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ALTERATIONS IN HUMAN B CELL CALCIUM HOMEOSTASIS BY POLYCYCLIC AROMATIC HYDROCARBONS: POSSIBLE ASSOCIATIONS WITH CYTOCHROME P450 METABOLISM AND INCREASED TYROSINE PHOSPHORYLATION

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by

BARBARA J. MOUNHO

B.S. Biology/Psychology - University of New Mexico, 1991

DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy
Pharmaceutical Sciences

The University of New Mexico
Albuquerque, New Mexico

August, 1997

Barbara J. Mounho
Candidate

Pharmaceutical Sciences (Toxicology)
Department

This dissertation is approved, and it is acceptable in quality and form for publication on microfilm:

Approved by the Dissertation Committee:

, Chairperson

Accepted:

Dean, Graduate School

JUN 16 1997
Date

DEDICATION

This work is dedicated to my parents, Jean and Genevieve Mounho, for their love, encouragement, and endless support.

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Most of all, I thank God for the strength He has given me that has allowed me to persevere and make my dream become a reality. I now know that "I can do all things through Christ who strengthens me", *Philippians 4:4, 13.*

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs), are known immunotoxicants in animals, and are suspect toxins to the human immune system. The mechanism(s), however, by which PAHs exert immunosuppression have not been fully elucidated. Previous studies conducted in our laboratory have shown that PAHs, such as 7,12-dimethylbenz(a)-anthracene (DMBA) and benzo(a)pyrene (BaP) may exert their immunotoxic effects by altering intracellular calcium (Ca^{2+}) homeostasis in lymphocytes. Intracellular Ca^{2+} is an important second messenger in the immune response, and the mobilization of Ca^{2+} is critical in the transduction of intracellular signals from the plasma membrane to the nucleus. The overall objective of this project was to examine the effects of PAHs on intracellular Ca^{2+} levels in human immune cells, particularly B lymphocytes. In these studies, it was shown that highly immunosuppressive PAHs, such as DMBA and BaP, produced a time-dependent increase in intracellular Ca^{2+} in normal human peripheral blood

B cells, T cells, and monocytes. Benzo(e)pyrene (BeP) and anthracene, however, did not produce a statistically significant elevation in intracellular Ca^{2+}. While studies have shown that certain PAHs, such as DMBA and BaP, exert their immunomodulatory effects directly, other researchers have shown that PAH-induced immunotoxicity may be mediated by the reactive metabolites of PAHs. Our laboratory has previously shown that the suppression of T cell mitogenesis produced by PAHs and certain BaP metabolites is reversed by treatment with the cytochrome P450 inhibitor, α-naphthoflavone. Therefore, a second objective in this research was to evaluate the potential role of cytochrome 450 and certain BaP metabolites in PAH-induced Ca^{2+} elevation in human B lymphocytes. Both of the BaP metabolites examined, BaP-7,8-diol and BaP-7,8-diol-9,10-epoxide, were more potent in producing an elevation in free intracellular Ca^{2+} than the parent compound. Additionally, the Ca^{2+} elevation produced by BaP-7,8-diol was completely blocked by pretreatment with ANF, suggesting a role for P450 activation. ANF had no effect on the Ca^{2+} elevation produced by BaP-7,8-diol-9,10-epoxide, suggesting that this may be the active metabolite responsible for Ca^{2+} elevation. The activation of PTKs is a significant intermediary step involved in B cell receptor-mediated proliferation and activation. Previous work in our laboratory has demonstrated that PAHs, such as DMBA, activate Src-family protein tyrosine kinases. The final goal of this project was to examine the role of protein tyrosine kinase (PTK) activity in PAH-induced alterations in Ca^{2+} homeostasis. Pretreatment of cells with a potent PTK inhibitor, herbimycin A, resulted in a significant reduction of the Ca^{2+} elevation produced by BaP-7,8-diol and BaP-7,8-diol-9,10-epoxide, suggesting that PTKs play a role in Ca^{2+} elevation. These two BaP metabolites also produced an increase in tyrosine phosphorylation of Syk and Lyn tyrosine kinases, which

correlates with the activation of these PTKs. In summary, these results suggest that PAHs mediate immunosuppression by disrupting intracellular Ca^{2+} homeostasis and these alterations in Ca^{2+} are possibly associated with cytochrome P450 and protein tyrosine kinase activity.

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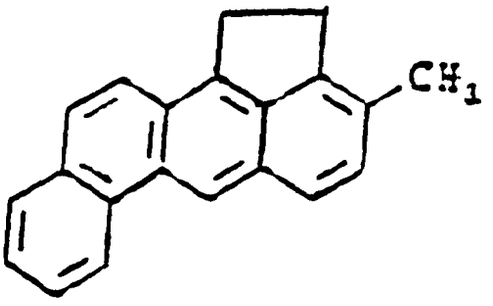
GENERAL INTRODUCTION

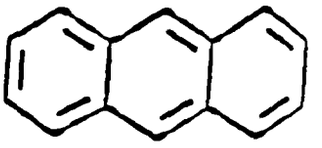
POLYCYCLIC AROMATIC HYDROCARBONS

Polycyclic aromatic hydrocarbons (PAHs) are a diverse class of ubiquitous environmental and occupational contaminants that have potentially important human health implications. PAHs are formed primarily by the incomplete combustion of organic materials and are found throughout the environment in air, water, and soil (U.S. DHHS, 1993). As lipophilic compounds, PAHs occur in anthracene-, phenanthracene-, or pyrene-based forms which can be methylated, nonmethylated, or nitro-substituted. Anthracene, is a relatively biologically inert PAH, and is often considered to be one form of the structural backbone of PAHs, which can vary by the addition and arrangement of aromatic rings and/or aliphatic constituents (Dipple, 1994; Krieger *et al.*, 1995). The PAHs most extensively used in models of immunotoxicity and carcinogenicity are benzo(a)pyrene (BaP) and 3-methylcholanthrene (3-MC), which are found environmentally, and 7,12-dimethylbenz(a)anthracene (DMBA), a methylated, synthetic PAH prototype (reviewed in Davila *et al.*, 1995). The structures of various PAHs, as well as a halogenated aromatic hydrocarbon, 2,3,7,8-tetrachlorodibenzo(p)dioxin, are shown in Figure 1. As a general rule, PAHs that are carcinogenic are immunotoxic, while noncarcinogenic PAHs are typically not immunotoxic (Table 1) and this correlation between PAH carcinogenicity and immunotoxicity is observed *in vivo* (Silkworth *et al.*, 1984; White & Holsapple, 1984; Ward *et al.*, 1985). This information suggests that both PAH-induced immunotoxicity and carcinogenicity involve common mechanism(s). Evaluating PAHs that are structurally similar, but vary in levels of immunotoxicity, may

Figure 1. Structures of various polycyclic aromatic hydrocarbons (PAHs) and halogenated aromatic hydrocarbons. 3-methylcholanthrene (3-MC), anthracene (ANTH), 7,12-dimethylbenz(a)anthracene (DMBA), benz(a)anthracene (BA), benzo(a)pyrene (BaP), benzo(e)pyrene (BeP), 2,3,7,8-tetrachlorodibenzo(p)dioxin (TCDD), and dibenzo(a,h)anthracene (DAH).



3-Methylcholanthrene



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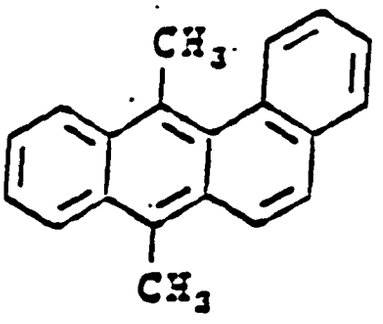
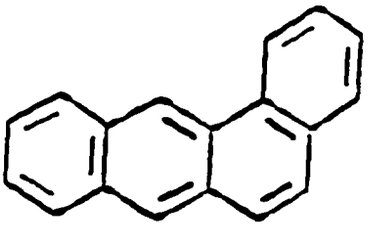


Dimethylbenz(a)anthracene



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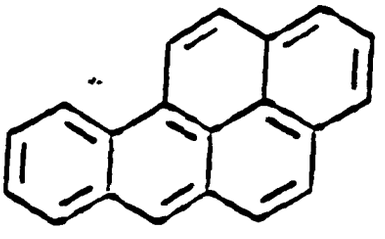
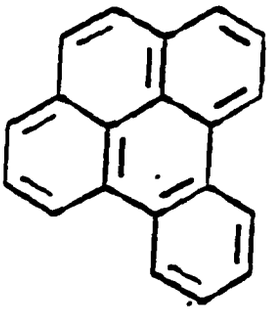
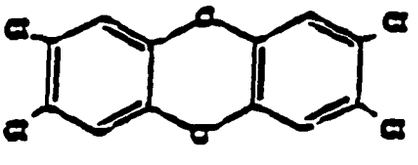


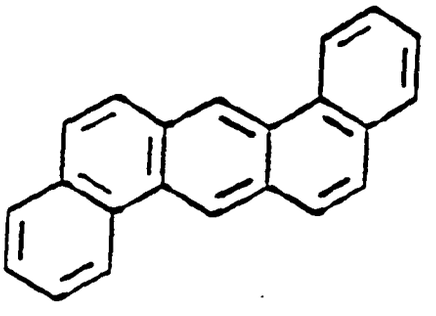
Benzo(a)pyrene



Benzo(e)pyrene



2,3,7,8-tetrachlorodibenzo-p-dioxin



Dibenzo(a,h)anthracene

TABLE 1

ASSOCIATION BETWEEN PAH/HAH IMMUNOTOXICITY AND CARCINOGENICITY

PAH	IMMUNOTOXICITY	CARCINOGENICITY
7,12-Dimethylbenz(a)anthracene (DMBA)	HIGH	HIGH
Benzo(a)pyrene (BaP)	HIGH	HIGH
3-Methylcholanthrene (3-MC)	HIGH	HIGH
2,3,7,8-tetrachloro-dibenzo(p)dioxin (TCDD)	MODERATE/HIGH	HIGH
Dibenz(a,h)anthracene (DAH)	MODERATE	MODERATE
Dibenzo(a,c)anthracene (DAC)	MODERATE	LOW
Anthacene (ANTH)	LOW	LOW
Benzo(e)pyrene	LOW	LOW

provide insight in identifying specific structural moieties responsible for the toxicity of a particular compound or class of compounds.

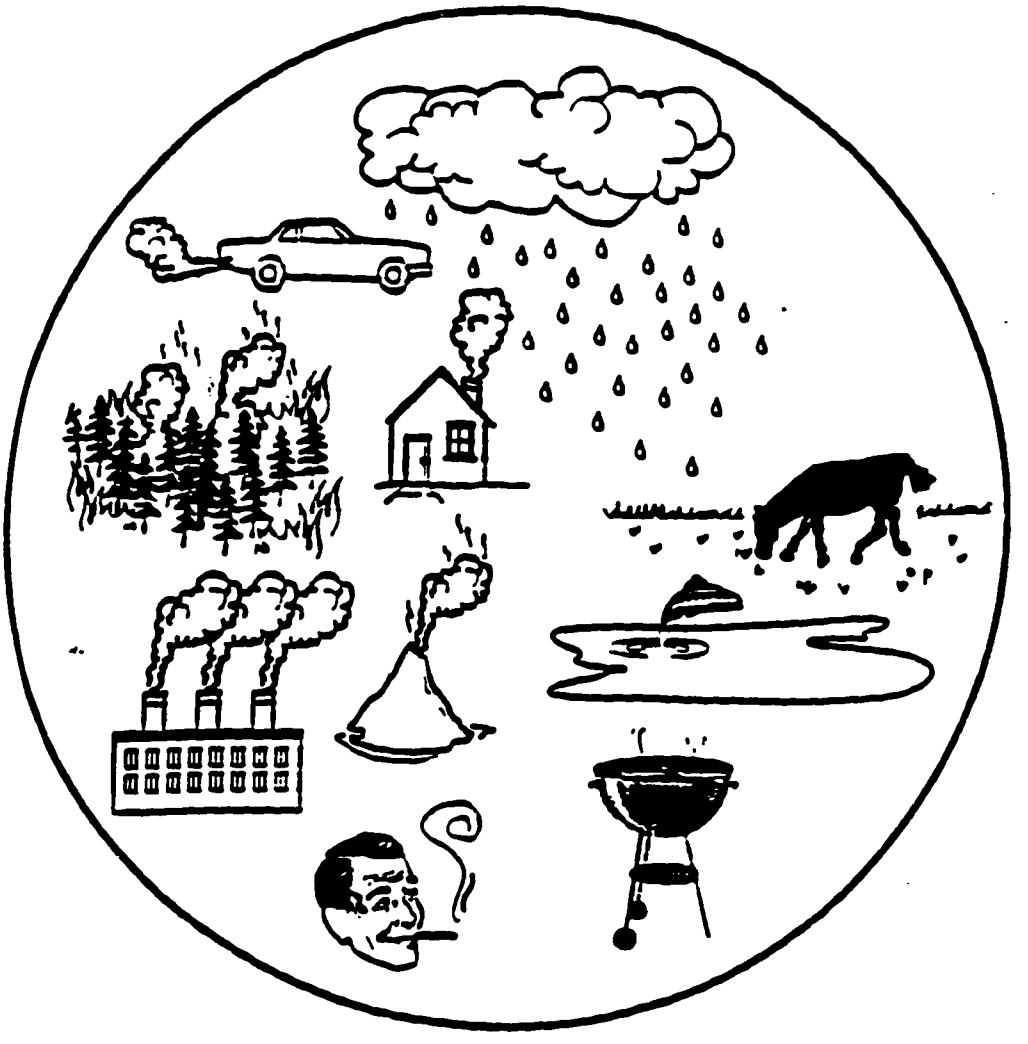
Sources of PAHs

PAHs enter the environment by various natural sources (Figure 2) including volcanic eruptions, forest fires and the decay of organic materials. The principal sources contributed by humans include the combustion of fossil fuels for power generation (particularly coal and diesel fuel), cigarette smoke, heat, transportation, and agricultural and residential burning of wood (reviewed by White *et al.*, 1994; Davila *et al.*, 1997). Residential wood burning and forest fires are the primary sources of PAHs released into the environment. The human contributions of PAHs to the environment exceed the volume released by natural sources (U.S. DHHS, 1993). These compounds gain entry into the environment generally through emissions into the atmosphere, where they integrate with particulate matter which deposits in surface waters and then can be distributed throughout the environment. PAHs primarily concentrate in sediments and soil due to their low solubility in water and strong adsorption to particulate matter. Additional routes by which PAHs enter the environment include discharges from industrial outflows, municipal waste water, and used motor oil (Davila *et al.*, 1997). The widespread distribution of PAHs poses the extreme difficulty of complete elimination of these compounds from the environment.

Routes of PAH Exposure

Animal and human exposure to PAHs is largely through a mixture of compounds rather than to a single PAH, and these compounds can be absorbed following inhalation, oral, or dermal exposure. For the United States human population, significant factors of

Figure 2. Various contributions of polycyclic aromatic hydrocarbons (PAHs) into the environment. PAHs enter the environment by natural sources including volcanic eruptions and forest fires. Human contributions would include automobile exhaust and residential wood burning. Routes of exposure would include the inhalation of cigarette and wood smoke, skin contact with soot and tars, and the ingestion of contaminated grains and charcoal-broiled meats. From: Davila *et al.*, (1997). *Toxicol. Ecotoxicol. News* **4**, 5-9.



exposure include the inhalation of cigarette and wood smoke, and air pollutants, skin contact with soot and tars, and the ingestion of charcoal-broiled foods and contaminated water (Zedeck, 1980). PAH exposure is estimated to be higher in particular members of the population, such as individuals whose occupation involves coke and gas production, working with tar, coal, mining or chimney sweeping, smokers, and people living with smokers, and persons living in a highly industrialized, polluted cities or near hazardous waste sites.

Surprisingly, the major source of exposure to PAHs is through ingestion (U.S. DHHS, 1993). The main factors contributing to the presence of PAHs in the food supply are the growth of plants in contaminated soils, which can absorb PAHs through their roots, and the atmospheric deposition of PAH on plant leaves. Additionally, the preparation of certain foods can also augment exposure to PAHs. Smoked or charcoal-broiled meats, for example, contain larger amounts of PAHs than oven-cooked or boiled foods. A charcoal-broiled steak may contain between 6 and 50 μg of BaP/kg, and for the average 200 g (8 oz) steak, ranges from 1.2 - 10 μg (Lijinsky, 1991). The quantity of carcinogenic PAHs in the average American diet has been estimated to be 1-5 μg/day, with charcoal-broiled or smoked meats or contaminated grains being the major contributing sources (U.S. DHHS, 1993).

PAHs have been detected in surface waters, and PAHs concentrating in the sediment can bioaccumulate in bottom-dwelling aquatic organisms, including fish, shrimp, and clams. The ability of certain aquatic organisms to metabolize and eliminate PAHs, however, usually inhibits the biomagnification of PAHs in the food chain. In U.S. groundwaters, it has been estimated that the average total daily intake of PAHs from

drinking water for an individual in the general population is 0.027 μg. For the non-smoking individual, the inhalation of ambient air is a major secondary source of PAH exposure, which contributes an average of 0.16 μg/day. A person who smokes one pack of unfiltered cigarettes per day, however, inhales an additional 2-5 μg of PAHs (U.S. DHHS, 1993).

IMMUNOTOXICITY OF PAHs

Several PAHs have been found to be potent immunosuppressants, and for this reason, these environmental contaminants have been studied with regard to their persistent disruption of normal immune function. Numerous investigators have demonstrated the immunosuppressive effects of PAHs on both humoral and cell-mediated immune responses (Table 2), primarily using rodent tissue exposed both *in vivo* and *in vitro* (White *et al.*, 1994; Davila *et al.*, 1995). In general, PAHs at high doses are cytotoxic, resulting in lymphoid organ atrophy, while low-dose PAH exposure may induce immunosuppression without evident cytotoxicity.

Cell-Mediated Immunity

T lymphocytes, natural killer (NK) cells, and macrophages are responsible for and involved in cell-mediated immune responses. Cell-mediated immunity is primarily responsible for the elimination of cancerous cells, foreign graft tissue, cellular pathogens, and virus-infected cells. PAHs produce a multitude of toxic effects on the cell-mediated immune system and the immunosuppressive effects of PAHs have been extensively characterized in functional tests of cell-mediated immunity by several investigators. *In vitro* exposure of murine spleen cells to immunotoxic PAHs such as DMBA (the prototype methylated PAH), results in the suppression of several cell-mediated immune

TABLE 2

IMMUNOTOXIC EFFECTS OF PAHs ON DIFFERENT SYSTEMS

IMMUNOTOXICITY	REFERENCE
Suppression of B cells to T-dependent antigens	Dean *et al*, 1983, 1985 Burchiel, 1988 Kawabata & White, 1987 White *et al*, 1985 Thurmond *et al*, 1988
Suppression of B cells to T-independent antigens	Ward *et al*, 1986 Wood & Holsapple, 1993 Burchiel *et al*, 1990 Ladics *et al*, 1992b
Apoptosis	Hardin *et al*, 1992 Burchiel *et al*, 1992, 1993
Decreased proliferation to T cell mitogens	Dean *et al*, 1983, 1985, 1986 Burchiel *et al*, 1990 Pallardy *et al*, 1988, 1992 Krieger *et al*, 1995 Davila *et al*, 1996 Mudzinski, 1993
Decreased cell-mediated responses	Wojandi *et al*, 1984 Wojandi & Alfred, 1984 House & Dean, 1987 Cornacoff *et al*, 1988
Lymphoid cell death	Burchiel *et al*, 1988, 1990, 1992 Davis *et al*, 1991 White *et al*, 1985

responses, including the production of interleukin 2 (IL-2), proliferation in response to the T-cell mitogens, phytohemagglutinin (PHA) and concanavalin A (Con A), and the production of cytotoxic T lymphocytes (CTL) (House *et al.*, 1987a, 1989; Pallardy *et al.*, 1988; Thurmond *et al.*, 1988; Burchiel *et al.*, 1990).

In vivo studies have shown that DMBA suppresses delayed-type hypersensitivity responses and increases host susceptibility to bacterial and tumor challenge (Dean *et al.*, 1985, 1986; Ward *et al.*, 1984, 1985), as well as depresses NK cell activity, CTL activity, and T cell proliferation to mitogens (House *et al.*, 1987; Pallardy *et al.*, 1988; Thurmond *et al.*, 1988). Persistent suppression of cell-mediated immune responses is also observed with BaP exposure. *In vivo*, BaP decreases CTL activity while *in vitro* exposure to BaP leads to the suppression of mixed lymphocyte responses (MLR) (Wojdani *et al.*, 1984; House & Dean, 1987; Thurmond *et al.*, 1988). The non-immunotoxic PAH, BeP, however, only moderately inhibits CTL activity and has no outstanding effects on the cell-mediated aspects of immune function (Wojandi & Alfred, 1984). Although the majority of studies conducted examining the effects of PAHs on the immune system have been performed in the murine model, there is limited information characterizing the effects of PAHs on human immune function. *In vitro* incubations of human lymphocytes with BaP and DMBA result in the suppression of mitogen-stimulated T-cell proliferation responses (Pallardy *et al.*, 1992; Mudzinski, 1993; Krieger *et al.*, 1995). In addition, our laboratory has recently demonstrated that immunotoxic and carcinogenic PAHs such as BaP, DMBA, 3-MC, and certain BaP metabolites, inhibit mitogen-induced human peripheral blood T lymphocyte proliferation (Davila *et al.*, 1996).

Humoral Immunity

The effects of PAHs on humoral immunity have been studied extensively because humoral immune function is the most sensitive indicator of the immunotoxicity produced by PAHs (Blanton *et al.*, 1986; Ward *et al.*, 1986; Luster *et al.*, 1992; Davila *et al.*, 1995). Several investigators have demonstrated *in vivo* and *in vitro* that DMBA suppresses both the IgG and IgM B cell response to T-dependent antigens as well as the IgM response to T-independent antigens (Ward *et al.*, 1984; Dean *et al.*, 1985; White *et al.*, 1985; Burchiel *et al.*, 1988; Ladics *et al.*, 1991). Additionally, the IgM and IgG antibody response to the T-dependent antigen, sheep red blood cell (SRBC), is persistently inhibited (4-8 weeks) by DMBA exposure (Ward *et al.*, 1986). *In vitro* measures of T-dependent B cell antibody responses to antigen stimulation is also suppressed after both *in vivo* and *in vitro* exposures to BaP and DMBA (White *et al.*, 1985; Ladics *et al.*, 1991, 1992b). Studies involving T-independent (lipopolysaccharide- or pokeweed mitogen-stimulated) B cell responses have demonstrated that both *in vivo* and *in vitro* treatments with DMBA produce a decrease in T-independent polyclonal B cell antibody production (Dean *et al.*, 1986; Ward *et al.*, 1986). Additionally, *in vivo* exposure to BaP results in suppression of antibody responses to particular T-independent antigens in mature, but not immature, murine splenic B cells (Ladics *et al.*, 1992a, 1992b).

It has been documented that other highly carcinogenic PAHs, including BaP and 3-methylcholanthrene (3-MC), and moderately carcinogenic PAHs, including dibenz(a,c)anthracene (DAC) and dibenz(a,h)anthracene (DAH), also decrease antibody production to SRBC after *in vivo* subchronic exposures (Malmgren *et al.*, 1952; Lubet *et al.*, 1984; White *et al.*, 1985). Alternatively, noncarcinogenic PAHs, including

anthracene and benzo(e)pyrene (BeP), do not produce a suppression in antibody responses (Stjernsward *et al.*, 1965, 1966; Dean *et al.*, 1983; White *et al.*, 1985).

Wood and Holsapple (1993) demonstrated that DMBA produced a suppression of superantigen toxic shock syndrome toxin-induced B cell proliferation and antibody secretion. Our lab has shown that anti-IgD-stimulated proliferation of murine B lymphocytes following *in vivo* exposure to DMBA is significantly suppressed, as measured by [^{3}H]thymidine incorporation (Davis & Burchiel, 1992). In addition, *in vivo* subchronic exposure of DMBA resulted in a reduction of the number of mature B cells and T cells in the spleens of treated B6C3F1 mice (Burchiel *et al.*, 1988).

Although numerous studies have demonstrated the immunotoxic effects of PAHs on both humoral and cell-mediated immune responses, the cellular and molecular mechanism(s) responsible for PAH-induced immunosuppression have not been fully characterized, and there is high probability that a combination of intracellular signaling mechanisms are involved.

MECHANSIMS OF PAH ACTION

While the molecular mechanism(s) by which PAHs exert their immunotoxic effect, have not been definitively characterized in either animal or human systems, it is very likely that several mechanisms play an important role in PAH-induced immunosuppression.

Metabolism and PAH Carcinogenicity

Polycyclic aromatic hydrocarbons induce cancer in rodents in the absence of known additional environmental or chemical signals and, therefore, are considered to be complete carcinogens capable of tumor initiation, promotion, and progression (Conney *et al.*, 1994; Dipple, 1994). The carcinogenic and mutagenic actions of PAHs are mediated by the

formation of reactive metabolites via cytochrome P450 metabolism rather than the parent compound. These metabolites are reactive electrophiles which are not only capable of binding cellular nucleophilic sites such as deoxyribonucleic acid (DNA), but proteins that play a pivotal role in signaling pathways involved in cellular function. These metabolites can be reactive and, therefore, attack sulfhydryl sensitive proteins involved in Ca^{2+}-dependent signal transduction pathways. It has been suggested by several investigators, that the immunosuppressive effects of PAHs, such as DMBA and BaP, are also mediated by their reactive metabolites (Kawabata & White, 1989; Ladics *et al.*, 1991). Studies evaluating the correlation between metabolism and immunosuppression have shown that PAHs known to be carcinogenic, required metabolic activation to carcinogenic and mutagenic metabolites, while noncarcinogenic PAHs did not undergo metabolic transformation (White *et al.*, 1985; White, 1986). Cytochrome P450 1A1, 1A2, and 1B1are major P450 isozymes involved in PAH metabolism, and each is induced by exposure to PAHs, such as BaP and dioxins including 2,3,7,8-tetrachlorodibenzo(p)dioxin (Whitlock, 1987; Sterling *et al.*, 1994; Shimada *et al.*, 1996).

Calcium Homeostasis

Cytosolic Ca^{2+} is an important intracellular second-messenger, and alterations in the concentration of Ca^{2+} can affect various physiological processes (Berridge, 1993). It has been well established that the mobilization of intracellular Ca^{2+} plays a prominent role in T and B cell receptor-mediated signaling pathways involved in cell activation (Cambier & Ransom, 1987; Weiss & Littman, 1994). Studies using Ca^{2+}-indicators have shown that the concentration of cytosolic Ca^{2+} in unstimulated cells is maintained around 100 nM, which is approximately 10^5 times lower than extracellular Ca^{2+} levels (Carafoli, 1989;

Tymianski, 1996). This significant difference between extracellular and intracellular Ca^{2+} levels produces an inwardly directed movement of Ca^{2+} into the cell, which is balanced by extrusion of Ca^{2+} through the plasma membrane and Ca^{2+}-sequestering systems distributed in mitochondrial, nuclear, and endoplasmic reticular membranes (Orrenius *et al.*, 1996). Although mitochondria and the nucleus can accumulate large amounts of Ca^{2+}, it is the endoplasmic reticulum that is the major intracellular Ca^{2+} store. Generally, increases in cytosolic Ca^{2+} is initiated by the transient release of Ca^{2+} from storage organelles such as the endoplasmic reticulum, followed by a sustained influx of extracellular Ca^{2+} (Premack & Gardner, 1992). This elevation in intracellular Ca^{2+} is a fundamental component of the signaling pathway, driving the resting levels of cytosolic Ca^{2+} up to concentrations as high as several μM, which are required for the activation of critical Ca^{2+}-dependent proteins involved in cellular function. The regulatory effects of Ca^{2+} are most often mediated by these Ca^{2+}-binding/dependent proteins by way of altering the phosphorylation status of target proteins (Premack & Gardner, 1994).

In establishing that Ca^{2+} is a key intracellular regulator of numerous cellular processes, there has come a greater understanding that Ca^{2+} can be an important mediator of pathological and toxicological processes. This thought has led to the generation of the calcium hypothesis of cell injury which proposes that disturbance of cytoplasmic Ca^{2+} may be directly involved in the development of cytotoxic cell death (Metcalfe *et al.*, 1980; Orrenius *et al.*, 1996). Researchers have shown that disruptions in Ca^{2+} homeostasis is often associated with cell injury and may be a central component in the development of cytotoxicity (Schanne *et al.*, 1979; Tymianski, 1996). Various environmental xenobiotics,

such as PAHs, can interfere with Ca^{2+} homeostasis and Ca^{2+}-dependent signaling pathways resulting in a loss of normal cellular activation and function.

RESEARCH OBJECTIVES

The objective of these studies was to examine the hypothesis that polycyclic aromatic hydrocarbons exert their immunomodulatory effects on human B lymphocytes by interfering with Ca^{2+} homeostasis which ultimately leads to the disruption of Ca^{2+}-dependent signaling pathways and biochemical events responsible for cellular activation and functioning.

