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2-Chloro- and 2-Bromopalmitic acids inhibit mitochondrial function in airway epithelial cells

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Abstract

2-Chloropalmitic acid (2-ClPA) and 2-bromopalmitic acid (2-BrPa) increase in inflammatory lung disease associated with formation of hypochlorous or hypobromous acid, and exposure to halogen gases. Moreover, these lipids may elicit cell responses that contribute to lung injury, but the mechanisms remain unclear. Here, we tested the hypothesis that 2-ClPA and 2-BrPA induce metabolic defects in airway epithelial cells by targeting mitochondria. H441 or primary human airway epithelial cells were treated with 2-ClPA or 2-BrPA and bioenergetics measured using oxygen consumption rates and extracellular acidification rates, as well as respiratory complex activities. Relative to vehicle or palmitic acid, both 2-halofatty acids inhibited ATP-linked oxygen consumption and reserve capacity, suggestive of increased proton leak. However, neither 2-ClPA nor 2-BrPA altered mitochondrial membrane potential, suggesting proton leak does not underlie inhibited ATP-linked oxygen consumption. Interestingly, complex II activity was significantly inhibited which may contribute to diminished reserve capacity, but activity of complexes I, III and IV remain unchanged. Taken together, the presented data highlight the potential of 2-halofatty acids to disrupt bioenergetics and in turn cause cellular dysfunction.

Keywords

Chlorinated lipids; Brominated lipids; Mitochondrial respiration

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CRediT authorship contribution statement

Karina Ricart: Writing – review & editing, Methodology, Formal analysis, Data curation. **Kyle S. McCommis:** Writing – review & editing, Conceptualization. **David A. Ford:** Writing – review & editing, Writing – original draft, Methodology, Funding acquisition, Conceptualization. **Rakesh P. Patel:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.arres.2024.100118.

Introduction

Myeloperoxidase and eosinophil peroxidase enhance the reactive oxygen species oxidizing potential by converting hydrogen peroxide to hypochlorous acid and hypobromous acid in activated neutrophils and eosinophils, respectively [1,2]. Hypochlorous or hypobromous acids target host plasmalogens liberating 2-chloro and 2-bromofatty aldehydes, which are subsequently oxidized to 2-chloro and 2-bromofatty acids [3–6]. Halogenated lipids are also formed, at higher concentrations, after exposure to Cl_2 or Br_2 gas that may occur during accidental or intentional release settings [7–9]. Several distinct chloro- and bromo-lipids, of sixteen carbon (palmitate) or eighteen carbon (stearate) derived from initial 2-chloro- and 2-bromofatty aldehydes including fatty acids, fatty alcohols and fatty aldehyde-glutathione adducts have been detected in biological systems [10,11].

Detection of halogenated lipids can serve as a specific biomarker to indicate hypohalous acid production in disease or halogen gas exposure [8,12,13]. In particular, 2-chlorofatty acid levels are associated with acute respiratory distress syndrome and associated mortality in septic patients [13]. Levels of 2-chlorofatty acid in the lungs are also elevated within 4 h following the induction of sepsis in the rat cecal slurry model [14]. Moreover, biological effects of these lipids have also been reported; for example, we and others have shown that 2-chloropalmitate (2-ClPA) can stimulate endothelial and epithelial dysfunction ex vivo and in vivo; direct administration to the lung leads to reactive airways in mice [8,15–17]. Taken together, these findings suggest that halogenated lipids derived from plasmalogens may transduce cellular effects that contribute to inflammatory tissue injury after the initial insult (e.g. systemic infection or halogen gas exposure) has abated.

Less understood are the biochemical and molecular mechanisms by which halogenated lipids modulate cellular dysfunction. Previous studies have demonstrated that exposure of airway epithelial cells to Cl_2 gas induces bioenergetic dysfunction that was prevented by mitochondrially targeted antioxidants [18]. Similarly effects for this therapeutic approach have been noted for preventing hypochlorous acid toxicity also [19]. Moreover, our recent studies using the click chemistry analog of the 2-chlorofatty aldehyde, 2-chlorohexadecanal identified covalently modified proteins associated with pathways that affect cellular bioenergetics, which led to identifying 2-chlorohexadecanal inhibition of mitochondrial respiration and pyruvate metabolism in human small airway epithelial cells [16]. In this study, our goal was to better understand the mechanisms by which 2-chloro and 2-bromofatty acids attenuate mitochondrial function in airway epithelial cells.

Material and methods

Cell culture

Primary human bronchial epithelial cells (HBE) were isolated from tissue from healthy (nonsmoker) donors in the UAB Cystic Fibrosis Research Center Clinical and Translational Core according to UAB Institutional Review Board (IRB) approved protocols (IRB # 080,625,002). Cells were cultured in BEGM complete growth media (Lonza, Texas). Human airway H441 cells were cultured in RPMI 1640 media (11,875–093,Gibco) with 10 % FBS and 1 % penicillin-streptomycin. Cells were kept at 37 °C in a humidified incubator with 5

% CO_2. The 2-chlorofatty acid, 2-chloropalmitic acid (2-ClPA) was synthesized and purified as previously described [20]. The 2-bromofatty acid, 2-bromopalmitic acid (2-BrPA) was purchased from Sigma (Cat # 238,422) and stock solutions of 100 mM prepared in ethanol.

