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

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Abstract

Polychlorinated biphenyls (PCBs) are ubiquitous, persistent environmental contaminants that affect a number of cellular systems, including neutrophils. Among the effects caused by the noncoplanar PCB 2,2',4,4',-tetrachlorobiphenyl (2244-TCB) in granulocytic HL-60 cells are increases in superoxide anion production, activation of phospholipase A₂ with subsequent release of arachidonic acid (AA), and upregulation of the inflammatory gene cyclooxygenase-2 (COX-2). The objective of this study was to determine the signal transduction pathways involved in the upregulation of COX-2 by 2244-TCB. Treatment of HL-60 cells with 2244-TCB led to increased expression of COX-2 mRNA. This increase was prevented by the transcriptional inhibitor actinomycin D in cells pretreated with 2244-TCB for 10 minutes. The increase in COX-2 mRNA was associated with release of ³H-AA, phosphorylation of p38 and extracellular signal-regulated kinase (ERK) mitogen-activated protein (MAP) kinases, increased levels of nuclear NF-κB and increased superoxide anion production. Bromoenol lactone, an inhibitor of the calcium-independent phospholipase A₂, reduced ³H-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 ³H-AA release. Treatment with PD 98059 or U 0126, inhibitors of the MAP/ERK (MEK) 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 c-Jun N-terminal kinase (JNK) phosphorylation. 2244-TCB exposure increased the amount of nuclear NF-κB. This increase was prevented by pretreatment with p38 MAP kinase inhibitors, but not by pretreatment with MEK inhibitors. Pretreatment with inhibitors of NF-κB prevented the 2244-TCB-mediated induction of COX-2 mRNA. 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 2244-TCB-induced changes in COX-2 mRNA levels or ³H-AA release. Taken together these data suggest that p38 MAP kinase-dependent activation of NF-κB is critical for the 2244-TCB-mediated upregulation of COX-2 mRNA.

Keywords

: polychlorinated biphenyl; HL-60; cyclooxygenase-2; p38; NF-κB

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Introduction

Polychlorinated biphenyls (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 2,2',4,4'-tetrachlorobiphenyl (2244-TCB), a noncoplanar congener. Noncoplanar PCBs are generally considered to be less toxic than coplanar or dioxin-like PCBs, but they occur in the environment in larger concentrations than the dioxin-like PCBs (WHO, 2003).

Noncoplanar PCBs cause a variety of adverse effects, including 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). PCBs also affect the regulation of a number of genes, the most studied being cytochromes P450 in liver (Chubb *et al.* 2004). In particular, 2244-TCB upregulates expression of the gene cyclooxygenase-2 (COX-2) in a neutrophil-like cell line (Bezdecny *et al.* 2005).

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 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, among which are NF- κ B (Ghosh *et al.* 1998) and elements of the mitogen-activated protein (MAP) kinase cascades (Dean *et al.* 1999; Lasa *et al.* 2000; Moon and Pestka, 2002; Steer *et al.* 2006; Su and Karin, 1996). In addition, reactive oxygen species (ROS; Amma *et al.* 2005) and activated phospholipase A₂ (PLA₂) and its product, free arachidonic acid (AA; Pawliczak *et al.* 2004) have been implicated in the upregulation of COX-2 in various cell systems. 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. Accordingly, the above-mentioned pathways were examined. Furthermore, since crosstalk among these pathways has been reported (Nakano *et al.* 2006; Wullaert *et al.* 2006), their interdependence was evaluated in granulocytic HL-60 cells exposed to 2244-TCB.

Materials and Methods

Chemicals

2,2',4,4'-Tetrachlorobiphenyl (2244-TCB; >99% pure) was purchased from ChemService (West Chester, PA). [³H-5,6,8,9,11,12,14,15]-Arachidonic acid (³H-AA; 180–240 Ci/mmol) was purchased from DuPont NEN (Boston, MA). PD 98059 and U 0126 were purchased from Calbiochem (San Diego, CA). SB-202190, SB-203580, oleyloxyethyl phosphorylcholine (OP), bromoenol lactone (BEL), Bay 11-7082 and helanalin were purchased from Biomol (Plymouth Meeting, PA). Actinomycin D was purchased from Invitrogen (Carlsbad, CA). 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.

HL-60 cells

HL-60 cells were obtained from American Type Culture Collection (ATCC; 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% CO₂/95% air. Cells were induced to differentiate along the granulocyte pathway by culturing in the presence of 1.25% dimethyl sulfoxide (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).

