



Early View

Original article

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Allergen inhalation generates pro-inflammatory oxidized phosphatidylcholine associated with airway dysfunction

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Abstract:

Rationale: Oxidized phosphatidylcholines (OxPC) are produced under conditions of elevated oxidative stress and can contribute to human disease pathobiology. However, their role in allergic asthma is unexplored.

Objectives: Characterize the OxPC profile in the airways after allergen challenge of people with airway hyperresponsiveness (AHR) or mild-asthma. Determine the capacity of OxPC to contribute to pathobiology associated with asthma.

Methods: Using bronchoalveolar lavage (BAL) from two human cohorts, OxPC species were quantified using ultra high performance liquid chromatography tandem mass spectrometry. Murine thin cut lung slices (TCLS) were used to measure airway narrowing caused by OxPCs. Human airway smooth muscle (HASM) cells were exposed to OxPCs to assess concentration-associated changes in inflammatory phenotype and activation of signaling networks.

Measurements and Main Results: OxPC profiles were different in the airways of people with or without AHR and correlated with methacholine responsiveness. Exposure of mild-asthmatics to allergens produced unique OxPC signatures that were associated with the late asthma response severity. OxPCs dose-dependently induced 15% airway narrowing in murine TCLS. In HASM, OxPCs dose-dependently increased the biosynthesis of cyclooxygenase-2, IL-6, IL-8, GM-CSF, and the production of oxylipins via protein kinase C-dependent pathways.

Conclusions: Data from human cohorts and primary HASM culture we show that OxPCs are present in the airways, increase after allergen challenge, and correlate with metrics of a dysfunction. Furthermore, OxPCs may contribute to asthma pathobiology by promoting

airway narrowing and inducing a pro-inflammatory phenotype and contraction of airway smooth muscle. OxPCs represent a potential novel target for treating oxidative stress-associated pathobiology in asthma.

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Abbreviations: AHR – Airway Hyperresponsiveness, COX – Cyclooxygenase, DiHETE - Dihydroxyeicosatetraenoic acid, GAPDH - Glyceraldehyde 3-phosphate dehydrogenase, HASM – Human airway smooth muscle, HDiA-PC - 4-hydroxy-7-carboxyhex-5-enoic acid ester of 1-stearoyl-2-linoleoyl-sn-glycero-phosphocholine, HETE - Hydroxyeicosatetraenoic acid, KODA-PPC - 1-palmitoyl-2-(9,12-dioxododec-10-enoyl)-sn-glycero-3-phosphocholine, LTB4 – Leukotriene B4, OxPC – Oxidized phosphatidylcholine, OxPAPC – Oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine, PAPC-keto – 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine ketone, PGPC - 1-palmitoyl-2-glutaryl-sn-glycero-3-phosphocholine, PLPC-OH - 1-Palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine hydroxide, PLPC-OOH,OH - 1-Palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine hydroxy aldehyde, PLS-DA – partial least squares discriminant analysis, PSPC - 1-palmitoyl-2-stearoyl-sn-glycero-3-phosphocholine, ROS – reactive oxygen species, SGPC - 1-stearoyl-2-glutaroyl-sn-glycero-3-phosphocholine, sPLS-DA – sparse PLS-DA, TCLS – thin cut lung slice, UBC – Ubiquitin C, YWHAZ - Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta.

Introduction

Oxidative stress is a major feature of asthma pathophysiology, but is not targeted by current treatments. Chronic inflammation involving neutrophils, eosinophils and alveolar macrophages, and exposure to environmental pollutants, allergens and oxidants in cigarette smoke contribute to the production of reactive oxygen species (ROS) that can overwhelm the body's antioxidant mechanisms (1). ROS such as superoxide and hydrogen peroxide, and other biomarkers of oxidative stress (eg. 8-isoprostane, exhaled nitric oxide, and lipid peroxides), are associated with severity of asthma and airway hyperresponsiveness (AHR), airway mucus secretion and inflammation, emergency department visits, and corticosteroid insensitivity (2–6). People with severe asthma and COPD have decreased plasma levels of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase, and reduced plasma glycine and glutamine that is needed for the synthesis of the antioxidant, glutathione (7). Despite the evidence that ROS play a key role in asthma severity, we have no effective therapies that directly target oxidative stress in asthma, thus ROS-associated pathobiology is a persistent threat to disease management.

ROS oxidize and damage DNA, as well as modifying proteins and lipids leading to bio-activation of otherwise inert of homeostatic molecules (8). The airway lining is particularly susceptible to the effects of ROS due to its large surface area and contact with the environmental pollutants and atmospheric oxygen. ROS generated in the airways can oxidize the phospholipid-rich lining of the airways, including pulmonary surfactant (9), resulting in the production of bioactive oxidized phospholipids. Phosphatidylcholine, the most abundant phospholipid in mammals (10), can undergo oxidation of the sn-2 polyunsaturated fatty acid chain, with subsequent fragmentation or cyclization to generate

hundreds of different oxidized phosphatidylcholines (OxPC) (9). OxPCs alter biological membrane structure and function, and activate signaling pathways that exacerbate and drive oxidative stress and persistent inflammation that promotes ageing and disease pathogenesis, for example atherosclerosis and renal ischemia reperfusion injury (11–14). OxPCs are also causally linked to acute lung injury and acute respiratory distress syndrome, and regulate antimicrobial mechanisms in sepsis (13, 15). Despite the potential for OxPCs to contribute to inflammatory disease, there is little information about whether they are present and contribute to asthma pathobiology. Therefore, in this study, using human cohort data and human cell culture models, we reveal that OxPCs are present in airways, correlate with measures of airway hyperresponsiveness (AHR) and lung dysfunction after allergen challenge, induce a pro-inflammatory phenotype in human airway smooth muscle, and promote airway constriction in murine lung slices.

Materials and Methods

Detailed methods can be found in the online supplement.

Human experiments. Taking advantage of existing samples from two established human cohorts available through the Canadian Respiratory Research Network, we are able to explore changes in the OxPC profile of the lungs associated with AHR or allergen exposure. We set out to identify the OxPC profile in subjects with or without AHR and after allergen challenge in subjects with asthma. The DE3 cohort (Diesel Exhaust 3, Vancouver, BC) is comprised of subjects with (n=8) and without (n=5) AHR to methacholine (16). These are otherwise healthy individuals with no smoking history, and are without a diagnosis of airways disease, thus none of these subjects were on inhaled corticosteroid therapy (Table 1). Following characterization of methacholine responsiveness, human bronchoalveolar lavage (BAL) samples were collected from the 13 participants by bronchoscopy (16). The DC cohort (Hamilton, Ontario) is a second study group comprised of mild-asthmatic, non-smoking subjects (n=10) not on inhaled corticosteroids or long acting β_2 -agonists (17) (Table 2). BAL samples were collected 24-hours after allergen or diluent challenge, then were filtered (40 μ m mesh filter), and cell-free supernatants were stored at -80°C for analysis of oxidized phospholipids (18). Together, these cohorts had unique features that enabled us to address critical issues, including, the capacity to: assess OxPCs in BALF from atopic asthmatics in direct response to allergen challenge (DC cohort); test for an association between BALF OxPCs and clinically validated AHR (DC and DE3 cohorts); and, discriminate whether BALF OxPCs are associated with airway dysfunction, independent of asthma diagnosis (DE3 cohort).

Oxidized phospholipid quantification. OxPC content in BAL from humans was extracted using the “Folch Method”, then identified and quantified by ultra-high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) using multiple reaction monitoring standards as previously described (14).

Cell culture. Primary human airway smooth muscle (HASM) cells (passage 4-6) isolated from lung tissue resections were grown to confluence, serum starved for 7-days in the presence of insulin-transferrin-selenium to induce a phenotype that partially resembles HASM cells *in vivo* (19–21). For experiments, cultures were exposed to increasing concentrations of oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (OxPAPC) for 24 hours unless otherwise indicated.

Lipid Peroxidation. 25mg of 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (PAPC) and 1-palmitoyl-2-stearoyl-sn-glycero-3-phosphocholine (PSPC), were oxidized in room air for 4-days and then re-suspended at a concentration of 1mg/mL in chloroform:methanol (2:1). A small sample of OxPAPC was tested for degree of oxidation using HPLC/electrospray ionization tandem mass spectrometry system as previously described (14). This OxPAPC represents a mixture of OxPC species that can be used to recapitulate the OxPC profile seen in human samples.

Cytokine analysis. Cytokine analysis on cell culture supernatants was performed using multiplex arrays from Eve Technologies (Alberta, Canada) and Mesoscale Discovery Platform (Maryland, USA).

qPCR. Cyclooxygenase-2 (COX2) mRNA abundance was quantified using qPCR on RNA from or HASM cells. Housekeeping genes used were YWHAZ, UBC, and GAPDH.

Western Blotting. 10µg of total protein isolated from HASM cells were run on a 10% gel, blotted onto nitrocellulose, and probed with COX2 and COX1 antibodies.

Oxylipin quantification. Cell supernatant, collected in phenol-free DMEM media was analyzed using HPLC-MS/MS as previously described (22).

Kinome Array. Cell lysates collected from cells exposed to OxPC 80µg/mL for 1, 3, or 6-hours were collected in phosphoarray lysis buffer and run on a peptide array as previously described (23) to quantify the OxPC induced cellular kinome. Specifically, lysates were run on human kinome peptide arrays (JPT Technologies, Berlin, Germany) which are comprised of 308 unique kinase recognition sites. Results were visualized using PRO-Q Diamond Phosphoprotein stain and subsequently scanned on a PowerScanner (Tecan, Morrisville, NC, USA) microarray scanner with a 580nm filter to detect fluorescence. Signal intensity values were collected with Array-Pro Analyzer version 6.3 software (Media Cybernetics, Rockville, MD, USA).

Thin Cut Lung Slice (TCLS) studies. Lungs from 8-10 week old female BALB/c mice were inflated with low-melting point agarose, and after cooling, cut into ~180µm thick sections as we previously described (24). TCLSs were mounted in a perfusion chamber on an inverted microscope and exposed to increasing concentrations of OxPAPC at room temperature. Video images were captured for 3 minutes, and acute airway narrowing calculated as a percent decrease from the initial lumen volume. Exposure to PSPC or methacholine was used as negative and positive controls, respectively.

Statistics. We used a sparse partial least squares discriminant analysis (sPLS-DA, mixOmics 6.10.4) to identify OxPCs important for distinguishing subjects with AHR from those without AHR or allergen response from diluent response. The optimal number of OxPCs required to

discriminate each group was determined through an iterative tuning process in the sPLS-DA algorithm. OxPCs selected from the sPLS-DA analysis were then correlated to the clinical parameters of each subject using a Spearman's correlation matrix in R (corrplot version 0.8.4). Spearman's correlation was used because the data did not fit a normal distribution. TCLS data was fit with a non-linear dose response curve (Graphpad Prism 8) and EC₅₀ calculated from the curve. Maximal airway narrowing induced by OxPC 80 µg/mL was compared, by an unpaired t-test, to the narrowing induced by PSPC at the equivalent concentration. All other data was analyzed using a one-way ANOVA with a Dunnet's post-hoc comparison. Kinome differential phosphorylation results were calculated using the Platform for Integrated, Intelligent Kinome Analysis 2 software (25). Significantly upregulated phospho-signals were analyzed using Kinase Enrichment Analysis (KEA) (26), to identify underlying activated kinases in the kinome data. Significant threshold set at $p < 0.05$. Experiments were completed in at least triplicate. Data analysis was done in R (version 3.5.1) and graphs made using ggplot2 package (version 3.1.1) or superheat (version 0.1.0).

Results

OxPCs in the DE3 cohort. PLS-DA analysis was able to cluster individuals with or without AHR separately based on their OxPC profiles (Figure 1A). Using sPLS-DA, we were able to identify the most significant discriminative OxPCs of each group (Figure 1B). There were eight OxPCs that discriminated the AHR group and five that discriminated the no-AHR group. PGPC and KODA-PPC were the most important discriminators of the two groups (highest loading weights, Figure 1B). PGPC had the highest mean value in the group without AHR and KODA-PPC had the highest mean value in the group with AHR. There was no correlation between sex and weight with any of the eight OxPCs that discriminated groups. There was a significant negative correlation between subject age and the abundance of PLPC-OH (Figure S1). To identify the relationships between OxPCs identified by PLS-DA and the degree of AHR, we performed a correlation analysis. For the full cohort, the slope of the methacholine dose response curve (dose-response slope) was positively correlated with HDiA-PC ($R=0.63$) and KODA-PPC ($R=0.64$) abundance, and negatively correlated with PGPC abundance ($R=-0.87$) ($p<0.05$, Figure 1C-E). Furthermore, methacholine PC_{20} negatively correlated with SGPC abundance ($R=-0.6$, $p<0.05$, Figure 1F) and positively correlated with PGPC abundance (Figure S1). Finally, FEV_1 positively correlated with PGPC abundance and FEV_1/FVC ratio positively correlated with PGPC and PLPC-keto abundance (Figure S1). A correlation matrix for all clinical measures listed in Table 1 is shown in Figure S1.

OxPCs in the DC cohort. PLS-DA analysis segregated and clustered response to allergen challenge from diluent challenge in individuals based on their OxPC profile (Figure 2A). Using sPLS-DA analysis, we identified seven OxPCs in BAL that were associated with the allergen challenge. Seven different BAL OxPCs emerged as the most significant discriminating

components for each group (Figure 2B). KDiA-PC was the most important OxPC for separating the groups, and was most abundant in the BAL post allergen challenge. The discriminating OxPCs significantly correlated with the severity of the late asthma response (percent fall in FEV₁). Specifically, PAPC-keto (R=0.69), PLPC-OH (R=0.66), and PLPC-OOH,OH (R=0.78) are all positively correlated with the percent fall in FEV₁ during the late asthma response (p<0.05, Figure 2C-E). There was no clear association between the type of allergen used for inhaled challenge and the abundance of specific OxPCs. Furthermore, there was no association between sex or age and any of the selected OxPCs. A correlation matrix for all clinical measures listed in Table 2 is shown in Figure S2.