Cellular bioenergetics

Oxygen consumption rates (OCRs) and extracellular acidification rates (ECAR) were determined using a Seahorse Extracellular flux analyzer (XF96) (Agilent, Santa Clara, CA). Cells were cultured in Seahorse 96-well plate at 25,000 cells / well in regular growth media. After two days, the media was removed and replaced with Seahorse assay media (DMEM (90–113-PB, Corning); 5 mM Glucose; 4 mM l-glutamate; 1 mM pyruvate, pH 7.4). OCR and ECAR were measured every 10 min for 2.5 h At each measurement time, mixing was performed for 3 min, followed by 2 min equilibration and then measurement of OCR and ECAR over 3 min. Ethanol (<4μl/ml) PA, 2-ClPA or 2-Br-PA (1μM,10 μM or 25 μM) were added after 30 min of starting OCR and ECAR measurements. After 2.5 h of treatment with lipids, oligomycin (1 μg/ml), FCCP (1 μM), and antimycin A (10 μM) were injected sequentially and effects on OCR and ECAR measured the addition of each compound as previously described [16]. ATP linked OCR was calculated as the difference in OCR before and after addition of oligomycin; maximal OCR was calculated as a difference in OCR with FCCP and antimycin A. The OCR after oligomycin is ascribed to proton leak. The bioenergetic reserve capacity is the difference between the maximal and basal OCR. The remaining OCR after antimycin A reflects non-mitochondrial processes that consume oxygen. The glycolysis reserve capacity is the ECAR measured after the injection of oligomycin. Cells were observed by light microscopy before and after each assay; there were no evident changes in cell viability over the indicated time frames (not shown). In some experiments, cells were pre-incubated with 50 μM genipin (to inhibit uncoupling protein 2 (UCP2)) or 2 μM cyclosporin A (to inhibit the permeability transition pore) 30 min before the lipid injection. Data are expressed as a percentage of the basal OCR or ECAR after 2.5 h to allow comparison between experimental replicates. Each experiment was conducted at least 3 times, with 3–6 replicates within each experiment.

Mitochondrial respiratory complex activity

Cells were permeabilized with 2 nM XF plasma membrane permeabilizer (XF PMP, Agilent, Santa Clara, CA) and substrates added to measure complex I, II, III or IV activity [21]. Briefly, for complex I activity, cells were permeabilized in the presence of pyruvate (10 mM) and malate (1 mM) with FCCP (0.6 μM) in MAS buffer (70 mM sucrose, 220 mM mannitol, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 2 mM HEPES, and 1 mM EGTA, pH 7.2). OCR was measured 3 times and then the complex I inhibitor rotenone was injected (1 μM) and OCR re-measured; Activity of complex I was calculated as the difference in OCR before and after rotenone. For complex II activity, cells were permeabilized in the presence of succinate (10 mM), rotenone (1 μM), FCCP (0.6 μM) in MAS buffer and activity calculated as the difference in OCR before and after addition of the complex III inhibitor antimycin A (10 μM). For complex III activity, cells were permeabilized in the presence of duroquinol (0.5 mM), rotenone (1 μM), FCCP (0.6 μM) in MAS buffer and activity assessed before and after antimycin A. For complex IV, ascorbate (2 mM) and N,N,N’,N’-tetramethyl-p-phenylenediamine (TMPD, 0.5 mM), and FCCP (0.6 μM) were added and activity measured

before and after addition of sodium azide (20 mM). All data are expressed as a percentage of control (vehicle).

Measurement of mitochondrial membrane potential (ΔΨ)

Mitochondrial inner membrane electrochemical potential (ΔΨ) was measured using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazo-lylcarbocyamine iodide (JC-1) (Invitrogen) or tetramethylrhodamine (ThermoFisher). For JC-1, cells were treated with 2-ClPA or 2-BrPA for 2.5 h in Seahorse media, and then replaced with Seahorse media containing JC-1 (7.4 μM, 30 min). Cells were then washed with the same media and fluorescence measured red (535/590 nm) and green (485/528 nm) using a fluorescence plate reader (Synergy H4, Agilent, Santa Clara, CA). The ratio of red to green fluorescence was calculated and data expressed as percentage of control (cells exposure to vehicle). Additionally, cells were treated in Seahorse media as described above and incubated with TMRM (100 nM) for 30 min. After washing with media, cells were imaged with texas red filter cube (586/647) in a Lionheart FX (Agilent, Santa Clara, CA) (10X). For these experiments, FCCP (10 μM) was added at the same time with JC-1, as a positive control.

DMNQ treatment

H441 cells were treated with vehicle, PA, 2-ClPA or 2-BrPA (each at 25 μM) for 2.5 h in seahorse media and then treated with vehicle (ethanol) or 2,3-dimethoxy-1,4-naphthalenedione (DMNQ) (300 μM; DMNQ concentration was calculated using ε_{340nm}=2770cm $^{-1}$M^{-1}) for a further 12 h and then viability assessed.

ATP levels

H441 cells were cultured in RPMI 1640 media until confluent, then changed to seahorse media and treated with vehicle, PA, 2-ClPA or2-BrPA (each at 25 μM). After 2.5 h equal volume of CellTiter-Glo 2.0 reagent (Promega, Madison, WI) was added and contents mixed for 2 min on an orbital shaker to induce cell lysis. Lysates were incubated at room temperature for 10 min and then luminescence measured in a Synergy H4 hybrid reader (Agilent Bio-tek, CA).

