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SIGNAL TRANSDUCTION PATHWAYS INVOLVED IN THE UPREGULATION OF CYCLOOXYGENASE-2 BY 2,2',4,4'-TETRACHLOROBIPHENYL

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ABSTRACT

SIGNAL TRANSDUCTION PATHWAYS INVOLVED IN THE UPREGULATION OF CYCLOOXYGENASE-2 BY 2,2′,4,4′-TETRACHLOROBIPHENYL

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Polychlorinated biphenyls (PCBs) are ubiquitous, persistent environmental contaminants that affect a number of cellular systems, including neutrophils. PCBs can be divided into two broad classes based on the presence or absence of chlorine atoms in the ortho- position. Congeners that lack chlorines in the ortho-positions are coplanar and bind with high affinity to the aryl hydrocarbon receptor to induce changes in cellular function. Congeners containing chlorines substituted at the ortho- positions cannot attain a coplanar configuration and are, in general, poor ligands for the aryl hydrocarbon receptor. Among the effects caused by noncoplanar PCBs is the alteration in function of polymorphonuclear neutrophils. These alterations include increases in superoxide anion production, activation of phospholipase A_2 and subsequent release of arachidonic acid (AA). Accordingly, the overall goal of the work outlined in this dissertation was to test the hypothesis that 2,2′,4,4′,-tetrachlorobiphenyl (2244-TCB) increases COX-2 expression in granulocytic HL-60 cells, and that this increase depends on activation of intracellular signaling pathways including arachidonic acid release, superoxide anion production and activation of the p38 mitogen-activated protein (MAP) kinase. 2244-TCB increased superoxide anion production, AA release

and levels of COX-2 mRNA, protein and enzyme activity. Neither superoxide anion nor free AA was involved in the 2244-TCB-mediated increase in COX-2 mRNA. Nonetheless, the increases in AA occur at the same time as the increases in COX-2 protein, raising the possibility that the AA may be used by the enzyme for eicosanoid production. The 2244-TCB-mediated increases in COX-2 mRNA, protein and enzyme activity were prevented by pretreatment with inhibitors of p38 mitogen-activated protein (MAP) kinase. 2244-TCB also caused the nuclear levels of NF-κB and C/EBP beta to increase. These increases were also prevented by inhibition of p38 MAP kinase. Additionally, inhibition of NF-κB prevented the 2244-TCB-mediated increase in COX-2 mRNA. Inhibition of transcription with actinomycin D reduced the 2244-TCB-mediated increase in COX-2 mRNA. Taken together, these results suggest that the 2244-TCB-mediated upregulation of COX-2 involves increased transcription of the COX-2 gene via a pathway requiring p38 MAP kinase and NF-κB.

To my wonderful wife Kaye

to my son Andrew

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My wife and son, who make every day worth fighting for

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LIST OF ABBREVIATIONS

2244-TCB	2,2′,4,4′,-tetrachlorobiphenyl
3344-TCB	3,3′,4,4′-tetrachlorobiphenyl
33445-PCB	3,3′,4,4′,5-pentachlorobiphenyl
^{3}H-AA	[^{3}H-5,6,8,9,11,14,15]-Arachidonic acid
AA	Arachidonic acid
ActD	Actinomycin D
Ah	Aryl hydrocarbon
BEL	Bromoenol lactone
C/EBP	CCAAT/enhancer binding proteins
COX	Cyclooxygenase
DMF	N,N′-dimethylformamide
DMSO	Dimethylsulfoxide
ERK	Extracellular signal-regulated kinase
fMLP	formyl-methionyl-leucyl-phenylalanine
HBSS	Hank's Balanced Salt Solution
IL-1	Interleukin-1
IL-8	Interleukin-8
IP_3	inositol-1,4,5-triphosphate
$iPLA_2$	Ca^{2+}-independent PLA_2
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
MAP	Mitogen-activated protein
MEK	MAP kinase kinases
MPO	Myeloperoxidase
NF-κB	nuclear factor-kappaB
OP	oleyloxyethyl phosphorylcholine
NSAID	nonsteroidal anti-inflammatory drug
PCB	Polychlorinated Biphenyl
PG	Prostaglandin

PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
ROS	Reactive oxygen species
SB1	SB-202190
SB2	SB-203580
SER	Smooth endoplasmic reticulum
SOD	superoxide dismutase
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TEMPO	4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl
TNF-α	tumor necrosis factor-α
Tx	Thromboxane

Chapter I

Introduction

I.1 Preface

Polychlorinated biphenyls (PCBs) are toxic, environmental contaminants that elicit a wide range of biological effects in mammals. PCBs cause dermal and ocular effects, hepatotoxicity, neurotoxicity, and endocrine effects, effects on the reproductive system and effects on both the specific and non-specific branches of the immune system (Safe, 1994; Van den Berg *et al*, 2006).

PCBs can be divided into two classes based on the presence or absence of chlorine in the ortho- position. Congeners that lack chlorines in the ortho-positions are coplanar and bind with high affinity to the aryl hydrocarbon (Ah) receptor to induce changes in cellular function. Congeners containing chlorines substituted at the ortho- positions are not coplanar (noncoplanar) and are, in general, poor ligands for the Ah receptor. The mechanisms by which PCBs in this latter group cause functional changes in cells are not well understood.

Among the effects caused by ortho-substituted, noncoplanar PCB congeners is the alteration in function of polymorphonuclear neutrophils (Brown and Ganey, 1995; Kristoffersen *et al*, 2002; Olivero and Ganey, 2001; Olivero-Verbel and Ganey, 1998; Voie *et al*, 2000), one of the key cells of the non-specific immune system. The primary role of neutrophils is to attack and destroy invading microorganisms. They are normally quiescent and exhibit their biological activity when activated. Alteration in neutrophil function can have serious consequences for the organism. Failure of neutrophils to activate results in compromise of the innate immune system, leading to increased risk of infection. At the other end of the spectrum, inappropriate activation of

neutrophils can lead to inflammatory disease states and injury to host tissue (Repo, 1987).

Noncoplanar PCBs cause many changes in function of primary rat neutrophils including stimulation of degranulation, production of reactive oxygen species (ROS) such as superoxide anion, release of arachidonic acid (AA), changes in gene regulation, including upregulation of cyclooxygenase-2 (COX-2), and altered response to subsequent stimulation with other agents (Bezdecny *et* al, 2005; Brown and Ganey, 1995; Ganey *et al*, 1993; Olivero and Ganey, 2001; Tithof *et* al, 1998).

As mentioned above, the mechanisms by which noncoplanar PCBs cause toxicity is unknown. By seeking to understand the mechanisms by which noncoplanar PCBs evoke functional changes in a human, neutrophil-like cell line, we may be able to predict potential toxicity from these and similar xenobiotics as well as gain a better understanding of the functioning of human neutrophils. Accordingly, the overall goal of the work outlined in this dissertation was to test the hypothesis that 2,2',4,4',-tetrachlorobiphenyl (2244-TCB) increases COX-2 expression in granulocytic HL-60 cells, and that this increase depends on activation of intracellular signaling pathways including AA release, superoxide anion production and activation of the p38 MAP kinase.

To provide the background and rationale for these studies, in the remainder of this Introduction what is known about PCBs, especially noncoplanar PCBs, their chemistry and toxicity, and their effects on neutrophil and HL-60 cell function will be presented. In addition, what is known about

neutrophils and granulocytic HL-60 cells, with particular emphasis on markers of activation, will be reviewed. The function and regulation of the cyclooxygenase gene in neutrophils and HL-60 cells will also be discussed.

I.2 Polychlorinated biphenyls

I.2.A Occurrence and exposure

PCBs are ubiquitous, man-made, environmental contaminants that affect a number of cellular systems. PCBs were widely used for over 40 years due their heat-resistant properties, low conductivity and chemical inertness. These properties made PCBs a very useful component of safety fluids used to insulate and cool heavy electrical equipment such as transformers and capacitors (Hutzinger *et al*, 1974). PCBs as a class display a wide range of chemical properties, some of which include low vapor pressure, low water solubility and a relatively high resistance to chemical transformation (Shiu and Mackey, 1986). However the very same properties that made PCBs attractive for use in electrical equipment, i.e. their low water solubility and chemical inertness, allow PCBs to bioaccumulate and biomagnify in the food chain (Kannan *et al*, 1998).

There are no natural sources of PCBs; they only result from human activities. Between 1929 and 1977 over 700,000 tons of PCBs were produced in the United States by Monsanto Chemical Company (St. Louis, MO), according to the U.S. Environmental Protection Agency (USEPA, 1999). PCB production in the U.S. was halted in 1977 as the toxicity of PCBs began to be appreciated;

however an estimated one and a half million tons of PCBs have been released into the environment worldwide (Tanabe, 1987). Currently, PCBs can be found far from production sites due to their extensive use, persistence and wide distribution (Armitage *et al*, 2006; Subramanian *et al*, 1983). Remote sites where PCBs have been found include Antarctica (Kallenborn *et al*, 1998; Sobek *et al*, 2006), Alaska (Rubin *et al*, 2006) and lake water in Nepal (Galassi, 1997).

There are three current sources of PCBs in the environment. Many PCB-containing items produced before the ban on PCB production, particularly transformers and capacitors, are still in use around the world. As these items age the possibility for leakage of PCBs is increased. In addition, PCBs can be produced as an inadvertent byproduct in any industrial process that involves chlorine, carbon and high temperatures. Finally, the majority of PCBs that were disposed of before the production ban in 1977 were sent to landfills and other storage and disposal facilities that were not adequate to prevent release of PCBs into the environment. These facilities represent another source of environmental PCB contamination (USEPA, 1999).

PCBs are found environmentally as mixtures, the sources of which are commercial preparations (Tanabe *et al*, 1987). Commercially available PCB preparations comprise several congeners, both coplanar and noncoplanar, and these mixtures are labeled based on the total chorine content. For example, Aroclor 1254 consists of a mixture of PCB congeners that is approximately 54% chlorine by weight.

Due to the ubiquitous nature of PCBs, detectable levels of PCBs have been found in human blood, milk (Giesy and Kannan, 1998) and other tissues, such as adipose and placental tissue (Laden *et al*, 1999). The majority of human exposures are due to consumption of contaminated foods, particularly seafood (De Roos *et al*, 2005; Kostyniak *et al*, 2005), though occupational exposures also occur (Bosetti and Weerasinghe, 2003).

I.2.B Chemistry and structure

PCBs consist of a biphenyl ring with one to ten chlorine atoms attached (Figure I.1). There are 209 possible PCB congeners, depending on the number and position of the chlorine atoms (Safe *et al*, 1985). Approximately 130 possible congeners have been identified in commercially available PCB preparations (Giesy and Kannan, 1998). The metabolism, toxicity and mechanism of action vary among PCB congeners (Seegal, 1996). Generally PCBs exist as oily liquids or solids. They are colorless to light yellow in color and have no known smell or taste (ATSDR, 2001).

PCBs can be divided into two broad categories based on the chemical structure. PCB congeners that do not contain chlorine atoms at the ortho- positions (2, 2′, 6 or 6′) of the biphenyl ring can rotate freely around the central bond. These congeners can achieve a flat or "coplanar" conformation, in which both phenyl rings lie in the same plane. "Noncoplanar" PCB congeners do contain chlorine atoms substituted at the ortho- positions. The presence of these chlorine atoms causes steric hindrance that limits rotation around the central

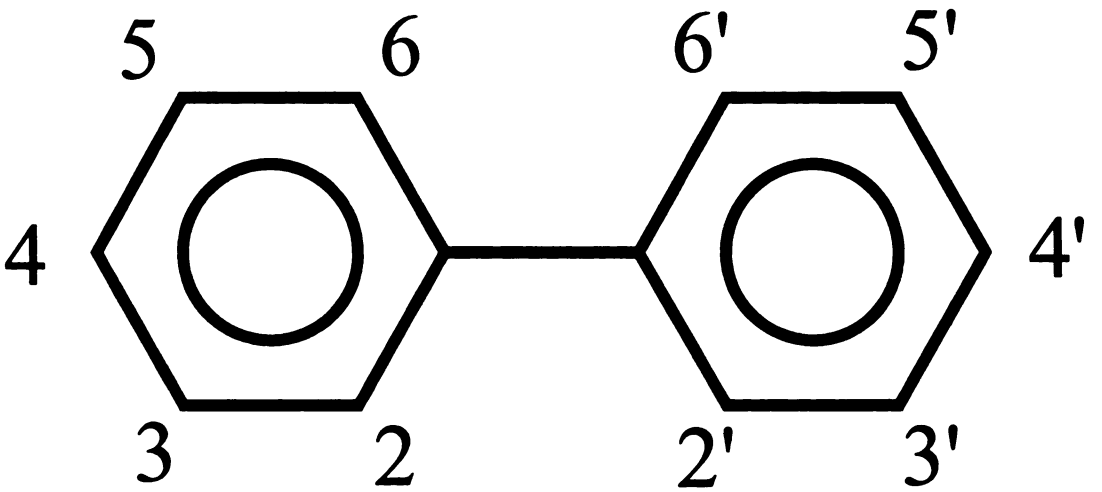

Figure I.1. Structure of the biphenyl ring of a PCB with attachment points for chlorine molecules indicated.

bond. This prevents both phenyl rings from lying in the same plane, forcing the molecule into a noncoplanar conformation. Coplanar and noncoplanar PCB congeners can cause different effects and act via different mechanisms (Safe *et al*, 1985).

In mammals, cytochrome P450 enzymes, including the 1A1, 1A2, 2B1 and 2B2 enzyme isoforms, can metabolize PCBs to intermediate arene oxides. These oxides are further metabolized into hydroxyl or methyl sulphone metabolites that are then converted into sulphate or glucuronide conjugates and excreted from the organism (Bergman *et al*, 1994). Metabolism of PCBs to more polar compounds is required for proper clearance; however rates of PCB metabolism vary greatly with species and with the degree and positions of chlorination. The most readily metabolized congeners have adjacent unsubstituted carbon atoms at the 3-4 positions. Congeners that lack these unsubstituted carbon atoms are metabolized very slowly and therefore cleared very slowly. PCBs that are not readily cleared tend to concentrate in adipose tissue (Matthews and Dedrick, 1984; Minh *et al*, 2006). The highly unstable arene oxides also react to form dihydrodiols, various hydroxyl metabolites as well as covalently bound protein, RNA and DNA adducts. These metabolites are generally less toxic then their parent compounds but still evince a variety of biological activities, including mitochondrial uncoupling, inhibition of various P450-dependent enzyme activities. Furthermore, they can interfere with proper hormone function, both by mimicking hormones such as estrogen, or binding to proteins involved in hormone transport and binding, such as prealbumin, a

major serum thyroxine-binding protein (Ahmad *et al*, 2003; Ghisari and Bonesfield-Jorgenson, 2005; Patterson *et al,* 1994; Safe, 1994; You *et al*, 2006).

Many toxicology studies have been performed using the seven different Aroclors that were produced commercially (ATSDR, 2001). The information derived from these studies, while useful, is limited in terms of understanding the specific mechanisms by which PCBs act, as percentages of specific congeners can vary among different lots of the same Aroclor mixture. Also, little is known about how individual PCB congeners in a mixture may interact to potentially cause synergistic or inhibitory biological effects.

Coplanar and noncoplanar PCBs are degraded in nature to differing extents. Coplanar PCBs tend to have shorter half-lives and are removed from the environment more rapidly than are noncoplanar congeners (Patterson *et al*, 1994). This leads to the enrichment of noncoplanar PCB congeners in the environment when compared to the commercially produced mixtures (Johansen *et al*, 1996). This enrichment of noncoplanar PCBs in the environment, coupled with the generally poor understanding of how these congeners mediate their effects, warrants further investigation into the specific mechanisms by which noncoplanar PCBs mediate their effects.

I.2.C Biological effects

I.2.C.1 General Effects

PCBs exhibit a wide range of biological effects in animals. PCBs are considered by the U.S. to be probable human carcinogens (Cogliano, 1998) and have been demonstrated to cause a number of non-cancer effects including dermal lesions and ocular effects (Nakayama, 1999), changes in hepatic glutathione regulation and hepatotoxicity (Twaroski *et al*, 2001; Sanderson *et al*, 1996) and endocrine effects including alterations in rat brain thyroid hormone status and reductions in progesterone levels (Augustowska *et al*, 2001; Morse *et al*, 1996; Safe, 2004). In addition PCBs have effects on the male rat reproductive system including reduced sperm production and increased numbers of abnormal sperm, as well as increased testicular and prostate weights (Faqi *et al*, 1998). Effects on the nervous system caused by PCB exposure include poorer gross motor function, poorer infant visual recognition memory and general cognitive deficits (Jacobson and Jacobson, 1997). Impaired cardiovascular function (Carpenter, 2006; Warshaw *et al*, 1979) and modulation of both the specific and nonspecific branches of the immune system also occur due to PCB exposure (Bezdecny *et al*, 2005; Carpenter, 2006; Vos and Van Loveren, 1998).

Coplanar and noncoplanar PCB congeners cause different effects via different mechanisms. Coplanar PCBs are good ligands for the Ah receptor and can cause gene induction via an Ah receptor-dependent mechanism (Safe *et al*, 1985). Indeed, the structure and toxic effects of coplanar congeners are similar

to those of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the prototypical ligand for the Ah receptor (Kafafi *et al*, 1993). For example, like TCDD, 3,3',4,4',5-pentachlorobiphenyl (33445-PCB), one of the best studied PCB congeners, induces hepatic enzymes involved in xenobiotic metabolism, such as aryl hydrocarbon hydroxylase (Sanderson *et al*, 1996), and promotes hepatotoxicity by reducing hepatic levels of glutathione and glutathione-related enzymes (Twaroski *et al*, 2001). 33445-PCB has also been implicated in endocrine disruption, including decreases in progesterone levels (Augustowska *et al*, 2001) and increases in thyroid hormone metabolism (Van Birgelen *et al*, 1995). In addition, 33445-PCB causes behavioral effects and learning deficits in rats (Rice, 1996). Immunosuppression, a hallmark of PCB exposure, has also been linked to 33445-PCB exposures. Specifically, reductions in the antibody response (Regala *et al*, 2001) and thymic atrophy with decreases in CD4 and CD8 T-cell levels (Grasman and Whitacre, 2001; Fox and Grasman, 1999) have been demonstrated. These effects are all mediated via activation of the Ah receptor, and TCDD exposure causes similar effects (Birnbaum and Tuomisto, 2000; Camacho *et al,* 2004; Fujimaki *et al,* 2002; Kakeyama and Tohyama, 2003; Kim and Yang, 2005; Viluksela *et al,* 2000; Watanabe *et al,* 2004)

In contrast to coplanar PCBs, noncoplanar PCBs do not make good ligands for the Ah receptor due to their inability to form the flat, coplanar conformation required to bind to the receptor. Because of this fact, the toxicity of noncoplanar PCBs has been considered secondary to that of the coplanar congeners for many years. It has now been shown that noncoplanar PCBs can

cause a number of effects on biological systems, including effects on the nervous, endocrine, reproductive, and immune systems (Fischer *et al*, 1998; Loch-Caruso, 2002). Some studies have suggested that noncoplanar PCBs can interact directly with cellular receptors (Angus and Contreras, 1995; Pessah *et al*, 2006; Wong *et al*, 1997); however a receptor-mediated mechanism of action for the toxicity of noncoplanar PCBs has yet to be elucidated. The effects noncoplanar PCBs have on tissues and organs include increased release and decreased synthesis of insulin in rat insulinoma cells (Fischer *et al*, 1996), changes in the concentrations and distribution of dopamine and 5-hydroxytryptamine in female rat brains (Chu *et al*, 1996), increases in rat liver weight (Lecavalier *et al*, 1997), reduced phagocytosis by neutrophils and monocytes in marine mammals (Levin *et al*, 2004), and modulation of superoxide anion production in rat neutrophils (Brown and Ganey, 1995).

On a cellular level there are many effects of exposure to noncoplanar PCBs. One such effect is an increase in intracellular free Ca^{2+} levels. Two distinct pathways have been described whereby noncoplanar PCBs increase intracellular Ca^{2+} concentrations. The first pathway involves activation of phospholipase C (PLC), leading to increased production of inositol-1,4,5-triphosphate (IP_3). IP_3 then interacts with IP_3 receptors on the smooth endoplasmic reticulum (SER), causing the release of Ca^{2+} from intracellular stores (Tithof *et al*, 1995). The second route by which intracellular Ca^{2+} can be increased by noncoplanar PCBs involves stabilization of ryanodine receptors by an immunophilin FKBP12-dependent mechanism. Exposure to noncoplanar

PCBs facilitates interaction between FKBP12 and ryanodine receptors, leading to conformational changes in the ryanodine receptor. These conformational changes cause an increased probability of the ryanodine receptor being in an open configuration; leading to increased release of calcium from SER stores (Wong and Pessah, 1997).

Alterations in Ca^{2+} homeostasis caused by exposure to PCBs have been demonstrated in neutrophils (Brown and Ganey, 1995), in cerebellar granule cells (Kodavanti *et al*, 1993), in uterine tissue (Bae *et al*, 1999; Loch-Caruso, 2002) and in mammalian brain (Wong and Pessah, 1997). Changes in Ca^{2+} homeostasis induced by noncoplanar PCBs have been shown to alter the synthesis and release of insulin in a cultured cell line (Fischer *et al*, 1996) as well as to change dopamine levels in areas of the brain (Seegal *et al*, 1990).

I.2.C.2 Effects in neutrophils

The focus of this dissertation is to better understand the impact of exposure to noncoplanar PCBs on neutrophil function. Exposure to noncoplanar PCBs causes increased kinase activity, activation of phospholipase A_2 (PLA_2), increased superoxide anion production, modulation of degranulation and increased production of inositol phosphates in rat neutrophils (Brown *et al*, 1998; Brown and Ganey, 1995; Olivero and Ganey, 2000; Tithof *et al*, 1997; Tithof *et al*, 1996; Tithof *et al*, 1995). Additionally, in neutrophils isolated from marine mammals, such as beluga whales and bottlenose dolphins, PCB exposure decreased phagocytosis (Levin *et al*, 2004). Interestingly, the same

PCB exposure did not affect neutrophil phagocytosis in mice (Levin *et al*, 2005). In human neutrophils, PCB exposure caused increased activation of NADPH oxidase and subsequent increases in superoxide anion production (Fonnum *et al*, 2006) as well as modulation of leukotriene production in neutrophils stimulated with other agents (Raulf and Konig, 1991).

Human exposure to PCBs can result in increased risk of disease due to PCB-mediated suppression of the immune system (Aoki, 2001; Carpenter, 2006; Levin *et al*, 2005). Human exposure to PCBs causes suppression of the delayed-type skin response to bacterial proteins (Chang *et al*, 1982), decreased phagocytosis in leukocytes (Levin *et al*, 2005) and decreased immune response to allergens (Noakes *et al*, 2006). Additionally, PCBs decrease overall antibody levels (Nakanishi *et al*, 1985), but increase the levels of autoantibodies, increasing the risk of autoimmune disease (Schoenroth *et al*, 2004). PCBs also increase the carcinogenic effects of other chemicals, increasing the risk of tumor formation (Carpenter, 2006).

To understand the mechanisms by which noncoplanar PCBs affect function of neutrophils, it is important to understand neutrophil function under physiologic conditions.

I.3 Neutrophils: Physiological function

Neutrophils are the predominant white blood cell in the human circulatory system and are one of the most important cells of the innate immune system. The primary role of neutrophils is to attack and destroy invading

microorganisms. They exhibit the most rapid response to tissue injury of any immune cell. Neutrophils are also one of the major cell types involved in the inflammatory response. During tissue injury neutrophils are recruited from the bloodstream to the site of damage. This recruitment involves activation of both neutrophils and vascular endothelial cells, leading to expression of cellular adhesion molecules on both cell types. E- and P-selectins expressed by endothelial cells weakly interact with mucin-like or sialyl Lewis adhesion molecules on the neutrophil, leading to the neutrophil rolling along the endothelial cell lining of the blood vessel. During rolling the neutrophil typically becomes activated by chemoattractant factors, leading to increased interaction between neutrophil integrin molecules and Ig-superfamily adhesion molecules on the endothelium. This interaction stabilizes the neutrophil on the endothelium, and it then migrates through the vessel wall into the tissue (Tonnesen, 1989). Once at the site of injury, neutrophils can destroy invading microorganisms by releasing cytotoxic enzymes and stored mediators from intracellular granules, as well as by phagocytosis of microorganisms and production of reactive oxygen species. This process is referred to as neutrophil "activation" (Clark, 1990; Cohen, 1976; McDonald, 2004).

I.4 Neutrophils: Activation

Neutrophils are normally quiescent, only exhibiting their biological activity when activated. Given the role of neutrophils in host defense and the requirement for activation, the mechanisms by which neutrophils become

activated are of great importance. Failure of neutrophils to activate results in severe compromise of the immune system, leading to increased risk of infection. At the other end of the spectrum, inappropriate activation of neutrophils can lead to inflammatory disease states, as the activated neutrophils damage host tissue (Kulberg *et al,* 1999). Neutrophils can be activated by inflammatory stimuli such as interleukin-1 (IL-1), interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α) as well as by products released by bacteria such as lipopolysaccharide (LPS) and formyl-methionyl peptides (Ellis and Beaman, 2004; Wilson, 1985). Activation of neutrophils involves a series of intracellular biochemical events in response to exposure to activating stimuli. These events are responsible for the changes observed in neutrophil function following activation, such as degranulation and production of superoxide anion. The end result of this activation is the migration of neutrophils to sites of inflammation and production of mediators that can degrade and destroy invading microorganisms (Becker, 1976; Haag-Weber *et al,* 1989). Gaining a better understanding of neutrophil function is a critical step in understanding the mechanisms by which neutrophils participate in both host defense and inflammation.

Of the variety of stimuli that can activate neutrophils the peptidyl agent formyl-methionyl-leucyl-phenylalanine (fMLP) is the most widely studied and understood and serves to illustrate the biochemical processes involved in neutrophil activation. The agent fMLP activates neutrophils, leading to ROS production and degranulation. fMLP is also a total degranulation stimulus; that

is, exposure to fMLP induces degranulation of all three classes of neutrophil granules (see section I.6.A). As such, fMLP is useful in demonstrating the biochemical processes involved in neutrophil activation.

The process of fMLP-induced activation is mediated via a membrane G-protein-coupled receptor, which leads to Ca^{2+} mobilization (Lad *et al*, 1985) and activation of second messengers such as PLC, tyrosine kinases, protein kinase C (PKC), phospholipase D (PLD) and PLA_2 (Krause *et al*, 1985).

