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Polychlorinated biphenyls induce arachidonic acid release in human platelets in a tamoxifen sensitive manner via activation of group IVA cytosolic phospholipase A_2-α

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PCB, polychlorinated biphenyls
PLA_2, phospholipase A2
$cPLA_2$, cytosolic PLA
12-HETE, 12(*S*)-hydroxy-5,8-*cis*-10-*trans*-14-*cis*-eicosatetraenoic acid
12-HHT, 12(*S*)-hydroxy-5-*cis*-8,10-*trans*-heptadecatrienoic acid
$AACOCF_3$, arachidonyl trifluoromethyl ketone
12-LO, 12-lipoxygenase
COX1, cyclooxygenase 1

ABSTRACT

Polychlorinated biphenyls (PCBs) are stable compounds commonly found in nature as environmental pollutants. PCBs can affect the endocrine function of hormones such as steroid-hormones. Also, PCBs are known to be inducers of arachidonic acid release in various cells. We report, here, the effects of PCBs on eicosanoid formation, arachidonic acid release and cytosolic phospholipase A_2-α ($cPLA_2$-α) activation in human platelets. *Ortho*-substituted PCBs induced a time and dose-dependent release of arachidonic acid and the concomitant formation of 12(*S*)-hydroxy-5,8-*cis*-10-*trans*-14-*cis*-eicosatetraenoic acid (12-HETE) and 12(*S*)-hydroxy-5-*cis*-8,10-*trans*-heptadecatrienoic acid (12-HHT) in human platelets. The release of arachidonic acid and the formation of 12-HETE was completely blocked by the $cPLA_2$-α inhibitors $AACOCF_3$ or pyrrolidine-1. PCB-treatment of platelets demonstrated that the $cPLA_2$-α protein as well as PLA_2 activity translocated to the membrane fraction, independent of a rise in intracellular Ca^{2+}. Furthermore, electrophoretic gel mobility shift analysis of $cPLA_2$-α on SDS-PAGE demonstrated a PCB-dependent phosphorylation of $cPLA_2$-α. The effects of 17β-estradiol and two structurally unrelated anti-estrogens, nafoxidin and tamoxifen on PCB-induced arachidonic acid release in platelets were also investigated. Both nafoxidin and tamoxifen inhibited PCB-induced arachidonic acid release as well as 12-HETE and 12-HHT formation. Interestingly, platelets incubated with PCBs did not aggregate despite the fact that robust release of arachidonic acid was observed. In summary, these results demonstrate that certain PCBs induce activation of $cPLA_2$-α independent of a rise in intracellular calcium and a robust release of arachidonic acid release with resulting eicosanoid formation in human platelets.

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TXA_2, thromboxane A2
PGH_2, prostaglandin H2
BAPTA-AM, 1,2-bis(o-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra-(acetoxymethyl)ester
BEL, (E)-6-(bromomethylene)3-3(1-naphtalenyl)-2H-tetrahydropyran-2-one

1. Introduction

Phospholipase A_2 (PLA_2) enzymes catalyze the hydrolysis of the *sn*-2 ester bond in phospholipids and hence, the release of a free fatty acid [1]. PLA_2 enzymes are a diverse family consisting of at least 16 different enzymes [2]. The intracellular group IV cytosolic PLA_2-α ($cPLA_2$) [3,4] has attracted much interest in the biosynthesis of eicosanoids since it preferentially release arachidonic acid from membrane phospholipids. Interestingly, macrophages from mice deficient in $cPLA_2$-α produced no prostaglandins or leukotrienes and various disease models have now been studied in these animals [5]. These mice demonstrate reduced fertility and reduced brain injury after cerebral ischaemia [6–8]. Three human paralogs of the $cPLA_2$-α enzyme have also been identified, termed $cPLA_2$-β, $cPLA_2$-γ and $cPLA_2$-δ [9–11]. Crystallographic studies of $cPLA_2$-α demonstrated the presence of two domains, an N-terminal calcium-phospholipid-binding (C2) domain and a catalytic domain [12]. Calcium promotes cytosol-to-membrane relocation of the $cPLA_2$-α protein by binding to the C2-domain [13–15]. However, $cPLA_2$-α targeting to membranes by calcium ions is complex since it is dependent both on the amplitude and the duration of the calcium increase [16,17]. The catalytic domain can affect membrane association of the enzyme by modulating the rate of association and also the residence time at membranes [18]. Furthermore, there is now convincing evidence that calcium-independent pathways for cytosol-to-membrane translocation of $cPLA_2$-α exists [19–24], thus, suggesting alternative mechanisms for membrane association. One such mechanism could involve calcium-independent association of $cPLA_2$-α to anionic phospholipids [21,24,25].

Phosphorylation of $cPLA_2$-α has been reported in several cell types and on several amino acid residues [26,27]. The role of phosphorylation in membrane association of $cPLA_2$-α is unclear but is has been demonstrated that phosphorylation events can, at least partially, contribute to $cPLA_2$-α activation [27–30].

Human platelets metabolize free arachidonic acid via cyclooxygenase/thromboxane synthase and lipoxygenase pathways. Cyclooxygenase 1 (COX1) produces prostaglandin H_2 (PGH_2) from arachidonic acid. PGH_2 is rearranged to thromboxane A_2 (TXA_2) by thromboxane A_2 synthase. Thromboxane A_2 synthase catalyzes in a parallel and almost equimolar reaction, the fragmentation of PGH_2 into 12(*S*)-hydroxy-5-*cis*-8,10-*trans*-heptadecatrienoic acid (12-HHT) and malondialdehyde. Thus, the formation of 12-HHT can be used as a marker of TXA_2 synthesis. The major lipoxygenase metabolite of arachidonic acid found in platelets is 12(*S*)-hydroxy-5,8-*cis*-10-*trans*-14-*cis*-eicosatetraenoic acid (12-HETE), formed by 12-lipoxygenase (12-LO).

Polychlorinated biphenyls (PCBs) are a family of relatively chemical stabile environmental pollutants. Many of the biological effects observed for PCBs are due to their ability to mimic hormones such as thyroid and steroid hormones [31–33]. Planar PCBs congeners, i.e. non-*ortho*-substituted biphenyls, are high-affinity ligands for the aromatic-hydrocarbon (Ah) receptor while the non-planar PCB congeners, i.e. *ortho*-substituted biphenyls, display no or weak affinity for the Ah-receptor [31]. Some of the toxicity observed for PCBs involves reproductive toxicity [31], immunotoxicity [34], neurotoxicity [35] as well as induction of hepatic xenobiotic-metabolizing enzymes [36]. PCB exposure is also associated with a modest decrease in maternal and umbilical cord serum arachidonic acid concentrations [37]. Several reports describe cellular activation by PCBs as well as induction of arachidonic acid release from different mammalian cells including rat neutrophils, myometrial cells and neuronal cells [38–44]. In neuronal cell culture model, PCBs induce arachidonic acid release and perturbed intracellular signaling [43,45], suggesting a possible link between arachidonic acid metabolism and neurotoxicity. Also, PCBs increases frequency of uterine contractions possibly by a mechanism involving PLA_2-dependent arachidonic acid release [40]. Phosphorylation events are, at least partially contributing to PCB-induced arachidonic acid release in rat neutrophils [46].