OxPAPC Effects on inflammatory cytokine production by HASM. To determine the effect of OxPAPC, a family of OxPCs that includes many identified in Figures 1 & 2, we exposed HASM cells to OxPAPC over a range of concentrations that included levels we detected in BAL. This challenge induced secretion of IL-6, IL-8, and GM-CSF (Figure 3 A-C). Specifically, OxPAPC 160 µg/mL significantly increased IL-6 (349.9 ± 200.3 pg/mL), IL-8 (107.2 ± 95.8 pg/mL) and GM-CSF (67.3 ± 52.2 pg/mL) abundance in cell culture media compared to the response to a non-oxidizable control phospholipid, PSPC (p<0.05). OxPAPC (80 µg/mL) also induced a significant accumulation of secreted IL-8 (104.8 ± 61.5 pg/mL). Conversely, we detected no change in the abundance of IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12(p70), IL-13, MCP-1, or TNF-α in response to any concentration of OxPAPC.

OxPAPC effects on COX2 and oxylin production by HASM. Exposure of cells to OxPAPC resulted in a dose-dependent increase in COX2 mRNA abundance compared to PSPC exposed HASM (Figure 3D). Specifically, there was a significant elevation in COX2 mRNA abundance 24-hours after exposure to 40 µg/mL (10.5 ± 7.5 fold increase), 80 µg/mL (23.7 ±

11.0 fold increase), and 160 $\mu\text{g}/\text{mL}$ (102.1 ± 82.5 fold increase) of OxPAPC ($p < 0.05$). COX2 mRNA abundance increased to a peak 1-hour after OxPAPC exposure and remained elevated at least 24-hours later (Figure 3E). Induction of COX2 was confirmed by immunoblotting, showing a significant accumulation of COX2 protein at OxPAPC 160 $\mu\text{g}/\text{mL}$ compared to PSPC and no treatment (Figure 3F). Exposure to OxPAPC 80 $\mu\text{g}/\text{mL}$ also resulted in accumulation of COX2 protein, but it was necessary to employ more sensitive chemiluminescence reagent to reveal this response. There was no change in COX1 protein abundance in response to OxPAPC. Concomitantly, a significant dose-dependent accumulation of oxylipins, including prostaglandins and leukotrienes, was measured in response to 24-hours exposure to OxPAPC (Figure 4A). In total, we detected 72 different oxylipin species, and 32 were significantly increased by exposure to OxPAPC ($p < 0.05$, Figure 4A). A full table of all detected oxylipins is included in Table S2 of the online supplement. Figure 4 also shows the four oxylipins with the steepest dose response relationship with different concentrations of OxPAPC challenge. These included: 6-trans leukotriene B4 (6-trans LTB4, Figure 4B), 12(S)-LTB4 (Figure 4C), 5,15-Dihydroxyeicosatetraenoic acid (5,15 DiHETE, Figure 4D), and 12-Hydroxyeicosatetraenoic acid (12-HETE, Figure 4E).

OxPAPC induces airway narrowing. We show that OxPCs associate with AHR in human subjects, thus, we tested whether OxPAPC were sufficient to induce airway narrowing in murine TCLS. We found dose-dependent airway narrowing to OxPAPC, with an EC_{50} of 29.5 $\mu\text{g}/\text{mL}$ and a maximum closure of $15.4\% \pm 2.5\%$ (Figure 5). This was significantly different from PSPC, which failed to induce any airway narrowing. As a positive control we also measured methacholine dose-response curves, which revealed an EC_{50} of 250nM, and indicated that maximum airway narrowing achieved by OxPAPC was equivalent to that induced by 34.4nM of methacholine.

OxPAPC signaling networks in HASM. In an attempt to decipher the receptors that may mediate the HASM response to OxPAPC, we screened a number of small molecular inhibitors, based on published predicted targets for OxPCs (27). Figure 6A shows that inhibitors of the prostaglandin receptor EP1, EP2, TLR2/4, platelet-activating factor receptor, or the macrophage scavenger receptor, CD36, had no impact on OxPAPC induced COX2 mRNA accumulation. To gain insight about the signaling networks that are activated in HASM in response to OxPAPC exposure, we performed a kinome analysis 1, 3, and 6 hours after treatment with OxPAPC (Table 3). As 80 µg/mL OxPAPC falls within predicted physiological range of OxPCs in BALF after allergen challenge, we chose this concentration for kinomic and inhibitor studies. The phospho-signature that emerged revealed that the activity of 15 kinases was enriched after 1 hour exposure ($p < 0.05$), and the activity of 20 kinases was enriched after 3 and 6 hours OxPAPC exposure ($p < 0.05$). Only two kinases activated at one hour remained so through 6 hours: pyruvate dehydrogenase kinase 1 (PDK1) and protein kinase C delta (PRKCD).

To validate the role of these predictions in the biological response to OxPAPC, we assessed the effect of PKC inhibition using GF-109203X. We targeted PKC because it is a confirmed signaling hub for OxPAPC effects on human endothelial cells (28). As a negative control we assessed the effects Y-27632 to inhibit ROCK1 and ROCK2, which were not identified by our kinase enrichment array. PKC inhibition, but not ROCK1/2 inhibition, significantly decreased OxPAPC-induced COX2 mRNA accumulation by $64.8 \pm 16.3\%$ ($p < 0.05$) (Figure 6B). Moreover, PKC inhibition decreased OxPAPC-induced IL-6, IL-8 and GM-CSF by $87.5 \pm 3.8\%$, $97.0 \pm 1.9\%$ and $98.6 \pm 0.2\%$, respectively ($p < 0.05$, Figure 6C-E). To assess the potential confounding effects of COX2 metabolites to induce inflammatory cytokines biogenesis, we inhibited either COX2 activity using indomethacin, or prostaglandin signaling via E1/2 receptors using

AH-6089. Indomethacin selectively prevented OxPAPC-induced GM-CSF production by $81.8 \pm 9.0\%$. Similarly, inhibition of prostaglandin receptors signaling with AH-6089 selectively reduced OxPAPC-induced GM-CSF production by $79.5 \pm 2.7\%$. Indomethacin and AH-6089 were without effect on OxPAPC-induced IL-6 or IL-8 release.

Discussion

Combining data from human cohorts, murine TCLS, and primary cultured airway cells, we reveal the presence of oxidized phospholipids in the airways of people with asthma and AHR, and their role in promoting airway narrowing and to induce a pro-inflammatory phenotype in human airway smooth muscle cells. Inhalation challenge by clinically relevant aeroallergens in humans results in the accumulation of a profile of OxPCs. Several OxPC variants associate with key features of asthma and AHR, including methacholine dose response slope, PC20, and the severity of fall in FEV₁ during the late asthmatic response to inhaled allergen. These data, combined with the ability of OxPAPCs to narrow murine airways *ex vivo*, suggest an interaction between OxPCs and airway cells such as airway smooth muscle, resulting in greater airway narrowing and responsiveness. For this reason, we validated a potential role for OxPCs in asthma pathobiology using HASM. We found that OxPCs promote the expression of COX2, and the release of pro-inflammatory mediators, including IL-6, IL-8, GM-CSF and a panel of oxylipins replete with prostaglandins, leukotrienes, and epoxyeicosatrienoic acids. Using a novel kinome array approach, we uncover a complex signaling response to OxPCs wherein diverse kinases are temporally induced, suggesting sequential signaling activation to orchestrate broad biological responses. We reveal sustained activation of protein kinase C, and using a selective inhibitor validate its role in OxPC-induced inflammatory mediator release by HASM cells. Collectively, our study identifies OxPCs as new mediators of allergic asthma pathobiology and pathophysiology, and provides direction for translational and clinical research of their role in existing needs in asthma management.

We demonstrate that inhaled allergen challenge of atopic asthmatics induces airway OxPC accumulation that correlates with clinically relevant early and delayed airway constriction. This suggests that OxPCs can contribute directly to a bronchoconstrictor response, a scenario we validated with murine TCLS preparations, using OxPAPC at a concentration equivalent that in BALF after allergen challenge to induce 15% airway narrowing. Poiseuille's equation predicts this could increase airflow resistance by as much as 40%, and thus, account for the 20% drop in FEV1 observed in subjects after allergen challenge. OxPC accumulation in the airways is consistent with the compromised antioxidant systems in the lungs of asthmatics (7), and that oxidative stress markers (29), including serum malondialdehyde - a product of OxPC formation (30) - are elevated in mild-moderate asthma (31). The asthmatic subjects we investigated were not treated with ICS prior to sample collection. In any case, salmeterol and fluticasone reportedly do not reduce serum malondialdehyde - and presumably OxPC formation - to levels found in healthy controls (31). A novel aspect of our work is that we investigated non-asthmatic individuals with AHR (DE3 cohort), and revealed that OxPCs were elevated and correlate with airway dysfunction compared to non-AHR subjects. This indicates that increased levels of OxPCs may be a pre-disposing factor for airway dysfunction, even in subjects without a clinical diagnosis of asthma. The effects of OxPCs are complex, as low concentrations can enhance endothelial barrier function (12, 32) and have anti-inflammatory effects (15, 33). Thus, low-abundance OxPAPC in the airways may have natural homeostatic benefit, but an elevation in OxPAPC levels, as we report here, is sufficient to promote airway hyperreactivity in asthmatic and non-asthmatic individuals.

OxPCs are associated with multiple pathogenic pathways linked to chronic human diseases. OxPAPC promotes atherosclerotic lesion development and endothelial dysfunction (34–40).

They are also exacerbating factors in acute lung injury (13, 41), renal ischemia-reperfusion injury (12), and acute or chronic inflammatory pain (42). Cigarette smoke exposure produces lung OxPAPCs that impair macrophage phagocytosis and bacterial clearance (43). A general role for OxPCs in pathobiology likely lies in the formation of oxidation specific epitopes that are recognized by soluble and cell associated pathogen recognition receptors, such as TLR2/4/6, CD36, C-reactive protein, and the complement system (44). Although OxPAPC can interact with G-protein coupled receptors (GPCRs), including prostaglandin receptors and platelet-activating factor receptor (45, 46), using selective inhibitors we were unable to identify a specific surface receptor that mediates effects in HASM. This suggests a complex interface that required broad profiling of OxPAPC cell signaling cascades.

Using kinome array we identified a temporally dynamic panel of OxPAPC-induced kinases in HASM cells. This could reflect interactions with multiple receptors and direct effects on cell membranes (47). OxPAPC had disparate activation effects on kinases, some rapidly, but only transiently induced, and others only active six hours after exposure. This suggests that feed forward signaling stemming from an initial response, but deciphering pathway integration is not possible based on our current study design. Our primary objective here was instead, to validate the biological relevance of kinome array findings. For this purpose we targeted PKC in subsequent studies for three reasons. First, PKC was activated rapidly and remained active for up to six hours. Second, Birukov et al. (28) and others (48) show that PKC is an important signaling hub for OxPAPCs in endothelial cells. Third, OxPC's may disrupt lipid rafts that localize GPCRs and Gαq/11 subunits that are directly upstream of PKC (49). Future work is needed to unravel the role of individual OxPC subtypes, and the receptor or non-receptor mediated processes that they trigger. Based on our findings here, this could include the exploration of other kinases that are associated with asthma pathobiology,

including Tyrosine Kinase 2 and Janus Kinase 1 (50), which was activated rapidly, and Pyruvate Dehydrogenase Kinase 1 (51), which underwent sustained activation after OxPAPC exposure.

OxPAPC exerted a strong, but somewhat selective, pro-inflammatory response in HASM cells. This included the release of GM-CSF, IL-6 and IL-8, which are associated with eosinophilic and neutrophilic airways inflammation in allergic asthma (52–54), with IL-8 and GM-CSF also being associated with severe asthma (55, 56). This suggests OxPCs could be linked to severe, steroid resistant asthma, a point that should be addressed in future. We further showed that PKC activation is necessary for OxPAPC induction of IL-6, -8 and GM-CSF. This confirms the biological significance of our kinome assay, and is consistent with multiple reports that PKC regulates cytokine transcription and secretion in smooth muscle and other airway cells (57–60). The 13-plex panel used in this study includes the relatively narrow array of asthma-associated cytokines produced by HASM cells, but a wider profile of mediators from *in vivo* asthma models is needed to fully establish how OxPCs may orchestrate airway inflammation and immunity.

Our experiments with HASM showed that OxPAPCs, even at relatively low concentrations, induce oxylipin biosynthesis, including products that require COX2 activity. On this basis we specifically investigated the effects on COX2, and found that biologically relevant concentrations of OxPAPC were sufficient to increase COX2 mRNA and protein, though immunoblotting was not sensitive enough to detect changes induced by concentrations below 80µg/mL. OxPAPC exposure also increased the abundance of the 5-lipoxygenase product, LTB₄, which promotes inflammation and the late asthma response, and is elevated in severe asthma (61–64). Furthermore, OxPAPC induced the production of 15/12-

lipoxygenase products, 5,15-diHETE and 12-HETE, which have less well defined roles in asthma but have been implicated in modulating inflammation, with chemoattractant roles for eosinophils (65, 66). Future research should focus on how OxPCs promote the production of these potent lipid mediators and their role in inflammation and AHR.

Highlighted in Figure 7, we have uncovered broad effects of OxPAPC on HASM, suggesting that processes for PKC activation are linked to lipid and protein mediator biosynthesis.