I.5 The HL-60 cell line

Due to the difficulty in obtaining and using human neutrophils, human cell culture lines are often used as a substitute in experimental procedures. HL-60 cells are one such cell line. The HL-60 cell line is a human, promyelocytic leukemia cell line. HL-60 cells are predominately promyelocytes and exhibit distinct morphological and histochemical myeloid characteristics (Collins *et al*, 1979). HL-60 cells, especially those under passage 60, display spontaneous differentiation, which can be increased by exposure to polar-planar agents, like dimethylsulfoxide (DMSO) or other agents such as retinoic acid (Breitman *et al*, 1980; Collins *et al*, 1978). HL-60 cells can be differentiated into four general types of cells: these are (a) granulocytes, (b) monocytes, (c) macrophage-like cells and (d) eosinophils (Collins, 1987). These categories are somewhat arbitrary, as certain differentiating agents give rise to cells that exhibit characteristics from multiple categories. In this Introduction I will focus on the granulocyte differentiation used in this dissertation research.

Several compounds can be used to induce granulocytic differentiation. In addition to polar-planar compounds and retinoic acid, actinomycin D (ActD), hypoxanthine, tunicamycin, 6-thioguanine and L-ethionine can induce granulocytic differentiation of HL-60 cells (Collins, 1987). Different inducing compounds act via different mechanisms and can cause differing degrees of cellular maturation (Dufer *et al*, 1989). Of the compounds that induce granulocytic differentiation in HL-60 cells, DMSO is the most widely used. As little as 8-18 hours of exposure to DMSO is enough to induce terminal differentiation of a significant subset of the HL-60 cells exposed to the treatment (Tsiftsoglou, 1985). Initiation of differentiation leads to a dramatic decrease in cell proliferation; cell division comes almost to a halt after three days of exposure to DMSO (Brackman *et al*, 1995) and differentiated cells die via apoptosis within two weeks (Martin *et al*, 1990). While differentiation can be initiated in as little as 8 hours, HL-60 cells are typically treated with DMSO for 5-7 days. After this period of treatment the majority of the treated cells have differentiated into morphologically mature myelocytes, metamyelocytes and banded or segmented neutrophils (Collins, 1978).

Being that these differentiated cells have their origin in an immortalized cell line, they are not identical to true human neutrophils. In fact by several standards granulocytic differentiation of HL-60 cells is deficient. They have decreased numbers of secondary granules; they are predominantly metamyelocytes and banded neutrophils as opposed to fully differentiated, multilobed neutrophils; and their LDH isoenzyme profile differs quantitatively

from true human neutrophils, all of which are consistent with incomplete differentiation (Collins, 1987). However, despite their leukemic origin and karyotypic abnormalities (Collins *et al*, 1977), differentiated HL-60 cells display the functional characteristics commonly associated with normal human blood granulocytes, including chemoattractant responses, phagocytosis, superoxide anion production, microorganism killing and expression of cell surface receptors (Collins, 1987; Newburger *et al*, 1979; Collins *et al*, 1978). Additionally, differentiated HL-60 cells produce a number of eicosanoids upon activation. These eicosanoids include PGE_2, $PGF_{1\alpha}$, $PGF_{2\alpha}$,, PGD_2 and thromboxane B_2 (Sadler and Badwey, 1988; Sanduja *et al*, 1988). All these properties are either absent in undifferentiated HL-60 cells, or are greatly reduced in comparison to granulocytically differentiated cells. Granulocytic HL-60 cells are not, however, as efficacious in these functions as are normal human granulocytes. The maximal performance of each of these functions in HL-60 granulocytes is 50-100% compared to a normal neutrophil (Newburger *et al*, 1979).

While HL-60 cells have some differences from true human neutrophils, they remain very similar functionally and can serve as a useful model to study human neutrophil function. HL-60 cells undergo activation events similar to those in neutrophils such as oxidative burst, degranulation and the cyclooxygenase/arachidonic acid cascade. As these activation events are relevant to this thesis, they will be discussed here.

I.6 Markers of neutrophil activity

I.6.A Degranulation

The degranulation response in neutrophils can be triggered by chemical factors such as fMLP and the complement protein C5a (Henson *et al*, 1978) and is one of the most studied cellular processes that neutrophils undergo. It involves the exocytosis of three types of intracellular vesicles, or granules, which contain hydrolytic enzymes such as elastase, β-glucuronidase, lysozyme and myeloperoxidase (MPO). Different types of granules contain different components and exhibit different kinetics of degranulation.

Primary azurophillic granules contain MPO, lysozyme and the membrane protein CD63 (Kuijpers *et al*, 1991). MPO generates hypochlorous acid and other halogenated agents as a host defense mechanism. However these compounds can also cause host tissue injury (Hazen *et al*, 1996). Secondary granules, also called secretory vesicles, are more easily mobilized than are the primary azurophillic granules. Secondary granules contain cytochrome B_{558} and the CD11b/CD18 heterodimeric glycoprotein (Calafat *et al*, 1993). Tertiary granules store gelatinase, and release of these granules has been implicated in the upregulation of the CD11b/CD18 complex on the plasma membrane (Lacal *et al*, 1988).

I.6.B Oxidative burst

Neutrophils phagocytose invading microorganisms, and phagocytosis leads to increased oxygen consumption by the cell, due to ROS

production. ROS then destroy the phagocytosed microorganism. The main enzyme involved in ROS production in neutrophils is NADPH oxidase. For NADPH oxidase to function properly, both cytoplasmic as well as membrane-associated proteins have to be assembled into a membrane-bound complex. Once assembled this complex converts molecular oxygen into superoxide anion. A variety of factors facilitate assembly of the NADPH oxidase complex in neutrophils, including linoleic acid and AA (Bouzidi and Doussiere, 2004; Henderson *et al*, 1993) which increase the number of assembled NADPH oxidase complexes as well as the affinity of these complexes for molecular oxygen. Eicosanoids, such as leukotriene B promotes p47phox phosphorylation and translocation (Serezani *et al,* 2005), and homocysteine which increases translocation of p47phox and p67phox subunits of NADPH oxidase to the plasma membrane (Alvarez-Maqueda *et al,* 2004). In addition, diacylglycerol and phoshpatidic acid interact directly with NADPH oxidase components (Palicz *et al,* 2001).

I.6.C Arachidonic acid release and the eicosanoid cascade

AA is a fatty acid that plays a very important role in the neutrophil, both as a second messenger and as a substrate for eicosanoid production. In resting cells, AA is stored within the cell membrane, esterified to glycerol in phospholipids. A receptor-dependent event, requiring a transducing G protein, initiates phospholipid hydrolysis and releases the fatty acid into the intracellular medium. Three enzymes are capable of mediating this deacylation

reaction: PLA_2, PLC, and PLD. PLA_2 catalyzes the hydrolysis of phospholipids at the sn (stereospecific numbering)-2 position. Therefore, this enzyme can release AA in a single-step reaction. By contrast, PLC and PLD do not release free arachidonic acid directly. Rather, they generate lipid products containing AA (diacylglycerol and phosphatidic acid, respectively), which can be released subsequently by diacylglycerol- and monoacylglycerol-lipases (Dennis *et al*, 1991).

Once released, free AA has three possible fates: reincorporation into phospholipids, diffusion outside the cell, and metabolism into eicosanoids. Metabolism is carried out by three distinct enzyme pathways expressed in neural cells: cyclooxygenase, lipoxygenases, and cytochrome P450 (Figure I.2.). Several products of these pathways act within cells to modulate the activities of ion channels, protein kinases, ion pumps, and neurotransmitter uptake systems. The newly formed eicosanoids may also exit the cell of origin and act at a distance, by binding to G-protein-coupled receptors present on nearby cells. Finally, the actions of the eicosanoids are terminated by diffusion, uptake into phospholipids, or enzymatic degradation (Bos *et al*, 2004).

Examples of eicosanoids include the prostaglandins (PGs), thromboxanes, leukotrienes, and epoxyeicosatrienoic acids. They play roles in inflammation, fever, regulation of blood pressure, blood clotting, control of reproductive processes and tissue growth, and regulation of the sleep/wake cycle (Abramson and Weissmann, 1989; Bos *et al*, 2004; Dennis *et al*, 1991).

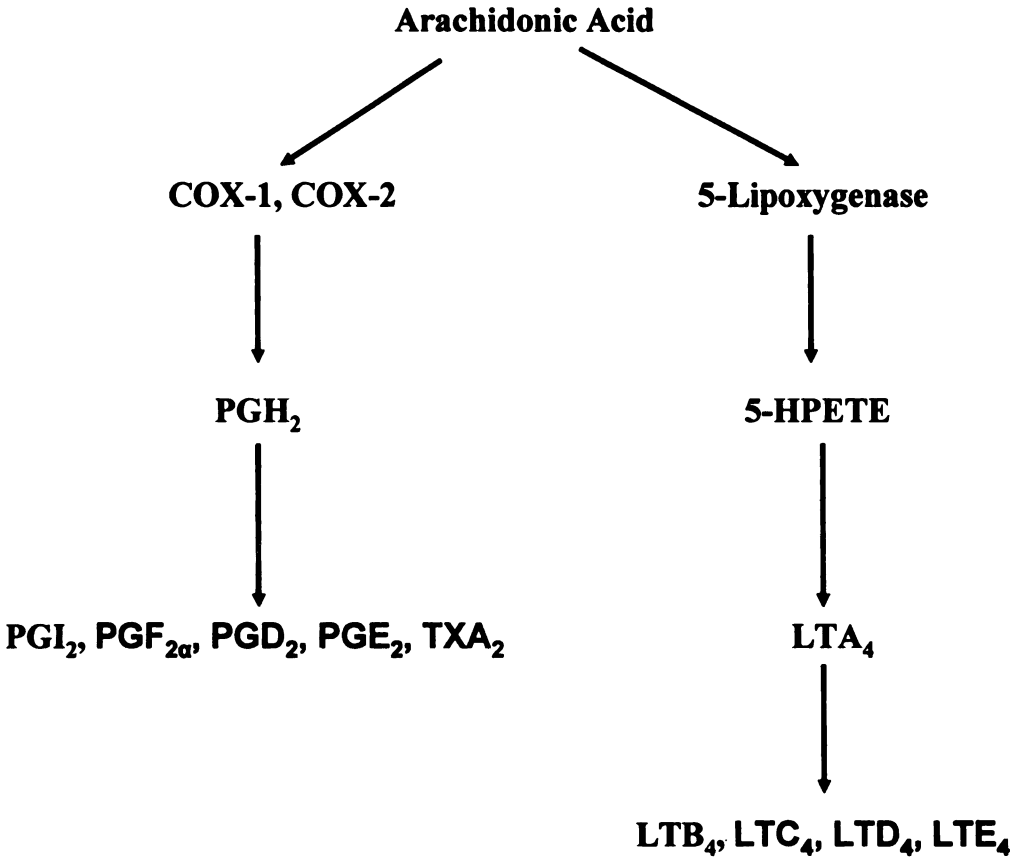

Figure I.2. Steps in the synthesis of eicosanoids from arachidonic acid.

AA can also act alone as a second messenger upon release. Free AA modulates the activity of a number of cellular components, including increasing PKC activation (Chalimoniuk *et al,* 2004). Release of calcium from intracellular stores (Peppiatt *et al,* 2004) is also caused by free AA. In addition downregulation of nitric oxide formation (Palomba *et al,* 2004), mediation of muscarinic inhibition and enhancement of N-type calcium current in sympathetic neurons (Liu and Rittenhouse, 2003) as well as activation of p42 MAP kinase, c-Jun kinase, and p38 MAP kinase (Hernandez *et al,* 1998).

I.6.D Cyclooxygenases

I.6.D.1 Cyclooxygenase Function

COX-1 and COX-2 are important enzymes that catalyze the committed step in the synthesis of prostaglandins and thromboxanes. Cyclooxygenases are particularly important, as they are the main targets of nonsteroidal anti-inflammatory drugs (NSAIDS) such as aspirin, ibuprofen and COX-2-specific inhibitors like celecoxib. Inhibition of cyclooxygenases acutely reduces inflammation, pain and fever. Cyclooxygenases have also been implicated in fatal thrombotic events, as well as in the development of Alzheimer's disease and several types of cancers (Smith *et al*, 2000).

The first step catalyzed by cyclooxygenases involves the oxidation of AA to hydroperoxy endoperoxide PGG_2 by means of the cyclooxygenase activity of the COX enzyme. The second step is the reduction of this intermediate into hydroxy endoperoxide PGH_2 by means of the peroxidase

activity of the COX enzyme. PGH_2 can then be transformed by a wide range of enzymatic and non-enzymatic mechanisms into the primary prostanoids, which include PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2 and TxA_2 (Smith *et al*, 2000; Vane *et al*, 1998). These products then exit the cell via a carrier-mediated process (Chan *et al*, 1998) where they can act on G protein-linked prostanoid receptors, or in some cases these products may act on nuclear receptors.

COX-1 and COX-2, while arising from different genes, are very similar both in structure and in their catalytic activity and can be co-expressed in the same cell. COX-1 activity is found in most cells of the body but is of particular importance in cells of the gastrointestinal tract, the kidney, in platelets and in regions of the CNS, notably in the forebrain. COX-1 resides in the endoplasmic reticulum, and its activity is constitutive and is typically found at constant levels (Vane *et* al, 1998; Smith *et al*, 1996). Products of COX-1 act as "local" hormones (Smith *et al*, 1996).

COX-2 activity is typically present at low to negligible levels in normal cells; however, it is strongly induced by a variety of pro-inflammatory stimuli, such as TNF-α, IL-1, IL-2 and LPS. COX-2 is an immediate early gene: protein levels can increase and decrease within hours after exposure to a single stimulus (Smith *et al*, 2000; Vane *et al*, 1998). Both *de novo* synthesis and post-transcriptional regulation have been shown to contribute to the magnitude and duration of COX-2 mRNA expression (Smith *et al*, 1996). COX-2 activity is most commonly observed in inflammatory cells and is also constitutively present in several regions of the central nervous system (cortex, hippocampus,

hypothalamus, spinal cord) where its products appear to mediate fever and pain responses. COX-2 is considered to serve two roles in cells where it is expressed. The first is to augment the activity of COX-1 in the endoplasmic reticulum or to substitute for COX-1 in cells that do not express it. The second role for COX-2 takes place on the lumenal surface of the inner membrane of the nuclear envelope. Prostanoids synthesized here do not get exported from the cell, but instead act on nucleoplasmic or nuclear membrane targets. It is thought these prostanoids play a role in cell differentiation and replication (Smith *et al*, 2000).

I.6.D.2 Cyclooxygenase-2 Regulation

A number of studies have been performed to determine the mechanisms by which COX-2 is induced, and from these some general conclusions can be drawn about regulation of this gene. The most commonly elevated growth factors and mediators of inflammation, such as IL-1, TNF-α, and LPS, are the factors to which the COX-2 gene is most responsive (Smith *et al*, 2000; Vane *et al*, 1998). A number of shared or convergent pathways have been described that are likely involved in transcriptional regulation of COX-2 in response to inflammatory mediators. These include pathways involving nuclear factor-kappaB (NF-κB) and the CCAAT/enhancer binding proteins (C/EBP; Poli, 1998; Ghosh *et al*, 1998). In addition, one or more of three MAP kinase pathways may be involved (Su and Karin, 1996).

In mammalian cells activity of NF- κB is regulated by its association with its inhibitory subunit, IκB. NF-κB is activated by many of the same agents that

activate neutrophils, such as IL-1, TNF-α and endotoxin. Whereas the initial steps involved in NF-κB activation vary among cell types these compounds ultimately activate kinases that phosphorylate the IκB subunit, causing it to dissociate from NF-κB. NF-κB is then translocated into the nucleus where it binds to transcriptional response elements (Wulczyn *et al*, 1996). There are four NF-κB binding sites on the COX-2 promoter and NF- κB has been repeatedly shown to play an important role in the regulation of COX-2, particularly in neutrophils and granulocytic HL-60 cells (Han *et al,* 2005; Keum *et al,* 2003; Takada and Aggarwal, 2004).

C/EBP is a family of transcription factors containing six known members. In neutrophils C/EBPs play an important role in cellular maturation. The regulation of C/EBPs seems to involve transcription of both full-length and truncated isoforms of the specific C/EBP. The full-length isoforms bind to and activate C/EBP binding domains in the nucleus, changing gene transcription. The truncated isoforms bind to the DNA, but lack the transactivation domain required for their transcriptional activity, both failing to change gene transcription as well as preventing "active", full-length C/EBP from binding (Lekstrom-Himes, 2001). C/EBP activation in regulation of the COX-2 gene has been shown in murine macrophages (Chen *et al,* 2004), human endometrial stromal cells (Tamura *et al,* 2003), human T-cells (Barat and Tremblay, 2003) as well as osteoblastic MC3T3-E1 cells (Harrison *et al,* 2000).

MAP kinase pathways have also been implicated in COX-2 regulation. There are three major families of MAP kinases: the p38 kinase family, the

extracellular signal-regulated kinase (ERK) family and the c-Jun N-terminal kinase (JNK) family. All three of these regulate COX-2 expression. MAP kinase signaling cascades can be activated by a variety of different cellular stimuli and mediate a wide range of responses (Chang and Karin, 2001). The ERK-pathway is activated in response to cytokines and growth factors and primarily mediates mitogenic and anti-apoptotic signals. The p38 family of MAP kinases is primarily activated by stress stimuli and also by the activation of some cytokine receptors. The p38 MAP kinase family is involved in the regulation of apoptosis and cell cycle arrest, induction of cellular differentiation, cytokine production and inflammation. The JNK MAP kinase family is also activated in response to stress and growth factors and mediates signals regulating apoptosis, cytokine production and cell cycle progression (Kyriakis and Avruch, 1996).

Activation of MAP kinases requires a dual phosphorylation on threonine and tyrosine residues found in specific motifs for each kinase family. The phosphorylation of each MAP kinase group is regulated by upstream kinases called MAP kinase kinases (MEKs) which are relatively specific for their target MAPK. MEK1 and 2 act on the ERK family, MEK3, 4 and 6 act on the p38 family and MEK4 and 7 act on the JNK family of kinases. Activation of MEK is regulated by another upstream group of serine/threonine kinases, called MEK kinases, which phosphorylate MEKs on specific serine residues. Some of the MEK kinases that have been identified include the Raf family of kinases, Mos and Tpl2, which are specific for ERK activation, and the mixed-lineage kinases,

Mekk kinases, Tak1, Ask1 and Ask2, which act on both the p38 and JNK families of kinases (Platanias, 2003).

MAP kinase-dependent regulation of COX-2 expression has been shown in numerous systems, involving all three MAP kinase cascades. The p38 MAP kinase regulates COX-2 transcription as well as mRNA stability in human monocytes (Dean *et al,* 1999) and Hela cells (Lasa *et al,* 2000). In addition p38 activation is required for increased COX-2 expression in human chondrocytes (Nieminen *et al,* 2005), ciliary and intestinal epithelial cells (Rosch *et al,* 2005; Shafer and Slice, 2005), as well as in human synovial fibroblasts and macrophages (Faour *et al,* 2003). Activation of the ERK cascade leads to upregulation of COX-2 in human colon and prostate cancer cell lines (Wang *et al,* 2005), human keratinocytes (Kanda *et al,* 2005), intestinal epithelial cells (Slice *et al,* 2004), rat cortical astrocytes (Brambilla *et al,* 2002) and in RAW 264.7 cells (Moon and Pestka, 2002). JNK activation is also involved in the regulation of COX-2 in a wide variety of systems, including RAW 264.7 cells (Uto *et al,* 2005; Wadleigh *et al,* 2000), human keratinocytes (Cho *et al,* 2005), human synoviocytes (Crofford *et al,* 2005), in a mouse model of Parkinson's disease (Hunot *et al,* 2004), in bronchial epithelial cells (Mizumura *et al,* 2003) and in HeLa cells and human fibroblasts (Holzberg *et al,* 2003).

I.7 Summary

In summary, noncoplanar PCBs are highly toxic compounds that impact neutrophil function via poorly understood mechanisms. One major effect that

noncoplanar PCBs have upon neutrophil function is the upregulation of the inflammatory gene COX-2 (see below). Accordingly, studies have been undertaken to elucidate the signal transduction pathways involved in noncoplanar PCB-mediated changes in regulation of the COX-2 gene. These studies were performed using the HL-60 cell line as a model of the human neutrophil and are described in detail in the following chapters. The overall hypothesis tested in these studies was that 2244-TCB increases COX-2 expression in granulocytic HL-60 cells and this increase depends on activation of intracellular signaling pathways including arachidonic acid release, superoxide anion production and activation of the p38 MAP kinase. To test this hypothesis, the following specific aims were addressed:

1) To determine the effects PCBs have on the transcription, translation and function of the COX-2 gene. This aim was addressed by determining the levels of COX-2 mRNA, the levels of COX-2 protein and the activity of COX-2 protein present in differentiated HL-60 cells treated with PCBs. These results will be discussed in chapters 2 and 3 of this dissertation.

2) To determine the signaling pathway(s) involved in the elevation of COX-2 mRNA in PCB-exposed HL-60 cells. This aim was addressed by assessing expression of COX-2 mRNA, protein and function in differentiated HL-60 cells treated with or without PCBs in the presence of pharmacological inhibitors of specific pathways of interest. These results will be discussed in chapters 3 and 4.

3) To determine if PCB-induced production of reactive oxygen species is linked causally to changes in COX-2 mRNA levels in differentiated HL-60 cells. This aim was addressed by measuring production of superoxide anion in differentiated cells treated with PCBs in the presence or absence of superoxide dismutase and examining whether inhibition of superoxide anion by the inhibitors apocynin and 5-hydroxy TEMPO affects COX-2 mRNA levels. These results will be discussed in chapter 3.

Chapter II

Effects of 2,2′,4,4′-Tetrachlorobiphenyl on Granulocytic HL-60 cell Function and Expression of Cyclooxygenase-2

II.1 Summary

PCBs are persistent environmental contaminants that affect a number of cellular systems, including neutrophils. It has been demonstrated that noncoplanar PCBs alter function of primary rat neutrophils. The objectives of these experiments were to determine if responses in a human, neutrophil-like cell line exposed to PCBs were similar to those reported for rat neutrophils and to explore further PCB-mediated alterations in neutrophil function. The human promyelocytic leukemia cell line (HL-60) was differentiated with DMSO to a neutrophil-like phenotype. Treatment of differentiated HL-60 cells with 2244-TCB, a noncoplanar, ortho-substituted PCB congener, caused an increase in fMLP-induced degranulation, as measured by release of MPO. Treatment with the coplanar, non-ortho-substituted congener 3,3′,4,4′-tetrachlorobiphenyl (3344-TCB) had no effect on MPO release. 2244-TCB caused a time- and concentration-dependent release of [^{3}H]-arachidonic acid (^{3}H-AA). A significant increase in ^{3}H-AA release was observed after 60 minutes of exposure, and concentrations of 10 μM or greater increased ^{3}H-AA release. In contrast, 3344-TCB had no effect on ^{3}H-AA release. The effect of PCBs on mRNA levels for COX-2 was examined using semiquantitative RT-PCR. COX-2 mRNA was significantly elevated in response to 2244-TCB in a concentration-dependent manner. COX-2 expression was maximal by 30 minutes of exposure to 2244-TCB. COX-2 protein and activity were also increased after exposure to 2244-TCB; COX-1 protein and activity were unaffected. 3344-TCB did not increase COX-2 mRNA levels. These results

demonstrate that a noncoplanar PCB alters the functional status of granulocytic HL-60 cells, causing enhanced degranulation and upregulation of COX-2, whereas a coplanar PCB lacks this activity. These data suggest that noncoplanar PCBs alter HL-60 cell function and COX-2 expression via an Ah-receptor-independent mechanism.

II.2 Introduction

PCBs are ubiquitous, man-made, environmental contaminants that have been shown to affect a number of cellular systems. PCBs were used widely for over 40 years due to their heat resistant properties, low conductivity and chemical inertness. These properties made PCBs a very useful component of safety fluids used to insulate and cool heavy electrical equipment such as transformers and capacitors (Hutzinger *et al*, 1974). The lipophilic nature of PCBs and their resistance to chemical transformation allow them to bioaccumulate in the food chain (Kannan *et al*, 1998). This has led to exposures of humans and wildlife to PCBs. PCBs have been detected in human blood, milk, adipose tissue and placental tissue (Giesy and Kannan, 1998; Laden *et al*, 1999).

PCBs exhibit a wide range of biological effects in animals. They are probable human carcinogens (Cogliano, 1998) and cause a number of non-cancer effects, including dermal lesions and ocular effects in humans, and hepatotoxicity and endocrine effects in rats (Morse *et al*, 1996; Takamatsu *et al*, 1985; Twaroski *et al*, 2001). In addition, PCBs have effects on the reproductive

system (Faqi *et al*, 1998), nervous system (Jacobson and Jacobson, 1997), cardiovascular system (Warshaw *et* al, 1979) and both the specific and non-specific branches of the immune system (Vos and Loveren, 1998).

The specific effects PCBs produce are related to their structure. PCBs can be divided into two broad classes based on the presence or absence of chlorine atoms in the ortho- position. Congeners that lack chlorines in the ortho-positions are coplanar and bind with high affinity to the aryl hydrocarbon (Ah) receptor to induce changes in cellular function. Congeners containing chlorines substituted at the ortho- positions cannot attain a coplanar configuration and are, in general, poor ligands for the Ah receptor. The mechanisms by which these noncoplanar PCBs cause functional changes in cells are not well understood (Bandiera *et al*, 1982).

Among the effects caused by ortho-substituted, noncoplanar PCB congeners is the alteration in function of polymorphonuclear neutrophils (Brown and Ganey, 1995; Kristoffersen *et al*, 2002; Olivero and Ganey, 2001; Olivero-Verbel and Ganey, 1998; Voie *et al*, 2000). The primary role of neutrophils is to attack and destroy invading microorganisms. They are normally quiescent and exhibit their biological activity when activated. Alteration in neutrophil function can have serious consequences for the organism. Failure of neutrophils to activate results in compromise of the innate immune system, leading to increased risk of infection. At the other end of the spectrum, inappropriate activation of neutrophils can lead to inflammatory disease states and injury to host tissue. Noncoplanar PCBs cause many changes

in the function of primary rat neutrophils including stimulation of degranulation, production of ROS such as superoxide anion, release of AA and altered response to subsequent stimulation with other agents (Brown and Ganey, 1995; Ganey *et al*, 1993; Olivero and Ganey, 2001; Tithof *et* al, 1998). Given the role of neutrophils in host defense and the requirement for activation, understanding the mechanisms by which neutrophils become activated in response to exposure to PCBs is important.