The mechanism behind PCB-induced arachidonic acid release is still unclear. Several reports describe PCB-induced arachidonic acid release to be independent or just marginally affected by chelation of extracellular and intracellular calcium [39,43,44,47]. Thus, the conclusion in several studies is that a calcium-independent PLA_2 ($iPLA_2$) is involved in PCB-induced arachidonic acid release. Despite the fact that arachidonic acid release has been observed for many cell types, few reports describe the formation of eicosanoids. In myometrial cells, less than 1% of arachidonate was converted to eicosanoids [40], whereas in a rat neutrophil preparation approximately 18% was metabolized into TXB_2, 12-HHT and 12-HETE, i.e. eicosanoids primarily produced by platelets [39].

The objective of this study was to investigate if PCBs induce arachidonic acid release in human platelets and whether this fatty acid is converted into eicosanoids. With the recent

availability of highly potent and specific inhibitors of PLA_2 enzymes, the role of these enzymes in PCB-mediated arachidonic acid release was explored. Additional studies are also presented that define the mechanism of PCB-induced arachidonic acid release in human platelets.

2. Materials and methods

2.1. Materials

Arachidonic acid, arachidonyl trifluoromethyl ketone ($AACOCF_3$), Fura2-AM, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra-(acetoxymethyl)ester (BAPTA-AM) and (*E*)-6-(bromomethylene)3-3(1-naphtalenyl)-2H-tetrahydropyran-2-one (BEL), were obtained from Biomol (Plymouth Meeting, Pa., USA). Tamoxifen, (*E*)-1-(4-(2-(*N*,*N*-dimethylamino)ethoxy)phenyl)-1,2-diphenylbut-1-ene, and nafoxidin, 1-(2-(P-(3,4-dihydro-6-methoxy-2-phenyl-1-naphtyl)phenoxy)-ethyl)pyrrolidine hydrochloride were obtained from Sigma. Pyrrolidine-1 was synthesized as previously described [48,49]. 1-palmitoyl-2-[1-^{14}C]arachidonyl phosphatidylcholine (PtdCho) (57 mCi/mmol) and 1-palmitoyl-2-[1-^{14}C]arachidonyl phosphatidylethanolamine (PtdEtn) (57 mCi/mmol) were from New England Nuclear. Rabbit anti-human $cPLA_2$-α polyclonal antibody MF142 (amino acids 731–749 as immunogen) was kindly provided by Nathalie Tremblay, Merck Frosst, Canada. Rabbit anti-human $iPLA_2$ polyclonal antibody PLF2 ($iPLA_2$ peptide CPRFNQNVNLRPPAQPSDQ as immunogen) was produced by Innovagen, Lund, Sweden. $iPLA_2$ cDNA was cloned into pFastBac1 (Bac-to-Bac, Gibco Life Technologies) and the enzyme was expressed in Sf9 insect cells using baculoviruses. Individual PCB congeners, 2,2′,4,4′-tetrachlorobiphenyl (CB47), 2,2′,5,5′-tetrachlorobiphenyl (CB52), 2,3′,4,4′,5-pentachlorobiphenyl (CB118), 2,2′,4,4′,5,5′-hexachlorobiphenyl (CB153), were synthesized as previously described [50,51]. The PCB mixtures, Clophen A30, Clophen A40, Clophen A50 and Clophen A60 are former commercially available PCB products (Bayer AG, Germany) [52].

2.2. Preparation of platelets

Human blood from healthy volunteers was obtained by vein puncture and blood was collected into EDTA-containing vaccutainer tubes. The platelet-rich plasma was obtained after centrifugation at 200 × *g* for 15 min. The platelets were washed with PBS (w/o Ca^{2+} and Mg^{2+}) supplemented with 1 mM EDTA and centrifuged at 1000 × *g* for 15 min. The cell pellet was gently resuspended in PBS with or without calcium as indicated, to a final concentration of 2–4 × 10^8 platelets/ml. In experiments where Ca^{2+} effects were studied, platelets were incubated with or without 10 μM BAPTA-AM for 45 min at 20 °C, washed and resuspended in PBS (w/o Ca^{2+} and Mg^{2+}) supplemented with 2 mM EGTA.

2.3. Measurement of ^{14}C-arachidonic acid release

Human platelets were resuspended in PBS containing 50 μM acetylsalicylic acid, 100 μM NDGA and 0.25 μCi/ml ^{14}H-arachidonic acid and incubated for 60 min at 37 °C. Thereafter, cells were centrifuged and washed thrice to remove unincorporated arachidonic acid. Labeled platelets were resuspended in PBS containing 50 μM acetylsalicylic acid, 100 μM NDGA. The samples were incubated with or without tamoxifen or nafoxidin for 10 min at 37 °C prior to the addition of 35 μM CB52 or 17β-estradiol and thereafter incubated for another period of 10 min. The reaction was terminated with one volume of methanol. The samples were thereafter stored at −20 °C and processed by solid phase extraction and analyzed by RP-HPLC as described.

2.4. Incubation of intact platelets and broken cell assay

Human platelets in 1 ml PBS was preincubated at 37 °C for the indicated times with DMSO or inhibitor as indicated. Subsequently, PCBs or calcium ionophore A23187 was added and the samples were incubated for 10 min at 37 °C. The reaction was terminated by the addition of 1 ml methanol. Samples were stored at −20 °C until eicosanoid content was analyzed. Cells (4 × 10^8) for the homogenate assay were centrifuged and resuspended in PBS (w/o Ca^{2+} and Mg^{2+}) supplemented with 1 mM EDTA and sonicated for 2 × 5 s. The samples were preincubated with the indicated compounds at 37 °C for 20 min. Thereafter, 20 μM arachidonic acid was added and the samples were incubated for another 10 min before termination.

2.5. Platelet aggregation

Platelet aggregation was measured by using an aggregometer. Platelets, suspended in PBS with Ca^{2+} and Mg^{2+}, were incubated at 37 °C with continuous stirring, and percent light transmission was recorded using a buffer sample as blank. Aggregation was initiated by the addition of 1 μM calcium ionophore A23187 or PCBs as indicated. In some experiments, samples were incubated with PCB at 37 °C for 10 min prior to aggregometer measurements.