Viability assay

Cell viability was measuried by lactate dehydrogenase (LDH) release. Media was collected and cell lysates prepared by adding 40 μl of ice-cold lysis buffer (PBS, 0.1 % triton X-100) and incubation on ice for 5min. Lysates (5μl) or culture media (200 μl) were incubated with NADH (300 μM) and pyruvate (10mM). The conversion of NADH into NAD was followed for 4 min at 340 nm in a UV–Vis spectrophotometer. Percent cell survival was calculated by LDH activity in the lysates divided by total LDH activity (lysates+ media) x100.

Protein assay

Cell lysates were prepared by rinsing plates with ice cold phosphate buffered saline (PBS) and incubating on ice for 5 min with 80 μl of lysis buffer (10mM tris, 1 % triton, 1X cOmplete Protease inhibitor cocktail (Sigma) pH 7.4). Protein concentrations were measured by the Bradford assay (Bio-Rad) using a BSA standard curve.

Statistical analysis

All results are expressed as mean ± SD. Each data point represents an average of an independent experiment, with 3–6 technical replicates within each experiment. One way ANOVA with Tukey's multiple comparison test was used to assess significant differences, with $p < 0.05$ indicating a significant difference. All statistical analyses were performed with GraphPad Prism 9.

Results

Effects of 2-ClPA and 2-BrPA on oxygen consumption rates in H441 cells

H441 cells were treated with 2-ClPA or 2-BrPA and effects on oxygen consumption kinetics and pH measured to assess mitochondrial metabolic or glycolysis flux. Data in Fig. 1A show representative traces of oxygen consumption rates (OCR) after addition of ethanol vehicle or 25 μM of either palmitic acid (PA), 2-ClPA or 2-BrPA. Rates were measured over 2.5 h, after which the effects of oligomycin and then FCCP were assessed to determine the role of ATP synthase and maximal rates of respiration, respectively. Several parameters related to the role of mitochondrial function and glycolytic flux were calculated from these data with salient observations being: i) neither 2-ClPA nor 2-BrPA affected basal OCR (Fig. 1B); ii) oligomycin dependent decreases in the OCR were significantly attenuated in cells treated with 2-ClPA or 2-BrPA resulting in close to complete inhibition of ATP-linked respiration (Fig. 1C) and suggestive of increased proton leak (Fig. 1D); iii) both 2-ClPA and 2-BrPA inhibited maximal respiration by ~50 % (Fig. 1E); iv) the reserve capacity (calculated by the differences between groups in FCCP-dependent maximal versus basal OCR) was greatly diminished by 2-ClPA and 2-BrPA (Fig. 1F); and vi) neither halofatty acid affected non-mitochondrial OCR indicating their effects are selective to mitochondrial respiration (Fig. 1G).

Data in Fig. 1H show representative traces for extracellular acidification rates (ECAR), a parameter that reflects glycolytic flux. Compared to the parent lipid, both 2-ClPA and 2-BrPA increased basal ECAR after 2.5 h (Fig. 1I). The glycolytic reserve capacity, determined after adding oligomycin and comparing to basal, was also calculated (Fig. 1J). Both 2-ClPA and 2-BrPA significantly inhibited this parameter. Under these conditions, all cells remained viable (based on light microscopic assessment). Also, no changes in protein levels were observed across treatments (Ethanol: 3.97 ± 0.41, PA: 3.63 ± 0.25, 2-ClPA: 4.3 ± 0.35, 2-BrPA: 3.6 ± 0.06; all values in mg/ml and mean ± SD).

Supplementary Fig. 1A–H show effects of lower doses, 1 μM and 10 μM 2-ClPA and 2-BrPA on bioenergetic function in H441 cells. Similar effects on inhibiting ATP-linked and maximal OCR, and reserve capacity with concomitant increases in proton leak were observed with both 2-ClPA and 2-BrPA at 10μM. No effect of either lipid was observed at the lowest dose tested.

2-ClPA and 2-BrPA do not affect mitochondrial membrane potential

The lack of inhibition in OCR by oligomycin suggests increased proton leak, and if so, lower mitochondrial membrane potentials are expected. However, Fig. 2A–B show that

neither lipid affected membrane potential measured by JC-1 or TMRM, whereas the positive control, FCCP led to significant inhibition. Moreover, genipin (50 μM), an inhibitor of UCP2 had no effect on proton leak (mean percent proton leak relative to basal were 71.9 % and 77.3 % for 2-ClPA without and with genipin respectively, and 67.5 % and 67.8 % for and 2-BrPA without and with genipin, respectively). Similarly, cyclosporin A (CsA, 2 μM), an inhibitor of the mitochondrial permeability transition pore had no effect (mean percent proton leak relative to basal were 70.8 % and 76.1 % for 2-ClPA without and with CsA, and 49.9 % and 57.5 % for and 2-BrPA without and with CsA respectively).

Effects of 2-ClPA and 2-BrPA on respiratory chain complex activities

We next measured activity of Complexes I, II, III and IV. Fig. 3 shows that neither 2-ClPA nor 2-BrPA affected complex I, III or IV activity. However, complex II activity was significantly inhibited by 2-BrPA, with effects of 2-ClPA being close to significant (p = 0.077 relative to PA).

