The Small Molecule Antipsychotic Aripiprazole Potentiates Ozone-Induced Inflammation in Airway Epithelium

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Abstract

Inhaled ground level ozone (O_3) has well described adverse health effects, which may be augmented in susceptible populations. While conditions, such as pre-existing respiratory disease, have been identified as factors enhancing susceptibility to O_3-induced health effects, the potential for drug x pollutant interactions in sensitizing populations or further exacerbating preexisting conditions has not yet been studied. In the airway, inhaled O_3 is able to interact with the lipid rich airway lining to generate reactive and electrophilic oxysterol species capable of causing cellular dysfunction and inflammation. Therefore, drugs which modify cholesterol synthesis could play a deleterious biological role in the presence of O_3. Recent chemical analysis studies established the capacity for a variety of small molecule antipsychotic drugs, like Aripiprazole (APZ), to elevate circulating 7-dehydrocholesterol (7-DHC) levels by competitively inhibiting 7-dehydrocholesterol reductase, the enzyme involved in the final step of cholesterol biosynthesis. 7-DHC serves as a highly reactive target for lipid peroxidation and increased oxidative stress. Our results show that APZ administered at clinically relevant concentrations increase 7-DHC levels and potentiate O_3-induced pro-inflammatory cytokine expression in airway epithelial cells. Additionally, we find that ozonized 7-DHC enhances *IL-8* expression more than ozonized cholesterol. Overall, the evidence we provide describes a potential mechanistic basis for a drug x pollutant interaction between APZ and O_3, posing significant adverse public health implications considering the prevalence of O_3 and APZ use.

Introduction

Despite the capacity for certain drugs to biochemically alter the targets for pollutants and potentially exacerbate injury, little research has been conducted on the subject. Previous studies

have demonstrated that in mice, a combination of supratherapeutic doses of acetaminophen with ozone (O_3) exacerbate drug-induced liver injury, while a combination of acetaminophen with environmental tobacco smoke exposure exacerbates respiratory oxidative stress responses (1, 2). Nonetheless, the inflammatory potential of other drug x O_3 interactions in the lung remains completely unknown. Currently, more than a third of the U.S. population lives in areas exceeding the 2015 National Ambient Air Quality Standard for O_3 of 0.07ppm for 8-hour average, which are exposure levels known to decrease lung function and cause inflammatory responses in humans (3-5). O_3 is a highly reactive oxidant gas, capable of damaging organic molecules in human tissue. More specifically, O_3 is capable of reacting with pulmonary surfactant and epithelial cell membranes to produce lipid peroxides and oxidant species (6). In the airway, the O_3-induced oxidation of cholesterol is known to lead to the formation of electrophilic oxysterols, capable of forming protein adducts, potentially perturbing normal cellular signaling, and increasing inflammation (7-9). Therefore, it would be important to pay specific interest to drug-induced cholesterol modification and its effect on modulating O_3-induced inflammation in the lung. Indeed, significant epidemiological data exists indicating that cholesterol-modifying drugs mitigate oxidant stress and air pollutant-induced adverse health effects, yet, little clinical evidence has been presented specifically for O_3 (10, 11).

In particular, one of the final steps in the synthesis of cholesterol involves the reduction of 7-dehydrocholesterol (7-DHC) to cholesterol by the enzyme 7-dehydrocholesterol reductase (DHCR7). The precursor molecule to cholesterol, 7-DHC, contains two double bonds on the primary carbon ring, making it more susceptible to O_3-induced oxidation and lipid peroxidation chain propagation (12). Considering the high ozonization potential of this sterol, any cell or fluid having elevated levels of 7-DHC could be sensitized to O_3-induced oxidative stress (13).

Consequently, the effects of drugs which modify cholesterol synthesis by elevating the 7-DHC/Cholesterol ratio needs to be understood.

In the United States, small molecule antipsychotic and antidepressant drugs are widely prescribed. In 2014, the drug Aripiprazole (APZ), often prescribed off-label, accounted for 8 billion dollars in sales and was ranked 2nd in the U.S. for top prescription drugs (14). Notably, APZ reduces the activity of DHCR7 in an off-target effect, thereby modifying cholesterol metabolism and leading to accumulation of 7-DHC (15, 16). However, whether drugs, such as APZ, known to modify cholesterol synthesis leading to the accumulation of reactive precursors, could potentially exacerbate O_3-induced inflammation, presents a knowledge gap.