Several studies conducted in our laboratory have demonstrated in both T and B lymphocytes that PAH-induced immunosuppression involves disrupting intracellular Ca^{2+} homeostasis and signaling pathways. We have observed that *in vivo* and *in vitro* exposure to DMBA results in an elevation of baseline Ca^{2+} in resting cells, and inhibits Ca^{2+} mobilization after PHA stimulation in the Jurkat human T cell line and murine T cells (Burchiel *et al.*, 1990; Burchiel *et al.*, 1991). In the HPB-ALL human T cell line, both highly immunosuppressive PAHs, including DMBA and BaP, and moderately immunosuppressive PAHs, including dibenz(a,h)anthracene (DAH) and 9,10-dimethylanthracene (DMA), produced a sustained (at least 4 hours) rise in intracellular Ca^{2+}, while PAHs that are minimally immunosuppressive, including DAC, BeP, anthracene, and benz(a)anthracene (BA), produced only a small and transient (3 minutes) elevation in Ca^{2+} (Krieger *et al.*, 1994). It appears that the methylation of anthracene at the 9,10-(DMA) or the 7,12-(DMBA) positions increased the duration of the Ca^{2+} elevation suggesting that PAHs may affect different constituents of signal transduction pathways in a structure-dependent manner (Davila *et al.*, 1995). A PAH-induced disruption in Ca^{2+} mobilization also occurs in nylon wool purified T lymphocytes (~85%

pure) isolated from human peripheral blood following Ficoll-Hypaque density gradient centrifugation (Mounho *et al.*, unpublished data).

In the B lymphocyte system, both *in vivo* and *in vitro* treatment with DMBA produced alterations in baseline Ca^{2+} levels (Burchiel *et al.*, 1990, 1992; Davis & Burchiel, 1992). Collectively, these findings suggest that the immunomodulatory effects of PAHs involves alterations in intracellular Ca^{2+} ultimately leading to a disruption of Ca^{2+}-dependent signaling pathways, and that B cells may be an important cellular target.

A second objective of these studies was to examine the role of cytochrome P450 metabolism and P450-derived BaP metabolites in PAH-induced alterations in free intracellular Ca^{2+} in human peripheral B cells and in the Daudi human B cell line. While studies have shown that PAHs, such as DMBA and BaP maybe directly immunosuppressive (Ladics *et al.*, 1991), others have demonstrated the PAH-induced immunotoxicity and carcinogenicity is mediated by the formation of reactive metabolites (Thakker *et al.*, 1977; Ladics *et al.*, 1991; Mudzinski, 1993). The biotransformation of BaP, for example, is necessary for its conversion to reactive electrophiles capable of binding DNA and tumor initiation (Bauer *et al.*, 1995). The most important metabolites involved in the metabolic oxidations of BaP by cytochrome P450 are known as the bay-region dihydrodiol-epoxides (Thakker *et al.*, 1985; Dipple *et al.*, 1994). It has been shown that the major human cytochrome P450 proteins involved in the metabolism of BaP are 1A1 and 1A2, 1B1, and 3A4 (McManus *et al.*, 1990; Bauer *et al.*, 1995; Shimada *et al.*, 1996).

Murine spleen cells are more sensitive to the reactive metabolites of BaP than the parent compound and lymphoid tissues are capable of generating these metabolites for the

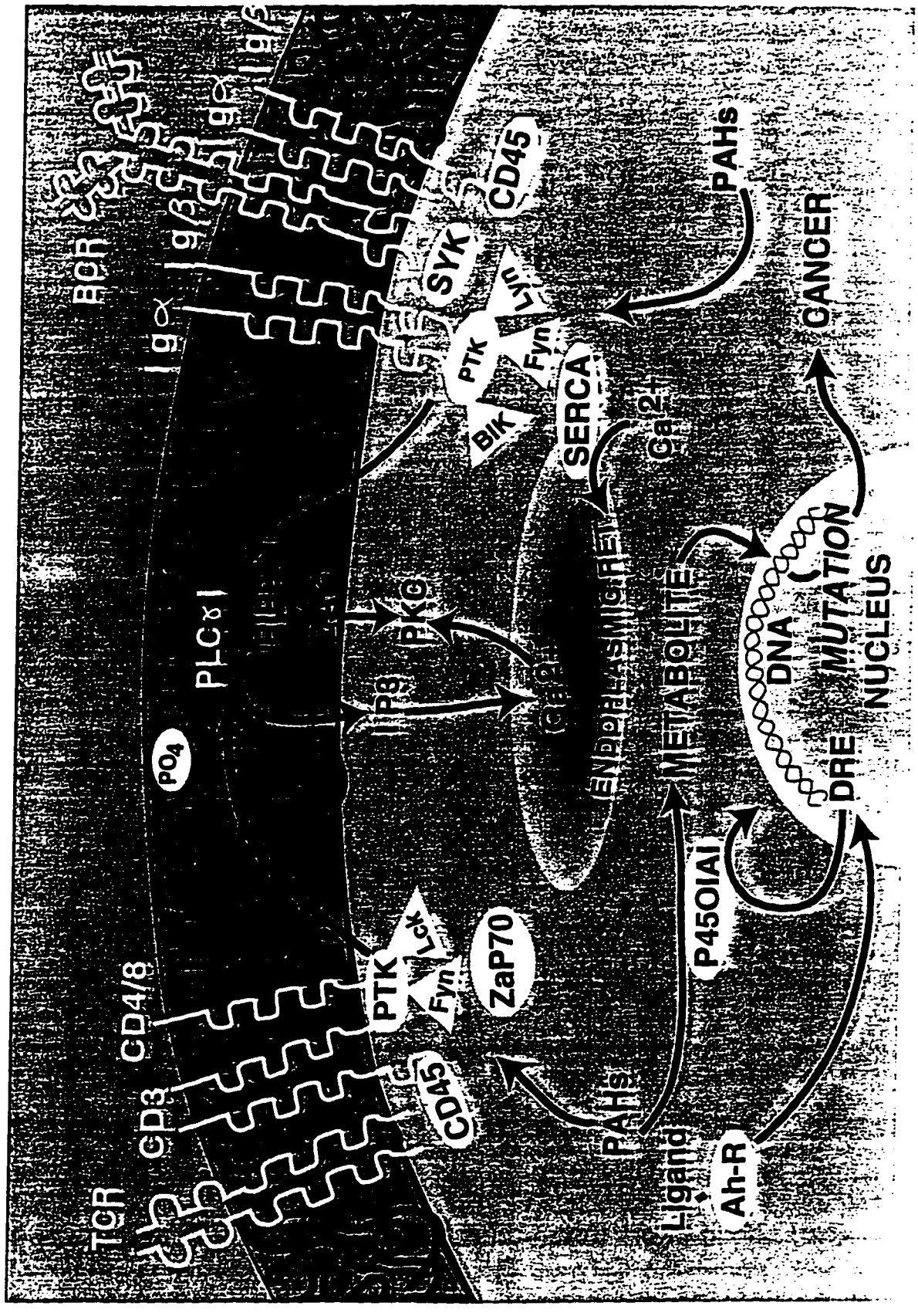
parent compound (Kawabata & White, 1989; Ladics *et al.*, 1991). Recent studies in our laboratory have demonstrated the reactive metabolites of DMBA and BaP play a role in the immunosuppressive effects of DMBA and BaP in human peripheral blood mononuclear cells (Davila *et al.*, 1996; Romero *et al.*, 1997). It has also been demonstrated that cytochrome P450 metabolism is required for the immunotoxicity of BaP and DMBA and that certain metabolites are potent inhibitors of murine immune responses (Ladics *et al.*, 1991, 1992b). It appears, therefore, that the immunotoxic and carcinogenic potential of PAHs may be associated with a common metabolic mechanism leading to the formation of reactive metabolites.

A third objective of these studies was to examine the association between PAH-induced alterations in Ca^{2+} homeostasis and protein tyrosine kinase activity in human B cells. It has been well established that the activation of protein tyrosine kinases is a significant intermediary step involved in membrane antigen receptor-mediated B cell proliferation and function (Pure' & Tardelli, 1992; Yamanashi *et al.*, 1992; Harwood & Cambier, 1993). In light of these observations, work in our laboratory has been conducted to determine the effects of PAHs on kinases responsible for stimulating downstream signaling events involved in B and T cell function. It has been observed in HPB-ALL human T cells that the rapid elevation in intracellular Ca^{2+} produced by DMBA is accompanied by a transient increase in inositol 1,4,5-triphosphate (IP_3) formation and tyrosine phosphorylation of PLCγ-1. Additionally, the activation of the T cell receptor-associated Src-family kinases, Fyn and Lck, is also modulated by DMBA. Collectively, these findings suggest that Fyn and Lck kinases may be responsible for the phosphorylation of PLCγ-1 resulting in IP_3 formation and mobilization of intracellular

Ca^{2+}, and these events may be altered by DMBA exposure (Archuleta *et al.*, 1993). Figure 3 represents both T and B cell receptor-mediated signaling pathways and metabolism of xenobiotics which may be disrupted by PAHs, as well as HAHs, such as TCDD. Genistein, a protein tyrosine kinase (PTK) inhibitor, partially inhibits both the rapid and sustained elevation in Ca^{2+} produced by PAHs, while staurosporine and calphostin C, protein kinase C (PKC) inhibitors, have no effect (Krieger *et al.*, 1995). Based on these data, it appears that DMBA-induced tyrosine kinase activation also plays a role in producing both an early and sustained rise in intracellular Ca^{2+} in the HPB-ALL cell line. In the murine B-lymphoma A20.1 cell line, both immunosuppressive and non-immunosuppressive PAHs produce an increase in tyrosine phosphorylation. This implies that PAHs may modulate intracellular signaling pathways in B lymphocytes by enhancing kinase activity (Davila *et al.*, 1995).

In summary, these studies have provided initial insights into the putative mechanisms involved in PAH-induced immunomodulatory effects in B lymphocytes. Collectively, these data suggest that PAHs may exert their immunomodulatory effects in B lymphocytes by producing a disruption of kinase activity leading to an elevation in intracellular Ca^{2+}, which eventually alters signaling pathways involved in cell growth and activation.

Figure 3. Schematic of both T cell and B cell antigen receptor mediated signaling pathways which may be susceptible to the effects of PAHs. While there may be several similarities between T and B cell receptor mediated signaling, there may also be some significant differences.



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CENTRAL HYPOTHESES

- Polycyclic aromatic hydrocarbons (PAHs) exert immunotoxic effects in human B lymphocytes by interfering with calcium (Ca^{2+}) homeostasis, ultimately leading to a disruption in Ca^{2+}-dependent signal transduction mechanisms involved in B cell activation and proliferation.

- PAH-induced alterations in Ca^{2+} homeostasis may be associated with cytochrome P450 metabolism and increased protein tyrosine phosphorylation.

SPECIFIC AIMS

1. Determine the differential sensitivity of human peripheral blood mononuclear cells (HPBMCs), including light scatter-gated and/or surface marker defined monocytes, B cells, and T cells, to intracellular Ca^{2+} changes produced by PAHs using multiparameter flow cytometric analysis.

a. Determine the dose- and time-dependence for PAH-induced Ca^{2+} elevation in HPBMCs (T cells, B cells, and monocytes) using the Ca^{2+}-chelating dyes, Fluo-3 and Fura Red in conjunction with phycoerythrin (PE)-conjugated murine monoclonal antibodies to human CD19 (B cells), CD3 (T cells), and CD14 (monocytes).

b. Determine structure activity relationships for various PAHs (DMBA, BaP, BeP, ANTH) and the halogenated aromatic hydrocarbon (HAH), 2,3,7,8-tetrachlorodibenzo(p)dioxin (TCDD) for Ca^{2+} elevation in HPBMCs.

2. Develop *in vitro* models of human B cell activation that can be utilized to examine the mechanisms of PAH-induced Ca^{2+} mobilization.

a. Perform selective structure-activity and kinetic studies of PAH-induced (BaP and BaP metabolites) Ca^{2+} elevation in NCL-BL2126, Daudi, Ramos, and Raji human B cell lines, and characterize which cell line is best for the studies.

b. Characterize and establish a human B cell line to serve as an *in vitro* model for human B cell protein tyrosine kinase phosphorylation and metabolism studies.

3. Evaluate the effects of cytochrome P450-mediated BaP metabolites and the potential role of P450 metabolism in PAH-induced alterations in free intracellular Ca^{2+} mobilization in human B lymphocytes.

a. Determine the dose- and time-dependence of BaP-induced Ca^{2+} elevations in light scatter gated human peripheral lymphocytes and monocytes and examine the effect of α-naphthoflavone (ANF), an inhibitor of cytochrome P450 activity, on altered Ca^{2+} mobilization produced by BaP.

b. Establish a dose- and time-dependent response of BaP and two BaP metabolites, benzo(a)pyrene-trans-7,8-dihydrodiol (±) (BaP-7,8-diol), and benzo(a)pyrene-r-7,t-8-dihydrodiol-t-9,10-epoxide (±), (anti) (BaP-7,8-diol-9,10-epoxide), and BeP on intracellular Ca^{2+} in the Daudi human B cell line.

c. Compare the direct effect of BaP and two BaP metabolites, BaP-7,8-diol and BaP-7,8-diol-9,10- epoxide, and BeP on intracellular Ca^{2+} in Daudi cells.

d. Evaluate the effects of ANF on intracellular Ca^{2+} alterations produced by BaP-7,8-diol and BaP-7,8-diol-9,10-epoxide in Daudi cells.

e. Establish a dose-response relationship and compare the direct effect of BaP, BaP-7,8-diol and BaP-7,8-diol-9,10-epoxide, and BeP on intracellular Ca^{2+} in CD19+ human peripheral B lymphocytes.

4. **Evaluate the association between disruptions in intracellular Ca^{2+} homeostasis produced by BaP metabolites (BaP-7,8-diol and BaP-7,8-diol-9,10-epoxide) and protein tyrosine kinase activity.**

   a. Establish a dose and pretreatment response of herbimycin A, a specific protein tyrosine kinase inhibitor, in Daudi cells.

   b. Evaluate the role of protein tyrosine kinase activity in PAH-induced Ca^{2+} mobilization by examining the effects of protein tyrosine kinase inhibition by herbimycin A on altered Ca^{2+} mobilization produced by BaP-7,8-diol and BaP-7,8-diol epoxide using multiparameter flow cytometric analysis.

   c. Establish a dose- and time-dependent response in increased protein tyrosine phosphorylation produced by BaP-7,8-diol and BaP-7,8-diol epoxide using immunoblot analysis.

   d. Establish a dose- and time-dependent response and evaluate increased protein tyrosine kinase activity of Syk and Lyn protein kinases by examining increased autophosphorylation using immunoprecipitated phosphotyrosine proteins and immunoblot analysis.

CHAPTER I

Characterization of Intracellular Calcium Responses Produced by Polycyclic Aromatic Hydrocarbons in Surface-Marker Defined Human Peripheral Blood Mononuclear Cells

BACKGROUND AND SIGNIFICANCE

The humoral and cell-mediated immune systems are common targets for the toxic effects of polycyclic aromatic hydrocarbons (PAHs) (White *et al.*, 1994; Davila *et al.*, 1995). Studies have shown that 7,12-dimethylbenz(a)anthracene (DMBA) and benzo(a)pyrene (BaP) suppress T-dependent B cell responses after *in vivo* or *in vitro* antigen stimulation (Ward *et al.*, 1984; White *et al.*, 1985; Burchiel *et al.*, 1988; Ladics *et al.*, 1991, 1992b). T-independent B cell responses have also been shown to be suppressed by *in vivo* or *in vitro* treatments with DMBA or BaP resulting in a decrease in antibody production (Ward *et al.*, 1984; Ladics *et al.*, 1992). Several T cell-dependent immune responses have also been shown to be sensitive to PAH-induced immunosuppression (Ward *et al.*, 1986; House *et al.*, 1987; Pallardy *et al.*, 1988, 1992).

Our laboratory has shown that both murine T and B cell proliferation is inhibited by DMBA and the effect of the PAH is quite persistent, producing immunosuppression for eight or more weeks (Burchiel *et al.*, 1990). Suppression of murine immune responses occurs at doses of DMBA that are not cytotoxic to lymphocytes, demonstrating that this long-lived effect may resemble the induction of tolerance. It has been shown that DMBA

increases intracellular Ca^{2+} in murine and human B and T lymphocytes, and our laboratory has hypothesized that the elevation of intracellular Ca^{2+} occurs by way of activation of protein tyrosine kinases (Archuleta *et al.*, 1993) or inhibition of sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) activity (Krieger *et al.*, 1995).

As an intracellular second-messenger, calcium is an important component of the immune response and alterations in intracellular Ca^{2+} affect both humoral (B cell) and cell-mediated (T cell) immunity (Aldo-Benson & Tsao, 1987). The mobilization of intracellular Ca^{2+} plays an essential role in signaling pathways involved in lymphocyte activation and gives the T cell or B cell receptor (TCR, BCR) the ability to transduce intracellular signals to the nucleus (Choquett *et al.*, 1994). Elevation of intracellular Ca^{2+} leads to a disruption in antigen and mitogen receptor signaling and may cause tolerance in lymphocytes and it is well known that excess Ca^{2+} signaling in lymphocytes in the absence of co-stimulatory signals leads to the induction of tolerance (Aldo-Benson & Tsao, 1987).

Past studies have shown that certain PAHs produce an elevation of free intracellular Ca^{2+} in the Jurkat and HPB-ALL human T cell lines, as well as nylon wool T lymphocytes isolated from human peripheral blood (Burchiel *et al.*, 1990; Krieger, *et al.*, 1994; Mounho *et al.*, unpublished data). In addition, a disruption of baseline Ca^{2+} has also been observed in resting murine splenic B cells and the murine B cell lymphoma A20.1 cell line exposed to DMBA (Davis & Burchiel, 1992; Burchiel *et al.*, 1993). In structure-activity studies, our laboratory has demonstrated in human T cells that there is a strong relationship between the immunotoxicity of PAHs and their ability to maintain elevated levels of intracellular Ca^{2+} (Krieger *et al.*, 1994).

The mechanism(s) by which PAHs exert their immunotoxic effects have not been well characterized in normal human peripheral lymphocytes and monocytes. For this reason, we and others have attempted to define the underlying cellular mechanisms of PAH-induced immunotoxicity of various human T and B cell lines, as well as in normal human peripheral blood mononuclear cells (HPBMC) (Cornacoff *et al.*, 1988).

Our data suggests that PAHs exert their immunomodulatory effects by interfering with Ca^{2+}-dependent intracellular signal transduction pathways involved in cell proliferation and activation. Since we have not previously characterized the effects of various PAHs or the halogenated aromatic hydrocarbon (HAH), 2,3,7,8-tetrachlorodibenzo(p)dioxin (TCDD) on Ca^{2+} signaling pathways in normal human immune cells, the purpose of these studies was to determine whether PAHs increase intracellular Ca^{2+} in HPBMC and to determine the differential sensitivity of various subsets of cells to the Ca^{2+} elevation using multiparameter flow cytometry. In preliminary studies, we determined the optimal doses and timing for examining the effects of PAHs on HPBMC Ca^{2+} elevation. We also noted significant variability in the responses of donor HPBMCs. Therefore, in the present studies, we report the results of ten consecutive normal donors analyzed under identical conditions, in an attempt to control for as many parameters as possible to limit variability. We examined the effects of two immunosuppressive PAHs (BaP and DMBA), two non-immunosuppressive PAHs, benzo(e)pyrene (BeP) and anthracene (ANTH), and TCDD on intracellular Ca^{2+} levels in surface marker-defined human peripheral blood mononuclear cells at 20, 42, and 66 hour time points. In the past, our laboratory has focused on the effects of PAHs on intracellular Ca^{2+} at shorter time points, and in these studies, we have focused on longer exposure periods.

MATERIALS AND METHODS

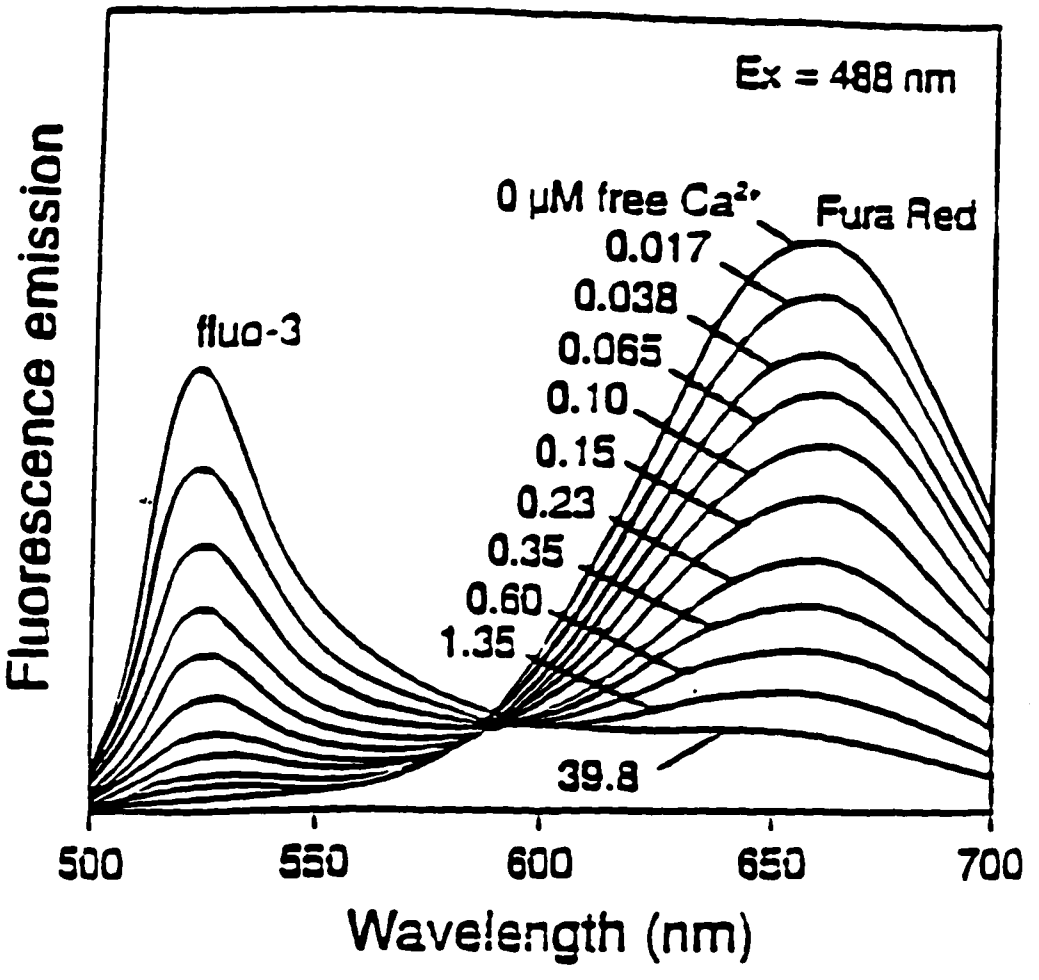
Reagents. All polycyclic aromatic hydrocarbons (PAHs; ≈ 95% purity as assessed by the manufacturer), including 7,12-dimethylbenz(a)anthracene, benzo(a)pyrene, benzo(e)pyrene, and anthracene were purchased from Sigma Chemical Co. (St. Louis, MO). 2,3,7,8-tetrachlorodibenzo(p)dioxin (TCDD; 99% purity) was purchased from Cambridge Isotopes Labs (Andover, MA). PAHs and TCDD were dissolved in tissue culture grade (> 99% purity) anhydrous dimethylsulfoxide (DMSO; Sigma, St. Louis, MO), which also served as the vehicle control. PAHs were prepared immediately prior to use, shielded from light, and visually inspected to affirm that the compounds were dissolved in DMSO. The final concentration of DMSO in all cell cultures was ≤ 0.1%. Control cultures without DMSO did not differ from cultures containing DMSO, and it was concluded that DMSO at the concentrations used did not produce any measurable effect on intracellular Ca^{2+} responses.

Preparation of Human Peripheral Blood Mononuclear Cells (HPBMC). Human peripheral blood was obtained from the Clinical Research Center (CRC) at the University of New Mexico Hospital in Albuquerque, NM. Volunteer donors were clinically normal adults in the normal range for peripheral blood differential cell counts (Complete Blood Cell, CBC analysis), and pre-screened to exclude individuals taking prescribed or over the counter medications, having chronic or acute illness, and had not donated a unit of blood for at least 8 weeks. The CRC volunteer donors were between the ages of 23 and 44, who were all non-smokers except one donor. Venous blood was collected into sterile, heparinized bags and centrifuged 2 times for 5 minutes each (1200 x g; 24 °C) to enrich

for leukocytes. The leukocyte layer was diluted with equal parts of sterile phosphate buffered saline (PBS), and layered over a ficoll density gradient solution (Fico/Lite-LymphoH, Atlanta Biological, Norcross, GA). After centrifugation (500 x g; 24 °C; 30 min), the cell layer at the ficoll-plasma interface (mononuclear cell layer) was collected, washed 3 times in PBS to remove any excess ficoll, and resuspended in RPMI 1640 medium (BioWhittaker, Walkersville, MD) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT) and 1% penicillin/streptomycin (pen/strep; BioWhittaker, Walkersville, MD). The total time from collection of blood to culturing of HPBMC was typically three hours. The percentage of monocytes in the HPMC ficoll preparation was typically around 19% (Davila *et al.*, 1996).

Flow Cytometry Measurement of Calcium. For the comparison of the effects of PAHs on intracellular Ca^{2+} in human peripheral T cells, B cells, and monocytes, ficoll density isolated mononuclear cells resuspended in complete media were treated with PAHs and plated at a concentration of 1 x 10^6 cells/ml into 24-well or 12-well plates in a volume of 1-3 mls. Quadruplicate samples were prepared for each control and treatment group, and alterations in free intracellular Ca^{2+} levels were measured using a Coulter ELITE flow cytometer (Hialeah, FL) and the Ca^{2+}-chelating dyes, Fluo-3 AM and Fura-Red AM (Molecular Probes, Eugene, OR), as a ratiometric indicator of Ca^{2+}. The acetoxymethyl (AM) ester form of Fluo-3 and Fura Red was used because once the lipophilic AM esters enter the cell, they are hydrolyzed to the active free dye in the cytoplasm by intracellular esterases (Haugland, 1993). Both of these indicator dyes excite at 488 nm, and Fluo-3 fluoresces with increasing intensity in the green region when bound to Ca^{2+} and Fura-Red fluoresces most intensely in the red region when it is not bound to Ca^{2+} (see Figure 4).

Figure 4. Fluorescence emission spectra of Fluo-3 and Fura Red Ca^{2+} indicators simultaneously excited at 488 nm in solutions containing zero to 39.8 μM free Ca^{2+}. Adapted from: Haugland, R.P. (1996). *Handbook of Fluorescent Probes and Research Chemicals, 6th Edition,* 507-521.



When used simultaneously, Fluo-3 and Fura Red exhibit reciprocal shifts in fluorescence intensity upon binding Ca^{2+}, and a low noise, highly sensitive Fluo-3 to Fura-Red ratiometric measurement of free intracellular Ca^{2+} is obtained (Novak & Rabinovitch, 1994). Unlike with other Ca^{2+}-chelating fluorescence dyes, such as Indo-1, we have found that the presence of PAHs did not interfere with the Fluo-3/Fura-Red fluorescence signal. Samples were pelleted and resuspended in complete media containing 6 μM Fluo-3 and 13 μM Fura-Red in a volume of 100 μl and incubated at 37 °C for 1 hour, with cells being resuspended ≈ every 20 minutes to ensure even loading. Samples were then incubated for an additional 30 min with phycoerythrin (PE)-conjugated murine monoclonal antibodies at 1 μg/1 x 10^6 cells (Caltag Laboratories, Burlingame, CA) to either CD3 (T cells), CD14 (monocytes), or CD19 (B cells) cell surface antigens. PE fluoresces in the orange region and like Fluo-3 and Fura Red, excites at 488 nm. Matching isotype controls (IgG-2a for T cells and monocytes, IgG-1 for B cells) was also used to account for non-specific binding and to assist in gating on the specific cell populations. Results are shown as the change in the Mean Channel Fluo-3/Fura-Red Fluorescence Ratio (MCFR) which is obtained by subtracting the MCFR of DMSO vehicle controls from the MCFR of PAH-treated samples. The MCFR for control DMSO samples was typically electronically set at channel 250 - 350, and the increase in channel number following PAH treatment is an indication in the change of this ratio. Trypan blue (Sigma) was used to check cell viability in all experiments which ranged from 92% - 99%. To ensure cells, chelators, and flow cytometry detection systems were functioning correctly, 1 μM thapsigargin (Sigma), which is a potent inhibitor of the sarcoplasmic/endoplasmic reticulum calcium ATPase

(SERCA) and induces Ca^{2+} mobilization without the generation of IP_3 (Premack & Gardner, 1992), was used in all experiments as a positive control for Ca^{2+} mobilization.

