Attenuation of the pulmonary inflammatory response following butylated hydroxytoluene treatment of cytosolic phospholipase A2 null mice

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cytosolic phospholipase A2 (cPLA2) hydrolyzes arachidonic acid, an intracellular regulator of apoptosis (10), from the sn-2 position of membrane phospholipids (11, 36). Although multiple forms of PLAs have been described, cPLA2 is the major enzyme responsible for arachidonic acid (AA) release and represents the rate-limiting step in eicosanoid production (18). The lysophospholipid product of cPLA2 catalysis is also biologically active and can be converted to platelet-activating factor (PAF) by a specific acetyltransferase (15). cPLA2 catalysis thus produces several different classes of inflammatory lipid mediators. Lung inflammation after LPS and HCl administration was attenuated in cPLA2 null mice (31) as was chemically induced lung tumor formation (30). cPLA2 null mice exhibited reduced ovalbumin-induced anaphylaxis characterized by attenuated thickening of the alveolar lumen and airway hyperresponsiveness (41).

cPLA2 activity is regulated by calcium binding and phosphorylation (11, 12). Compounds that inhibit cPLA2 and secretory PLA2 (sPLA2) activities block chemotaxis of human leukocytes in culture (40). Chemokines are a low-molecular-weight subclass of cytokines that promote leukocyte chemotaxis. Chemokines with a C-C (cysteine-cysteine) peptide weight subclass of cytokines that promote leukocyte chemoaxis in culture (40). Chemokines with a C-C (cysteine-cysteine) peptide structure at their NH2 terminus primarily attract monocytes to sites of injury (26) and include monocyte chemotactic protein-1 (MCP-1/also known as CCL2) and macrophage inflammatory proteins-1α and -2 (MIP-1α and MIP-2). MCP-1 is important in acute respiratory distress syndrome (ARDS) where patients present with injury at the alveolar capillary interface and exudative infiltration of predominately polymorphonuclear (PMN) cells at early stages of the disease (26). ARDS patients who additionally recruit monocytes secrete high levels of MCP-1 into alveolar air spaces and have an especially poor prognosis (34). Mice treated with LPS and MCP-1 in experimental models of ARDS recruit PMNs and monocytes to the lungs (28) and the NFκB and MAPK-2 concentrations in bronchoalveolar lavage (BAL) fluid (29).

Exposing mice to butylated hydroxytoluene (BHT) causes reversible lung injury and inflammation. The model described more than 30 years ago (43) has been used to study molecular mechanisms underlying ARDS and the chronic inflammatory state that characterizes chronic obstructive pulmonary disease and asthma. A single injection of BHT results in alveolar epithelial injury that is repaired by compensatory hyperplasia of type II pneumocytes that in turn differentiate into type I cells lining the alveolar walls (1). As a result of this injury, vascular permeability increases and leukocytes (especially macrophages) are recruited within 6 days after BHT administration (6). These events mimic the key features of ARDS in humans (28, 34). MCP-1, MIP-1α, and/or MIP-2 may mediate this BHT-induced monocyte recruitment, and their production and/or cellular responsiveness to them may be cPLA2 dependent. For example, human bronchioloalveolar carcinoma-derived A549 cells secrete MCP-1 in response to smoke extracts (27) and after exposure to conditioned media obtained from...
LPS-activated macrophages (38), indicating that epithelial cells secrete MCP-1 in response to inflammatory signals. Additionally, inflammation is associated with lung cancer (25), and MCP-1 and MIP-1α levels are elevated in non-small cell lung cancer patients (2). In this study, we used cPLA2 null mice to examine interactions between lipid mediators and chemokines during the inflammation resulting from BHT damage.

MATERIALS AND METHODS

Mice. BALB/cByJ mice, an inbred strain especially sensitive to the inflammatory effects of BHT (5), were obtained from The Jackson Laboratory (Bar Harbor, ME). cPLA2 null mice (8) in a C57BL/6-129/Sv chimeric background and their wild-type littermates were bred in the Center for Laboratory Animal Care at the University of Colorado Health Sciences Center. Mice were fed Harlan Teklad 225 rodent chow (Harlan, Madison, WI), given water ad libitum, and housed on hardwood bedding with a 12-h light/12-h dark cycle in a climate-controlled facility. Because the homozygous null females have a parturition defect, breeding was conducted with heterozygotes (35). Lung inflammation was induced in BALB mice by injection with 200 mg/kg body wt BHT ip (Sigma, St. Louis, MO); controls received Mazola corn oil vehicle. The BHT dose injected into cPLA2 null mice and their wild-type littermates was 165 mg/kg body wt. All procedures were approved by the Animal Care and Use Committee of the University of Colorado Health Sciences Center.

