Impairment of human neutrophil oxidative burst by polychlorinated biphenyls: inhibition of superoxide dismutase activity

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Abstract: We report evidence of a novel mechanism by which polychlorinated biphenyls might act as potent inducers of inflammation. Aroclor 1242 (A1242), a polychlorinated biphenyl mixture, and 2,2',4,4'-tetrachlorobiphenyl (PCB47), a constituent of A1242 that produces the same patterns of effects, impaired the oxidative burst of human neutrophils by inhibiting the antioxidant enzyme superoxide dismutase, which converts O_2^- to H_2O_2. Pre-incubation of neutrophils with A1242 or PCB47 before stimulation with phorbol 12-myristate 13-acetate heightened the respiratory burst, producing a significant increase in intracellular O_2^- production along with a significant decrease in H_2O_2 production compared with unexposed agonist-stimulated neutrophils. This was also evident in a physiologically relevant situation in which neutrophils pre-incubated with A1242 were subsequently stimulated with a combination of *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine and tumor necrosis factor-α. Incubation of bovine copper-zinc superoxide dismutase (EC 1.15.1.1) with A1242 or PCB47 in a cell-free system reversed the enzyme-mediated inhibition of 6-hydroxydopamine autoxidation, indicating that polychlorinated biphenyls inhibited superoxide dismutase activity. Low superoxide dismutase activity in neutrophils leads to imbalances between production of free radicals and antioxidant defense mechanisms, which can in turn induce tissue damage and hasten the onset of neutrophil apoptosis. *J. Leukoc. Biol.* 63: 216–224; 1998.

Key Words: *Aroclor · flow cytometry · PCB47 · neutrophil apoptosis*

INTRODUCTION

Neutrophils form the predominant component of the circulating leukocyte pool in human peripheral blood and constitute the first line of defense against infectious agents and foreign stimuli (particulate and soluble) that manage to cross the body's physical barriers [1]. Although the importance of the neutrophil in counteracting infectious pathogens is well recognized [1], much less is known about its involvement with other external influences, including chemicals of environmental concern. The environmental persistence of these toxins, especially polychlorinated biphenyls (PCBs), which are ubiquitous and potent immunotoxins, raises serious questions about their effects on human health. Previous studies have demonstrated that exposure to PCBs affects immune system components, mainly T lymphocytes and macrophages, in both human and animal models [2–5]. A notable feature in PCB-exposed humans, however, is a reduction in the number of circulating neutrophils with a concomitant increase in the number of circulating lymphocytes, monocytes, and eosinophils [2].

On interaction with endogenous and exogenous stimuli, neutrophils undergo a respiratory burst. Increased oxygen consumed during this process is reduced to superoxide (O_2^-) via membrane NADPH oxidase, a multicomponent system formed by the assembly of cytosolic and membrane proteins upon appropriate stimulation [6]. Superoxide can engender more harmful reactive oxygen species (ROS) when two molecules of O_2^- react together or enzymatically as a consequence of the activity of superoxide dismutase (SOD), to form dioxygen and hydrogen peroxide (H_2O_2) and finally hydroxyl radical ($OH\cdot$) [7]. With regard to PCBs, previous studies on rat neutrophils have demonstrated that Aroclor 1242 (A1242) and 2,2',4,4'-tetrachlorobiphenyl (PCB47) can increase extracellular O_2^- generation through the inositol phosphate pathway in a dose-dependent manner [8, 9]. Because the current understanding of neutrophil oxidant production on interaction with PCBs is

Abbreviations: PCBs, polychlorinated biphenyls; ROS, reactive oxygen species; SOD, superoxide dismutase; HE, hydroethidine; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; PMA, phorbol 12-myristate 13-acetate; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; DDC, diethyldithiocarbamide; DPI, diphenyleneiodonium; 6-OHDA, 6-hydroxydopamine; HBSS, Hanks' balanced salt solution; BSA, bovine serum albumin; PBS, phosphate-buffered saline; ELS, erythrocyte lysing solution; DMSO, dimethyl sulfoxide; EB, ethidium bromide; TNF-α, tumor necrosis factor-α; PI, propidium iodide.

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limited to extracellular generation of O_2^- in rodents, the goal of this study was to determine the effects of A1242 (a mixture of PCBs) and PCB47 (a PCB congener present in A1242) on the function of both quiescent and stimulated human neutrophils in terms of intracellular generation of O_2^- and H_2O_2. The neutrophil oxidative burst was monitored by flow cytometry [10, 11] with the use of hydroethidine (HE) and 2',7'-dichlorofluorescin diacetate (DCFH-DA) as fluorescent indicators of intracellular O_2^- [12, 13] and H_2O_2 production [14], respectively. This report presents evidence that PCBs not only activate ROS production in neutrophils directly, but also produce an imbalanced oxidative burst in stimulated neutrophils. Pre-incubation with both A1242 or PCB47 enhanced O_2^- generation in phorbol 12-myristate 13-acetate (PMA)-stimulated and tumor necrosis factor α (TNF-α)-primed, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP)-stimulated neutrophils, while diminishing H_2O_2 production in a dose-dependent manner. These effects could be secondary to either increased activity of NADPH oxidase, decreased activity of SOD, or both. We therefore investigated the ability of these toxins to modulate the oxidative burst in stimulated neutrophils by comparing their effects to inhibitors of NADPH oxidase and SOD: diethyldithiocarbamate (DDC) and diphenyleneiodonium (DPI). In addition, the effects of the above compounds on SOD activity were also evaluated in a cell-free system utilizing the autoxidation of 6-hydroxydopamine (6-OHDA) as a test model.