One fate of AA released in response to PCB exposure is that it may serve as a precursor for eicosanoid synthesis, which is catalyzed by the cyclooxygenase enzymes. One isoform of cyclooxygenase, COX-2, is present in negligible amounts in quiescent neutrophils but can be induced by a variety of inflammatory stimuli (Smith *et al*, 2000; Vane *et al*, 1998). It was of interest to determine whether COX-2 expression is affected by PCBs. In this work we used the human, promyelocytic leukemia cell line, HL-60, to examine the effect of *in vitro* exposure to PCBs on COX-2 mRNA levels. HL-60 cells have the capacity to differentiate to a number of different, functionally and morphologically distinct, forms (Collins, 1987) including a neutrophil-like phenotype, and differentiated HL-60 cells have been a useful model with which to study neutrophil responses (Arroyo *et al*, 2002; Mullick *et al*, 2004).

II.3. Materials and Methods

II.3.A. Chemicals.

2,2',4,4'-Tetrachlorobiphenyl (>99% pure) and 3,3',4,4'-tetrachlorobiphenyl (>99% pure) were purchased from ChemService (West Chester, PA). fMLP was purchased from Sigma Chemical Co. (St. Louis, MO). ^{3}H-AA (180-240 Ci/mmol) was purchased from DuPont (Boston, MA). All other chemicals were of the highest grade commercially available.

II.3.B. HL-60 cells.

HL-60 cells were obtained from American Type Culture Collection (Manassas, VA). Cells between passage 20 and 45 were grown in Iscove's Modified Dulbecco's Medium supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 0.1% gentamicin and 0.9% antibiotic-antimycotic (Invitrogen Corporation, Carlsbad, CA). Cultures were maintained in a humidified incubator at 37°C in a controlled atmosphere of 5% CO_2. Cells were induced to differentiate along the granulocyte pathway by culturing in the presence of 1.25% DMSO for 5 days. Cells were then maintained an additional two days in the absence of DMSO. After this procedure, approximately 80% of the cells had nuclear appearance characteristic of neutrophils. In addition, these cells reduced nitro blue tetrazolium, a functional marker of differentiation to a granulocytic phenotype (Hua *et al*, 2000).

II.3.C. Exposure to polychlorinated biphenyls.

Stock solutions of the PCBs were prepared by dissolution in N,N'-dimethylformamide (DMF). The differentiated HL-60 cells were suspended in HBSS in 12 x 75 mm borosilicate glass test tubes (VWR, Chicago, IL), and 1 μL of the stock solution was added per mL of cells to achieve the desired concentration. Control cells received 1 μL of DMF per mL of cells. Exposure to vehicle had no significant effects. The concentrations of PCBs used in the current study were selected based on their previously described activity in neutrophils and their minimal cytotoxicities (Olivero *et al*, 2002).

II.3. D. Cellular degranulation.

Degranulation was measured by the release of the enzyme MPO. Differentiated HL-60 cells (2 x 10^6) were suspended in Hank's Balanced Salt Solution (HBSS) and exposed to PCBs at 37°C for 30, 60, 90 or 120 minutes. fMLP (10 nM) was added for the final 30 minutes of treatment to stimulate degranulation. Immediately after treatment, cells were centrifuged for 10 minutes at 4000 g. The cell-free supernatant fluids were collected, and MPO activity was measured according to the method of Henson *et al.* (1978). Release of MPO in the medium was expressed as the percentage of total MPO activity that was present in an equivalent number of cells lysed with 10 μL of Triton X-100 and ultrasonication.

II.3.E. Superoxide anion production.

Superoxide anion generation by HL-60 cells was measured as the reduction of cytochrome c in the presence or absence of superoxide dismutase (SOD; Babior et al, 1976). Differentiated HL-60 cells were suspended in Ca^{2+}- and Mg^{2+}-containing HBSS in the presence of cytochrome c (10 mg/mL) and of PCB or vehicle. Experiments were performed in 96-well plates, and for every sample two wells were incubated: one to which SOD (840 U/mL) was added and one to which an equal volume of vehicle was added. The amount of superoxide anion produced was estimated from the amount of cytochrome c reduced as determined from the difference in absorbance between the two wells, using an extinction coefficient of 18.5 cm^{-1} mM^{-1}.

II.3.F. Labeling of HL-60 cells with ^{3}H-arachidonic acid.

Differentiated HL-60 cells (10 x 10^6/mL) were suspended in HBSS containing 0.1% bovine serum albumin (BSA) and incubated for 120 minutes at 37°C with 0.5 μCi/mL ^{3}H-AA. At the end of the labeling period, cells were washed twice and resuspended in HBSS containing 0.1% BSA. At the end of the incubation period, radioactivity in an aliquot of cells was determined in a scintillation counter to determine cellular uptake of radiolabel. Uptake was routinely ~70% of added ^{3}H-AA.

II.3.G. Determination of arachidonic acid release.

Cumulative release of ^{3}H-AA was measured in HL-60 cells (2 x 10^{6}) exposed for 30, 60, 90 or 120 minutes at 37°C to PCBs. At the end of the incubations, samples were placed on ice and centrifuged, and radioactivity in the cell-free supernatant fluids was determined by liquid scintillation counting. Release is expressed as a percentage of the total radioactivity in the labeled cells.

II.3.H. Determination of COX-2 mRNA.

Differentiated HL-60 cells (5 x 10^{6}) were suspended in HBSS and treated at 37°C for 30, 60, 90 or 120 minutes with 3344-TCB or 2244-TCB at the concentrations indicated in the figure legends. At the end of the incubation period, total cellular RNA was isolated using Tri-Reagent (Sigma Chemical Co., St. Louis, MO). Concentration of isolated RNA was determined spectrophotometrically by measuring absorbance at 260 nm. cDNA was synthesized by reverse transcription at 42°C for 45 minutes in a 20 μL reaction mixture containing 1 μg total RNA and 100 units MMLV reverse transcriptase (Promega Corp., Madison, WI). After heating at 99°C for 5 minutes for denaturing, followed by cooling at 5°C, the cDNA was used for amplification. For PCR reactions, 5 μL of denatured cDNA was amplified in a 25-μL final volume with 2.5 units of *Taq* DNA polymerase (Invitrogen Corp., Carlsbad, CA) 1mM of each primer and *Taq* polymerase buffer containing 25mM $MgCl_2$ with 2mM of each dNTP (dATP, dCTP, dGTP and dTTP; Promega Corp.). PCR

was performed in a thermal cycler (GeneAmp 9700, Applied Biosystems, Foster City, CA), using a program of 35 cycles at 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute, followed by a 10-minute extension at 72°C. The amplified products were subjected to electrophoresis on a 1.5% agarose gel and detected and photographed under UV light. Densitometry on detected bands was performed using Bio-Rad Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA).

The following primers were used for COX-2: 5'-TTCAAATGAGATTGTGGGAAAATTGCT-3' (forward primer), 5'-AGATCATCTCTGCCTGAGTATCTT-3' (reverse primer). The predicted size of the fragment was 301 base pairs.

For β-actin, the primers were 5'-GACGAGGCCCAGAGCAAGAGAG-3' (forward primer), 5'-ACGTACATGGCTGGGGTGTTG-3' (reverse primer). The predicted size of the fragment was 284 base pairs (Vergne et al, 1998).

II.3.I. COX-2 Western blotting.

Differentiated HL-60 cells (50 x 10^6) were suspended in HBSS and treated at 37°C for 30, 60, 90 or 120 minutes with 2244-TCB at the concentrations indicated in the figure legends. Immediately after treatment, cells were centrifuged for 10 minutes at 4000 g. Supernatant was discarded and the cell pellet was lysed with 100 µL of 2% SDS. Proteins (30 µL) were separated on 10% Bis-Tris polyacrylamide gels (NuPAGE, Invitrogen Corporation, Carlsbad, CA) and electrophoretically transferred onto polyvinylidene

difluoride membranes (Bio-Rad Laboratories, Hercules, CA). After blocking, the membranes were incubated with a primary anti-human COX-2 monoclonal antibody (1:1000 dilution; Cayman Chemical, Ann Arbor, MI) and then a secondary antibody (peroxidase-conjugated goat anti-human IgG; 1:1000 dilution; Santa Cruz biotechnology, Santa Cruz, CA). The blots were developed using an ECL detection kit (Amersham Biosciences, Little Chalfont, UK). The blots were stripped and successively reprobed with an anti-human B-actin monoclonal antibody (Sigma Chemical Co. St. Louis, MO) then with a corresponding secondary antibody. The levels of COX-2 bands were measured densitometrically as described above and corrected using levels of B-actin as an internal standard.

II.3.J. Cyclooxygenase activity assay.

Differentiated HL-60 cells (15 x 10^6) were suspended in HBSS and treated at 37°C for 30, 60, 90 or 120 minutes with 2244-TCB at the concentrations indicated in the figure legends. Immediately after treatment, cells were centrifuged for 10 minutes at 4000 g. Supernatant was discarded and the cell pellet was resuspended in 200 µL then lysed via sonication. COX activity was then measured using a COX activity assay (Cayman Chemical, Ann Arbor, MI) according to manufacturer's instructions. This assay utilizes the peroxidase component of cyclooxygenase. The peroxidase activity is assayed colorimetrically by monitoring the appearance of oxidized N,N,N',N'-tetramethyl-p-phenylenediamine at 590 nm. Activities of COX-1 and COX-2

were differentiated using the isoform-specific inhibitors DuP-697 and SC-560 according to manufacturer's instructions.

II.3.K. Statistical analysis.

Data are expressed as mean ± SEM. Results were analyzed by one-way analysis of variance. Group means for the superoxide anion production results were compared using Dunnett's post hoc test; all other data were compared using Tukey's post hoc test. Appropriate transformations were performed on all data that did not follow a normal distribution (e.g., percent data). For all studies, the criterion for statistical significance was $p < 0.05$.

II.4 Results

II.4.A. Degranulation of HL-60 cells.

In the absence of PCB exposure differentiated HL-60 cells released ~27% of total MPO in response to fMLP, a peptide that activates neutrophils. Exposure to the noncoplanar PCB congener 2244-TCB resulted in an increase in degranulation that was significant by 90 minutes (Figure II.1). 2244-TCB concentrations greater than 3 μM were required to affect increases in degranulation (Figure II.2). 3344-TCB did not affect fMLP-induced degranulation at any time or dose examined (Figures II.1 and II.2).

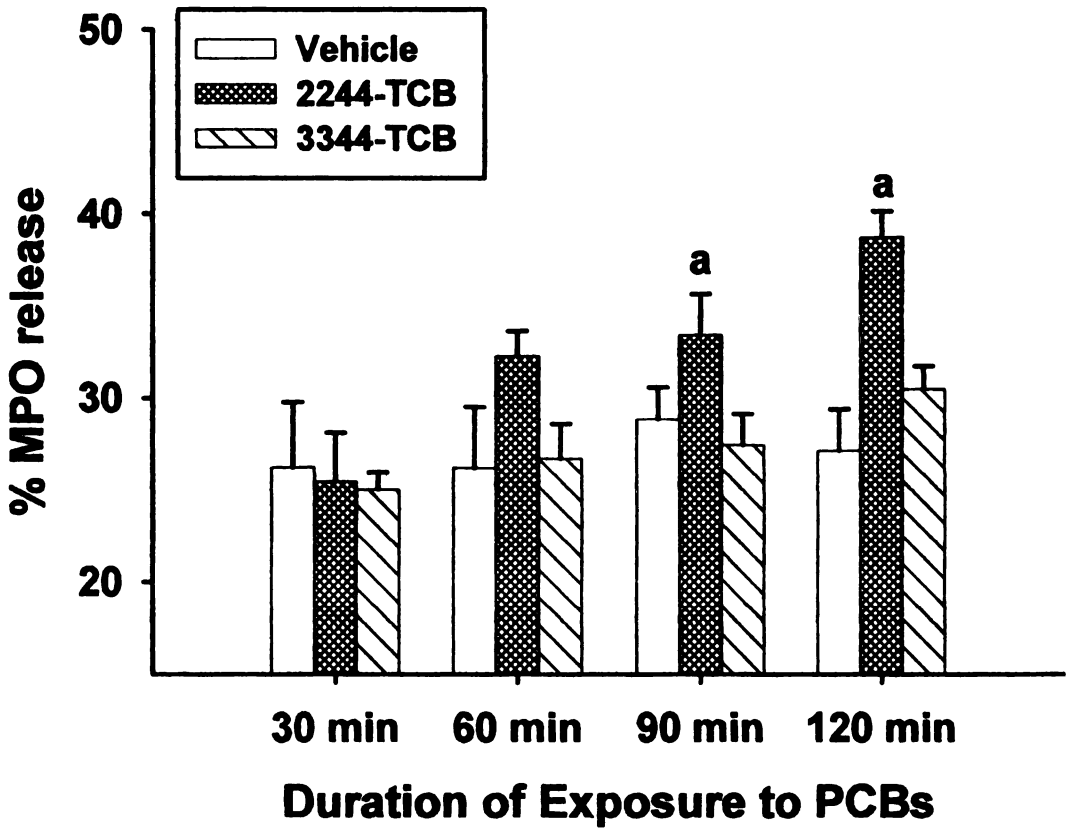

Figure II.1. Time course for PCB-induced degranulation. Differentiated HL-60 cells ($1x10^6$ cells/mL) were treated with 30 μM 2244-TCB, 30 μM 3344-TCB or vehicle (DMF) for 30, 60, 90 or 120 minutes. The degranulation stimulus fMLP was added for the last 30 minutes of treatment. Degranulation was estimated by determining percent of total cellular myeloperoxidase (MPO) released into culture medium. N = 5 separate experiments. a = Significantly different from all other treatments at that time.

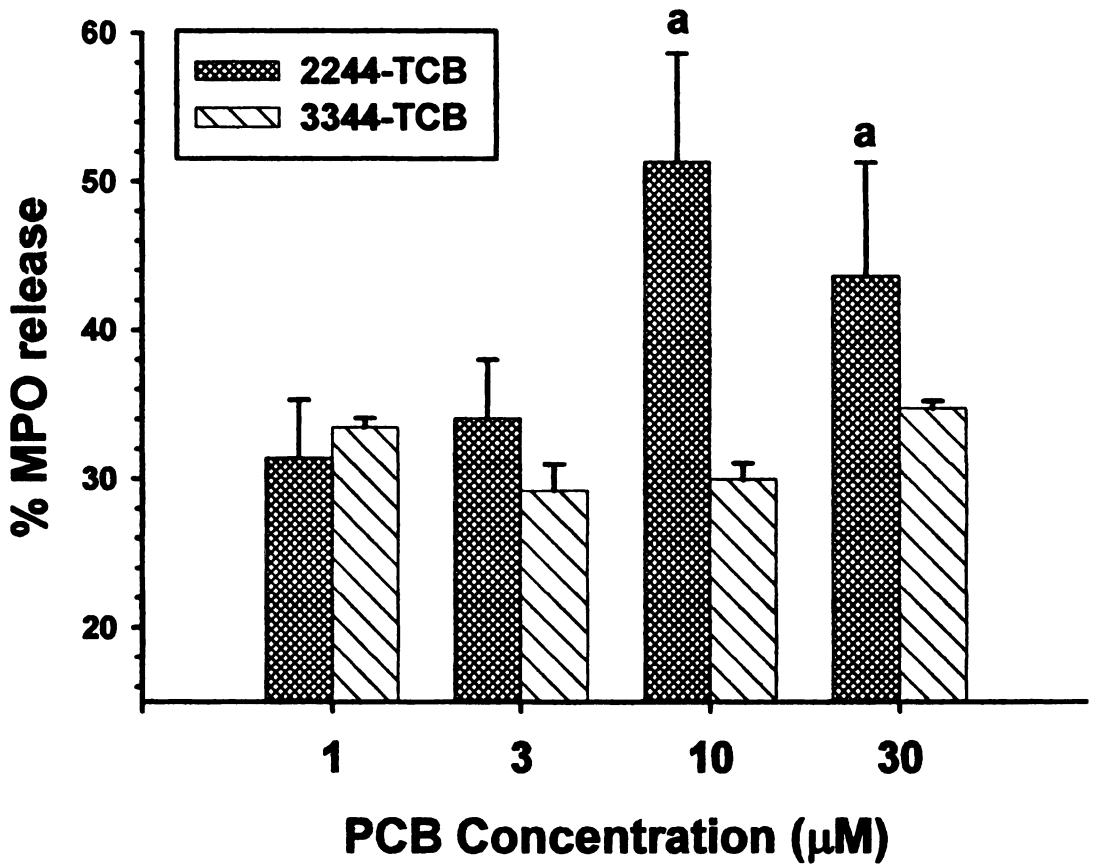

Figure II.2. Concentration-response relationship for PCB-induced degranulation. HL-60 cells ($1x10^6$ cells/mL) were treated with 1, 3, 10 or 30 μM 2244-TCB, 3344-TCB or vehicle (DMF) for 60 minutes, then fMLP was added and cells were incubated for an additional 30 minutes. Degranulation was estimated as the percent of total cellular MPO released into culture medium. Cells exposed to vehicle released 27% ± 3.5% MPO. N = 5 separate experiments. a = significantly different from 3344-TCB- or vehicle-treated cells at the same concentration.

II.4.B. Superoxide anion production.

In the absence of PCB exposure, differentiated HL-60 cells produced < 5 nmol of superoxide per 10^6 cells (Figure II.3). Exposure to 2244-TCB resulted in a significant increase in superoxide anion production. The coplanar PCB congener 3344-TCB did not affect superoxide anion production (Figure II.3).

II.4.C. ^{3}H-AA release.

In the absence of PCB exposure, HL-60 cells released ~10% of total ^{3}H-AA (Figure II.4) after two hours. Exposure of differentiated HL-60 cells to 2244-TCB resulted in an increase in the release of ^{3}H-AA. Statistically significant increases in ^{3}H-AA release were observed in cells exposed to 2244-TCB for longer than 60 minutes (Figure II.4). Concentrations of 2244-TCB greater than 3 μM produced a significant increase in ^{3}H-AA release from HL-60 cells (Figure II.5). Exposure to 3344-TCB did not cause statistically significant changes in ^{3}H-AA release at any time or concentration examined (Figure II.4 and II.5).

II. 4. D. Effects of PCBs on COX-2 mRNA expression.

COX-2 mRNA levels did not change over two hours in vehicle-treated HL-60 cells. A significant increase in the level of COX-2 mRNA was observed after 30 or 60 minutes exposure to 2244-TCB (Figure II.6.A). In cells

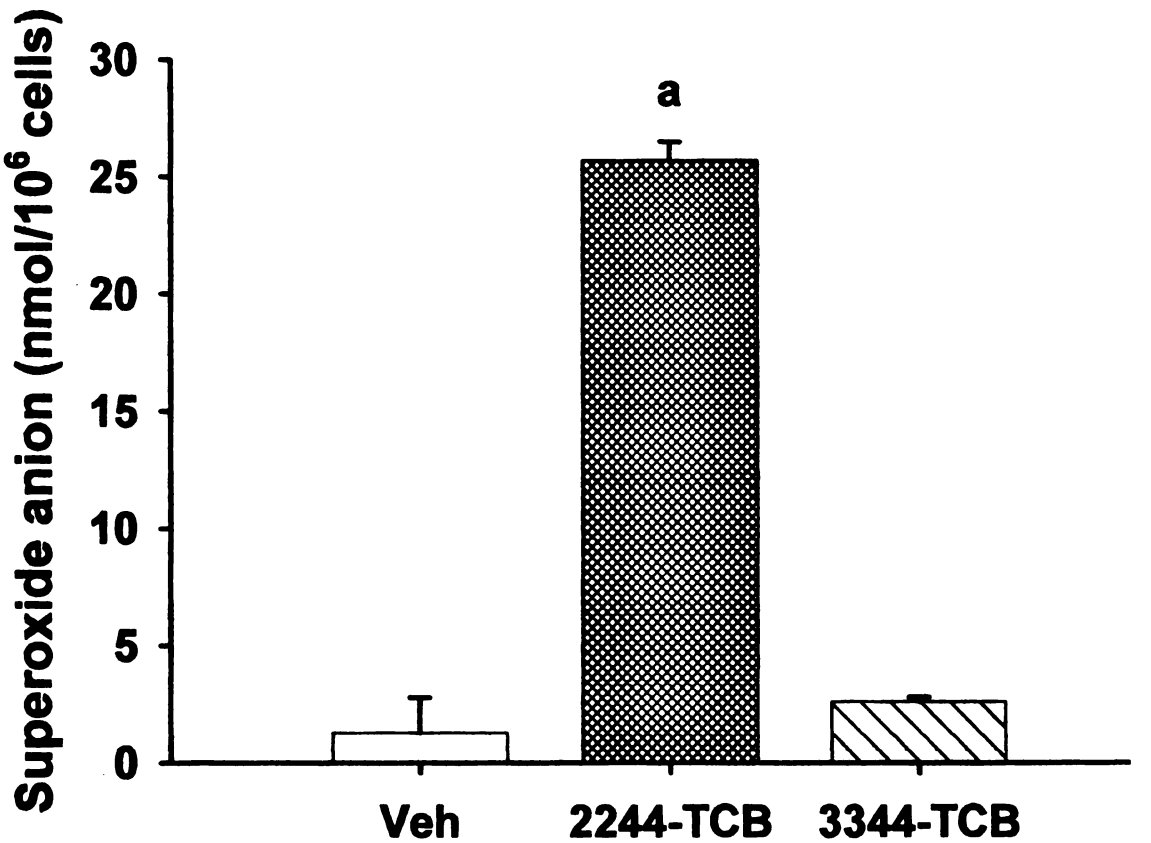

Figure II.3. Superoxide anion production in PCB-treated cells. Differentiated HL-60 cells (1 x10^6) were exposed to 30 μM 2244-TCB, 30 μM 3344-TCB or vehicle (DMF) for 30 minutes, and superoxide anion production was determined as described in Materials and Methods. N=3-6 separate experiments. a = Significantly different from vehicle control.

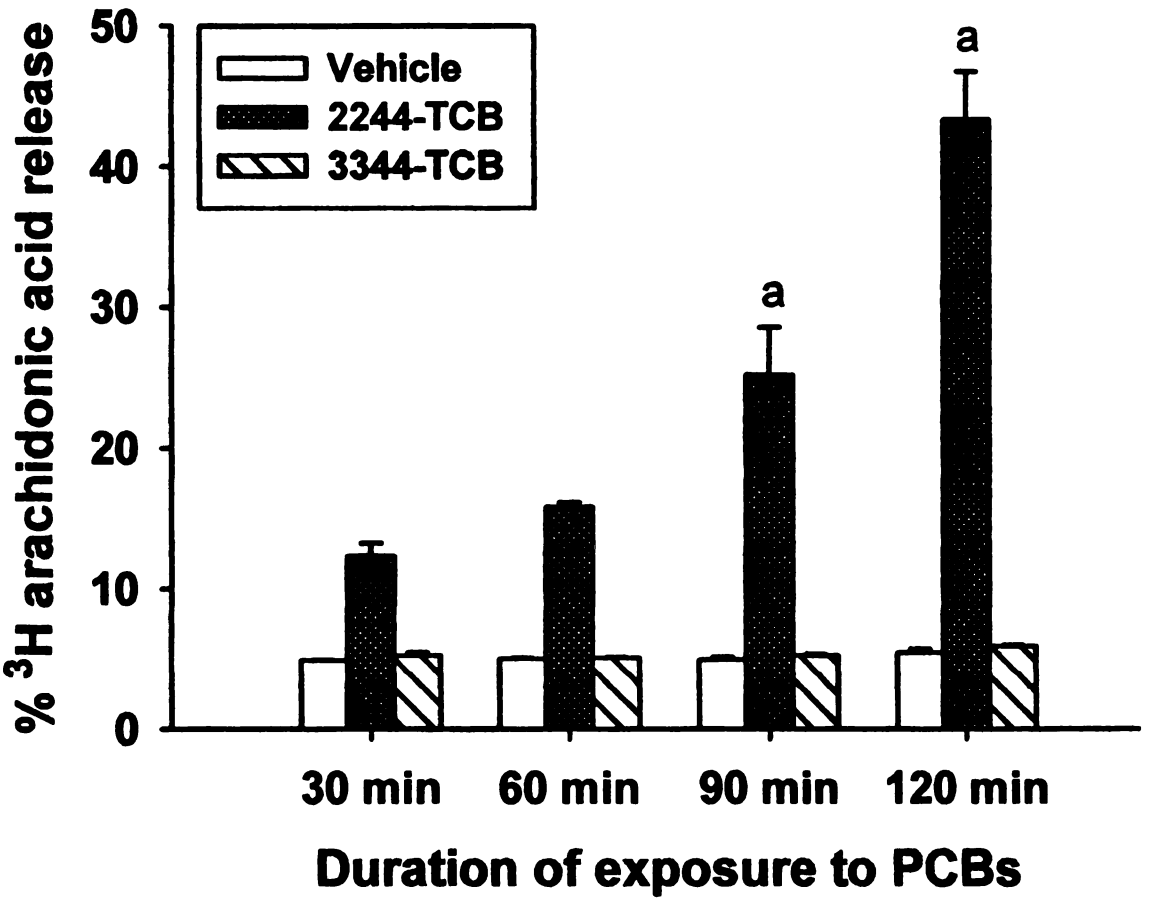

Figure II.4. Time course for ^{3}H-AA release in PCB-treated cells. Differentiated HL-60 cells were labeled with ^{3}H-AA as described in Materials and Methods. Labeled cells ($1x10^{6}$ cells/mL) were treated with 30 μM 2244-TCB, 30 μM 3344-TCB or vehicle (DMF) for 30, 60, 90 or 120 minutes. ^{3}H-AA release is presented as the percent of total cellular 3H-AA released into culture medium. N = 3 separate experiments. a = significantly different from other treatments at same time.