2.6. Measurement of intracellular Ca^{2+}

Platelets were incubated with 10 μM Fura2-AM for 45 min at 20 °C. The incubation mixture was occasionally gently shaken. Fura2 loaded platelets were washed twice to remove extracellular Fura2-AM. Fluorescence was measured with continuous stirring of platelets. Excitation wavelengths were 335 and 363 nm with emission wavelength set at 510 nm using a Shimadzu spectrofluorimeter.

2.7. Subcellular fractionation

Platelets (10^9) were incubated with vehicle or PCB for 10 min at 37 °C and subsequently centrifuged at 1000 × *g* for 10 min. The pellet was resuspended in buffer A [20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 0.5 mM dithiothreitol and 10% glycerol] supplemented with 1 mM phenylmethanesulfonyl fluoride and homogenized by sonication twice for 5 s. The resulting homogenate was centrifuged at 100,000 × *g* for 60 min. The 100,000 × *g* supernatant was designated as cytosolic fraction; the 100,000 × *g* pellet was washed once, thereafter resuspended in buffer A and sonicated to obtain the

membrane fraction. The samples were thereafter either subjected to SDS-PAGE or analysed for calcium-dependent or calcium-independent PLA_2 activity. Protein content was measured with a kit (Bio-Rad) against bovine serum albumin as the standard protein.

2.8. Analysis of PLA_2 activity

Calcium-dependent and calcium-independent PLA_2 activity was assayed with 2 μM palmitoyl-2-[1-^{14}C]-arachidonyl PtdCho and 1-palmitoyl-2-[1-^{14}C]-arachidonyl PtdEtn, at a 1:1 molar ratio, as substrate. The substrates were dried under nitrogen and for measurement of calcium-dependent PLA_2 activity, resuspended in 80 mM glycine pH 9.0, 5 mM $CaCl_2$, 0.5 mM DTT, 1 mg/ml albumin and 10% glycerol (vol.%). For the calcium-independent PLA_2 assay, substrates were resuspended in buffer A supplemented with 1 mg/ml albumin. These preparations were vortexed and sonicated in a water bath for 10 min at 4 °C. The reaction was initiated by adding 75 μl aliquots of sample and allowed to proceed for 60 min at 37 °C with agitation before termination by the addition of two volumes of methanol containing 0.5% acetic acid and 40 μM stearic acid.

2.9. Analysis of 12-HHT, 12-HETE and arachidonic acid

Samples were centrifuged and the resulting supernatants were applied to Sep-Pak® C18 cartridges (Waters associated, Milford, USA). After washing, bound compounds were eluted with 400 μl methanol. The eluates were analysed by reverse-phase (RP)-HPLC essentially as described [53]. Qualitative analysis was performed by comparison with retention times of authentic standards and by spectrum analysis using a diode-array spectrophotometer. Radioactivity was detected with a β-RAM HPLC flow-through monitor system (Inus System Inc., USA) coupled on-line to the HPLC.

2.10. SDS-PAGE and immunoblotting

Samples were mixed with loading buffer [50 mM Tris (pH 6.8), 10% glycerol, 0.1% SDS, 3 mM 2-mercaptoethanol and 0.005% bromophenol blue] heated for 3 min at 95 °C and loaded onto a 10% SDS-PAGE. For phosphorylation-induced electrophoretic mobility shift analysis of $cPLA_2$-α, the gel was run an additional 45 min after the front had migrated out of the gel. Afterwards, the gels were subjected to immunoblotting. Briefly, the samples were transferred to Hybond™-C nitrocellulose sheets (Amersham, UK) and thereafter blocked with 5% milk powder in Tris-buffered saline with 0.1% Tween for 60 min. Membranes were incubated over night at 4 °C with polyclonal rabbit anti-human $cPLA_2$ antiserum. Enhanced chemiluminescence, ECL-PLUS™, was used for detection (Amersham, UK).

2.11. Analysis of results

Results are expressed as mean ± S.E. Statistical significance was assessed using Student's paired *t*-test. Inhibitory activity was analyzed using GraphPad Prism 3 and IC50-values were obtained by non-linear regression analysis.

3. Results

3.1. Effects of PCBs on the biosynthesis of 12-HETE and 12-HHT in intact platelets

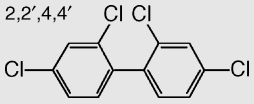
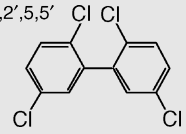
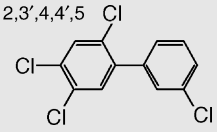
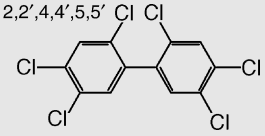
Arachidonic acid released from platelets is rapidly converted to either 12-HETE or TXA_2 and 12-HHT [54]. Therefore, the level of free arachidonic acid is usually very low in platelets even after stimulation. To circumvent this, several investigators block the metabolism, as well as the reacylation processes, of arachidonic acid by the use of general inhibitors such as indomethacin, eicosa-5,8,11,14-tetraynoic acid (ETYA) or lipoxygenase inhibitors such as BW4AC [54,55]. We used a similar approach and included the lipoxygenase inhibitor NDGA (50 μM) and the cyclooxygenase inhibitors indomethacin (5 μM) or acetylsalicylic acid (50 μM). Also, to avoid the use of inhibitors and thereby to get a more physiological relevant model, we measured arachidonic acid release indirectly by measuring the levels of two major metabolites of arachidonic acid in platelets, i.e. 12-HETE and 12-HHT.

To investigate if platelets release arachidonic acid after stimulation with PCBs, intact human platelets were incubated with four different isolated PCBs congeners and four former commercial PCB mixtures (Table 1). Two of the congeners, CB47 and CB52, induced the release of arachidonic acid and the formation of eicosanoid formation to the same extent as the calcium ionophore A23187 (Table 1). The sum of the production of 12-HETE and 12-HHT was approximately 2.2-fold higher in CB52-treated platelets than in A23187-treated platelets. Also, three out of four PCB mixtures, i.e. Clophen 30, Clophen 40 and Clophen 50, induced eicosanoid formation. Interestingly, no formation of leukotrienes could be observed after incubation of human neutrophils or B-lymphocytes with PCBs, showing that PCBs are not sufficient enough stimuli to induce leukotriene formation in these cells (data not shown).