Effects of 2-ClPA and 2-BrPA on ATP and cell viability after stress

Fig. 4A shows that ATP levels were not affected by treatment with 2-ClPA whereas 2-BrPA decreased levels slightly (~15 %) compared to FCCP, which completely ablated ATP levels. While these data suggest minimal or no ATP deficit, potential changes in ATP flux may still be occurring. To gain insights into whether 2-ClPA or 2-BrPA mediated changes in epithelial cell bioenergetics modulate cellular responses to secondary stress, we pre-treated H441 cells (in seahorse media) with vehicle, 2-ClPA or 2-BrPA (25 μM) for 2.5 h and then assessed DMNQ dependent toxicity (measured at 12 h post treatment). Fig. 4B shows that 2-ClPA alone induced ~50 % toxicity under these conditions whereas 2-BrPA had little effect. At the dose tested, DMNQ alone had little affect on viability at the time tested; however in combination with either 2-ClPA or 2-BrPA viability decreases to <10 %, suggesting that both 2-ClPA and 2-BrPA sensitize cells to a secondary stress.

Effects of 2-ClPA and 2-BrPA on oxygen consumption rates in primary airway epithelial cells

Finally, to validate observations with H441 cells, we repeated experiments using primary human airway epithelial cells. Data in Fig. 5A–J show that 2-ClPA and 2-BrPA (25 μM) treatment led to similar changes in OCR and ECAR in these cells with significant increases in proton leak, decreases in ATP-linked and maximal respiration, decreased reserve OCR capacity, increased basal ECAR, and decreased glycolytic reserve capacity noted. Supplementary Fig. 2 shows that lower doses of 2-ClPA (10μM) but not 2-BrPA elicited similar effects in inhibiting OCR and reserve capacity and increasing proton leak. Trends towards decreased glycolytic reserve capacity were also observed (p < 0.07).

Discussion

Neutrophils are early immune responders during sepsis and we have shown 2-chlorofatty acid levels are elevated in plasma in human sepsis and in the lung and plasma of rodent sepsis models [13,14]. Furthermore, in human sepsis elevated plasma levels of 2-chlorofatty acid associate with acute respiratory distress syndrome. 2-Chlorofatty acid also has profound

effects on human lung microvascular endothelial cells leading to barrier dysfunction [13]. Lung injury with 2-chlorofatty acid accumulation also occurs with Cl_2 exposure [8,22]. Similarly Br_2 gas is toxic with increased lung inflammation and permeability barrier dysfunction being primary causes of morbidity and mortality [12]. Previously, we and others demonstrated that exposure to Cl_2 or Br_2 gas results in the formation of chlorinated or brominated proteins (on tyrosine) and lipids [7,8,23], and we have further suggested that 2-ClPA/2-BrPA, and corresponding aldehyde derivatives are specific mediators of post halogen gas exposure injury. Based on their potential roles as mediators of sepsis and halogen associated lung injury, we tested whether airway epithelial bioenergetics are affected by 2-ClPA and 2-BrPA.

Both 2-ClPA and 2-BrPA similarly induced bioenergetic dysfunction characterized by uncoupling, decreased maximal respiration and reserve capacity. A concomitant increase in glycolysis flux was observed, a classical cellular response to ensure adequate ATP levels when mitochondrial ATP production is compromised. The lack of a decrease in OCR in the presence of oligomycin suggests increased proton leak. Surprisingly however, no decreases in membrane potential were observed. While we cannot exclude small changes in membrane potential that are undetectable within the sensitivity of the assays used, a lack of membrane potential decrease argues against elevated proton leak. Supporting this conclusion were data that neither UCP2 nor permeability pore transition inhibition prevented 2-ClPA or 2-BrPA effects. We did not test other potential pathways of proton leak including activation of the adenine nucleotide transporter or possibly direct intercalation of 2-halofatty acids into mitochondrial membranes, but note that if applicable these should also have led to decreases in membrane potential. Also, we and others have shown the click chemistry analog of 2-ClPA localizes to the mitochondria in human coronary endothelial cells and hCEM/D3 cells (a human brain microvascular endothelial cell line) [24,25]. Interestingly, in brain microvascular endothelial cells, 2-ClPA did decrease mitochondrial membrane potential [24]. The differences in membrane potential effect could be due to the cell type (endothelial versus epithelial) and/or organ (brain versus lung) differences. Another possible explanation for why no changes in membrane potential were observed in airway epithelial cells, is that 2-ClPA and 2-BrPA modify mitochondria so that oligomycin can no longer bind nor inhibit ATP synthase. Oligomycin binds to the proton-channel F_0 ATP synthase subunit, and our recent studies with 2-chloropalmitaldehyde showed that not only is mitochondrial metabolism modulated, but identified the F_o submit of ATP synthase as one (of many) potential target modified by these lipids [16]. Direct modification and inhibition of ATP synthase by 2-ClPA and 2-BrPA may also explain why basal ECAR increases. We cannot exclude other explanations for these results including that 2-ClPA and 2-BrPA increase the rate of respiration or a competing pathway that increases membrane potential. The relatively unique profile of increased OCR when ATP synthase is inhibited, without changes in membrane potential warrants further investigation.

A key observation was that both 2-ClPA and 2-BrPA significantly decreased the reserve capacity. A larger reserve capacity is associated with enhanced capacity for cells to withstand stress. Indeed, we observed that 2-ClPA or 2-BrPA pretreatment sensitized epithelial cells to killing by DMNQ which generates superoxide/ hydrogen peroxide via redox cycling. The observation that reserve capacity was essentially depleted in 2-ClPA and

2-Br-PA treated cells suggests that these lipids will sensitize epithelial cells to injury, and or limit recovery/ resolution, all possible features of sepsis or post-halogen gas toxicity [14,26].