Thus far, the potential for drug x pollutant interactions in sensitizing populations to ground level O_3 has not yet been studied (17). The increased prevalence of O_3 air pollution and prescription small molecule drugs, such as APZ, further illuminates the scope and capacity for this adverse drug x pollutant interaction. Our study is designed to uncover the impact of the cholesterol-modifying drug APZ on O_3-induced inflammation in the human airway. We hypothesize that APZ inhibition of DHCR7 increases pulmonary levels of 7-DHC, leading to the formation of highly reactive oxysterol species and potentiation of O_3-induced inflammation.

Methods

Cell Culture – 16HBE14o (16HBE) cells, a SV-40 transformed human bronchial epithelial cell line were a gift from Dr. D. C. Gruenert (University of California San Francisco, San Francisco, CA). For experiments performed at air-liquid interface (ALI), 16HBE cells were plated on fibronectin-coated (LHC Basal Medium [Life Technologies, Carlsbad, CA], 0.01% BSA [Sigma, St. Louis, MO], 1% Vitricol [Advanced Bio Matrix, San Diego, CA], and 1%

human fibronectin [BD Biosciences, San Jose, CA]) 0.4μm Transwell® plates(Costar, Corning, NY), and grown submerged in minimal essential media (Gibco) with 10% FBS, 1% penicillin-streptomycin, and 1% L-glutamine (Life Technologies) until confluent for 6 days. For experiments performed with submerged cultures, 16HBEs were plated on fibronectin coated 12 or 24 well plates and given 10% FBS minimal essential media. For both types of *in vitro* experiments, the serum concentration was reduced to 2% FBS, apical media was removed (if at ALI), and the cells were grown for an additional day before treatment and subsequent challenges.

APZ treatment of 16HBE cells – APZ was reconstituted in DMSO to create a 10mM stock solution. Serial dilutions were performed in 2% FBS minimal essential media to make 5μM and 1μM treatment solutions. Cells grown at ALI were given 1mL of the APZ treatment on the basolateral side. Submerged cells were given 1mL and 500μL of APZ treatment for 12 and 24 well plates, respectively. APZ treatment was replenished daily and immediately before O_3 exposure.

TNFα agonist treatment – Submerged 16HBE cells were grown in 24 well plates until confluent and given 500μL of 0.5 to 2.5μM of APZ in 2% FBS minimal essential media. TNFα was reconstituted in PBS to create a 10μg/mL stock solution; serial dilutions were performed in 2% FBS minimal essential media to make 20ng/mL and 5ng/mL solutions. After 24hr pre-treatment with APZ, cells were stimulated with TNFα for 4hrs. Immediately afterwards, RNA was collected for qPCR analysis.

Ozonization of sterols in solution – Compressed Gas (UN3156) was bubbled through Hanks' balanced salt solution with calcium and magnesium (HBSS++) (Life Technologies) containing either 20μM cholesterol, 7-DHC, or DMSO for 30mins. The conditions were then repeated by sending the carrier gas through an ozone generator (AquaZone, Red Sea). Solutions

were immediately applied to submerged 16HBE cultures for 1hr. Afterwards, RNA lysates were collected and analyzed for *IL-8* expression.

In Vitro O_3 Exposure – Cultures at ALI were exposed to either filtered air or 0.4ppm O_3 for 4hrs in exposure chambers operated by the U.S. EPA in order to mimic the 8hr average exposure of an individual. This dose of O_3 is shown to have minimal cytotoxicity and maximal innate immune response in our 16HBE cells (18). 1hr post exposure, basolateral media was collected, apical sides of the transwells were washed with 110µL of Hanks' balanced salt solution (HBSS) (Life Technologies). RNA was collected in lysis buffer provided by the Pure Link RNA Mini Kit (Life Technologies, Carlsbad, CA) prepared with 1% 2-Mercaptoethanol.

Real-time qPCR – Total RNA was isolated from the 16HBE lysates using the RNA kit listed above. cDNA preparation and real-time qPCR were performed as previously described (19, 20). The β-actin primer was purchased from Applied Biosystems, Foster City, CA. Human IL-8: 5'-FAM-CCTTGGCAAAACTGCACCTTCAC-TAMRA-3' (probe), 5'-TTGGCAGCCTTCCTGATTTC-3' (sense), and 5'-TATGCACTGACATCTAAGTTCTTTAGCA-3' (antisense) and IL-6: 5'-FAM-CCAGCATCAGTCCCAAGAAGGCAACT-TAMRA-3' (probe), 5'-TATGAAGTTCCTCTCTGCAAGAGA-3' (sense), and 5'-TAGGGAAGGCCGTGGTT-3' (antisense) were prepared in-house. Expression was determined by the ΔΔCt method using β-actin for normalization. ΔCt values were computed by subtracting the threshold cycle (Ct) values for β-actin from the Ct values for the gene of interest. The ΔCt for the control was then subtracted from each sample's value to determine the ΔΔCt values. The fold change in gene expression was then calculated as $2^{-\Delta\Delta Ct}$.