BAL fluid preparation and analysis. BAL cells were collected by low-speed centrifugation from vehicle or BHT-treated mice, as described (6). Briefly, the trachea of an anesthetized mouse was cannulated, and the lungs were lavaged with three instillations of 1 ml each of PBS, pH 7.2, containing 0.6 mM EDTA. Cells were pelleted from the first ml at 2,000 g for 5 min, and the supernatant used to determine total3H-AA uptake. Data are presented as the ratio of sample cpm (AA release) to total3H-AA uptake.

Immunohistochemistry. Lung tissue sections were prepared for immunohistochemistry (IH) as described (7). In brief, lungs were perfused through the pulmonary artery with saline, fixed by inflation with 10% formalin, dehydrated, embedded in paraffin, and cut into 4-μm sections. After rehydration, endogenous endoperoxidase activity was inhibited by incubation with 3% H2O2 in methanol for 15 min, followed by antigen retrieval using warm 100 mM citrate buffer, pH 6.0. A 1:5 dilution of mouse monoclonal cPLA2 antibody (Santa Cruz, Santa Cruz, CA) was used for immunostaining after blocking endogenous mouse immunoglobulins with the Mouse-On-Mouse kit (Vector Laboratories, Burlingame, CA). Samples were treated with biotin-conjugated anti-mouse IgG or anti-goat IgG secondary antibody (Vector) followed by peroxidase-conjugated, streptavidin, tertiary antibody complex (Vector). 3,3-Diaminobenzidine (Sigma, St. Louis, MO) was used as the peroxidase substrate for cPLA2 detection, and hematoxylin (Sigma) was the counterstain.

Results

Inflammatory response following acute BHT injection. Repetitive BHT injections increase the number of BAL macrophages and lymphocytes for at least 45 days, raise BAL protein content, and induce pulmonary COX-1 and COX-2 expression (5). We examined biomarkers of inflammation in BAL fluid at various times after BALB mice, an inbred strain particularly responsive to pulmonary inflammation (6, 21), were administered a single BHT or corn oil vehicle injection. Increased...
Fig. 1. Effect of a single butylated hydroxytoluene (BHT) injection on inflammatory cell recruitment and secretion of total protein and monocyte chemotactic protein (MCP)-1 into bronchoalveolar lavage (BAL) fluid. BALB mice were injected with BHT and the number of macrophages (A), lymphocytes (B), and neutrophils (C) in BAL fluid determined as a function of time (n = 5 mice per day). Mean cell number ± SE per ml of BAL fluid is shown. D: protein concentration in BAL fluid was determined as a measure of transudation. *P < 0.05 vs. day 0 (vehicle-treated mice). E: the concentration of MCP-1 in BAL fluid was determined by enzyme immunoassay (n = 5 mice per day). The limit of detection for MCP-1 was 16 pg/ml. *P < 0.05 vs. all other days.

Fig. 2. Immunohistochemical localization of cytosolic phospholipase A2 (cPLA2) in lungs from mice treated with vehicle or BHT. Lungs removed from control or BHT-treated BALB mice 6 days after injection were fixed, and tissue sections were prepared. cPLA2 immunostained in alveolar and bronchiolar epithelia in both vehicle-treated (A) and BHT-treated (C) mice (arrowhead A, alveolar type II cell; arrowhead M, macrophage; and arrowhead B, bronchiolar Clara cell). B and D are ×5 magnifications of A and C, respectively. Images are representative of several fields on slides containing sections from 2 corn oil-treated and 2 BHT-treated mice. Black bar in A represents 50 μm.
numbers of BAL macrophages were detectable 3 days after BHT treatment and remained elevated for at least 30 days (Fig. 1A). To identify chemokines responsible for recruiting macrophages, BAL titers of MCP-1, MIP-1α, and MIP-2 were assessed. MCP-1 levels transiently increased 2 days following BHT (Fig. 1E), while MIP-1α and MIP-2 did not significantly change after BHT treatment (baseline levels of 4.5 ± 0.15 and 6.5 ± 0.28 pg/ml, respectively; data not shown). Importantly, MCP-1 levels in BAL fluid rose a few days before monocyte infiltration, consistent with an evocative role.