The labelling of the PCB mixtures can roughly be a measure of the degree of chlorination of the biphenyl moiety. The major constituent in the PCB products A30, A40, A50, and A60 are *tri*-chlorinated biphenyls (48%), *tetra*-chlorinated biphenyls (52%), *penta*-chlorinated biphenyls (55%) and *hexa*-chlorinated biphenyls (54%), respectively [52]. Further, the percentage of low-chlorinated biphenyls (*tetra*-chlorinated or lower) in the four PCB products A30, A40, A50, and A60, are 93%, 70%, 19%, and 1%, respectively. Thus, the observed effects (Table 1) are most pronounced after treatments with the *tetra*-chlorinated PCBs (CB47 and CB52) and the low-chlorinated PCB products (A30 and A40), indicating that the effects are linked to the degree of chlorination.

In order to investigate the effects of PCBs on enzyme activity in a broken cell assay, the effects of CB52 on conversion of exogenous arachidonic acid into 12-HETE and 12-HHT was determined. Homogenate samples incubated for 10 min with 35 μM (10 μg/ml) CB52 and subsequently stimulated with 20 μM arachidonic acid demonstrated a 30 ± 6.5% reduction of 12-HHT levels, as compared to vehicle treated samples. The arachidonic acid seemed to be shunted to the 12-LO pathway since the production of 12-HETE increased by 31 ± 5.7% (data is mean ± range from two separate experiments performed in duplicate). Because of its high capacity to induce arachidonic acid release and its relatively small

Table 1 – Effects of PCBs on the formation of 12-HETE, 12-HHT and arachidonic acid in platelets

Stimuli	Chlorine position (compound structure)	12-HETE (pmol/mg protein)	12-HHT (pmol/mg protein)	[1-^{14}C]-20:4 (cpm/sample)
Vehicle	Negative control	63 ± 14	98 ± 14	<200
A30	Mixture	8955 ± 816	165 ± 17	nd
A40	Mixture	7595 ± 1677	164 ± 39	nd
A50	Mixture	5872 ± 910	144 ± 16	nd
A60	Mixture	977 ± 120	115 ± 18	nd
CB-47	2,2′,4,4′ 	7515 ± 1231	842 ± 177	1109
CB-52	2,2′,5,5′ 	7814 ± 1124	1552 ± 305	1379
CB-118	2,3′,4,4′,5 	730 ± 115	175 ± 23	787
CB-153	2,2′,4,4′,5,5′ 	461 ± 79	189 ± 30	780
A23187	Positive control	2931 ± 364	2703 ± 216	1211

Platelets were incubated with the indicated PCB compound (10 μg/ml) for 10 min at 37 °C. Control incubations contained either ethanol (negative control) or 1 μM calcium ionophore A23187 (positive control). The results are expressed as the formation of 12-HETE or 12-HHT in pmol/mg protein. Data show the mean ± S.E. of three separate experiments performed in duplicates except for the determination of ^{14}C-arachidonic acid (^{14}C-20:4), which is one representative experiment out of three. nd = not determined; <200 = below detection limit.

inhibitory effect on COX/TXA_2 pathways, CB52 was selected for further studies.

A time curve for CB52 induced 12-HETE and 12-HHT synthesis is shown in Fig. 1A. The production of 12-HETE showed a lag phase, and production reached a plateau after about 20 min. In contrast, no lag phase is seen for the production of 12-HHT.

For comparison, calcium ionophore-induced 12-HETE and 12-HHT production in human platelets is very rapid and is almost completed within 1 min (data not shown). A dose–response curve for CB52 is shown in Fig. 1B. Half maximal response for both 12-HETE and 12-HHT formation occurred with a dose of about 35 μM CB-52.

Platelets studied by light microscopy after stimulation with CB52 revealed no gross morphological changes as compared to vehicle-treated cells (data not shown).

3.2. *PCB-induced arachidonic acid release in human platelets is mediated by cPLA$_2$-α*

PCBs are suggested to activate two distinct PLA_2 activities, i.e. calcium-dependent and calcium-independent [39]. Therefore, we investigated the involvement of PLA_2 enzymes in PCB-induced release of arachidonic acid in human platelets. To discriminate between secretory PLA_2 ($sPLA_2$) and intracellular PLA_2-mediated arachidonic acid release, we employed the PLA2 inhibitors pyrrolidine-1, $AACOCF_3$ and BEL. Pyrrolidine-1 is a potent and selective inhibitor of cPLA$_2$-α [48,49]. BEL is an inhibitor of $iPLA_2$ [56,57] whereas $AACOCF_3$ inhibits both cPLA$_2$-α and $iPLA_2$ [56,58]. Furthermore, $AACOCF_3$ is known to inhibit thrombin- and calcium ionophore-induced arachidonic acid release and 12-HETE synthesis in human platelets [54,59].

Preincubation of human platelets for 10 min with increasing concentrations of pyrrolidine-1, $AACOCF_3$ or BEL led to decreased amounts of 12-HETE in a dose-dependent manner after stimulation with CB52 (Fig. 2A). Pyrrolidine-1 was approximately 150 times more potent than $AACOCF_3$ and displayed an IC_{50} value of 38 nM. BEL demonstrated a reduction of 12-HETE synthesis by 52% at the highest dose tested (30 μM). However, the results obtained with 30 μM BEL is likely not due to a specific inhibition of $iPLA_2$, since other intact cell assays demonstrate IC_{50} values of less than 5 μM BEL on arachidonic acid release or eicosanoid synthesis [53,60]. In line with this, the broken cell assay demonstrated that 30 μM BEL inhibited the conversion of exogenous arachidonic acid into 12-HETE by 42.1 ± 3.7%. $AACOCF_3$ at 10 μM had no effect on the formation of 12-HETE in a broken cell assay (98.6 ± 1.2% of vehicle-treated samples (data is mean ± range from two separate experiments performed in duplicate)). This is consistent with an earlier study showing that 12-HETE formation induced by addition of arachidonic acid to platelets was not blocked by $AACOCF_3$ [54].

Direct measurement of arachidonic acid release was performed by using platelets labeled with [1-^{14}C]-arachidonic acid. Fig. 2B demonstrates that pyrrolidine-1 inhibited the release of CB52-induced arachidonic acid with an apparent IC_{50} of 5 nM.

