibits both EC and SMC replication (much like BaP and DMBA) *in vitro* but is associated with an increase in atherosclerosis in mice.

The reported changes in cell number following BaP or DMBA treatment could be attributed to cell death or to a decrease in cell proliferation. Thus, endothelial LDH release was monitored to assess cytotoxicity and changes in cell proliferation were further studied by evaluating colony forming ability following treatment with BaP or DMBA. Results indicate that the concentrations of BaP and DMBA used in these studies did not cause significant LDH release (overt cytotoxicity) at any time after treatment. Thus, the failure of ECs to increase in cell number and the lack of cytotoxicity suggest that proliferation is inhibited with chemical treatment. Results from the colony forming assay show that the ability of individual ECs to divide and form colonies is significantly reduced with treatment of DMBA or BaP (Figure 13), thus providing evidence that BaP and DMBA are capable of inhibiting endothelial cell proliferation. It was important to note that individual cells remained attached to the plates in all treatment groups of this assay, suggesting that while replication was inhibited, the lack of colony formation was not a result of cell mortality.

DNA synthesis and cellular protein content were evaluated in PAH treated ECs to gain some initial insight into the intracellular changes that result from PAH treatment and culminate in the inhibition of cell replication. DNA synthesis was determined following a growth arrest of monolayers such that cells were synchronized into the same phase of the cell cycle. Results from BaP treated cells show that cellular DNA synthesis increases in all treatment groups throughout the first 48 hours when synchronized cells are in the S phase of the cell cycle.

Thymidine incorporation was significantly elevated in 0.01 μM BaP-treated cells at 24 and 48 hours, while thymidine incorporation was significantly decreased in 1 μM BaP-treated cells at 24 hours. These results suggest that there may be a concentration threshold at which BaP induces different cellular responses; 0.01 μM BaP may initiate DNA synthesis while 1 μM BaP may inhibit DNA synthesis, possibly through alterations in the proteins which catalyze this process (Alberts *et al.*, 1994). The decrease in DNA synthesis in the 1 μM BaP treated cells may also affect the triggering of signaling events which allows cells to proceed into the M phase of the cell cycle, such that cell replication is inhibited. DNA synthesis decreases in all BaP treatment groups between 48 hours and 96 hours. This decrease occurs as cell numbers increase; DNA synthesis has been completed such that thymidine incorporation returns to zero hour levels and cells have proceeded into the G_2 phase of the cell cycle. There is little fluctuation in DNA synthesis throughout the rest of the experimental period in any of the treatment groups, although cell number continues to increase in controls and 0.01 μM BaP-treated cells. This suggests that cells may have become unsynchronized by 168 and 240 hours, such that any net changes in DNA synthesis are not seen. It is also possible that at least part of the cell population may have become quiescent by 168 and 240 hours.

Results from DMBA-treated cells show trends in DNA synthesis similar to BaP-treated cells. DNA synthesis decreases in all treatment groups throughout the first 12 hours following synchronization, after which synthesis increases in all treatment groups until 48 hours (Figure 12). At 48 hours all treatment groups show elevated DNA synthesis (significant in 0.01 μM DMBA treated cells) in comparison to vehicle controls, suggesting that DMBA induces DNA replication. DNA synthesis decreases in all treatment groups at 96 hours, although levels are significantly higher in 0.01 and 0.1 μM DMBA-treated cells. The decrease in

synthesis in controls correlates to the increase in cell number at 96 hours, after DNA synthesis has been completed and cells have completed the remainder of the cell cycle. However, cell numbers from 0.01 and 0.1 μM DMBA-treated cells are decreased in comparison to controls. This suggests that prolonged exposure to low concentrations of DMBA may stimulate DNA synthesis, although cells are unable to complete the cycle and divide. These alterations in synthesis during the S phase of the cell cycle may affect the signaling which allows cells to proceed into the M phase where cytokinesis occurs (Alberts, 1994). Damage to the DNA as a result of chemical treatment may also inhibit cell cycle progression if damage is too extensive for repair (Rogers and Kavlock, 1996). Synthesis continues to decrease throughout the remainder of the experiment in all treatment groups. Again, this suggests that cells may have become unsynchronized by 168 hours, such that any net changes in DNA synthesis are not observed. Our results are similar to those reported by Zwijsen *et al.*, (1990), where BaP was found to contribute to unscheduled DNA synthesis in smooth muscle, culminating in a growth inhibitory response *in situ*.