Novel mechanisms for OxPC effects are likely, since even though COX2 and oxylipins are induced, pro-inflammatory mediators such as IL-1 β or TNF that are typically associated with COX2 (67, 68) are not. We show that OxPC activation of PKC is needed to induce COX2, and this is consistent with responses to other stimuli (69, 70). We also show that inhibiting COX2 activity or E1/E2 receptor-mediated response to COX2 metabolites blocks GM-CSF, but not IL-6 or IL-8 biosynthesis, whereas PKC inhibition effectively prevents release of all three cytokines. This diversity in pathway activation and integration is also evident in the kinome response to OxPC that we tracked in HASM. It appears that OxPAPC-induced oxylipins may potentiate cytokine release in a paracrine fashion, with the potential for positive feedback pathways to perpetuate COX2 expression (71). Notably, we could not identify a receptor that directly mediates OxPC-induced COX2. Thus, feed forward signaling may be critical, resulting from disruption of cell membranes, the creation of oxidation specific epitopes, and formation of lipid and peptide products, and cellular debris that can activate various receptors. Identifying these secondary products and their receptors are important future areas for study. In this regard, the requirement of PKC activity for perpetuating the effects of OxPCs suggests that Gq/11- and Gi-coupled GPCR agonists such as prostaglandins, prostacyclins, and thromboxane, and 5-lipoxygenase products (eg. LTB₄), may be candidates. Coincidentally, many Gq/11 GPCRs for 5-lipoxygenase and COX2 products, and receptors for

some cytokines, including GM-CSF, mediate MAPK activity, which is linked to sustained COX2 activation (72–74). Collectively, our findings create need for new research to identify receptor and non-receptor processes effected by OxPCs in HASM, other lung structural cells, and immune cells in the context of allergic asthma and AHR.

Our study design leaves some limitations on the interpretation of our data. Surprisingly we found no significant change in in the total OxPC abundance in our human samples, even after allergen challenge. The natural biologic variability of individual human subjects may explain this, but it is also possible that the OxPC profile is the critical determinant of biological significant of OxPC production. This creates need for more precise analysis of associations between clinical traits and the abundance of individual OxPCs in larger cohorts. Our study was not designed to directly compare broad differences in total OxPC abundance between subjects; rather our goal was to highlight the presences of OxPCs in the human lung and to profile the association of differences in AHR and response to allergen challenge on the profile of OxPC species. For this reason, the subjects in the two cohorts we studied do not have severe airways disease and the subjects with diagnosed asthma (DC cohort), were well-controlled at the time of sample collection. To more effectively determine the influence of asthma severity, asthma treatment, environmental exposures, and sex on OxPC profile and abundance, an investigation of sufficiently-powered cohorts is needed.

Nonetheless, our current work is the first that reveals an association, and testable biological mechanisms, for oxidized phospholipids in pathophysiology of chronic airways disease.

In summary, we show for the first time that airway OxPCs are present in the airways and correlate with lung dysfunction in individuals with AHR and in subjects with mild asthma after allergen challenge. Our data using cultured HASM indicates that airway structural cells

exhibit a coordinated secretory response involving lipid mediators, cytokines and chemokines. This discovery opens new avenues to consider exploring the role of bioactive molecules linked with oxidative stress in airways disease, and suggests that OxPCs could represent one mechanism for persistent inflammation that requires new options for asthma management.

Table 1: Patient characteristics for DC cohort. AHR – Airway hyperresponsiveness. Data presented as mean (SD).

	DC Cohort (Hamilton, Ontario)
	Mild-Asthma
Age (years)	34.0 (15.5)
Sex (% Male)	70
FEV₁ Pre-challenge (% Predicted)	92.4 (9.3)
Methacholine PC₂₀ (mg)	5.5 (3.8)
Late Asthma Response (% Fall FEV₁ Allergen-Diluent)	20.1 (10.1)

Table 2: Patient characteristics DE3 cohort. AHR – Airway Hyperresponsiveness. Data presented as mean (SD). All patients were never smokers. Chi-square test performed on categorical data, t-test performed on continuous data.

	DE3 Cohort (Vancouver, British Columbia)		
	No AHR	AHR	p-value
Age (years)	35.4 (10.6)	29.0 (6.7)	0.2
Sex (% Male)	40	50	0.6
FEV₁ Pre-challenge (% Predicted)	107.4 (8.2)	101.0 (10.2)	0.5
FEV₁/FVC Pre-challenge	0.85 (0.038)	0.78 (0.054)	<0.05
Methacholine PC₂₀ (mg)	>128	3.3 (2.6)	<0.05
Dose Response Slope	1.07 (2.00)	21.8 (27.0)	<0.05
Weight (kg)	67.6 (17.7)	71.4 (11.2)	0.7
Height (cm)	165.2 (14.8)	173.6 (7.4)	0.2
BMI	24.3 (2.0)	23.6 (2.3)	0.6
Inhaled Corticosteroids (%)	0	0	NA

Table 3: Kinase enrichment results following 1, 3, or 6 hours OxPAPC exposure in HASM.

Bolded kinases are common across all three time points.

<u>1hr Exposure</u>		<u>3hr Exposure</u>		<u>6hr Exposure</u>	
<u>Protein</u>	<u>p-value</u>	<u>Protein</u>	<u>p-value</u>	<u>Protein</u>	<u>p-value</u>
TYK2	0.0006	MAP2K2	0.00004	MAP3K7	0.0001
JAK1	0.002	MAP3K1	0.0001	MAPK1	0.0001
RPS6KA5	0.005	IRAK1	0.0005	EGFR	0.0005
LCK	0.006	MAP2K1	0.0005	MAP3K11	0.0007
DYRK2	0.01	MAP3K2	0.0005	MAPK3	0.001
<u>PDK1</u>	<u>0.01</u>	MAP3K8	0.0009	MAP3K4	0.003
SYK	0.02	MAPK3	0.001	BRAF	0.004
INSR	0.02	EGFR	0.01	MAP3K8	0.004
IRAK1	0.02	<u>PRKCD</u>	<u>0.01</u>	MAP2K7	0.005
ABL2	0.03	AURKA	0.01	TYK2	0.005
CAMKK1	0.03	DYRK2	0.02	MAP2K2	0.007
PTK2B	0.04	LCK	0.02	JAK2	0.01
MAP3K14	0.04	ZAP70	0.02	PDPK1	0.01
<u>PRKCD</u>	<u>0.04</u>	FGFR2	0.02	<u>PDK1</u>	<u>0.02</u>
HIPK2	0.04	PDPK1	0.02	CDK4	0.02

<u>PDK1</u>	<u>0.03</u>	MAP3K1	0.02
TXK	0.03	<u>PRKCD</u>	<u>0.02</u>
ABL2	0.04	PRKG2	0.02
SYK	0.04	MTOR	0.02
FGFR4	0.04	MAPK13	0.03

Figure Legends.

Figure 1: Oxidized phospholipid profiles differ in the lung dependent on airway

hyperresponsiveness. A) PLS-DA discriminated individuals with airway hyperresponsiveness (AHR, blue) and those without AHR (orange) in the DE3 cohort based on OxPC profile. B) 13 OxPC species were identified using sparse PLS-DA analysis to be significant discriminators of individuals with and without AHR. X-axis is a relative scale indicating the importance of each OxPC (higher absolute number is more important) in each group (AHR, blue; no AHR, orange). C) Correlation between HDiA-PC, D) KODA-PPC, E) PGPC with the methacholine dose response slope (an index of methacholine responsiveness). F) Correlation between SGPC and methacholine PC20. Data plotted on log scale. Subjects with AHR are blue and without AHR are orange. Spearman correlation coefficients and p-values noted on graphs. n=5 no-AHR and 8 AHR.

Figure 2: Oxidized phospholipid profiles change after allergen challenge in subjects with

mild-asthma. A) PLS-DA discriminated allergen (blue) and diluent (orange) challenge in individuals with mild asthma based on their OxPC profile. B) Seven OxPC species were identified using a sparse PLS-DA analysis to be significant discriminators of subjects, post-allergen or post-diluent challenge. X-axis is a relative scale indicating the importance of each

OxPC (higher absolute number is more important) in each group (allergen (blue) and diluent (orange) challenge). C) Correlation between PAPC-keto, D) PLPC-OH, E) PLPC-OOH, OH and the fall in FEV₁ during the late asthma response. Data plotted on log scale. Allergens used for each challenge are color coded according to the legend in the panels. Spearman correlation coefficients and p-values noted on graphs. n=10 subjects with pre- and post-allergen BAL.

Figure 3: OxPAPC exposure of HASM cells induces the pro-inflammatory cytokine release.

Abundance in cell culture media of A) IL-6, B) IL-8, C) GM-CSF in response to increasing concentrations of OxPAPC for 24 hours (the numbers in X-axis labels indicate concentration in µg/mL). Control = vehicle only exposure. PSPC 160 = negative control (160µg/mL). D) Dose-dependent increase in COX2 mRNA abundance in response to OxPAPC, E) Temporal effect of 80µg/mL OxPAPC on COX2 mRNA abundance for up to 24 hours exposure, F) Immunoblot showing the effects of OxPAPC on protein abundance for COX2 and COX1 in HASM. PSPC used as negative control. NT = no treatment, vehicle-only control. Enhanced ECL was required to detect COX2 protein in OxPAPC 80µg/mL treated HASM. * p<0.05 determined by one-way ANOVA with Dunnett's post-test versus PSPC treated cells. n=3 primary HASM cell cultures from different donors.

Figure 4: OxPAPC exposure of HASM cells significantly increases the production of oxylipin metabolites.

A full list of detected oxylipins is provided in Supplemental Table 2. A) Heatmap showing the profile of oxylipin production in cell culture media of three different primary HASM cell cultures after 24 hour exposure to increasing doses of OxPAPC (concentration in µg/mL, indicated in X-axis labels). As a negative control cells were challenged with PSPC (80µg/mL). NT= non treatment (vehicle-only control). Each column

represents a single cell culture. Individual oxylipins, 72, are represented in each row (labeled on left). Abundance is demonstrated by coloring from yellow to red (low abundance to high abundance), using Z-score (-2 minimum, +4 maximum). Panels B) 6-trans leukotriene B4 (6-trans LTB4), C) 12(S)-LTB4, D) 5,15-Dihydroxyeicosatetraenoic acid (5,15 DiHETE), and E) 12-Hydroxyeicosatetraenoic acid (12-HETE) show the most significant concentration-dependent relationship that were revealed in response to OxPAPC exposure. * $p < 0.05$ one way ANOVA with Dunnett's post-test vs PSPC treatment. $n = 3$ primary HASM cell cultures from different donors.

Figure 5: OxPAPC exposure causes dose-dependent airway narrowing in murine TCLS.

Dose response curve comparing effect of increasing concentration of OxPAPC on airway narrowing in a TCLS. * $p < 0.05$ vs. PSPC 80 $\mu\text{g/mL}$. Dashed line indicates airway narrowing caused by 0.1 μM methacholine (MCh). Insert shows a reference MCh dose response curve. EC_{50} concentration for OxPAPC – 29.5 $\mu\text{g/mL}$ and MCh – 0.24 μM .

Figure 6: Receptors and signaling pathways that regulate OxPAPC-induced COX2, oxylipins, cytokines and chemokines in primary HASM cells. A) Relative abundance of OxPAPC induced COX2 mRNA following 2-hour pre-incubation with inhibitors for: prostaglandin E2 receptor (PF-04418948, 1 μM), prostaglandin E1/2 receptor (AH-6089, 10 μM), TLR2/4 receptor (sparstolonin B, 10 μM), platelet-activating factor receptor (WEB-2086, 10 μM) or CD36 receptor (sulfosuccinimidyl oleate 20 μM). B) Relative abundance of OxPAPC induced COX2 mRNA following 2-hour pre-incubation with inhibitors for PKC (GF-109203X, 10 μM) or ROCK1/2 (Y-27632, 10 μM). C) IL-6, D) IL-8, E) GM-CSF abundance following 2-hour pre-inhibition with GF-109203X, indomethacin (10 μM), or AH-6089 for 2

hours prior to the addition of OxPAPC. * $p < 0.05$ one way ANOVA with Dunnett's post-test versus OxPAPC 80 $\mu\text{g/mL}$. N=3 primary HASM cell cultures from different donors.

Figure 7: Schematic overview of signaling pathways orchestrating OxPAPC induced COX2

and inflammatory cytokine synthesis. OxPAPC causes airway narrowing through an unidentified cell membrane receptor or direct effects on the plasma membrane. By similar receptor or membrane-linked mechanisms, OxPAPC also activates PKC, which leads to production of IL-6 and IL-8 and expression of COX2. COX2 products (oxylipins), signal through EP1/2 receptor and are required for OxPC-induced production of GM-CSF. PKC – Protein Kinase C, COX2 – Cyclooxygenase 2, EP1/2 – Prostaglandin E2 Receptor 1/2.