EXPERIMENTAL PROCEDURES

Buffers and reagents

Hanks' balanced salt solution (HBSS) containing 12.2 mM glucose, 1.87 mM Ca^{2+}, 0.8 mM Mg^{2+}, and 0.1% bovine serum albumin (BSA), pH 7.4, was used to incubate neutrophils for subsequent stimulation with PMA, fMLP (1–1000 nM final; Sigma Chemical Co., St. Louis, MO), and human recombinant TNF-α (10 ng/mL final; Sigma). Phosphate-buffered saline (PBS), pH 7.4 (Difco Laboratories, Detroit, MI) was used for reagent dilutions. Erythrocyte lysing solution (ELS) containing 150 mM NH_4Cl, 10 mM $NaHCO_3$, and 1 mM ethylenediaminetetraacetate was used to eliminate change in SOD autoxidation of 6-OHDA. Bovine Cu-Zn SOD and 6-OHDA (hydrobromide salt) were obtained from Sigma. DCFH-DA (Molecular Probes, Eugene, OR) was dissolved in absolute ethanol to a concentration of 20 mM (final concentration of 20 μM). HE (Molecular Probes) was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10 mM (final concentration 10 μM). PMA (LC Services Corp., Woburn, MA) was dissolved in DMSO at 2 mg/mL (3.25 mM) and used at a final concentration of 50 ng/mL [15]. A1242 and PCB47 (Chem Service, West Chester, PA) were dissolved in DMSO as stock solutions of 1 mg/mL and stored at room temperature. Further dilutions were made in PBS so that the final concentration of DMSO in the reaction mixture was less than 0.1%. DPI (Molecular Probes, Eugene, OR), a specific inhibitor of NADPH oxidase (2 μM), and DDC (1 mM; Sigma) were used as positive controls to inhibit O_2^- and H_2O_2 production, respectively, in PMA-stimulated neutrophils.

Collection of neutrophils

Peripheral blood was obtained from healthy adult volunteers in a sterile tube containing preservative-free heparin by standard venipuncture methods after informed consent was obtained in compliance with the regulations of the Committee on the Use of Human Subjects Research, Purdue University. Three milliliters of heparinized whole blood were added to a 50-mL conical tube followed by 47 mL of ELS. The tubes were rotated for 10 min at room temperature on a bench rocker and then centrifuged at 400 *g* for 10 min at 4°C. The supernatant fluid was decanted and the pellet resuspended by drawing the tube gently across a test tube rack. The cells were washed with HBSS, centrifuged at 250 *g* for 10 min at 4°C, and resuspended in 4 mL HBSS. Cells were counted with a Coulter Counter (Coulter Corp., Hialeah, FL) and concentration adjusted to 2.0×10^6 cells/mL. Detection of ROS O_2^- and H_2O_2 was performed according to the procedures described elsewhere [16]. Direct intracellular measurement of O_2^- utilized HE, the sodium borohydride-reduced derivative of ethidium bromide (EB). In our hands oxidation of HE is predominantly a measurement of O_2^- generation based on cell-free assays employing potassium superoxide (KO_2), H_2O_2, and horseradish peroxidase in HBSS with 0.1% BSA [13]. However, there is no conclusive evidence that O_2^- is the only free radical that can convert HE to EB in all experimental systems. Intracellular H_2O_2 generation was detected with the use of neutrophils loaded with DCFH-DA [14]. Cell suspensions were pre-incubated 15 min at 37°C for DCFH-DA and 5 min at 37°C for HE to incorporate the probes into the cells. A positive control with PMA alone (50 ng/mL) was run in parallel with cells exposed to A1242 and PCB47 (1 and 10 μg/mL) alone in order to make a comparative evaluation between PCB- and PMA-treated samples. In experiments that assessed the effect of DPI (2 μM), DDC (1 mM), A1242, or PCB47 (0.01–10 μg/mL) on PMA-stimulated neutrophils, the reagents were added to the respective tubes 5 min before stimulation with PMA. In experiments employing TNF-α, neutrophils were incubated in the presence or absence of TNF-α (10 ng/mL) for 5 min at 37°C before the addition of stimulants such as fMLP (1–1000 nM). When A1242 was included in the protocol, neutrophils were incubated with the PCB mixture for 5 min before the addition of TNF-α and/or fMLP.