Exposure to polychlorinated biphenyls

Stock solutions of 2244-TCB were prepared by dissolution in dimethylformamide (DMF). The differentiated HL-60 cells were suspended in Hanks' balanced salt solution (HBSS) in 12 × 75 mm borosilicate glass test tubes (VWR, Chicago, IL), 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 DMF. The concentration of 2244-TCB used in the current study was selected based on their previously described effects on HL-60 cells and their minimal cytotoxicities (Bezdecny *et al.* 2005).

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. 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).

Actinomycin D studies

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

Determination of COX-2 mRNA levels

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 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., Fullerton, 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 Moloney Murine Leukemia Virus (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), µM of each primer and Sybr green PCR buffer containing 25 mM MgCl₂ with 10 mM 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'-TTCAAATGAGATTGTGGGAAAATTGCT-3' (forward primer), 5'-AGATCATCTCTGCCTGAGTATCTT-3' (reverse primer). The predicted size of the fragment was 301 bp. For β -actin, the primers were 5'-GACGAGGCCAGAGCAAGAGAG-3' (forward primer), 5'-ACGTACATGGCTGGGGTGTG-3' (reverse primer). The predicted size of the fragment was 284 bp. 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. All results were compared to β -actin and expressed as fold induction versus vehicle control. Fold induction was calculated by first determining the ratio of COX-2 to β -actin in cells treated only with vehicle. All results from treated cells were then divided by this value to give the fold increase in COX-2 mRNA over that of untreated cells.

Labeling of HL-60 cells with ^3H -arachidonic acid

Differentiated HL-60 cells ($10 \times 10^6/\text{mL}$) were suspended in HBSS containing 0.1% bovine serum albumin (BSA) and incubated for 120 minutes at 37°C with 0.5 $\mu\text{Ci}/\text{mL}$ ^3H -AA. At the end of the labeling period cells were washed twice and resuspended in HBSS containing 0.1% BSA. An aliquot of cells was subjected to scintillation counting to determine cellular uptake of radiolabel; uptake was routinely $\sim 70\%$ of added ^3H -AA.

Determination of arachidonic acid release from HL-60 cells

Cumulative release of ^3H -AA was measured in HL-60 cells (2×10^6) pretreated with the appropriate inhibitor or vehicle for the indicated time, then exposed to 30 μM 2244-TCB or vehicle for 90 minutes at 37°C. Previous studies indicated a significant increase in ^3H -AA release after 90 minutes of exposure to 2244-TCB (Bezdecny *et al.* 2005). At the end of the incubations, samples were placed immediately on ice and then 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.

Western blotting for COX-2 and p38

Differentiated HL-60 cells (50×10^6) were suspended in HBSS and pretreated at 37°C with the appropriate inhibitor or vehicle for 30 minutes, then exposed to 30 μM 2244-TCB or vehicle for 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 Bio-Rad Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA). β -actin was used as an internal standard.

Cyclooxygenase activity assay

COX activity was measured using a COX Activity Assay (Cayman Chemical, Ann Arbor, MI) according to manufacturer's instructions. Differentiated HL-60 cells (15×10^6) were suspended in HBSS and pretreated at 37°C with the appropriate inhibitor or vehicle for 30 minutes, then treated for 90 minutes with 30μM 2244-TCB. Immediately after treatment, cells were centrifuged for 10 minutes at 4000 g. Supernatant was discarded, and the cell pellet was resuspended in 200μL of assay buffer then lysed via sonication. Activities of COX-1 and COX-2 were determined using the isoform-specific inhibitors DuP-697 and SC-560 according to manufacturer's instructions.

Measurement of phospho-p38 and phospho-ERK

Levels of phosphorylated and total p38 and ERK (extracellular signal-regulated kinase) 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 30μM 2244-TCB. For determination of levels of phospho-p38, cells were treated for 90 minutes with vehicle or 30μM 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.

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 30μM 2244-TCB. After 30 minutes, nuclear protein was isolated using a nuclear extract kit (Active Motif, Carlsbad, CA) according to manufacturer's instructions.

Determination of nuclear NF-κB

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 30μM 2244-TCB for 30 minutes, after which nuclear protein was isolated. Amounts of nuclear NF-κB were measured using a TransAM NF-κB ELISA kit (Active Motif, Carlsbad, CA) according to manufacturers instructions.