Our results also demonstrate that complex II activity was significantly inhibited by 2-BrPA with a trend towards a decrease with 2-ClPA. Complex II does not pump protons from the mitochondrial matrix to intermembrane space likely precluding any direct role of complex II inhibition on increased oligomycin-sensitive OCR. Interestingly, polychlorinated biphenyls, a class of environmental toxins, inhibit complex II activity leading to increased cell death [27], and conversely metabolic sensors including AMP kinase, activate complex II activity leading to increased bioenergetic reserve capacity and survival of cardiac myocytes to hypoxic stress [28]. We posit that the lower reserve capacity in cells exposed to 2-ClPA or 2-BrPA is secondary to inhibition of complex II activity. Proteomics studies with the click chemistry analog of 2-ClPA have not identified direct modification of complex II. However, Davda et al. using the click chemistry analog of 2-BrPA demonstrated 2-BrPA covalently modifies the succinate dehydrogenase C and D subunits of complex II in HEK293T cells [29]. Since 2-BrPA is a stronger electrophilic lipid compared to 2-ClPA, it will be of interest to examine differences in succinate dehydrogenase modification by 2-ClPA and 2-BrPA in future studies using primary human lung epithelial cells. Indeed, we have observed major differences in the extent of protein modification of 2-bromofatty aldehyde compared to that of 2-chlorofatty aldehyde [7]. Further experiments comparing the potency by which 2-ClPA or 2-BrPA modulated bioenergetic function are warranted. Another consideration is which compartments these lipids localize to. Previous studies and those of others with endothelial cells demonstrate that 2-ClPA localizes to the mitochondria [24,30]. We posit a similar effect in airway epithelial cells may underlie the complex II inhibition. It should be noted that complex II inhibition can also lead to protective effects by inhibiting superoxide formation (e.g. in cardioprotection) [31]. While our studies were not designed to test this directly, future studies need to determine whether 2-ClPA or 2-BrPA modulate complex II activity in other cells and tissues, and if so discern functional consequences.

2-Bromofatty acid is a known inhibitor of fatty acid oxidation and protein palmitoylation [29,32,33]. 2-Chlorofatty acid also interferes with protein palmitoylation and in hCEM/D3 cells decreases the mitochondrial membrane potential [24]. The results herein provide new insights into the role of 2-halofatty acids in cell metabolic function by detailing their role as modulators of lung epithelial cell mitochondrial respiration. It remains to be determined which proteins are modified by these 2-halofatty acids in epithelial cells, which may explain differences in 2-chlorofatty acid alterations in mitochondrial membrane potential in hCEM/D3 endothelial cells and primary human lung epithelial cells.

In conclusion, this study shows that 2-chloropalmitic acid and 2-bromopalmitic acid alter mitochondrial respiration and glycolysis in lung epithelial cells, which may contribute to epithelial dysfunction and lung injury in settings where these halogenated lipids are increased.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

The original data is available in Chemical Effects in Biological Systems (CEBS) database.

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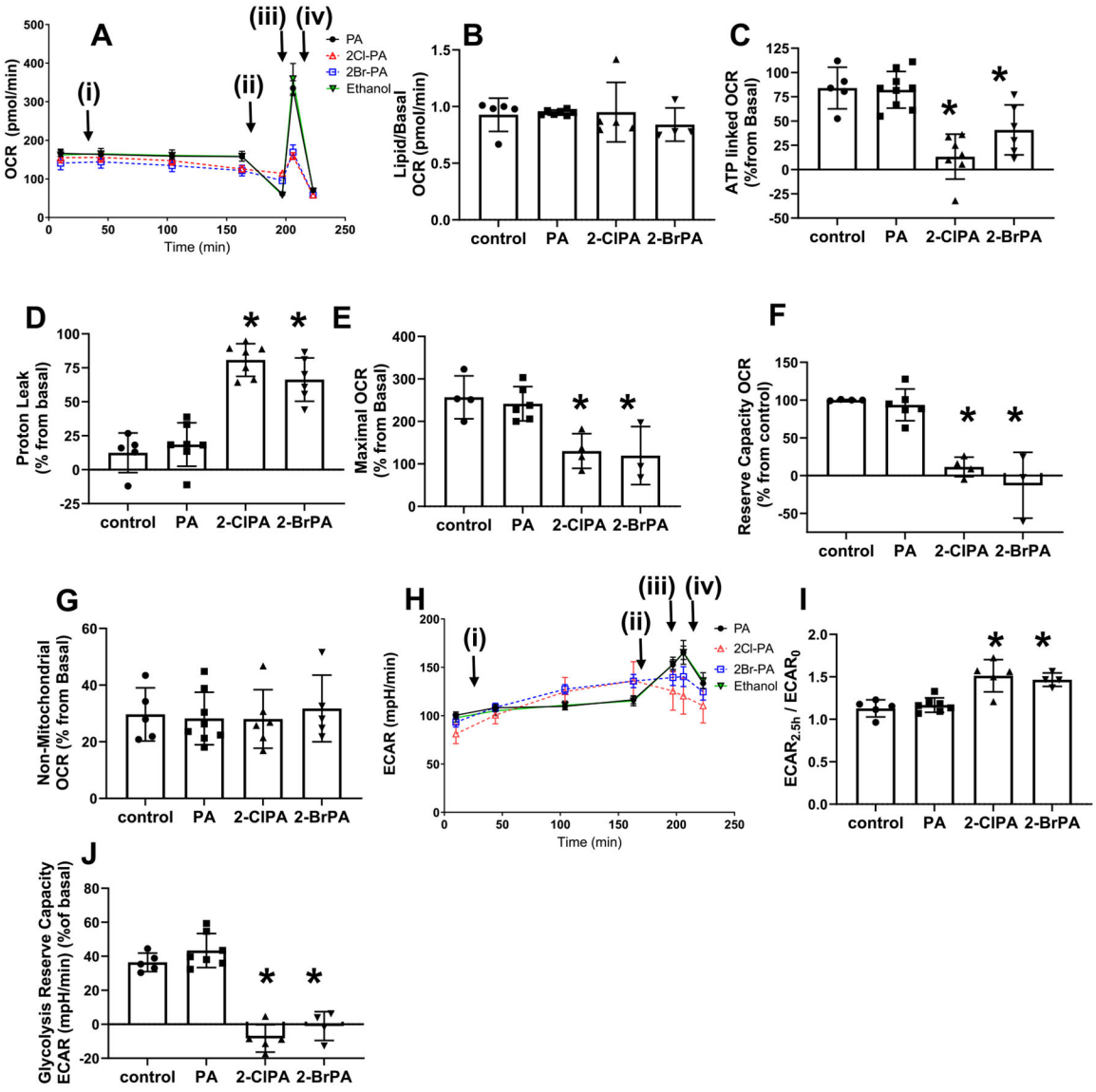