Flow cytometry

Flow cytometric data were collected on an ELITE flow cytometer (Coulter Cytometry) using instrument-standard computer, optics, and electronics. Two argon lasers (a Cyonics Uniphase and a Coherent Innova 300) were used as excitation sources. The first laser was used for the excitation of ROS-specific dyes (HE and DCFH) and propidium iodide (PI) at 488 nm. Optical filters placed in the fluorescence collection pathway included a 488-nm dichroic filter for 90°LS, a 525-nm band pass for DCF, and a 610-nm long pass for EB and PI. A previously defined assay technique was used to collect kinetic measurements on individual cells [17]. Briefly, samples were activated immediately after being run on the cytometer for the first measurement (zero time) to ensure that each sample's zero time measurement was a true zero for that tube. Neutrophils were distinguished from lymphocytes and monocytes by forward angle light scatter

(FALS) and orthogonal light scatter (90°LS) by virtue of the increased granularity in the neutrophil cytoplasm [18]. A bitmap (gate) was drawn around the cell population with the largest FALS and 90°LS to identify the neutrophils; subsequent fluorescent measurements were collected from those cells. The histograms of mean EB and DCF fluorescence of neutrophils exposed to A1242 and PCB47 alone (depicted in **Figs. 1** and **2**) were generated using the flow cytometry software program WinMDI® (Joseph Trotter, Scripps Research Institute, La Jolla, CA). Neutrophils exposed to A1242 and PCB47 alone or in combination with fMLP, TNF-α, and PMA were assessed for viability through their ability to exclude PI, a well-characterized property of live cells [19]. Cell viability did not drop below 90% for all samples (data not shown).

Assay for superoxide anion production in a cell-free system, autoxidation of 6-OHDA

This assay system was adopted to measure O_2^- production in place of the much more specific xanthine-xanthine oxidase O_2^- detection system [20] for the primary reason that both A1242 and PCB47 interacted with xanthine oxidase in experiments performed in the absence of SOD, giving a false positive result (data not shown). Autoxidation of 6-OHDA results in the production of O_2^- and a quinone with an absorption maximum of 490 nm. Measurements were made on a Lambda3 Series spectrophotometer (Perkin-Elmer Corp.). Because the resulting O_2^- also oxidizes 6-OHDA to the same quinone, the presence of SOD results in slower formation of the quinone because of the removal of O_2^- [21]. This retardation of 6-OHDA autoxidation was used to examine the role of Aroclor in the inactivation of SOD. In preliminary experiments the concentration of SOD required to provide half-maximal retardation of quinone formation was determined to be 0.3 U/mL; this concentration was used in subsequent experiments. The change in absorbance at 490 nm in the presence of SOD was taken as 0% SOD inhibition, whereas the change in absorbance in a sample containing no SOD was taken as 100% SOD inhibition. SOD was incubated with varying concentrations of A1242 (0.01–2 μg/mL) for 30 min at 37°C (data not shown). The autoxidation reaction was begun by the addition of 1 mL of 10^{-2} M 6-OHDA (final concentration 10^{-5} M), and the rate of change in absorbance over the first 30 s was measured at 490 nm. The inhibitory effect of A1242 (1 μg/mL) was compared to that of DDC (1 mM) and PCB47 (1 μg/mL).

Statistical analyses

Triplicate samples were run on the flow cytometer and spectrophotometer and repeated several times for all assays. Between 5,000 and 10,000 cells were analyzed from each tube. Data were expressed as mean ± SEM and were analyzed by one-way analysis of variance followed by Tukey's procedure for multiple comparisons. The acceptable level of significance was $P < 0.05$.

RESULTS

Effect of A1242 and PCB47 on intracellular ROS production in neutrophils

Neutrophils stimulated with PMA (50 ng/mL) were used as a positive control to monitor both O_2^- (EB fluorescence) and

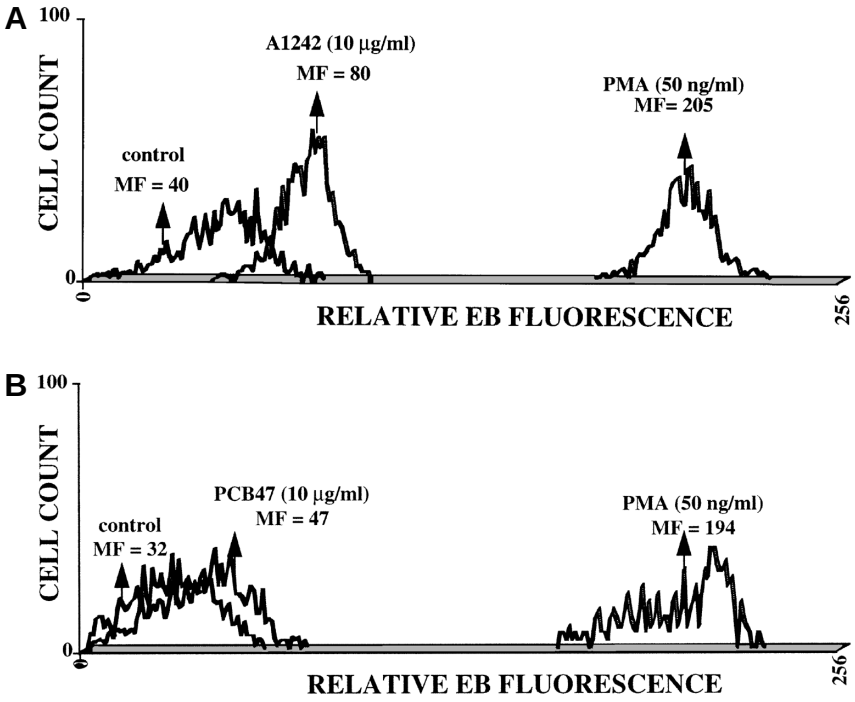

Fig. 1. Flow cytometric histograms of intracellular superoxide production (relative EB fluorescence) in human neutrophils exposed to A1242 or PCB47. Human peripheral blood neutrophils (2×10^6/mL) were incubated for 30 min in the absence or presence of A1242 (A) or PCB47 (B) at the concentration indicated. Superoxide production was determined as described in Materials and Methods.