Cytokine Analysis, ELISAs – Concentration of IL-6 and IL-8 in the apical wash of 16HBE cells was determined by enzyme-linked immunosorbent assay (ELISA) per the manufacturer instructions (BD Biosciences, San Jose, CA).

NanoString Gene Expression Analysis – 16HBE mRNA was isolated, with the previously mentioned method, and analyzed for gene expression via Nanostring™ (Seattle, WA) nCounter® PanCancer Immune Profiling Panel. Nanostring™ gene expression data were normalized to the geometric mean of stable housekeeping genes and to positive and negative control genes, and analyzed using the nSolver™ software provided by the manufacturer. The heat map was generated using Spearman correlation and Average Euclidian Distance.

Statistical Analysis – All *in vitro* data were performed in at least 3 separate experiments, each with multiple technical replicates. Data shown are mean ± SEM. See figure legends for further information on the specific statistical analysis used for each experiment.

LC-MS/MS 7-DHC measurement – Samples were chromatographed by RP-HPLC using a UPLC BEH C18 column (1.7μm, 2.1mm × 100mm) in Waters Acquity UPLC system equipped with an autosampler (Waters, Milford, MA) and either ESI or APCI in positive ion mode. For ESI, the sterols were separated by 95% solvent B in an isocratic method with a flow rate of 200μL/min, and the mobile phase solvents consisted of 2mM NH_4OAc (solvent A) in water and 2mM NH_4OAc in MeOH (solvent B). The injection volume was 10μL using a partial loop with needle overfill mode. MS detections were done using a TSQ Quantum Ultra tandem mass spectrometer (ThermoFisher, Waltham, MA), and data was acquired and analyzed using a Thermo Xcalibur™ 2.2 software package.

Results

APZ increases 7-DHC levels in human airway epithelial cells – Our experimental paradigm treated 16HBE cells with increasing concentrations of APZ for 24 or 72h. No morphological changes were observed at any concentration, and our 72h 1μM APZ treatment showed no overt cytotoxicity, in either air or O_3 exposed cells (Fig. 2A). We also quantified the level of 7-DHC increase in 16HBE cells treated with APZ. Although the 24h treatments of APZ increased the 7-DHC/Cholesterol Ratio, the 72h 1μM APZ treatment yielded the greatest significant increase in 7-DHC/Cholesterol to a 1-1 ratio (Fig. 2B).

Ozonized 7-DHC increases IL-8 expression – O_3 was bubbled through buffer containing 20μM cholesterol or 7-DHC for 30 minutes. Buffer solutions were immediately added to submerged 16HBE cultures for 1 hour, which were subsequently analyzed for *IL-8* expression. Cells treated with an ozonized buffer containing cholesterol modestly elevated *IL-8* expression in comparison to the DMSO control, however the ozonized buffer containing 7-DHC significantly increased *IL-8* expression compared to the DMSO control but not to the cholesterol buffer (Fig. 2C). No significant changes in *IL-8* expression were observed between the DMSO, cholesterol, or 7-DHC buffers exposed to the carrier gas alone.

APZ treatment causes O_3-specific increases in pro-inflammatory cytokine levels – As shown in previous studies, exposure to O_3 caused an inflammatory response in 16HBE cells, marked by increased gene expression and protein levels of pro-inflammatory cytokines IL-6 and IL-8 (Fig. 3A-F). Additionally, APZ treatment with 5μM for 24hr and 1μM for 72hr prior to O_3 exposure potentiated *IL-6* and *IL-8* expression 2 to 4.5 times higher when compared to vehicle (0.1% DMSO) treated cells exposed to O_3 with p values less than 0.001 and 0.01 respectively (Fig.3A-D). Protein concentrations of these cytokines followed the same trend, but did not yield

significant differences between the APZ and DMSO treated cells in the O_3 exposure group (Fig. 3E-F). Notably, treatment with APZ at any concentration did not significantly increase pro-inflammatory cytokine levels when exposed to filtered air. Moreover, the APZ-induced potentiation appears to be O_3 specific as 16HBE cells challenged with various concentrations of another NF-κB agonist, TNFα, did not exhibit the same APZ-induced exacerbated expression of *IL-6* and *IL-8* (Fig. 4A-B).