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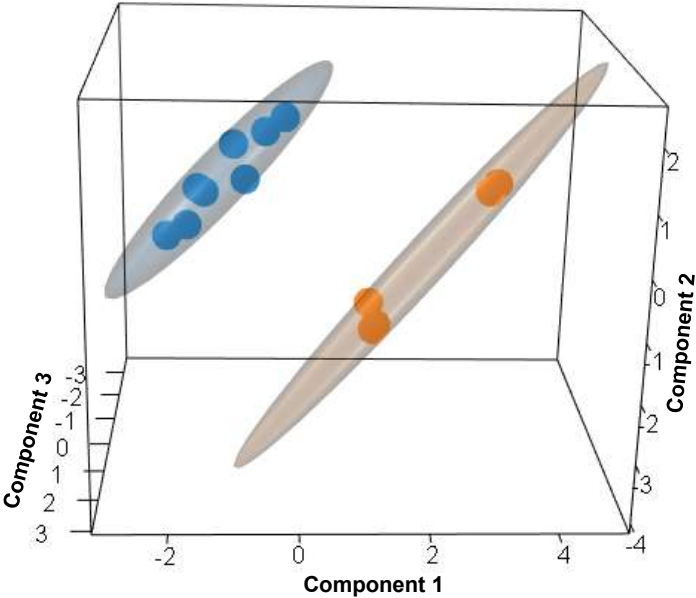
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Figure 1

A



B

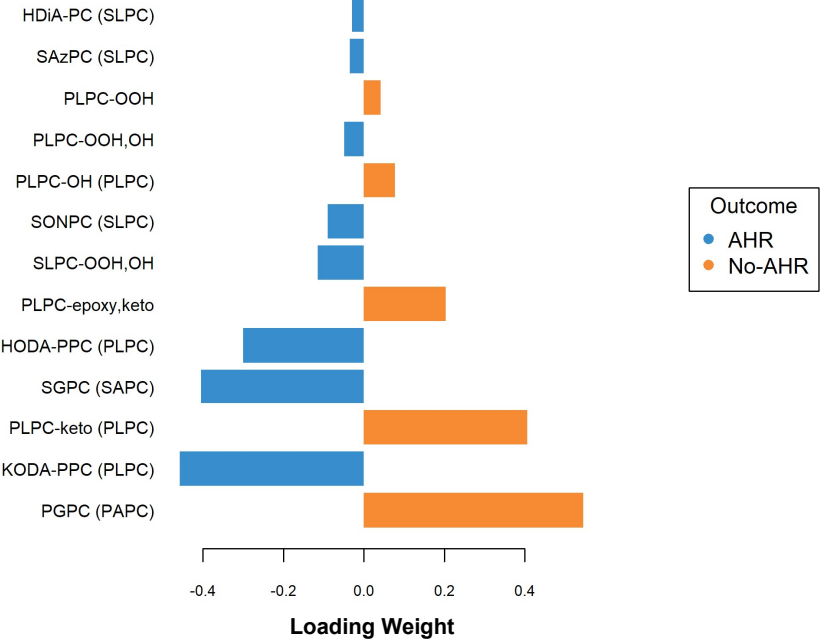


Figure 1

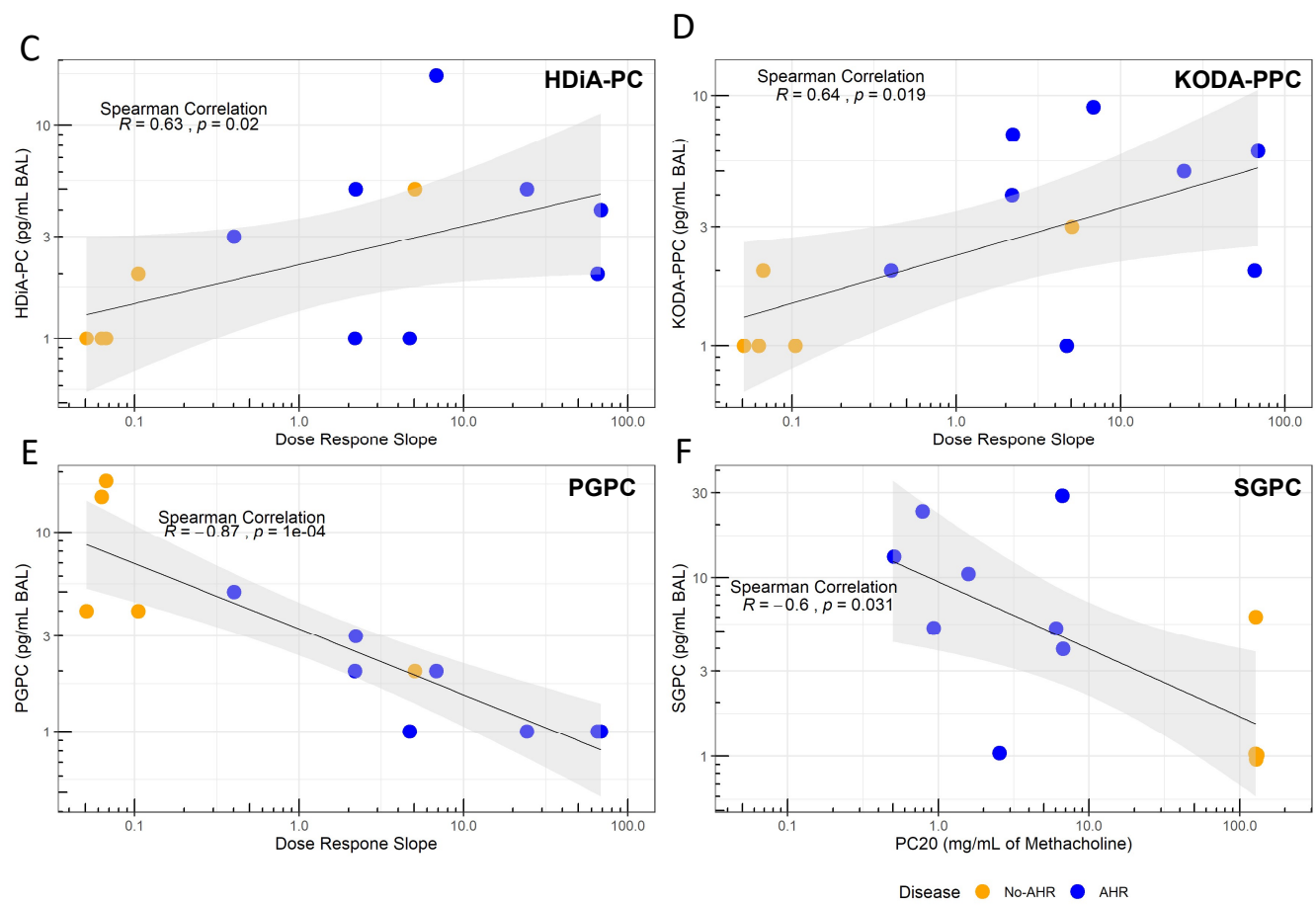


Figure 2

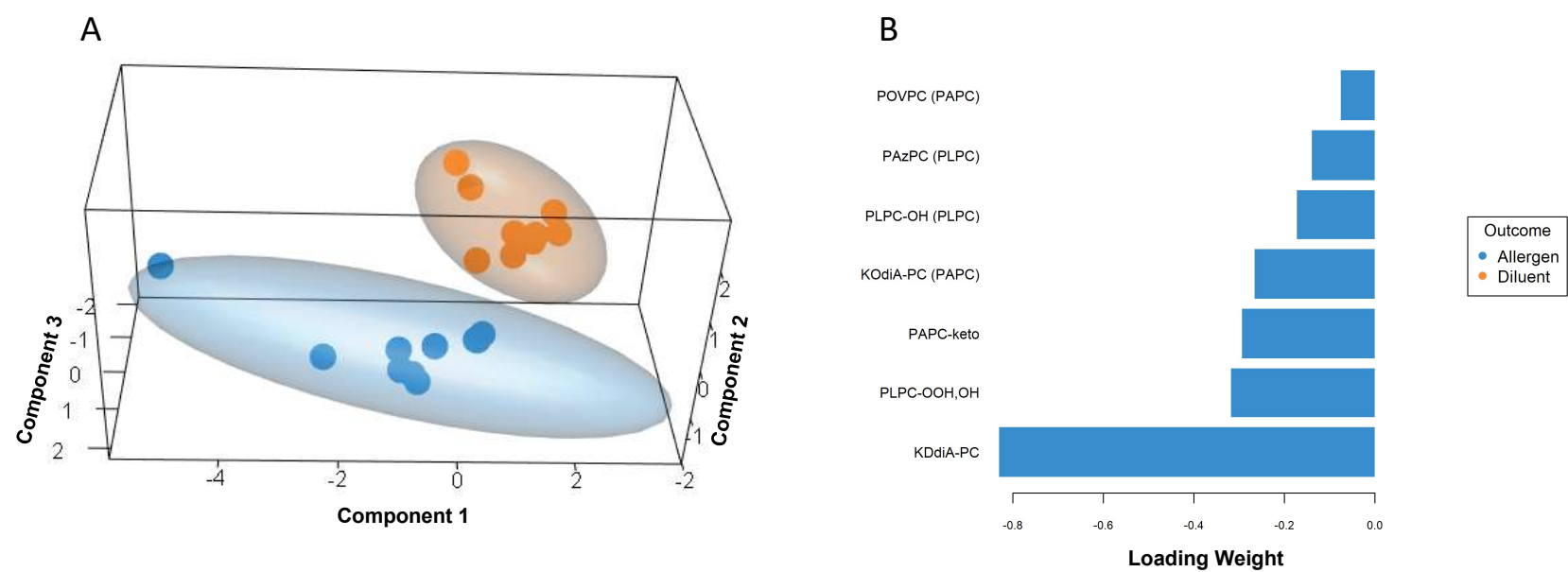


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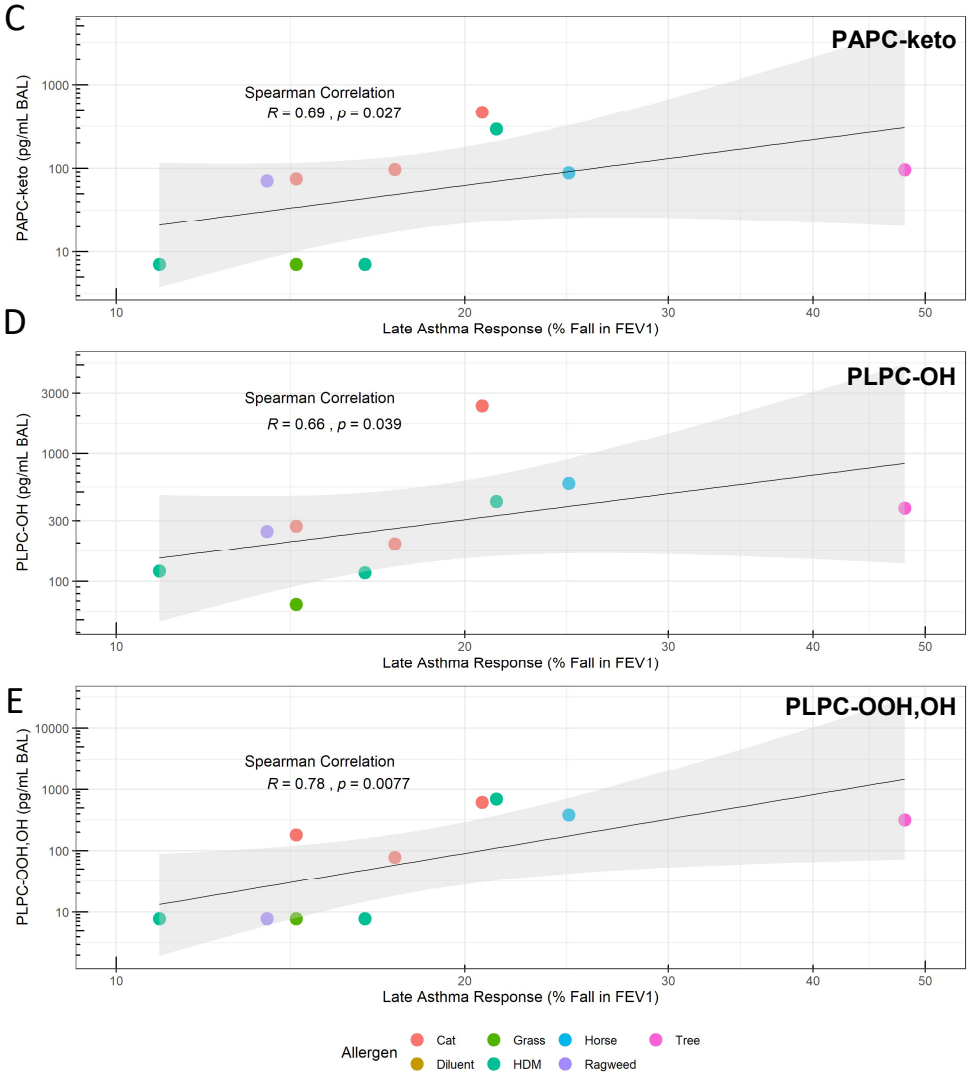