Results from protein data suggest that BaP does not have any significant effects on cellular protein content (Figure 9). The changes in cellular protein content throughout the 240 hour experimental period may be explained by cell growth and division; cellular protein increases as cells grow and decreases following division (Alberts *et al.*, 1994). Cellular protein in DMBA treated monolayers decreased throughout the first 12 hours (Figure 10). At 24 hours, 0.01 μM DMBA treated cells showed a significant decrease in protein content in comparison to vehicle controls, while all other treatment groups increased in cellular protein content. At 24 hours, 0.01 μM DMBA treated cells showed a decrease in protein, while all other treatment groups showed an increase. These changes in protein content may be different from those induced by BaP. Results suggest that 0.01 μM

DMBA treated cells may be inhibited in their ability to synthesize protein; experiments to evaluate leucine or methionine incorporation could be done to prove or disprove this hypothesis. This suggests that DMBA may induce at least some of its effects on mRNA synthesis or on protein synthesis at this concentration. All treatment groups showed an increase in cellular protein throughout 168 hours, followed by a decrease at 240 hours which may be attributed to cell quiescence. 1 μM DMBA treated cells showed cellular protein levels to be significantly elevated in comparison to controls at both 168 and 240 hours. This may be explained by the increase in cell size that occurs with 1 μM DMBA treatment at 168 hours when cells are incapable of dividing (Figure 6). Post-translational damage to cellular protein as a result of PAH treatment may lead to the inactivation of some proteins thus preventing the appropriate signaling necessary for cells to proceed into the M phase of the cell cycle (Alberts *et al.*, 1994). Additionally, phenotypic changes in cells and extracellular matrix changes which occur in response to PAH treatment may affect the cytoskeletal control of gene expression, thus inducing changes in protein content (Alberts *et al.*, 1994; Gimbrone *et al.*, 1995).

The endothelium is known to be a critical factor in maintaining vascular homeostasis. In addition to the regulation of permeability, hemostasis, vasoactivity and vascular cell growth, the endothelium is also actively involved in the inflammatory response, wound repair, vascular dysfunction and disease (van Hinsberg, 1992). Atherosclerosis has been characterized in its early stages by lipid accumulation within the vessel intima, which occurs as a result of altered endothelial ultrastructure (i.e., increased permeability) and increased uptake of LDL (Penn and Chisolm, 1994; Poumay and Ronveaux-Dupal, 1989; Guretski *et al.*, 1994). If BaP and DMBA are capable of inducing proatherogenic changes in the endothelium, one of these changes may be an increase in cellular uptake of modified

LDL. Thus, we measured cellular LDL uptake for monolayers treated with BaP and DMBA throughout a 168 hour experimental period (Figures 14 and 15). Results from BaP treatment showed a slight increase in LDL uptake at 24 hours in 1 μM BaP treated cells and at 96 hours in 0.1 and 1 μM BaP treated cells. All BaP treatment groups showed an increase in cellular LDL uptake at 168 hours in comparison to controls; 1 μM BaP treated cells showed a significant increase. This data suggests that BaP is capable of inducing an increase in LDL uptake with prolonged exposure and increasing concentration of chemical. DMBA treatment showed a slight increase in LDL uptake in all treatment groups at 24 and 48 hours; the increase in 1 μM DMBA-treated cells at 48 hours was significant. At 96 hours, all treatment groups showed significant elevation in cellular LDL uptake in comparison to vehicle controls. At 168 hours, 0.01 and 0.1 μM DMBA treated cells showed that LDL uptake was increased in comparison to controls, although control levels were elevated in comparison to those at previous time points. This data suggests that like BaP, DMBA is capable of inducing an increase in EC uptake of LDL. DMBA, however, is capable of inducing this response more quickly and with lower concentrations of chemical, although effects are most substantial with prolonged exposure. Holland and coworkers (1992) and Penn and Chisolm (1994) have shown that increases in the uptake of modified LDL occur due to an increase in endocytic activity. Holland *et al.*, (1992) have shown that increased EC endocytosis of LDL is accompanied by cytoskeletal remodeling with increased stress fiber formation; these changes may further contribute to alterations in cellular function. It has also been shown that EC are capable of being maintained in this new functional state and is consistent with the concept of endothelial dysfunction (Holland *et al.*, 1992). This increase in LDL uptake may in turn induce signaling for monocyte recruitment and adhesion in addition to altering cell migration and growth (Penn and Chisolm, 1994), thus