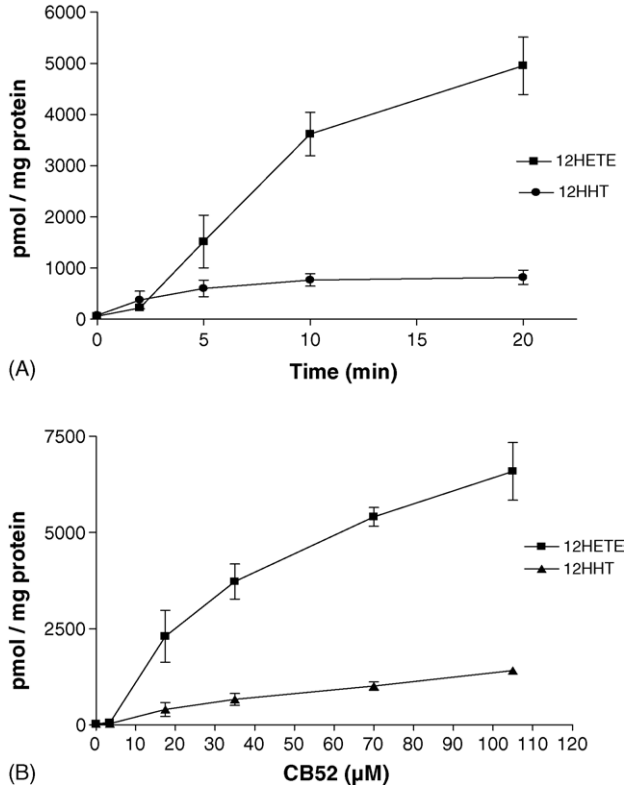


Fig. 1 – Effects of CB52 on eicosanoid biosynthesis. (A) Platelets were treated with 35 μM CB52 for the indicated time. Data show the mean ± S.E. of three separate experiments performed in duplicates. (B) Platelets were treated with increasing amounts of CB52 for 10 min. Data show the mean ± S.E. of three separate experiments performed in duplicates. The results are expressed as the formation of 12-HETE (solid square) or 12-HHT (solid circle) (pmol/mg protein).

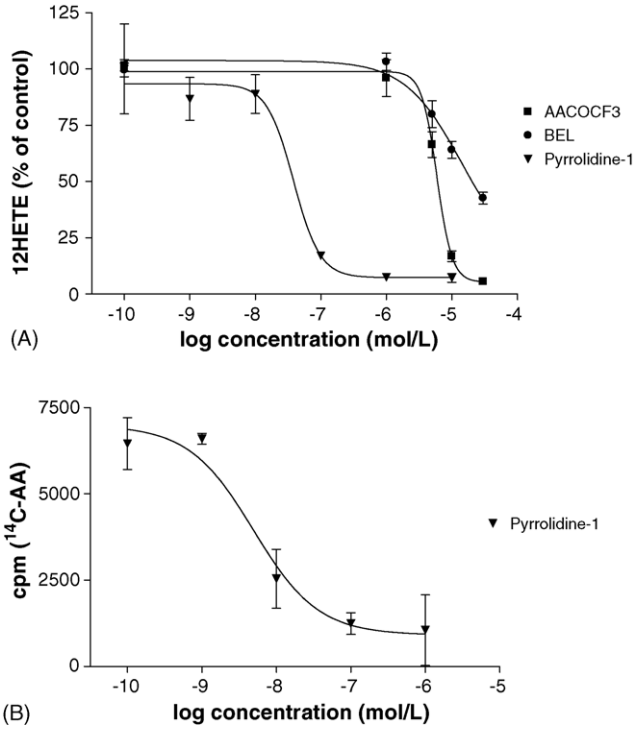


Fig. 2 – Effects of PLA_2 inhibitors on CB52 induced 12-HETE biosynthesis and arachidonic acid release. (A) Platelets were incubated for 10 min with the indicated amount of pyrrolidine-1 (solid triangle) $AACOCF_3$ (solid square) or BEL (solid circle) and thereafter stimulated with 35 μM CB52 for 10 min. The results are expressed as percentage of 12-HETE formation in vehicle treated samples. Data show the mean ± S.E. of three separate experiments performed in duplicates. The amount of 12-HETE in control samples was 6927 ± 455 pmol/mg protein (mean ± S.E.). (B) Platelets were labeled with [1-^{14}C]-arachidonic acid, washed and subsequently incubated with the indicated amount of pyrrolidine-1 for 10 min and thereafter stimulated with 35 μM CB52 for 10 min. The results are expressed as released ^{14}C-arachidonic acid in cpm. Data show the mean ± range of one representative experiment out of two.

In summary, these pharmacological results suggest a role for $cPLA_2$-α in PCB-induced arachidonic acid release in human platelets.

3.3. *Effects of CB52 on $cPLA_2$-α translocation and phosphorylation*

We further explored the role of $cPLA_2$-α in PCB-induced arachidonic acid release by measuring PLA_2 activity as well as $cPLA_2$-α and $iPLA_2$ protein content in platelets treated with or without CB52. Homogenate, cytosolic and membrane fractions were obtained from untreated or CB52-treated platelets. As depicted in Fig. 3A (upper panel, gray bars), the majority of PLA_2 activity was found in the cytosolic fraction of non-stimulated platelets. Interestingly, the PLA_2 activity translocated to the membrane fraction after stimulation of platelets with CB52 (Fig. 3A, upper panel, black bars). To investigate if the observed shift of PLA_2 activity from the cytosolic to the membrane fraction could be ascribed to different $cPLA_2$-α content, immunoblotting was performed on these fractions. Fig. 3A (lower panel) demonstrates that $cPLA_2$-α indeed translocated from the cytosolic to the membrane fraction upon CB52 treatment of intact platelets.

It is established that phosphorylation of $cPLA_2$-α leads to reduced electrophoretic mobility on SDS-PAGE [61]. Interestingly, a change in the ratio between phosphorylated and unphosphorylated $cPLA_2$-α protein was observed after CB52-treatment (Fig. 3A). As seen in the two most left lanes containing control homogenate and homogenate from CB52 treated platelets, respectively, a majority of the $cPLA_2$-α protein shifted its electrophoretic mobility to a slower migrating form after CB52 treatment, suggesting that the $cPLA_2$-α protein became phosphorylated after CB52 treatment.