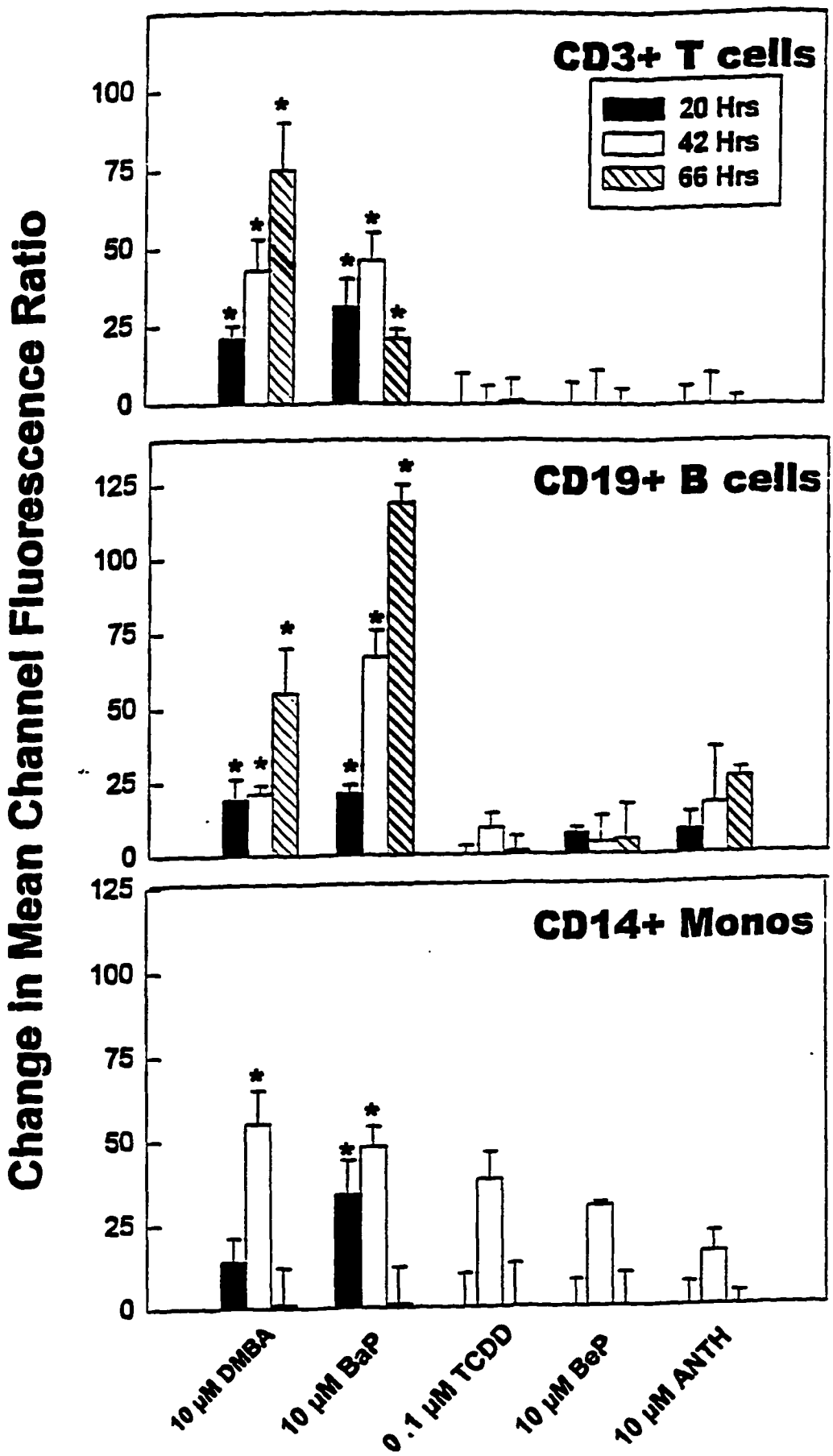
Statistical Design and Analysis. Data were analyzed for statistical differences between DMSO control and treated groups using SigmaStat statistical software (Jandel Scientific, San Rafeal, CA). ANOVA followed by Dunnett's *t*-tests were performed on sample means, with use of a Mann-Whitney test when equal variance test failed. Statistical significance is defined as a p value of $p < 0.05$.

RESULTS

Time-Dependence of Ca^{2+} Elevation by PAHs in Surface Marker-Defined HPBMC.

The time-dependence for PAH-induced Ca^{2+} elevation in subsets of HPBMC was assessed using HPBMC obtained from five separate donors. Results from a representative donor are shown in Figure 5. BaP and DMBA, but not BeP and ANTH, were found to produce a statistically significant increase in intracellular Ca^{2+} following 20, 42, and 66 hours of exposure in $CD3^+$ T cells, $CD\ 19^+$ B cells, and at 42 hours in $CD14^+$ monocytes. Results are shown as the change in the mean channel fluorescence ratio (MCFR) which was obtained by subtracting the MCFR of DMSO controls for each subset from the MCFR of PAH-treated samples. At the 20 hour time point, 10 μM DMBA produced a significant ($p < 0.05$) rise in intracellular Ca^{2+} in the T and B cells, but this rise was not significant in monocytes. BaP at 10 μM produced an elevation in Ca^{2+} in all three cell populations. At 42 hours, both DMBA and BaP significantly ($p < 0.05$) increased intracellular Ca^{2+} in all three cell populations. Interestingly, the BaP response appeared to peak in T cells at 42 hours, but appeared to be higher at 66 hours in B cells and perhaps monocytes. TCDD (0.1 μM), BeP, and ANTH (both at 10 μM) also produced a rise in Ca^{2+} in monocytes, but due to the variability in the monocyte DMSO control samples for Ca^{2+} measurements in this experiment, the effects of these agents were not statistically significant. At 66 hours, DMBA caused a significant Ca^{2+} rise only in T and B cells, but not in monocytes. At all three time points, TCDD (0.1 μM) and 10 μM BeP and ANTH did not produce a significant rise in Ca^{2+} in any of the cell populations.

Figure 5. Intracellular calcium elevation in human peripheral blood T cells ($CD3^-$), B cells ($CD19^+$) and monocytes ($CD14^+$) treated for 20, 42, and 66 hours. Mononuclear cell cultures were treated with either DMBA, BaP, BeP, or ANTH at a 10 μM concentration or 0.1 μM TCDD. Cells were incubated with Fluo-3 and Fura Red and phycoerythrin-conjugated murine monoclonal anti-human antibodies to either CD3, CD19, or CD14. Results are shown a the change in Mean Channel Fluorescence (Fluo-3/Fura Red) Ratio (MCFR) which is obtained by subtracting the MCFR of DMSO solvent controls from the MCFR of PAH-treated samples analyzed in quadruplicate. Values are mean ± S.E.M. for one representative donor. *Indicates significant ($p < 0.05$) increases in free intracellular Ca^{2+} DMSO solvent control. Actual MCFR for samples having a change in MCFR ≤ 0 are as follows: **CD3+ T cells** - 20 hrs : DMSO = 238 ± 6.5, TCDD = 228 ± 9.7, BeP = 226 ± 7.4, ANTH = 236 ± 6.2; 42 hrs: DMSO = 328 ± 11.0, TCDD = 318 ± 6.0, BeP = 319 ± 11.0, ANTH = 301 ± 10.0; 66 hrs: DMSO = 265 ± 5.6, BeP = 233 ±5.4, ANTH = 244 ± 3.3; **CD19+ B cells** - 20 hrs: DMSO = 210 ± 3.8, TCDD = 203 ± 3.0; 42 hrs: DMSO = 225 ± 2.0; 66 hrs: DMSO = 271 ± 8.0; **CD14+ Monocytes** - 20 hrs: DMSO = 338 ± 12, TCDD = 331 ± 9.7, BeP = 322 ± 8.3, ANTH = 328 ± 6.5; 42 hrs: DMSO = 237 ± 5.0; 66 hrs: DMSO = 317 ± 2.9, TCDD = 309 ± 13.1, BeP = 310 ± 10.0, ANTH = 307 ± 4.4.



33b

Ca^{2+} Elevation by Immunotoxic PAHs in HPBMC Obtained from Ten Normal Donors. Because the broadest Ca^{2+} responses following PAH exposure in all three cell populations were observed following 42 hour exposures in several donors, this time point was chosen for comparing the effects of various PAHs and TCDD in HPMC obtained from several separate donors. In Figure 6, the pooled results are shown for ten replicate studies performed using HPBMC obtained from different donors. The statistical results from the ten individual experiments are shown in Table 3. As in Figure 5, all PAHs were examined at 10 μM concentrations following a 42 hour exposure period, whereas TCDD studies were performed a 0.1 μM. DMBA and BaP produced a significant ($p < 0.05$) increase in free intracellular Ca^{2+} compared to the DMSO control in all $CD3^+$ T cells, $CD19^+$ B cells, and $CD14^+$ monocytes. Interestingly, while TCDD produced a statistically significant increase in intracellular Ca^{2+} in B cells in only 2 of 10 donors, the results for the pooled 10 donors demonstrated an overall statistically significant change in Ca^{2+} responses in B cells, but not T cells.

In analyzing the pooled data for the relatively non-immunotoxic PAHs, BeP and ANTH were not found to produce a significant Ca^{2+} elevation in any of the three HPBMC populations. However, as shown in Table 3, these PAHs were found to occasionally produce a statistically significant increase in T cells, B cells, and/or monocytes in HPBMC obtained from individual donors, following a 42 hour exposure. It is unclear why relatively non-immunotoxic PAHs occasionally increase Ca^{2+} in some donors. One possible explanation is some individuals may be more sensitive to the effects of PAHs than others. However, there was a clear difference in the number of responders for the

Figure 6. Calcium elevation in human peripheral blood T cells ($CD3^+$), B cells ($CD19^+$), and monocytes ($CD14^+$) from 10 different donors treated for 42 hours and analyzed in quadruplicate. 10 μM DMBA, BaP, BeP, ANTH, and 0.1 μM TCDD , were added to human peripheral blood mononuclear cell cultures. Alterations in Ca^{2+} were analyzed using flow cytometry and the Ca^{2+}-indicator dyes Fluo-3 AM and Fura Red AM, and changes in MCFR were determined as previously described in Fig. 5. Cell populations were identified using murine monoclonal anti-human antibodies to CD3, CD19, or CD14. Between 70-80% of the cells gated as lymphocytes on the flow cytometer were T cells and approximately 15% were B cells, whereas approximately 60-75% of the cells gated in the monocyte region were monocytes. Values are Mean ± S.E.M. for 10 different donors. *Indicates significant ($p < 0.05$) increase in intracellular Ca^{2+} compared to DMSO control. The actual MCFR of samples a change in MCFR ≤ 0 are as follows: **CD3+ T cells** - DMSO = 283 ± 12.3, BeP = 279 ± 12.6; **CD19+ B cells** - DMSO = 263 ± 11.5; **CD14+ Monocytes** - DMSO = 329 ± 14.8.

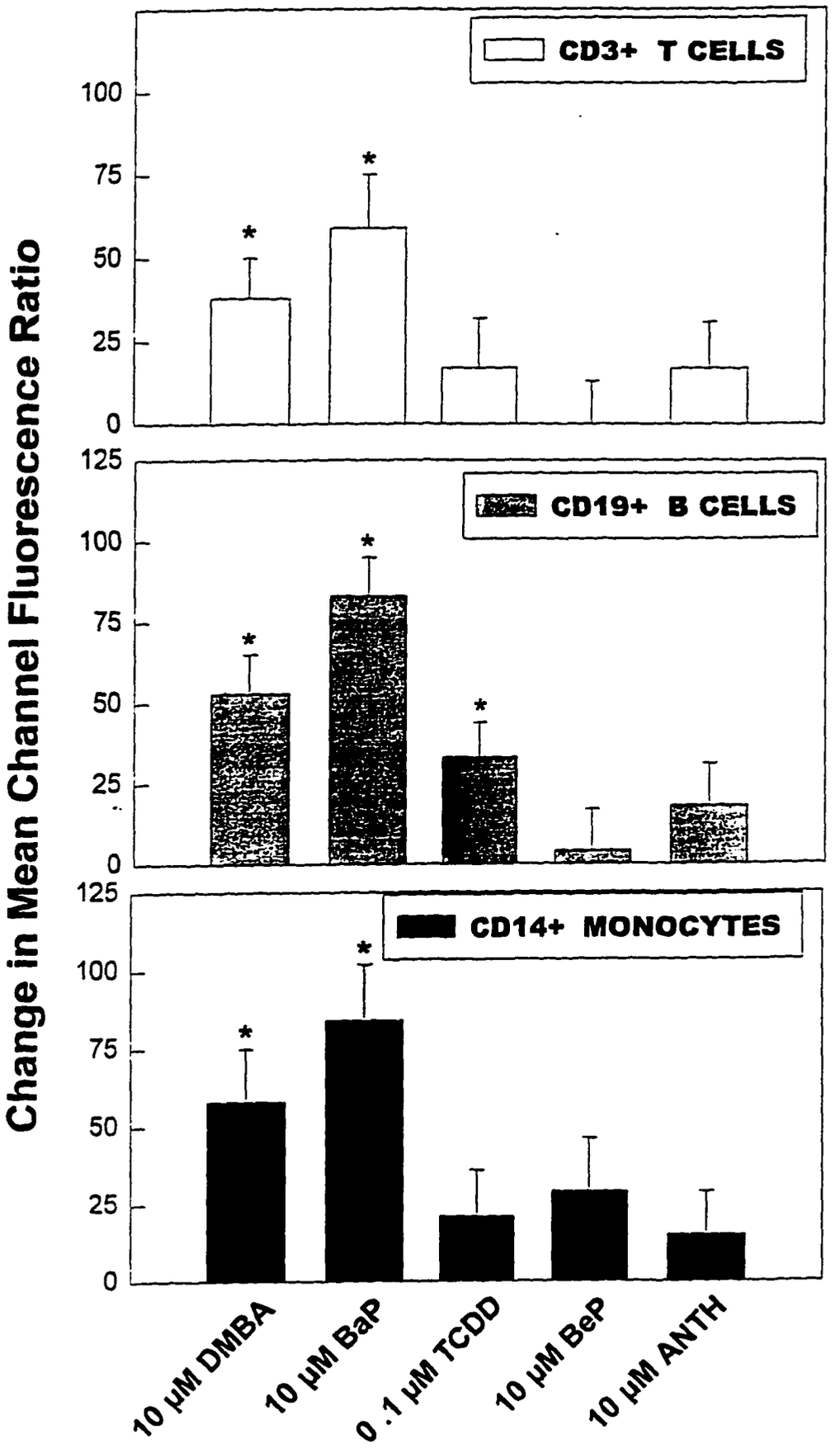


TABLE 3: Summarized Results for Ten Donors in the Average Change in Mean Channel Fluorescence Ratio (MCFR) for Surface Marker-Defined HPBMC Treated for 42 Hrs with PAHs[a]

PAH Tested	T Cells	B Cells	Monocytes
10 μM DMBA	38.4 ± 12.1	53.4 ± 11.7	57.8 ± 17.1
	7 of 10*	8 of 10*	6 of 10*
10 μM BaP	59.1 ± 16.0	83.2 ± 11.6	83.6 ± 17.9
	8 of 10*	9 of 10*	8 of 10*
0.1 μM TCDD	17.2 ± 14.9	32.5 ± 10.6	20.8 ± 14.5
	NR	2 of 10*	3 of 10*
10 μM BeP	0.0 ± 12.6	3.97 ± 12.8	28.5 ± 16.8
	NR	1 of 10*	1 of 10*
10 μM ANTH	16.7 ± 14.0	17.7 ± 13.4	14.6 ± 14.3
	1 of 10*	1 of 10*	3 of 10*

[a]Intracellular calcium elevation in HPBMC T cells, B cells, and monocytes from 10 different donors treated with PAHs for 42 hours. Human mononuclear cells were incubated with DMSO solvent control or PAHs at the concentrations shown and alterations in Ca^{2+} were determined using flow cytometry, as described in Fig. 1. Cell populations were identified using phycoerythrin-conjugated antibodies to CD3 (T cells), CD19 (B cells), or CD14 (monocytes). Values are mean ± S.E.M. for the total number of donor tested. *Indicates the number of donors that showed a MCFR significantly ($p < 0.05$) higher than DMSO control. NR = No response (*i.e.*, no donors showed a MCFR significantly higher than DMSO control).

immunotoxic PAHs compared to the non-immunotoxic PAHs, with the great majority of donors responding to BaP and DMBA.

In order to determine if the same Ca^{2+} response could be reproduced in donors that were re-analyzed after several months, we performed the same flow cytometric analysis of Ca^{2+} mobilization in human peripheral B and T lymphocytes and monocytes in donors who had been previously studied. Again, HPBMC were treated for 42 hours with PAHs and TCDD, and the effects on intracellular Ca^{2+} were compared to DMSO controls (Figure 7). Figure 7a is a graph of the first experiment, and Fig 7b represents the same experiment performed approximately 6 months later. In both experiments, DMBA and BaP produced a significant ($p < 0.05$) elevation in intracellular Ca^{2+} in the T and B cell populations. The reproducibility of these results suggests that this donor may be consistently sensitive to these two PAHs. Interestingly, TCDD produced a small, but statistically significant Ca^{2+} response in the monocytes in the first experiment but not in the second. Additionally, the magnitude of the change in MCFR in the DMBA and BaP samples was considerably higher in the second experiment. We believe that these results reflect the day to day individual changes that occur in the human population which can be contributing factors to the variability and complexity often encountered in human studies.

Figure 7. Reproducibility of PAH-induced Ca^{2+} elevation obtained in the same donor after a 6 month interval. This is a representative experiment of 3 separate experiments performed in different individual donors. DMBA, BaP, BeP, and ANTH (all 10 μM) and 0.1 μM TCDD were added to HPBMC cultures for 42 hours and alterations in Ca^{2+} were analyzed as described as previous described. Cell populations were identified using phycoerythrin-conjugated antibodies as previously described. Figure 7a represents the first experiment and 7b is the graph of the same experiment performed in the same donor and manner approximately 6 months later. Values are Mean ± S.E.M. for a single donor. *Indicates significant ($p < 0.05$) increase in Ca^{2+} compared to DMSO control. Actual MCFR of samples having a change in MCFR ≤ 0 is as follows: Figure 7a - **T cells**: DMSO = 367 ± 4.0, TCDD = 351 ± 14.5, BeP = 339 ± 9.5, ANTH = 362 ± 12.0; **B cells**: DMSO = 348 ± 5.5, TCDD = 324 ± 3.4, BeP = 335 ± 7.8, ANTH = 333 ± 4.9; **Monocytes**: DMSO = 321 ± 8.5, DMBA = 314 ± 5.1, BeP = 311 ± 5.2, ANTH = 313 ± 4.4. Figure 7b - **T cells**: DMSO = 327 ± 5.0; **B cells**: DMSO = 348 ± 9.5, TCDD = 344 ± 6.9, ANTH = 340 ± 4.6; **Monocytes**: DMSO = 363 ± 9.5, BaP = 345 ± 5.3, TCDD = 329 ± 13.5, BeP = 362 ± 1.5, ANTH = 330 ± 3.8.

DISCUSSION

The mobilization of intracellular Ca^{2+} plays an essential role in signaling pathways involved in lymphocyte activation, facilitating the transduction of signals from both the T and B cell antigen receptors to the nucleus (Weiss & Imboden, 1987; Cambier *et al.*, 1994; Choquet *et al.*, 1994). For this reason, it is likely that agents that alter Ca^{2+} mobilization in immune cells may also disrupt intracellular signaling pathways.

Previous studies conducted in our laboratory have demonstrated that PAHs may exert their immunomodulatory effects by interfering with intracellular Ca^{2+} homeostasis. We have shown that *in vivo* and *in vitro* exposure to DMBA results in an elevation of baseline Ca^{2+} in resting cells, and inhibits Ca^{2+} mobilization after PHA stimulation in the Jurkat human T cell line and murine T cells (Burchiel *et al.*., 1990, 1991). Using the HPB-ALL human T cell line, both highly immunosuppressive PAHs, including DMBA and BaP, and moderately immunosuppressive PAHs, including dibenzo(a,h)anthracene (DAH) and 9,10-dimethylanthracene (DMA), have been found to produce a sustained Ca^{2+} elevation for at least 4 hours, while PAHs that are minimally immunosuppressive, including dibenzo(a,c)anthracene (DAC), benz(a)anthracene (BA), BeP, and ANTH, produced only a small and transient (3 minutes) rise in intracellular Ca^{2+} (Krieger *et al.*., 1995). It appears that the methylation of anthracene at the 9,10-(DMA) or the 7,12-(DMBA) positions increased the duration of the Ca^{2+} elevation suggesting that different PAHs may affect different constituents of signal transduction pathways in a structure-dependent manner (Davila *et al.*., 1995). In the B lymphocyte system, a disruption of baseline Ca^{2+} has been observed in resting murine splenic B cells and the murine B cell lymphoma A20.1

cell line after treatment with DMBA (Davis & Burchiel, 1992; Burchiel *et al.*, 1993). Various PAHs were found to increase tyrosine phosphorylation in A20.1 (Davila *et al.*, 1995), and DMBA was also found to induce apoptosis in the murine B cell line (Burchiel *et al.*, 1993).

In this report, we demonstrate that PAHs produce structure-dependent alterations in Ca^{2+} mobilization in human peripheral blood T and B lymphocytes and monocytes identified by monoclonal antibodies and flow cytometry in a standard HPBMC preparation. The effects of the highly immunotoxic PAHs, DMBA and BaP, and TCDD were compared to anthracene and BeP, which are two PAHs that are relatively non-immunosuppressive in mice (White *et al.*, 1985). In determining the differential sensitivity of human monocytes, B cells, and T cells to intracellular Ca^{2+} changes produced by PAHs, we found that a 20 hour exposure to BaP (10 μM) produced a variable degree of intracellular Ca^{2+} elevations in B cells and T cells, perhaps due to differences in the metabolic activity of HPBMC obtained from different donors. The most significant responses to PAHs appeared to occur following 42 hours or longer exposures, possibly due to the increased formation of PAH metabolites. In the pooled data obtained from 10 donors, both DMBA and BaP produced a significant increase in intracellular Ca^{2+} in T cells, B cells, and monocytes treated for 42 hours, while TCDD produced a significant Ca^{2+} response only in B cells. BeP and ANTH did not cause a significant elevation in any of the cells populations. These results demonstrate that immunotoxic PAHs, and perhaps TCDD, produce both a structure and time-dependent increase in intracellular Ca^{2+} in HPBMC, but there may be subtle differences in the responses of subsets of lymphoid cells.

To determine if the alterations of intracellular Ca^{2+} produced by certain PAHs in donors could be reproduced and to get some idea of the variability between experiments in Ca^{2+} responses, repeat experiments were performed in several donors. In one representative experiment ($n = 3$ using different individual donors) using a donor studied at a 6 month time interval, DMBA and BaP (10 μM) produced a significant elevation in Ca^{2+} in the T and B cell populations treated for 42 hours. TCDD (0.1 μM), however, produced a significant ($p < 0.05$) Ca^{2+} response in the monocyte population in the first experiment but not second, performed approximately 6 months later.

The effects of TCDD, an environmentally persistent contaminant formed as a by-product in the manufacture of herbicides (Esser, 1994), on B cell Ca^{2+} levels seen in the 10 donor pooled data are potentially interesting, based upon the hypothesis presented by Holsapple that this agent may disrupt Ca^{2+} homeostasis in B cells (Karras & Holsapple, 1994; Holsapple *et al.*, 1996). Current evidence suggests that PAHs and perhaps TCDD produce immunotoxicity by both Ah receptor (AhR)-dependent and independent mechanisms (Luster *et al.*, 1987; Holsapple *et al.*, 1991). We observed a small, but statistically significant difference in the response of human B cells to TCDD in the present studies. It is unclear at the present time whether these differences are due to AhR-dependent or independent factors. However, it is notable that we have recently observed a marked difference in the expression of AhR by HPBMC T cells and B cells with T cells lacking significant expression of mRNA for AhR detected using RT-PCR (Mounho *et al.*, 1996). Future studies will evaluate the potential role of AhR in PAH-induced signaling in human lymphocytes. These results demonstrate the variability in PAH responses to HPBMC that can be expected from normal donors.

As a general rule, PAHs that are carcinogenic are typically immunosuppressive, while non-carcinogenic PAHs are generally non-immunosuppressive (White & Holsapple, 1984; Ward *et al.*., 1985). This information suggests that both PAH-induced immunotoxicity and carcinogenicity may involve common mechanism(s). Evaluating PAHs that are structurally similar, but vary in levels of immunotoxicity, may provide insight in identifying specific structural moieties responsible for the toxicity of a particular compound or class of compounds.

In summary, these studies have demonstrated that known immunotoxic PAHs/HAHs, such as DMBA and BaP, produce alterations in Ca^{2+} mobilization in human peripheral T cells, B cells, and monocytes. These results provide further evidence that intracellular Ca^{2+} elevation produced by PAHs/HAHs may be an important mechanism associated with PAH/HAH-induced immunosuppression.

CHAPTER II

The Potential Role of Cytochrome P450 Metabolism in Polycyclic Aromatic Hydrocarbon-Induced Alterations in Intracellular Calcium Homeostasis in Human B Lymphocytes

BACKGROUND AND SIGNIFICANCE

It has been known for at least 30 years that PAHs, such as DMBA, BaP, and 3-MC are immunosuppressive to both rodent and human lymphocytes (Mudzinski, 1993; White *et al.*, 1994; Davila *et al.*, 1996). However, the biochemical mechanism(s) by which PAHs produce immunosuppression has not been definitively established. Using structure-activity relationships, it has been demonstrated that there is a correlation between PAH immunotoxicity and carcinogenicity. White and Holsapple (1984), for example, have shown in mice that two noncarcinogenic PAHs (BeP and anthracene) are not immunosuppressive, whereas carcinogenic PAHs suppress the *in vivo* IgM antibody-forming colony response (AFC) to sheep red blood cells (SRBC). This relationship has also been observed using *in vitro* splenocyte cultures (Dean *et al.*, 1983; White & Holsapple, 1984; Ward *et al.*, 1985; Dean *et al.*, 1986).

While the relationship between immunotoxicity and carcinogenicity of PAHs has been noted by previous investigators, it is unclear whether there are common biochemical mechanisms which may be responsible for both effects. Therefore, a question that could be posed is whether the mechanism of cancer induction is related to the mechanism of

immunosuppression. This is a relevant question to address since the mechanism by which PAHs produce cancer has been more extensively studied that the mechanism by which PAHs produce immunosuppression.

For cancer induction, it is well known that PAHs must be metabolized by cytochrome P450 to reactive metabolites which are capable of interacting with DNA (Miller, 1970; Thakker *et al.*, 1985). The metabolism of BaP occurs primarily in the bay region (Macleod *et al.*, 1982) and the ultimate carcinogen of BaP is believed to be BaP-7,8-diol-9,10-epoxide (Dipple, 1994). In contrast, little or no bay region metabolism has been shown to occur in BeP. The lack of bay region metabolism of BeP, therefore, may explain the noncarcinogenic properties of BeP compared to its carcinogenic congener, BaP (Macleod *et al.*, 1982).

In order to determine if the suppressive actions of PAHs on lymphoid cells is mediated by the parent compound or by the reactive metabolites of PAHs, *in vitro* studies using splenocyte cultures have been performed. While both DMBA and BaP exposure resulted in the suppression of the AFC response to SRBC, the BaP-7,8-diol and DMBA-3,4-diol metabolites were found to be more immunosuppressive that the parent compounds (Kawabata & White, 1987; Ladics *et al.*, 1991). Therefore, these studies provide some initial evidence that PAH metabolites may be responsible for the immunotoxicity of certain PAHs.

An alternative approach to examine the influence of PAH metabolism on immunotoxicity is to utilize a P450 inhibitor that blocks the formation of these metabolites. One such agent, α-naphthoflavone (ANF), has previously been shown to inhibit P450 1A and 1B1 activity (Goujon *et al.*, 1972; Santostefano *et al.*, 1992;

Masubuchi *et al.*, 1994; Bowes *et al.*, 1996). Kawabata and White (1987) have shown that ANF inhibits the immunotoxicity of BaP in mice. Our laboratory, and others, have also demonstrated in HPBMC *in vitro* that T cell mitogenesis is suppressed by BaP and DMBA, and that this suppression is reversed by ANF (Mudzinski, 1993; Davila *et al.*, 1996). These results also support the concept that reactive metabolites of DMBA and BaP play a role in the immunosuppressive effects of these agents. However, while these results suggest that P450 metabolism may be necessary for the immunotoxicity of certain PAHs, the interpretation of these findings is complicated by the fact that ANF is also a partial antagonist of the cytosolic aromatic hydrocarbon receptor (AhR) (Merchant *et al.*, 1993; Wilhelmsson *et al.*, 1994).

Our laboratory has previously shown a strong correlation between the ability of PAHs to alter Ca^{2+} homeostasis and to suppress mitogen and antibody responses in murine and human lymphocytes (Burchiel *et al.*, 1988; Krieger *et al.*, 1994; Davila *et al.*, 1996). While most of our previous work has focused on the effects of PAHs on human T cells, it is clear from Chapter 1 that PAHs also exert important effects on intracellular Ca^{2+} homeostasis in human B cells and monocytes. These Ca^{2+} modulations, however, have not been well-characterized. The effects of PAHs on B cells may be especially critical since humoral immunity, a B cell-dependent response, is known to be the most sensitive immune response inhibited by PAHs (Davila *et al.*, 1995).