H_2O_2 (DCF fluorescence) production in assays testing the stimulatory capacity of both A1242 and PCB47. An increase in intracellular O_2^- production was demonstrated by an increase in mean fluorescence (MF) after 30 min in A1242- and PCB47-stimulated neutrophils (Fig. 1, A and B). A1242 (10 µg/mL) caused a twofold increase in O_2^- production compared with that of unstimulated neutrophils. PCB47 at the same concentration elicited a smaller increase in O_2^- production than did A1242. Pretreatment of human neutrophils with DPI (2 µM) for 10 min at 37°C decreased the formation of O_2^- after stimulation with 10 µg/mL A1242 and PCB47 (data not shown). On the other hand, the mitochondrial respiratory inhibitor azide did not influence the PMA- and PCB-stimulated O_2^- production in any significant manner. An increase in intracellular H_2O_2 production was also seen at 30 min in neutrophils stimulated with 10 µg/mL A1242 and PCB47 (Fig. 2, A and B).

Intracellular oxidation of HE and DCFH in PMA-stimulated neutrophils pre-incubated with A1242 and PCB47

There was a dose-dependent increase in intracellular O_2^- production indicated by percent EB fluorescence at 30 min in PMA-stimulated neutrophils pre-incubated with A1242 and PCB47 (**Fig. 3**, **A** and **B**). All concentrations of A1242 employed in this study caused a significant increase in O_2^- production compared with the control (PMA alone). A similar phenomenon was also evident with the congener PCB47. The increase in O_2^- production was significant but smaller than the response elicited by A1242. In contrast, intracellular H_2O_2 production decreased in a dose-dependent manner at 30 min in PMA-stimulated neutrophils pre-incubated with A1242 and PCB47 (Fig. 3, A and B). This decrease in H_2O_2 production was significant at concentrations from 0.1 to 10 µg/mL for both A1242 and PCB47.

Effect of inhibition of intracellular O_2^- and H_2O_2 production by DPI and DDC

To obtain a flow cytometric delineation of intracellular O_2^- and H_2O_2 production mechanisms, we made use of DPI and DDC, inhibitors of NADPH oxidase and SOD, respectively. In this study, DPI added to cells 5 min before HE assay caused nearly 50% inhibition of PMA-induced O_2^- production (**Fig. 4**). A half-maximal inhibition (52%) of PMA-induced O_2^- production was achieved at 30 min with a DPI concentration of 2 µM. Accompanying this decrease in O_2^- production was a precipitous decline in PMA-induced H_2O_2 production (35%) in the presence of DPI. DDC (1 mM), an inhibitor of SOD, caused a significant increase in O_2^- production (190%) in neutrophils stimulated with PMA. Concomitantly, the H_2O_2 production at that concentration was significantly decreased (52%) at 30 min.

Functional inactivation of SOD by A1242 and PCB47, autoxidation of 6-OHDA

SOD (0.3 U/mL) inhibition of 6-OHDA autoxidation was reversed by pre-incubating the enzyme with A1242 at concentrations ranging from 0.01 to 1 µg/mL (data not shown). There was no interference by A1242 alone at any concentration in the assay. When the above concentration of SOD was incubated with DDC (1 mM), A1242 (1 µg/mL), and PCB47 (1 µg/mL), there was an increase in percent change in absorbance from 0 (SOD alone) to 63, 51, and 42%, respectively (**Fig. 5**). Heat-inactivated SOD did not inhibit the autoxidation of 6-OHDA.