Figure 3

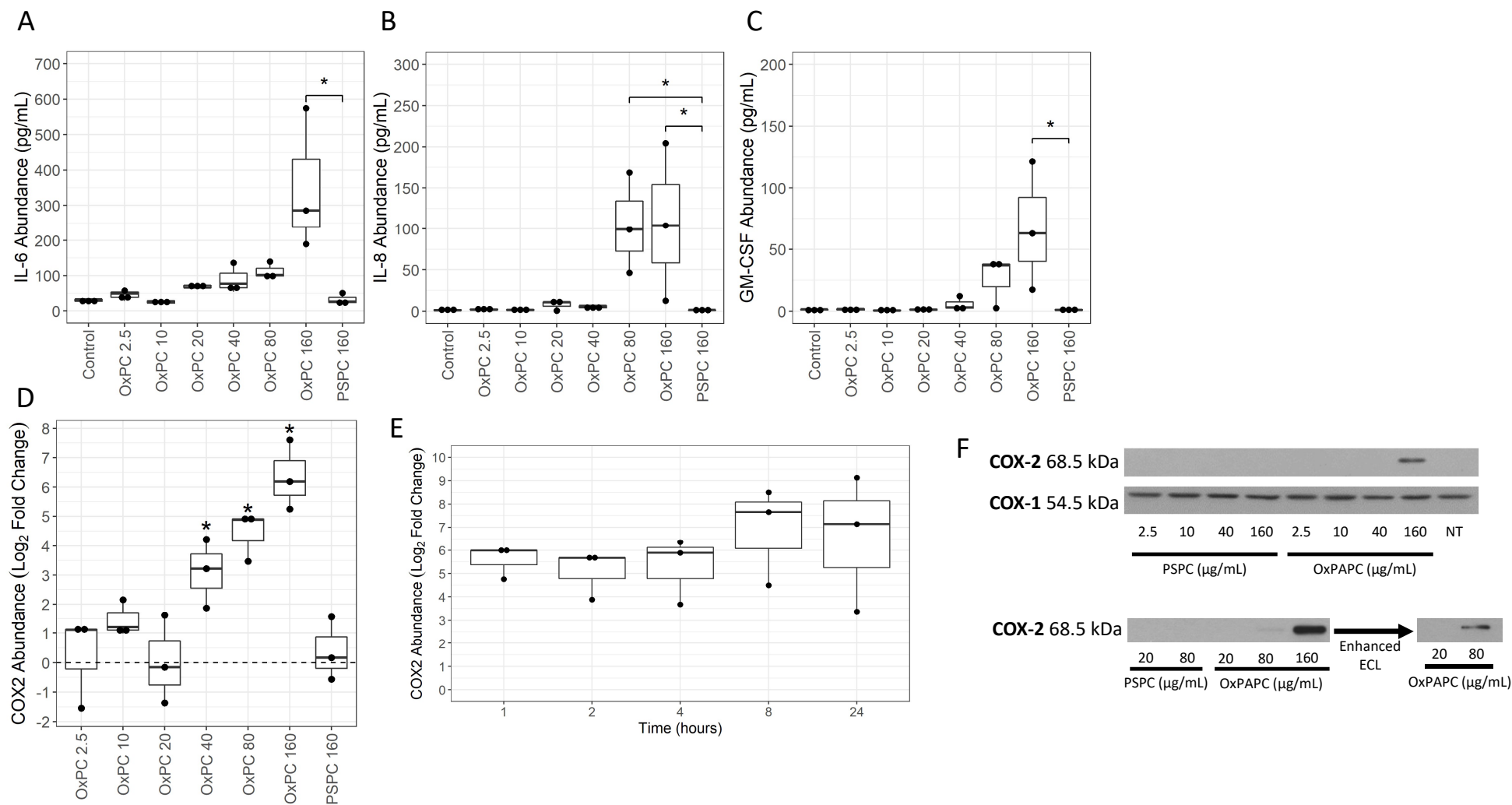
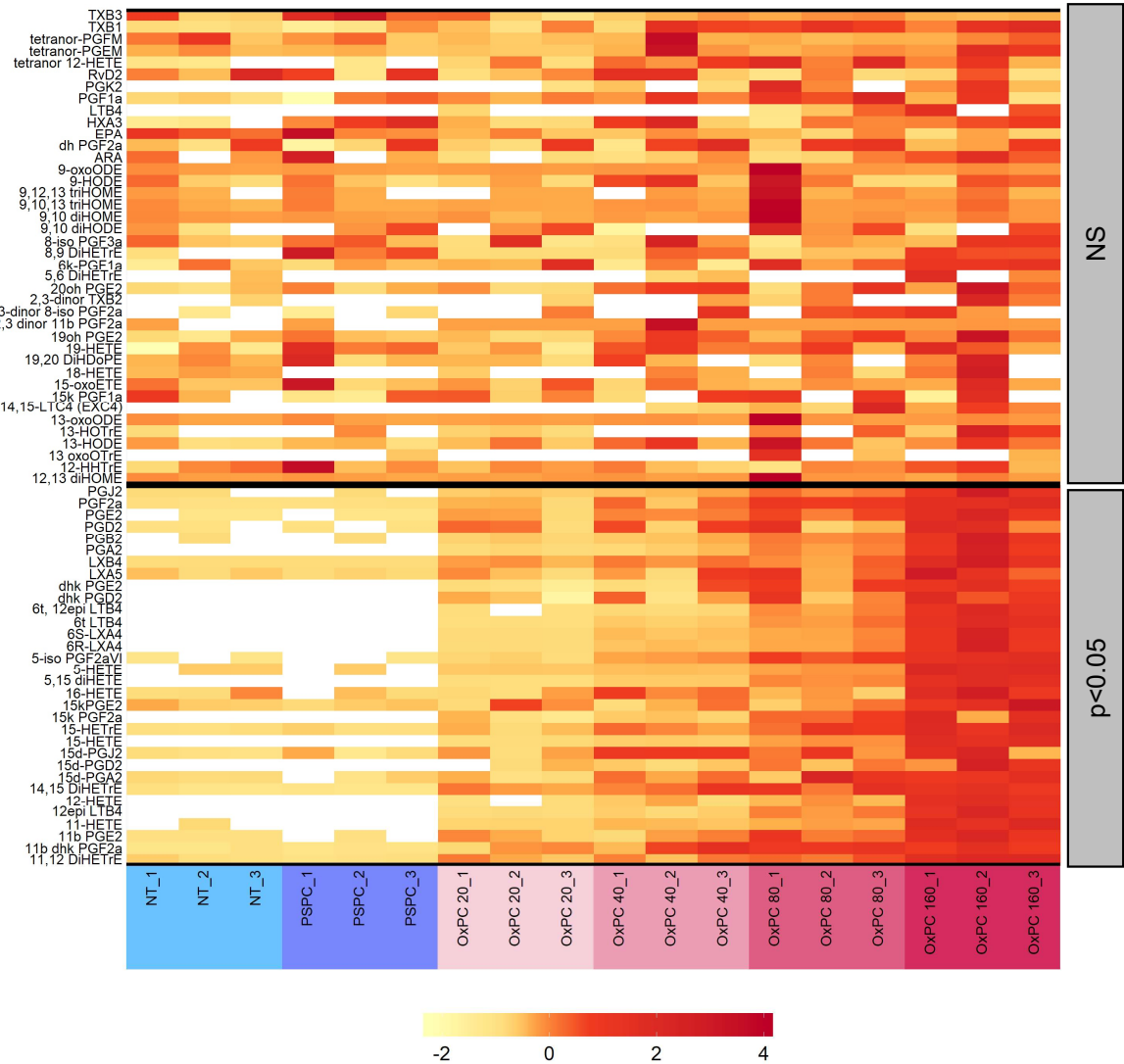
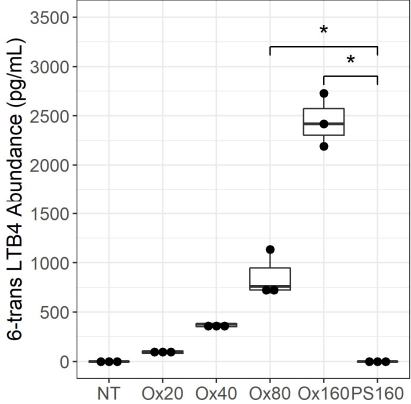


Figure 4

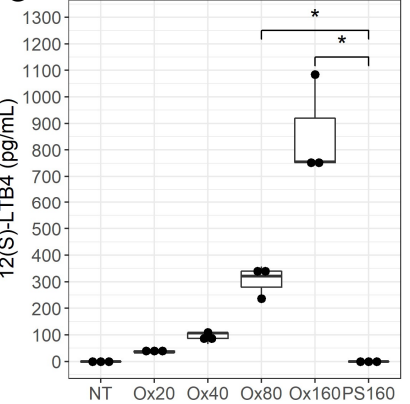
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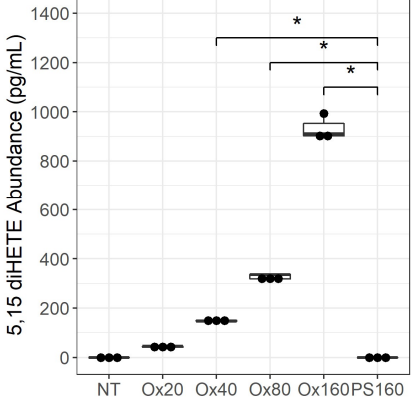
B