Superoxide anion production

Superoxide anion generation by HL-60 cells was measured as the reduction of cytochrome c in the presence and absence of superoxide dismutase (SOD; Babior *et al.* 1976). Differentiated HL-60 cells were suspended in Ca²⁺- and Mg²⁺-containing HBSS and pretreated with the appropriate inhibitor or vehicle for the indicated time. Cytochrome c (10 mg/mL) and 2244-TCB or vehicle was then added to the pretreated cells. 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.

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 (e.g., percent data). For all studies, the criterion for statistical significance was $p < 0.05$.

Results

COX-2 mRNA expression in HL-60 cells exposed to 2244-TCB in the absence and presence of actinomycin D

Treatment with 2244-TCB caused a time-dependent increase in COX-2 mRNA levels that peaked at ~4-fold after 30 minutes exposure and declined thereafter (Fig. 1). To evaluate whether an increase in transcription contributes to increased levels of COX-2 mRNA after exposure to 2244-TCB, HL-60 cells were pretreated for 10 minutes with 2244-TCB, then ActD was added. The 2244-TCB-induced increase in COX-2 mRNA was significantly smaller in cells treated with ActD compared to vehicle-treated cells. These results suggest that exposure to 2244-TCB causes transcription of the COX-2 gene in granulocytic HL-60 cells.

Effects of signal transduction inhibitors on 2244-TCB-mediated $^3\text{H-AA}$ release

In the absence of 2244-TCB exposure, vehicle-treated HL-60 cells released ~5% of $^3\text{H-AA}$ after 90 minutes. Exposure to 2244-TCB produced a significant increase in $^3\text{H-AA}$ release (Table 1). BEL, an inhibitor of the calcium-independent PLA_2 (Ackermann *et al.* 1995), attenuated the 2244-TCB-mediated increase in $^3\text{H-AA}$ release. Incubation with either OP, an inhibitor of the cytosolic and secretory isoforms of PLA_2 (Magolda and Galbraith, 1989), or SB-202190, an inhibitor of p38 MAP kinase (Lee *et al.* 1994), had no effect on 2244-TCB-induced $^3\text{H-AA}$ release.

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 SB-202190, SB-203580, OP or BEL (data not shown). In untreated cells, levels of phosphorylated (active) p38 were negligible. Levels of phospho-p38 increased after 60 (data not shown) or 90 minutes of exposure to 2244-TCB (Fig. 2). The p38 MAPK inhibitors SB-202190 and SB-203580 attenuated this increase. The 2244-TCB-induced increase in phospho-p38 protein level was not affected by exposure to OP or BEL.

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 (Fig. 3A). Incubation with either SB-202190 or SB-203580 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 (data not shown). By 90 minutes of exposure to 2244-TCB, COX-2 protein was increased to detectable levels (Fig. 3B; Bezdecny *et al.*, 2005). This increase was reduced by incubation of the cells with SB-202190 or SB-203580. 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 (Fig. 3C). Treatment with 2244-TCB for 90 minutes increased COX-2 activity approximately 4-fold. Pretreatment with SB-202190 significantly decreased 2244-TCB-stimulated COX-2 activity. Neither OP nor BEL had a significant effect on COX-2 activity.

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 (Fig. 4). Pretreatment with either MEK (MAP/ERK) inhibitor, PD 98059 or U 0126, significantly decreased 2244-TCB-stimulated phosphorylation of ERK (Fig. 4A). Additionally, pretreatment with either p38 inhibitor significantly decreased 2244-TCB-stimulated phosphorylation of ERK (Fig. 4B). None of these inhibitors affected phosphorylation of ERK in the absence of 2244-TCB. The 2244-TCB-mediated increase in the ratio of phospho-p38 to total p38 was not affected by MEK inhibitors in the absence or presence of 2244-TCB (Fig. 4C). Phosphorylation of JNK (c-Jun N-terminal kinase) was not affected by 2244-TCB (data not shown).

Lack of effect of MEK inhibitors on 2244-TCB-mediated COX-2 mRNA expression

COX-2 mRNA expression was modest in vehicle-treated cells and was unaffected by exposure to either PD 98059 or U 0126 (Fig. 5). As noted above, 2244-TCB increased COX-2 mRNA as compared to control. Pretreatment with either PD 98059 or U 0126 did not affect this increase in COX-2 mRNA.

Effects of 2244-TCB on nuclear NF- κ B

Exposure to 2244-TCB increased the amount of nuclear NF- κ B approximately 1.7 fold after 15 minutes of exposure (Fig. 6A). It remained greater than the vehicle-treated value for 75 minutes (data not shown). Pretreatment with either SB-202190 or SB-203580 prevented the 2244-TCB-mediated increase in nuclear NF- κ B (Fig. 6A). Neither SB-202190 nor SB-203580 affected nuclear NF- κ B 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- κ B or the amount of nuclear NF- κ B in vehicle-treated cells (Fig. 6B).