In the present chapter, studies were pursued to define the relationship between intracellular $Ca^{2=}$ elevation produced by BaP and two important P450-derived metabolites, BaP-7,8-diol and BaP-7,8-diol-9,10-epoxide. In addition, the effects of ANF on BaP and BaP metabolite-induced Ca^{2+} elevation in HPBMC lymphocytes and monocytes, and

Daudi human B cells were also evaluated. The goal of these studies was to provide initial evidence that immunosuppression produced by BaP metabolites, and its reversal by ANF, can be correlated with effects on intracellular Ca^{2+} homeostasis in human lymphocytes, and more specifically in human B cells

MATERIALS AND METHODS

Reagents. Benzo(a)pyrene (BaP), benzo(e)pyrene (BeP), and α-naphthoflavone (ANF) (purchased from Sigma Chemical Co., St. Louis, MO), and the BaP metabolites, benzo(a)pyrene-r-7,*t*-8-dihydrodiol-*t*-9,10-epoxide(+/-) (*anti*) (BaP-7,8-diol-9,10-epoxide) and benzo(a)pyrene-trans-7,8-dihydrodiol(+/-) (BaP-7,8-diol) (purchased from the NCI Repository, Midwestern Research Institute, Kansas City, MO) were greater than 95% purity as assessed by the manufacturer. The molecular formula of BaP-7,8-diol is $C_{20}H_{14}O_2$ (m.w. = 286.3) and for BaP-7,8-diol9,10-epoxide is $C_{20}H_{14}O_3$ (m.w. = 302.3). All PAHs and ANF were dissolved in fresh, sterile, tissue culture grade (> 99% purity) anhydrous dimethylsulfoxide (DMSO; Sigma), which also served as the solvent control. All compounds were prepared and used under protective yellow light and visually inspected to affirm that the compounds were completely dissolved. After BaP metabolites were dissolved in DMSO, the compounds were aliquoted and stored under nitrogen gas in glass vials at -20° C until use. The final concentration of DMSO in all cell cultures was ≤ 0.1%. Cultures containing DMSO did not differ from controls without DMSO, and it was concluded that at this concentration, DMSO did not produce any measurable effect on intracellular Ca^{2+} responses.

Preparation of Human Peripheral Mononuclear Cells (HPBMC). Human peripheral blood was obtained from the Clinical Research Center (CRC) at the University of New Mexico Hospital in Albuquerque, NM. Volunteer donors were clinically normal prescreened adults and the isolation of normal human peripheral blood mononuclear cells

is previously described in Chapter 1. Viability, which ranged from 95 - 99%, was assessed by trypan blue dye (Sigma) exclusion.

Establishing an in vitro Model of Human B Cell Activation. Since a high degree of variability was previously observed in Chapter 1 using HPBMC, a human B cell line was chosen as a model in these studies in parallel with the HPBMC assays. In order to define an appropriate human B cell line suitable for the Ca^{2+} mobilization and ANF studies, several human B cell lines were evaluated, including NCI-BL2126, and the Burkitt's lymphoma lines, Ramos, Raji, and Daudi cell lines (all purchased from ATCC, Rockville, MD). All cell lines were cultured according to ATCC protocol. In separate and individual experiments, cells from each cell line were treated with either DMBA, BaP, or BeP for 18 hours, and alterations in free intracellular Ca^{2+} were measured as described below. The Ramos cells were unable to retain Fura Red within the cytoplasm, resulting in a highly fluorescent but calcium-insensitive data that was inconsistent and variable. The NCI-BL2126 and Raji cell lines are characteristic of growing in cell clumps, which made flow cytometric analysis difficult since the flow cytometer reads individual cells one at a time, rather than populations. The cell clumps would often block the flow cytometer lines, in which the experiment would be terminated until the lines were flushed clean. Additionally, the NCI-BL2126 cell line is an EBV transformed lung carcinoma line , and therefore, considered an extremely hazardous cell line. The Daudi B lymphoblastoid cell line grows as single cells in suspension and retains Fluo-3 and Fura Red Ca^{2+} indicators and, therefore, are ideal cells for flow cytometric analysis. The Daudi cell line is a mature B cell line that is surface Igm+ and derived from a patient with Burkitt's lymphoma (Klein *et al.*, 1968; Kuwahara *et al.*, 1993). This cell line behaves as normal B cells with regard to B

cell receptor-mediated signaling events, including phosphatidylinositol turnover, mobilization of intracellular Ca^{2+}, and tyrosine phosphorylation of proteins (Carter *et al.*, 1991; Yamanashi *et al.*, 1992; Brent, *et al.*, 1993). Our laboratory has also recently demonstrated that Daudi cells express both cytochrome P450 1A1 and 1A2 (unpublished data), which are two P450 isozymes involved in the metabolism of PAHs.

Cell Culturing Conditions of Daudi cells. Daudi B lymphoblastoid cells were maintained at 37° C (5% CO_2) in RPMI-1640 (Sigma) supplemented with 20% fetal bovine serum (Sigma), 2 mM L-glutamine, and 1% penicillin-streptomycin (stock = 10 U/100 ml pen, 10 mg/100ml strep; Sigma).

Treatment of Cells. In all intracellular Ca^{2+} studies using Daudi cells, 10^6/ml cultured cells were incubated in the presence of different amounts of PAHs (1.0 - 10.0 μM), ANF (0.3 - 3.0 μM), or DMSO, the vehicle control. The incubation periods and treatment conditions varied in the different assays and they are given in the Results and Figure Legends. For ANF studies using the Daudi cell line, cells were pretreated with ANF for 4 hours and then treated with PAHs. Based on previous studies (Kawabata & White, 1987; Ladics *et al.*, 1991; Bowes *et al.*, 1996) and studies performed in our laboratory examining the effects of ANF pretreatment (0.5 - 4 hours) in Daudi, optimal reduction in the Ca^{2+} elevation produced by PAHs occurred at the 4 hour pretreatment. Therefore, the 4 hour pretreatment time was used in all subsequent ANF studies in the Daudi cell line. For the ANF studies using normal human HPBMC, BaP (10 μM) was added to isolated HPBMC (plated a 1 x 10^6 cells/ml) alone or in combination with 10 μM ANF.

Measurement of Calcium using Flow Cytometry. Alterations in free intracellular Ca^{2+} produced by PAHs were measured using Fluo-3 and Fura-Red acetoxymethyl esters

(Molecular Probes, Eugene, OR), and a Coulter ELITE flow cytometer (Hialeah, FL) as previously described in Chapter 1. Treated cells were harvested, centrifuged, and resuspended in complete medium containing 13 μM Fura-Red and 6 μM Fluo-3 in a volume of 100 μl and incubated at 37° C for 1.5 hours, with cells being resuspended ≈ every 20 minutes to ensure even dye loading. To compare the effects of BaP in gated-lymphocytes and monocytes, the specific cell populations were identified based on forward and side scatter flow cytometric parameters. The forward scatter (1.5°-19°)° measures the cell size while the side scatter (90°) measures the granularity of the cells. Monocytes are larger and more granular than lymphocytes and, therefore, can be easily targeted using these flow cytometric parameters. To compare the effects of PAHs on intracellular Ca^{2+} in human peripheral B lymphocytes, PAH-treated HPBMCs were incubated with the Ca^{2+} indicators, followed by an incubation for an additional 30 minutes with a phycoerythrin (PE)-conjugated murine monoclonal antibody at 1 μg/1 x 10^6 cells to CD19 (a surface marker for B cells) as previously described in Chapter 1. Results are shown as the change in the Mean Channel Fluorescence Ratio (MCFR), which is obtained by subtracting the MCFR of DMSO vehicle controls from the MCFR of PAH-treated samples. The MCFR, therefore, represents the elevations in intracellular Ca^{2+} produced by the PAHs. As described in Chapter 1, thapsigargin (1 μM) (Sigma) was used in all experiments to ensure cells, chelators, and flow cytometry detection systems were functioning correctly.

Statistical Design and Analysis. SigmaStat statistical software (Jandel Scientific, San Rafael, CA) was utilized to analyze data for statistical differences between DMSO control and treated samples as previously described in Chapter 1. Statistically significant samples were defined as $p < 0.05$.

RESULTS

Elevation of intracellular Ca^{2+} by BaP in human lymphocytes and monocytes and its prevention by α-naphthoflavone (ANF). To determine the effects of BaP on intracellular Ca^{2+} levels in HPBMC, cells were treated for 18 or 36 hours with 10 μM BaP and were then analyzed for changes in intracellular Ca^{2+} using flow cytometric analysis in light-scatter gated lymphocytes (T and B cells) and monocytes. While in the past our laboratory has focused on the effects of PAHs at early time points, in these studies we were interested in evaluating the effects of these compounds at longer exposures, 18 and 36 hours. As shown in Figure 8, BaP produced a statistically significant ($p < 0.05$) increase in intracellular Ca^{2+} in HPBMC lymphocytes at 36 hours and a trend toward an increase at 18 hours. The increase in intracellular Ca^{2+} produced by BaP was significantly ($p < 0.05$) blocked by coexposure of HPBMC to BaP with an equimolar (10 μM) concentration of ANF. While the effects of ANF are very dose dependent (Wilhelmsson *et al.*, 1994), in these HPBMC coincubation studies, optimal reduction of PAH-induced Ca^{2+} elevation by ANF occurred at 10 μM. Figure 9 shows BaP-induced elevations in intracellular Ca^{2+} in HPBMC (obtained from the same donor) monocytes identified using flow cytometry. At 18 and 36 hours, BaP produced a statistically significant ($p < 0.05$) elevation in intracellular Ca^{2+} in HPBMC monocytes, and this increase in Ca^{2+} produced by BaP was significantly ($p < 0.05$) blocked by ANF. Since ANF is a known P450 inhibitor and was found to block the BaP-induced Ca^{2+} increase seen in HPBMC lymphocytes and monocytes, these results provide initial evidence that BaP-induced Ca^{2+} elevation in HPBMC may somehow involve cytochrome P450. Values for Figures 8 and 9

Figure 8. BaP increases intracellular Ca^{2+} in human peripheral blood lymphocytes identified using flow cytometry. HPBMC were treated with 10 μM BaP for 18 or 36 hours in the presence or absence of 10 μM α-naphthoflavone (ANF). Results are shown as the change in Ca^{2+} Mean Channel Fluorescence (Fluo-3/Fura Red) Ratios (MCFR) obtained by subtracting the MCFR for DMSO control from the MCFR of PAH-treated samples. BaP was found to produce a statistically significant increase in Ca^{2+} at 36 hours ($p < 0.05$), and ANF was found to reverse this Ca^{2+} effect of BaP. *Indicates significant ($p < 0.05$) increase in intracellular Ca^{2+} compared to DMSO solvent control. §Indicates BaP + ANF produced a significant ($p < 0.05$) reduction in intracellular Ca^{2+} compared to BaP alone. Actual MCFR of DMSO (18 hrs) = 316 ± 7.6, and at 36 hrs DMSO = 250 ± 5.2.

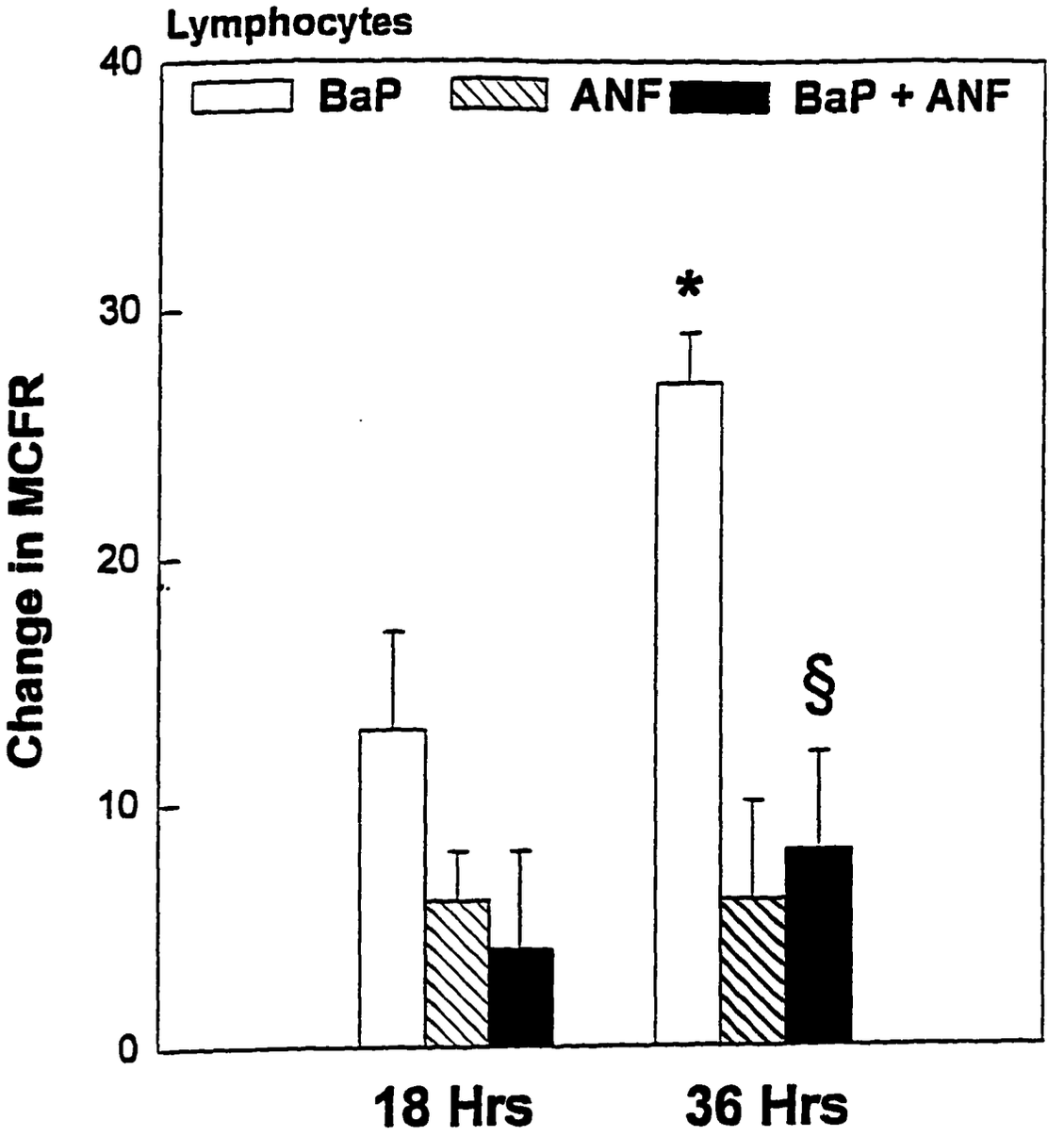
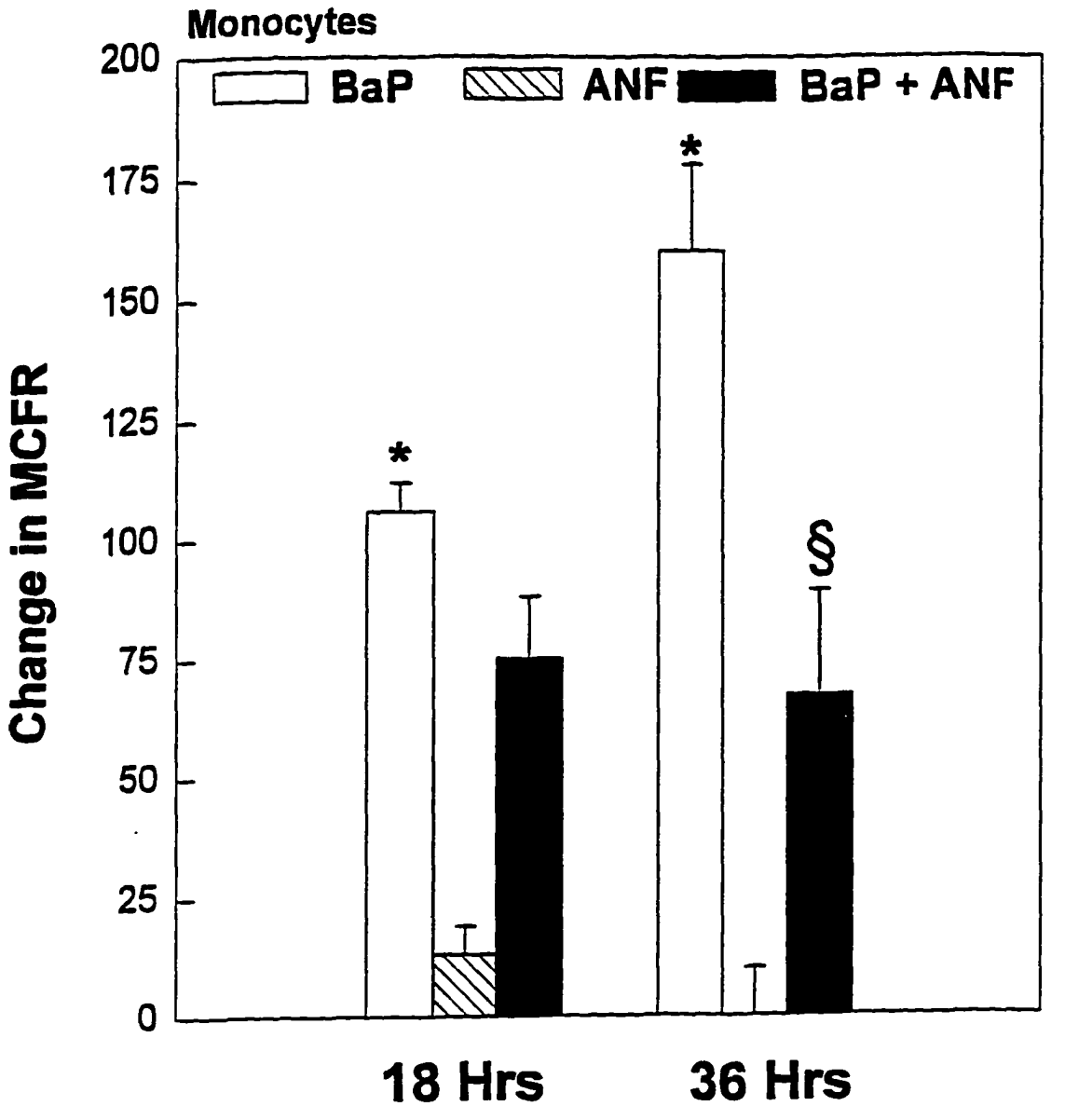


Figure 9. BaP-induced elevation in intracellular Ca^{2+} in human peripheral blood monocytes identified using flow cytometry. HPBMC were treated with 10 μM BaP for 18 or 36 hours in the presence or absence of equimolar concentrations of α-naphthoflavone ANF. Results are shown as the change in Ca^{2+} MCFR as previously described. BaP produced a statistically significant increase in Ca^{2+} at both 18 and 36 hours ($p < 0.05$), and ANF reversed the Ca^{2+} elevation produced by effect BaP. *Indicates significant ($p < 0.05$) increase in intracellular Ca^{2+} compared to DMSO solvent control. §Indicates BaP + ANF produced a significant ($p < 0.05$) reduction in intracellular Ca^{2+} compared to BaP alone. Actual MCFR of samples haviing a change in MCFR ≤ 0 is as follows: 18 hrs: DMSO = 368 ± 13.8; 36 hrs: DMSO = 327 ± 12.3, ANF = 325 ± 9.7.



are the means for quadruplicate determinations ± S.D. for a single donor (note: these data are representative of experiments repeated 3 times using different donors).

Intracellular Ca^{2+} Elevation by BaP Metabolites in Daudi Human B Cells. Daudi cells were treated with either BaP, BaP metabolites, or BeP for 1, 4, or 18 hours. Alterations in free intracellular Ca^{2+} were measured by multiparameter flow cytometric analysis using the Ca^{2+}-chelating dyes, Fluo-3 and Fura-Red. In previous studies determining a dose-response relationship, it was found that a 10 μM concentration of BaP-7,8-diol and especially BaP-7,8-diol-9,10-epoxide killed most of the cells, while at a 1 μM concentration, the Ca^{2+} response often did not occur. A 3 μM concentration, however, produced a consistent and optimal Ca^{2+} response, and for this reason, was the concentration used in these studies. Both BaP-7,8-diol and BaP-7,8-diol-9,10-epoxide at 3 μM concentrations produce a significant ($p < 0.05$) elevation in intracellular Ca^{2+} compared to DMSO vehicle control at 1 (Figure 10), 4 (Figure 11), and 18 (Figure 12) hours. The various time points were chosen to observe if there was a time-dependent effect of the BaP metabolites on intracellular Ca^{2+} in the Daudi cell line. At 10 μM concentrations, both the parent compound, BaP, and BeP, a nonimmunotoxic PAH, did not produce a significant rise in Ca^{2+} at any of the time points. Results are shown as the change in the MCFR as previously described and values shown are means for quadruplicate determinations ± S.D.

Reduction of BaP metabolite-induced intracellular Ca^{2+} elevation by α-naphthoflavone (ANF) in Daudi. To further investigate the potential role of P450 in PAH-induced Ca^{2+} elevations and immunosuppression, the effects of ANF on BaP metabolite-induced Ca^{2+} elevation were examined. Daudi cells were pretreated for 4 hours

Figure 10. Intracellular Ca^{2+} Elevation by BaP Metabolites in Daudi Human B Cells treated for 1 hour. Daudi cells were treated with either BaP or BeP at 10 μM concentrations, or BaP-7,8-diol, BaP-7,8-diol-9,10-epoxide, at 3 μM concentrations. Free intracellular Ca^{2+} was measured by multiparameter flow cytometric analysis using the Ca^{2+}-chelating dyes Fluo-3 and Fura Red. Results of alterations in intracellular Ca^{2+} concentrations are shown as the change in the MCFR and values are means for quadruplicate determinations $\pm$ S.D. *Indicates significant ($p < 0.05$) increase in free intracellular Ca^{2+} compared to DMSO vehicle control. Actual MCFR of samples having a change in MCFR ≤ 0 is as follows: DMSO = 228 ± 2.7, BaP = 227 ± 1.3, BeP = 225 ± 1.5.

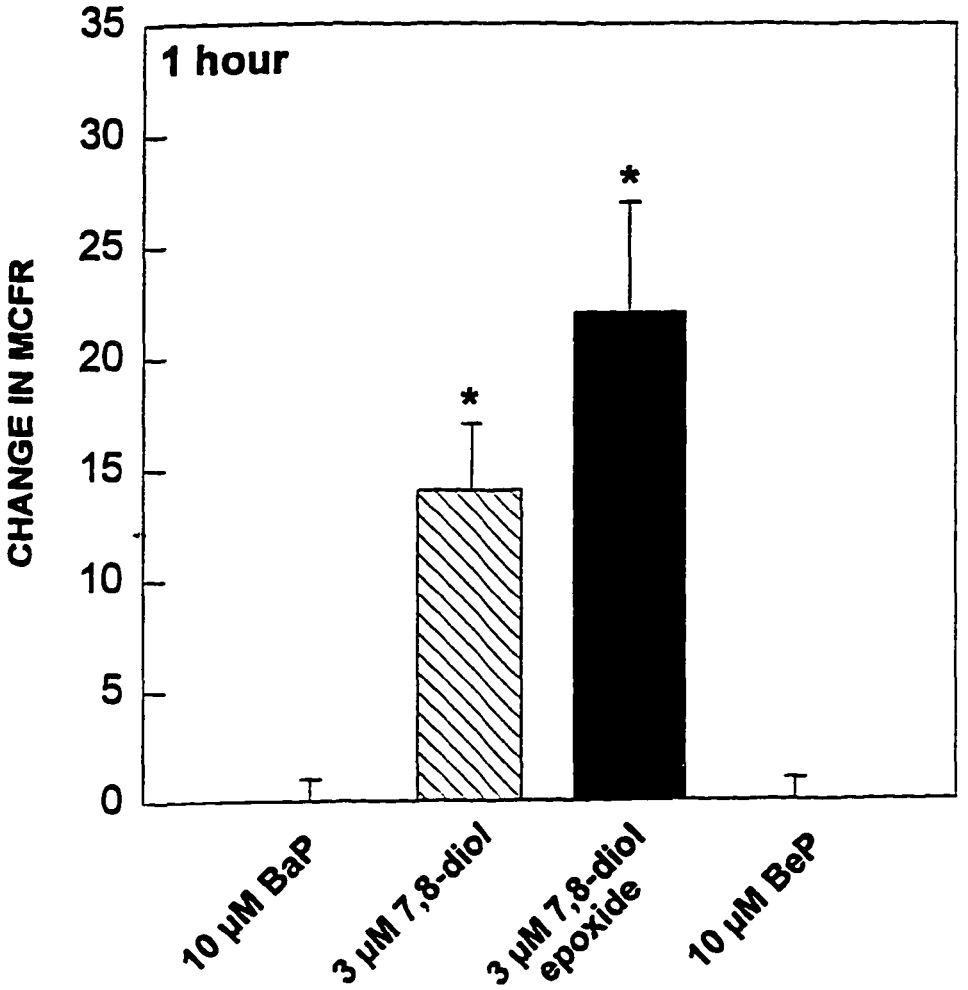


Figure 11. Intracellular Ca^{2+} Elevation produced by BaP Metabolites in Daudi Human B Cells treated for 4 hours. Daudi cells were treated with either 10 μM BaP or BeP, or BaP-7,8-diol and BaP-7,8-diol-9,10-epoxide at 3 μM concentrations. Free intracellular Ca^{2+} was measured by multiparameter flow cytometric analysis and results of alterations in intracellular Ca^{2+} concentrations are shown as the change in MCFR as previously described. Values are means for quadruplicate determinations ± S.D. and *indicates significant ($p < 0.05$) increase in free intracellular Ca^{2+} compared to DMSO vehicle control. Actual MCFR of DMSO = 232 ± 5.9 and BeP = 228 ± 4.3.