**Effects of BHT administration on pulmonary cPLA2**. Because cPLA2 is the upstream enzyme that provides substrate to COX, whose expression increases following BHT (5), we tested whether cPLA2 expression is also induced by BHT. cPLA2 localizes to the alveolar and bronchiolar epithelia and macrophages in normal lungs (30), and we found that cPLA2 expression following BHT administration increased (Fig. 2). This increase was particularly notable throughout the bronchiolar epithelium, which includes ciliated and nonciliated Clara cells. Accordingly, we quantified cPLA2 in Clara cell primary isolates. cPLA2 expression in Clara cells isolated 3 and 6 days after vehicle or BHT injection increased fourfold, as determined by immunoblotting (Fig. 3A). cPLA2 in whole lung extracts rose only slightly (data not shown), probably reflecting the relatively small contribution of Clara cells to the lung cell population. Because COX expression in whole lung homogenates prepared from BHT-treated mice increases considerably (5), we examined COX-1 and COX-2 in Clara cells isolated from BHT and control mice. In contrast to cPLA2, Clara cell COX-1 (Fig. 3B) and COX-2 (Fig. 3C) contents did not increase after BHT treatment. Other cell types, such as type II cells and macrophages, may account for the increased COX-1 and COX-2 expression detected in whole lung homogenates, since COX-1 and COX-2 are also expressed in these cell types (5, 42). To determine whether the elevated cPLA2 in Clara cells is concomitant with an increased cPLA2 activity, H3-labeled AA release into media of isolated Clara cells was measured. The calcium ionophore, Ionomycin, increases uptake of the calcium necessary for cPLA2 translocation to phospholipid membranes (33). Clara cells from mice treated with BHT secreted more H3-AA than those isolated from control mice (Fig. 4), suggesting that Clara cell cPLA2 pro-
vides some of the lipids that mediate BHT-induced inflammation.

**Effect of cPLA2 ablation on BHT-induced pulmonary inflammation.** Inbred strains of mice vary in their pulmonary responsiveness to BHT (6, 23). The cPLA2 deletion was made in mice on a B6/129 genetic background. BHT-induced lung toxicity, as assessed by the lung weight to body weight ratios (23), was similar in cPLA2 null and wild-type mice, with both groups increasing their lung wt/body wt ratio twofold. To examine the role of cPLA2 in the inflammatory response arising in conjunction with this BHT-induced injury, wild-type and cPLA2 null mice were injected with corn oil vehicle or BHT, and the leukocyte and protein contents in BAL fluid were determined 6 days later. The macrophage titer rose 11-fold in wild-type mice following BHT administration, an increase that was significantly attenuated in cPLA2 null mice (Fig. 5A). BAL neutrophil levels also increased when wild-type 129/B6 chimeric mice were treated with BHT, but not in cPLA2 null mice (Fig. 5B). In contrast, cPLA2 ablation did not affect lymphocyte infiltration (Fig. 5C), indicating a myeloid-specific requirement for cPLA2 function that is not necessary for attracting lymphoid cells. Protein concentration in BAL fluid after BHT treatment increased to similar extents in wild-type and cPLA2 null mice (Fig. 5D).

Wild-type and cPLA2 null mice were treated with vehicle control or BHT, Clara cells were isolated, and effects of cPLA2 genetic ablation on arachidonate release were determined. Clara cell AA release from BHT-treated wild-type B6/129 mice rose 40% (Fig. 6), analogous to that observed in BALB mice (Fig. 4). However, Clara cells from BHT-treated and

![Fig. 5](image-url)  
**Fig. 5.** Inflammatory cells and protein in BAL fluid from control or BHT-treated wild-type (WT) or cPLA2 null mice. Cell counts were performed 6 days after vehicle or BHT treatment, and percentages of each cell type were determined for each mouse [n = 10 corn oil-treated mice, n = 19 BHT-treated WT mice, n = 16 BHT-treated knockout (KO) mice]. The average cell number per ml of BAL fluid per mouse is shown. Macrophage (A), neutrophil (B), and lymphocyte (C) concentrations in lavage fluid. D: protein concentration in lavage fluid (n = 5 corn oil-treated mice, n = 9 BHT-treated WT mice, n = 5 BHT-treated KO mice) (P < 0.05 vs. corn oil groups). In A, B, and D: *P < 0.05 vs. corn oil, #P < 0.05 vs. WT BHT. In C, P < 0.05 vs. all other groups.

![Fig. 6](image-url)  
**Fig. 6.** AA release from Clara cells isolated from WT and cPLA2 null mice after control (CO) or BHT injections. H3-labeled AA release is expressed as a ratio of total cell uptake from Clara cells isolated from vehicle control WT (○) or cPLA2 null (□) mice or BHT-treated WT (●) or cPLA2 null mice (●) over time (n = 3 samples for each).