C



D



E

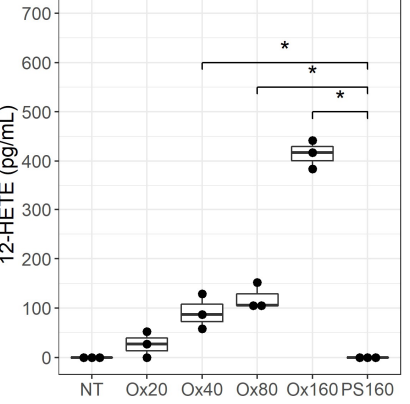


Figure 5

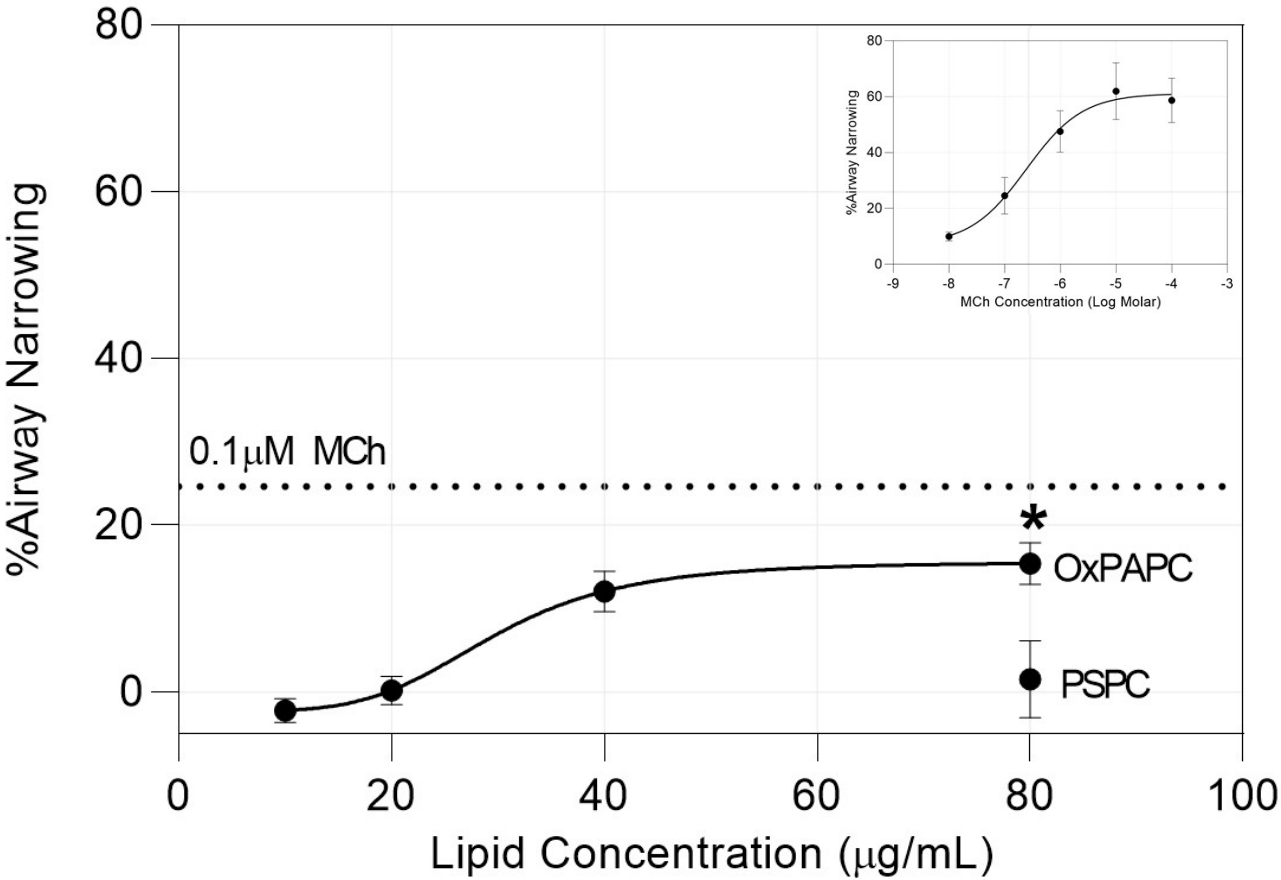


Figure 6

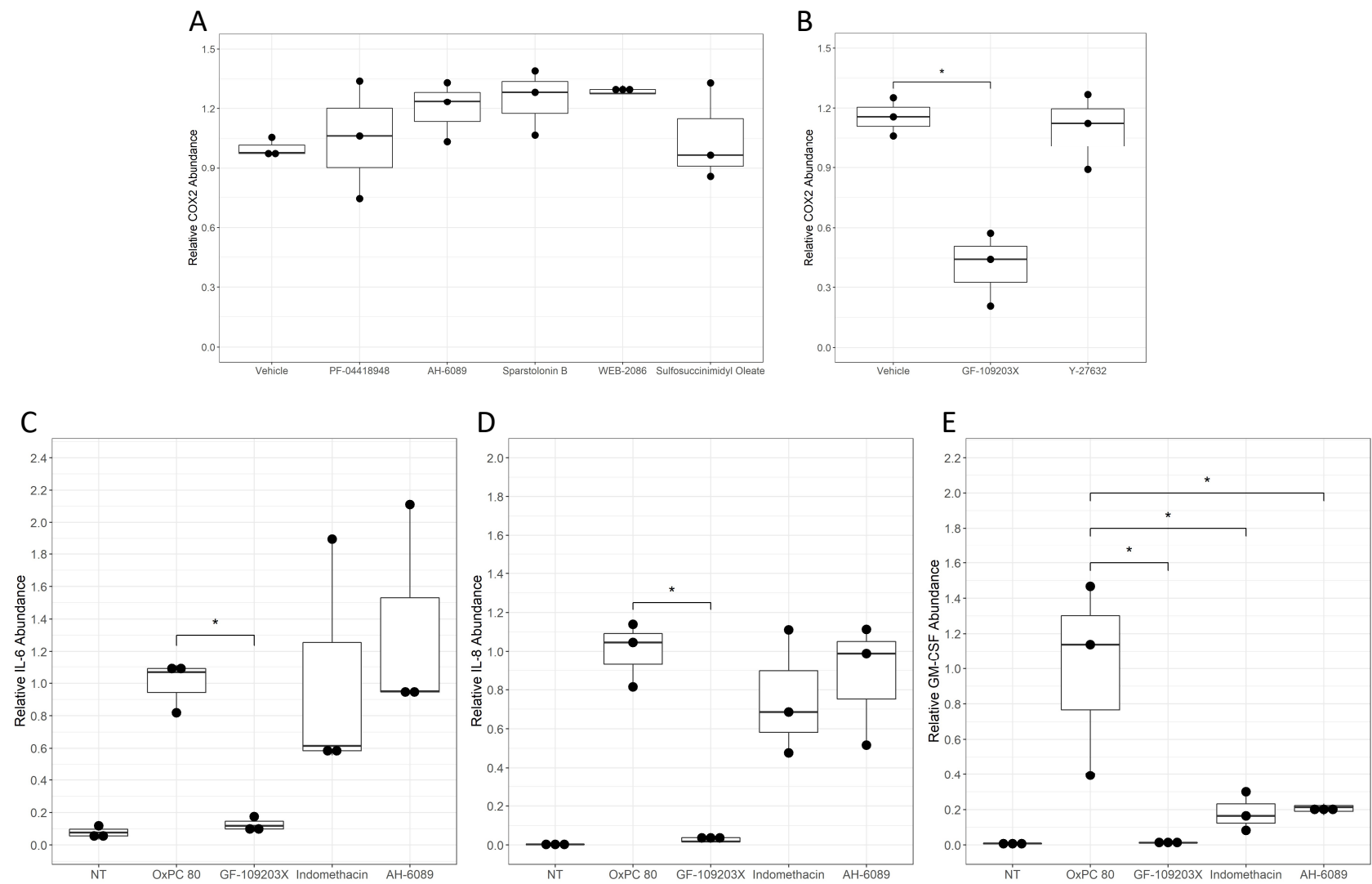
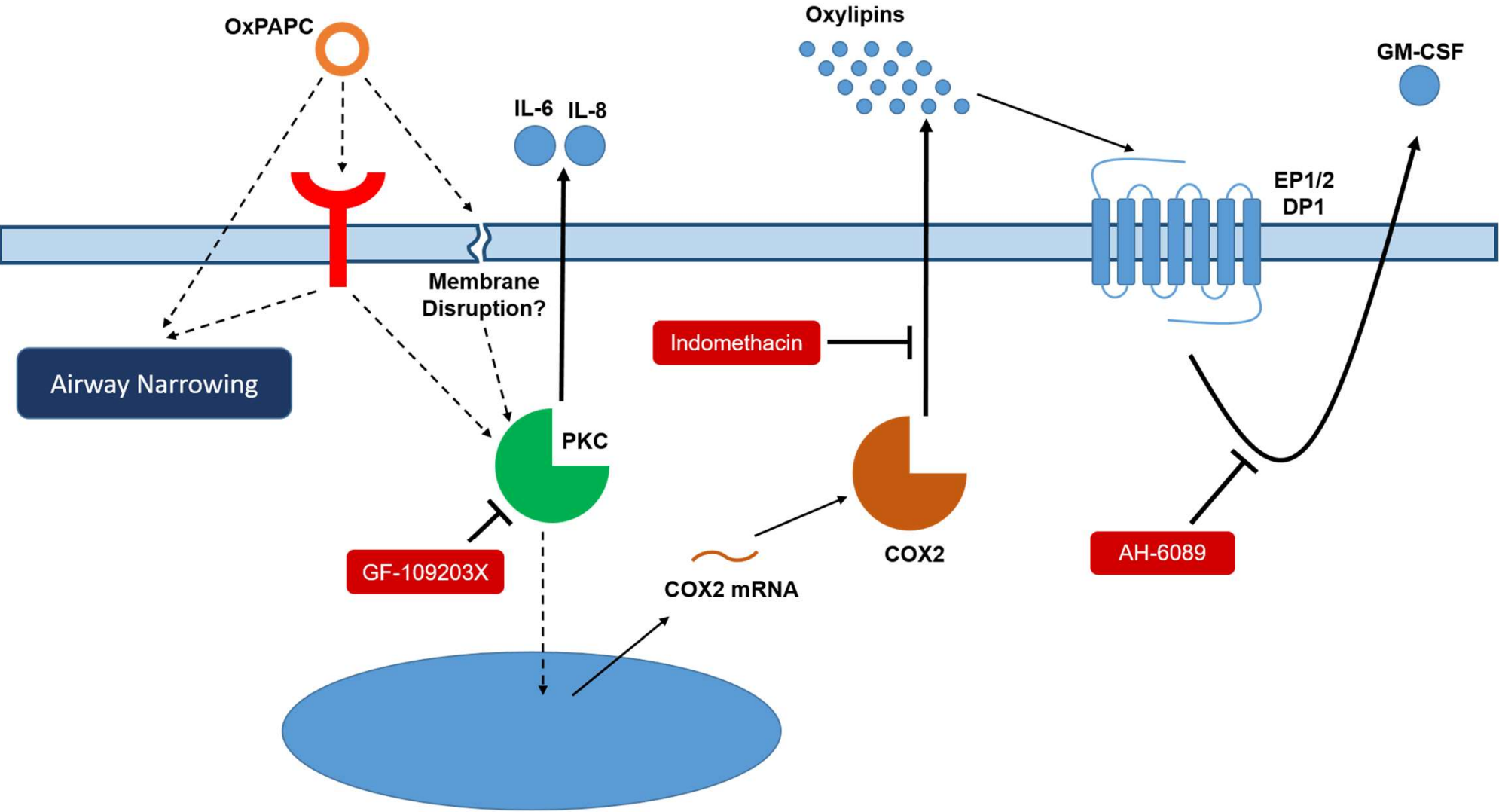


Figure 7



Supplementary Methods:

Materials. Dulbecco's Modified Eagle Medium (DMEM) with and without Phenol red and Insulin-Transferrin-Selenium (ITS) were purchased from ThermoFisher Scientific (Massachusetts, United States). Phospholipids 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (PAPC) and 1-palmitoyl-2-stearoyl-sn-glycero-3-phosphocholine (PSPC) were purchased from Avanti Polar Lipids (25mg in chloroform, Alabama, USA). PureLink RNA Mini kit was purchased from ThermoFisher Scientific for isolation of total RNA and iScript[™] Reverse Transcription Supermix kit purchased from Bio-Rad. Forward and reverse primers for qPCR were purchased from Integrated DNA Technologies (IDT, Iowa, United States) and suspended in TE buffer for storage at -20°C. SSoFast[™] EvaGreen Supermix from Bio-Rad (California, United States) was used to perform qPCR. A table of primer sequences and information can be found in table S1. COX2 antibody (#12282) and COX1 antibody (#4841) were purchased from Cell Signaling Technology (Massachusetts, United States). Protein kinase C (PKC) inhibitor GF-109203X (Cat. No. 0741) was purchased from Tocris (Bristol, United Kingdom). Cytokine analysis in cell supernatants were performed using bead-based Luminex technology (Eve Technologies, Calgary Canada) and Meso Scale Discovery (MSD, Maryland, USA) System custom U-Plex panel. Protein concentration in cell lysates was quantified using the Bio-Rad protein assay. For kinomics array, PRO-Q Phosphoprotein stain was purchased from ThermoFisher/Invitrogen.

DE3 Study design: Thirteen allergen-sensitized volunteers participated in the randomized, double-blinded, controlled human exposure crossover study taking place between April 2013 and April 2017 (Clinical Trials ID: NCT02017431). All participants gave written informed consent to the study protocol, which was approved by the University of British Columbia Research Ethics Board (H11-01831), Vancouver.

For inhaled allergen challenge, standardized allergenic extracts for HDM (Dermatophagoides pteronyssinus), Timothy grass, and Birch mix were purchased from Hollister-Stier Laboratories

(Spokane, WA, USA). On two occasions separated by minimum 4-weeks washout period, participants inhaled either saline (0.9% NaCl) or diluted skin prick test allergen (HDM, grass or birch mix). 2 mL of diluted allergen or saline was delivered to participants using VixOne™ Nebulizer Mask Kit (Westmed, Tucson, AZ, USA) with flow rate of medical air set at 5 L/min for 2 minutes. The concentration of diluted allergen used for the inhalation challenge was allergen provocative concentration (PC)₂₀, the concentration causing a 20% forced expiratory volume in one second (FEV₁) fall. Allergen PC₂₀ was calculated by methacholine PC₂₀ and skin prick wheal size (1), and then was confirmed at one of the screening visits. Research participants, research coordinators, medical staff, and the study physician were blinded to allergen inhalation challenge.

2 days after allergen inhalation challenge, each participant underwent flexible bronchoscopy. Bronchoalveolar lavage (BAL) was collected by instilling and suctioning 20 mL of sterile saline (0.9% NaCl) twice and pooled them together to obtain bronchial wash (BW) and instilling and suctioning 50 mL of normal saline twice and pooled them together to obtain bronchoalveolar lavage (BAL). Both fractions were passed through a mesh filter (40 µm pore size) and centrifuged for 15 mins at 475 x g. Acellular supernatant of BW and BAL was separated, aliquoted and stored at -80 °C. For the purpose of this study, only BAL was assayed for O_xPCs.

DC Study Design: Ten subjects (aged 22-66 years) with positive skin test responses to relevant aeroallergens (>2 mm in diameter) were recruited. All subjects had mild, stable allergic asthma with FEV₁ values greater than or equal to 70%, and baseline methacholine PC₂₀ of less than or equal to 16 mg/mL. All subjects were nonsmokers, not on inhaled steroid with or without long acting inhaled β₂ agonist therapy and infrequently used inhaled β₂ agonists.

The study was a randomized, diluent-controlled, crossover design approved by the Hamilton Integrated Research Ethics Committee (Project 14-122). On day 1 subjects provided consent, completed a skin prick test, a medical history and methacholine PC₂₀ was measured. On day 2 subjects were randomized to inhale diluent or allergen and spirometry was measured until 7h post-

challenge. On day 3, 24 hours after the inhalation challenge, bronchoscopy was performed to obtain BAL fluid. This triad was repeated after a 4-week recovery period.

Airway challenges: Methacholine inhalation challenges were performed using Wright nebulizer and the tidal breathing method, as previously described (2). PC₂₀ was calculated as the provocative concentration of methacholine inducing a 20% decrease in FEV₁.

Allergen inhalation challenge was performed as previously described (3). Briefly, doubling doses of allergen extract was delivered by a Wright nebulizer and the tidal breathing method. Subjects inhaled doubling concentrations of allergen extract for 2 minutes using a nebulizer with a mouthpiece on a one-way Hans-Rudolph valve, until a greater than 20% fall in FEV₁ at 10 minutes post allergen was reached. The FEV₁ was then measured at 10, 20, 30, 45, 60, 90, and 120 minutes after inhalation challenge, and each hour until 7 hours after challenge. The early bronchoconstrictor response (EAR) is taken to be the largest fall in FEV₁ within 2 hours after allergen inhalation, and the late response (LAR) is taken to be the largest fall in FEV₁ between 3 hours and 7 hours after allergen inhalation. LAR was defined as at least 15% fall in FEV₁ between 3 and 7 hours after allergen challenge. Diluent challenge consisted of 3 inhalations of normal saline (0.9% NaCl) followed by spirometry for 7 hours.

Bronchoscopy: Fiberoptic bronchoscopy was performed as previously described (4) using a Pentax fiberoptic bronchoscope passed through the vocal cords and introduced into the airways and wedged into a segmental or subsegmental bronchus. BAL was collected from the right middle lobe using 3X60ml aliquots of normal saline (0.9% NaCl) introduced via the bronchoscope and then aspirated through the bronchoscope using a closed, sterile collecting system.

Primary cell culture. Primary HASM cells were isolated from lung tissue obtained through lung resection surgery following approval from the Human Research Ethics Board (University of Manitoba). Subjects had no previous history of chronic lung disease (asthma/COPD) and were never

smokers or had quit 30 years prior to lung resection surgery. Detailed demographics along with information about which experiments utilized which subjects are presented in the online supplement (Table S1). HASM were grown to confluence on 6-well plates in DMEM media supplemented with 5% fetal bovine serum (FBS) and 1% penicillin-streptomycin. To induced phenotype differentiation, cells were serum-deprivation in DMEM supplemented with ITS for 7 days. Cells were used between passage 4-6 for all experiments.

Lipid Peroxidation. 25mg of PAPC and PSPC, were oxidized in room air for 4 days and then re-suspended at a concentration of 1mg/mL in chloroform:methanol (2:1). A small sample of oxidized PAPC (OxPAPC) was tested for degree of oxidation using HPLC/electrospray ionization tandem mass spectrometry system as previously described (29684044). PSPC, which lacks fatty acid double bonds, does not undergo oxidation and therefore acts as the control lipid in these experiments. OxPAPC and PSPC were maintained in chloroform methanol at a stock concentrations of 1 mg/mL and stored under nitrogen gas at -20 °C. For exposure in cell culture, desired volumes of OxPAPC- and PSPC-containing chloroform:methanol were transferred to a falcon tube and, under nitrogen gas, the evaporated, leaving behind OxPAPC and PSPC in the tubes. Media was added to tubes containing OxPAPC and PSPC and sonicated to form liposomes prior to the addition to cells.