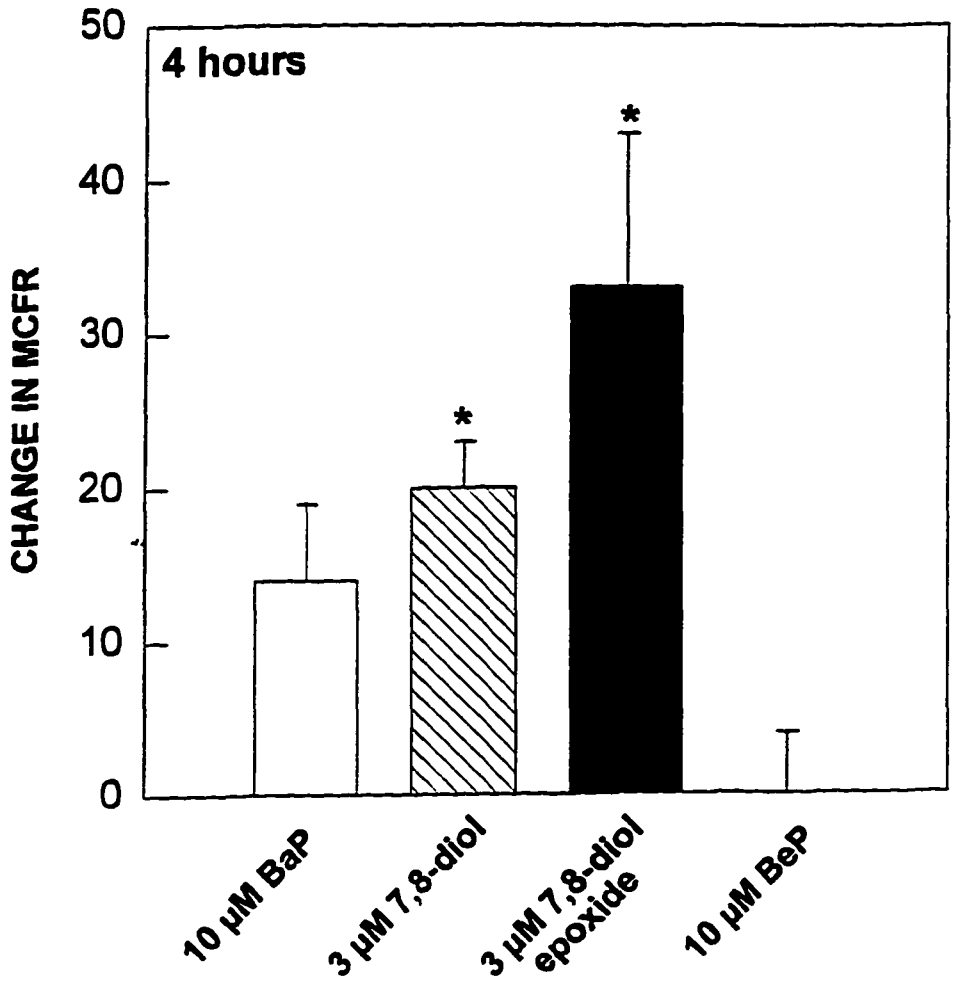
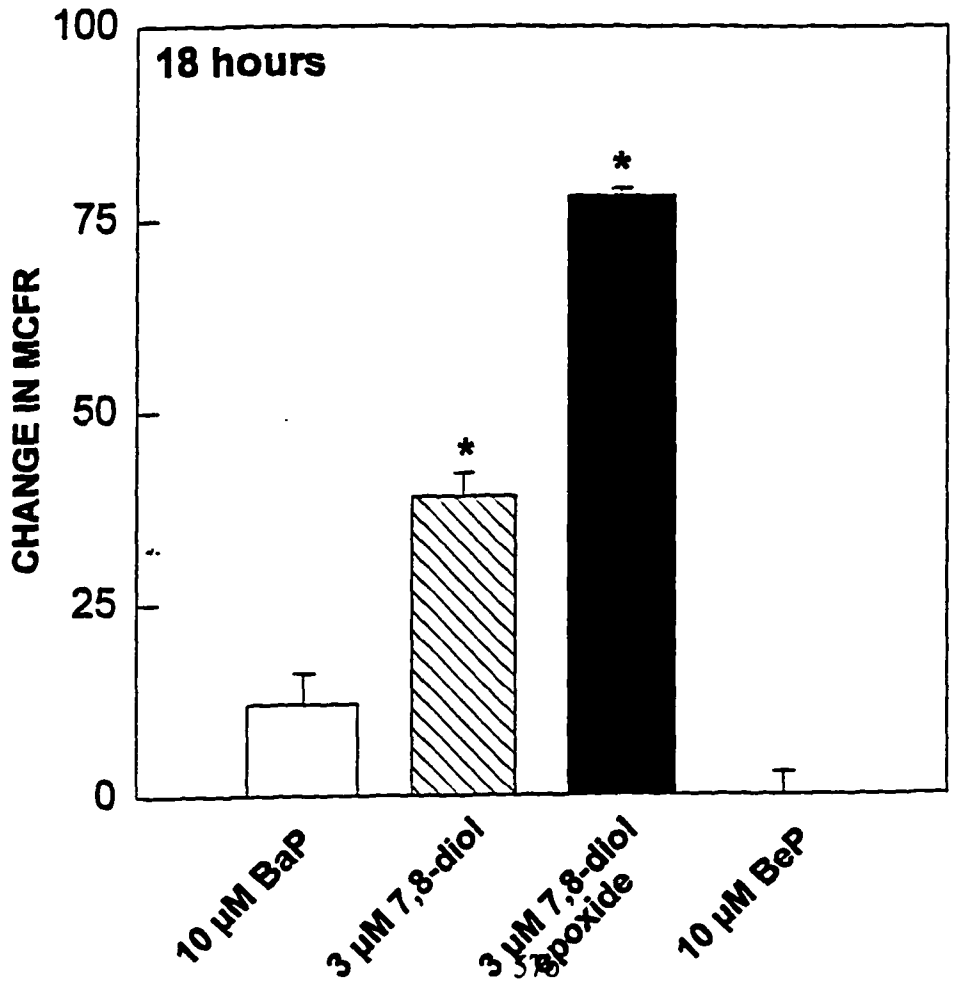


Figure 12. Elevations in intracellular Ca^{2+} by BaP Metabolites in Daudi Human B Cells treated for 18 hours. Daudi cells were treated with either 10 μM BaP or BeP, or BaP-7,8-diol and BaP-7,8-diol-9,10-epoxide at 3 μM concentrations. Of the three time points evaluated (1,4, and 18), the maximal Ca^{2+} response occurs at the 18 hour time point. Free intracellular Ca^{2+} was measured by multiparameter flow cytometric analysis and results of alterations in intracellular Ca^{2+} concentrations are shown as the change in MCFR as previously described. Values are means for quadruplicate determinations $\pm$ S.D. and *indicates significant ($p < 0.05$) increase in free intracellular Ca^{2+} compared to DMSO vehicle control. Actual MCFR of DMSO = 254 $\pm$2.0 and BeP = 253 $\pm$ 3.4.



with ANF, and were then treated for 18 hours with the BaP metabolites, BaP-7,8-diol and BaP-7,8-diol-9,10-epoxide. In separate experiments, pretreatment with ANF for 4 hours was found to give optimal inhibition of the Ca^{2+} elevation produced by PAHs in Daudi cells. Figure 13 demonstrates the dose-response trend of various concentrations of ANF (0.3 μM - 3 μM) that produce the largest reduction in the intracellular Ca^{2+} elevation produced BaP-7,8-diol (3 μM). Alone, ANF does not produce a significant response at any of the concentrations used. P450 inhibition by ANF pretreatment significantly ($p < 0.05$) reduced the rise in Ca^{2+} produced by BaP-7,8-diol, with the greatest reduction occurring at the 0.3 μM concentration. For this reason, this concentration of ANF used in all subsequent experiments. While pretreatment with 0.3 μM ANF completely blocks the BaP-7,8-diol-induced Ca^{2+} elevation, ANF had no effect on the Ca^{2+} response produced by BaP-7,8-diol-9,10-epoxide (Figure 14; a separate representative experiment of 5 assays). The fact that ANF had no effect on BaP-7,8-diol-9,10-epoxide-induced Ca^{2+} elevation was expected since it is the final P450-derived BaP metabolite in this pathway and does not require further P450 metabolism to be active (Figure 15). These results provide some preliminary evidence that of the two BaP metabolites examined, it is BaP-7,8-diol-9,10-epoxide that predominantly and directly alters intracellular Ca^{2+} in B cells. BaP never produced a statistically significant rise in intracellular Ca^{2+} at 1, 4, or 18 hours in the Daudi cell line, and for this reason, the effects of ANF on BaP-induced Ca^{2+} elevation in Daudi cells was not examined. Results in Figure 14 are shown as the change in the MCFR and values shown are means for quadruplicate determinations ± S.D.

BaP Metabolites elevate intracellular Ca^{2+} in normal HPBMC B lymphocytes. In observing that both the BaP metabolites, BaP-7,8-diol and BaP-7,8-diol-9,10-epoxide,

Figure 13. Dose-response of the reduction of BaP metabolite-induced intracellular Ca^{2+} mobilization by α-naphthoflavone (ANF) in Daudi. Daudi cells were pretreated for 4 hours with either 0.30, 1, or 3 μM ANF, and then treated for 18 hours with BaP-7,8-diol at 3 μM. Free intracellular Ca^{2+} was measured using flow cytometry and results are shown as the change in the MCFR. Values are means for quadruplicate determinations ± S.D. *Indicates significant ($p < 0.05$) increase in free intracellular Ca^{2+} compared to DMSO vehicle control. §Indicates a significant ($p < 0.05$) reduction in intracellular Ca^{2+} by ANF pretreatment compared to PAH-treatment alone. Actual MCFR of DMSO = 265± 12.4 and .3 μM ANF = 259 ± 6.1.

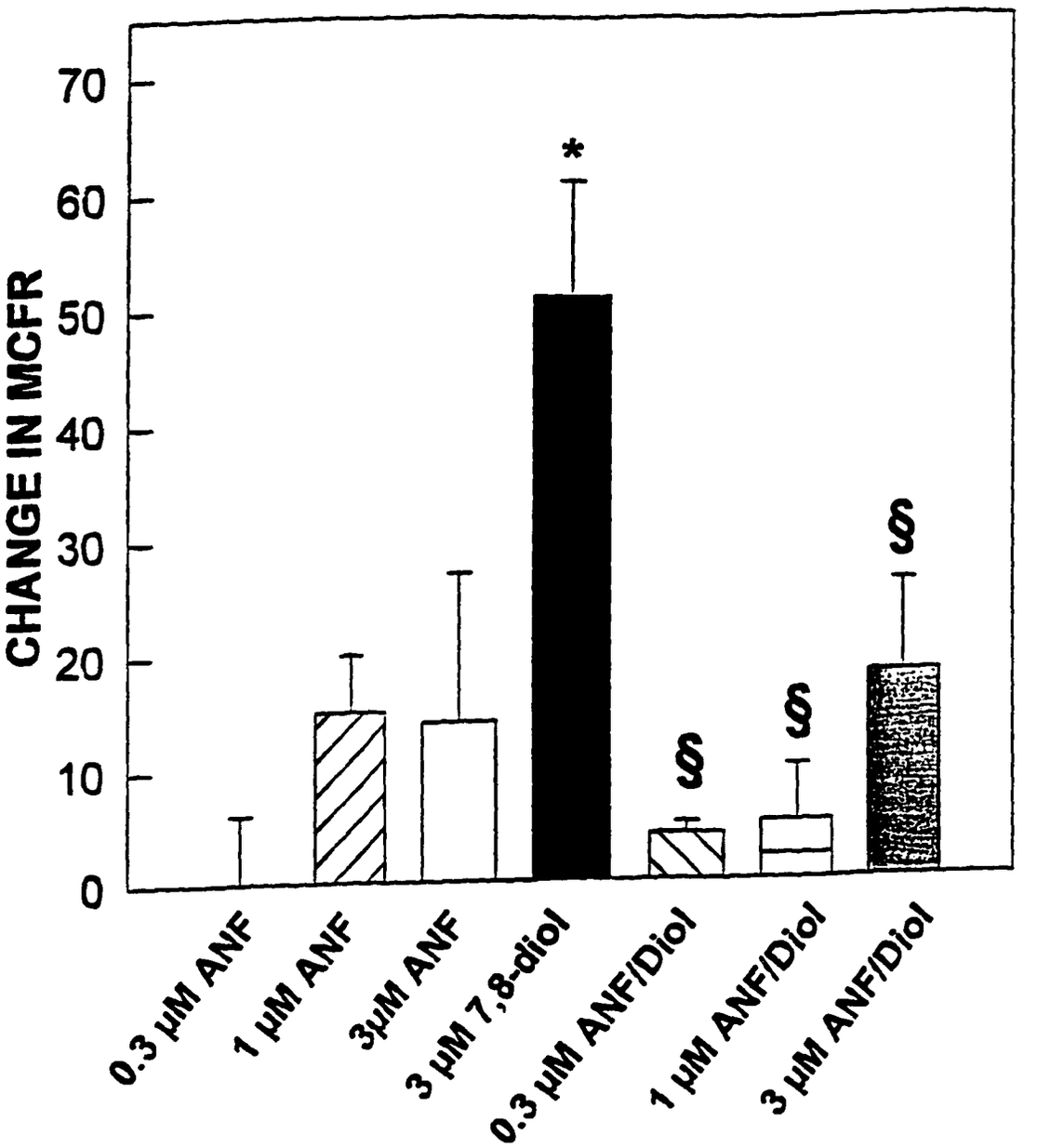


Figure 14. Inhibition of BaP-7,8-diol- but not BaP-7,8-diol-9,10-epoxide-induced Ca^{2+} by ANF. Daudi cells were pretreated for 4 hours with 0.30 μM ANF, followed by treatment with BaP-7,8-diol and BaP-7,8-diol-9,10-epoxide at 3.0 μM for 18 hours. Free intracellular Ca^{2+} was measured by multiparameter flow cytometric analysis using the Ca^{2+}-chelating dyes Fluo-3 and Fura Red. Results of alterations in intracellular Ca^{2+} concentrations are shown as the change in the MCFR and values are means for quadruplicate determinations ± S.D. *Indicates significant ($p < 0.05$) increase in free intracellular Ca^{2+} compared to DMSO vehicle control. §Indicates a significant ($p < 0.05$) reduction in intracellular Ca^{2+} by ANF pretreatment compared to PAH-treatment alone. Actual MCFR for samples having a change in MCFR ≤ 0 is as follows: DMSO = 235 ± 1.8, ANF = 226 ± 5.0, 7,8-diol/ANF = 230 ± 4.0.

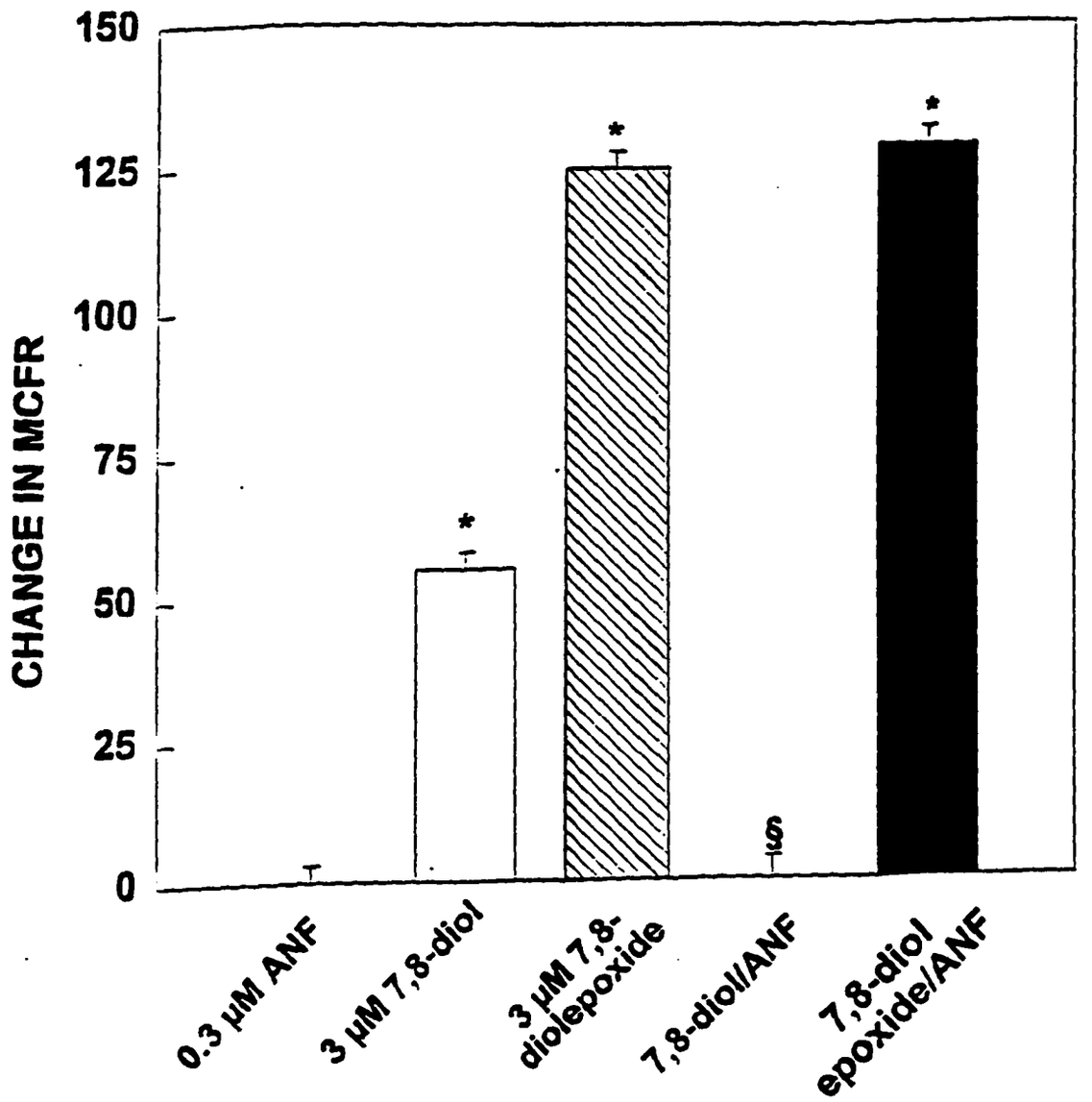


Figure 15. The metabolism of benzo(a)pyrene (BaP) by cytochrome P450. BaP, the parent compound, is metabolized by P450 to the BaP-7,8-oxide, which is then converted to the BaP-7,8-diol by epoxide hydrolase. The BaP-7,8-diol is then metabolized by P450 to the BaP-7,8-diol-9,10-epoxide, which is the last P450-derived metabolite in this pathway. Adapted from: White *et al.*, (1994). *Immunotoxicology and Immunopharmacology, 2nd Edition*, Figure 1, p. 127.

significantly elevated free intracellular Ca^{2+} in Daudi human B cells, we were interested in determining if these metabolites would produce the same Ca^{2+} response in normal human peripheral B lymphocytes. Alterations in intracellular Ca^{2+} were measured using flow cytometric analysis as previously described and $CD19^+$ positive B cells were identified using a PE-conjugated monoclonal antibody to CD19 cell surface antigens. A dose-response relationship of the two BaP metabolites is shown in Figure 16. Results are shown as the change in the MCFR as previously described, and values shown are means for quadruplicate determinations $\pm$ S.D. for a single donor (representative of $n = 3$). In this donor at 18 hours, neither BaP or BeP (10 μM) produced a significant rise in intracellular Ca^{2+}. Both BaP-7,8-diol and BaP-7,8-diol-9,10-epoxide significantly ($p < 0.05$) elevated intracellular Ca^{2+} levels at 1 μM compared to DMSO controls, and the magnitude of the Ca^{2+} elevation was even greater at 3 μM. The 3 μM concentration, therefore, was used for the remaining HPBMC studies. Figure 17 shows the Ca^{2+} elevation in HPBMC $CD19^+$ B lymphocytes in a different donor. As in the previous donor, BaP and BeP at 10 μM did not produce a significant Ca^{2+} rise, whereas both BaP-7,8-diol and BaP-7,8-diol-9,10-epoxide (3 μM) significantly ($p < 0.05$) elevated intracellular Ca^{2+} compared to DMSO controls. These data suggest that certain BaP metabolites, particularly BaP-7,8-diol-9,10-epoxide, do have a direct effect on intracellular Ca^{2+} homeostasis in normal human B lymphocytes.

Figure 16. Intracellular calcium elevation in normal human peripheral blood B cells ($CD19^+$) treated for 18 hours. Mononuclear cell cultures were treated with either 10 μM BaP or BeP, or BaP-7,8-diol and BaP-7,8-diol-9,10-epoxide at 1.0 and 3.0 μM concentrations. Alterations in intracellular Ca^{2+} were measured using flow cytometric analysis as previously described and $CD19^+$ positive B cells were identified using a PE-conjugated monoclonal antibody to CD19 cell surface antigens. Results are shown as the change in the MCFR and values are means for quadruplicate determinations ± S.D. for a single donor. *Indicates significant ($p < 0.05$) increase in free intracellular Ca^{2+} compared to DMSO solvent control. Actual MCFR of DMSO = 223 ± 2.5.

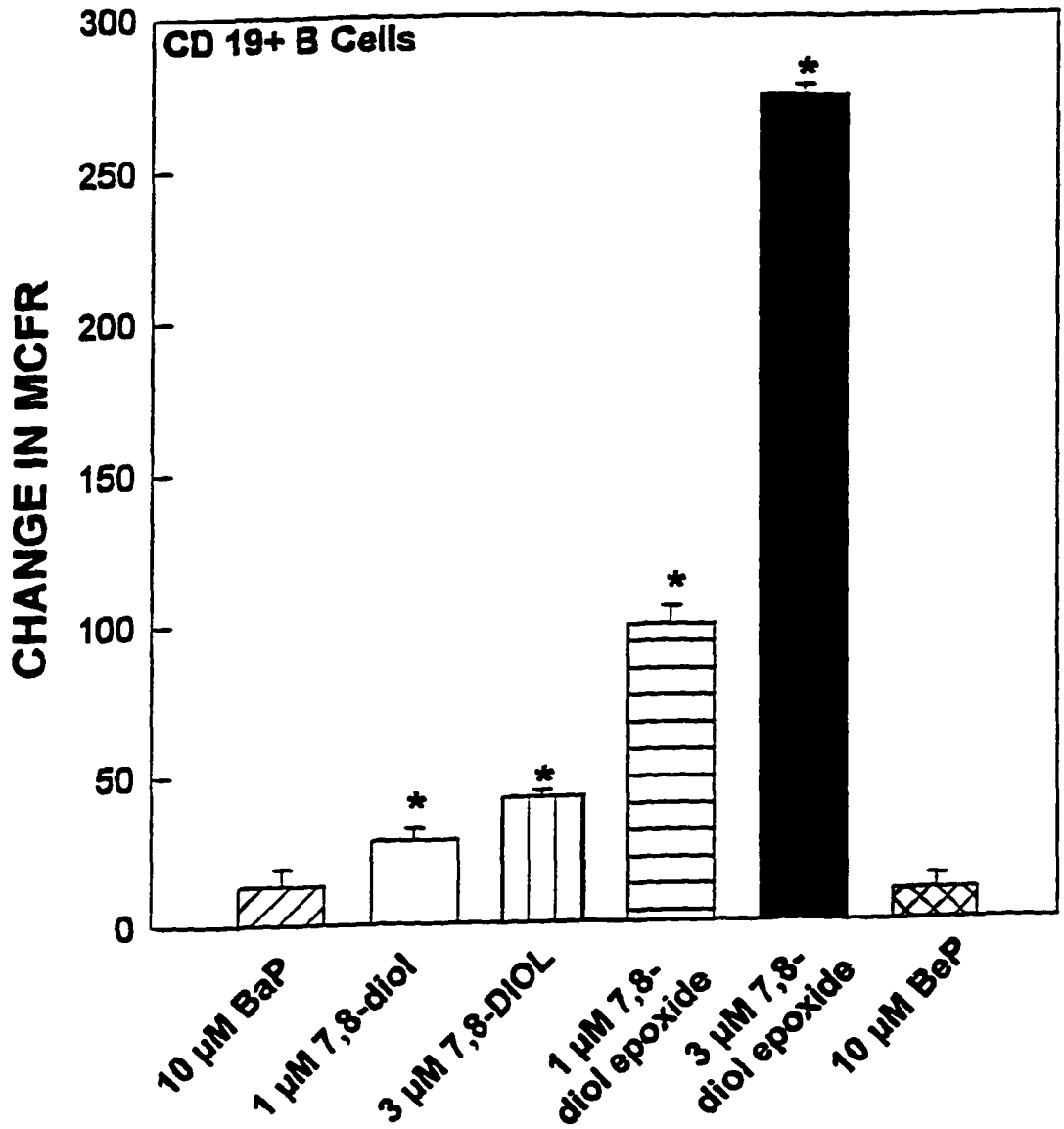
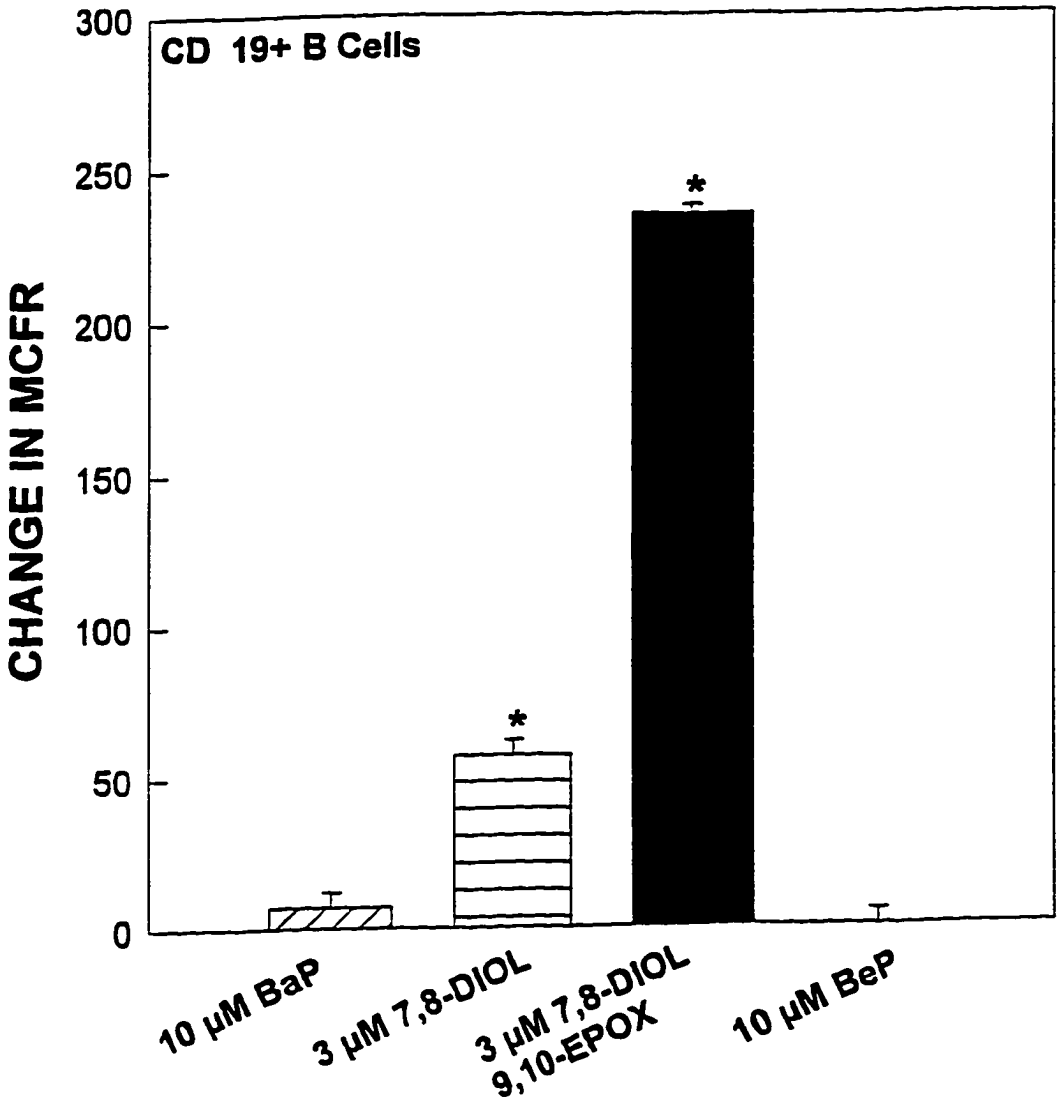


Figure 17. Intracellular calcium elevation in normal human peripheral blood B cells ($CD19^+$) treated for 18 hours. Mononuclear cell cultures were treated with either 10 μM BaP or BeP, or BaP-7,8-diol and BaP-7,8-diol-9,10-epoxide at 3.0 μM concentrations. Alterations in intracellular Ca^{2+} were measured using flow cytometry and the Ca^{2+}-chelating dyes, Fluo-3 and Fura Red and $CD19^+$ positive B cells were identified using a PE-conjugated monoclonal antibody to CD19 cell surface antigens. Results are shown as the change in the MCFR and values are means for quadruplicate determinations ± S.D. for one donor. *Indicates significant ($p < 0.05$) increase in free intracellular Ca^{2+} compared to DMSO solvent control. Actual MCFR of DMSO = 199 ± 6.0.



DISCUSSION

Several cytochrome P450 enzymes are involved in PAH metabolism and bioactivation and it is well recognized that the carcinogenic actions of BaP and other PAHs are mediated by P450-derived metabolites (Ladics *et al.*, 1992a, 1992b; White *et al.*, 1994; Bauer *et al.*, 1995; Shimada *et al.*, 1996; Kress & Greenlee, 1997). In studies examining the role of various P450s catalyzing the activation of different procarcinogenic chemicals, P450 1A1, 1A2, 3A4 and 1B1 were all shown to exhibit activity in the metabolism of certain PAHs (Bauer *et al.*, 1995; Shimada *et al.*, 1996). It has been shown that BaP induces cytochrome P450 levels (Ladics *et al.*, 1992b; Sterling *et al.*, 1994) and the carcinogenic and mutagenic actions of BaP and many other PAHs are known to be mediated by their reactive metabolites (Gelboin *et al.*, 1980; Osborne & Crosby, 1986).

While the characterization of cytochrome P450 isozymes involved in PAH activation has been primarily documented in rat hepatic microsomes, relatively little information is available regarding the bioactivation of PAHs in human tissues (Shimada *et al.*, 1989; Borlakoglu *et al.*, 1993). Jerina *et al.* (1984), have shown in rat hepatic microsomes that BaP is metabolized by P450 1A1 to BaP-7,8-oxide which is then hydrated to BaP-7,8-diol by epoxide hydrolase. Further metabolism by P450 1A1 results in the formation of BaP-7,8-diol-9,10-epoxide. Recent studies, however, have demonstrated that other P450 isozymes are also involved in the metabolism of PAHs, including P450 1A1, 1A2, 1B1, and 3A4 (McManus *et al.*, 1990; Bauer *et al.*, 1995; Shimada *et al.*, 1996). While P450 1A1 is primarily expressed in extrahepatic tissues and 1A2 is principally found in the liver, both of these P450 isozymes predominantly participate in the bioactivation of PAHs (Tuteja *et al.*, 1985; Shimada *et al.*, 1992, 1994). P450 1A1 is known to be expressed

and active in HPBMC following treatment with T cell mitogens and it has been demonstrated that human lymphocytes are capable of converting pre-carcinogenic hydrocarbons to their reactive forms (Whitlock *et al.*, 1972; Gupta *et al.*, 1988). Cytochrome P450 3A4, a major P450 isozyme found in human liver, also appears to be involved in the metabolism of PAHs (McManus *et al.*, 1990; Bauer *et al.*, 1994). Cytochrome P450 1B1 is a relatively new member of the "1" family, and has been characterized in rodent and human tissues and is constitutively expressed in several organs (Savas *et al.*, 1994; Sutter *et al.*, 1994). P450 1B1 has been shown to be inducible by several environmental carcinogens, including PAHs and TCDD, and just as P450 1A1 and 1A2, is inhibited by ANF (Brake & Jefcoate, 1995; Shimada *et al.*, 1996; Bowes *et al.*, 1996).

Since previous studies have shown that certain BaP metabolites elevate intracellular Ca^{2+} as well as deplete intracellular glutathione in HPBMC, and inhibition of P450 activity reduces the immunotoxicity of BaP (Davila *et al.*, 1996), we performed a series of experiments directly examining the effects of two known immunosuppressive BaP metabolites on intracellular Ca^{2+} in Daudi human B cells and HPBMC B lymphocytes. Both of the metabolites examined, BaP-7,8-diol and BaP-7,8-diol-9,10-epoxide, were much more effective than the parent compound, BaP, in increasing intracellular Ca^{2+} in Daudi B cells and in $CD19^+$ human B lymphocytes obtained from normal human peripheral blood.