Effects of NF- κ B 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. 7), confirming previous results (Fig. 1 and Bezdecny et al. 2005). Incubation with either BAY 11-7082 or helenalin, inhibitors of NF- κ B activity that act via two different mechanisms, significantly reduced the 2244-TCB-mediated increase in COX-2 mRNA. Inhibition of NF- κ B did not affect the expression of COX-2 mRNA in the absence of 2244-TCB.

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

We have reported previously that exposure of differentiated HL-60 cells to 2244-TCB causes a time- and dose-dependent increase in superoxide anion production (Bezdecny *et al.* 2005). In the present study, 2244-TCB increased superoxide production relative to control (Fig. 8A), 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 (Fig. 8B). Neither apocynin nor TEMPO pretreatment affected the 2244-TCB-mediated increase in COX-2 mRNA. Inhibition of ROS production also did not affect release of $^3\text{H-AA}$ (Table 2).

Discussion

Exposure of HL-60 cells to 2244-TCB leads to a modest and transient increase in transcription of the cyclooxygenase-2 gene. Inhibition of transcription with ActD reduced COX-2 mRNA levels in cells exposed to 2244-TCB. These results suggest that transcription of the COX-2 gene occurs in response to 2244-TCB exposure (Fig. 1). In the present study, signal transduction pathways involved in the upregulation of COX-2 by 2244-TCB were examined. One signal transduction pathway that was investigated is the PLA₂ 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 ³H-AA release, and this response was attenuated by pretreatment with BEL, an inhibitor of iPLA₂, but not by OP, an inhibitor of cytosolic and secretory PLA₂ (Table 1). HL-60 cells resemble primary neutrophils in many respects and have, therefore, been used as a model to study neutrophil responses. Many of the effects that 2244-TCB has on neutrophil function are similar in primary rat neutrophils and HL-60 cells. Indeed, iPLA₂-mediated increases in ³H-AA release similar to those observed here have been reported in primary rat neutrophils treated with the PCB mixture, Aroclor 1242, as well as with individual, noncoplanar PCB congeners (Olivero and Ganey, 2001; Tithof *et al.* 1998). Thus, these results suggest that HL-60 cells and rat neutrophils share a common pathway to PCB-stimulated AA release, i.e. one mediated via iPLA₂. Nonetheless, the origin and nature of HL-60 cells should be considered when interpreting these results.

Activation of PLA₂ 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 phosphatidylinositol-3-kinase (Monick *et al.* 2002). Interestingly, expression of COX-2 was decreased in brains of PLA₂-deficient mice relative to wild type mice (Bosetti and Weerasinghe, 2003). These reports suggest that PLA₂ 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 ³H-AA, had no effect on COX-2 mRNA, protein or enzyme activity (Fig. 3).

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 (Fig. 2). Activation of p38 MAP kinase was associated with increased levels of COX-2 mRNA and COX-2 protein in cells treated with 2244-TCB for similar times. Treatment with p38 MAP kinase inhibitors reduced levels of phospho-p38 as well as mRNA levels, protein and enzyme activity of COX-2 (Figs. 2 and 3). 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 prostaglandins (PGs) and thromboxane (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. Inhibition of iPLA₂ diminished ³H-AA release (Table 1) but not the 2244-TCB-mediated increase in COX-2 mRNA or activation of p38 (Figs. 2 and 3A). Conversely, activation of iPLA₂ and subsequent release of ³H-AA

was not affected by inhibitors of p38 MAP kinase (Table 1), which did reduce the 2244-TCB-mediated upregulation of COX-2 (Fig. 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. In the present study, 2244-TCB exposure had no effect on JNK activation at any time up to 90 minutes after exposure (data not shown). 2244-TCB increased levels of phosphorylated ERK, and this increase was prevented by pretreatment with inhibitors of p38, as well as by the MEK inhibitors PD 98059 and U 0126 (Fig. 4). However, pretreatment with MEK inhibitors did not affect the 2244-TCB-mediated increase in COX-2 mRNA (Fig. 5). 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.