Fig. 1. Effects of 2-ClPA or 2-BrPA on Bioenergetics in H441 Cells.

H441 cells were exposed to vehicle (ethanol), PA, 2-ClPA or 2-BrPA (each at 25 μM added at 25 min) (i) and oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) measured over 4 h Oligomycin (1 μg/ml, **ii**), FCCP (1 μM, **iii**) and antimycin A (10 μM, **iv**) were injected sequentially. (**A**) Representative average tracing from 6 replicates, from a single experiment, for changes in OCR. (**B**) Change in basal OCR after 2.5 h in the presence of lipids. (**C**) ATP-linked respiration calculated by the difference between OCR basal and OCR after oligomycin. (**D**) Proton Leak (**E**) Maximal OCR after FCCP. (**F**) Reserve Capacity determined by the differences between OCR after FCCP and basal OCR. (**G**) Non-mitochondrial OCR after antimycin A. (**H**) Representative tracing for changes in ECAR. (**I**) Changes in ECAR at 2.5 h relative to basal. (**J**) Glycolysis reserve capacity calculated after oligomycin. Data shown are means ± SD. Each data point represents mean from an independent experiment with 3–6 replicates per experiment. *$p < 0.05$ by one-way ANOVA with Tukey's multiple comparison post test relative to vehicle and PA.

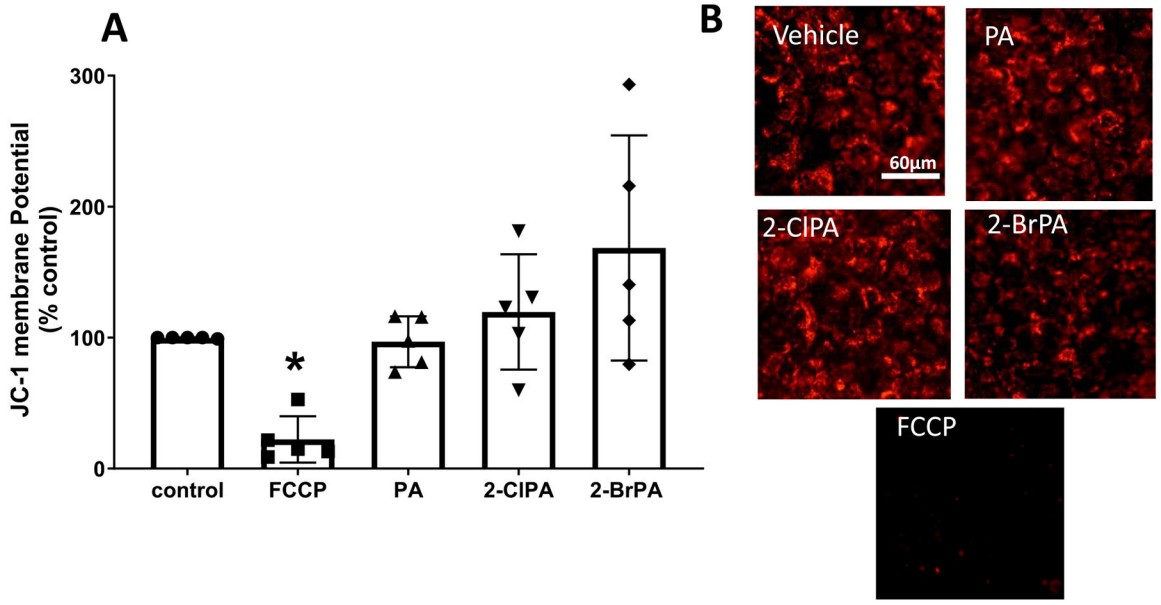

Fig. 2. Effects of 2-ClPA or 2-BrPA on Membrane Potential in H441 Cells.

H441 cells were exposed to vehicle, PA, 2-ClPA or 2-BrPA (each at 25 μM, for 2.5 h) and membrane potential measured by JC-1 or TMRM. (A) JC-1 fluorescence (red/green) (B) representative images of TMRM fluorescence. Data shown are means ± SD. Each data point represents mean from an independent experiment with 3 replicates per experiment. *$p < 0.05$ by one way ANOVA with Tukey's multiple comparison post hoc test relative to control.