BaP-7,8-diol-9,10-epoxide is a bay region diol epoxide and is thought to be the ultimate carcinogenic metabolite, forming nucleic acid adducts predominantly by reaction with N^2 of guanine in DNA at the benzylic 10 position (Miller, 1970; Dipple, 1994;

Peltonen & Dipple, 1995). An important characteristic of bay region diol epoxides is their resistance to hydrolyation by epoxide hydrolase, which is due to the steric hindrance from the adjacent dihyrodiol group (Parkinson, 1996). Additionally, the reactive metabolites of BaP are not only capable of DNA adduct formation, but may also covalently bind cysteine sulfhydryls or amine groups of cellular macromolecules such as proteins or peptides (Pruess-Schwartz *et al.*, 1986; Pitot & Dragan, 1996; Romero *et al.*, 1997).

Alpha-naphthoflavone (ANF), is known to inhibit cytochrome P450 isozyme activity, P450 mRNA transcription, and the metabolism of BaP (Merchant *et al.*, 1990; Hardin *et al.*, 1992). Additionally, ANF has been shown to antagonize the ability of TCDD to induce P450 1A1, immunosuppression, and down-regulation of the estrogen receptor (Goujon *et al.*, 1972; Gurtoo *et al.*, 1979; Blank *et al.*, 1987). Proposed mechanisms by which ANF mediates these effects include 1) competitive inhibition of the AhR, thereby blocking AhR-substrate translocation to the nucleus, and 2) acting as a metabolic antagonist to cytochrome P450 activity (Blank *et al.*, 1987; Gasiewiez & Rucci, 1991; Ladics *et al.*, 1991).

Kawabata and White (1987) have shown that ANF blocks the suppression of the *in vitro* humoral immune response produced by BaP and BaP metabolites in murine splenocytes. Our laboratory has recently shown that ANF reverses the suppression of T cell mitogenesis produced by PAHs and certain BaP metabolites (Davila *et al.*, 1996) and we have also observed that P450 inhibition by ANF blocks the elevation in intracellular Ca^{2+} produced by BaP in nylon wool-purified human peripheral blood T cells (unpublished data). In the present studies, we found in light scatter-gated HPBMC lymphocytes and monocytes that the elevation in intracellular Ca^{2+} produced by BaP was significantly

inhibited by ANF. Additionally, in Daudi cells, P450 inhibition by ANF pretreatment completely blocked the elevation of intracellular Ca^{2+} produced by BaP-7,8-diol, but had no effect on the Ca^{2+} rise produced by BaP-7,8-diol-9,10-epoxide, which is the last P450-derived metabolite in this pathway. By demonstrating that the Ca^{2+} elevation produced by BaP-7,8-diol is completely blocked by ANF, while having no effect on BaP-7,8-diol-9,10-epoxide, these data provide some preliminary evidence that cytochrome P450 may be associated with PAH-induced Ca^{2+} elevations in human B cells.

Based on the present studies, there are four independent observations which suggest that BaP metabolites are important in Ca^{2+} elevation, and suggest that HPBMC and Daudi cells are capable of metabolizing BaP to the reactive diol epoxide, which appears to be predominantly responsible for elevations in intracellular Ca^{2+}: 1) ANF inhibits BaP and BaP-7,8-diol-induced increases in intracellular Ca^{2+} in HPBMC (lymphocytes and monocytes) and Daudi, but not the Ca^{2+} elevations produced by BaP-7,8-diol-9,10-epoxide; 2) BaP requires relatively long exposure times (4 - 36 hours) to produce Ca^{2+} elevations in Daudi and HPBMC; 3) BaP metabolites are more potent in producing an increase in intracellular Ca^{2+} than the parent compound; 4) BaP-7,8-diol-9,10-epoxide acts more rapidly in increasing intracellular Ca^{2+} than BaP in HPBMC B lymphocytes and Daudi cells. These results further suggest that BaP-7,8-diol-9,10-epoxide is the ultimate immunotoxicant for Ca^{2+} elevation and provides a strong correlation with the known carcinogenicity of this metabolite. Thus, these results suggest that a common P450-derived metabolic product of BaP, such as BaP-7,8-diol-9,10-epoxide, may provide a biochemical association between immunotoxicity and carcinogenicity of this PAH.

At this time, the relative P450 activity in human lymphocytes and monocytes has not been definitively established. While it is known that human lymphocytes have P4501A activity (Whitlock *et al.*, 1972), it is unclear whether lymphocytes have adequate P450 activity to form sufficient concentrations of PAH metabolites to produce immunotoxicity. In addition, it has not been fully defined if PAH metabolism occurs predominantly via macrophages present in spleen cell preparations or other mixed cell cultures, such as HPBMC. For these reasons, our laboratory and others, have evaluated the effects of the parent compound and the reactive metabolites of PAHs on several potential mechanisms which may be associated with immunotoxicity (Kawabata & White, 1987; Ladics *et al.*, 1991; Davila *et al.*, 1996). Researchers have shown that in mixed cultures, macrophages are the primary class of immune cells which metabolize PAHs (Okano *et al.*, 1979; Ladics *et al.*, 1992b). Therefore, characterizing the relative role of immune cells involved in P450 metabolism, as well as the kinetics of P450 induction in various cells, will also need to be explored in future studies.

Studies have demonstrated that several immunotoxic PAHs, including BaP, bind to the AhR (Okey *et al.* 1984; Pliszczynska *et al.*, 1986; Nebert, 1989). PAHs bind to the AhR prior to metabolic activation by P450 and, therefore, some investigators postulate that PAH-induced immunotoxicity is associated with AhR-binding and subsequent activation of the Ah gene complex (Bast *et al.*, 1974; Silkworth *et al.*, 1984; Wojandi & Alfred, 1984; Thurmond *et al.*, 1988; Waithe *et al.*, 1991; Mudzinski, 1993; Nebert, 1994). Currently, we do not know whether ANF exerts its effects on AhR in Daudi cells and, therefore, the role of AhR in Ca^{2+} signaling can not be excluded. At a minimum, the AhR may be involved in P450 induction in B lymphocytes and other cells. Investigators have

also suggested, however, a role for AhR in signaling effects produced by PAHs (Matsumura *et al.*, 1993). It is probable that both AhR-binding and cytochrome P450 play a role in the immunosuppressive effects of PAHs and, therefore, polymorphisms in AhR expression and P450 activity may be important factors in determining human health risks associated with PAH exposure. For these reasons, future work in our laboratory will continue to investigate the potential association of AhR and cytochrome P450 with PAH-induced Ca^{2+} elevation and immunotoxicity in human lymphocytes.

Finally, we have not established the mechanism(s) by which PAHs and/or metabolites cause alterations in intracellular Ca^{2+} in human B cells. Our laboratory has recently shown that certain BaP metabolites deplete intracellular glutathione in HPBMC, suggesting that sulfhydryl attack or covalent binding may be associated with the immunotoxicity of PAHs (Romero *et al.*, 1997). Based upon previous studies, two potential mechanisms for PAH-induced Ca^{2+} elevation are likely: 1) activation of Src-related protein tyrosine kinases (Archuleta *et al.*, 1993) or 2) inhibition of sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPases (SERCA) (Krieger *et al.*, 1995). The first of these two possibilities was evaluated in Chapter 3 of this dissertation.

CHAPTER III

The Potential Association Between PAH-Induced Disruptions in Ca^{2+} Homeostasis and Increased Protein Tyrosine Kinase Activity in Human B Lymphocytes

BACKGROUND AND SIGNIFICANCE

There has been an increasing awareness over the past few years that PAHs exert their immunosuppressive effects by disrupting intracellular Ca^{2+} homeostasis in lymphocytes and other immune cells (Davila *et al.*, 1995; Holsapple *et al.*, 1996). In Chapters 1 and 2, it was demonstrated that certain PAHs, such as BaP, and the BaP metabolites, BaP-7,8-diol and BaP-7,8-diol-9,10-epoxide, elevated intracellular Ca^{2+} concentrations in both HPBMC $CD19^+$ B lymphocytes and Daudi B cells. PAH-induced alterations in Ca^{2+} homeostasis in B lymphocytes interrupts antigen receptor signaling that employs free intracellular Ca^{2+} as a second messenger. Acting as an important second messenger, Ca^{2+} plays a significant role in the regulation of signal transduction pathways (Carafoli, 1987; Cole & Kohn, 1994).

At this time, the actual molecular mechanisms by which certain PAHs and their reactive metabolites alter Ca^{2+} homeostasis and intracellular signaling in B cells have not been fully defined. One potential mechanism involved in PAH-mediated immunotoxicity is an increase in Src-family protein tyrosine kinase (PTK) activity. Previous studies performed in our laboratory have shown that certain PAHs increase both tyrosine kinase activation and tyrosine phosphorylation of phospholipase C-γ1 (PLCγ1) in HPB-ALL

(Archuleta *et al.*, 1993). These data suggest that PAHs may alter PTK activity which ultimately results in the elevation of intracellular Ca^{2+}. In the past, research on PTKs has focused on understanding their regulation and identifying their substrates. Only in the past few years have their roles in B cell signal transduction cascades been examined. The purpose of the studies presented in this Chapter, therefore, was to evaluate the potential role of PTKs, including Lyn (a Src-family kinase) and Syk, in PAH-induced intracellular Ca^{2+} alterations in human B lymphocytes.

The class of immune cells that is primarily responsible for the humoral immune response is B lymphocytes. When activated, B cells differentiate into plasma cells that eliminate extracellular pathogens by way of specific antibodies. Antibodies are immunoglobulins which have a structure of two low molecular weight (light) polypeptide chains and two high molecular weight (heavy) polypeptide chains linked by disulfide bonds. Immunoglobulins are divided into five classes designated IgM, IgG, IgA, IgD, and IgE. B lymphocytes have membrane-bound immunoglobulins (mIgs) on their surface that recognize and act as receptors for antigens. Although all immunoglobulin isotypes can act as surface receptors on mature peripheral B cells, mIgM and mIgD are predominately expressed (Reth *et al.*, 1991). The B cell antigen receptor (BCR) is a multimeric complex of membrane immunoglobulin which is noncovalently associated with transmembrane disulfide-linked heterodimers designated Ig-α and Ig-β (Reth, 1992). The Ig-α heterodimer has a long cytoplasmic tail containing 61 amino acid residues and tail of Ig-β contains 48 amino acids (Kuby, 1997). The cytoplasmic tails of both the Ig-α and Ig-β proteins contain a conserved sequence motif known as an "immunoreceptor tyrosine based activation motif" or ITAM (Sefton and Taddie, 1994; Johnson *et al.*, 1995). The ITAM

sequence consists of 18 amino acid residues and is defined as two tyrosine residues spaced 9 to 11 residues apart with isoleucine or leucine resides positioned three residues carboxy-terminal to each tyrosine (Chan & Shaw, 1995). The molecular weight of Ig-α is approximately 34 kDA and for Ig-β is 42 kDa. Ig-α and Ig-β have been shown to associate with Syk/ZAP-70 and Src-family kinases and appear to play a role in intracellular signaling following stimulation of the BCR (DeFranco, 1995). Upon antigen crosslinking of the BCR, the ITAMs are rapidly tyrosine phosphorylated by Src-family PTKs which subsequently results in the activation of Src- and Syk-family kinases (Cambell & Sefton, 1992; Clark *et al.*., 1992; DeFranco, 1992; Cambier, 1995; Johnson *et al.*, 1995).

Antigen crosslinking of mIgM or mIgD on mature B cells results in cell proliferation and activation, while antigen binding by mIgM on immature B cells results in cell death or inactivation (Nossal, 1983; DeFranco, 1987 Nemazee & Burki, 1989). In both mature and immature B cells, stimulation of resting B cells via the crosslinking of mIg results in the phosphorylation and activation of two intracellular signaling pathways: 1) protein tyrosine phosphorylation and 2) phosphoinositide hydrolysis resulting in the stimulation of protein kinase C (PKC) and an elevation of intracellular Ca^{2+} (Coggeshall & Cambier, 1984; Gold *et al.*, 1990; Yamanashi *et al.*, 1992; Harwood & Cambier, 1993). Several non-receptor PTKs mediate this phosphorylation, including the Src-family and the Syk/ZAP-70 family of PTKs (van Oers & Weiss, 1995; Law *et al.*, 1996; Pao & Cambier, 1997) and several studies utilizing PTK inhibitors have demonstrated that downstream signaling events in B cells are dependent on tyrosine phosphorylation of effector proteins (Padeh *et al.*, 1991; Pure' & Tardelli, 1992). Both mIgM and mIgD have short cytoplasmic tails (only three amino acids) and it is, therefore, highly unlikely

that these surface receptors directly stimulate intracellular signaling pathways (DeFranco, 1995). It is more conceivable that B cell antigen receptors associate with other proteins to transduce their intracellular signal to the nucleus.

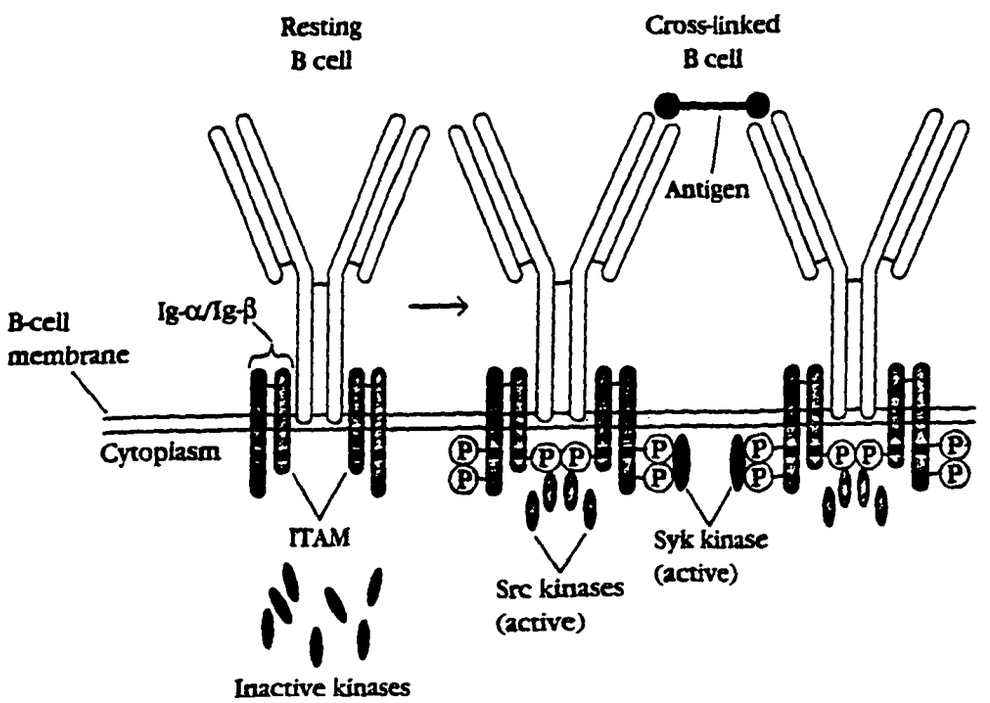
Crosslinking of the BCR rapidly activates receptor-associated kinases, resulting in an increase in tyrosine phosphorylation of numerous cytoplasmic effector proteins (DeFranco, 1992; Kurosaki *et al.*, 1994). After BCR ligation, the activation of these receptor-associated kinases is maximal between 15 and 60 seconds, and the resulting tyrosine phosphorylation of proteins is likely to be one of the earliest events in the mIg-mediated signaling cascade (Burkhardt *et al.*, 1991; Pleiman *et al.*, 1994). It appears that several receptor-associated tyrosine kinases mediate this phosphorylation of effector enzymes, including the Syk/ZAP-70 family and the Src-family kinases $p53/56^{lyn}$ (Lyn), $p55^{blk}$ (Blk), $p59^{fyn}$ (Fyn), and $p56^{lck}$ (Lck) (DeFranco, 1992; Hutchcroft *et al.*, 1992; Yamanashi *et al.*, 1992; Pleiman *et al.*, 1994). ZAP-70, a 70 kd PTK, is expressed exclusively in T cells and natural killer (NK) cells, while Syk (72 kd) is preferentially expressed in B cells, myeloid cells, and thymocytes (Taniguchi *et al.*, 1993; DeFranco, 1995). Both Syk/ZAP-70 and Src-family kinases are characterized by the motif HRDLAARN, which is exclusively associated with PTKs in addition to important regulatory sites or Src-homology domains which precede a highly conserved catalytic domain (Taniguchi *et al.*, 1991; Law *et al.*, 1994).

Spleen tyrosine kinase or Syk was initially purified from bovine thymus and is a 72 kDa non-receptor protein tyrosine kinase abundantly expressed in B lymphocytes (Taniguchi *et al.*, 1991). The structure homology of Syk is very similar (approximately 73% amino acid sequence homology) to ZAP-70 (zeta-associated protein), which is found primarily in T

cells. Syk has two Src-homology 2 (SH2) domains (110 amino acids) which are important regulatory sites that mediate protein-phosphoprotein interactions and serve as binding sites for other intracellular signaling proteins (Koch *et al.*, 1991). The SH2 domain recognizes a sequence motif consisting of a region of amino acids containing a phosphorylated tyrosine residue which, in conjunction with the surrounding residues, determines the specificity of these interactions (Pawson & Schlessinger, 1993; Zhou *et al.*, 1993).

Unlike Src-family kinases, Syk has no SH3 regulatory domain, no negative regulatory COOH-terminal tyrosine phosphorylation site, and no sites of N-myristylation. Therefore, Syk is probably not constitutively localized in the plasma membrane (Weiss & Littman, 1994). The conserved catalytic site of Syk consists of a glycine-rich motif and a downstream lysine residue, and the glycine residues form a loop and anchor the phosphates of ATP while lysine binds the nucleotide (Knighton *et al.*, 1991). Although the phosphorylation sites in Syk have not been definitively identified, it appears that Syk contains four tyrosine phosphorylation sites with the autophosphorylation motif of Syk comprising of the amino acid sequence YKAQ representing residues 526-529 (Watts *et al.*, 1994). Syk coimmunoprecipitates with components of the BCR, suggesting that Syk is associated with mIg complexes (Hutchcroft *et al.*, 1992; Cooper & Howell, 1993). Upon mIg receptor crosslinking and ITAM phosphorylation, Syk is rapidly tyrosine phosphorylated by a Src-family kinase which leads to the spatial reorientation, recruitment, and clustering of Syk into the receptor complex (Pleiman *et al.*, 1994; Weiss & Littman, 1994; van Oers & Weiss, 1995). This localization of Syk (Figure 18) into the receptor complex is achieved by way of Syk SH2 domains binding to doubly tyrosine

Figure 18. The structure of the B cell antigen receptor (BCR). The BCR is a complex of membrane immunoglobulin associated with two transmembrane heterodimers, Ig-α and Ig-β. Immediately upon BCR crosslinking, the immunoreceptor tyrosine based activation motifs (ITAMs) are tyrosine phosphorylated by Src-family kinases, which serve as docking sites for the SH2 domains of Syk and Src kinases. From: Kuby, J. (1997). *Immunology 3rd Edition*, Figure 8-7, p. 203, with permission.



phosphorylated ITAM sequences on Ig-α and/or Ig-β, resulting in the upregulation of Syk activity (Chan & Shaw, 1995; Rowley *et al.*, 1995; Zoller *et al.*; 1997).

Of the Src-family kinases, Lyn and Blk are preferentially expressed in B cells and other hematopoietic cells, while Fyn and Lck appear to be predominantly expressed in T cells (Burkhardt *et al.*, 1991; Yamanashi *et al.*, 1992). The Src-family kinases are associated with the cytoplasmic region of the plasma membrane and appear to act as intracellular signal transducers for surface receptors. While some investigators believe that Lyn and Blk are physically associated with mIg, others propose that these kinases are actually associated with components of the B cell antigen receptor, such as Ig-α or Ig-β (Burkhardt *et al.*, 1991; Campbell & Sefton, 1992). Src-family kinases, such as Lyn, possess one SH2 and one SH3 domain, an inhibitory tyrosine phosphorylation site in the C-terminal tail (Tyr-527), and a N-terminal myristylation site (Cooper & Howell, 1993; Sefton & Taddie, 1994). The SH3 domain enables Lyn to bind to proline-rich regions in other signal transducing proteins such as phosphatidylinositol 3 kinase (Ren *et al.*, 1993).

In B lymphocytes, the phosphorylation of Ig-α/Ig-β and Syk kinase is carried out by Src-family kinases, particularly Lyn (Sefton & Taddie, 1994; Kong *et al.*, 1996). Studies using Lyn-negative cells demonstrate that Lyn phosphorylates and modulates the enzymatic activity of Syk, suggesting that Lyn acts upstream of Syk (Kurosaki *et al.*, 1994). All Src-family kinases possess two regulatory sites for tyrosine phosphorylation: one site located in the kinase domain that serves to potentiate kinase activity, and a site within the COOH-terminal region that functions as a negative regulatory site. The up- and downregulation of Lyn activity is mediated by the tyrosine phosphorylation/ dephosphorylation of these specific regulatory sequences. The transmembrane tyrosine

phosphatase, CD45, activates Lyn kinase through the dephosphorylation of a specific tyrosine residue (Tyr527) in the negative regulatory COOH-terminal region (Koretzky, 1993; Burg *et al.*, 1994; Justement *et al.*, 1994; Thomas, 1994). Alternatively, the catalytic activity of Lyn kinase is inhibited by Csk kinase, which phosphorylates Tyr527 in the negative COOH-terminal domain (Erpel & Courtneidge, 1995). The autophosphorylation of Tyr416 within the catalytic domain of Lyn is also a main contributor to the upregulation of Lyn activity (Chan & Shaw, 1995).

It is well recognized that BCR cross-linking activates both Lyn and Syk tyrosine kinases which subsequently results in the activation of several second messenger pathways. Syk couples the BCR to the phosphatidylinositol/Ca^{2+} pathway through the direct phosphorylation and activation of PLC-γ, and in Daudi human B cells, Syk phosphorylates PLCγ1 on Tyr77, the major regulatory residue of PLCγ, and Tyr783 (Sillman & Monroe, 1995; Law *et al.*, 1996). Takata *et al.* (1994) has demonstrated in a Syk-negative cell line that the crosslinking of the BCR does not activate the PLCγ-dependent release of intracellular Ca^{2+}, which also supports the concept that Syk is involved in the activation of PLCγ. Once activated by Syk, PLCγ catalyses the breakdown of membrane phosphoinositides into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Leprince *et al.*, 1992; Richards *et al.*. 1996). IP_3 subsequently binds to the IP_3 receptor located on the membrane of the endoplasmic reticulum which stimulates the mobilization of free intracellular Ca^{2+} into the cytosol (McConnell *et al.*, 1992; Jayaraman *et al.*, 1996).

In addition to the mobilization of intracellular Ca^{2+}, the activation of both Syk and Lyn kinases results in the tyrosine phosphorylation of numerous effector proteins, including

GTPase-activating protein (GAP), phosphoinositide 3-kinase (PI 3-kinase), and mitogen-activated protein kinase (MAPK) (Yamanashi *et al.*, 1992; Kuruvilla *et al.*, 1993). These effector proteins are constituents of downstream signal transduction pathways intimately involved in B cell growth and activity. Activation of PTKs is a fundamental process in BCR-mediated signaling pathways involved in cell function (Brunswick *et al.*, 1991; Panayotou and Waterfield, 1993; Sidorenko *et al.*, 1995). Since PAHs alter B cell signaling and intracellular Ca^{2+} homeostasis, it is important to understand the effects of xenobiotics on PTKs and signal transduction pathways, especially those associated with Ca^{2+} signaling in B cells. Therefore, the focus of this chapter is to evaluate the effects of BaP and certain BaP metabolites on B cell PTKs, including Lyn and Syk, and to examine their potential role in alterations in B cell signaling.

MATERIALS AND METHODS

Monoclonal Antibodies and Reagents. Herbimycin A (Sigma Chemical Co., St. Louis, MO), BaP, BeP, and the BaP metabolites, benzo(a)pyrene-r-7,*t*-8-dihydrodiol-*t*-9,10-epoxide(+/-) (*anti*) (BaP-7,8-diol-9,10-epoxide) and benzo(a)pyrene-trans-7,8-dihydrodiol(+/-) (BaP-7,8-diol) (purchased from the NCI Repository, Midwestern Research Institute, Kansas City, MO) were greater than 95% purity as assessed by the manufacturer. BaP metabolites and herbimycin A were dissolved in tissue culture grade (> 99% purity) anhydrous dimethylsulfoxide (DMSO; Sigma) which also served as the solvent control. All compounds were prepared and used under protective yellow light and visually inspected to affirm that the compounds were completely dissolved in solution. After BaP metabolites were dissolved in DMSO, the compounds were aliquoted and stored under nitrogen gas in glass vials at -20° C until use. The final concentration of DMSO in all cell cultures was $\leq 0.1\%$. Cultures containing DMSO were analyzed for biochemical effects and did not differ from controls without DMSO. It was concluded that at this concentration, DMSO did not produce any measurable effect on intracellular Ca^{2+} or PTK responses. For immunoprecipitations, a murine monoclonal antibody to phosphotyrosine agarose conjugate (Santa Cruz Biotech., Inc., Santa Cruz, CA) was used. For immunoblots, murine monoclonal antibodies to Lyn (Transduction Laboratories, Lexington, KY), Syk (4D10), and phosphotyrosine (Santa Cruz) were used. The anti-Syk (4D10) antibody is a mouse monoclonal IgG_{2a} and reacts with Syk of human origin, and is noncross-reactive with ZAP-70 or other related protein tyrosine kinases. The anti-Lyn antibody is a mouse monoclonal IgG_1 and reacts with Lyn of human, rat, mouse, and chick

origin. Lyn is often observed as a doublet of about 56 and 53 kDa that may represent alternative splicing products of Lyn mRNA. The anti-phosphotyrosine (PY) antibody as well as the anti-PY antibody conjugate to agarose, is a mouse monoclonal IgG_{2b} antibody and reacts with phosphotyrosine-containing proteins and is noncross-reactive with phosphoserine or phosphothreonine. As a positive control for B cell activation, cells were stimulated with monoclonal antibody to human IgM at 20 $\mu g/10^6$ cells (μ chain; Sigma). Protease inhibitor cocktail tablets were purchased from Boehringer Mannheim (Indianapolis, IN) and used at a concentration of 1 tablet/50 mls lysis buffer.

Cell Culturing Conditions of Daudi cells. Daudi B lymphoblastoid cell line was used in all experiments and was maintained as described in Chapter 2.

Inhibition of Protein Tyrosine Kinase (PTK) activity by Herbimycin A. To determine the potential role of PTK activity in PAH-induced mobilization of free intracellular Ca^{2+}, Daudi cells were pretreated with herbimycin A, which inhibits Src-related PTKs by irreversibly binding to the sulfhydryl groups of the kinase and depleting the intracellular level of active enzyme (Knox & Gordon, 1994). Based on previous studies (Carter *et al.*, 1991), we decided to pretreat cells with Herbimycin A for 18 hours to achieve optimal PTK inhibition. Cells were pretreated with herbimycin A for 18 hours, followed by treatment with BaP-7,8-diol and BaP-7,8-diol-9,10-epoxide for 18 hours.

Measurement of Calcium using Flow Cytometry. Alterations in free intracellular Ca^{2+} produced by BaP metabolites were measured using the Ca^{2+}-chelating dyes, Fluo-3 and Fura-Red acetoxymethyl esters (AM) (Molecular Probes, Eugene, OR), and a Coulter ELITE flow cytometer (Hialeah, FL) as described in the previous chapters. Results are shown as the change in the Mean Channel Fluo-3/Fura-Red Fluorescence Ratio (MCFR)

as described in Chapter 1. Cell viability, which ranged from 95% - 99% was determined in all experiments using trypan blue (Sigma). Thapsigargin (Sigma), was used in all experiments (1 μM) as a positive control for Ca^{2+} elevation and to ensure that cells and flow cytometry detection systems were functioning correctly.

Immunoprecipitations and Immunoblotting. Following treatment with BaP metabolites or anti-IgM antibody (positive control for B cell activation), cells were pelleted and resuspended in lysis buffer (1% Nonidet P-40, 150 mM NaCl, 10 mM Tris, pH 7.5, protease inhibitor cocktail, 2 mM sodium orthovanadate, 0.4 mM EDTA, and 10 mM NaF). Lysates were incubated 15 - 30 minutes on ice and cleared of particulated nuclear/cytoskeletal components by centrifugation at 15,000 x *g* for 20 minutes. After centrifugation, whole cell lysates of treated Daudi cells were immunoprecipitated using an anti-PY antibody agarose conjugate overnight at 4° C with constant mixing by rotation. For standardization purposes, the same amount of protein (2000 μg) in all treatment groups was added to 40 μl of a 50:50 slurry of anti-PY antibody-conjugated agarose (stock = 500 μg IgG_{2b}/0.25 ml agarose). Following immunoprecipitation, absorbates were washed three times with lysis buffer, and eluted by resuspension in 200 μl of reducing SDS-PAGE sample buffer. Either whole cells lysates (30 μg) or PY immunoprecipitates (50 μl) were boiled 5 minutes and then separated by SDS-PAGE (12%) gels (200 constant volts for approximately 3 hours) with prestained (rainbow) m.w. markers (BioRad Life Technologies, Hercules, CA). Size fractionated proteins were then transferred to polyvinylidene difluoride membranes (PVDF; NEN Research Products, Boston, MA) by electroblot transfer (Hoefer Scientific Instruments, San Francisco, CA) for 2.5 hours (750 constant amperage).