Nanostring gene expression analysis of APZ and O_3 – Using the NanoString nCounter® Analysis System, 16HBEs treated for 24 hours with 5μM of APZ or DMSO and exposed to air or O_3 were examined for 730 genes, of which 454 were detectable at baseline in our DMSO/Air treated cells. O_3 exposure revealed 91 genes with significantly altered expression. Within the O_3 exposed cells, APZ treatment showed 72 genes with altered expression when compared to the DMSO treated cells (Fig. 5A). As before, *IL-6* and *IL-8* expression was significantly elevated in APZ treated cells exposed to O_3 with *p* values less than 0.05 and 0.01 respectively (Fig. 5E-F). Additionally, *NFKB-1A* expression, the gene controlling the alpha subunit of the IKK protein complex, was significantly increased in APZ/O_3 cells with a *p* value less than 0.05 when compared to DMSO/O_3 cells (Fig. 5G). Conversely, the expression of S100 proteins, calcium-binding proteins with intra- and extracellular functions associated with inflammation (Takashi Ebihara), were reduced in APZ treated cells compared to vehicle control cells with *p* values less than 0.05 (Fig. 4D). Overall, O_3 exposure increased the expression of chemokines and cytokines; cells receiving APZ prior to exposure demonstrated the greatest expression (Fig. 5B-C).

Discussion

Using a combination of different approaches, this study examines the potential adverse drug x pollutant interaction between APZ and O_3, two relevant and ubiquitous conditions within the population. Ultimately, we present compelling data demonstrating APZ-induced exacerbation of O_3-induced inflammation in 16HBE cells by qPCR and NanoString gene expression analysis. In order to thoroughly explain this primary observation, we endeavored to show the potential for APZ to inhibit DHCR7 and increase the ratio of 7-DHC/cholesterol in airway epithelial cells. Although we cannot present direct evidence of DHCR7 inhibition by APZ, APZ significantly increases levels of 7-DHC, a sterol that when ozonized in solution, elevates *IL-8* expression more than ozonized cholesterol. Finally, the drug-induced lipid peroxidative interaction between O_3 and 7-DHC was supported as being somewhat specific to O_3-induced inflammatory signaling, as treatment with the agonist TNFα did not exhibit the same drug-induced inflammatory effects.

To examine this potential drug x pollutant interaction, we first had to determine APZ's ability to affect sterol levels in the lung. Although the airway is not a significant contributor in the production of endogenous cholesterol, pharmacologically relevant doses of APZ were able to shift the balance of sterols, elevating the relative abundance of 7-DHC *in vitro* (Fig 2B). Considering the increased reactivity of 7-DHC compared to cholesterol is two-fold: 7-DHC reacts with O_3 more readily and produces more reactive oxysterols than cholesterol (12), although minor, this shift in sterol levels in the airway could prove to prompt significant health implications. To further demonstrate the link between 7-DHC, O_3, and enhanced inflammatory gene expression we conducted experiments directly supplementing cells with ozonized sterols derived from either 7-DHC or cholesterol (Fig. 2C). Our data demonstrate that the ozonized 7-DHC products elevated *IL-8* more than the ozonized cholesterol products. While this does not

guarantee the formation of oxysterols, the expected response of elevated *IL-8* expression compared to carrier gas controls and the DMSO/O_3 control suggests the generation of these highly reactive species.

Next, we determined whether APZ not only affected sterol levels, but also induced an inflammatory effect, marked by elevated downstream pro-inflammatory cytokine levels (Figs. 3 and 5). O_3-derived oxysterols are known to have pro-inflammatory effects by increasing NF-κB activity (7). We compared both acute chronic treatment regimens APZ, which more closely mimic clinically relevant conditions, in which cells were treated either with a high dose of 5μM for 24hrs or 1μM for 3 days. Both treatment schemes combined with O_3 exposure exhibited a significant increase in *IL-6* and *IL-8* expression when compared to the DMSO/O_3 control and no difference when combined with filtered air between treatment groups (Fig. 3A-D). The higher dose, shorter exposure showed a greater fold increase in the expression of cytokines than the lower dose, longer exposure, which is more pharmacologically relevant.