Cytokine analysis. Quantification of inflammatory mediators secreted by OxPAPC-stimulated HASM was performed on cell supernatants. Cytokines tested were GM-CSF, IFN- γ , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12(p70), IL-13, MCP-1, and TNF- α (Eve Technologies). MSD custom panel with IL-6, IL-8, and GM-CSF was used to test impact of inhibitors on cytokine secretion.

qPCR. Isolation of total RNA from HASM for qPCR was done according to kit instructions. A total of 1 μ g of extracted total RNA was reverse transcribed to cDNA using manufacturer's instructions. qPCR was performed according to the SsoFast[™] EvaGreen Supermix protocol on the CFX96 thermocycler (Bio-Rad) which included a melting curve.

Western Blotting. 10µg of total protein was run on a 10% SDS-PAGE gel and blotted onto nitrocellulose. Primary antibody exposure was done at 4 °C overnight at a concentration of 1:500 (COX1) and 1:750 (COX2) according to vendor specifications. Secondary anti-rabbit HRP antibody was used at a concentration of 1:3000 for both COX1 and COX2.

Oxidized Phospholipid and Oxylipin Quantification. Cells used for assessment of the levels of oxylipins were grown as mentioned above with the exception of phenol-free DMEM media used for supernatant collection. Analysis of oxylipin profile was conducted using high-performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS) as previously described (5). Oxidized phospholipids were measured in lipid extracts as previously described (6, 7).

Briefly, lipid extracts were reconstituted in RP eluent consisting of 60:40 acetonitrile:water, 10 mM ammonium formate and 0.1% formic acid immediately prior to injection. Thirty microlitres of the sample were injected onto an Ascentis Express C18 HPLC column (15 cm × 2.1 mm, 2.7 µm; Supelco Analytical, Bellefonte, Pennsylvania, USA) with separation by a Prominence UFLC system from Shimadzu Corporation (Canby, OR, USA). Elution was performed using a linear gradient of solvent A (acetonitrile/water, 60:40 v/v) and solvent B (isopropanol/acetonitrile, 90:10, v/v) with both solvents containing 10 mM ammonium formate and 0.1% formic acid. The mobile phase composition that was used is as follows: initial solvent B at 32% until 4.00 min; switched to 45% B; 5.00 min 52% B; 8.00 min 58% B; 11.00 min 66% B; 14.00 min 70% B; 18.00 min 75% B; 21.00 min 97% B; 25.00 min 97% B; and 25.10 min 32% B. A flow rate of 260 µL/min was used for analysis, and the sample tray and column oven were held at 4°C and 45°C, respectively.

Detection of OxPL was carried out by mass spectrometry in positive polarity mode. MRM scans were performed on 6 transitions using a product ion of 184.3 m/z, corresponding to the cleaved phosphocholine moiety. Ten nanograms of internal standard 9:0-9:0 PC was added to all samples during extraction. A 4000 QTRAP® triple quadrupole mass spectrometer system with a Turbo V

electrospray ion source from AB Sciex (Framingham, MA, USA) was coupled to the liquid chromatography system.

Kinomics Array. Following exposure of HASM to 80µg/mL of OxPAPC for 1, 3, or 6 hours, cell lysates were collected in a Phosphoarray lysis buffer and run on a peptide array as previously described (8). In brief, 100µg of protein from the cell lysates were loaded onto a peptide array in the presence of activation mixture containing 50µM ATP and incubated at 37°C for 2 hours. Results were visualized using PRO-Q Diamond Phosphoprotein stain and subsequently scanned on a PowerScanner (Tecan, Morrisville, NC, USA) microarray scanner with a filter at 580 nm to detect fluorescence. Signal intensity values were collected with Array-Pro Analyzer version 6.3 software (Media Cybernetics, Rockville, MD, USA).

Statistics. qPCR results were analyzed using the delta-delta CT method correcting for PCR efficiency and normalized to the geometric mean of the housekeeping genes (YWHAZ, UBC, and GAPDH). Relative abundance of cytokines and COX2 mRNA were calculated for comparison of PKC inhibitor effects. Differential phosphorylation results were calculated using the Platform for Integrated, Intelligent Kinome Analysis 2 (PIIKA2) software (9). Significantly upregulated phospho-signals at 1,3, and 6 hours were analyzed using Kinase Enrichment Analysis (KEA, (10)) to identify underlying activated kinases in the kinome data. Data was analyzed using a One-way ANOVA with a Dunnet's post-hoc comparison. Significant threshold set at $p < 0.05$. All results were completed in at least triplicate (3 different primary human cells). Data analysis was done in R (version 3.5.1) and graphs made using ggplot2 package (version 3.1.1).

Table S1: qPCR Primer Information.

Gene	NCBI Accession Number	Forward Primer	Reverse Primer
COX2 (PTGS2)	NM_000963.4	CAAATTGCTGGCAGGGTTG	GGTCAATGGAAGCCTGTGATA
UBC	NM_021009.7	TGGCACAGCTAGTTCCGTC	CACGAAGATCTGCATTGTCAAGT G
GAPDH	NM_001256799 .2	CTGACTTCAACAGCGACACC	CGTTGTCATACCAGGAAATGAG
YWHAZ	NM_001135699 .1	CTTCACAAGCAGAGAGCAAAG	CGACAATCCCTTTCTTGTCATC

Supplementary Results:

Table S2: Oxylipin abundance in OxPC treated ASM cell supernatant. CYP - Cytochrome P450, LOX - Lipoxygenase, COX- Cyclooxygenase. Data presented as mean $\pm$ SD (ng/mL). * $p < 0.05$ vs. PSPC 160

<u>Oxylipin</u>	<u>Enzyme</u>	<u>NT</u>	<u>PSPC 160</u>	<u>Ox 20</u>	<u>Ox 40</u>	<u>Ox 80</u>	<u>Ox 160</u>
11,12 DiHETrE	CYP	0.040 $\pm$ 0.017	0.025 $\pm$ 0.006	0.089 $\pm$ 0.030 *	0.103 $\pm$ 0.031 *	0.143 $\pm$ 0.022 *	0.290 $\pm$ 0.015 *
14,15 DiHETrE	CYP	0.043 $\pm$ 0.012	0.039 $\pm$ 0.003	0.133 $\pm$ 0.022	0.186 $\pm$ 0.054 *	0.228 $\pm$ 0.068 *	0.258 $\pm$ 0.028*
16-HETE	CYP	0.056 $\pm$ 0.037	0.034 $\pm$ 0.031	0.052 $\pm$ 0.027	0.128 $\pm$ 0.035	0.068 $\pm$ 0.018	0.254 $\pm$ 0.079 *
17-HETE	CYP	2.393 $\pm$ 4.145	0.000 $\pm$ 0.000	0.000 $\pm$ 0.000	0.000 $\pm$ 0.000	0.000 $\pm$ 0.000	4.274 $\pm$ 7.402
18-HETE	CYP	0.053 $\pm$ 0.010	0.000 $\pm$ 0.000	0.010 $\pm$ 0.018	0.044 $\pm$ 0.042	0.034 $\pm$ 0.047	0.145 $\pm$ 0.173
19-HETE	CYP	5.472 $\pm$ 1.641	8.505 $\pm$ 1.407	6.263 $\pm$ 0.971	8.222 $\pm$ 0.684	7.713 $\pm$ 0.994	8.450 $\pm$ 1.750
20-COOH AA	CYP	0.000 $\pm$ 0.000	0.078 $\pm$ 0.135	0.000 $\pm$ 0.000	0.000 $\pm$ 0.000	0.000 $\pm$ 0.000	0.321 $\pm$ 0.556
5,6 DiHETrE	CYP	0.008 $\pm$ 0.014	0.000 $\pm$ 0.000	0.000 $\pm$ 0.000	0.014 $\pm$ 0.013	0.000 $\pm$ 0.000	0.037 $\pm$ 0.039

8,9 DiHETrE	CYP	0.004 ± 0.007 *	0.083 ± 0.052	0.017 ± 0.001 *	0.041 ± 0.021	0.022 ± 0.007	0.065 ± 0.007
12-Epi LTB4	LOX	0.000 ± 0.000	0.000 ± 0.000	0.038 ± 0.007	0.094 ± 0.025	0.305 ± 0.062 *	0.863 ± 0.192 *
12-HETE	LOX	0.000 ± 0.000	0.000 ± 0.000	0.026 ± 0.026	0.091 ± 0.036 *	0.121 ± 0.027 *	0.414 ± 0.029 *
14,15-LTC4 (EXC4)	LOX	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.061 ± 0.055	0.357 ± 0.397	0.288 ± 0.131
15-HETE	LOX	0.000 ± 0.000	0.000 ± 0.000	0.162 ± 0.060	0.340 ± 0.013 *	0.711 ± 0.058 *	2.370 ± 0.153 *
15-oxoETE	LOX	2.212 ± 1.917	6.584 ± 9.975	3.075 ± 2.613	2.293 ± 2.123	2.132 ± 0.811	5.486 ± 6.068
6R-LXA4	LOX	0.000 ± 0.000	0.000 ± 0.000	1.456 ± 0.255	4.175 ± 0.366	7.399 ± 2.081	26.454 ± 9.451*
6S-LXA4	LOX	0.000 ± 0.000	0.000 ± 0.000	0.553 ± 0.062	1.673 ± 0.175	2.851 ± 0.675	10.648 ± 3.610 *
6-trans LTB4	LOX	0.000 ± 0.000	0.000 ± 0.000	0.095 ± 0.026	0.364 ± 0.034	0.860 ± 0.240 *	2.445 ± 0.271 *
6-trans, 12(S) LTB4	LOX	0.000 ± 0.000	0.000 ± 0.000	0.058 ± 0.051	0.207 ± 0.033	0.598 ± 0.120 *	1.724 ± 0.304 *
8,15 diHETE	LOX	0.074 ± 0.128	0.000 ± 0.000	0.000 ± 0.000	0.112 ± 0.194	0.060 ± 0.104	0.000 ± 0.000

8-HETE	LOX	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.213 ± 0.186 *
5,15 diHETE	LOX	0.000 ± 0.000	0.000 ± 0.000	0.043 ± 0.005	0.148 ± 0.005 *	0.325 ± 0.021 *	0.932 ± 0.053 *
5-HETE	LOX	0.037 ± 0.034	0.010 ± 0.017	0.119 ± 0.031	0.355 ± 0.036	1.231 ± 0.200 *	8.224 ± 0.570 *
HXA3	LOX	0.030 ± 0.034 *	0.546 ± 0.221	0.201 ± 0.059	0.537 ± 0.323	0.267 ± 0.172	0.466 ± 0.096
HXB3	LOX	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.036 ± 0.062	0.024 ± 0.042
LTB4	LOX	0.000 ± 0.000	0.000 ± 0.000	0.069 ± 0.119	0.000 ± 0.000	0.287 ± 0.285	0.568 ± 0.530
LXB4	LOX	0.093 ± 0.042	0.101 ± 0.030	1.251 ± 0.319	1.947 ± 0.453	1.825 ± 0.802	6.102 ± 1.549 *
Tetranor 12-HETE	LOX	0.055 ± 0.060	0.017 ± 0.029	0.412 ± 0.256	0.779 ± 0.237	1.232 ± 0.506	0.746 ± 0.376
11-HETE	Non-enz/ LOX/COX	0.004 ± 0.006	0.000 ± 0.000	0.050 ± 0.002	0.150 ± 0.032	0.241 ± 0.036 *	1.408 ± 0.178 *
9-HETE	Non-enz/ LOX/COX	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.582 ± 0.630
2,3-dinor-8-	Non-enz	0.040 ±	0.110 ±	0.133 ±	0.220 ±	0.339 ±	0.329 ±

iso PGF2 alpha		0.069	0.123	0.231	0.381	0.294	0.323
5-iso PGF2 alpha VI	Non-enz	0.038 ± 0.036	0.040 ± 0.070	3.630 ± 0.826 *	7.291 ± 0.680 *	13.893 ± 1.151 *	20.738 ± 1.402 *
8-iso 15-keto PGF2 beta	Non-enz	0.000 ± 0.000	0.000 ± 0.000	0.070 ± 0.067	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
11-beta Dihydroxy- PGF2 alpha	COX	0.311 ± 0.054	0.321 ± 0.022	0.722 ± 0.166	1.172 ± 0.500 *	1.417 ± 0.081 *	1.417 ± 0.211 *
11-beta PGE2	COX	0.181 ± 0.032	0.063 ± 0.110	4.469 ± 1.888	4.557 ± 2.619	9.595 ± 3.231 *	16.978 ± 3.457 *
11-delta TXB2	COX	0.149 ± 0.259	0.000 ± 0.000	0.553 ± 0.480	0.725 ± 0.678	0.597 ± 1.034	0.000 ± 0.000
12-HHTrE	COX	0.191 ± 0.096	0.000 ± 0.000	0.554 ± 0.480	0.725 ± 0.678	0.597 ± 1.034	0.000 ± 0.000
15-deoxy PGA2	COX	1.464 ± 0.185	0.814 ± 0.967	1.581 ± 0.852	3.696 ± 0.907	6.090 ± 3.966	6.764 1.055 *
12-deoxy PGD2	COX	0.000 ± 0.000	0.000 ± 0.000	0.037 ± 0.036	0.053 ± 0.011	0.104 ± 0.036	0.223 ± 0.129 *
15-deoxy PGJ2	COX	0.042 ± 0.010	0.055 ± 0.041	0.086 ± 0.032	0.199 ± 0.003	0.152 ± 0.056	0.225 ± 0.123 *
12-keto PGF2	COX	0.000 ±	0.000 ±	0.817 ±	1.103 ±	2.080 ±	2.724 ±

alpha		0.000	0.000	0.419	0.065	0.231 *	1.214 *
15-keto PGE2	COX	0.160 ± 0.042	0.133 ± 0.029	0.278 ± 0.187	0.207 ± 0.140	0.236 ± 0.104	0.797 ± 0.307 *
19oh PGE2	COX	0.505 ± 0.151	0.665 ± 0.139	0.555 ± 0.054	0.900 ± 0.165	0.850 ± 0.214	1.155 ± 0.637
2,3-dinor 11- beta PGF2 alpha	COX	0.002 ± 0.004	0.003 ± 0.005	0.028 ± 0.004	5.166 ± 8.864	0.083 ± 0.023	0.180 ± 0.114
2,3-dinor TXB2	COX	0.008 ± 0.013	0.000 ± 0.000	0.008 ± 0.013	0.010 ± 0.017	0.020 ± 0.019	0.038 ± 0.038
2,3-dinor-6 keto PGF1 alpha	COX	0.004 ± 0.008	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.003 ± 0.006
6-keto PGF1 alpha	COX	0.314 ± 0.122	0.326 ± 0.037	0.442 ± 0.176	0.272 ± 0.119	0.494 ± 0.156	0.564 ± 0.020
20oh PGE2	COX	0.116 ± 0.022	0.142 ± 0.032	0.129 ± 0.027	0.217 ± 0.026	0.181 ± 0.080	0.253 ± 0.141
Bicyclo PGE2	COX	0.000 ± 0.000	0.034 ± 0.069	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Dihydroxy PGF2 alpha	COX	223.139 ± 51.046	209.954 ± 80.004	225.266 ± 54.268	257.601 ± 83.962	262.032 ± 53.537	234.956 ± 36.726
Dihydroxy-	COX	0.000 ±	0.000 ±	1.250 ±	1.644 ±	2.010 ±	2.619 ±