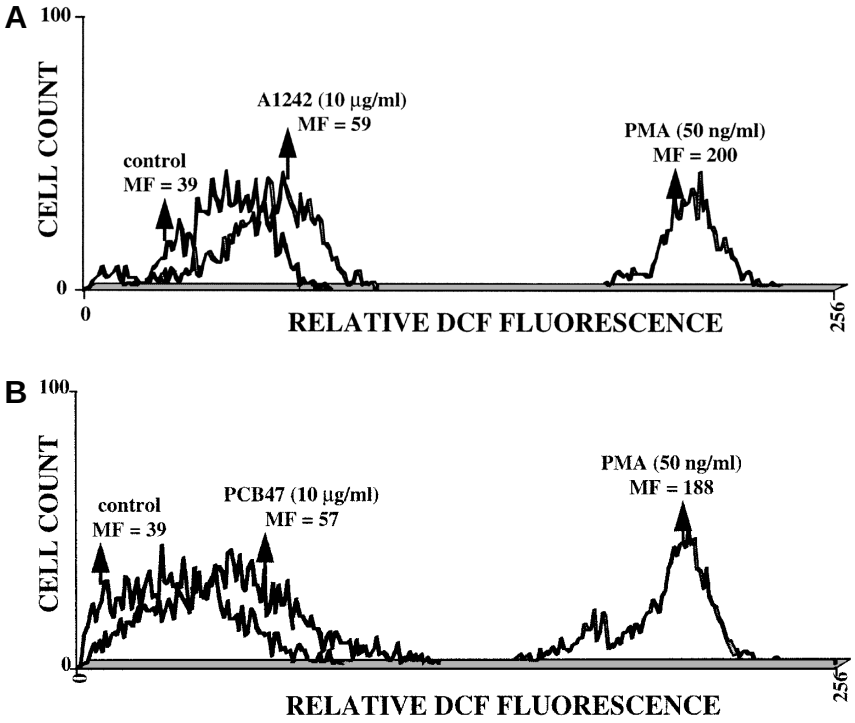

Fig. 2. Flow cytometric histograms of intracellular hydrogen peroxide (relative DCF fluorescence) production in human neutrophils exposed to A1242 or PCB47. Human peripheral blood neutrophils (2×10^6/mL) were incubated for 30 min in the presence of A1242 (A) and PCB47 (B) at the concentration indicated. Hydrogen peroxide production was determined as described in Materials and Methods.

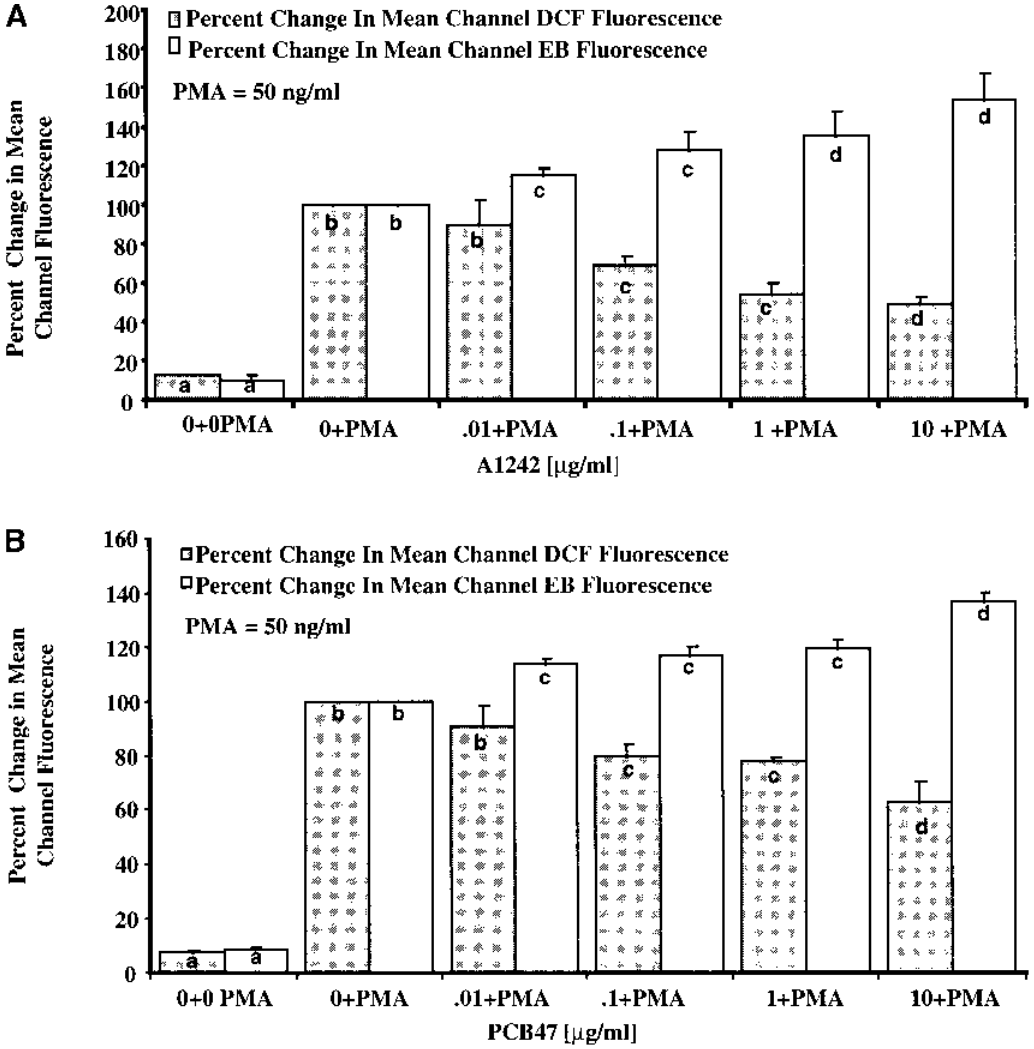

Fig. 3. (A) PMA-induced superoxide and hydrogen peroxide production by human neutrophils pre-incubated with A1242. Human peripheral blood neutrophils (2×10^6/mL) were pre-incubated for 5 min with A1242 followed by a 30-min incubation with PMA (50 ng/mL). Superoxide production and H_2O_2 production were determined as described in Materials and Methods ($n = 3$). [a–d]Significant differences ($P < 0.05$). (B) PMA-induced superoxide and hydrogen peroxide production by human neutrophils exposed to PCB47. Human peripheral blood neutrophils (2×10^6/mL) were pre-incubated for 5 min with PCB47 followed by a 30-min incubation with PMA (50 ng/mL). Superoxide and H_2O_2 production were determined as described in Materials and Methods ($n = 3$). [a–d]Significant differences ($P < 0.05$).