Finally, we are able to support our data showing that the observed inflammatory effect between APZ and O_3 is lipid-peroxidation specific compared to another NF-kB agonizing agent. In contrast to the results produced between APZ and O_3, when APZ treatment was followed by exposure to various concentrations of TNFα, cells exhibited no APZ-induced increase in *IL-6* and *IL-8* expression (Fig. 4A-B). These data suggest that the effect of APZ-induced enhancement of *IL-6* and *IL-8* expression is not generally applicable to all pro-inflammatory stimuli, but potentially specific to oxidants, such as O_3.

Overall, these data support the hypothesis that APZ-induced inhibition of DHCR7 and subsequent elevated 7-DHC levels potentiates the already described deleterious effects of O_3-induced inflammation in the human airway. While the primary results of APZ increasing 7-DHC

and pro-inflammatory levels is promising, we acknowledge the limitations of this study. Primarily, the direct impact of 7-DHC O_3-derived oxysterols to cells was not measured and the work is limited to *in vitro* applications. Additionally, the comparison between the inhibition of DHCR7 and the use of APZ prior to O_3 exposure would further support this narrative.

While APZ is an atypical antipsychotic, it is commonly prescribed in combination with other drugs to treat depression, tic disorders, and irritability associated with autism (21). In the U.S. from 2013 to 2014 APZ was the best-selling pharmaceutical with over 9 million prescriptions filled (14). Considering the pervasiveness of O_3 pollution and APZ use, the drug x pollutant interaction between these two players could pose major public health implications. Based on the conclusions of these results, we believe that determining APZ's effect on O_3-induced inflammation in humans *in vivo* is necessary. Our work here serves to highlight the importance of drug x pollutant interactions and should be considered by primary physicians and future clinical studies.

Conclusion

It is of great public health importance for us to describe the potential for commonly prescribed therapeutic drugs, such as APZ, to elevate levels of 7-DHC, the more O_3-reactive precursor molecule to cholesterol. Treatment with APZ prior to O_3 exposure increases pro-inflammatory cytokine expression. Stimulation with NF-κB agonist TNFα does not prompt the same APZ-induced inflammatory effects. Finally, ozonized 7-DHC elevates *IL-8* expression in bronchial epithelial cells more than ozonized cholesterol. These findings emphasize the need to examine the drug x pollutant interactions between O_3 and cholesterol-modifying drugs like APZ.

Figures

Figure 1. **APZ inhibition of cholesterol synthesis pathway, 7-DHC and cholesterol structures. (**A) Cholesterol synthesis pathway and impact of APZ on DHCR7. (B) Chemical structure of cholesterol and (C) 7-DHC.

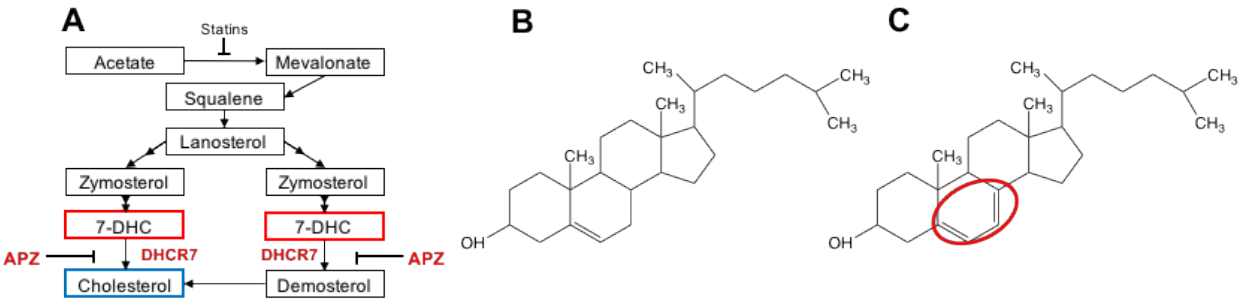


Figure 2. APZ effects on 7-DHC levels in airway epithelial cells. Ozonized 7-DHC and cholesterol induction of *IL-8* expression. (A) LDH release assay revealed no overt cytotoxicity at 1μM APZ for 72hr, independent of air or O_3 exposure. (B) LC-MS/MS analysis of the ratio of 7-DHC to cholesterol in 16HBE cells grown at air-liquid interface for 24hrs and 72hrs with increasing concentrations of APZ. (C) 7-DHC, cholesterol and the DMSO vehicle control were ozonized in buffer for 30 minutes and immediately added apically to submerged 16HBE cultures for 1 hour. *IL-8* expression was determined with qPCR. n=3-4. Data are presented as mean ± SEM. Statistical analysis was performed using a one-way ANOVA for (B) and a two-way ANOVA for (C). *p<0.05, ****p<0.00001.