further contributing to vascular injury.

While these findings provide some initial information on the endothelial response to BaP and DMBA, they have not addressed the issue of LDL oxidation and the effects on cellular uptake. LDL oxidation is a key factor in the atherogenic process (Alexander *et al.*, 1991; Penn and Chisolm, 1994), and the ability of BaP or DMBA to generate reactive oxygen species that oxidize LDL may be important in determining if the findings from this study are truly indicative of proatherogenic changes. It is of interest to determine the extent to which BaP or DMBA may induce LDL oxidation; an increase in LDL uptake may be explained in part by oxidation as there are several receptor-mediated pathways through which modified LDL can be internalized in the endothelium, while native LDL is thought to be recognized only by the B/E LDL receptor (Penn and Chisolm, 1994). Oxidized LDL has also been shown to be chemotactic for monocytes and SMC, but is inhibitory to EC migration (Penn and Chisolm 1994). Additionally, oxidized LDL has been shown to have direct cytotoxic effects on the endothelial monolayer (Alexander *et al.*, 1991), such that LDL modification may play a significant role in inducing and/or prolonging endothelial dysfunction.

Endothelial injury resulting in focal cell detachment and alterations in proliferation may result in an increase in the permeability of the monolayer, thus allowing the passage and accumulation of various macromolecules into the subendothelial space (DiCorleto and Chisolm, 1986; van Hinsberg, 1992; Coomber and Gottlieb, 1990). Monolayers under repair have been also been found to have increased permeability (Coomber and Gottlieb, 1990). It was thus expected that endothelial injury as a result of BaP or DMBA treatment might induce an increase in monolayer permeability. Such changes would correlate to atherogenic changes, as an increase in monolayer permeability to macromolecules such as LDL and monocytes

are some of the first occurrences in lesion development. Preliminary results from monolayers treated with BaP or DMBA show that the monolayer permeability of vehicle controls did not change much throughout the 168 hour experimental period (Figures 16 and 17). Monolayers treated with 1 μM BaP mirrored the changes seen in controls, with the exception of the 96 hour measurements. At 96 hours, monolayer permeability appears to be increased in 1 μM BaP treated cells. However, the variability in monolayer response at this time point suggests that this increase may not be real; further investigation will be necessary to prove or disprove the validity of this increase. 1 μM DMBA treated monolayers showed trends in monolayer permeability to be similar to controls. At 48 hours, 1 μM DMBA treated monolayers showed an increase in permeability, although again the large variability suggests that this increase is not real. Therefore, it appears that BaP and DMBA do not affect monolayer permeability although this does not mean that these chemicals are incapable of playing a role in atherogenesis. It is possible that longer treatment periods are required for substantial effects on permeability, or that a model which more closely mimics an *in vivo* situation is required (i.e., a co-culture system including smooth muscle cells and endothelium) in order to better evaluate the effects of PAH treatment on monolayer permeability.