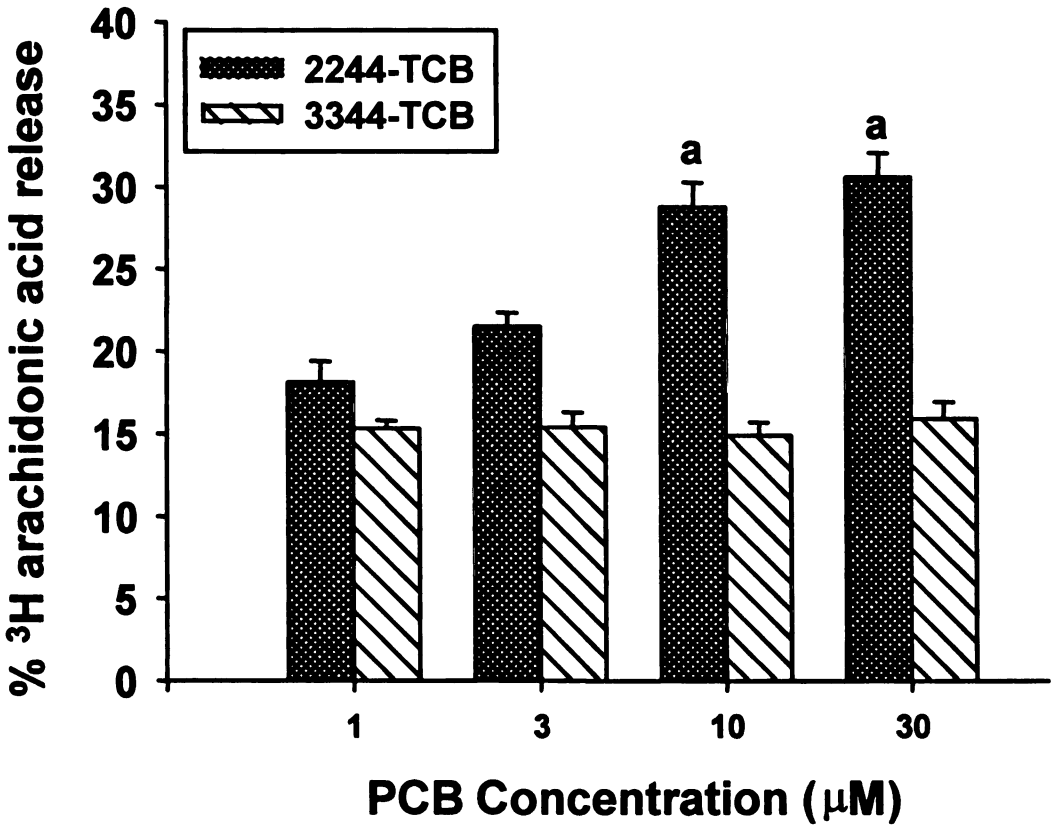

Figure II.5. Concentration-response relationship for ^{3}H-AA release in PCB-treated cells. Differentiated HL-60 cells were labeled with ^{3}H-AA as described in Materials and Methods. Labeled cells ($1x10^{6}$ cells/mL) were treated with 1, 3, 10 or 30 μM 2244-TCB, 30 μM 3344-TCB or vehicle for 90 minutes. ^{3}H-AA release is presented as the percent of total cellular ^{3}H-AA released into culture medium. Cells exposed to vehicle released 16% ± 0.3% ^{3}H-AA. N = 3 separate experiments. a = significantly different from 3344-TCB- or vehicle-treated cells at the same concentration.

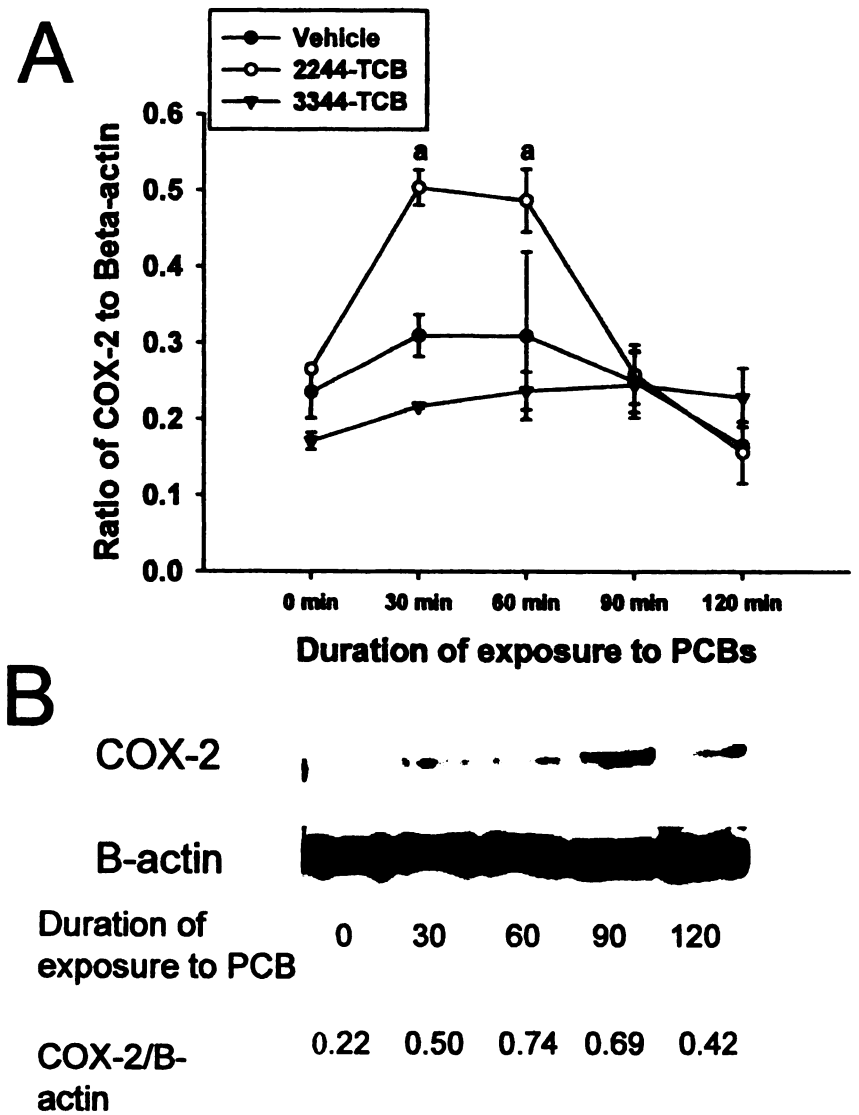

Figure II.6. Time course for the expression of COX-2 mRNA and protein in PCB-treated cells. A) COX-2 mRNA was determined in HL-60 cells (5 x10^6) treated with 30 μM 2244-TCB, 30 μM 3344-TCB or vehicle for 0, 30, 60, 90 or 120 minutes as described in Materials and Methods. β-actin was used as a loading control. N = 5 separate experiments. a = Significantly different from the same treatment at time 0. B) COX-2 protein was determined in HL-60 cells (50 x10^6) treated with 30 μM 2244-TCB for 0, 30, 60, 90 or 120 minutes as described in Materials and Methods. β-actin was used as a loading control. COX-2/B-actin ratios were determined by densitometry.

treated longer then 60 minutes, expression of COX-2 mRNA returned to baseline. This effect was observed with 10 μM or 30 μM 2244-TCB (Figure II.7.A). 3344-TCB had no statistically significant effect on COX-2 mRNA levels at any time or concentration examined.

II. 4. E. Effects of PCBs on COX-2 protein expression.

COX-2 protein levels remained undetectable over two hours in vehicle-treated HL-60 cells. COX-2 protein was increased after 30, 60 or 90 minutes exposure to 2244-TCB. After 120 minutes exposure to 2244-TCB, protein levels were decreased compared to previous time points, but still elevated compared to untreated cells (Figure II.6.B). This effect was only observed with 30 μM 2244-TCB (Figure II.7.B).

II. 5. F. Effects of PCBs on COX-2 activity.

The activity of the peroxidase component of COX was used as a measure of total COX activity. Activity of COX-2 was undetectable over two hours in vehicle-treated HL-60 cells. An increase in the levels of COX activity was observed after 60, 90 or 120 minutes exposure to 2244-TCB (Figure II.8.A). This effect was only observed with 30 μM 2244-TCB (Figure II.8.B). Activity of COX-1 did not change at any concentration of 2244-TCB examined.

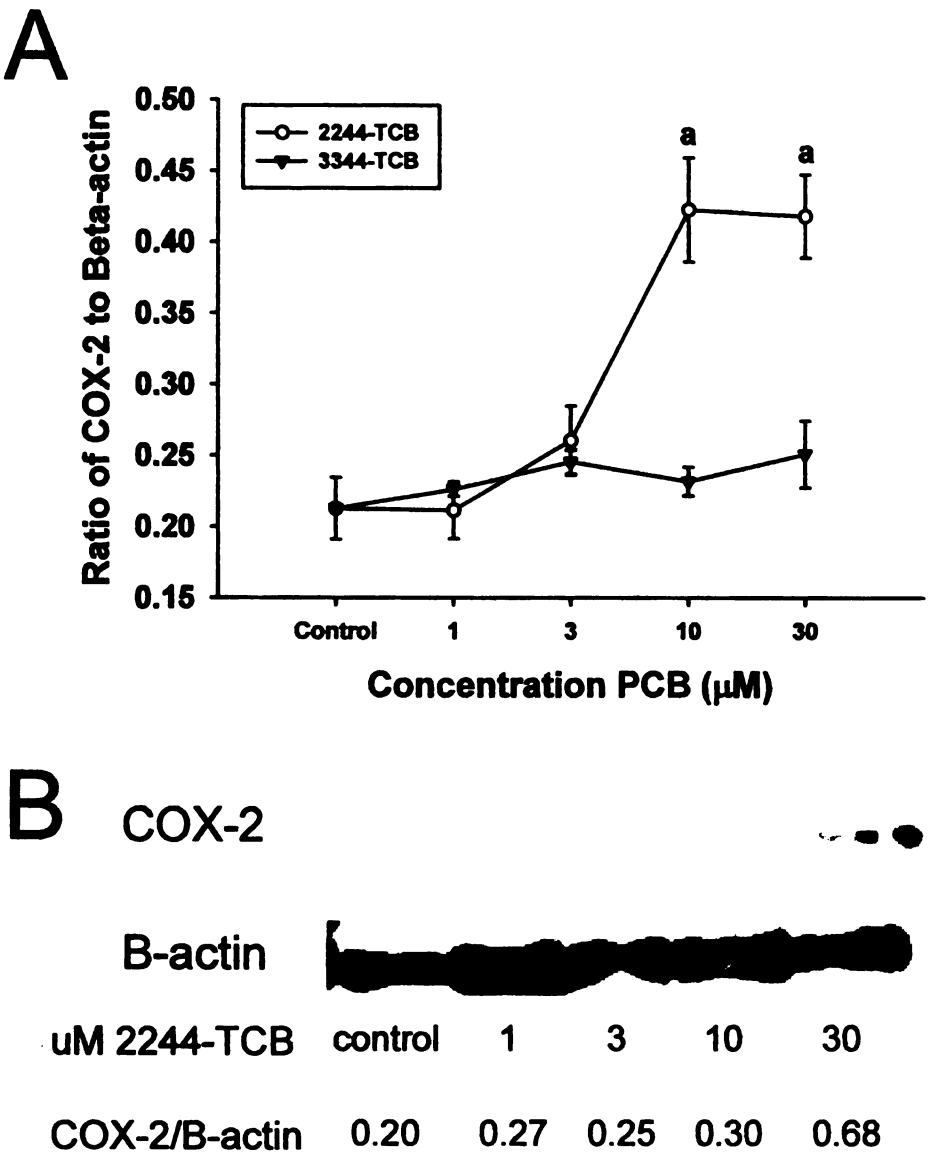

Figure II.7. Dose-response relationship for PCB-induced increases in COX-2 mRNA and protein. A) COX-2 mRNA was determined in HL-60 cells (5 x10^6) treated with vehicle, 1, 3, 10 or 30 μM 2244-TCB or 3344-TCB for 30 minutes as described in Materials and Methods. β-actin was used as a loading control. N = 5 separate experiments. a = Significantly different from vehicle-treated control. B) COX-2 protein was determined in HL-60 cells (50 x10^6) treated with vehicle, 1, 3, 10 or 30 μM 2244-TCB for 30 minutes as described in Materials and Methods. β-actin was used as a loading control. COX-2/B-actin ratios were determined by densitometry.

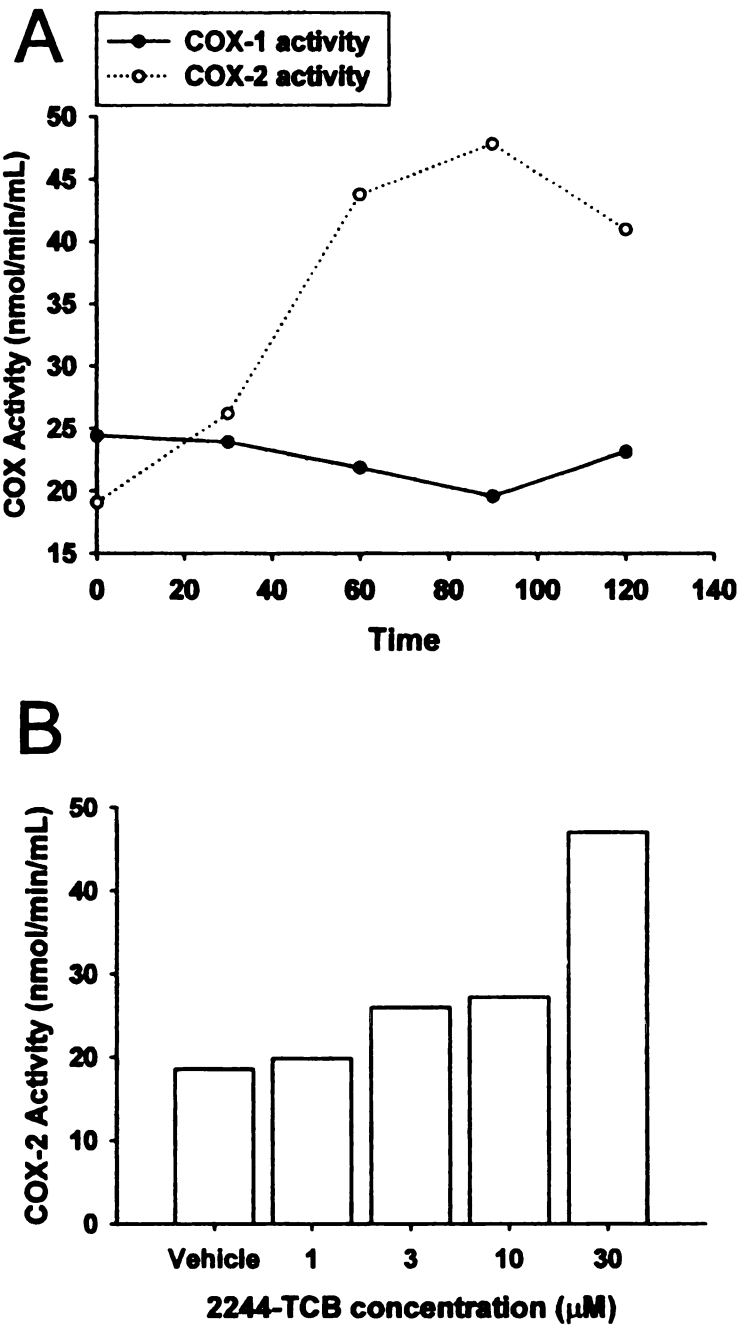

Figure II.8. COX activity in PCB-treated HL-60 cells. A) COX activity was determined in HL-60 cells (15 x10^6) treated with 30 μM 2244-TCB for 0, 30, 60, 90 or 120 minutes. COX activity was measured as described in Materials and Methods. B) COX activity was determined in HL-60 cells (15 x10^6) treated with vehicle, 1, 3, 10 or 30 μM 2244-TCB for 60 minutes. COX activity was measured as described in Materials and Methods.

II. 6 Discussion

In the present study, the function of a human cell line differentiated to a neutrophil-like phenotype was affected by exposure to the noncoplanar congener 2244-TCB, but not by the coplanar congener 3344-TCB. These results have not been previously described in the human neutrophillic HL-60 cell line. 2244-TCB caused a time- and concentration-dependent increase in cellular degranulation and induced superoxide anion production (Figures II.1, II.2 and II.3). These results are similar to previous observations of the effects of PCBs on primary rat neutrophils, in which several mono- or di-ortho-substituted PCBs stimulated superoxide anion production and/or increased superoxide anion generation in response to phorbol myristate acetate (Brown *et al*, 1998; Ganey *et al*, 1993). None of a variety of coplanar PCBs, including 3344-TCB, affected the function of rat neutrophils (Brown *et* al, 1998; Brown and Ganey, 1995). In primary human neutrophils noncoplanar PCB congeners, including 2244-TCB, increased oxidative burst (Voie *et al*, 1998; Voie *et al*, 2000) confirming that differentiated HL-60 cells respond similarly to primary human neutrophils. 2244-TCB and 2,3,4,5-TCB increase degranulation in rat neutrophils (Brown and Ganey, 1995), but changes in degranulation caused by PCBs have not been reported previously in human cells. Thus, with respect to oxidative burst and degranulation, responses in granulocytic HL-60 cells, a human model of the neutrophil, are similar to those observed in rat neutrophils as well as in PCB-treated primary human neutrophils.

Several potential mediators of PCB-induced superoxide anion production have been described, including increased intracellular Ca^{2+} with subsequent activation of Ca^{2+}-dependent proteins such as calmodulin (Olivero and Ganey, 2001) and release of AA due to activation of phospholipases (Tithof *et al*, 1998). In the present study, the noncoplanar PCB congener 2244-TCB, but not the coplanar congener 3344-TCB, increased the release of ^{3}H-AA in a time- and concentration-dependent manner (Figures II.4 and II.5). Similar time- and concentration-dependent increases in ^{3}H-AA release have been reported in primary rat neutrophils, but not in human cells, treated with Aroclor 1242 as well as with individual, noncoplanar PCB congeners (Olivero *et al*, 2001; Olivero and Ganey, 2001; Tithof *et al*, 1998). Release of ^{3}H-AA, similar to that observed in PCB-treated HL-60 cells, has also been observed in rat uterine strips (Bae *et al*, 1999) and in cerebellar granule cells treated with Aroclor mixtures (Kodavanti and Derr-Yellin, 2002; Kodavanti and Tilson, 2000).

One major fate of released AA is its conversion into eicosanoids, such as prostaglandins and thromboxanes, by cyclooxygenases or cytochrome P450 monooxygenases. In neutrophils, the eicosanoids produced by COX-2 play an important modulatory role in inflammation. COX-2 is an immediate early gene that is present in negligible amounts in quiescent neutrophils. Upon activation of the cell, COX-2 is rapidly upregulated. In the present study 2244-TCB rapidly and transiently increased levels of COX-2 mRNA and protein in a time- and concentration-dependent manner (Figures II.6 and II.7). Additionally, time- and concentration-dependent increases in COX activity were observed (Figure

II.8), and these were greatly inhibited by the COX-2-specific inhibitor DuP-697. Previously observed effects of PCBs on COX-2 regulation have involved activation of the Ah receptor (Kietz and Fischer, 2003; Kwon *et al*, 2002). As 2244-TCB, a poor ligand for the Ah receptor, caused changes in COX-2 whereas 3344-TCB, a more potent Ah receptor ligand, did not, it is unlikely that Ah receptor activation is involved in the increases in COX-2 observed in the present study. In mast cells, another immune cell critical in the initiation of inflammatory responses, COX-2 mRNA was markedly induced after exposure to the noncoplanar PCB congener 2,2',4,4',5,5'-hexachlorobiphenyl (Kwon *et al*, 2002). A similar induction of COX-2 mRNA was observed in rabbit blastocysts that were exposed to a mixture of 7 different noncoplanar PCB congeners (Kietz and Fischer, 2003). Interestingly, COX-2 mRNA was also elevated in rabbit blastocysts by treatment with a mixture of three coplanar PCB congeners. This was not the case in differentiated HL-60 cells exposed to 3344-TCB, indicating differences in COX-2 regulation between HL-60 cells and rabbit blastocysts.

In summary, exposure to a noncoplanar PCB *in vitro* alters the function of granulocytic HL-60 cells in several ways. Exposing differentiated HL-60 cells to the noncoplanar PCB congener 2244-TCB increased degranulation and superoxide anion production, stimulated the release of ^{3}H-AA, and induced COX-2 mRNA, protein and activity. Exposure to 3344-TCB had no effect on any of these endpoints in HL-60 cells. These effects mirror those seen with other noncoplanar PCB congeners in a variety of cell systems. Neutrophils play a vital role in the immune response as well as in inflammation, and any

alterations in neutrophil function can have dire implications, either leading to increased susceptibility to infection, or contributing to destruction of healthy host cells.

Based on these observations, studies were undertaken to evaluate the mechanisms and signaling pathways involved in these responses. These are described in the following chapters.

Chapter III

Signal Transduction Pathways Involved in the Induction of Cyclooxygenase-2 by 2,2′,4,4′-Tetrachlorobiphenyl in HL-60 cells

III.1. Summary

PCBs are ubiquitous, persistent environmental contaminants that affect a number of cellular systems including neutrophils. Effects in neutrophils are mediated by ortho-substituted, noncoplanar PCBs like 2244-TCB. These effects include changes in the expression of inflammatory genes such as COX-2. The objective of this study was to determine the signal transduction pathways involved in 2244-TCB-mediated upregulation of COX-2 in neutrophilic HL-60 cells. Treatment of HL-60 cells with 2244-TCB led to increased expression of COX-2 mRNA. This was associated with release of ^{3}H-AA, increased superoxide anion production and phosphorylation of p38 and ERK MAP kinases. Bromoenol lactone (BEL), an inhibitor of the Ca^{2+}-independent PLA_2 ($iPLA_2$), reduced ^{3}H-AA release but had no effect on COX-2 mRNA, protein or activity. Pretreatment with SB-202190 or SB-203580, inhibitors of the p38 MAP kinase pathway, prevented the 2244-TCB-mediated induction of COX-2 and phosphorylation of p38 and ERK MAP kinases. These inhibitors did not alter ^{3}H-AA release. Treatment with PD 98059 or U 0126, inhibitors of the ERK MAP kinase pathway, prevented the 2244-TCB-mediated activation of ERK but had no effect on COX-2 induction or p38 phosphorylation. 2244-TCB treatment did not affect JNK phosphorylation. 2244-TCB-mediated increases in superoxide anion were prevented by the NADPH oxidase inhibitor apocynin or the free radical scavenger 4-hydroxy TEMPO, but neither of these inhibitors affected the PCB-induced changes in COX-2 mRNA levels or ^{3}H-AA release. Taken together these data suggest that activation of the p38 MAP kinase

pathway is involved in the 2244-TCB-mediated upregulation of COX-2 mRNA.

III.2. Introduction

PCBs are persistent, widespread environmental contaminants associated with functional changes in a variety of cellular systems. They are probable human carcinogens (Cogliano, 1998) and cause a number of non-cancer effects, including dermal lesions and ocular dysfunction in humans and hepatotoxicity and endocrine disruption in rats (Morse *et al*, 1996; Takamatsu *et al*, 1985; Twaroski *et al*, 2001). In addition, PCBs have effects on the reproductive system (Faqi *et al*, 1998), nervous system (Jacobson and Jacobson, 1997), cardiovascular system (Warshaw *et* al, 1979) and both the specific and nonspecific branches of the immune system (Vos and Loveren, 1998).

PCBs exist as 209 different congeners based on the number and distribution of chlorine atoms on the biphenyl rings. Noncoplanar PCBs that contain ortho- substituted chlorines alter cellular function through effects on signal transduction pathways, including alterations of kinases and phospholipases, disturbance of Ca^{2+} homeostasis and modulation of gene expression (Tilson and Kodavanti, 1997; Brown *et al*, 1998; Fischer *et al*, 1998; Oakley *et al*, 2001). In polymorphonuclear neutrophils, which are important in host defense against invading microorganisms, noncoplanar PCBs stimulate degranulation and release of AA (Brown and Ganey, 1995; Kristoffersen *et al*, 2002; Olivero and Ganey, 2001; Olivero-Verbel and Ganey, 1998; Voie *et al*, 2000). In addition, exposure to PCBs results in altered responses to subsequent

stimulation with other agents (Brown and Ganey, 1995; Ganey *et al*, 1993; Olivero and Ganey, 2001; Tithof *et* al, 1998). These alterations have been linked to changes in signal transduction, such as kinase activation (Olivero and Ganey, 2000; Tithof *et al*, 1997), increased phospholipase activity (Bezdecny *et al*, 2005; Tithof *et al*, 2000; Tithof *et al*, 1996), changes in regulation of calcium and calcium-dependent proteins (Olivero and Ganey, 2001) and changes in the expression of genes, such as COX-2 (Bezdecny *et al*, 2005).

COX enzymes catalyze the conversion of arachidonic acid to prostaglandins and thromboxane, and some of these lipid mediators have immunomodulatory effects. The isoform COX-1 is constitutively expressed and primarily plays a cytoprotective role. The COX-2 isoform is generally found in negligible amounts in normal cells, such as quiescent neutrophils, but expression of its gene is induced by a wide variety of inflammatory stimuli. In activated neutrophils, COX-2 expression is an important component of the inflammatory response (Smith *et al*, 2000; Vane *et al*, 1998). Given the observation that PCBs increase COX-2 expression (Bezdecny *et al*, 2005), it was of interest to determine the signal transduction pathways involved in this alteration. A number of pathways were examined, including a pathway involving ROS; a pathway involving activated PLA_2 and its product, free AA; and three MAP kinase systems, p38, ERK and JNK. Each of these pathways has been implicated previously in the upregulation of COX-2 in other cell systems (Amma *et* al, 2005; Dean *et al*, 1999; Lasa *et al*, 2000; Moon and Pestka, 2002; Pawliczak *et al*, 2004; Steer *et al*, 2006).

III.3. Materials and Methods

III.3.A. Chemicals.

PD 98059 and U 0126 were purchased from Calbiochem (San Diego, CA). SB-202190 (SB1), SB-203580 (SB2), oleyloxyethyl phosphorylcholine (OP) and BEL were purchased from Biomol (Plymouth Meeting, PA). 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPO) and apocynin were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of the highest grade commercially available.

III.3.B. HL-60 cells.