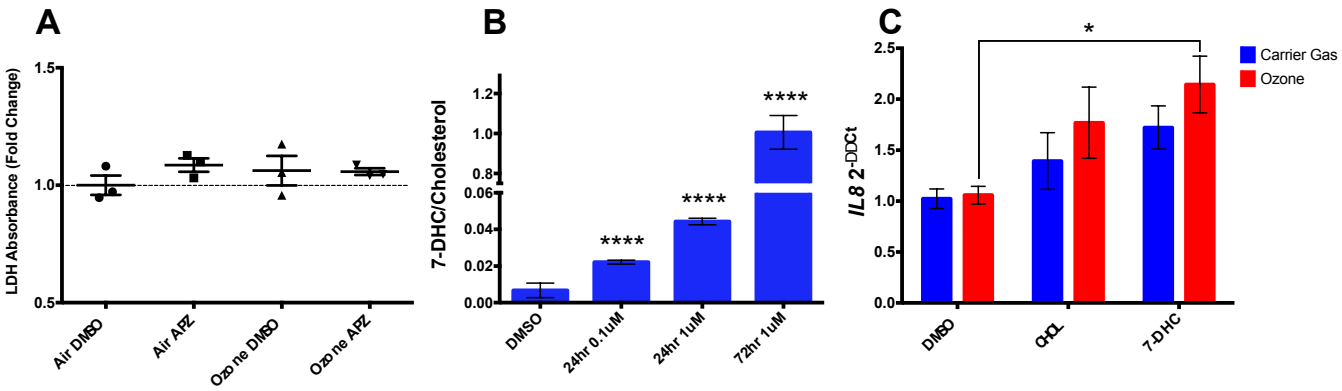


Figure 3. APZ potentiates O_3-induced *IL-6* and *IL-8* expression. 16HBE cells grown at ALI treated basolaterally with increasing concentrations of APZ 24h prior to 0.4ppm O_3 exposure yielded enhanced expression of (A) *IL-6* and (B) *IL-8* with significance at the 5µM APZ concentration. Prolonged APZ treatment at 1µM over 3 days prior to O_3 exposure increased (C) *IL-6* and (D) *IL-8* gene expression and (E) IL-6 and (F) IL-8 protein levels. (A-B) n=5-6, (B-E) n=3. Data are presented as mean ± SEM. Statistical analysis was performed using a two-way ANOVA. **p<0.01, ****p<0.00001.