The time course of $cPLA_2$-α cytosol-to-membrane translocation was investigated by Western blot analysis of platelets treated with CB52 for various times. As outlined in Fig. 3B, the $cPLA_2$-α content in the membrane fraction reached a plateau

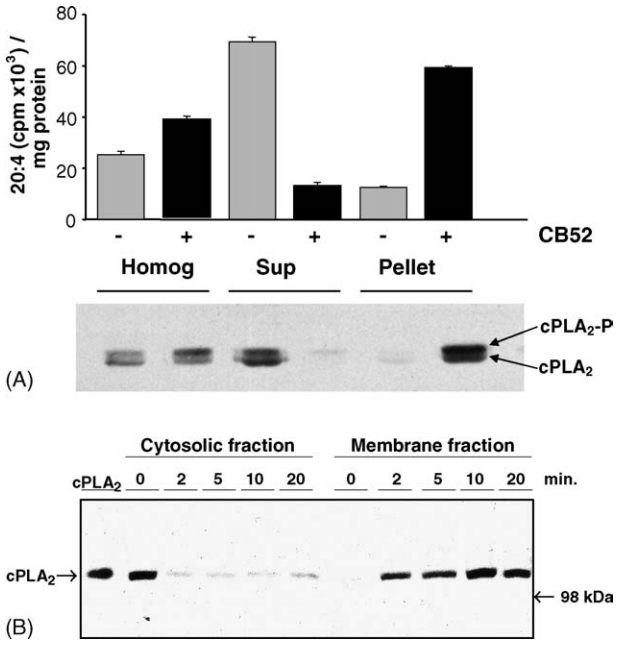


Fig. 3 – PLA$_2$ activity and western blot analysis of cPLA$_2$-α protein in subcellular fractions of platelets. (A) Intact platelets were incubated with vehicle (gray bars) or CB52 (35 μM) (black bars) for 10 min prior to homogenization. Aliquots of homogenate and subcellular fractions were assayed for PLA$_2$ activity (upper panel). Thirty micrograms of protein of the same fractions as used in the upper panel were subjected to Western blot analysis using cPLA$_2$ antiserum (lower panel). Vehicle treated samples are indicated with (−); CB52-treated samples are indicated with (+). Arrows on the right indicate phosphorylated and non-phosphorylated form of cPLA$_2$. Data is from one representative experiment, performed in duplicate, out of two. (B) Intact platelets were incubated with or without CB52 (35 μM) for the indicated time prior to homogenization. Aliquots (30 μg protein) of subcellular fractions were separated on a 4–12% SDS-PAGE gel and subsequently analyzed by western blot for cPLA$_2$ content.

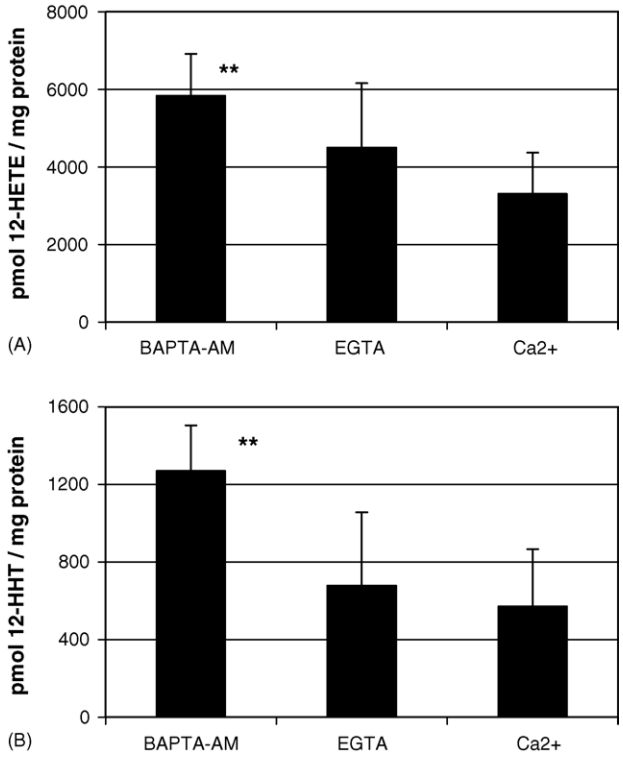


Fig. 4 – Effect of Ca^{2+}-chelation on CB52 induced 12-HETE and 12-HHT biosynthesis. Platelets were treated with BAPTA-AM (10 μM) and EGTA (2 mM), EGTA (2 mM) or 1 mM CaCl$_2$ for 45 min. Thereafter, platelets were stimulated with 35 μM CB52 for 10 min. The results are expressed as the formation of 12-HETE (A) or 12-HHT (B) in nmol/mg protein. Data show the mean ± S.E. of three separate experiments performed in duplicates ($^{**}p < 0.005$ as compared to 1 mM CaCl$_2$).

at 10 min. At 20 min, there was a tendency for dissociation of cPLA$_2$-α from the membrane.

The cytosol-to-membrane translocation of cPLA$_2$-α correlates well with the time course for eicosanoid synthesis and the tendency to dissociation is in line with eicosanoid synthesis reaching a plateau at 20 min (Fig. 1A).

By activity measurements or by Western blot analysis, we were unable to detect any calcium-independent PLA$_2$ activity or iPLA$_2$ protein in platelet homogenate or subcellular fractions thereof (data not shown).

3.4. *Effect of CB52 on intracellular Ca^{2+} concentrations*

Since Ca^{2+} is known to induce cytosol-to-membrane translocation of cPLA$_2$-α in many cell types, we investigated the effects of Ca^{2+}-chelation on eicosanoid formation by using EGTA and the intracellular Ca^{2+}-chelator BAPTA-AM. Platelets were treated with 2 mM EGTA or EGTA and 10 μM BAPTA-AM and thereafter stimulated with CB52. The formation of 12-HETE and 12-HHT increased significantly after treatment of platelets with EGTA/BAPTA-AM as compared to platelets incubated in the presence of 1 mM extracellular Ca^{2+} (Fig. 4). This was surprising since BAPTA-AM previously has been demonstrated to suppress calcium-dependent release of arachidonic acid from human platelets [61]. However, several reports describe PCB-induced arachidonic acid release to only be marginally or unaffected by chelation of both extra- and intra-cellular calcium [39,40,43,44,47].

To more thoroughly investigate the role of Ca^{2+} in CB52-induced cPLA$_2$-α cytosol-to-membrane translocation, intracellular Ca^{2+} changes were measured using Fura2-AM. Platelets loaded with Fura2 were treated either with vehicle, CB52 or the calcium ionophore ionomycin. Fig. 5 demonstrates representative traces of one such experiment from platelets incubated in the presence of 1 mM extracellular CaCl$_2$. CB52 did not induce a significant increase in the fluorescence ratio (Fig. 5, trace A), indicating that CB52 did not induce an increase in intracellular Ca^{2+}. For comparison, the traces of ionomycin or vehicle-treated platelets are also shown (Fig. 5, traces B and C).