Several transcription factors have been implicated in the regulation of COX-2 in different cell systems. In particular, there is a large body of literature supporting a role for NF- κ B in the regulation of COX-2. For example, NF- κ B 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) and in triptolide-treated astrocytes (Dai *et al.* 2006). In addition, NF- κ B 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- κ B levels (Figure 6 and data not shown) suggesting activation of this transcription factor. Additionally, nuclear levels of NF- κ B were elevated at the same times that 2244-TCB-mediated increases in COX-2 mRNA were observed, and inhibition of NF- κ B prevented the 2244-TCB-mediated increase in COX-2 mRNA (Fig. 7), suggesting a link between NF- κ B activation and increases in COX-2 mRNA.

Inhibition of p38 reduced the 2244-TCB-mediated increase in nuclear NF- κ B (Fig. 6A). The p38 MAP kinase is involved in phosphorylation of I κ B, the inhibitory subunit of NF- κ B, leading to the subsequent activation and nuclear translocation of NF- κ B (Sweeney and Firestein, 2004). Activation of p38 and its subsequent activation of NF- κ B 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-1 β (Lin *et al.* 2004). Interestingly, in human pulmonary epithelial cells treated with phorbol 12-myristate 13-acetate, ERK and NF- κ B, but not p38 MAP kinase, were required for upregulation of COX-2 (Chang *et al.* 2005).

In contrast to p38, MEK inhibitors did not affect the amount of nuclear NF- κ B in the present study (Fig. 6B). Activation of ERK and subsequent activation and nuclear translocation of NF- κ B 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- κ B. Taken together, the results suggest that 2244-TCB activates NF- κ B in a p38-dependent manner, and that this is an important component in the 2244-TCB-mediated upregulation 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. 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- κ B (Amma *et al.* 2005; Kim *et al.* 2005; Singer *et al.* 2003). In the present study, 2244-TCB caused an increase in superoxide anion production, similar to results reported previously for rat neutrophils (Ganey *et al.* 1993). This increase was prevented by apocynin as well as by TEMPO (Fig. 8A), however, neither of these treatments affected 2244-TCB-mediated upregulation of COX-2 mRNA (Fig. 8B). These results suggest that ROS do not play a critical role in this response in HL-60 cells. However, they could still play a role in eicosanoid formation by COX-2, since this enzyme requires ROS and/or peroxides for full activity (Smith *et al.* 2000; Vane *et al.* 1998).

In summary, several different signal transduction pathways are affected in HL-60 cells by exposure to 2244-TCB; however, not all of these pathways play a role in 2244-TCB-mediated upregulation of COX-2 mRNA in HL-60 cells. Neither reactive oxygen species, PLA₂ 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. ERK was activated in response to 2244-TCB exposure, but this activation is not needed for the upregulation of COX-2. In contrast, the modest and transient increases in COX-2 mRNA, protein and activity that were observed 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. p38 activation also led to an increase in nuclear levels of NF- κ B, and inhibition of NF- κ B prevented 2244-TCB-induced upregulation of COX-2. Taken together, these results support a mechanism (Fig. 9) in which activation of neutrophils by 2244-TCB enhances p38 MAP kinase-dependent biosynthesis of COX-2 enzyme via an NF- κ B-dependent mechanism and also activates iPLA₂ to provide AA as substrate for prostaglandin synthesis.

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Figure 1. Effect of inhibition of transcription on 2244-TCB-mediated COX-2 mRNA expression
Differentiated HL-60 cells (5×10^6) were treated with $30\mu\text{M}$ 2244-TCB for 10 minutes. Cells were then treated with $10\mu\text{g/mL}$ of the transcriptional inhibitor actinomycin D (ActD) or its vehicle, 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 at the same time.

Figure 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 (50×10^6) pretreated for 30 minutes with vehicle, 5 μ M SB-202190 (SB1), 15 μ M SB-203580 (SB2), 15 μ M OP or 25 μ M BEL, then treated with 30 μ M 2244-TCB for 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. Levels of phospho-p38 were negligible in the absence of 2244-TCB. N = 3 separate experiments. a= significantly different from 2244-TCB in the absence of inhibitor.