PGD2		0.000	0.000	0.548	0.511	0.634	0.469
Dihydroxy- PGE2	VOC	0.000 ± 0.000	0.000 ± 0.000	2.403 ± 0.711	3.704 ± 1.611	5.176 ± 1.226	6.505 ± 0.939
PGA2	COX	0.000 ± 0.000	0.000 ± 0.000	2.008 ± 0.751	3.673 ± 1.351	9.176 ± 1.819	26.413 ± 11.077 *
PGB2	COX	0.500 ± 0.866	0.269 ± 0.466	29.719 ± 9.917	53.479 ± 19.729	141.215 ± 22.135	426.067 ± 161.622 *
PGD2	COX	0.040 ± 0.037	0.020 ± 0.018	2.949 ± 2.037	4.027 ± 2.464	3.754 ± 3.809	7.132 ± 3.582 *
PGE2	COX	0.028 ± 0.024	0.028 ± 0.026	1.342 ± 0.470	2.043 ± 0.138 *	2.867 ± 0.617 *	4.982 ± 1.128 *
PGF2 alpha	COX	0.400 ± 0.071	0.448 ± 0.067	0.950 ± 0.204	1.513 ± 0.550 *	2.515 ± 0.142 *	3.455 ± 0.174 *
PGJ2	COX	0.054 ± 0.051	0.017 ± 0.029	2.783 ± 0.600	5.220 ± 1.618	13.085 ± 3.006	37.341 ± 14.232 *
PGK2	COX	0.000 ± 0.000	0.000 ± 0.000	0.007 ± 0.012	0.036 ± 0.033	0.124 ± 0.136	0.124 ± 0.076
Tetranor- PGEM	COX	1.469 ± 0.419	1.164 ± 0.225	0.936 ± 0.079	4.110 ± 4.514	1.568 ± 0.308	3.828 ± 2.058
Tetranor- PGFM	COX	1.096 ± 0.722	0.751 ± 0.421	0.403 ± 0.071	1.799 ± 2.273	0.517 ± 0.064	0.833 ± 0.388

TXB2	COX	0.000 ± 0.000	0.016 ± 0.028	0.015 ± 0.025	0.038 ± 0.066	0.000 ± 0.000	0.000 ± 0.000
12,13 diHOME	CYP	0.101 ± 0.027	0.088 ± 0.018	0.090 ± 0.010	0.098 ± 0.012	0.509 ± 0.699	0.112 ± 0.055
12,13 EpOME	CYP	0.037 ± 0.032	0.037 ± 0.064	0.014 ± 0.024	0.017 ± 0.029	0.045 ± 0.047	0.000 ± 0.000
13-HODE	LOX	0.338 ± 0.150	0.364 ± 0.118	0.480 ± 0.161	0.732 ± 0.312	0.985 ± 0.813	0.733 ± 0.243
13-oxoODE	LOX	1.002 ± 1.068	1.302 ± 1.362	0.863 ± 0.229	1.481 ± 0.566	17.041 ± 27.178	1.815 ± 0.793
15-oxoEDE	LOX	0.176 ± 0.305	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.744 ± 1.289	0.000 ± 0.000
8-HEPE	LOX	0.000 ± 0.000	0.000 ± 0.000	0.045 ± 0.039	0.000 ± 0.000	0.000 ± 0.000	0.056 ± 0.097
LXA5	LOX	0.539 ± 0.259	0.373 ± 0.124	0.829 ± 0.626	1.534 ± 1.452	2.363 ± 1.434	4.436 ± 2.381 *
8-iso PGF3 alpha	Non-enz	2.901 ± 0.979	3.514 ± 0.918	3.212 ± 3.010	4.261 ± 3.354	2.253 ± 1.019	4.472 ± 1.703
D17 6-keto PGF-1 alpha	COX	0.000 ± 0.000	0.000 ± 0.000	0.004 ± 0.008	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
PGD3	COX	0.000 ± 0.000	0.037 ± 0.065	0.157 ± 0.183	0.224 ± 0.205	0.757 ± 1.312	0.627 ± 1.086

TXB3	COX	3.786 ± 1.270	6.929 ± 2.280	3.336 ± 1.222	3.433 ± 0.322 *	3.768 ± 0.214 *	3.404 ± 0.056 *
13-oxoOTrE	LOX	0.000 ± 0.000	0.904 ± 1.566	0.000 ± 0.000	0.000 ± 0.000	28.138 ± 44.669	2.664 ± 4.614
13-HOTrE	LOX	2.646 ± 4.582	5.291 ± 9.164	9.259 ± 1.212	0.000 ± 0.000	12.698 ± 11.252	26.984 ± 17.514
9-HOTrE	LOX	0.000 ± 0.000	0.013 ± 0.023	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
17-keto DHA	LOX	0.000 ± 0.000	0.000 ± 0.000	0.052 ± 0.090	0.102 ± 0.176	0.000 ± 0.000	0.000 ± 0.000
10S,17S- DiHDoHE (PDX)	LOX	0.010 ± 0.009	0.003 ± 0.006	0.013 ± 0.013	0.010 ± 0.017	0.018 ± 0.019	0.000 ± 0.000
RvD2	LOX	1.000 ± 0.399	0.914 ± 0.389	0.694 ± 0.116	1.075 ± 0.356	0.669 ± 0.177	0.722 ± 0.235
19,20 DiHDoPE	CYP	0.053 ± 0.009	0.079 ± 0.069	0.042 ± 0.009	0.048 ± 0.048	0.032 ± 0.032	0.079 ± 0.088
20HDoHE	Non- enz/CYP	0.108 ± 0.188	0.000 ± 0.000	0.192 ± 0.332	0.000 ± 0.000	0.295 ± 0.511	0.000 ± 0.000
9-HODE	LOX	0.326 ± 0.132	0.310 ± 0.134	0.287 ± 0.057	0.525 ± 0.228	0.614 ± 0.485	0.423 ± 0.159
9-oxoODE	LOX	1.571 ±	1.175 ±	1.405 ±	1.646 ±	24.136 ±	1.790 ±

		1.235	0.546	0.352	0.585	39.195	1.332
9,10,13 triHOME	LOX/CYP	0.133 ± 0.118	0.183 ± 0.169	0.114 ± 0.016	0.182 ± 0.051	1.112 ± 1.643	0.303 ± 0.192
9,12,13 triHOME	LOX/CYP	0.120 ± 0.127	0.209 ± 0.253	0.101 ± 0.087	0.130 ± 0.123	1.295 ± 1.866	0.286 ± 0.270
9,10 diHODE	CYP	0.008 ± 0.007	0.010 ± 0.009	0.010 ± 0.009	0.002 ± 0.004	0.018 ± 0.005	0.009 ± 0.008
9,10 diHOME	CYP	0.063 ± 0.011	0.055 ± 0.006	0.045 ± 0.018	0.055 ± 0.006	0.231 ± 0.298	0.067 ± 0.024
9,10 EpODE	CYP	0.009 ± 0.008	0.004 ± 0.008	0.007 ± 0.006	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
9,10 EpOME	CYP	0.138 ± 0.078	0.066 ± 0.115	0.066 ± 0.114	0.000 ± 0.000	0.068 ± 0.119	0.000 ± 0.000
15-HETrE	LOX	0.023 ± 0.010	0.034 ± 0.023	0.071 ± 0.026	0.134 ± 0.036	0.225 ± 0.058 *	0.352 ± 0.062 *
8-HETrE	LOX	0.000 ± 0.000	0.000 ± 0.000	0.011 ± 0.020	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
PGF1 alpha	COX	0.507 ± 0.018	0.523 ± 0.118	0.558 ± 0.022	0.601 ± 0.067	0.686 ± 0.074	0.563 ± 0.094
TXB1	COX	0.287 ± 0.025	0.267 ± 0.34	0.349 ± 0.102	0.532 ± 0.211	0.609 ± 0.073	0.638 ± 0.117

15-keto PGF1 alpha	COX	0.207 ± 0.190	0.219 ± 0.094	0.281 ± 0.052	0.190 ± 0.178	0.251 ± 0.217	0.193 ± 0.229
Arachidonic Acid	NA	0.001 ± 0.001	0.002 ± 0.003	0.000 ± 0.000	0.001 ± 0.001	0.001 ± 0.001	0.003 ± 0.001
DHA	NA	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.088 ± 0.152	0.000 ± 0.000	0.000 ± 0.000
EPA	NA	0.085 ± 0.022	0.107 ± 0.095	0.042 ± 0.014	0.041 ± 0.009	0.040 ± 0.014	0.030 ± 0.013
17-keto DPA	LOX	0.003 ± 0.006	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000

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Figure S1

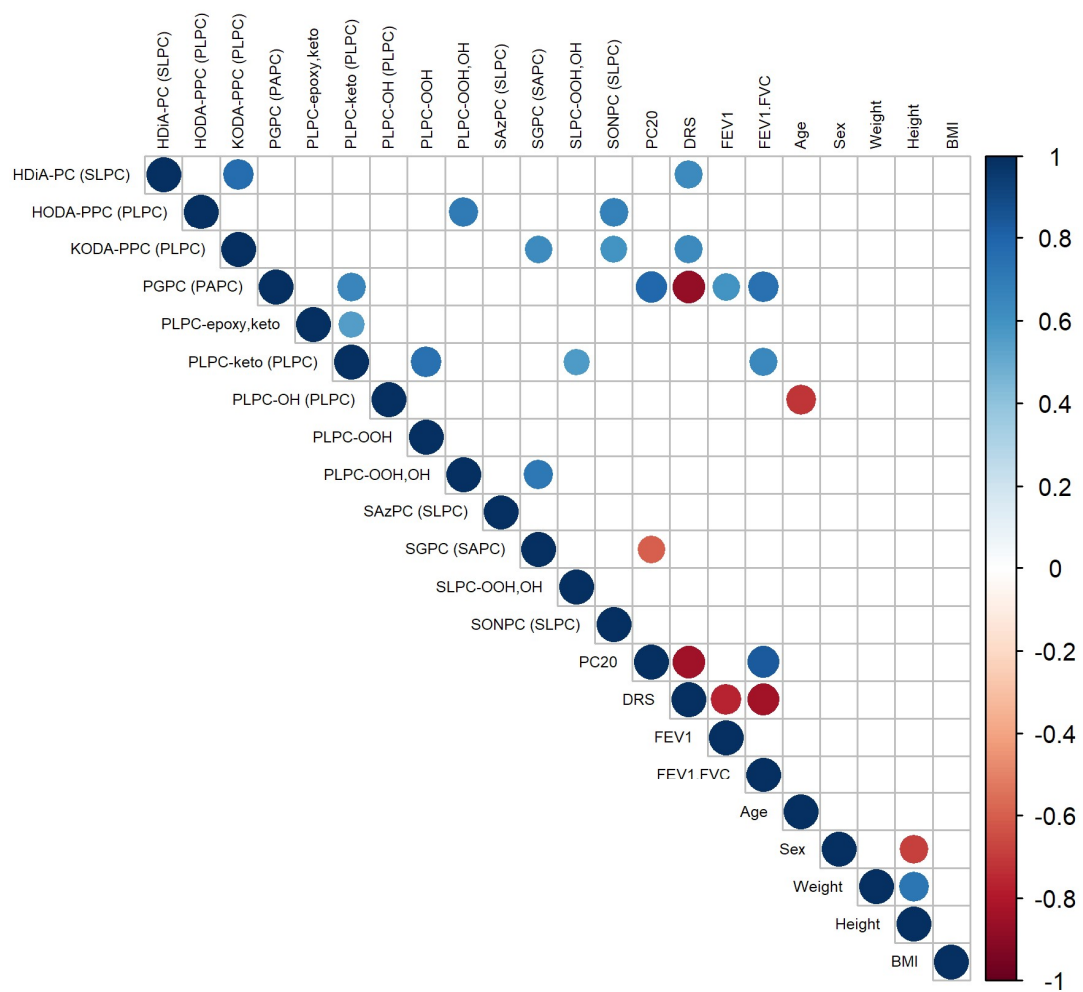


Figure S2