HL-60 cells were obtained from American Type Culture Collection (Manassas, VA). Cells between passage 20 and 50 were grown in Iscove's Modified Dulbecco's Medium supplemented with 10% Cosmic Calf Serum (HyClone; Logan, UT), 0.1% gentamicin and 0.9% antibiotic-antimycotic. Cultures were maintained in a humidified incubator at 37°C in a controlled atmosphere of 5% CO_2/95% air. Cells were induced to differentiate along the granulocyte pathway by culturing in the presence of 1.25% DMSO for 5 days. Cells were then maintained an additional two days in the absence of DMSO. After this procedure, approximately 80% of the cells had nuclear appearance characteristic of neutrophils. In addition, they reduced nitroblue tetrazolium, a functional marker of differentiation to a neutrophilic phenotype (Hua *et al*, 2000).

III.3.C. Exposure to inhibitors.

Stock solutions of inhibitors were prepared by dissolution in DMSO, and 1 μL/mL of the stock solution was added to the cells to achieve the desired concentration. Control cells received 1 μL/mL of DMSO. Exposure to vehicle had no significant effects. The concentrations of inhibitors used in the current study were selected based on their IC50 values and previously described activities (Ackermann *et* al, 1995; Lee *et al*, 1994; Magolda and Galbraith, 1989).

III.3.D. Determination of COX-2 mRNA levels.

Differentiated HL-60 cells (5 x 10^6) were suspended in HBSS and pretreated with the appropriate inhibitor or vehicle for 30 minutes. They were then treated with vehicle or 30 μM 2244-TCB for 30 minutes. Previous studies indicated a significant increase in COX-2 mRNA levels after 30 minutes of exposure to 2244-TCB (Bezdecny *et* al, 2005). At the end of the incubation period, total cellular RNA was isolated using Tri-Reagent (Sigma Chemical Co., St. Louis, MO). The concentration of isolated RNA was determined by spectrophotometry at 260 nm using a Beckman DU 640 spectrophotometer (Beckman Coulter Inc., Fullertom, CA.). cDNA was synthesized by reverse transcription at 42°C for 45 minutes in a 20 μL reaction mixture containing 1 μg total RNA and 100 units MMLV reverse transcriptase (Promega Corp., Madison, WI). After heating at 99°C for 5 minutes for denaturing, followed by cooling at 5°C, the cDNA was used for amplification. For PCR reactions, 3 μL

of denatured cDNA was amplified in a final volume of 30 μL with 0.75 units of AmpliTaq Gold (Applied Biosystems, Foster City, CA), 1μM of each primer and Sybr green PCR buffer containing 25mM $MgCl_2$ with 10mM of each dNTP (dATP, dCTP, dGTP and dTTP; Applied Biosystems, Foster City, CA). Primers were constructed using primer express. The following primers were used for COX-2 5'-TGATCCCCAGGGCTCAAAC -3' (forward primer), 5'-AGCTGGCCCTCGCTTATGAT -3' (reverse primer). For β-actin, the primers were 5'- TCACCCACACTGTGCCCATCTACGA -3' (forward primer), 5'-GGATGCCACAGGATTCCATACCCA -3' (reverse primer). PCR was performed in a thermal cycler (Applied Biosystems 7500) using a program of 50°C for 2 minutes, 95°C for 5 minutes and 40 cycles at 95°C for 15 seconds, 60°C for 1 minute. Results were analyzed using the program ABI 5700 SDS (Applied Biosystems, Foster City, CA). All results were compared to β-actin and expressed as fold induction versus vehicle control.

III.3.E. Western blotting for COX-2 and p38.

Differentiated HL-60 cells (50 x 10^6) were suspended in HBSS and pretreated at 37°C with the appropriate inhibitor or vehicle for the indicated time, then exposed to 30 μM 2244-TCB or vehicle for 60 or 90 minutes. Immediately after treatment, cells were centrifuged for 10 minutes at 4000 g. Supernatant was discarded, and the cell pellet was lysed with 100 μL of 2% SDS. Proteins (30 μL) were separated on 10% Bis-Tris polyacrylamide gels (NuPAGE, Invitrogen Corporation, Carlsbad, CA) and electrophoretically

transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). After blocking, the membranes were incubated with a primary anti-human COX-2 monoclonal antibody (1:1000 dilution; Cayman Chemical, Ann Arbor, MI), or a primary anti-human phospho-p38 monoclonal antibody (1:1000 dilution for each; both from Cell Signaling Technology, Beverly, MA) and then a secondary antibody (peroxidase-conjugated goat anti-mouse IgG; 1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). The blots were developed using an ECL detection kit (Amersham Biosciences, Little Chalfont, UK). The blots were stripped and successively reprobed with an anti-human β-actin monoclonal antibody (Sigma Chemical Co. St. Louis, MO) then with a corresponding secondary antibody. The intensities of bands of interest were measured densitometrically and corrected using Scion Image software (Scion Corporation, Frederick, MD). β -actin was used as an internal standard.

III.3.F. Measurement of phospho-p38 and phospho-ERK.

Levels of phosphorylated and total p38 and ERK were determined using FACE cell-based ELISAs from Active Motif (Carlsbad, CA). Differentiated HL-60 cells (20×10^6) were suspended in HBSS and pretreated at 37°C with the appropriate inhibitor or vehicle for 30 minutes. For determination of levels of phospho-ERK cells were then treated for 45 minutes with vehicle or 30 μM 2244-TCB. For determination of levels of phospho-p38 cells were treated for 90 minutes with vehicle or 30 μM 2244-TCB. Levels of total and

phospho-ERK and p38 were then determined according to manufacturer's instructions and presented as a ratio of phospho-protein to total protein.

III.3.G. Statistical analysis.

Data are expressed as mean ± SEM. Results for RT-PCR studies were analyzed by two-way repeated measures analysis of variance. All other results were analyzed by one-way analysis of variance. Group means for all data were compared using Tukey's post hoc test. Appropriate transformations were performed on all data that did not follow a normal distribution. For all studies, the criterion for statistical significance was $p < 0.05$. For Materials and Methods for exposure to PCBs, labeling with ^{3}H-AA, measurement of AA release, measurement of COX activity and production of superoxide anion see Chapter II.

III.4. Results

III.4.A. Effects of signal transduction inhibitors on 2244-TCB-mediated ^{3}H-AA release.

In the absence of PCB exposure, HL-60 cells released ~5% of total ^{3}H-AA after 90 minutes. Exposure to 2244-TCB produced a significant increase in ^{3}H-AA release (Figure III.1). BEL, an inhibitor of $iPLA_2$ (Ackermann *et al*, 1995), attenuated the 2244-TCB-mediated increase in ^{3}H-AA release. Incubation with either OP, an inhibitor of the cytosolic and secretory

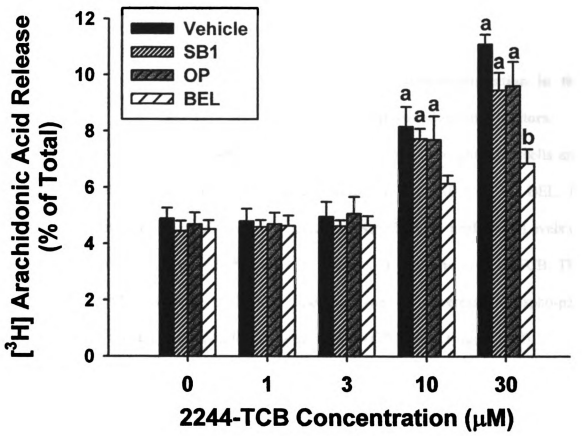

Figure III.1. 2244-TCB-mediated ^{3}H-AA release in the presence of signal transduction inhibitors. Differentiated HL-60 cells were labeled with ^{3}H-AA as described in Materials and Methods. Labeled cells ($1x10^{6}$ cells/mL) were pretreated with vehicle, 5 μM SB1, 15 μM OP, or 25 μM BEL. They were then treated with vehicle (DMF) or 1, 3, 10 or 30 μM 2244-TCB for 90 minutes. ^{3}H-AA release is presented as the percent of total cellular ^{3}H-AA released into culture medium. N = 3 separate experiments. a= significantly different from respective group in the absence of PCB; b= significantly different from the same PCB concentration in the absence of inhibitor.

isoforms of PLA_2 (Magolda and Galbraith, 1989), or SB1, an inhibitor of p38 MAP kinase (Lee *et al*, 1994) had no effect on 2244-TCB-induced ^{3}H-AA release.

III.4.B. p38 MAP kinase protein phosphorylation in the presence of 2244-TCB and signal transduction inhibitors.

Total levels of p38 protein were similar in untreated cells and 2244-TCB-treated cells and were unaffected by SB1, SB2, OP or BEL. In untreated cells, levels of phosphorylated (active) p38 were negligible. Levels of phospho-p38 increased after 60 or 90 minutes of exposure to 2244-TCB. The p38 MAPK inhibitors SB1 and SB2 attenuated this increase. Phospho-p38 protein levels were not affected by exposure to OP or BEL (Figure III.2).

III.4.C. Effects of signal transduction inhibitors on 2244-TCB-mediated changes in COX-2 mRNA, protein and activity.

Exposure to 2244-TCB increased COX-2 mRNA as compared to control (Figure III.3.A). Incubation with either SB1 or SB2 significantly reduced this increase. Neither OP nor BEL treatment affected the 2244-TCB-mediated increase in COX-2 mRNA. COX-2 protein was not detectable in untreated cells. By 60 or 90 minutes of exposure to 2244-TCB, COX-2 protein was increased to detectable levels (Figure III.3.B). This increase was prevented

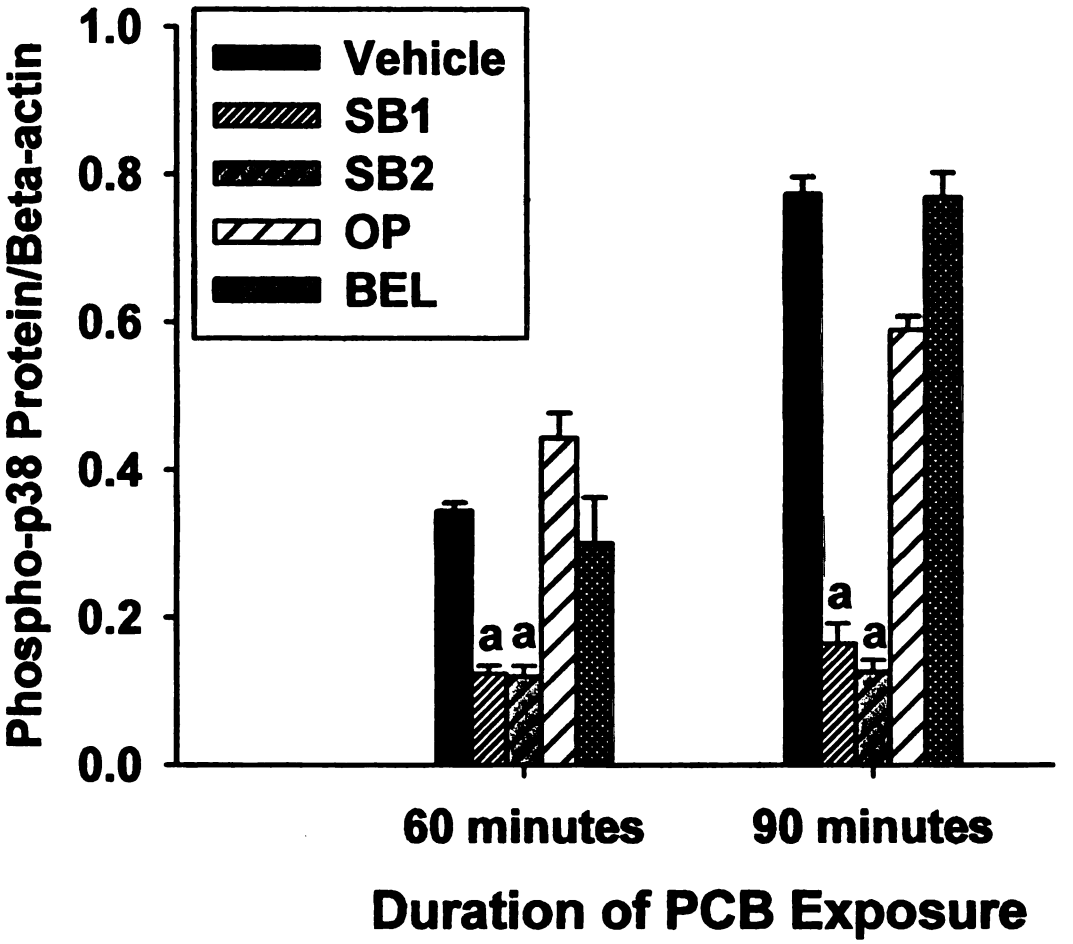

Figure III.2. 2244-TCB-mediated activation of p38 in the presence of signal transduction inhibitors. Levels of active, phospho-p38 protein were determined by Western analysis in HL-60 cells (50 x10^6) pretreated for 30 minutes with vehicle, 5 μM SB1, 15 μM SB2, 15 μM OP or 25 μM BEL, then treated with 30 μM 2244-TCB for 60 or 90 minutes as described in Materials and Methods. β-actin was used as a loading control. Phospho-p38/β-actin ratios were determined by densitometry. N = 3 separate experiments. a= significantly different from 2244-TCB in the absence of inhibitor at the respective time.

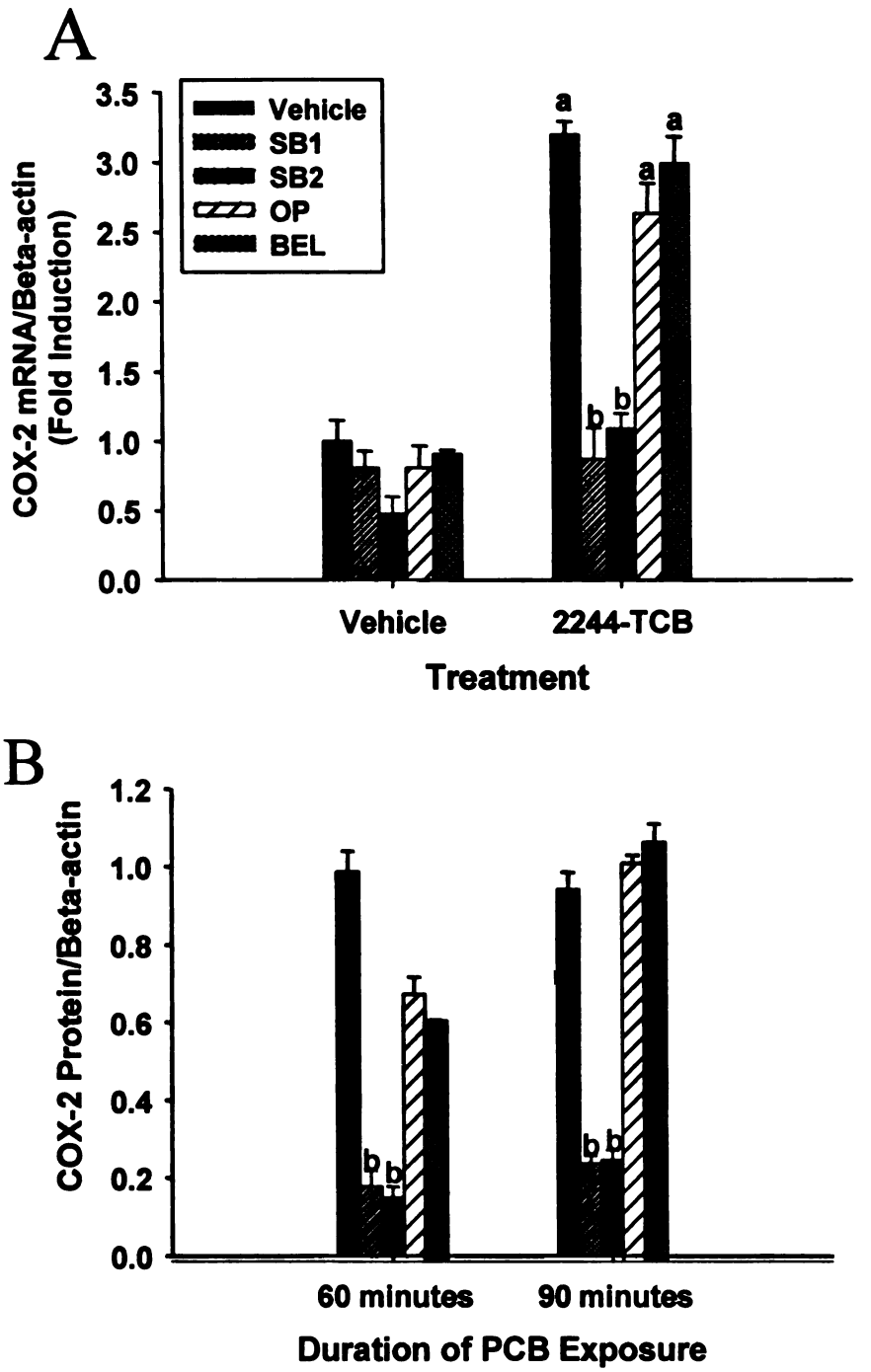

Figure III.3. 2244-TCB-mediated COX-2 mRNA expression in the presence of signal transduction inhibitors. HL-60 cells (A: 5 x10^6; B: 50 x10^6) were pretreated for 30 minutes with vehicle, 5 μM SB1, 15 μM SB2, 15 μM OP or 25 μM BEL, then treated with vehicle or 30 μM 2244-TCB. A) COX-2 mRNA was determined by RT-PCR as described in Materials and Methods after 30 minutes exposure to 2244-TCB. B) COX-2 protein was determined after 60 or 90 minutes exposure to 2244-TCB as described in Materials and Methods. β-actin was used as a loading control. N= 5 separate experiments. a= significantly different from respective group in the absence of 2244-TCB; b= significantly different from 2244-TCB in the absence of inhibitor.

by incubation of the cells with SB1 or SB2. The 2244-TCB-mediated increase in COX-2 protein was unchanged by exposure to OP or BEL.

COX-2 activity in untreated HL-60 cells was ~10 units/mL (Figure III.4). Treatment with 2244-TCB for 90 minutes increased COX-2 activity approximately 4-fold. Pretreatment with SB1 significantly decreased 2244-TCB-stimulated COX-2 activity. Neither OP nor BEL had a significant effect on COX-2 activity.

III.4.D. Effects of inhibitors on p38 and ERK phosphorylation.

In untreated cells, the ratio of phospho-ERK to total ERK was small. Treatment with 2244-TCB caused a significant increase in phospho-ERK (Figure III.5). Pretreatment with either ERK inhibitor, PD 98059 or U 0126, significantly decreased 2244-TCB-stimulated phosphorylation of ERK (Figure III.5.A). Additionally, pretreatment with either p38 inhibitor significantly decreased 2244-TCB-stimulated phosphorylation of ERK (Figure II.5.B). None of these inhibitors affected phosphorylation of ERK in the absence of 2244-TCB. The ratio of phospho-p38 to total p38 was increased by exposure to 2244-TCB (Figure III.6). ERK inhibitors did not affect phosphorylation of p38 in the absence or presence of 2244-TCB. Phosphorylation of JNK was not affected by 2244-TCB (Figure III.7).

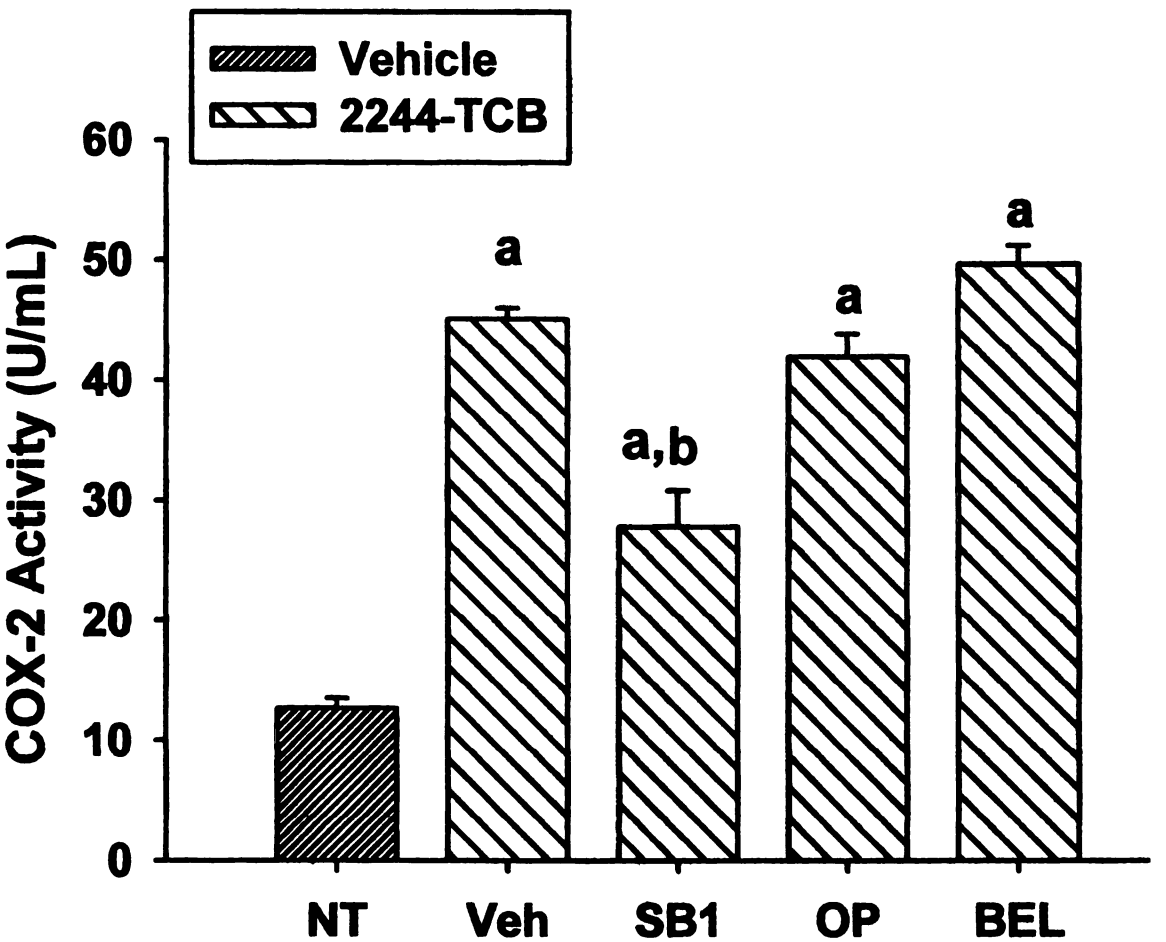

Figure III.4. 2244-TCB-mediated activation of COX-2 in the presence of signal transduction inhibitors. COX-2 activity was determined in HL-60 cells (15×10^6) pretreated for 30 minutes with vehicle, 5 μM SB1, 15 μM OP or 25 μM BEL, then treated with 30 μM 2244-TCB for 90 minutes. COX activity was measured as described in Materials and Methods. N= 3 separate experiments. NT= no treatment. a= significantly different from untreated cells. b= significantly different from 2244-TCB-treated cells in the absence of inhibitor (i.e. vehicle-treated).

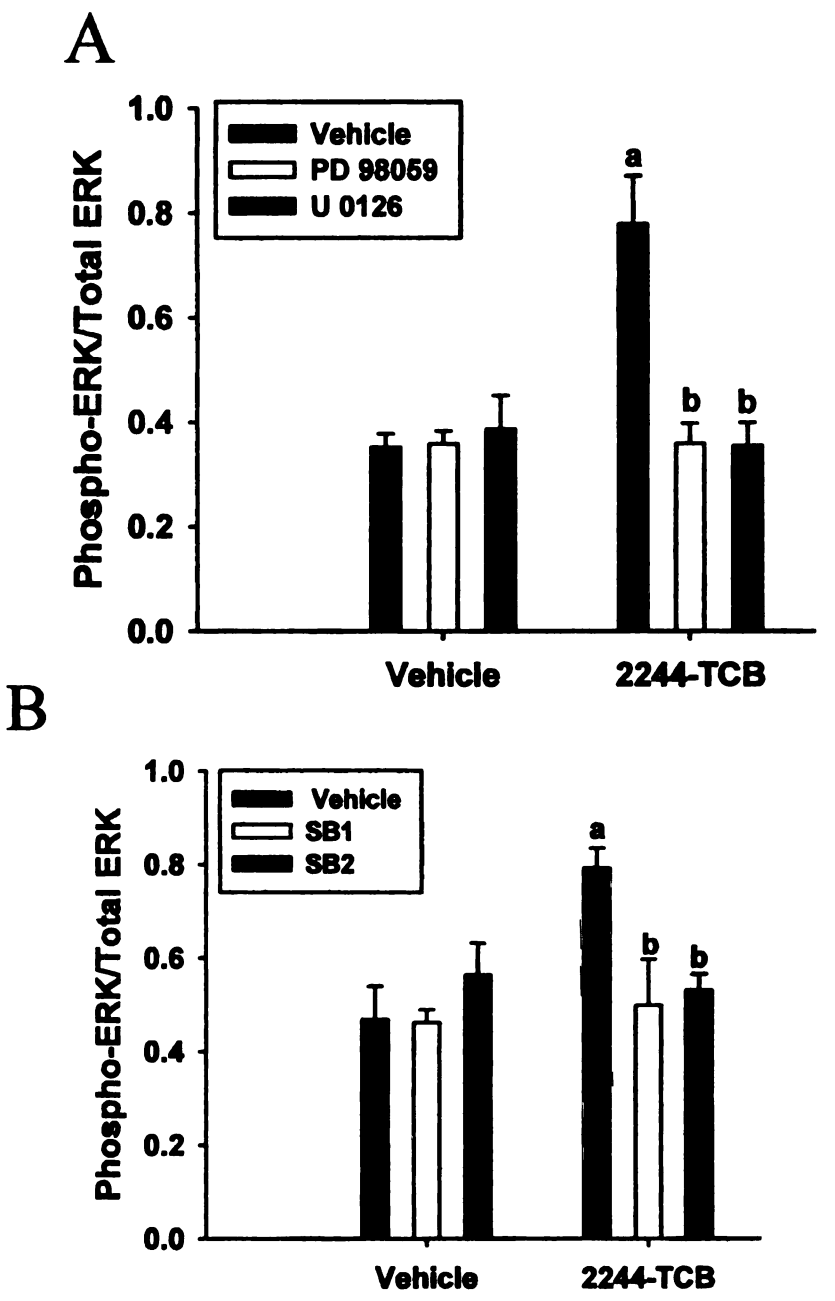

Figure III.5. Effects of inhibitors on ERK phosphorylation. Phosphorylated and total ERK were determined by ELISA in HL-60 cells (20 x 10^6) pretreated for 30 minutes with vehicle, 10 μM PD 98059, 5 μM U 0126, 5 μM SB1 or 15 μM SB2 then treated with vehicle or 30 μM 2244-TCB for 45 minutes. N = 3 separate experiments. a= significantly different from respective group in the absence of 2244-TCB; b= significantly different from 2244-TCB in the absence of inhibitor.