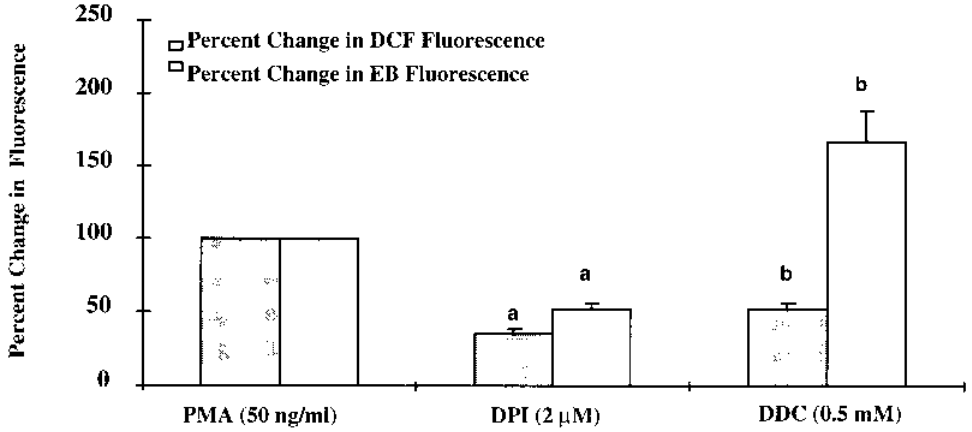

Fig. 4. Intracellular superoxide and hydrogen peroxide production in human neutrophils pre-incubated with DPI (2 μM) and DDC (1 mM) before PMA stimulation. Human peripheral blood neutrophils (2×10^6/mL) were incubated for 30 min in the absence or presence of DPI or DDC at the concentrations indicated. Superoxide and H_2O_2 production were determined as described in Materials and Methods ($n = 3$). [a]Significantly different from the untreated control.

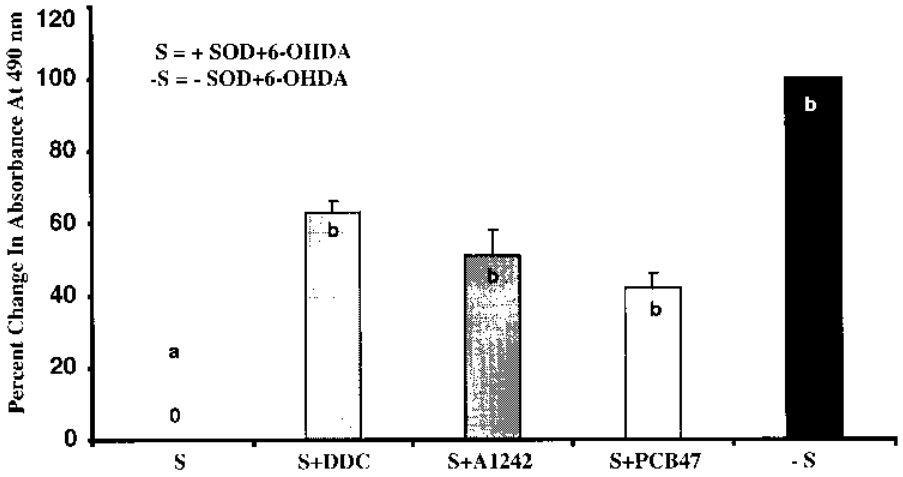

Fig. 5. Inhibition of SOD in vitro by A1242, PCB47, and DDC. Reaction mixtures containing SOD (0.3 U/mL), A1242 (1 µg/mL), PCB47 (1 µg/mL), and DDC (1 mM) were incubated for 30 min at 37°C, and the reaction was started by the addition of 6-OHDA (10^{-5} M final concentration). Absorbance was measured at 490 nm. The control mixtures did not contain A1242, PCB47, DDC, or SOD ($n = 3$). [b]Significantly different from samples with SOD only.