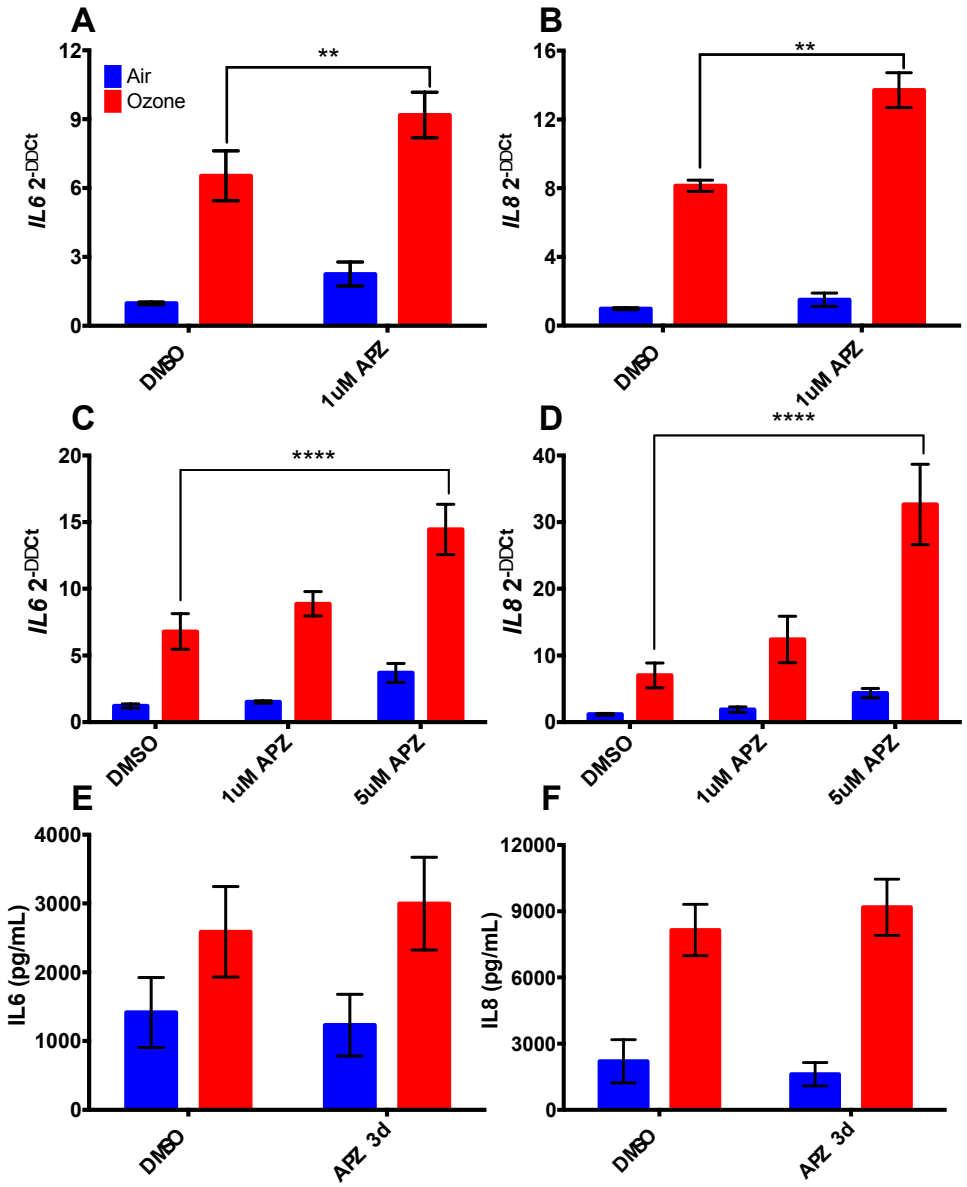


Figure 4. Stimulation with TNFα does not incite APZ-induced *IL-6* and *IL-8* increased expression. Treatment with increasing concentrations of APZ for 24hrs and TNFα for 4hrs did not exhibit the same drug-induced potentiation of (A) *IL-6* and (B) *IL-8* expression.