Figure 3. 2244-TCB-mediated COX-2 mRNA expression in the presence of signal transduction inhibitors

(A) HL-60 cells (5×10^6) were pretreated for 30 minutes with vehicle, 5 μ M SB-202190 (SB1), 15 μ M SB-203580 (SB2), 15 μ M OP or 25 μ M BEL, then treated with vehicle or 30 μ M 2244-TCB for 30 minutes. COX-2 mRNA was determined by RT-PCR as described in Materials and Methods. (B) HL-60 cells (50×10^6) were pretreated as described in panel A, and then treated with vehicle or 2244-TCB for 90 minutes. COX-2 protein was determined as described in Materials and Methods. β actin was used as a loading control. Levels of COX-2 protein were negligible in the absence of 2244-TCB. (C) HL-60 cells (15×10^6) were pretreated as described in Panel A, and then treated with vehicle or 2244-TCB for 90 minutes. COX-2 activity was

determined as described in Materials and Methods. NT= no pretreatment. N= 5 (A) or 3 (B and C) 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 4. Effects of inhibitors on p38 and ERK phosphorylation

Phosphorylated and total ERK (A,B) and p38 (C) were determined by ELISA in HL-60 cells (20×10^6) pretreated for 30 minutes with vehicle, 10 μ M PD 98059, 5 μ M U 0126, 5 μ M SB-202190 (SB1) or 15 μ M SB-203580 (SB2) then with vehicle or 30 μ M 2244-TCB for 45 minutes (A,B) or 90 minutes (C). 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 5. Effect of MEK inhibitors on 2244-TCB-mediated COX-2 mRNA expression
COX-2 mRNA was determined by RT-PCR in HL-60 cells (5×10^6) pretreated for 30 minutes with vehicle, 10 μ M PD 98059 or 5 μ M U 0126, then treated with 30 μ M 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 6. The 2244-TCB-mediated increase in nuclear NF- κ B is reduced by p38 inhibitors, but not by MEK inhibitors

Nuclear NF- κ B was determined in HL-60 cells (10×10^6) pretreated with vehicle, 5 μ M SB-202190 (SB1) or 15 μ M SB-203580 (SB2) (A) or 10 μ M PD 98059 or 5 μ M U 0126 for 30 minutes, then treated with 30 μ M 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 7. NF- κ B 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 μ M Bay 11-7082 or 10 μ M helenalin for 30 minutes, then treated with 30 μ M 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 8. Lack of effect of reactive oxygen species inhibition on 2244-TCB-induced upregulation of COX-2 mRNA expression

(A) HL-60 cells (1×10^6) were pretreated with vehicle, 1mM apocynin (30 minutes) or 200 μ M TEMPO (10 minutes) then exposed to 30 μ M 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 respective group in the absence of 2244-TCB; b=significantly different from 2244-TCB-treated cells in the absence of inhibitor (i.e. vehicle-treated).

Figure 9. Working hypothesis for the 2244-TCB-mediated 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. These increases are caused by the activation of transcription factors such as NF- κ B. In addition, 2244-TCB activates iPLA₂ by a pathway independent of p38 MAP kinase, leading to the release of free AA, which can then be converted by COX-2 into prostaglandins and thromboxane.

Table 12244-TCB-mediated ^3H -AA release in the presence of inhibitors of p38 and PLA₂.

Pretreatment	% ^3H -Arachidonic Acid Release	
	Vehicle	2244-TCB
Vehicle	4.9 ± 0.4	11.1 ± 0.3 ^a
BEL	4.5 ± 0.3	6.8 ± 0.5 ^b
OP	4.7 ± 0.4	9.6 ± 0.9 ^a
SB-202190	4.4 ± 0.4	9.5 ± 0.6 ^a

Differentiated HL-60 cells were labeled with ^3H -AA as described in Materials and Methods. Labeled cells (1×10^6 cells/mL) were pretreated with vehicle, 25 μM BEL, 15 μM OP, or 5 μM SB-202190 for 30 minutes. They were then treated with vehicle (DMF) or 30 μM 2244-TCB for 90 minutes. ^3H -AA release is presented as the percent of total cellular ^3H -AA released into culture medium. 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.

Table 22244-TCB-mediated ^3H -AA release in the presence of inhibitors of ROS.

Pretreatment	% ^3H -Arachidonic Acid Release	
	Vehicle	2244-TCB
Vehicle	3.8 $\pm$ 0.2	6.5 $\pm$ 0.4 ^a
Apocynin	3.0 $\pm$ 0.2	5.8 $\pm$ 0.3 ^a
TEMPO	3.8 $\pm$ 0.2	7.4 $\pm$ 0.4 ^a

Differentiated HL-60 cells were labeled with ^3H -AA as described in Materials and Methods. Labeled cells (1×10^6 cells/mL) were pretreated with vehicle, 1 μM 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. ^3H -AA release is presented as the percent of total cellular ^3H -AA released into culture medium. N = 3 separate experiments. a=significantly different from respective group in the absence of 2244-TCB.