Intracellular oxidation of HE and DCFH in TNF-α-primed, fMLP-stimulated neutrophils pre-incubated with A1242

To assess whether A1242 can impair the oxidative burst response to physiological stimuli, we challenged neutrophils with a combination of TNF-α (10 ng/mL) and fMLP (100 nM) after a 5-min pre-incubation with A1242 (10 µg/mL). To increase the magnitude of response, we primed the neutrophils with TNF-α for 5 min at 37°C before stimulation with fMLP. This resulted in significant and marked increases in O_2^- production at all concentrations of fMLP (data not shown). Pre-incubation with A1242 increased the percent EB fluorescence of fMLP/TNF-α-stimulated neutrophils at least sixfold at 30 min and decreased the percent DCF fluorescence by half (**Fig. 6**). Such results indicate that pre-incubation with A1242 can elicit a response with physiologically relevant agonists, in terms of O_2^- and H_2O_2 production, which is similar to results obtained previously with non-physiological stimulants such as PMA. A fivefold increase was observed in O_2^- production by neutrophils pretreated with PCB47 followed by fMLP/TNF-α stimulation. Hydrogen peroxide production, on the other hand, decreased significantly (50%) in neutrophils pre-incubated with either A1242 or PCB47.

DISCUSSION

To determine whether PCBs induce or alter ROS production in unstimulated and stimulated neutrophils, we evaluated several different activation mechanisms. Initial results demonstrate that treatment of neutrophils with A1242 and PCB47 in vitro in the absence of any additional stimulus is sufficient to induce

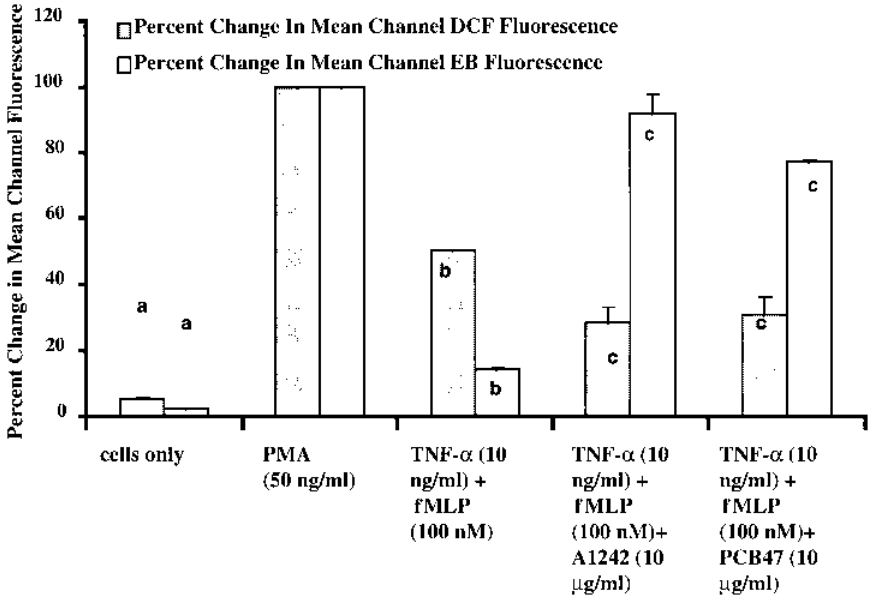

Fig. 6. TNF-α-primed, fMLP-stimulated superoxide and hydrogen peroxide production by neutrophils exposed to A1242 and PCB47. Human peripheral blood neutrophils (2×10^6/mL) were pre-incubated for 5 min with A1242 or PCB47 (10 µg/mL) followed by TNF-α for 5 min before incubation with fMLP (100 nm). Superoxide and H_2O_2 production were determined as described in Materials and Methods ($n = 3$). [a–c]Significant differences ($P < 0.05$).

significant intracellular O_2^- and H_2O_2 production. Cell-free spectrofluorometric studies performed to rule out any interaction between the PCBs at the concentrations employed and the ROS-specific probes indicated no evidence in the alterations of the intensity of fluorescent signals from the probes (data not shown). We compared these effects to those produced by PMA because of the substantially higher levels of ROS released in relation to fMLP, a suboptimal stimulator of O_2^- production by itself [22]. PMA is known to substitute for diacylglycerol, which maximally activates protein kinase C [23–25]. Protein kinase C catalyzes the phosphorylation of specific cytosolic proteins that are translocated to the plasma membrane and interacts with the cytochrome b_{558} component of NADPH oxidase [6].

Pre-incubation of neutrophils with either A1242 or PCB47 before PMA stimulation resulted in a significant increase in O_2^- production, even after maximal stimulation was achieved with a high dose of PMA. The percentage increase in O_2^- production by PMA-stimulated neutrophils pre-incubated with A1242 was larger than that of PMA-stimulated neutrophils pre-incubated with PCB47. This indicates that the PCB mixture is more effective than a single PCB congener in inducing this phenomenon. In contrast, A1242 and PCB47 caused a significant down-regulation of PMA-stimulated H_2O_2 production in a dose-dependent manner.