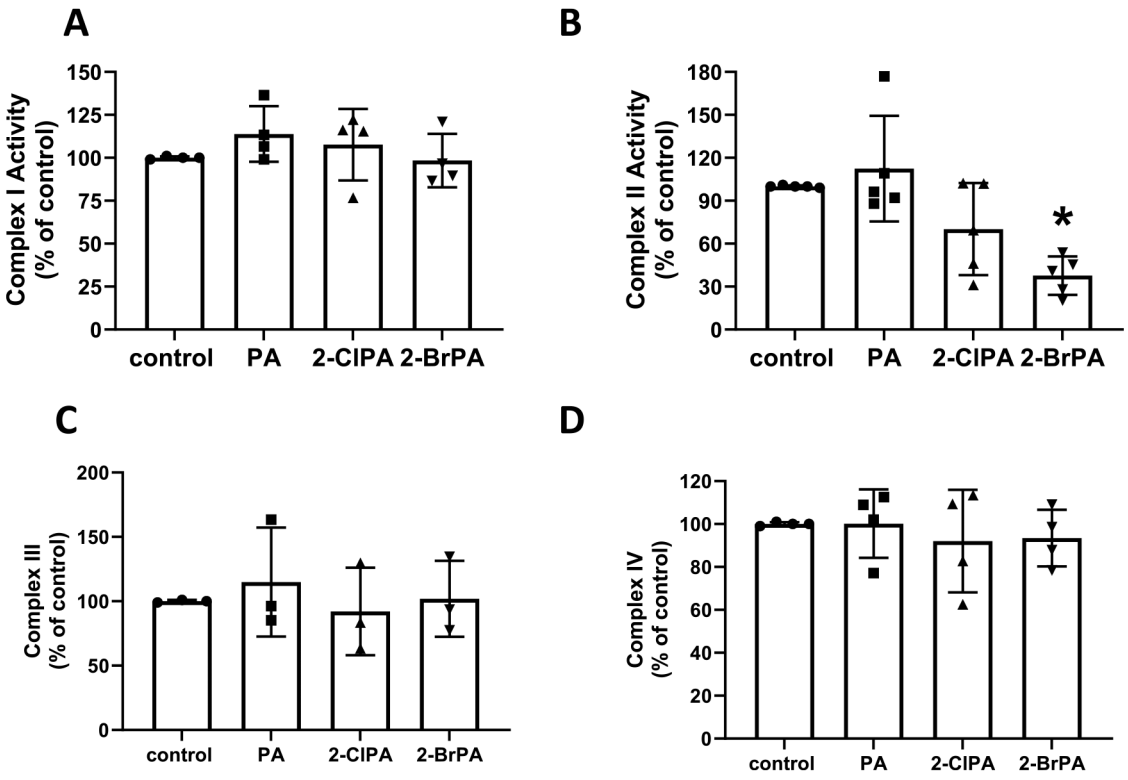

Fig. 3. Effects of 2-ClPA or 2-BrPA on respiratory complex activities in H441 cells.

H441 cells were exposed to vehicle, PA, 2-ClPA or 2-BrPA (each at 25 μM, for 2.5 h) and respiratory complexes I (A), II (B), III (C) and IV (D) measured as described in methods. Data shown are means ± SD. Each data point represents mean from an independent experiment with 3–6 replicates per experiment. *$p < 0.05$ by one-way ANOVA with Tukey's multiple comparison post hoc test relative to vehicle and PA.

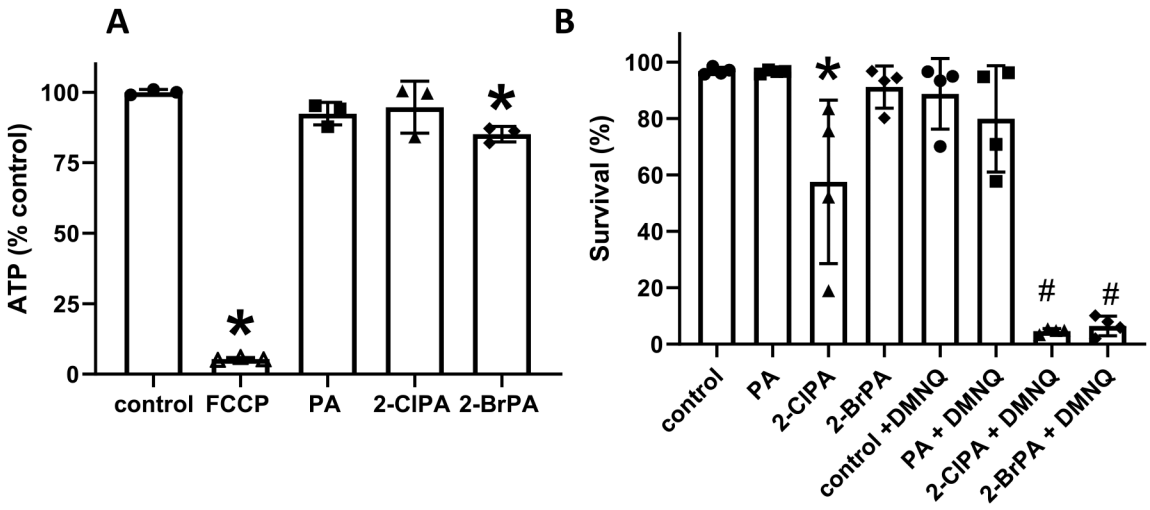

Fig. 4.