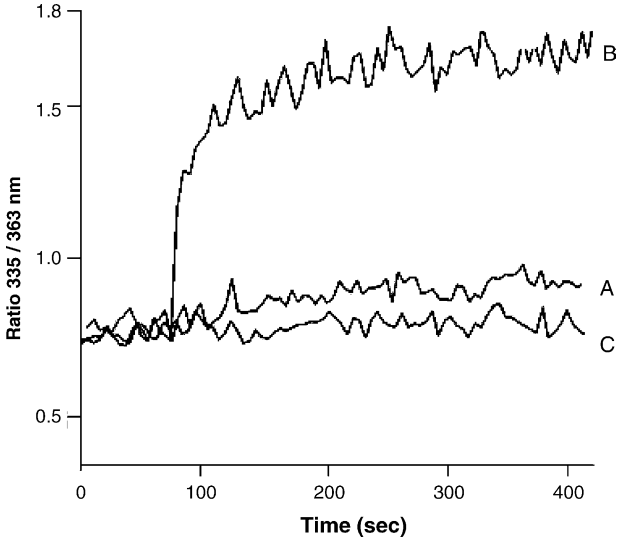


Fig. 5 – Effects of CB52 or calcium ionophore on intracellular Ca^{2+}-concentrations. The changes in intracellular Ca^{2+}-concentrations were followed using the fluorescent Ca^{2+}-indicator dye, Fura2, as outlined in Section 2. Traces are presented as the ratio of obtained fluorescence between 335 and 363 nm. Fura2 loaded platelets were treated with either 35 μM CB52 (trace A), 0.5 μM of the calcium ionophore ionomycin (trace B) or vehicle (ethanol) (trace C). Data shown are representative traces from two different experiments performed in duplicates.

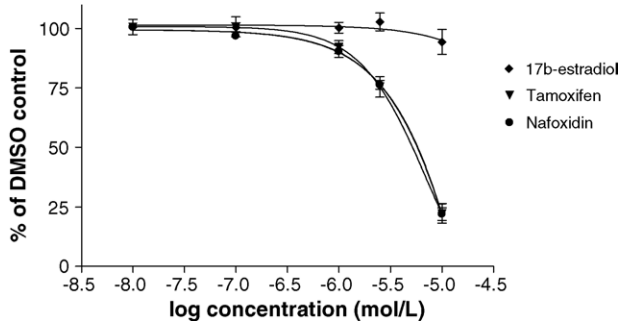


Fig. 6 – Dose–response of anti-estrogens on CB52 induced 12-HETE biosynthesis. Platelets were incubated for 20 min with nafoxidin (solid circle), tamoxifen (solid square) or 17β-estradiol (solid triangle) and thereafter stimulated with 35 μM CB52 for 10 min. The results are expressed as percentage of 12-HETE formation in vehicle treated samples. Data show the mean ± S.E. of three separate experiments performed in duplicates. The amount of 12-HETE in control samples were 10086 ± 1428 pmol/mg protein (mean ± S.E.).

3.5. *Effects of anti-estrogens on CB52-induced eicosanoid synthesis*

In order to determine the mechanism behind CB52-induced arachidonic acid release in human platelets, experiments were designed to investigate whether CB52 could function as a structural analogue of human hormones. It is a well-known fact that PCBs can mimic hormones such as steroids and their reactivity can be expressed in terms of estrogen equivalents [31]. The binding of PCBs to sites distinct from nuclear receptors such as the estrogen receptor and the Ah-receptor, could mediate arachidonic acid release in human platelets. Indeed, the estrogen-antagonist tamoxifen has been reported to decrease the amount of $cPLA_2$-α associated with membranes independent of the estrogen receptor [62]. Thus, 17β-estradiol and two anti-estrogens, tamoxifen and nafoxidin, were selected for this study. As depicted in Fig. 6, platelets pre-treated with tamoxifen or nafoxidin showed a dose-dependent inhibition of CB52 induced 12-HETE formation. Results for 12-HHT are not included in Fig. 6, due to inhibition of tamoxifen and nafoxidin on the COX/TXA_2 pathways in the broken cell assay. On the other hand, no inhibitory effect was observed for nafoxidin or tamoxifen on the 12-lipoxygenase activity in the broken cell assay (data not shown).

17β-estradiol had no effect on 12-HETE formation in intact platelets (Fig. 6). Furthermore, preincubation of platelets with dexamethasone (10 μM) for 20 min did not inhibit CB52-induced 12-HETE or 12-HHT formation (data not shown).

To investigate if the estrogen-antagonist nafoxidin or 17β-estradiol could affect the release of arachidonic acid, direct measurement of arachidonic acid release was performed by using platelets labeled with [1-^{14}C]-arachidonic acid as described in Section 2. Nafoxidin inhibited CB52-induced release of arachidonic acid from intact platelets by 67.7 ± 16.5% (data is mean ± S.D. from one experiment performed in triplicate). 17β-estradiol at 10 μM had no effect on the release of arachidonic acid from intact platelets (data not shown).

It has been reported that tamoxifen can reduce the amount of $cPLA_2$-α associated with membranes [62]. To investigate this in platelets, we examined the effects of both tamoxifen and nafoxidin on $cPLA_2$-α activity in an in vitro $cPLA_2$-α assay. Vehicle-treated samples released 48.8 ± 7.0 × 10^3 cpm [1-^{14}C]-arachidonic acid/mg protein. For comparison, when 10 μM nafoxidin or tamoxifen where present during the assay, the activities were 47.8 ± 3.9 or 49.9 ± 10.0 × 10^3 cpm [1-^{14}C]-arachidonic acid/mg protein, respectively (data is mean ± range from two experiments performed in duplicates). In summary, these results suggest that nafoxidin and tamoxifen impair CB52-induced arachidonic acid release without inhibiting $cPLA_2$-α.

3.6. *CB52 does not induce aggregation of human platelets*

The large induction of 12-HHT formation after incubation of platelets with CB52 suggested that PCBs could induce platelet aggregation due to the formation of TXA_2. Platelets resuspended in PBS were placed in the aggregometer and stimulated with the indicated reagent for up to 10 min at 37 °C with constant stirring. Fig. 7 shows representative traces from 5 min incubations. Trace A demonstrates stimulation of platelets with 35 μM CB52. Clearly, platelets did not aggregate after stimulation with CB52, despite the fact that platelets from the same batch produced similar amounts of 12-HETE