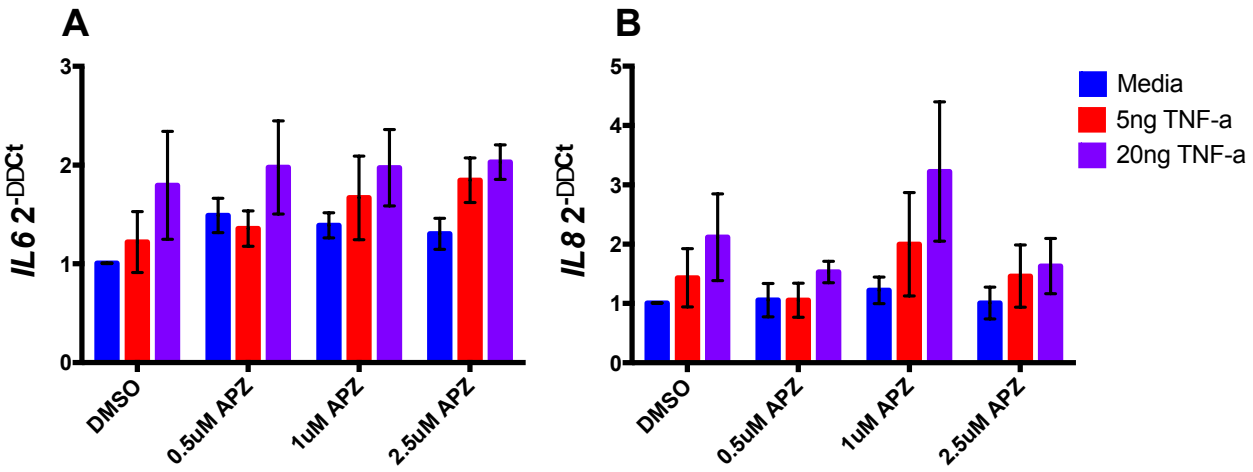
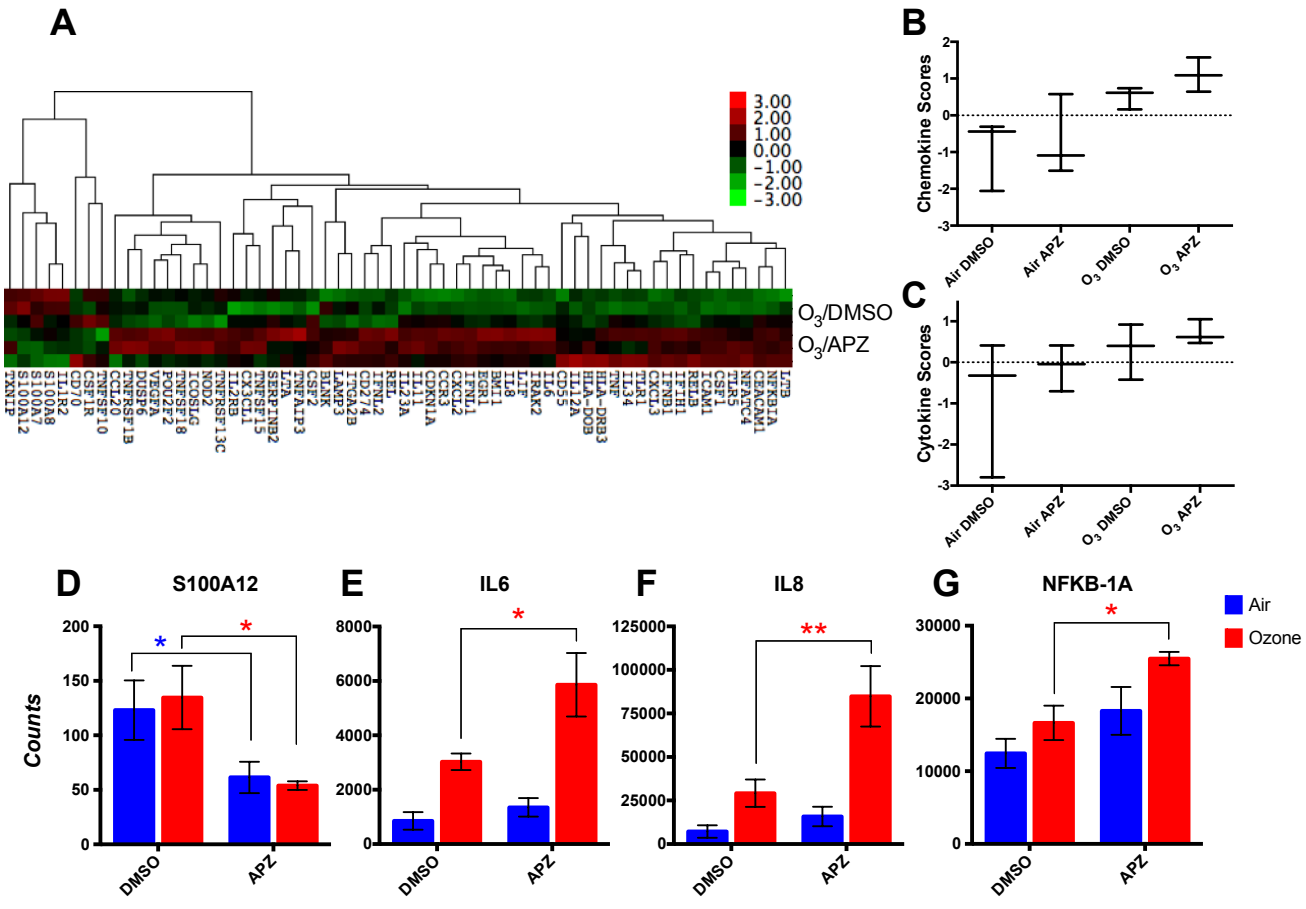


Figure 5. Nanostring analysis of gene expression in 16HBE cells treated with APZ (A) Unbiased heat map of gene expression for cells exposed to O_3. Cells were treated with DMSO or 5μM APZ for 24 hours and exposed to O_3. (B) Chemokine and (C) Cytokine scores based on groups of gene expression as determined by the NanoString n-solver™ software. Cells exposed to O_3 had higher expression of chemokines and cytokines. Cells treated with APZ prior to O_3 exposure had the highest expression of chemokines and cytokines. Unbiased gene counts are presented for (D) *S100A12*, (E) *IL-6*, (F) *IL-8*, and (G) *NFKB-1A*. APZ increases *IL-6*, *IL-8*, and *NF-kB* expression and decreases *S100A12* expression. n=3. Statistical analysis was performed using a two-way ANOVA for (D-G). *p<0.05, **p<0.01.



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