![Fig. 7](image-url)  
**Fig. 7.** MCP-1 release from Clara cells isolated from WT and cPLA2 null mice after control or BHT injections. MCP-1 concentration was measured by enzyme immunoassay in aliquots of media from cultured Clara cells 3 days after vehicle or BHT injection into WT and cPLA2 null mice. *P < 0.05 vs. other groups; n = 3 for each condition.
control cPLA₂ null mice released significantly less AA. The residual AA release from Clara cells isolated from cPLA₂ null mice may reflect compensation by other phospholipases, such as sPLA₂ and calcium-independent PLA₂ (iPLA₂). The molecular composition of the various PLA₂ enzymes in Clara cells is not known.

The enhanced macrophage infiltration that follows BHT administration is preceded by a concentration rise in a particular chemoattractant, MCP-1, in BAL fluid (Fig. 1E). Cell types that may contribute this chemokine include resident macrophages and epithelial cells, as we have shown in Fig. 2 (3, 4, 27). Similar to what we observed in BALB mice (Fig. 1), a time course of MCP-1 content in lavage fluid from wild-type B6/129 mice revealed a peak 3 days after BHT treatment (data not shown). We quantitated MCP-1 secretion from Clara cells isolated from wild-type and cPLA₂ null mice 3 days after treating mice with corn oil or BHT. Clara cells from BHT-treated wild-type mice secreted fivefold more MCP-1 than wild-type control Clara cells, but cPLA₂ null Clara cells showed no such enhancement (Fig. 7). Thus cPLA₂ mediates MCP-1 secretion from Clara cells isolated from mice undergoing injury-induced inflammation, and this may contribute to BHT-induced macrophage recruitment.

DISCUSSION

We have shown that the number of macrophages recovered in BAL fluid prepared from both BALB and 129/B6 mice dramatically increases after a single BHT treatment and remains elevated for several days. MCP-1 is at least in part responsible for this recruitment, since a rise in BAL MCP-1 levels precedes this recruitment (Fig. 1). cPLA₂ is expressed in alveolar and bronchiolar epithelia and in macrophages of normal and BHT-treated lungs, with the most intense staining in Clara cells (Fig. 2). BHT administration to mice dramatically induced Clara cell cPLA₂ expression. More AA was released from Clara cells following BHT treatment of mice, implying that this induced cPLA₂ is enzymatically active. This is consistent with the impaired ability to recruit monocytes into other organs lacking MCP-1 or its CCR2 receptor due to genetic ablation (9, 19, 20, 22). Clara cell-derived MCP-1 may thus mediate, at least in part, the monocyte recruitment to alveolar spaces in response to BHT. Consistent with our findings, transgenic mice overexpressing human MCP-1 driven by a human surfactant protein C promoter, and thus targeted to peripheral lung epithelia (17), contain more BAL macrophages (14).

cPLA₂ null mice recruited 40% fewer macrophages than their wild-type littermates in response to BHT treatment, and Clara cells isolated from these null mice were deficient in both AA release and MCP-1 secretion. These results suggest that cPLA₂ mediates the MCP-1 secretion that recruits macrophages. C₄-PAF (a stable analog of platelet-activating factor) injected into the pleural cavity of mice stimulated rapid monocyte recruitment, accompanied by increased synthesis of MCP-1 and leukotriene B₄ (37). Although cPLA₂ is not considered to be rate limiting in PAF production (16), the absence of cPLA₂ in knockout mice should inhibit PAF production by decreasing the lysophospholipid concentration available for the acetyltransferase. Decreased PAF may diminish MCP-1 production. PAF is involved in cervical ripening during parturition using human uterine cervical fibroblasts (39), and treatment of these fibroblasts with PAF stimulated the production and release of several cytokines, including MCP-1. Deficient PAF might account for why cPLA₂ homozygous null females are unable to give birth to live progeny (8, 41) and for the decreased MCP-1 secretion and subsequent macrophage recruitment.

Prominent features of acute BHT treatment-induced inflammation include sustained monocyte recruitment preceded by MCP-1 secretion and increased cPLA₂ expression and activity in bronchiolar Clara cells. We hypothesize that cPLA₂ expressed in Clara cells produces lipid mediators, including eicosanoids and/or PAF, which lead to MCP-1 secretion. This stimulates macrophage recruitment, as suggested by the lack of these effects in cPLA₂ knockout mice. cPLA₂ inhibitors might be beneficial in treating inflammatory lung diseases in which increased macrophages are associated with a poorer prognosis, such as ARDS (34) and lung cancer (2).

REFERENCES