Focal monocyte adherence to the endothelium and subsequent migration into the subendothelial space are observed in the first stages of atherosclerosis (Farqi and DiCorleto, 1993; Takahashi *et al.*, 1994). If PAH treatment enhances this process, it might be a way in which these chemicals contribute to the development of atherosclerosis. Thus, monocyte adherence to endothelial monolayers treated with BaP and DMBA was monitored throughout a 168 hour experimental period (Figures 18 and 19). Preliminary results showed little change in monocyte adherence in response to BaP or DMBA treatment at 24 hours in comparison to controls. At 48

hours, 0.01 and 0.1 μM BaP treated cells showed an increase in monocyte adherence in comparison to vehicle controls, while DMBA-treated cells showed an increase in adherence in all treatment groups. At 96 hours, monocyte adherence was not elevated in comparison to controls in any BaP treatment group, while all DMBA treatment groups showed an increase in adherence. All BaP treatment groups showed an increase in monocyte adherence at 168 hours in comparison to controls, while DMBA-treated cells only showed an increase with 0.01 or 0.1 μM DMBA. This data suggests that BaP and DMBA are capable of inducing an increase in EC monocyte adherence with increasing concentration and prolonged exposure. Further work will need to be done in order to determine if these results are reproducible. Increased monocyte adherence at 48 hours, 96 hours, and 168 hours may be due to elevated endothelial expression of ICAM-1 and/or VCAM-1 in response to chemical exposure. Both of these adhesion molecules are induced within 12 to 48 hours following stimulation (Beekhuizen and van Furth, 1993). ICAM-2 may not play a role in this process as it is constitutively expressed (Beekhuizen and van Furth, 1993), and results indicate that there is no increase in adherence at 24 hours with BaP or DMBA treatment; however it is possible that the expression of this adhesion molecule is upregulated with PAH treatment. Both P-selectin and E-selectin may have a role in tethering the monocytes to PAH-treated endothelium (Beekhuizen and van Furth, 1993). Further studies will need to be done to determine which of the adhesion molecules are involved in PAH induced monocyte adhesion to ECs. It is possible that a more complex model is necessary to accurately assess PAH induced changes in monocyte adherence because it is a multifactorial process, relying on the activation of both endothelial cells and monocytes. However, the results do provide initial evidence which supports the hypothesis that BaP and DMBA are capable of inducing a change in the endothelium with time and prolonged exposure which may be indicative

of a shift towards a proatherogenic state.

While descriptive and non-mechanistic, this study has provided valuable information on the concentration/response relationships between PAHs and the endothelium. Data from general toxicity assays in this study have defined the dose and length of PAH exposure which is necessary to induce an endothelial response. This study has demonstrated that these chemicals are capable of altering cell proliferation and cell function (including LDL uptake and monocyte adherence) without inducing acute cytotoxicity. Thus, it appears that BaP and DMBA could play a role in prolonged endothelial cell dysfunction and possibly in the initiation and/or promotion of the atherogenic process. Atherogenesis is considered to be a multifactorial process which occurs in response to some form of vascular injury. The cultured endothelium is a useful model for the study of vascular injury, although it is simplistic. Thus, the preliminary information obtained from this study must be interpreted carefully with regard to the ability of BaP and DMBA to induce atherogenic changes. Analysis of results from this study suggest that although PAHs did not induce large, overt changes in cell function, they did perhaps induce effects which may play a role in prolonged vascular injury and the initiation or promotion of atherosclerosis.

In conclusion, this study is the first to have demonstrated the effects of BaP and DMBA on the vascular endothelium with regard to cell toxicity and functional alterations. Better understanding of the effects that BaP and DMBA, as environmental contaminants and components of cigarette smoke, have on the endothelium may help to further elucidate the processes by which vascular disease develops. There are several approaches which may be used to further our understanding of PAH toxicity and vascular injury. Additional studies using ECs in co-culture with SMC may provide information which more accurately mimics an *in*

vivo situation. Other studies may also examine specific changes in the expression of adhesion molecules, both on the endothelium and on monocytes, in response to PAH treatment. Determining the role of PAHs in the generation of reactive oxygen intermediates (ROIs) as well as their role in the EC changes observed in this study may provide additional information about PAH induced vascular injury. Additionally, the assays used in this study could be repeated following treatment with PAHs given in an initiation-promotion sequence with a known initiator or promotor of atherosclerosis. This may help to determine if PAHs are capable of inducing more profound endothelial effects when acting as an initiator or a promotor of atherosclerosis as opposed to a complete atherogen.

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