Spectrofluorometric analysis of the supernatants from the DCFH-DA assays also demonstrated that the extracellular DCF fluorescence from PMA-stimulated neutrophils pre-incubated with the PCB mixture and the congener was significantly less compared with neutrophils stimulated with PMA alone (data not shown). This diminished H_2O_2 production was then hypothesized to be secondary to decreased metabolism of O_2^- via SOD. Before proceeding further, we obtained a clear depiction of intracellular ROS production by inhibiting SOD with DDC before PMA stimulation. DDC has previously been shown to inhibit SOD activity both in vitro and in vivo [26, 27]. For comparison, we also inhibited NADPH oxidase by DPI before PMA stimulation. DPI substantially decreased EB fluorescence, indicating that PMA-induced O_2^- production was inhibited. The decrease in O_2^- generation also resulted in a dramatic decrease in H_2O_2 production, manifested as decreased DCF fluorescence in PMA-stimulated neutrophils. Pre-incubation of neutrophils with DDC before PMA stimulation decreased H_2O_2 production with a concomitant increase in intracellular O_2^- generation. Because DDC is an inhibitor for SOD, the reduction in DCF fluorescence suggests a block in the efficient dismutation of O_2^-. This observation is in accord with earlier findings [28, 29], which demonstrated that SOD inhibition by DDC in isolated rat neutrophils and cerebellar neurons augmented O_2^- generation and inhibited H_2O_2 production. This finding strengthens the notion that PCBs, which can bind to proteins [30], have a definite role in inhibiting the activity of SOD, leading to inefficient dismutation of O_2^-. DDC has also been found to reduce the effect of the hydroperoxide-myeloperoxidase system [28].

One may argue two possibilities against the hypothesis that PCBs inhibited SOD in our study. First, A1242 and PCB47 may diminish the intracellular content of DCFH, attenuating the PMA-induced augmentation of DCF fluorescence. However, this is unlikely; even in the presence of 100 μg/mL A1242 and PCB47, addition of 200 μM H_2O_2 to a cell-free system caused an increase in the intensity of DCF fluorescence to a level similar to that observed in the absence of PCBs, indicating enough cellular content of DCFH trapped intracellularly for further oxidation. Second, PCBs conceivably could alter the viability of neutrophils, allowing greater leakage of fluorescent DCF. However, this was also shown to be unlikely because PMA-stimulated neutrophils pre-incubated with A1242 and PCB47 excluded PI and retained the light scatter properties of viable cells.

To prove our hypothesis that PCBs and DDC have a biochemically similar effect on SOD activity [31, 32], we determined whether PCBs could induce any change in the functional properties of purified bovine copper-zinc SOD (Cu-Zn SOD) similar to DDC inhibition. Cu-Zn SOD, a 32-kDa dimer, is the predominant type of SOD in the cytoplasm of a mature human neutrophil, whereas manganese SOD (Mn SOD, an 89-kDa tetramer) is the major form in the mitochondrial compartment [33]. Quantitative cuvette assays of sonicated leukocyte preparation have shown that about 65% of the total activity of SOD per cell is due to the cytoplasmic form [34]. Pre-incubation with A1242 and PCB47 inhibited the ability of SOD to scavenge O_2^- generated by autoxidation of 6-OHDA, suggesting that dismutation of O_2^- to H_2O_2 was slowed. It has been reported that halides weakly inhibit Cu-Zn SOD activity [35]. Nuclear magnetic resonance studies suggest that halides bind to the water coordination position of the copper ligand sphere, resulting in inhibition of enzyme activity [36].

To adapt the above findings to a physiological setting, neutrophils were pre-incubated with A1242 and PCB47 before stimulation with fMLP and TNF-α. Although fMLP is a weak or suboptimal stimulator of O_2^- production, pre-incubation of neutrophils with recombinant human TNF-α is known to enhance fMLP-induced O_2^- production in a concentration-dependent manner [37–39]. Pre-incubation with A1242 and PCB47 resulted in an approximately sixfold increase in O_2^- generation followed by a near twofold reduction in H_2O_2 production.

These studies indicate that PCBs can decrease O_2^- scavenging by interfering with the activity of SOD. The biological significance of SOD has been demonstrated via biochemical inactivators of SOD activity, for example thiocarbamic acid derivatives such as DDC [26, 31, 32, 40]. Removal of excess O_2^- by SOD is important in vivo not only because of the damaging potential of this oxygen radical per se, but also, and perhaps more importantly, because of its role in forming more reactive species such as hydroxyl radical (·OH) [7, 41]. Superoxide buildup along with significant H_2O_2 production on exposure to PCBs makes an ideal scenario for these ROS to react with each other to form ·OH. Hydroxyl radicals are known to attack DNA, inducing strand breaks, damaging cellular proteins, and initiating lipid peroxidation either directly or via intermediate radicals of longer half-life [42]. Decreased SOD content and activity have been shown to contribute to apoptotic death in neutrophils [16]. Chronic inhibition of SOD has also been demonstrated to produce apoptotic death in neurons [43]. In this regard it should be noted that low concentrations of

A1242 (0.01–1 μg/mL) hastened the onset of apoptosis in neutrophils [unpublished observations]. Whether the reduction in the number of circulating neutrophils in the peripheral blood of individuals exposed to PCBs [2] is attributable to the capacity of PCBs to induce premature apoptosis in neutrophils remains to be determined.

In conclusion, these studies demonstrate decreased SOD activity, a major antioxidant defense mechanism, in neutrophils exposed to PCBs. The reduced number of circulating neutrophils secondary to PCB exposure could explain, at least in part, the decreased immunocompetence caused by PCBs, leading to an increase in individual susceptibility to bacterial and viral infections [44, 45].

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