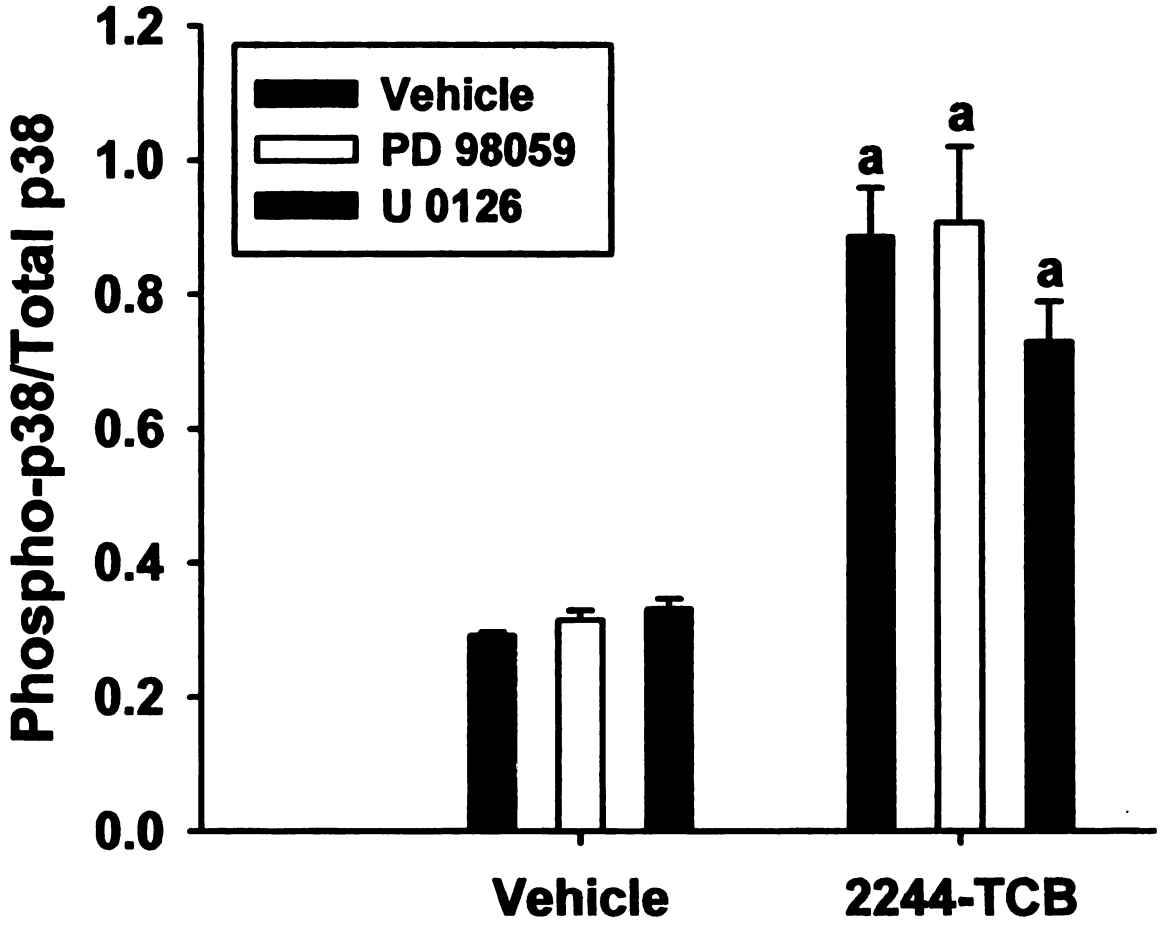

Figure III.6. Effects of inhibitors on p38 phosphorylation. Phosphorylated and total p38 was determined by ELISA in HL-60 cells (20×10^6) pretreated for 30 minutes with vehicle, 10 μM PD 98059 or 5 μM U 0126 then treated with vehicle or 30 μM 2244-TCB for 90 minutes. N = 3 separate experiments. a= significantly different from respective group in the absence of 2244-TCB.

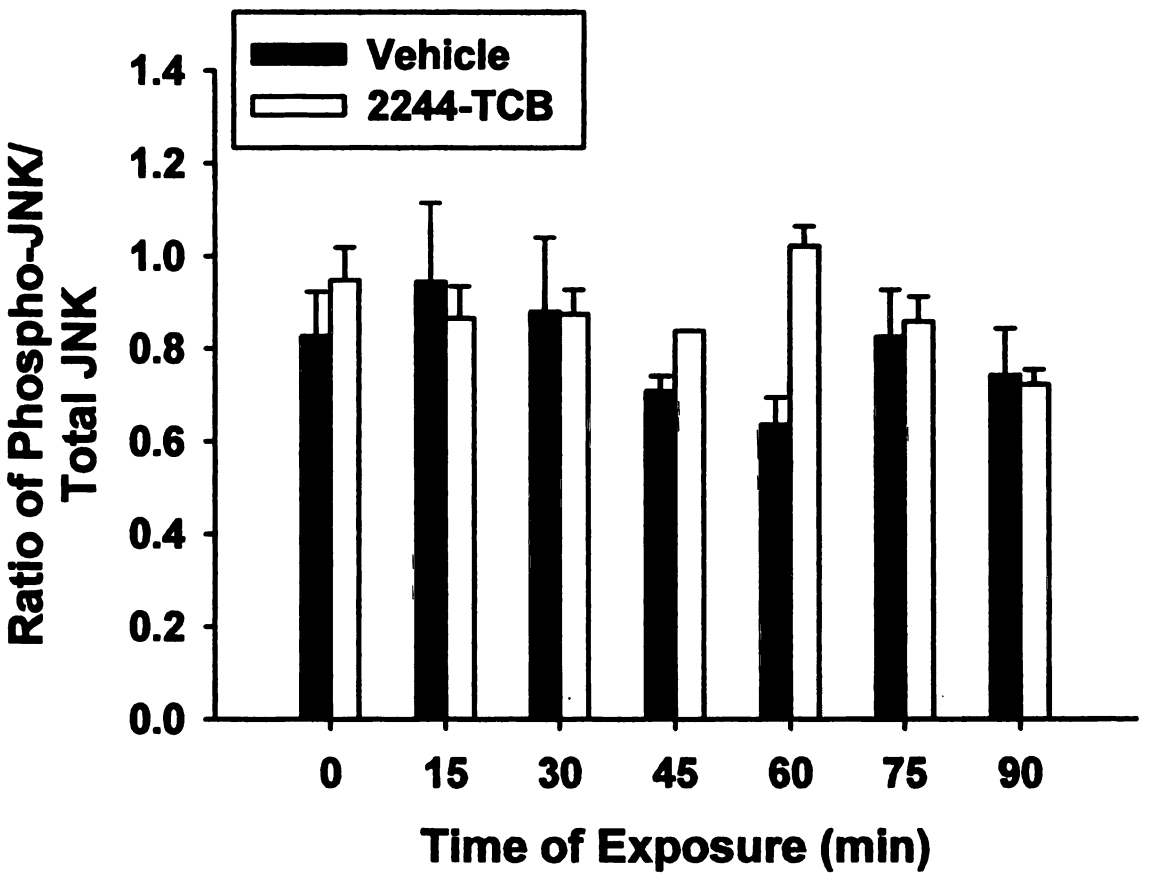

Figure III.7. Nuclear JNK is not affected by 2244-TCB. Phosphorylated and total JNK was determined by ELISA in HL-60 cells (20 x 10^6) treated with vehicle or 2244-TCB for 0, 15, 30, 45, 60, 75 or 90 minutes. N = 3 separate experiments.

III.4.E. Effects of ERK inhibitors on 2244-TCB-mediated COX-2 mRNA expression.

COX-2 mRNA expression was low in vehicle-treated cells and was unaffected by exposure to either PD 98059 or U 0126 (Figure III.8). As noted above, 2244-TCB increased COX-2 mRNA as compared to control. Pretreatment with either PD 98059 or U 0126 did not affect 2244-TCB-mediated increases in COX-2 mRNA.

III.4.F. Effect of inhibitors of superoxide anion production on 2244-TCB-induced changes in COX-2 mRNA expression.

In differentiated HL-60 cells, exposure to 2244-TCB causes a time- and concentration-dependent increase in superoxide anion production (Bezdecny *et al*, 2005). In the absence of 2244-TCB, cells produced ~300 nmol/min/million cells of superoxide anion. 2244-TCB increased superoxide production relative to control (Figure III.9.A), and pretreatment with either the NADPH oxidase inhibitor apocynin or the intracellular free radical scavenger TEMPO prevented the 2244-TCB-mediated increase in superoxide anion concentration.

2244-TCB increased COX-2 mRNA levels approximately fourfold after 30 minutes of exposure (Figure III.9.B). Neither apocynin nor TEMPO pretreatment affected the 2244-TCB-mediated increase in COX-2 mRNA.

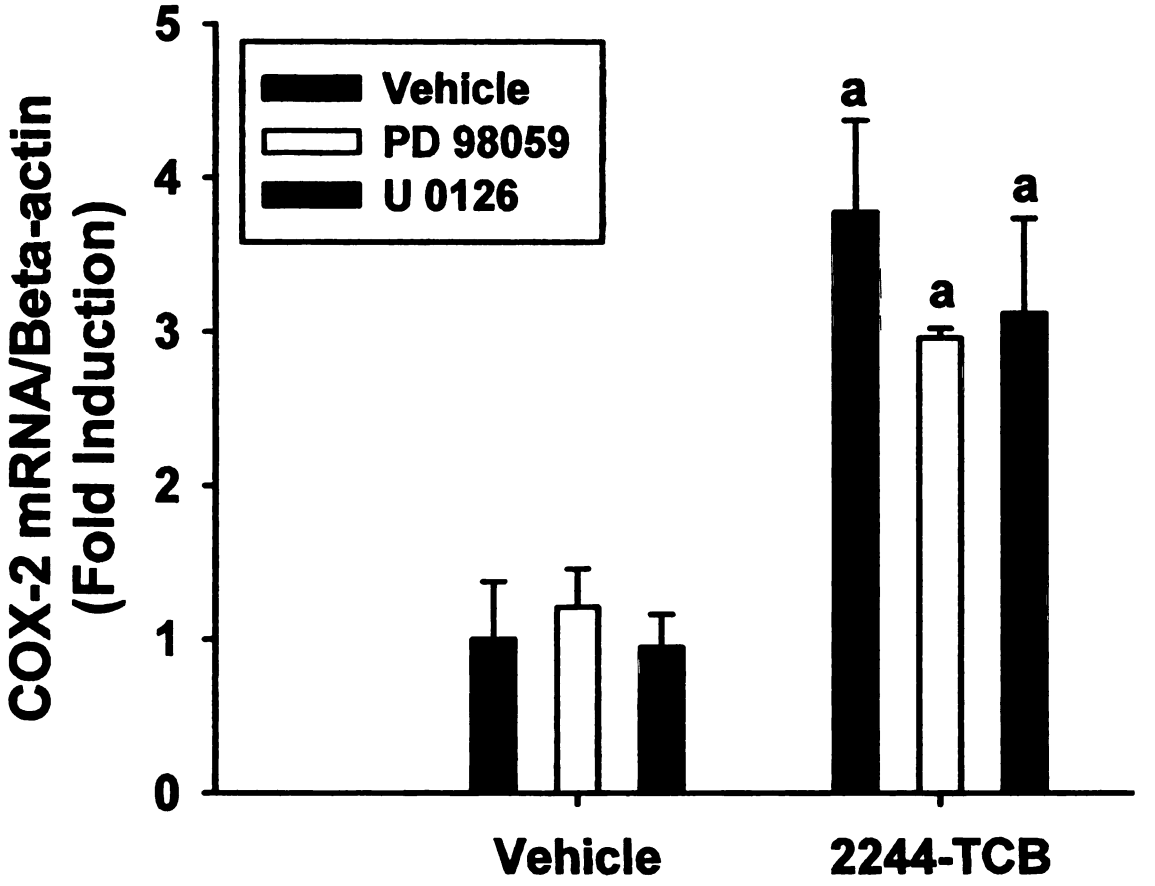

Figure III.8. Effects of ERK inhibitors on 2244-TCB-mediated COX-2 mRNA expression. COX-2 mRNA was determined by RT-PCR in HL-60 cells (5 x106) pretreated for 30 minutes with vehicle, 10 μM PD 98059 or 5 μM U 0126, then treated with 30 μM 2244-TCB for 30 minutes. β-actin was used as a loading control. N= 4 separate experiments. a= significantly different from respective group in the absence of 2244-TCB.

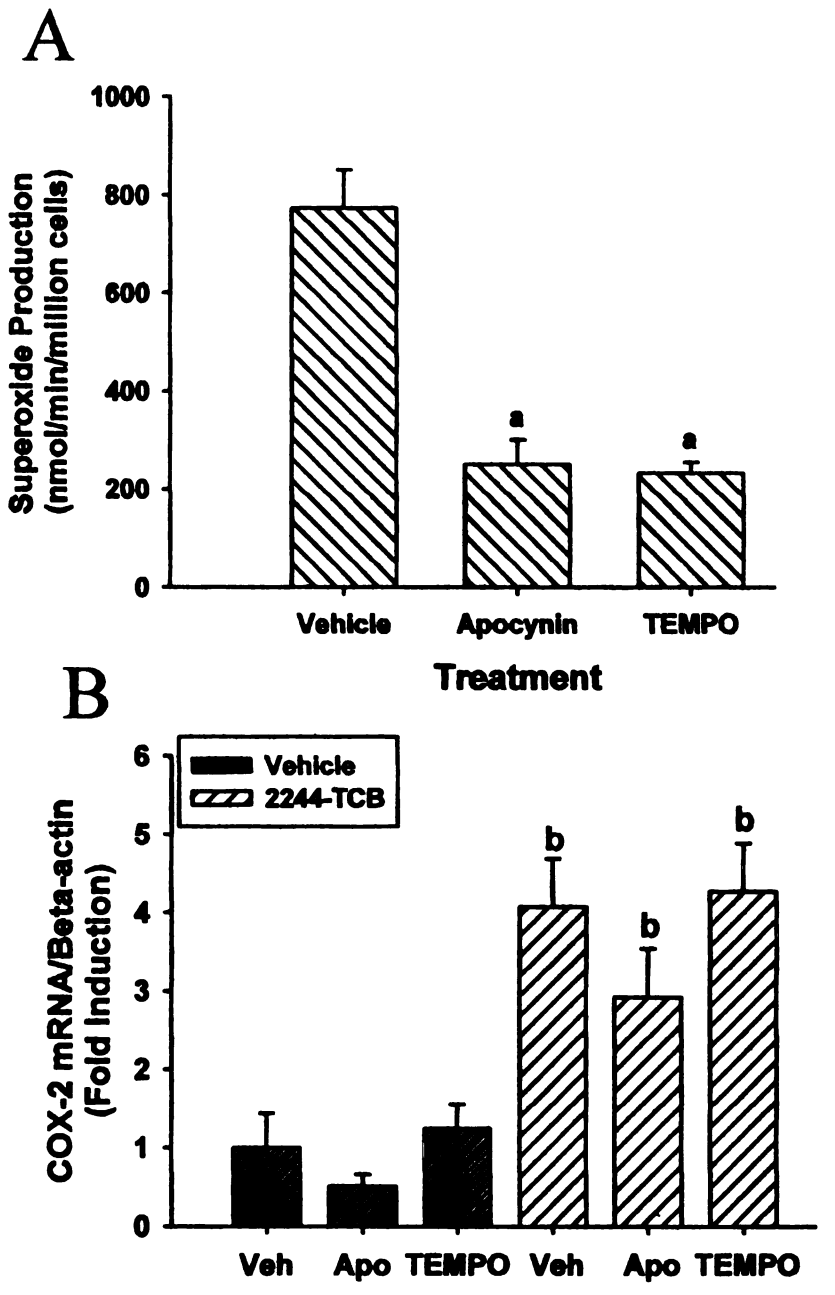

Figure III.9. Lack of effect of reactive oxygen species inhibition on 2244-TCB-induced upregulation of COX-2 mRNA expression. (A) HL-60 cells ($1x10^6$) were pretreated with vehicle, 1mM apocynin (30 minutes) or 200 μM TEMPO (10 minutes) then exposed to 30 μM 2244-TCB for 30 minutes after which superoxide anion production was determined. (B) HL-60 cells were pretreated with vehicle, apocynin or TEMPO as described, then with vehicle or 2244-TCB for 30 minutes. mRNA was collected for determination of COX-2 mRNA levels. β-actin was used as a loading control. N = 3 separate experiments. a= significantly different from 2244-TCB-treated cells in the absence of inhibitor (i.e. vehicle-treated). b= significantly different from respective group in the absence of 2244-TCB.

Inhibition of ROS production also did not affect release of ^{3}H-AA (Figure III.10).

III.5. Discussion

In the present study, three signal transduction pathways were investigated for potential involvement in 2244-TCB-mediated upregulation of COX-2 in differentiated HL-60 cells. One signal transduction pathway that was investigated is the PLA_2 pathway that leads to release of AA. AA acts as both the substrate for COX-mediated eicosanoid production as well as a cell signaling molecule. 2244-TCB increased ^{3}H-AA release, and this response was attenuated by pretreatment with BEL, an inhibitor of $iPLA_2$, but not by OP, an inhibitor of cytosolic and secretory PLA_2 (Figure III.1). Similar $iPLA_2$-mediated increases in ^{3}H-AA release and associated increases in eicosanoid production have been reported in primary rat neutrophils treated with Aroclor 1242 as well as with individual, noncoplanar PCB congeners (Olivero and Ganey, 2001; Tithof *et al*, 1998). Thus, rat neutrophils and human-derived HL-60 cells appear to share a common pathway to PCB-stimulated AA release, i.e. one mediated via $iPLA_2$.

Activation of PLA_2 contributed to upregulation of COX-2 in murine macrophages and human lung cells (Balsinde *et* al, 1999; Pawliczak *et al*, 2002, 2004). Furthermore, free AA activates a variety of proteins involved in the regulation of COX-2 expression, including p38 MAP kinase (Hii *et al*, 1998), peroxisome proliferator-activated receptor γ (Pawliczak *et al*, 2002, 2004) and

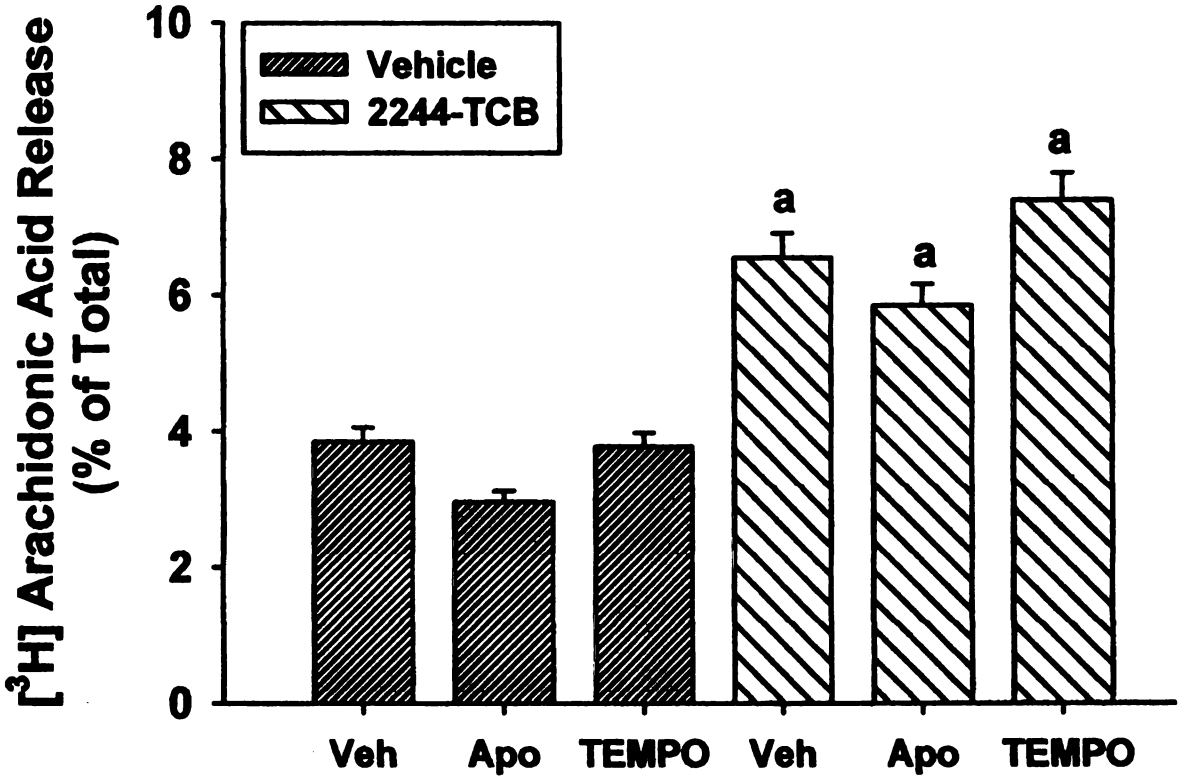

Figure III.10. 2244-TCB-mediated ^{3}H-AA release in the presence of ROS inhibitors. Differentiated HL-60 cells were labeled with ^{3}H-AA as described in Materials and Methods. Labeled cells ($1x10^{6}$ cells/mL) were pretreated with vehicle, 1mM apocynin for 30 minutes or 200 μM TEMPO for 10 minutes. They were then treated with vehicle (DMF) or 30 μM 2244-TCB for 90 minutes. ^{3}H-AA release is presented as the percent of total cellular ^{3}H-AA released into culture medium. N = 3 separate experiments. a= significantly different from respective group in the absence of PCB.

phosphoinositide 3-kinase (Monick *et* al, 2002). Interestingly, expression of COX-2 was decreased in brains of PLA_2-deficient mice relative to wild type mice (Bosetti and Weerasinghe, 2003). These reports suggest that PLA_2 or free AA can contribute to increased expression of COX-2. Despite this possibility, this pathway does not appear to be involved in the 2244-TCB-mediated upregulation of COX-2 in HL-60 cells because treatment with BEL, which inhibited release of ^{3}H-AA, had no effect on COX-2 mRNA, protein or enzyme activity (Figures III.3 and III.4).

The link between p38 activity and changes in COX-2 expression has been well established in a variety of systems, including rat hepatic macrophages (Ahmad *et al*, 2002), the HeLa cell line (Lasa *et al*, 2000), human airway myocytes (Singer *et al*, 2003) and human monocytes (Dean *et al*, 1999). p38 regulates COX-2 expression both at the transcriptional level, by activating transcription factors, as well as at the level of RNA stability, by activating cytoplasmic proteins that increase the half life of COX-2 mRNA (Dean *et al*, 1999; Lasa *et* al, 2000; Singer *et al*, 2003). In the present study, 2244-TCB exposure increased levels of phosphorylated, active p38 (Figure III.2). Activation of p38 MAP kinase was associated with increased levels of COX-2 protein in cells treated with 2244-TCB for similar times (Figure III.3.B). Treatment with the p38 MAP kinase inhibitors SB1 and SB2 reduced levels of phospho-p38 as well as mRNA levels, protein and enzyme activity of COX-2 (Figures III.2, III.3 and III.4). These results suggest that 2244-TCB exposure

leads to activation of the p38 MAP kinase system, and that this activation is required for the 2244-TCB-mediated induction of COX-2.

Exposure of neutrophils to PCBs leads to increased production of PGs and Tx (Tithof *et al*, 1998). Two components are necessary for generation of PGs and Tx: availability of free AA as substrate and COX activity. 2244-TCB provides both of these components by apparently independent pathways. Release of ^{3}H-AA was diminished by an inhibitor of $iPLA_2$, BEL (Figure III.1), but this inhibitor did not affect the 2244-TCB-mediated increase in COX-2 mRNA (Figure III.3.A). Conversely, activation of $iPLA_2$ and subsequent release of ^{3}H-AA was not affected by inhibitors of p38 MAP kinase (Figure III.1), which did reduce the 2244-TCB-mediated upregulation of COX-2 mRNA, protein and activity (Figure III.3).

p38 is not the only MAP kinase reported to be involved in induction of COX-2. Activation of ERK and JNK regulates COX-2 expression in several cell types, including mouse macrophages and RAW-264.7 cells (Moon and Pestka, 2002; Steer *et al*, 2006), cardiac myocytes (Wu *et al*, 2006), the HeLa cell line (Holzberg *et al*, 2003) and glomerular epithelial cells (Takano *et al*, 2001). Both ERK and JNK regulate COX-2 expression at the transcriptional level by activating transcription factors, but unlike p38, do not appear to regulate COX-2 mRNA stability (Holzberg *et al*, 2003; Moon and Pestka, 2002; Steer *et al*, 2006; Takano *et al*, 2001). In the present study, 2244-TCB exposure had no effect on JNK activation at any time up to 90 minutes after exposure (Figure III.7). 2244-TCB increased levels of phosphorylated ERK, and this increase was

prevented by pretreatment with the p38 inhibitors SB1 and SB2, as well as by the ERK inhibitors PD 98059 and U 0126 (Figure III.5). However, pretreatment with PD 98059 or U 0126 had no effect on the 2244-TCB-mediated increase in COX-2 mRNA (Figure III.8). These data suggest that ERK is activated by p38 in response to 2244-TCB exposure, but it is not involved in the 2244-TCB-mediated induction of COX-2.

Another pathway examined involved production of ROS. ROS, such as superoxide anion, contribute to changes in COX-2 gene regulation induced by a number of different stimuli in a variety of cell systems. For example, in human mesangial cells exposed to high glucose, in human monocytes undergoing differentiation, and in human fibroblasts subjected to cyclic stretch stimuli, increased COX-2 expression was dependent on generation of ROS (Amma *et* al, 2005; Barbieri *et* al, 2003; Kiritoshi *et al*, 2003). These changes occurred through activation of p38 MAP kinase and a variety of transcription factors including NF-κB (Amma *et al*, 2005; Kim *et al*, 2005; Singer *et al*, 2003*)*. In the present study, 2244-TCB caused an increase in superoxide anion concentration. This increase was prevented by apocynin, an inhibitor of NADPH oxidase, as well as by TEMPO, an intracellular free radical scavenger (Figure III.9.A). However, treatment with apocynin or TEMPO did not affect 2244-TCB-mediated upregulation of COX-2 mRNA (Figure III.9.B), suggesting that ROS do not play a critical role in this response in HL-60 cells.