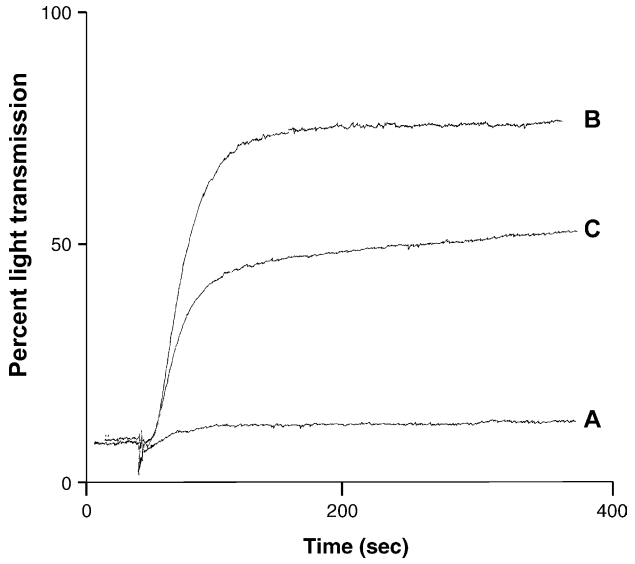


Fig. 7 – Effects of CB52 or calcium ionophore A23187 on platelet aggregation. Platelets (200 × 10^6/ml) were resuspended in PBS and incubated for 5 min at 37 °C. Aggregation of platelets was measured in an aggregometer when a stable baseline was obtained. Platelets were stimulated with 35 μM CB52 (trace A) or 1 μM calcium ionophore A23187 (trace B). Trace C represents A23187-induced aggregation of platelets pretreated with 35 μM CB52 for 10 min at 37 °C. Data shown are representative traces from three different experiments performed in duplicates.

and 12-HHT as those indicated in Table 1 (data not shown). Similar results were obtained for 10 min incubations. For comparison, platelets stimulated with 1 μM calcium ionophore A23187 did aggregate as shown in Fig. 7, (trace B). Interestingly, preincubation of platelets with 35 μM CB52 for 10 min at 37 °C before addition of A23187 reduced the aggregation by only approximately 30% (Fig. 7, trace C). This indicated that platelets incubated with CB52 for 10 min still could respond to stimuli that induce aggregation.

4. Discussion

We report, herein, that low-chlorinated PCBs activate cPLA$_2$-α, leading to arachidonic acid release and eicosanoid formation in human platelets. The PCB-induced release of arachidonic acid was clearly structure-dependent since CB118 or CB153 did not induce as much arachidonic acid release or eicosanoid synthesis as CB47 or CB52 (Table 1). This indicates that the effect of PCBs on arachidonic acid release is not due to a general disturbance of the membranes, but rather due to a specific activation. Furthermore, we demonstrate that CB52 induces phosphorylation and cytosol-to-membrane translocation of cPLA$_2$-α, independent of a rise in intracellular calcium. It is by now a well-known fact that calcium can promote cytosol-to-membrane translocation of cPLA$_2$-α. However, several papers describe a calcium-independent cytosol-to-membrane translocation of cPLA$_2$-α [19,21,63]. It has been suggested that cPLA$_2$-α can undergo interfacial binding by two different modes, i.e. calcium-dependent binding via the C2 domain [13-15] or calcium-independent binding through a phospholipid-binding region in the catalytic domain [21,19]. Furthermore, agents such as okadaic acid and phorbol myristate acetate (PMA) induces arachidonic acid release without increasing intracellular calcium concentration [22]. Also, it was reported earlier that thrombin-stimulated platelets produced more TXA_2 in the absence of extracellular calcium [64]. Taken together, these lines of evidence suggest an alternative mechanism for cPLA$_2$-α cytosol-to-membrane translocation, which is independent of increased calcium concentrations. It remains to be investigated if the observed phosphorylation of cPLA$_2$-α after PCB-treatment (Fig. 5) contributes to the calcium-independent cytosol-to-membrane translocation.

A structure–activity relationship among PCB congeners demonstrated a mechanism through which these compounds could affect calcium signaling in neuronal cells by binding to the ryanodine receptor in the endoplasmatic reticulum. This binding leads to release of Ca^{2+} from the endoplasmatic reticulum [65]. However, that mechanism is not likely the cause for the observed effects of PCBs on platelets since no increase in intracellular Ca^{2+} could be observed. Also, we found that ruthenium red, an inhibitor of PCB-induced Ca^{2+}-release mediated via the ryanodine receptor, had no effect on CB52-induced 12-HETE or 12-HHT formation (data not shown).

The mechanism behind PCB-induced eicosanoid formation in human platelets involves cytosol-to-membrane translocation of cPLA$_2$-α and possibly also phosphorylation of the enzyme. It has previously been reported that PCBs can induce cytosol-to-membrane translocation of cPLA$_2$-α in rat renal tubular cells after exposure of the cells to PCBs for 60 min [66]. This effect was partially dependent on de novo protein synthesis, suggesting a mechanism involving increased synthesis of cPLA$_2$-α. However, the signaling pathway leading to the activation of cPLA$_2$-α in human platelets is unknown. Since platelets lack a nucleus, it is unlikely that the observed effects of PCBs are mediated via de novo protein synthesis. Nevertheless, non-genomic effects could account for some of the observed effects of PCBs on arachidonic acid release [67]. Platelets have been found to possess both estrogen-receptor α and β [68]. The lack of effect of 17β-estradiol on arachidonic acid release observed in this report is in line with previous reported findings [69] and suggests that the estrogen receptors are not involved in PCB-induced arachidonic acid release. Also, the estrogen-like activities of CB47, CB52, CB118 and CB153 [70,71] does not correlate with their ability to induce eicosanoid synthesis and arachidonic acid release in platelets. Thus, the effects observed for anti-estrogens on CB52-induced eicosanoid formation suggest that PCBs might activate cPLA$_2$-α by binding to a binding site distinct from the estrogen receptors. A high-affinity binding site for tamoxifen, distinct from the estrogen receptor has indeed been described [72]. If this binding site, or a similar is involved in PCB-induced eicosanoid formation in platelets remains to be investigated. Tamoxifen has been reported to decrease the amount of cPLA$_2$-α associated with membranes in A549 lung adenocarcinoma cell line [62]. However, we were unable to detect any inhibitory effects of tamoxifen or nafoxidin on platelet cPLA$_2$-

α activity in an in vitro assay. This suggests that the effects observed for tamoxifen on human A549 cells and platelets is mediated through different mechanisms. Tamoxifen is currently one of the most commonly used drugs for endocrine therapy of breast cancer. The possibility that the inhibition of eicosanoid synthesis and arachidonic acid release by antiestrogens observed in this report is involved in the chemoprevention of breast cancer remains to be investigated. Interestingly, the 12-LO pathway has been suggested to play a key role in breast cancer cell growth as well as in prostate tumor motility [73,74].

In conclusion, low-chlorinated PCBs activate $cPLA_2$-α in human platelets leading to eicosanoid formation. Interestingly, $cPLA_2$-α play important roles in tissues or cells known to also be affected by PCB, such as reproductive and brain tissue as well as blood cells. PCBs might be useful tools in further studies of hormonal-induced arachidonic acid release.

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