Membranes containing transferred proteins were blocked using Tris-buffered saline (TBS) containing 1% BSA and 1% phosphate-free nonfat dry milk (Upstate Biotechnology, Lake Placid, NY) overnight at 4° C and subsequently probed with various antibodies diluted in 1% milk/BSA-TBS for 1 hour at room temperature. These antibodies included anti-Lyn, anti-Syk, or anti-PY at a dilution of 1:500. Membranes were then washed 4 times at 10 minutes each with TBS containing 0.05% Triton X-100 (Sigma). Membranes were then incubated for 1 hour with anti-mouse IgG conjugated to horseradish peroxidase (Santa Cruz) at a dilution of 1:5000 and washed again 4 time at 10 minutes each. Immunoreactive proteins were visualized by an enhanced chemiluminescence dectection system (NEN Research Products). Syk and Lyn immunoblots were quantitated using the GS-363 molecular imager system and Molecular Analyst software (BioRad Life Technologies).

Statistical Design and Analysis. For the Ca^{2+} elevation studies, sigmaStat statistical software (Jandel Scientific, San Rafael, CA) was utilized to analyze data for statistical differences between DMSO control and treated samples analyzed in quadruplicate as described in Chapter 1. Statistically significant samples were defined as $p < 0.05$.

RESULTS

Inhibition of Protein Tyrosine Kinase (PTK) activity by Herbimycin A. Herbimycin A was used to determine the potential role of PTK activity in PAH-induced alterations in Ca^{2+} homeostasis and immunosuppression. A dose-response for PTK inhibition by herbimycin A is shown in Figure 19. Daudi cells were pretreated for 18 hours with 0.08, 0.4, or 2 μM herbimycin A followed by treatment with BaP-7,8-diol-9,10-epoxide (3 μM) for 18 hours. Alone, herbimycin A did not produce a significant Ca^{2+} response at any of the concentrations used. Herbimycin A at 0.4 and 2 μM significantly ($p < 0.05$) reduced the elevation in intracellular Ca^{2+} produced by BaP-7,8-diol-9,10-epoxide. Inhibition of Ca^{2+} elevation by herbimycin A was greatest at 0.4 μM, and for this reason, this concentration was used in all subsequent experiments. In Figure 20, second experiment (representative of 6 separate assays performed) is shown in which herbimycin A pretreatment significantly ($p < 0.05$) reduced the Ca^{2+} elevation produced by both BaP-7,8-diol and BaP-7,8-diol-9,10-epoxide. These results suggest that the activation of PTKs is a necessary step in PAH-induced Ca^{2+} mobilization. Results are shown as the change in the MCFR as described in Chapter 1, and values are means for quadruplicate determinations $\pm$ S.D.

BaP Metabolite-Induced Protein Tyrosine Phosphorylation in Daudi Whole cell lysates. In order to address the role of PTK activity and the tyrosine phosphorylation status of proteins in PAH-induced alterations in intracellular Ca^{2+}, whole cell lysates from Daudi cells were treated with 10 μM BaP, 10 μM BeP, or 3 μM BaP-7,8-diol and BaP-7,8-diol-9,10-epoxide for 4 hours. These concentrations of PAHs were chosen because

Figure 19. Dose-response reduction of BaP metabolite-induced intracellular Ca^{2+} elevation by herbimycin A. Daudi cells were pretreated for 18 hours with the protein tyrosine kinase inhibitor, herbimycin A, at 0.08, 0.4, or 2 μM concentrations, and then treated with 3 μM BaP-7,8-diol-9,10-epoxide for 18 hours. Free intracellular Ca^{2+} was measured by multiparameter flow cytometric analysis using the Ca^{2+}-chelating dyes Fluo-3 and Fura Red. Results of alterations in intracellular Ca^{2+} concentrations are shown as the change in the Mean Channel Fluorescence (Fluo-3/Fura Red) Ratio (MCFR) which is obtained by subtracting the MCFR of DMSO solvent controls from the MCFR of PAH-treated samples. Values are means for quadruplicate determinations $\pm$ S.D. *Indicates significant ($p < 0.05$) increase in free intracellular Ca^{2+} compared to DMSO vehicle control. §Indicates a significant ($p < 0.05$) reduction in intracellular Ca^{2+} by herbimycin A pretreatment compared to BaP-7,8-diol-9,10-epoxide-treatment alone. Actual MCFR of samples having a change in MCFR ≤ 0 is as follows: DMSO = 215 $\pm$ 13.1, 0.08 μM herbimycin A = 208 $\pm$ 4.8, 0.4 μM herbimycin A = 205 $\pm$ 4.0, 2 μM herbimycin A = 209 $\pm$ 2.3.

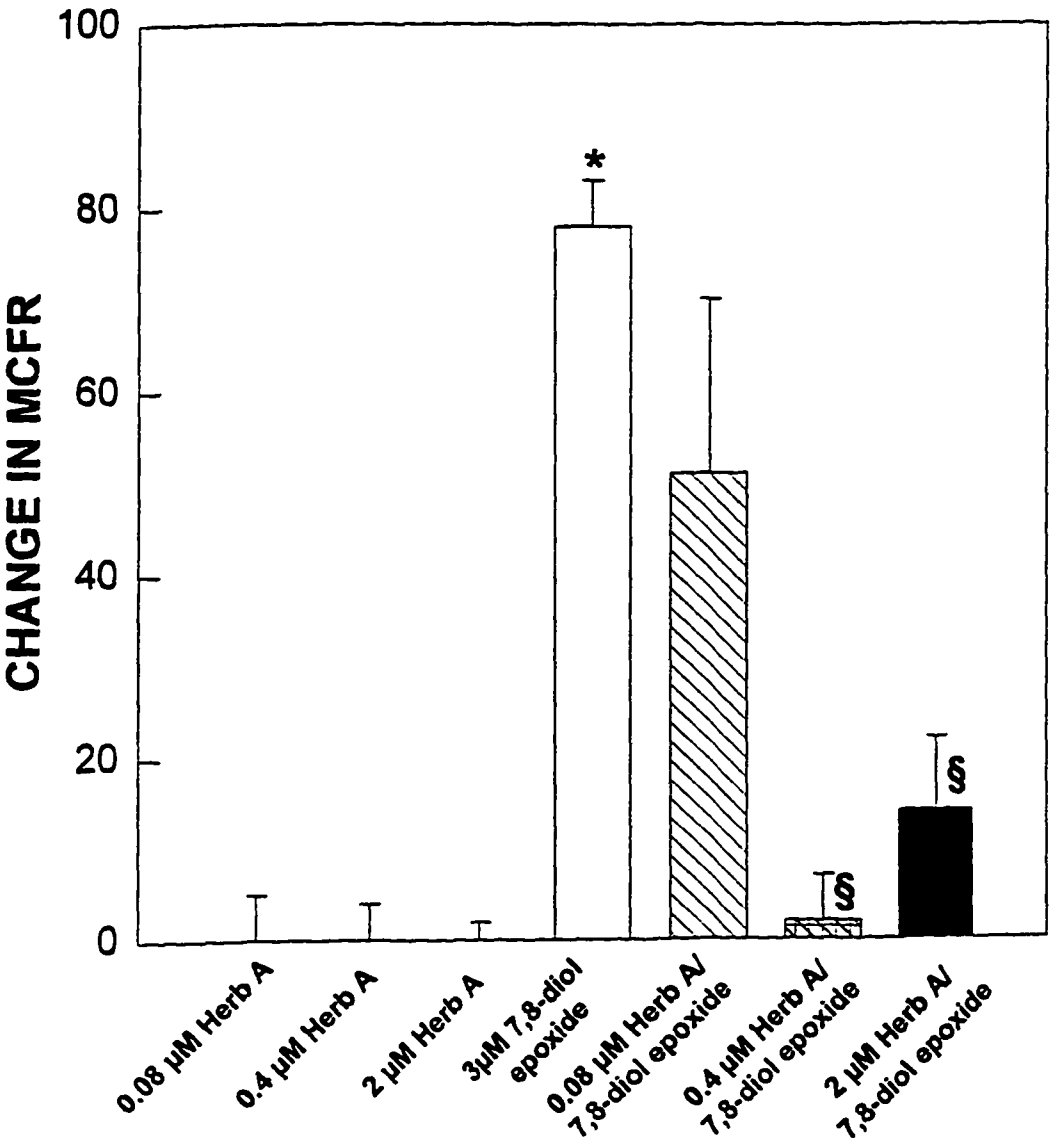
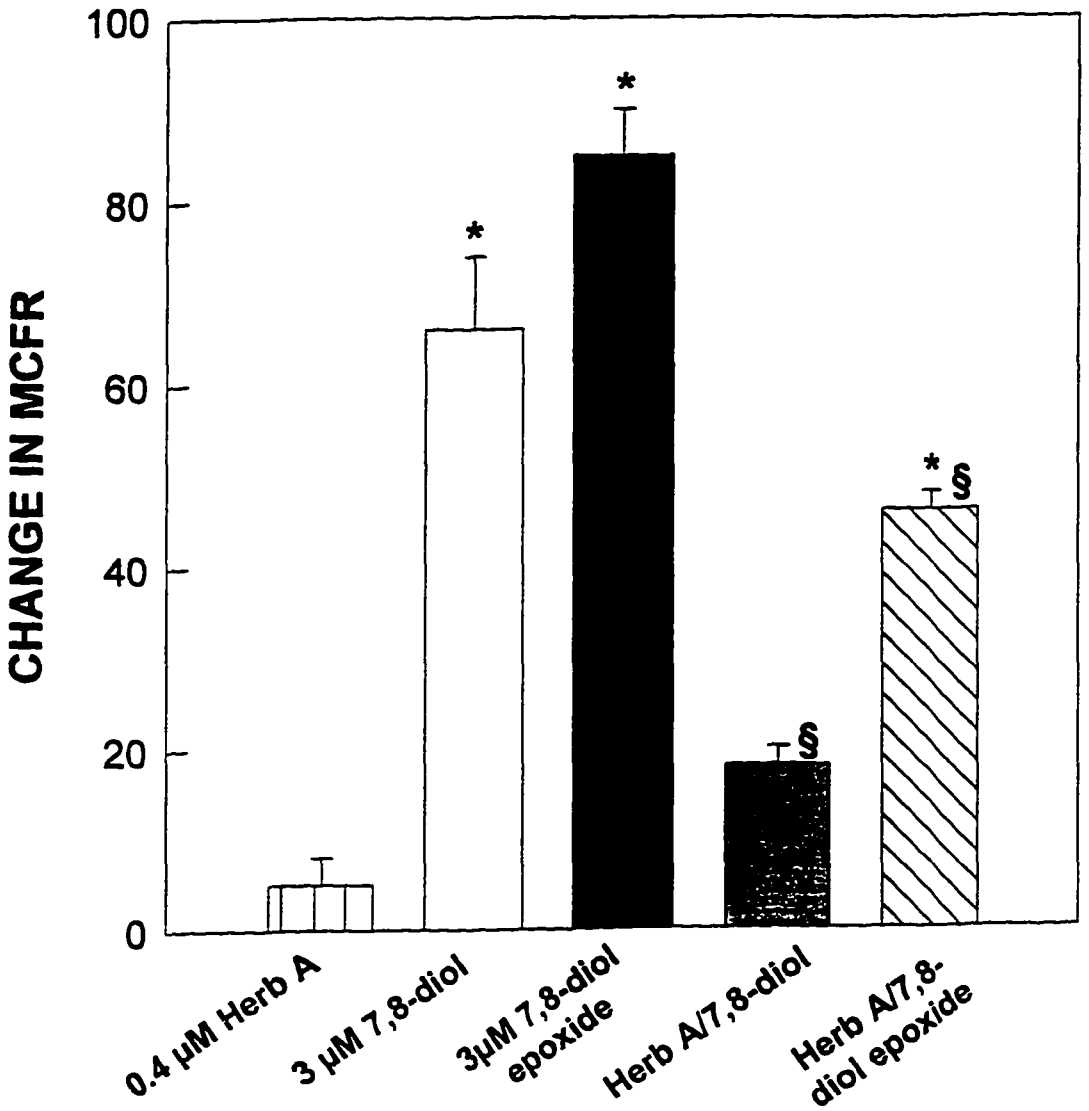


Figure 20. Reduction of BaP-7,8-diol- and BaP-7,8-diol-9,10-epoxide-induced intracellular Ca^{2+} elevation by herbimycin A. Daudi cells were pretreated for 18 hours with herbimycin A at a 0.4 μM concentration, and then treated with 3 μM BaP-7,8-diol or BaP-7,8-diol-9,10-epoxide for 18 hours. Free intracellular Ca^{2+} was measured by multiparameter flow cytometric analysis using the Ca^{2+}-chelating dyes Fluo-3 and Fura Red. Results of alterations in intracellular Ca^{2+} concentrations are shown as the change in the MCFR as previously described. Values are means for quadruplicate determinations ± S.D. *Indicates significant ($p < 0.05$) increase in free intracellular Ca^{2+} compared to DMSO vehicle control. §Indicates a significant ($p < 0.05$) reduction in intracellular Ca^{2+} by herbimycin A pretreatment compared to BaP-7,8-diol or BaP-7,8-diol-9,10-epoxide-treatment alone. Actual MCFR of DMSO = 242 ± 0.3.



they were found to be optimal for Ca^{2+} elevations in Daudi cells. The lysates were subjected to SDS-PAGE (12 %) electrophoresis, and membranes were then blotted with murine monoclonal anti-PY antibodies. As shown in Figure 21, both 3 μM BaP-7,8-diol and BaP-7,8-diol-9,10-epoxide produced an increased tyrosine phosphorylation in proteins in the 40 kDa and 55-65 kDa size range, which could possibly be Ig-α (34-36 kDa) and Lyn kinase (56 kDa). PAHs have previously been shown to increase tyrosine phosphorylation of murine B cell proteins in this same size range (Davila *et al.*, 1995). These data demonstrate that certain BaP metabolites induce tyrosine phosphorylation of proteins in human B lymphocytes which may be involved in BCR activation and Ca^{2+} mobilization.

In Figure 22, Daudi cells were treated for 10 minutes with either anti-IgM antibody (positive control) or BaP-7,8-diol at 1, 3, and 10 μM concentrations. Whole cell lysates were subjected to SDS-PAGE and blotted with anti-PY antibody as previously described. BaP-7,8-diol at all three concentrations stimulated protein tyrosine phosphorylation, particularly of a protein in the 60 kDa size range. This increased tyrosine phosphorylation (compared to DMSO control) of the 60 kDa protein was also observed in Daudi cells treated for 30 minutes (Figure 23). It is possible that this 60 kDa protein may be a Src-family kinase, such as Lyn, since it has a molecular weight of 56 kDa.

Increased tyrosine phosphorylation of Lyn and Syk tyrosine kinases by BaP-7,8-diol and BaP-7,8-diol-9,10-epoxide. In order to examine the effects of BaP metabolites on the tyrosine phosphorylation of Syk and Lyn PTKs, whole cell lysates from treated Daudi cells were immunoprecipitated with anti-PY-conjugated agarose, subjected to SDS-PAGE, and then blotted with either anti-Syk or anti-Lyn antibodies. Figure 24 represents

Figure 21. BaP metabolite-induced protein tyrosine phosphorylation in Daudi whole cell lysates. Whole cells lysates from Daudi cells treated for 4 hours with either no treatment (lane 1), 10 μM BaP (lane 2), 10 μM BeP (lane 3), 3 μM BaP-7,8-diol (lane 4) or 3 μM BaP-7,8-diol-9,10-epoxide (lane 5), were subjected to SDS-PAGE electrophoresis, and membranes were then blotted with monoclonal antibodies to phosphotyrosine (PY).

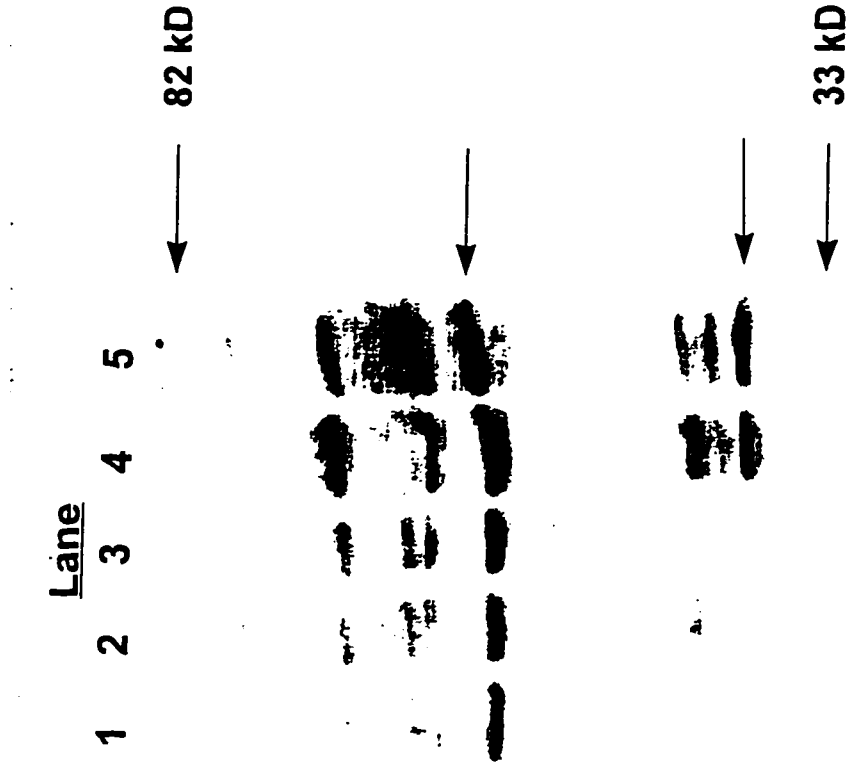


Figure 22. BaP metabolite-induced tyrosine phosphorylation of a protein in the 60 kDa size range. Whole cells lysates from Daudi cells stimulated for 10 minutes with either anti-IgM antibody (positive control, lane 1), DMSO solvent control (lane 2), or BaP-7,8-diol at 1 μM (lane 3), 3 μM (lane 4), or 10μM (lane 5), were subjected to SDS-PAGE electrophoresis, and membranes were then blotted with monoclonal antibodies to phosphotyrosine.

ANTI-PHOSPHOTYROSINE IMMUNOBLOT OF WHOLE CELL LYSATES FROM DAUDI CELLS TREATED FOR 10 MIN

1 = sIgM Ab
2 = DMSO
3 = 1 μM 7,8-Diol
4 = 3 μM 7,8-Diol
5 = 10 μM 7,8-Diol

Figure 23. BaP metabolite-induced tyrosine phosphorylation of a protein in the 60 kDa size range. Whole cells lysates from Daudi cells stimulated for 30 minutes with either anti-IgM antibody (positive control, lane 1), DMSO solvent control (lane 2), or BaP-7,8-diol at 1 μM (lane 3), 3 μM (lane 4), or 10μM (lane 5), were subjected to SDS-PAGE electrophoresis, and membranes were then blotted with monoclonal antibodies to phosphotyrosine.

ANTI-PHOSPHOTYROSINE IMMUNOBLOT OF WHOLE CELL LYSATES FROM DAUDI CELLS TREATED FOR 30 MIN

← ≈ 60 kD

1 2 3 4 5

1 = sIgM
2 = DMSO
3 = 1 μM 7,8-Diol
4 = 3 μM 7,8-Diol
5 = 10 μM 7,8-Diol

an immunoblot of Daudi cells treated for 10 minutes. While there was not an observable dose response between the different concentrations of BaP-7,8-diol-9,10-epoxide, there was an increase in tyrosine phosphorylation of Lyn kinase in all treatment groups as compared to the DMSO solvent control. As shown in Figure 25, the relative signal of Lyn tyrosine phosphorylation produced by BaP-7,8-diol-9,10-epoxide at 1 μM is 35, which is more than double the relative signal produced by DMSO control (relative signal = 14). These numbers were used as relative indicators of signal strength since no statistical comparisons can be performed on values from single samples. However, the relative consistency of findings demonstrate that the PAH effects are reproducible and it is known that a doubling of PTK phosphorylation is biologically relevant (Johnson *et al.*, 1995; Kurosaki *et al.*, 1995). Lyn kinase is constitutively and transiently phosphorylated and, therefore, the more than double increase in its tyrosine phosphorylation status produced by BaP-7,8-diol-9,10-epoxide may also be biologically significant. It is unclear as to why BaP-7,8-diol-9,10-epoxide at a 3 μM concentration produced a low relative signal of 18. One possible explanation is that the system is maximally stimulated at this concentration. However, this is difficult to assume, especially since in cells treated for 30 minutes (Figure 26), the relative signal produced by BaP-7,8-diol-9,10-epoxide at 3 μM is 28, which is considerably higher than DMSO control which produced a relative signal of 17 (Figure 27). At 30 minutes, the largest level of tyrosine phosphorylation is induced by BaP-7,8-diol-9,10-epoxide at 1 μM, which produced a relative signal of 32 and is nearly double compared to DMSO control.

Figure 28 demonstrates that by 18 hours, BaP-7,8-diol-induced tyrosine phosphorylation of Lyn returned to baseline and was no different from DMSO control.

Figure 24. Increased tyrosine phosphorylation of Lyn kinase by BaP-7,8-diol-9,10-epoxide. Whole cell lysates from treated Daudi cells were immunoprecipitated with anti-PY-conjugated agarose, subjected to SDS-PAGE, and then blotted with a monoclonal antibody to Lyn. Daudi cells were treated for 10 minutes with either anti-IgM antibody (positive control; lane 1), DMSO solvent control (lane 2), or BaP-7,8-diol-9,10-epoxide at 1 μM (lane 3) and 3 μM (lane 4) concentrations.

ANTI-LYN IMMUNOBLOT OF PHOSPHOTYROSINE IMMUNOPRECIPITATES FROM DAUDI CELLS TREATED FOR 10 MIN

1 2 3 4

1 = sIgM
2 = DMSO
3 = 1 µM 7,8-Diol Epoxide
4 = 3 µM 7,8-Diol Epoxide

Figure 25. The relative signal of Lyn tyrosine phosphorylation produced by BaP-7,8-diol-9,10-epoxide. Whole cell lysates from Daudi cells treated for 10 minutes were immunoprecipitated with anti-PY-conjugated agarose and probed with anti-Lyn antibody as previously described in Figure 24. The relative signal of tyrosine phosphorylation of Lyn kinase was determined using a molecular imager system. The quantitated relative signal is of a single band for each treatment group, and does not represent statistical analysis.

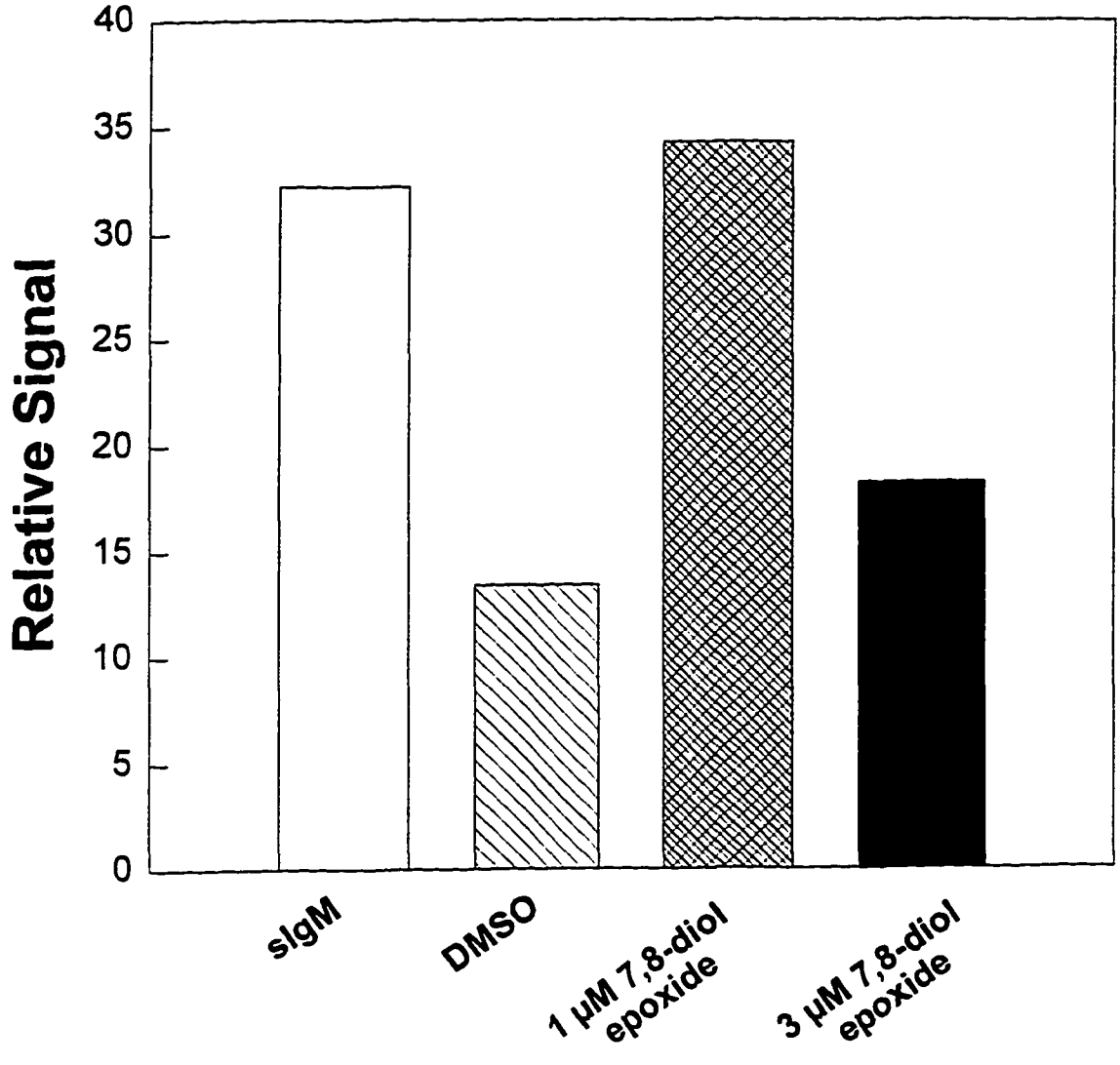
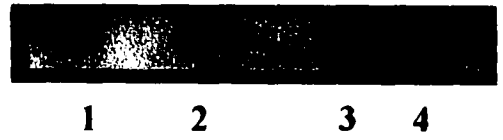


Figure 26. Increased tyrosine phosphorylation of Lyn kinase by BaP-7,8-diol-9,10-epoxide. Whole cell lysates from treated Daudi cells were immunoprecipitated with anti-PY-conjugated agarose, subjected to SDS-PAGE, and then blotted with a monoclonal antibody to Lyn. Daudi cells were treated for 30 minutes with either anti-IgM antibody (positive control; lane 1), DMSO solvent control (lane 2), or BaP-7,8-diol-9,10-epoxide at 1 μM (lane 3) and 3 μM (lane 4) concentrations.

ANTI-LYN IMMUNOBLOT OF PHOSPHOTYROSINE IMMUNOPRECIPITATES FROM DAUDI CELLS TREATED FOR 30 MIN



1 = sIgM
2 = DMSO
3 = 1 μM 7,8-Diol Epoxide
4 = 3 μM 7,8-Diol Epoxide

Figure 27. The relative signal of Lyn tyrosine phosphorylation produced by BaP-7,8-diol-9,10-epoxide. Whole cell lysates from Daudi cells treated for 30 minutes were immunoprecipitated with anti-PY-conjugated agarose and probed with anti-Lyn antibody as previously described in Figure 26. The relative signal of tyrosine phosphorylation of Lyn kinase was determined using a molecular imager system. The quantitated relative signal is of a single band for each treatment group, and does not represent statistical analysis.