In summary, several different signal transduction pathways are affected by exposure to 2244-TCB; however, not all of these pathways play a role in

2244-TCB-mediated upregulation of COX-2 mRNA. Neither reactive oxygen species, PLA_2 nor free AA appear to be involved in increased expression of COX-2 by 2244-TCB, though increased levels of free AA can still serve as a substrate for eicosanoid production (Figure III.11). ERK was activated in response to 2244-TCB exposure but this activation is not needed for the upregulation of COX-2. In contrast, increased COX-2 mRNA, protein and activity were associated with phosphorylation of p38 MAP kinase in 2244-TCB treated HL-60 cells, and these effects were attenuated by pretreatment with p38 MAP kinase inhibitors. Taken together, these results support a working hypothesis in which activation of neutrophils by 2244-TCB enhances prostaglandin production by increasing p38 MAP kinase-dependent biosynthesis of COX-2 enzyme and by activating $iPLA_2$ to provide AA as substrate for prostaglandin synthesis.

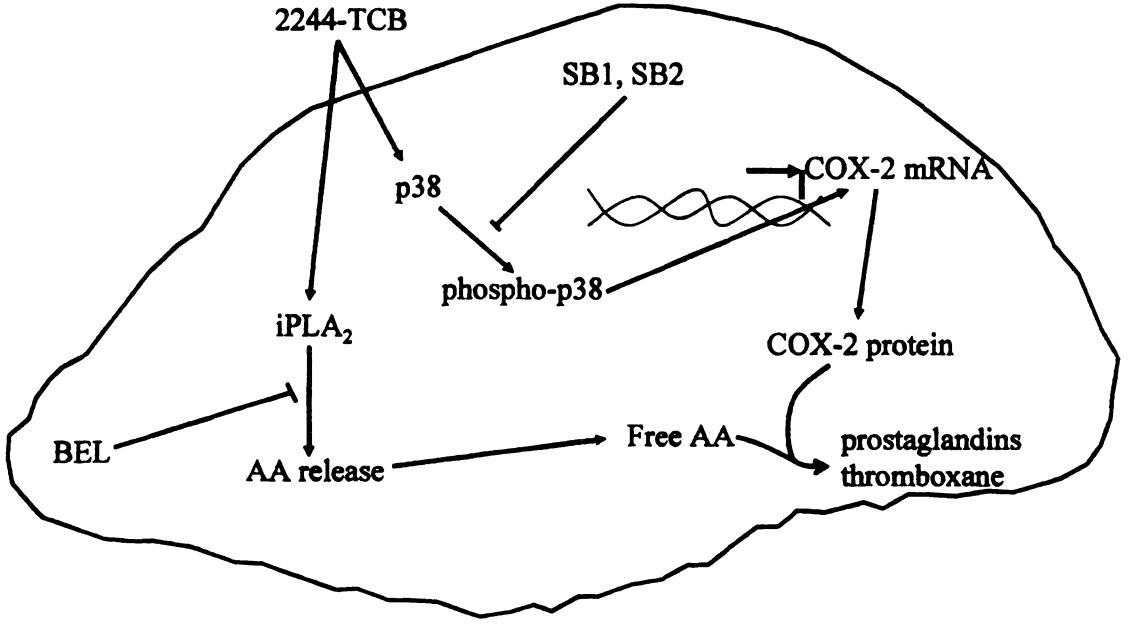

Figure III.11. Proposed mechanism by which 2244-TCB exposure leads to upregulation of the COX-2 gene. 2244-TCB exposure causes phosphorylation and activation of p38 MAP kinase. This activation leads to increases in COX-2 mRNA, protein and activity. In addition, 2244-TCB activates $iPLA_2$ by a pathway independent of p38 MAP kinase, leading to the release of free AA, which can then be converted by COX-2 into PGs and Tx.

Chapter IV

2,2′,4,4′-Tetrachlorobiphenyl Upregulates Cyclooxygenase-2 in HL-60 cells via p38 Mitogen-Activated Protein Kinase and NF-κB

IV.1 Summary

PCBs are persistent environmental contaminants that affect a number of cellular systems, including neutrophils. The objective of this study was to explore the roles that NF-κB and C/EBP play in the 2244-TCB-mediated upregulation of COX-2 and to evaluate the relationship of MAP kinase pathways to 2244-TCB-mediated changes. Treatment of granulocytic HL-60 cells with 2244-TCB increased the amount of nuclear C/EBP beta and NF-κB. These increases were prevented by pretreatment with either of the p38 MAP kinase inhibitors, SB1 or SB2, but not by pretreatment with either of the ERK MAP kinase inhibitors, PD 98059 or U 0126. Pretreatment with Bay 11-7082 or helenalin, inhibitors of NF-κB, prevented the 2244-TCB-mediated induction of COX-2 mRNA. Exposure to the transcriptional inhibitor ActD after 10 or 20 minutes exposure to 2244-TCB reduced the 2244-TCB-mediated increase in COX-2 mRNA. However, exposure to ActD after 30 minutes exposure had no effect upon COX-2 mRNA levels, indicating that transcription of the COX-2 gene occurs rapidly after exposure to 2244-TCB but is no longer significant after 30 minutes exposure. Taken together, these results suggest that p38 is involved in the increased transcription of the COX-2 gene via an NF-κB-dependent pathway.

IV.2 Introduction

PCBs are persistent, man-made pollutants that are widespread in the environment. Food is the main source of human exposure, and although PCB levels are declining due to legislation and preventive measures, people are still exposed (Turyk *et al*, 2006). The 209 PCB congeners differ with regard to the number and the location of the chlorine atoms on the two benzene rings. In the present study, we focused on 2244-TCB, a noncoplanar congener. Noncoplanar PCBs are generally considered to be less toxic than coplanar or dioxin-like PCBs, but occur in the environment in larger concentrations than the dioxin-like PCBs (WHO, 2003).

PCBs cause a variety of adverse effects, including alterations of the reproductive, nervous and cardiovascular systems (Faqi *et al*, 1998; Lamb *et al*, 2006; Pessah *et al*, 2006; Warshaw *et al*, 1976). In addition, they are probable human carcinogens (Cogliano, 1998) and cause a number of non-cancer effects, including dermal lesions and ocular toxicity in humans, and hepatotoxicity and endocrine effects in rats (Morse *et al*, 1996; Takamatsu *et al*, 1985; Twaroski *et al*, 2001; Vondracek *et al*, 2005). PCBs also affect the regulation of a number of genes, including cytochromes P450 in rat livers and COX-2 in a neutrophil-like cell line (Bezdecny *et al*, 2005; Chubb *et al*, 2004).

COX-2 is generally found in negligible amounts in normal cells, such as quiescent neutrophils, but is induced by a wide variety of inflammatory stimuli (Smith *et al*, 2000; Vane *et al*, 1998). COX-2 catalyzes the committed step in the synthesis of prostaglandins and thromboxanes. Once produced, these

mediators can cause a wide range of immunomodulatory effects by acting on G protein-linked prostanoid receptors or, in some cases, on nuclear receptors (Louis *et al*, 2005).

A number of shared or convergent pathways have been described that are involved in transcriptional regulation of COX-2 in response to inflammatory mediators. These include NF-κB and C/EBP, two signaling pathways commonly activated in an inflammatory response (Poli, 1998; Ghosh *et al*, 1998). In addition, elements of the MAP kinase cascades regulate COX-2 expression. These pathways are the ERK1/2, JNK and p38 pathways (Su and Karin, 1996). Both the p38 and ERK pathways are activated in HL-60 cells in response to 2244-TCB exposure, and inhibition of p38 prevents 2244-TCB-mediated upregulation of COX-2 (Figure III.3, III.4).

In mammalian cells, activity of NF-κB is regulated by its association with the inhibitory subunit, IκB. NF-κB is activated by many of the same agents that activate neutrophils, such as IL-1, TNF-α and endotoxin. Whereas the initial steps involved in NF-κB activation vary among cell types, pathways converge on kinases which, when activated, phosphorylate the IκB subunit, causing it to dissociate from NF-κB. NF-κB then translocates into the nucleus where it binds to transcriptional response elements (Wulczyn *et al*, 1996). One pathway that can activate NF-κB is the p38 MAP kinase system. For example, p38 activates NF-κB in endotoxin-treated human neutrophils (Cloutier *et al*, 2006), in human lung epithelium exposed to Legionella pneumophilia (N'Guessan *et al*, 2006) and in human dendritic cells exposed to CpG DNA (Osawa *et al*, 2006).

C/EBPs comprise a family of transcription factors containing six known members. In neutrophils, C/EBPs play an important role in cellular maturation. The regulation of C/EBPs involves transcription of both full-length and truncated isoforms of the protein. The full-length isoforms activate C/EBP binding domains in the nucleus. The truncated isoforms also bind to DNA but lack the transactivation domain required for transcriptional activity (Lekstrom-Himes, 2001). Like NF-κB, C/EBP is activated by p38 MAP kinase. p38-dependent activation of C/EBP occurs in proliferating cardiomyocytes (Ambrosino *et al*, 2006), mammary epithelial cells (Seymour *et al*, 2006) and murine macrophages (Chen *et al*, 2004). Given the observation that 2244-TCB increases COX-2 expression by a p38 MAP kinase-dependent mechanism (Bezdecny *et al*, 2005; Chapter III) and that both NF-κB and C/EBP-beta are activated by p38, it was of interest to determine the roles that NF-κB and C/EBP play in the 2244-TCB-mediated upregulation of COX-2 mRNA.

IV.3 Materials and Methods

IV.3.A. Chemicals

Bay 11-7082 and helanalin were purchased from Biomol (Plymouth Meeting, PA). Actinomycin D was purchased from Invitrogen (Carlsbad, CA). All other chemicals were of the highest grade commercially available.

IV.3.B. Exposure to inhibitors.

Stock solutions of inhibitors were prepared by dissolution in DMSO, and 1 μL/mL of the stock solution was added to the cells to achieve the desired concentration. Control cells received 1 μL/mL of DMSO. Exposure to vehicle caused no significant effects. The concentrations of inhibitors used in the current study were selected based on their IC50 values and previously described activities (Ackerman *et* al, 1995; Hsieh *et al*, 2006; Lee *et al*, 1994; Lee *et al*, 2006; Magolda and Galbraith, 1985).

IV.3.C. Preparation of nuclear extracts.

Differentiated HL-60 cells (5×10^6) were suspended in HBSS and pretreated with the appropriate inhibitor or vehicle for 30 minutes. They were then treated with vehicle or 30 μM 2244-TCB. After 30 minutes, nuclear protein was isolated using a nuclear extract kit (Active Motif, Carlsbad, CA) according to manufacturer's instructions.

IV.3.D. Determination of nuclear C/EBP and NF-κB.

For time course studies differentiated HL-60 cells (10×10^6) were suspended in HBSS and treated with vehicle or 2244-TCB. Nuclear protein was isolated after 0, 15, 30, 45, 60, 75 or 90 minutes. For inhibitor studies, differentiated HL-60 cells (10×10^6) were suspended in HBSS and pretreated with the appropriate inhibitor or its vehicle. 30 minutes later they were treated with vehicle or 30 μM 2244-TCB for 30 minutes, after which

nuclear protein was isolated. Amounts of nuclear C/EBP-beta or NF-κB were measured using TransAM C/EBP-beta or TransAM NF-κB ELISA kits (Active Motif, Carlsbad, CA) according to manufacturer's instructions.

IV.3.E. COX-2 mRNA stability assay.

Differentiated HL-60 cells (5 x 10^6) were suspended in HBSS and treated with 30 μM 2244-TCB for 10, 20 or 30 minutes at 37°C. Samples were then exposed to 10 μg/mL ActD or vehicle. Samples were collected 0, 10, 20, 30, 40, 50 and 60 minutes after exposure to ActD and centrifuged for 10 minutes at 4000 g. RNA was collected, and real-time RT-PCR was performed on the samples as described above.

IV.3.F. Statistical analysis.

Data are expressed as mean ± SEM. Results were analyzed by two-way repeated measures analysis of variance. Group means for all data were compared using Tukey's post hoc test. Appropriate transformations were performed on all data that did not follow a normal distribution. For all studies, the criterion for statistical significance was $p < 0.05$. For information on exposure to 2244-TCB, see Chapter II. For information on HL-60 cells and determination of COX-2 mRNA, see Chapter III.

IV.4 Results

IV.4.A. Effects of 2244-TCB on nuclear C/EBP-beta in the presence and absence of MAP kinase inhibitors.

In the absence of 2244-TCB, the amount of nuclear C/EBP-beta increased approximately 1.2 fold after 30 minutes of exposure to vehicle and remained at this value through 90 minutes. Exposure to 30 μM 2244-TCB caused the amount of nuclear C/EBP-beta to increase approximately 1.6 fold after 15 minutes of exposure. The amount of nuclear C/EBP-beta remained greater than values in the vehicle-treated group through 75 minutes of exposure (Figure IV.1). Pretreatment with either SB1 or SB2, inhibitors of the p38 MAP kinase pathway, prevented the 2244-TCB-mediated increase in nuclear C/EBP-beta (Figure IV.2.A). The inhibitors had no effect on the amount of nuclear C/EBP-beta in the absence of 2244-TCB. Pretreatment with either PD 98059 or U 0126, inhibitors of the ERK MAP kinase pathway, did not affect the amount of nuclear C/EBP-beta in the absence or presence of 2244-TCB (Figure IV.2.B).

IV.4.B. Effects of 2244-TCB on nuclear NF-κB.

In the absence of 2244-TCB, the amount of nuclear NF-κB increased approximately 1.2 fold after 30 minutes of exposure to vehicle and remained at this amount through 90 minutes. Exposure to 2244-TCB increased the amount of NF-κB approximately 1.7 fold after 15 minutes of exposure. The amount remained greater than the vehicle-treated value for 75 minutes (Figure IV.3). Pretreatment with either SB1 or SB2 prevented the 2244-TCB-mediated

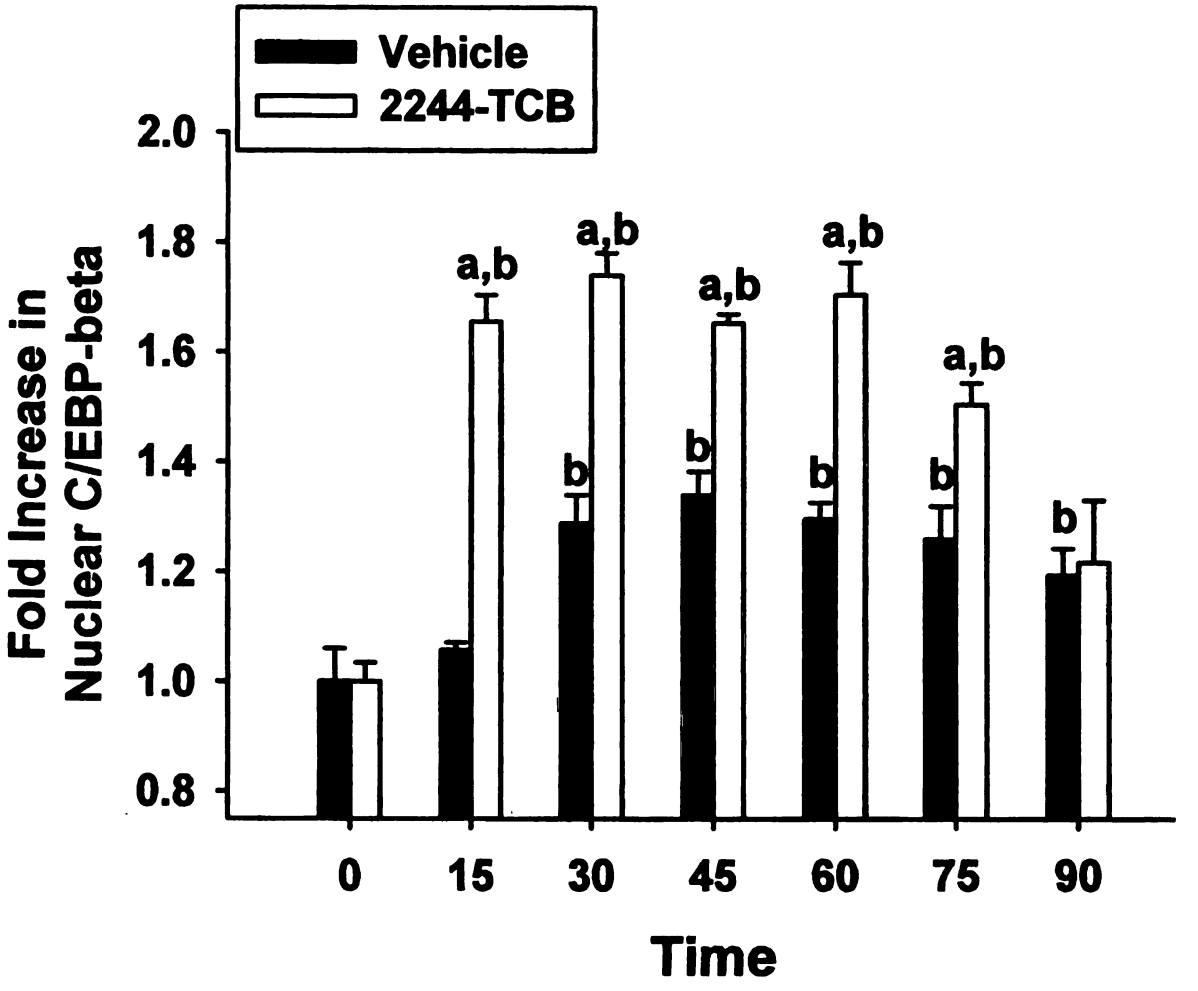

Figure IV.1. 2244-TCB increases nuclear C/EBP-beta. Nuclear C/EBP-beta was determined in HL-60 cells (10×10^6) treated with vehicle or 30 μM 2244-TCB for 0, 15, 30, 45, 60, 75 or 90 minutes. N = 3 separate experiments. a= significantly different from group treated for the same time in the absence of 2244-TCB; b= significantly different from respective group at time 0.

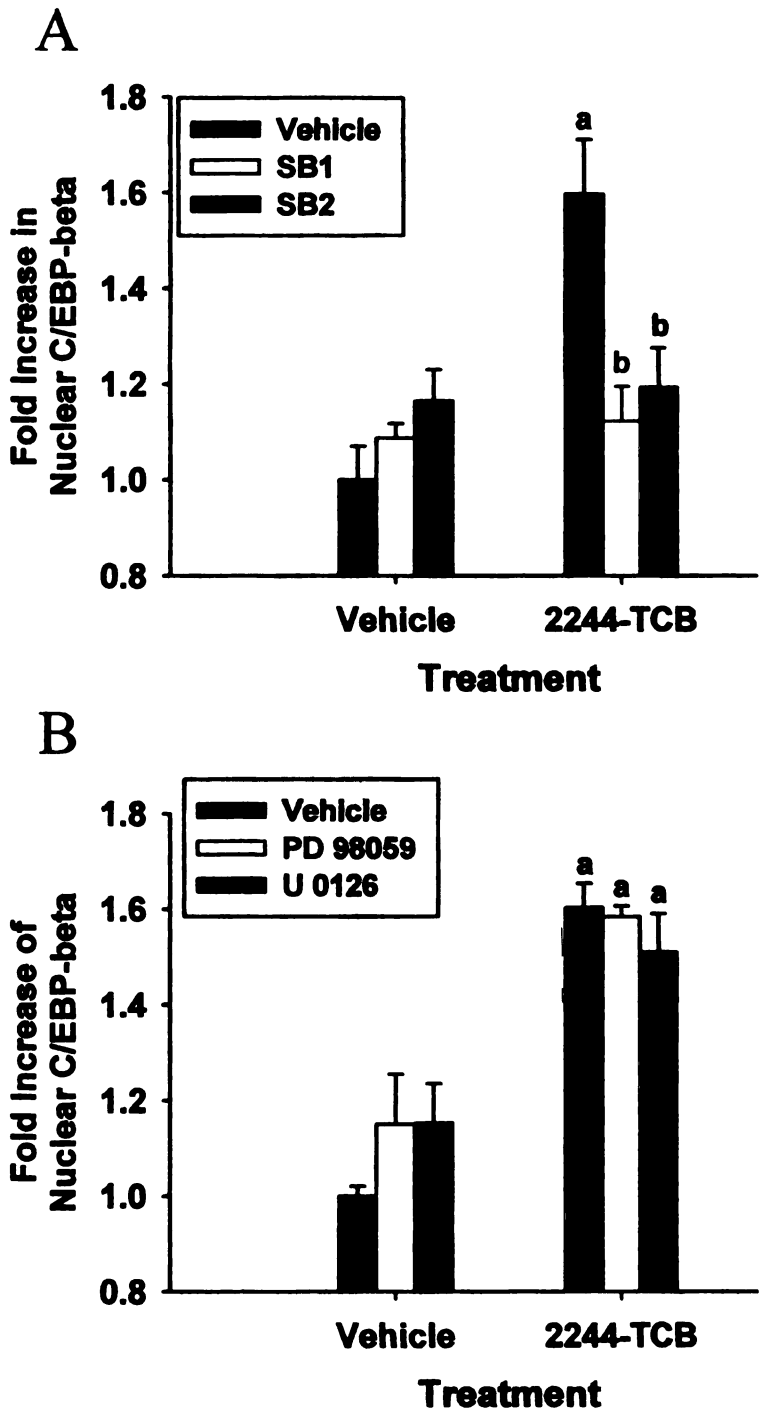

Figure IV.2. The 2244-TCB-mediated increase in nuclear C/EBP-beta is reduced by p38 inhibitors, but not by ERK inhibitors. Nuclear C/EBP-beta was determined in HL-60 cells (10 x 10^6) pretreated with vehicle, 5 μM SB1 or 15 μM SB2 (A) or 10 μM PD 98059 or 5 μM U 0126 (B) for 30 minutes, then treated with 30 μM 2244-TCB for 30 minutes. N = 3 separate experiments. a= significantly different from respective group in the absence of 2244-TCB; b= significantly different from respective group in the absence of inhibitor.

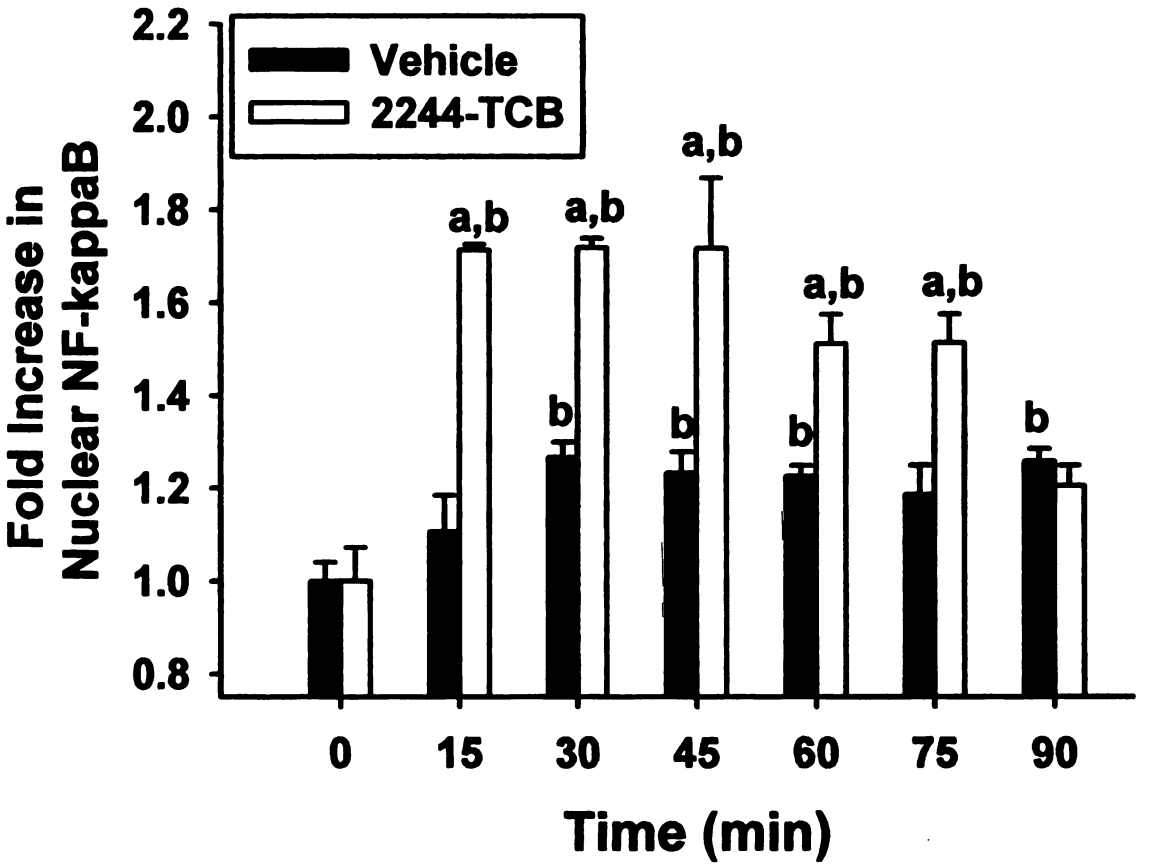

Figure IV.3. 2244-TCB increases nuclear NF-κB. The amount of nuclear NF-κB was determined in HL-60 cells (10 x 10^6) treated with vehicle or 2244-TCB for 0, 15, 30, 45, 60, 75 or 90 minutes. N = 3 separate experiments. a= significantly different from group treated for the same time in the absence of 2244-TCB; b= significantly different from respective group at time 0.

increase in nuclear NF-κB (Figure IV.4.A). Neither SB1 nor SB2 affected nuclear NF-κB in the absence of 2244-TCB. Pretreatment with either PD 98059 or U 0126 did not affect the 2244-TCB-mediated increase in nuclear NF-κB or the amount of nuclear NF-κB in vehicle-treated cells (Figure IV.4.B).

IV.4.C. Effects of NF-κB inhibitors on 2244-TCB-mediated COX-2 mRNA expression.