(A) Relative ATP levels (to control). (B) H441 cells were treated with vehicle, PA, 2-ClPA or 2-BrPA (each at 25 μM) in seahorse media. After 2.5 h cells were left untreated or treated with 2,3-dimethoxy-1,4-naphthalenedione (DMNQ) (300 μM) for 12 h and cell viability measured. Data shown are means ± SD. Each data point represents mean from an independent experiment. *$p < 0.05$ by one way ANOVA with Tukey's multiple comparison post hoc test relative to control, or #$p < 0.05$ relative to DMNQ alone.

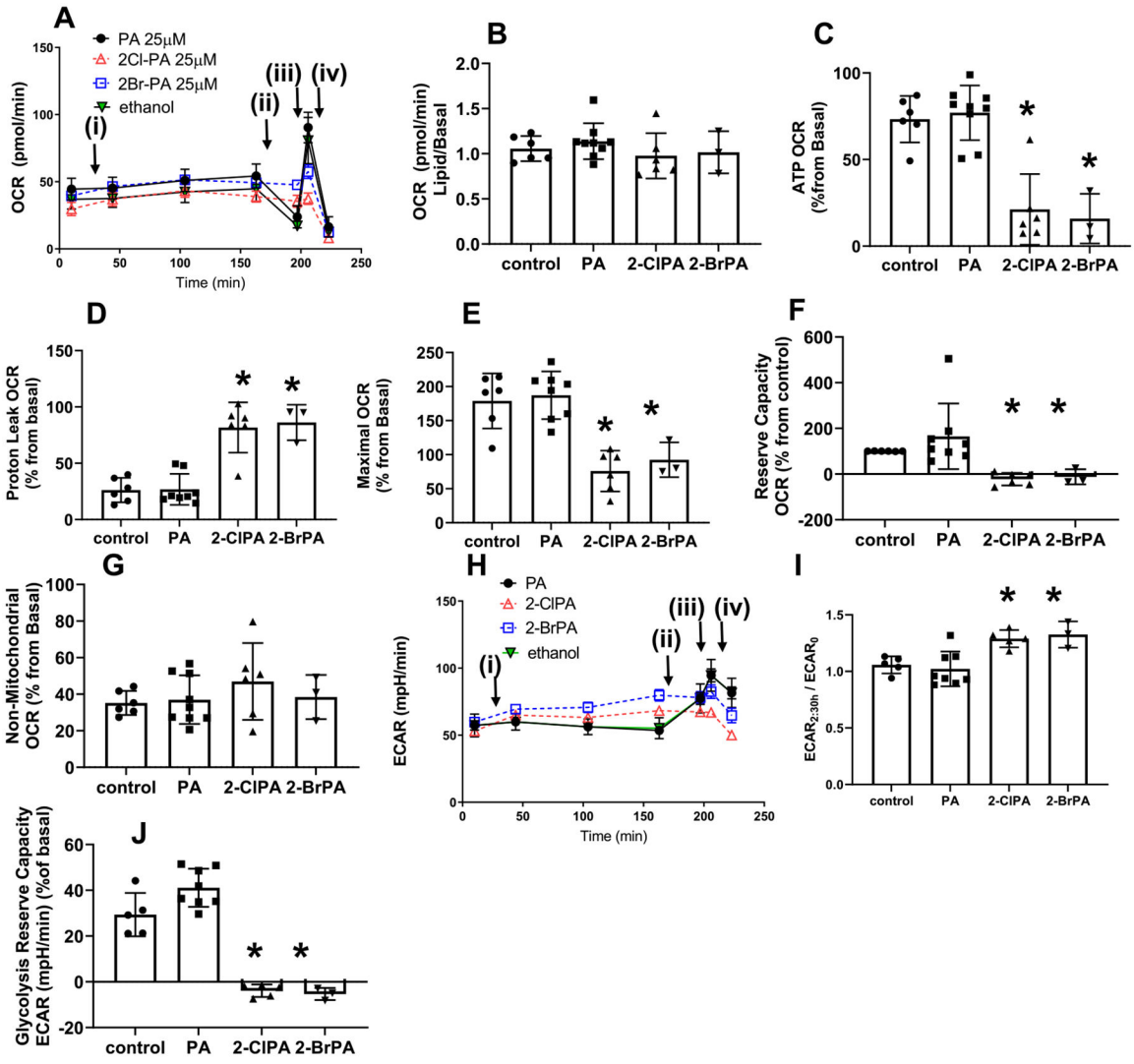

Fig. 5. Effects of 2-ClPA or 2-BrPA on Bioenergetics in Primary Human Airway Bronchial Epithelial Cells.

Cells were exposed to vehicle (ethanol), PA, 2-ClPA or 2-BrPA (each at 25 μM and added at 25 min) (i) and oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) measured over 4 h Oligomycin (1 μg/ml, ii), FCCP (1 μM, iii) and antimycin A (10 μM, iv) were injected sequentially. (A) Representative tracing for changes in OCR. (B) Change in basal OCR after 2.5 h in the present of lipids. (C) ATP-linked respiration and (D) proton leak. (E) Maximal OCR after FCCP. (F) Reserve capacity determined by the differences between OCR after FCCP and basal OCR. (G) Non-mitochondrial OCR after antimycin A. (H) Representative tracing for changes in ECAR. (I) Changes in ECAR at 2.5 h relative to basal. (J) Glycolysis reserve capacity calculated after oligomycin. Data shown are means ± SD. Each data point represents mean from an independent experiment with 3–6 replicates per experiment. *$p < 0.05$ by one-way ANOVA with Tukey's multiple comparison post hoc test relative to vehicle and PA.