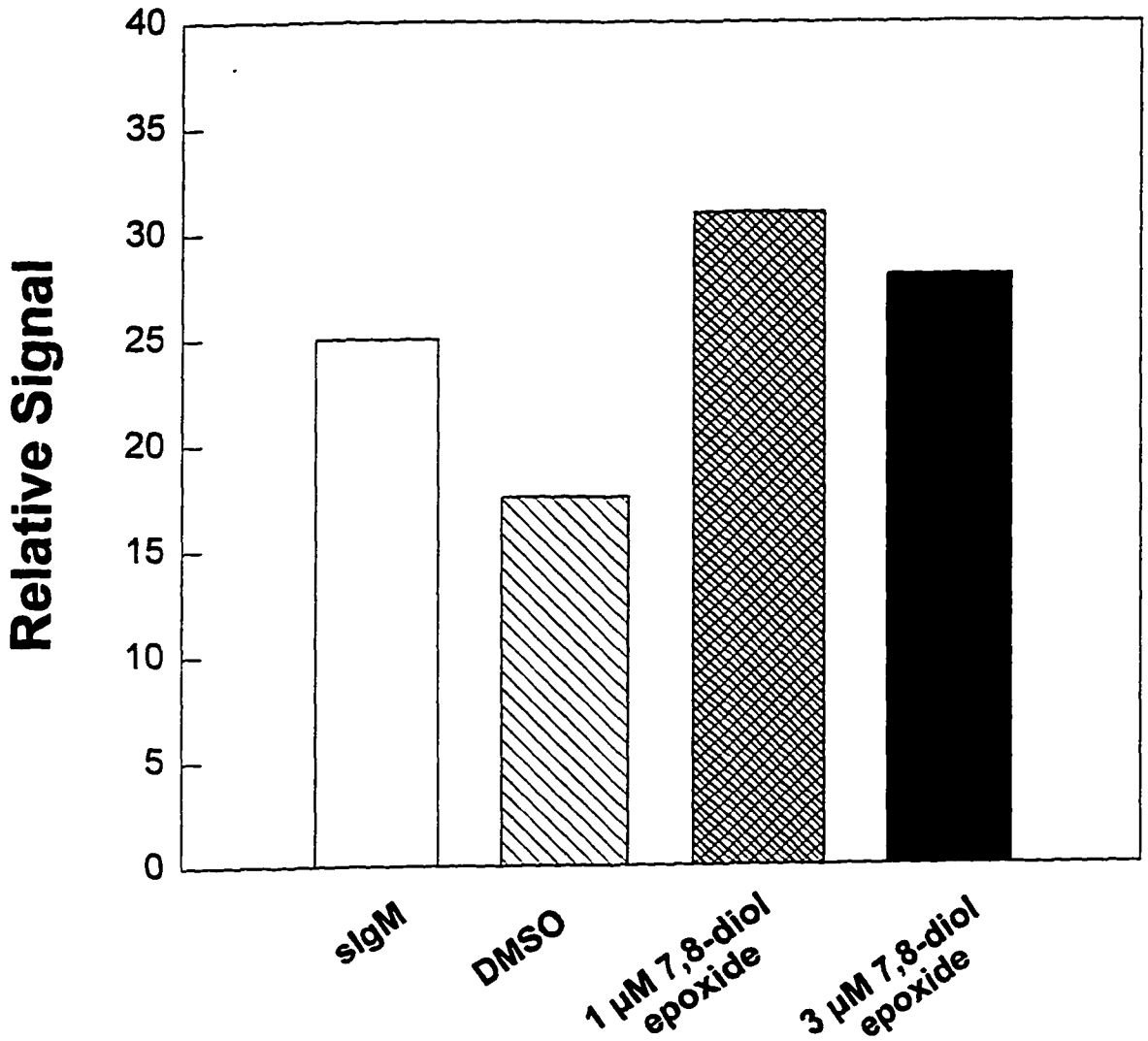


Figure 28. Tyrosine phosphorylation of Lyn kinase following 18 hours of exposure. Whole cell lysates from treated Daudi cells were immunoprecipitated with anti-PY-conjugated agarose, subjected to SDS-PAGE, and then blotted with a monoclonal antibody to Lyn. Daudi cells were treated with either DMSO solvent control (lane 1), or BaP-7,8-diol at 1 μM (lane 2), 3 μM (lane 3), or 10 μM (lane 4) concentrations.

ANTI-LYN IMMUNOBLOT OF PHOSPHOTYROSINE IMMUNOPRECIPITATES FROM DAUDI CELLS TREATED FOR 18 HRS

← Lyn 56 kD

1 2 3 4

1 = DMSO
2 = 1 μM 7,8-Diol
3 = 3 μM 7,8-Diol
4 = 10 μM 7,8-Diol

This return to baseline tyrosine phosphorylation at 18 hours was also observed with BaP-7,8-diol-9,10-epoxide and in Syk kinase (data not shown). One possible explanation for transient increases in tyrosine phosphorylation may be compensatory mechanisms that regulate phosphorylation levels and PTK activity, such as CD45. Thus, CD45, or other protein tyrosine phosphatase (PTPase) activity, may lead to dephosphorylation of target proteins.

At 10 minutes (Figure 29), BaP-7,8-diol-9,10-epoxide also stimulated the tyrosine phosphorylation of Syk kinase. While the relative signal of Syk (Figure 30) may not be as high as seen with BaP-7,8-diol-9,10-epoxide-induced tyrosine phosphorylation of Lyn, it is difficult to quantitatively compare results obtained from separate experiments. In Daudi cells treated for 30 minutes, there was no observable increase in tyrosine phosphorylation of Syk produced by BaP-7,8-diol-9,10-epoxide as compared to DMSO control (data not shown). Thus, these results demonstrate that tyrosine phosphorylation induced by certain BaP metabolites is rapid and relatively transient in Daudi human B cells.

Figure 29. Increased tyrosine phosphorylation of Syk kinase by BaP-7,8-diol-9,10-epoxide. Whole cell lysates from treated Daudi cells were immunoprecipitated with anti-PY-conjugated agarose, subjected to SDS-PAGE, and then blotted with a monoclonal antibody to Syk. Daudi cells were treated for 10 minutes with either anti-IgM antibody (positive control; lane 1), DMSO solvent control (lane 2), or BaP-7,8-diol-9,10-epoxide at 1 μM (lane 3) and 3 μM (lane 4) concentrations.

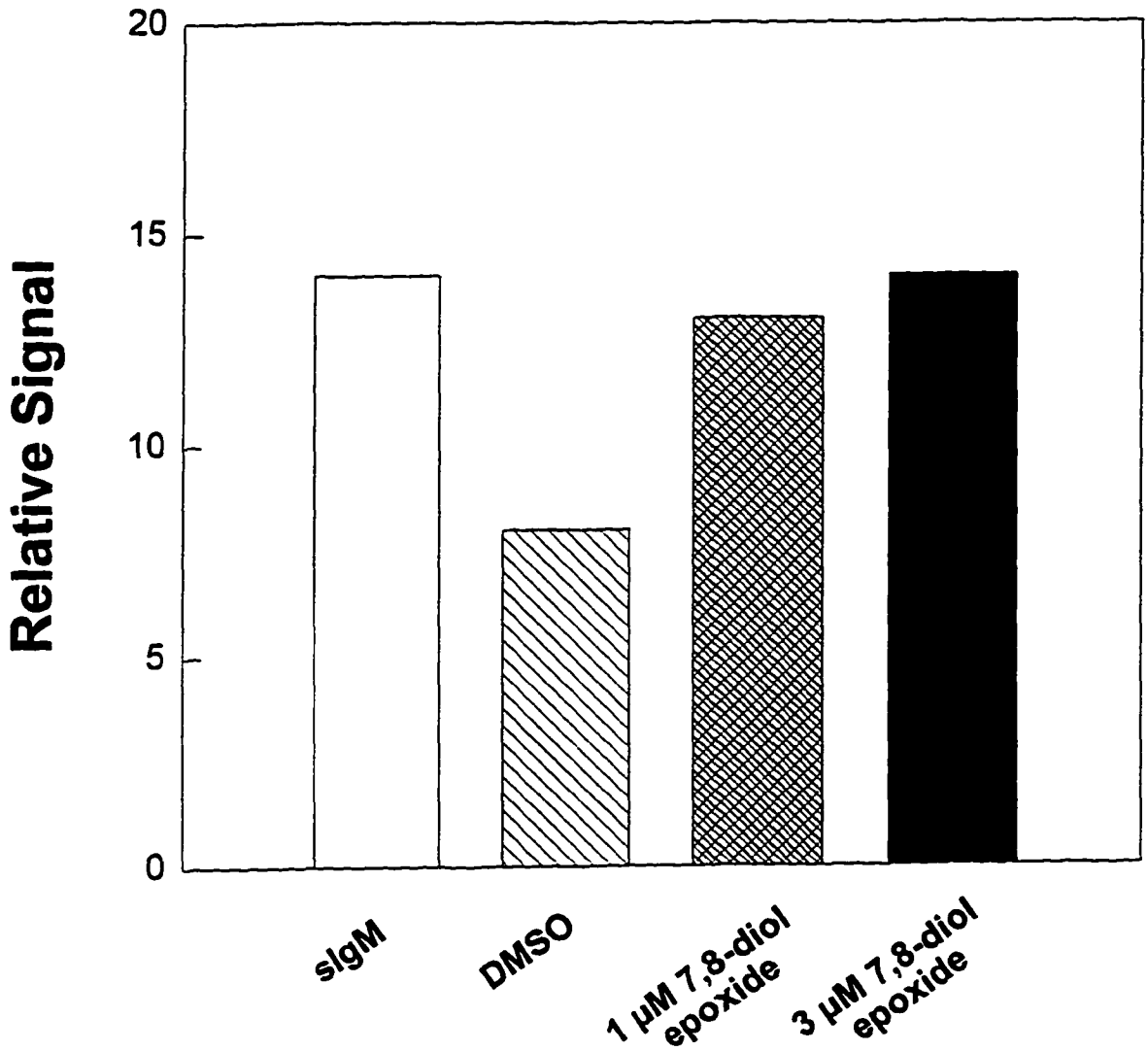
ANTI-SYK KINASE IMMUNOBLOT OF PHOSPHOTYROSINE IMMUNOPRECIPITATES FROM DAUDI CELLS TREATED FOR 10 MIN

← Syk 72 kD

1 2 3 4

1 = sIgM Ab
2 = DMSO
3 = 1 μM 7,8-Diol Epoxide
4 = 3 μM 7,8-Diol Epoxide

Figure 30. The relative signal of Syk tyrosine phosphorylation produced by BaP-7,8-diol-9,10-epoxide. Whole cell lysates from Daudi cells treated for 10 minutes were immunoprecipitated with anti-PY-conjugated agarose and probed with anti-Syk antibody as previously described in Figure 29. The relative signal of tyrosine phosphorylation of Syk kinase was determined using a molecular imager system. The quantitated relative signal is of a single band for each treatment group, and does not represent statistical analysis.



DISCUSSION

Since previous work in our laboratory has demonstrated that the increase in intracellular Ca^{2+} produced by PAHs is likely due to the activation of Src-family protein tyrosine kinases (Archuleta *et al.*, 1993; Krieger *et al.*, 1994) in T lymphocytes, we wanted to examine the role of PTKs, particularly Syk and Lyn, in PAH-induced elevations in intracellular Ca^{2+} in human B lymphocytes. Past studies have revealed that the activation of PTKs, such as Syk and Lyn, is an essential step in the propagation of signals from the BCR to the nucleus, and these activated kinases, in turn, stimulate several interrelated pathways of signal transduction. For example, immediately following BCR stimulation and activation of Syk, PLCγ1 and 2 are phosphorylated and activated, resulting in the activation of protein kinase C (PKC) and the mobilization of Ca^{2+} from intracellular stores (Carter *et al.*, 1991; Coggeshall *et al.*, 1992). Additionally, antigen receptor stimulation and PTK activation leads to the activation of GAP, which regulates the activity of the ras gene ($p21^{ras}$) involved in growth control (Panaytou & Waterfield, 1993). A third signaling protein, PI 3-kinase, is activated by direct interaction with Src-family kinases, and initiates the activation of other signaling cascades necessary for proliferation by phosphorylating the inositol ring of inositol phospholipids (Tseng *et al.*, 1994).

The studies presented in the previous chapters have demonstrated that both BaP-7,8-diol and BaP-7,8-diol-9,10-epoxide produce a significant elevation in intracellular Ca^{2+} in human peripheral blood $CD19^+$ B cells. BaP metabolites produce a more potent and rapid increase in intracellular Ca^{2+}, suggesting that they are ultimately responsible for Ca^{2+}

elevation. In this chapter, the intracellular Ca^{2+} elevation produced by BaP-7,8-diol and BaP-7,8-diol-9,10-epoxide was also observed in Daudi human B cells and was significantly blocked by inhibition of PTKs by herbimycin A. Additionally, exposure to BaP-7,8-diol and BaP-7,8-diol-9,10-epoxide also resulted in the stimulation of tyrosine phosphorylation of Syk and Lyn tyrosine kinases. The same doses of these BaP metabolites that increased Ca^{2+} in Daudi also increased tyrosine phosphorylation. These data suggest that there is a correlation between PTK tyrosine phosphorylation and intracellular Ca^{2+} elevations produced by these BaP metabolites. Both Lyn and Syk kinases are associated with BCR-mediated activation of intracellular signaling pathways (Ullrich & Schlessinger, 1990; Harwood & Cambier, 1993; van Oers & Weiss, 1995). Therefore, BaP-induced alterations in the tyrosine phosphorylation status of Lyn and Syk may be of critical importance in B cell activation and immunotoxicity.

While both BaP-7,8-diol and BaP-7,8-diol-9,10-epoxide produced an increase in intracellular Ca^{2+} at longer time points (18 hours) in Daudi cells (Chapter 2), these two metabolites increased the tyrosine phosphorylation of Lyn and Syk only at shorter exposure times (10 minutes - 4 hours), but not at 18 hours. Syk couples the BCR to the phosphatidylinositol/Ca^{2+} pathway and is associated with BCR-mediated activation of $PLC_{\gamma}2$, followed by the generation of IP_3 and the subsequent mobilization of intracellular Ca^{2+} (Takata *et al.*, 1994; Law *et al.*; 1996). The BCR-mediated mobilization of free intracellular Ca^{2+} occurs immediately following BCR stimulation and is transient (Premack & Gardner, 1994). Therefore, it is difficult to explain how PTKs maintain Ca^{2+} elevation at 18 hours when Lyn and Syk tyrosine phosphorylation has returned to baseline. One possibility is that the rise in intracellular Ca^{2+} produced by PAHs at the shorter time

points may be regulated by PTKs while the sustained Ca^{2+} elevations ($\geq$ 18 hours) may involve other mechanisms, such as opening the storage operated Ca^{2+} channels (SOCC) located in the plasma membrane of lymphocytes (Premack *et al.*, 1994) or inhibition of Ca^{2+} pumps, such as sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPases (SERCA) (Krieger *et al.*, 1995). Alternatively, PTK activation may continue at low levels or in a subset of cells at any given time point that cannot be assessed using immunoblot analysis of the overall population. Since we have not yet determined the cell cycle-dependence of Ca^{2+} elevation produced by PAHs, this is another potential source of variability in this system. Further studies are necessary to resolve these points.

The biochemical mechanisms by which PAHs interact and influence PTK signaling have not been defined in the present studies. One hypothesis is that the metabolism of BaP leads to the generation of reactive metabolites, such as BaP-7,8-diol-9,10-epoxide, capable of reacting with oxidant-sensitive protein sulfhydryls. Reactive oxygen intermediates (ROI) can readily attack cellular macromolecules directly or by initiating a chain reaction in which a free radical is passed along macromolecules, resulting in the damage of cellular components such as plasma membranes and regulatory proteins (Qin *et al.*, 1996). Studies have shown that an increase in intracellular Ca^{2+} and activation of PTKs, such as Syk, occurs in lymphocytes in response to oxidative stress, including H_2O_2 treatment and UV radiation (Schieven *et al.*, 1993a, 1993b, 1993c; Yang *et al.*, 1995; Suzuki *et al.*, 1996). Interestingly, the increase of PTK activity and tyrosine phosphorylation produced by oxidants has been shown to be indirect resulting from inhibition of protein tyrosine phosphatase activity (Schieven *et al.*, 1993a; Krejsa *et al.*, 1997). Our laboratory has recently demonstrated that BaP metabolites produce a

significant reduction in glutathione (GSH) (Romero *et al.*, 1997), which supports the current working hypotheses that BaP is metabolized to reactive oxidant species which could potentially produce a disruption in PTK activation by attacking/oxidizing sulfhydryl sensitive proteins. Therefore, the metabolism of PAHs by P450, resulting in the generation of reactive metabolites capable of oxidizing cellular sulfhydryls, may be one mechanism of PAH-induced PTK dysregulation, alterations in Ca^{2+} homeostasis, and immunotoxicity. (Gelboin, 1980; Conney *et al.*, 1994; Qin *et al.*, 1996). An alternative mechanism by which the reactive metabolites of certain PAHs, including BaP-7,8-diol-9,10-epoxdie, may interfere with PTK signaling and function is through the covalent binding amine groups of cellular proteins and peptides (Thakker *et al.*, 1977).

The data presented in this chapter provides some preliminary evidence that PAHs and particularly the reactive metabolites of PAHs, may modulate intracellular signaling in B cells through the disruption of PTK activation. The effects of these compounds on other signaling proteins, however, cannot be excluded. Future studies in our laboratory, therefore, will focus on the direct effects of PAHs on PTKs as well as other signaling proteins in human lymphocytes, including the transmembrane tyrosine phosphatase, CD45, and other protein tyrosine phosphatases.

GENERAL DISCUSSION

Summary and Conclusions

It is well recognized that, acting as a second messenger, the mobilization of intracellular Ca^{2+} is critical in the propagation of signals from external stimuli to the nucleus. The overall hypothesis that was evaluated in these studies is that polycyclic aromatic hydrocarbons (PAHs) produce immunotoxicity by altering intracellular Ca^{2+} homeostasis and Ca^{2+}-dependent signal transduction pathways involved in B cell activation and function. An extension of this hypothesis is that PAH-induced disruptions in intracellular Ca^{2+} homeostasis is associated with cytochrome P450 metabolism and protein tyrosine kinase activity.

Initial studies examining the mechanism(s) of PAH-induced immunosuppression demonstrated that immunotoxic and carcinogenic PAHs, including DMBA, BaP, and 3-MC, produce a sustained elevation in intracellular Ca^{2+} in murine and human T and B lymphocytes. In employing the very sensitive Ca^{2+}-indicators, Fluo-3 and Fura Red, in conjunction with PE-conjugated antibodies to cell surface antigens, these studies expanded on these findings and characterized the differential sensitivity of normal HPBMC in surface marker-defined monocytes, B cells, and T cells, to the intracellular Ca^{2+} changes produced by PAHs and HAHs. In characterizing PAH-induced elevations in intracellular Ca^{2+}, most work has been performed in cell lines and until now, the effects of PAHs and TCDD on intracellular Ca^{2+} homeostasis has not been evaluated in normal human monocytes, T lymphocytes, and B lymphocytes.

These studies showed that PAHs/HAHs produced structure-dependent alterations in Ca^{2+} homeostasis in the three different blood cell populations, and the highly immunotoxic PAHs, DMBA, BaP, and TCDD, compared to BeP and ANTH, two non-immunotoxic PAHs, produced a structure and time-dependent increase in intracellular Ca^{2+} in HPBMC. These data provided information that helps us gain a better understanding of the mechanism(s) of PAH/HAH-induced immunotoxicity.

The cytochrome P450 inhibition studies demonstrated that elevations in intracellular Ca^{2+} produced by BaP in normal human peripheral lymphocytes and monocytes, and by the BaP metabolite, 7,8-diol in Daudi human B cells, is reduced by ANF, an inhibitor of cytochrome P450 activity. Additionally, the BaP metabolites, BaP-7,8-diol and BaP-7,8-diol-9,10-epoxide were much more effective in producing intracellular Ca^{2+} elevations than the parent compound in both Daudi cells and human peripheral blood $CD19^+$ B lymphocytes. Based on these studies and others, (Ladics *et al.*, 1991; Dipple, 1994), it appears that the metabolism of PAHs by cytochrome P450 plays a significant role in PAH-induced intracellular Ca^{2+} elevation and immunosuppression. While the parent compounds of certain PAHs, such as DMBA and BaP, do produce alterations in Ca^{2+} homeostasis *in vitro*, these effects may involve both specific and nonspecific components, such as alterations in plasma membrane or Ca^{2+} channel activity. The effects of PAH metabolites, however, may be quite specific, involving reactive oxidants capable of oxidizing cellular sulfhydryls.

The reactive metabolites/reactive oxygen intermediates generated by P450 metabolism may readily attack cellular macromolecules resulting in the damage of cellular components and signaling proteins. Signaling proteins and pathways that may be susceptible to these

reactive oxidants would include the transmembrane tyrosine phosphatase, CD45, protein tyrosine kinases, including Syk and Src-family kinases, phospholipase C-γ (PLC-γ), protein kinase C (PKC), GTPase activation protein (GAP), phosphatidylinositol 3 kinase (PI-3 kinase), mitogen activated protein kinase (MAPK), and various transcription factors involved in growth regulation, including NF-κB and c-Jun (see Figure 31).

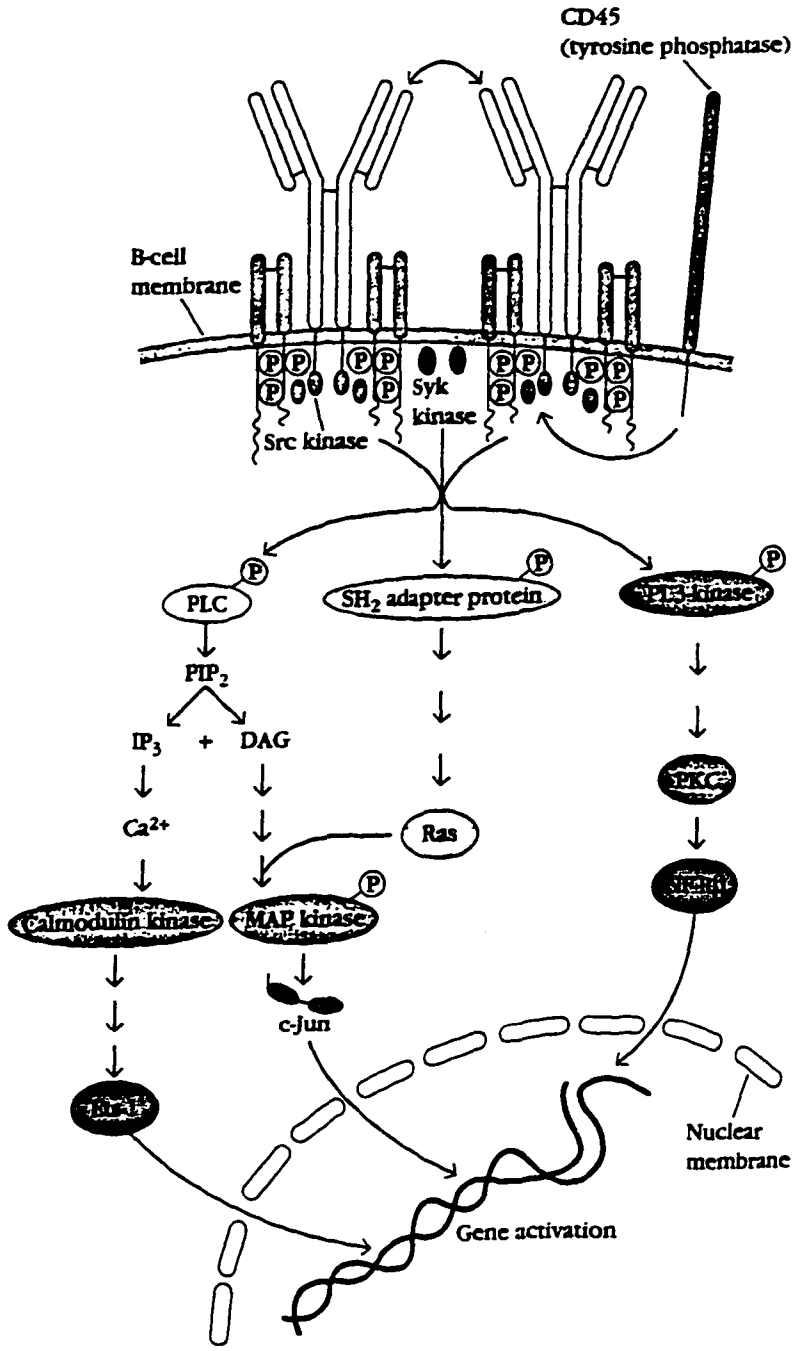
Two major signaling pathways important to B lymphocyte activation are protein tyrosine phosphorylation and intracellular Ca^{2+} mobilization. Antigen receptor stimulation results in the activation of protein tyrosine kinases which subsequently leads to the mobilization of Ca^{2+} from intracellular stores. Protein tyrosine inhibition by herbimycin A, a specific protein tyrosine kinase inhibitor, demonstrates that in human B lymphocytes, there is a strong correlation between protein tyrosine kinase activity and PAH-induced Ca^{2+} elevation, and therefore, any dysregulation in protein tyrosine activity produced by PAHs could ultimately lead to disruptions in Ca^{2+} homeostasis.

Additionally, immunoblot analysis showed that BaP-7,8-diol and BaP-7,8-diol-9,10-epoxide produced an increase in tyrosine phosphorylation of both Lyn and Syk tyrosine kinases, and possibly the Ig-α and Ig-β heterodimers. While future studies will focus on the effects of these compounds on kinase activity in conjunction with increased autophosphorylation and tyrosine phosphorylation, studies do show that Syk and Lyn autophosphorylation correlates with and is indicative of increased kinase activity (DeFranco 1995; Kurosaki *et al.*, 1995; Rowley *et al.*, 1995).

Future Studies

Evaluating and characterizing the mechanisms by which PAHs exert their immunosuppressive effects in lymphoid cells will help us gain a better understanding of

Figure 31. B cell antigen receptor (BCR)-mediated signal transduction pathways. The BCR is noncovalently associated with disulfide-linked heterodimers, Ig-α and Ig-β, which play an important role in BCR-mediated signaling pathways. Several protein tyrosine kinases, including Syk and Src-family kinases, such as Lyn, are associated with the Ig-α and Ig-β proteins. The tyrosine transmembrane phosphatase, CD45, activates Src-family kinases through the dephosphorylation of a specific tyrosine residue in the negative regulatory C-terminal domain. B cell antigen crosslinking activates Src and Syk kinases which subsequently results in the generation important second messengers, including Ca^{2+}, diacylglycerol (DAG), and inositol triphosphate (IP_3). The activation of various signaling cascades leads to the activation of transcription factors that stimulate the transcription of specific genes. From: Kuby, J. (1997). *Immunology 3rd Edition*, Figure 8-8, p. 204, with permission.



important health effects of PAHs on human immune function and may provide meaningful insight in identifying important genetic factors, such as polymorphisms, associated with PAH-induced immunotoxicity.

There are several other postulated mechanisms by which PAHs produce dysregulation in Ca^{2+} homeostasis and cell signaling not examined in these studies, and will be evaluated in the future. One area of focus will examine the potential influence of PAHs on Ca^{2+} influx associated with storage-operated Ca^{2+} channels (SOCC) in human T and B lymphocytes. Second, our laboratory will further characterize the inhibitory effects of PAHs and certain BaP metabolites on human sarcoplasmic/endoplasmic reticulum calcium ATPases (SERCA), which actively pumps cytosolic Ca^{2+} back into the endoplasmic reticulum (Krieger *et al.*, 1995).

These and past studies performed in our laboratory have demonstrated that the elevation in intracellular Ca^{2+} produced by PAHs and certain BaP metabolites strongly correlates with, and is likely due to the dysregulation and activation of protein tyrosine kinases. Using an *in vitro* kinase activity assay, future studies will examine the effects of these compounds on Syk/ZAP-70 and Src-family kinase, including Lyn, activity in human T and B cells, as well as characterize whether the effects of PAHs on kinase activity is direct or indirect. PAHs may activate protein tyrosine kinases indirectly by inhibiting phosphatases, and for this reason, we will also evaluate the effects of PAHs and specific BaP metabolites on the tyrosine phosphatase, CD45. CD45 selectively associates and regulates Lyn kinase activity (Brown *et al.*, 1994; Katagiri *et al.*, 1995), and has been shown to be sensitive to oxidative stress (Schieven *et al.*, 1994).

Future work will continue to further evaluate the role of cytochrome P450 metabolism, as well as characterize the role of the aryl-hydrocarbon receptor (AhR) in PAH signaling and immunotoxicity. It is likely a combination of biochemical mechanisms and interrelated signaling cascades crosstalk with Ca^{2+} pathways and are responsible for the immunotoxic effects mediated by PAHs. Future work focusing on the molecular mechanisms responsible for PAH immunosuppression, therefore, will help us gain a more complete understanding of PAH-induced immunotoxicity and potential environmental risk factors associated with PAH suppression of human immune function.

ABBREVIATIONS

AhR	aryl-hydrocarbon receptor
ANF	α-naphthoflavone
ANTH	anthracene
BA	benz(a)anthracene
BaP	benzo(a)pyrene
BaP-7,8-diol	benzo(a)pyrene-trans-7,8-dihydrodiol (±)
BaP-7,8-diol-9,10-epoxide	benzo(a)pyrene-r-7,t-8-dihydrodiol-t-9,10-epoxide (±), (anti)
BCR	B cell receptor
BeP	benzo(e)pyrene
Con A	concanavalin A
DAG	diacylglycerol
DMA	9,10-dimethylanthracene
DMBA	7,12-dimethylbenz(a)anthracene
DMSO	dimethylsulfoxide
GAP	GTPase-activating protein
HAH	halogenated aromatic hydrocarbon
HPBMC	human peripheral mononuclear cell
IgG	immunoglobulin G
IgM	immunoglobulin M
IP_3	inositol 1,4,5-trisphosphate

ITAM	immunoreceptor tyrosine based activation motif
MAPK	mitogen-activated protein kinase
MCFR	mean channel fluorescence ratio
PAH(s)	polycyclic aromatic hydrocarbon(s)
PE	phycoerythrin
PHA	phytohemagglutinin
PI 3-kinase	phosphoinositide 3-kinase
PLC	phospholipase C
PTK	protein tyrosine kinase
SERCA	sarcoplasmic/endoplasmic reticulum calcium ATPase
SH_2/SH_3	Src-homology domain
SRBC	sheep red blood cell
TCDD	2,3,7,8-tetrachlorodibenzo(p)dioxin
TCR	T cell receptor
3-MC	3-methylcholanthrene

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