2244-TCB caused an approximately four-fold increase in COX-2 mRNA as compared to control after 30 minutes of exposure (Fig. IV.5), confirming previous results (Bezdecny et al, 2005). Incubation with either BAY 11-7082 or helenalin, inhibitors of NF-κB activity, significantly reduced the 2244-TCB-mediated increase in COX-2 mRNA. Inhibition of NF-κB did not affect the expression of COX-2 mRNA in the absence of 2244-TCB.

IV.4.D. Effect of 2244-TCB on COX-2 mRNA stability.

2244-TCB caused an approximately four-fold increase in COX-2 mRNA, confirming previous results. Inhibition of transcription with ActD attenuated this increase in cells exposed to 2244-TCB for 10 or 20 minutes, but not in cells exposed for 30 minutes (Figure IV.6).

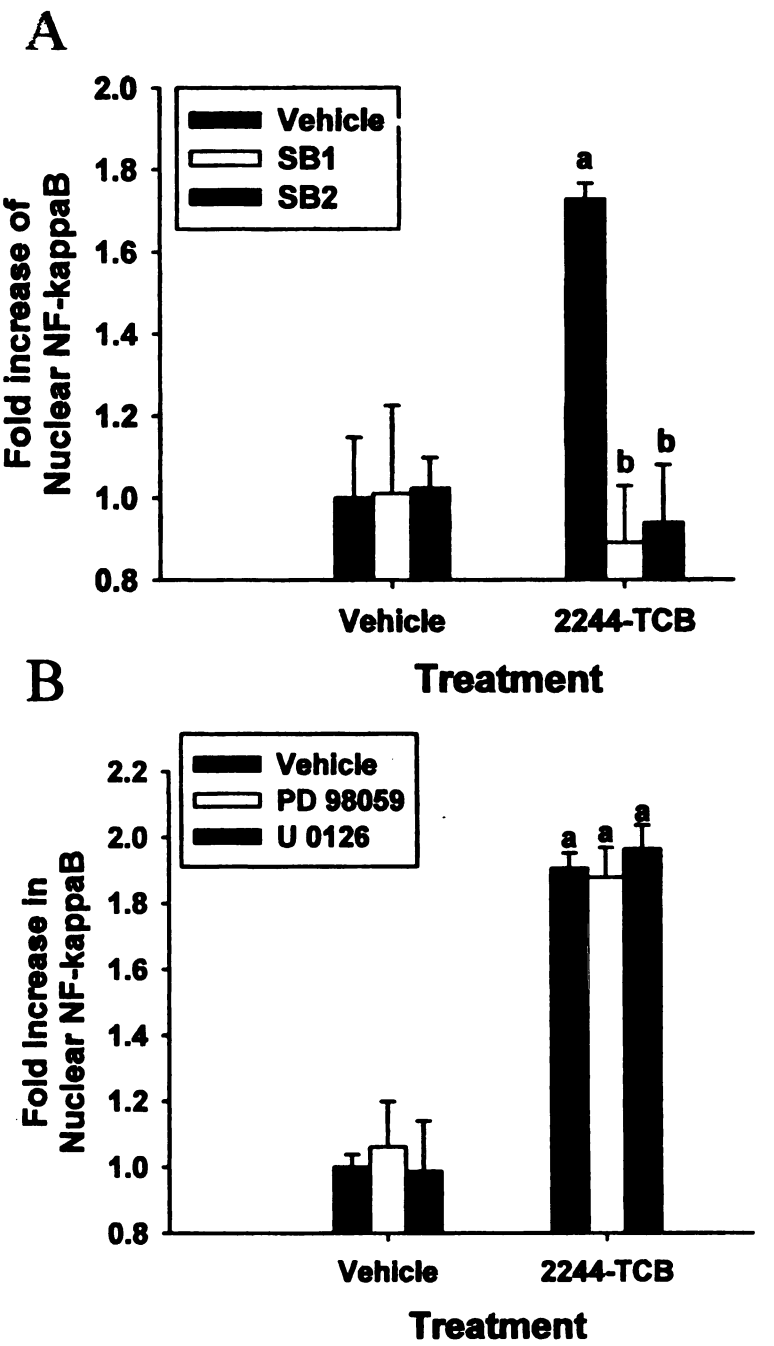

Figure IV.4. The 2244-TCB-mediated increase in nuclear NF-κB is reduced by p38 inhibitors, but not by ERK inhibitors. Nuclear NF-κB was determined in HL-60 cells (10×10^6) pretreated with vehicle, 5 μM SB1 or 15 μM SB2 (A) or 10 μM PD 98059 or 5 μM U 0126 for 30 minutes, then treated with 30 μM 2244-TCB for 30 minutes. N = 3 separate experiments. a= significantly different from respective group in the absence of 2244-TCB; b= significantly different from respective group in the absence of inhibitor.

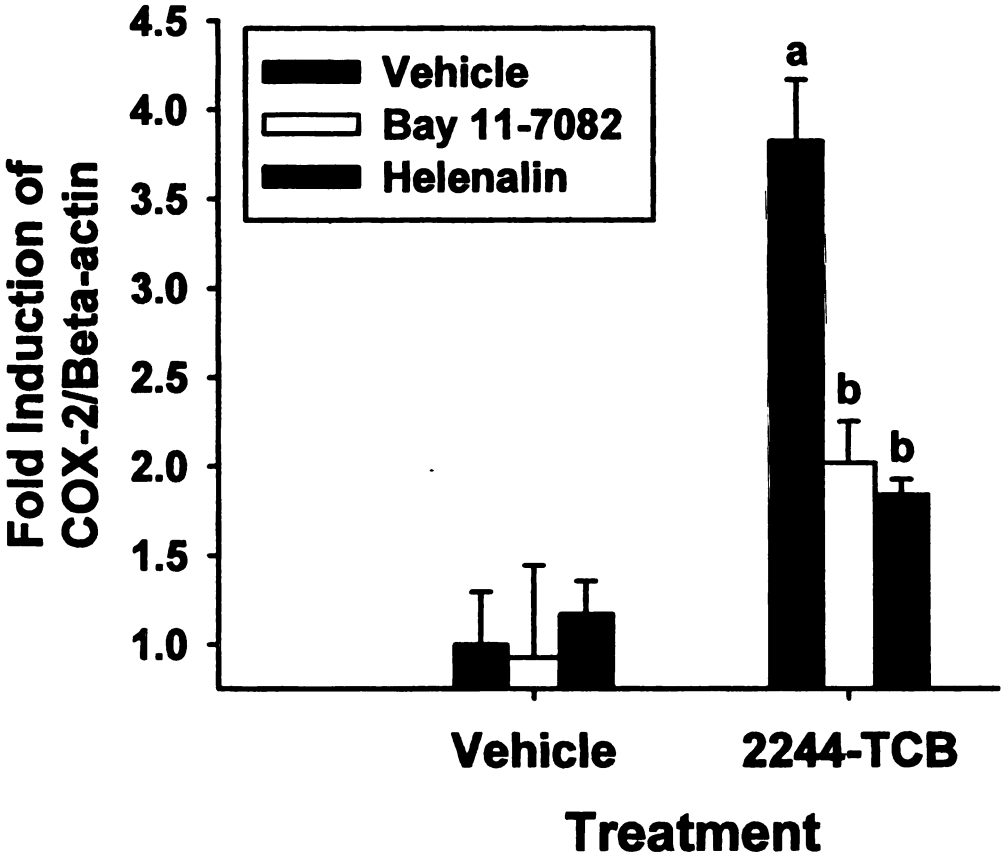

Figure IV.5. NF-κB inhibitors attenuate the 2244-TCB-mediated increase in COX-2 mRNA expression. COX-2 mRNA was determined in HL-60 cells (5×10^6) pretreated with vehicle, 10 μM Bay 11-7082 or 10 μM helenalin for 30 minutes, then treated with 30 μM 2244-TCB for 30 minutes. mRNA was quantified using real-time RT-PCR as described in Materials and Methods. β-actin was used as a loading control. N= 4 separate experiments. a= significantly different from respective group in the absence of 2244-TCB. b= significantly different from respective group in the absence of inhibitor.

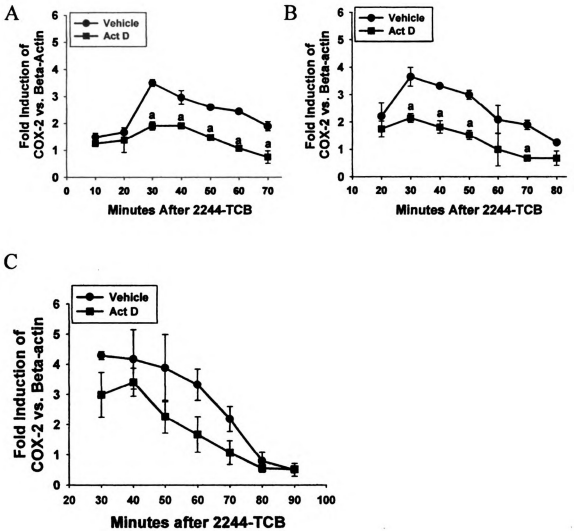

Figure IV.6. Effects of inhibition of transcription on 2244-TCB-mediated COX-2 mRNA expression. Differentiated HL-60 cells (5 x 10^6) were treated with DMSO as vehicle or with 30 μM 2244-TCB for 10 (A), 20 (B) or 30 (C) minutes. Cells were then treated with 10 μg/mL of the transcriptional inhibitor actinomycin D, and samples were collected every 10 minutes. COX-2 mRNA levels were determined by Sybr Green RT-PCR as described in Materials and Methods. All data were normalized to β-actin. N= 4 separate experiments. a= significantly different from vehicle-treated groups.

IV.5. Discussion

In the present study, the role of the transcription factors NF-κB and C/EBP in the 2244-TCB-mediated upregulation of COX-2 were examined, as was the effect that inhibition of the p38 and ERK MAP kinase pathways have upon these pathways. Each of these transcription factors has been implicated in the regulation of COX-2 in different cell systems. For example, NF-κB is involved in the upregulation of COX-2 in response to infection by *Legionella* bacteria in the lung and the parasite *Cryptosporidium* in the heart (Asaad and Sadek, 2006; N'Guessan *et al*, 2006). Additionally, NF-κB regulates COX-2 transcription in insulin-treated fibroblasts (Kitazawa *et al*, 2006), lipopolysaccharide-treated RAW 264.7 cells (Chen *et al*, 2006) and triptolide-treated astrocytes. C/EBP-beta is involved in the regulation of COX-2 in gastric tumors (Regalo *et al*, 2006), the human amnion (Lee *et al*, 2006) and in collagen-treated RAW 264.7 cells (Cho *et al*, 2004). 2244-TCB increased nuclear levels of C/EBP-beta in a time-dependent manner (Figure IV.1), suggesting increased transcriptional activity of C/EBP-beta. Levels of nuclear C/EBP-beta were increased after 15 minutes of exposure to 2244-TCB and this increase was maintained up to 75 minutes after exposure. COX-2 mRNA was also increased in response to 2244-TCB exposure shortly after the increase in nuclear C/EBP-beta was observed (Bezdecny *et al*, 2005). Taken together, this suggests that increased nuclear C/EBP-beta might play a role in the 2244-TCB-mediated upregulation of COX-2 mRNA.

p38 MAP kinase regulates activation and nuclear translocation of C/EBP-beta in proliferating cardiomyocytes (Ambrosino *et al*, 2006) and mammary epithelial cells (Seymour *et al*, 2006). In addition, p38-dependent activation of C/EBP-beta participates in the upregulation of COX-2 in murine macrophages (Chen *et al*, 2004). Similarly, the 2244-TCB-induced increase in nuclear translocation of C/EBP-beta was prevented by pretreatment with SB1 or SB2, indicating that activation of p38 is required for this response (Figure IV.2.A). SB2 was used to confirm the results of SB1, as SB1 may affect the JNK MAP kinase pathway, whereas SB2 is more selective for p38 MAP kinase inhibition (Cuenda *et al*, 1995).

ERK can also regulate the activation and translocation of C/EBP-beta. This has been demonstrated in differentiating HL-60 cells (Marcinkowska *et al*, 2006), in ceramide-treated rat hepatocytes (Giltiay *et al*, 2005) and in mouse fibroblasts (Park *et al*, 2004). However, ERK does not appear to be involved in the 2244-TCB-mediated activation and nuclear translocation of C/EBP-beta in HL-60 cells, as pretreatment with either PD 98059 or U 0126 had no effect on levels of nuclear C/EBP-beta (Figure IV.2.B).

Activation of NF-κB is important in the regulation of COX-2 in human adherent monocytes (Barbieri *et al*, 2004), in gastric ulcer healing in rats (Takahashi *et al*, 2001) and in rats challenged with lipopolysaccharide (Liu *et al*, 1999). In addition, NF-κB is critical to the regulation of COX-2 in HL-60 cells treated with andrographolide (Hidalgo *et al*, 2005) and in human neutrophils exposed to water-soluble bacterial proteins (Kim *et al*, 2001). In the

present study, exposure to 2244-TCB caused a time-dependent increase in nuclear NF-κB levels (Figure IV.3) suggesting activation of this transcription factor. Additionally, nuclear levels of NF-κB were elevated at the same times that 2244-TCB-mediated increases in COX-2 mRNA were observed, suggesting a link between NF-κB activation and increases in COX-2 mRNA.

As observed for C/EBP-beta, inhibition of p38 reduced the 2244-TCB-mediated increase in nuclear NF-κB (Figure IV.4.A). p38 MAP kinase is involved in the phosphorylation of IκB, the inhibitory subunit of NF-κB, leading to the subsequent activation and nuclear translocation of NF-κB (Sweeney and Firestein, 2004). Activation of p38 and its subsequent activation of NF-κB regulates COX-2 expression in linoleic acid-treated mouse skin (Hwang, *et al*, 2006), in RAW 264.7 cells treated with dipyridamole (Chen *et al*, 2006) and in human tracheal smooth muscle cells treated with interleukin-1beta (Lin *et al*, 2004). Interestingly, in human pulmonary epithelial cells treated with phorbol 12-myristate 13-acetate, ERK and NF-κB, but not p38 MAP kinase were required for upregulation of COX-2 (Chang *et al*, 2005). In contrast to p38, ERK inhibitors did not affect the amount of nuclear NF-κB (Figure IV.4.B). Activation of ERK and subsequent activation and nuclear translocation of NF-κB regulates COX-2 expression in resveratrol-treated mouse skin (Kundu *et al*, 2006), in RAW 264.7 cells exposed to Mycobacterium avium proteins (Pathak *et al*, 2004) and in human pulmonary epithelial cells (Chang *et al*, 2005). However, results presented here suggest that ERK does not participate in the 2244-TCB-mediated activation of NF-κB.

Taken together, the results suggest that 2244-TCB activates NF-κB and C/EBP-beta in a p38-dependent manner. How do these events relate to 2244-TCB-mediated increased expression of COX-2? Previously we demonstrated that p38 is critical to 2244-TCB-mediated upregulation of COX-2 (see Chapter III). Here we present data suggesting that NF-κB is also important in this response (Figure IV.5). Accordingly, the results suggest that activation of NF-κB by p38 is an important component in the 2244-TCB-mediated upregulation of COX-2. The link between p38 activity and regulation of COX-2 has been demonstrated in rat hepatic macrophages (Ahmad *et al*, 2002), the HeLa cell line (Lasa *et al*, 2000), human airway myocytes (Singer *et al*, 2003) and human monocytes (Dean *et al*, 1999).

p38 regulates the transcription of the COX-2 gene as well as stability of COX-2 mRNA. The latter effect is mediated by activation of cytoplasmic proteins, such as the mRNA stabilization factor HuR, that increase the half life of COX-2 mRNA (Dean *et al*, 1999; Dixon *et al*, 2001; Lasa *et* al, 2000; Singer *et al*, 2003). It seems likely that 2244-TCB regulates COX-2 at the level of transcription, since NF-κB was required for this upregulation, but the critical role of p38 raised the possibility that stabilization of mRNA also contributed to increases in COX-2. Inhibition of transcription with ActD reduced COX-2 mRNA levels in cells exposed to 2244-TCB for 10 or 20 minutes, but not in cells exposed to 2244-TCB for 30 minutes. These results suggest that p38 might regulate COX-2 mRNA levels both by activating transcription factors including NF-κB and C/EBP-beta rapidly upon exposure to 2244-TCB (Figure IV.6).

There is also the possibility that activation of p38 may contribute to increases in COX-2 mRNA by increasing the stability of existing COX-2 mRNA.

In summary, exposure to 2244-TCB upregulates COX-2 expression via a p38 MAP kinase-dependent mechanism. Activation of p38 leads to increases in nuclear levels of C/EBP-beta and NF-κB, and inhibition of NF-κB prevents 2244-TCB-induced upregulation of COX-2. Taken together, the results of these studies suggest that p38 is involved in the increased transcription of the COX-2 gene via an NF-κB-dependent pathway.

Chapter V

Summary

Neutrophils are the predominant white blood cell in the human circulatory system and are one of the most important cells of the innate immune system. The primary role of neutrophils is to attack and destroy invading microorganisms. They exhibit the most rapid response to tissue injury of any immune cell. Neutrophils are also one of the major cell types involved in the inflammatory response. Neutrophils are normally quiescent, only exhibiting their biological activity when activated. Given the role of neutrophils in host defense and the requirement for activation, the mechanisms by which neutrophils become activated are of great importance. Failure of neutrophils to activate results in severe compromise of the immune system, leading to increased risk of infection. At the other end of the spectrum, inappropriate activation of neutrophils can lead to inflammatory disease states, as the activated neutrophils damage host tissue. A number of cellular changes accompany neutrophil activation, including changes in cell surface proteins, increased chemotaxis, activation of cell signaling pathways and increased transcription of inflammatory genes such as COX-2.

Neutrophil function can be affected by a wide range of toxic compounds, including bacterial components such as endotoxin, naturally occurring compounds such as monocrotaline and man-made chemicals such as PCBs and other organochlorine compounds. These compounds cause numerous effects such as impairment of neutrophil function, impaired or inappropriate neutrophil activation and changes in cell signaling pathways. The effect of PCBs is particularly well studied in the rat neutrophil. In these cells PCB exposure

caused activation of NADPH oxidase and increased production of superoxide anion, activation of $iPLA_2$ and release of AA as well as activation of other signal transduction pathways.

Many of the responses seen in PCB-exposed rat neutrophils have also been observed in other cell types, notably human neutrophils and as presented in the preceding chapters, granulocytic HL-60 cells. All three of these cell types show increased oxidative burst and production of superoxide anion in response to PCB exposure. Far more work has been done in rat neutrophils and granulocytic HL-60 cells. Work presented in this dissertation demonstrates that rat neutrophils and granulocytic HL-60 cells respond similarly to PCB exposure. Both cell types show activation of $iPLA_2$ and subsequent release of free AA. Additionally, activation of protein kinases, changes in intracellular Ca^{2+} and induction of degranulation occur in both rat neutrophils and granulocytic HL-60 cells exposed to PCBs. These findings are important, as they demonstrate that neutrophils from different species, as well as a neutrophil-like human cell line, share common responses to PCB exposure.

As mentioned above, COX-2 is an important component of the inflammatory response. COX-2 is the key enzyme required for the conversion of AA to PGs and Tx. Once produced, these mediators can cause a wide range of immunomodulatory effects on multiple cell types by acting on G protein-linked prostanoid receptors, or in some cases these products may act on nuclear receptors. Some effects these mediators have upon the inflammatory response include increased vascular permeability and vascular dilation, as well as acting

as chemotactic factors for neutrophils. COX-2 activity is typically present at low to negligible levels in quiescent neutrophils. However, the COX-2 gene is strongly inducible by a variety of pro-inflammatory stimuli, such as TNF-α, IL-1, IL-2 and LPS. Inappropriate induction of COX-2 can have serious consequences, particularly on preexisting inflammatory states. Induction of COX-2, with the attendant increase in prostaglandin and thromboxane production, may lead to increased recruitment and activation of inflammatory cells such as neutrophils, increasing the severity of existing inflammatory conditions.

The goal of this thesis was to test the hypothesis that 2244-TCB increases cyclooxygenase-2 expression in granulocytic HL-60 cells, and that this increase depends on activation of intracellular signaling pathways such as AA release, superoxide anion production and activation of the p38 mitogen-activated protein kinase. Results presented in the previous chapters led to the working hypothesis presented in Figure V.1. 2244-TCB caused a time- and concentration-dependent increase in COX-2 mRNA, protein and enzyme activity as well as increased phosphorylation and activation of the p38 MAP kinase. These increases were prevented by inhibition of p38 MAP kinase. The 2244-TCB-mediated increase in COX-2 mRNA involved activation and nuclear translocation of the transcription factors NF-κB and C/EBP-beta by a p38 MAP kinase-dependent mechanism.

While the specific mechanism by which 2244-TCB causes these effects is unknown, several hypotheses have been put forth. One such hypothesis suggests

that 2244-TCB directly interacts with the enzymes involved. Another is that PCB exposure causes stress to the cellular membranes, leading to the activation of stress response mechanisms. However, this is unlikely as noncoplanar PCBs and not coplanar PCBs cause these effects and both types of PCBs would be expected to cause the same degree of cellular stress.

2244-TCB exposure also caused increased production of superoxide anion. This increase was caused by the activation of NADPH oxidase by free AA. While increased levels of ROS can lead to changes in gene expression via the activation of ROS-dependent kinases, ROS generation does not play a role in the 2244-TCB-dependent upregulation of COX-2 mRNA.

Additionally, 2244-TCB exposure caused increases in free AA. This release was mediated by $iPLA_2$. However, release of AA was not involved in the 2244-TCB-mediated upregulation of COX-2 mRNA. Despite not contributing to regulation of COX-2 in the presence of 2244-TCB, the increased levels of free AA might still be used as substrate for COX-2-mediated production of PGs and Tx, as significant increases of free AA occur at the same time as the 2244-TCB-mediated increases in COX-2 protein and activity.

Noncoplanar PCBs such as 2244-TCB are generally considered to be less toxic than coplanar or dioxin-like PCBs but are on the other hand detected at greater concentrations and are generally more persistent in the environment than the dioxin-like PCBs. Despite this fact little is known about the signal

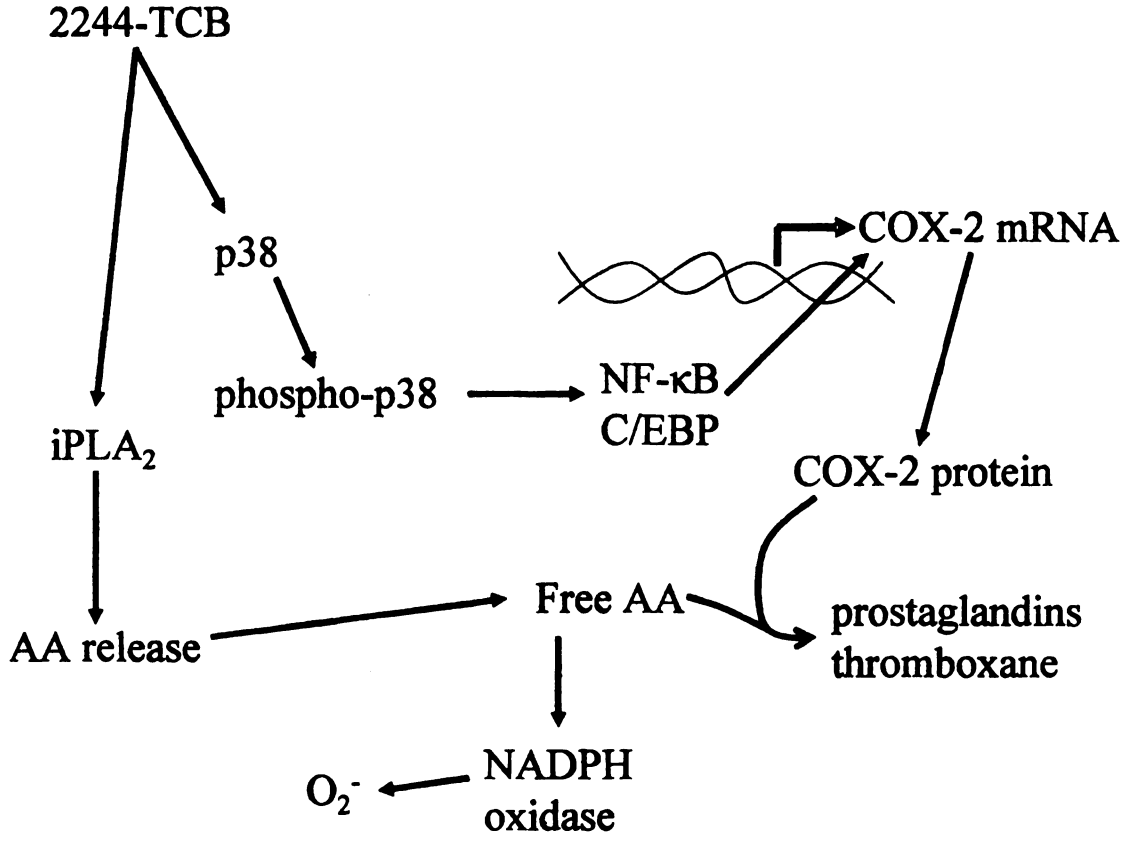

Figure V.1. Signal transduction pathways involved in the 2244-TCB-mediated upregulation of COX-2 in granulocytic HL-60 cells.

transduction pathways by which noncoplanar PCBs mediate their toxicity. As human exposure to PCBs, particularly noncoplanar PCBs will continue to be significant for decades to come, a better understanding of the effects noncoplanar PCBs have upon cell signaling pathways is needed. This thesis elucidates one such pathway by which a noncoplanar PCB changes neutrophil function by activating specific cell signaling mechanisms to cause changes in the expression of COX-2, a enzyme of critical importance in the inflammatory response and in overall neutrophil function. These results demonstrate several components of cell signaling are activated by 2244-TCB in HL-60 cells. These components, particularly the p38 MAP kinase pathway and the transcription factor NF-κB, are present in all mammalian cells. Therefore these noncoplanar PCBs can potentially affect the function of any cell type by interacting with these common pathways. p38 is important in responding to cellular stress while NF-κB is involved in the regulation of many inflammatory genes in addition to COX-2. Changes in the proper function of either of these components would cause serious consequences to cell function and survival. By understanding the mechanisms by which noncoplanar PCBs mediate their toxic effects a better knowledge of the potential toxicity of PCBs and other similar compounds can be reached.

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