PFOA induces alteration in DNA methylation regulators and SARS-CoV-2 targets Ace2 and Tmprss2 in mouse lung tissues

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\textbf{A B S T R A C T}

Perfluorooctanoic acid (PFOA), a ubiquitous environmental toxicant from the Per- and polyfluoroalkyl substances (PFAS) family has been implicated in toxicity of various organs. Several epidemiological studies have linked PFOA to different lung injuries and diseased conditions. However, the implication of PFOA in affecting epigenetic regulators and SARS-CoV-2 infection pathways in the lung are unknown. The present work explores the accumulation of PFOA in lungs and changes in mRNA expression of DNA methylation regulator genes DNA methyltransferases (\textit{Dnmts}) and ten-eleven translocation (\textit{Tets}) along with the membrane proteins angiotensin converting enzyme 2 (\textit{Ace2}) and transmembrane Serine Protease 2 (\textit{Tmprss2}) genes involved in the SARS-CoV-2 virus infection. CD1 mice were orally exposed to 5 and 20 mg/kg/day PFOA for 10 days and the lung tissues were analyzed using LCMS, qPCR, and pyrosequencing techniques. PFOA was shown to accumulate in the lung tissues and increase in a dose-dependent manner. \textit{Dnmts} and \textit{Tets} were significantly downregulated upon at least one of the PFOA dosing concentration, whereas \textit{Ace2} and \textit{Tmprss2} show significant increase in their expression level. Further, CpG islands in the promotior region of \textit{Tmprss2} exhibited significant hypomethylation in PFOA treated groups, which supports its increased gene expression level. Current study reveals the implication of PFOA induced DNA methylation changes in lungs and their possible role in upregulation of \textit{Ace2} and \textit{Tmprss2}. It is possible that increased expression of these membrane receptors due to PFOA exposure can lead to higher susceptibility of SARS-CoV-2 infections.

\section{1. Introduction}

Per- and polyfluoroalkyl substances (PFAS) are a class of synthetic chemicals which contain more than 4000 compounds \cite{1}. Perfluorooctanoic acid (PFOA), an 8-carbon PFAS has been widely used in household products and industrial applications since 1940s \cite{2}. Due to their extensive use and high chemical stability, PFAS compounds are widespread in the environment \cite{3}. PFOA has been detected in the blood serum of over 98 \% of the US population \cite{4,5}. Humans are exposed to PFOA through drinking water, agricultural products, inhalation of dust and medical materials and devices \cite{6,7}. The long elimination half-life of PFOA which is $\sim$2.4 years in humans \cite{8}, shows the low elimination rate from the body \cite{9}. It is readily absorbed in the body and is not known to undergo metabolism or biotransformation in the cells \cite{10}. The ubiquitous presence of PFOA in the environment and high bioaccumulation capability in body organs, make it a health concern for humans \cite{4,11}.

PFOA exposure has already been linked to the damage of different organs in several epidemiological, animal, and several in vitro studies. The primary toxicological effects observed in animal studies are liver, mammary, pancreatic, and testicular cancers \cite{12}, immune system suppression, obesity, and developmental toxicity \cite{13}. Several epidemiological studies have linked PFOA to different type of cancers, ulcerative colitis, thyroid diseases \cite{14}, reduced response to vaccines \cite{15}, cardiovascular abnormalities \cite{16}, and decreased birth weight \cite{17}. PFOA toxicity is well studied in the liver, kidney, and other body organs but very few experimental studies are reported on its toxicity in lungs. Lung is one of the primary exposed organs to this environmental contaminant by both inhalation \cite{18} and oral ingestion \cite{19}. Epidemiological studies link PFOA and other Per-fluorinated compounds to...
2. Methods

2.1. Chemicals and concentrations

Perfluorobutanoic acid (PFBA) to Covid-19 hospitalization in these patients [41]. The epigenetic regulation of ACE2 and TMPRSS2 genes have long been reported and most recently in Covid-19 patients in the respiratory tract and lungs tissue [42,43].

We hypothesize that, PFOA can alter the expression of epigenetic regulators in the lungs to dysregulate the expression of ACE2 and TMPRSS2 genes and affect the downstream associated pathways. We evaluated the accumulation of PFOA in mice lungs and monitored gene expression changes of the DNA methylating enzymes, Dnmts, and DNA demethylating enzymes Tets, and SARS-CoV-2 target genes Ace2 and TMPRSS2. Next, we analyzed the CpG methylation patterns of the promotor region of Tmprss2 upon exposure to PFOA.

2.2. Animals housing, dosing, and tissues collection

CD1 mice (an outbred strain) were used in this study to assess the possible effect of PFOA. Animal experiments were conducted with an approved protocol (Toxicology of Endocrine Disrupting Chemicals, Protocol# 19037) by the University of Illinois Urbana-Champaign, Institutional Animal Care and Use Committee (IACUC) per National Institute of Health (NIH) guidelines. Mice were acquired from Charles River, USA, and randomly divided into 3 groups (n = 5 mice per group) and housed in polysulphone, ventilated cages at room temperature on a 12:12 h light: dark cycle and given free access to the Teklad Rodent Diet 8604 and purified water.

PFOA dosing was initiated when mice were acclimatized to the new environment and reached the age of one month by oral gavage method. Two doses (5, and 20 mg/kg/day) of PFOA along with vehicle control were administered daily for 10 days. At the end of the dosing period, mice were euthanized with CO2 asphyxiation with a flow rate of 2.0 L/min to the mouse cage (8” x 13’’ x 5’’). Lung tissues were collected immediately and placed in cryotubes after euthanasia, and stored in liquid nitrogen at the site of surgery and later transferred to the –80 °C freezer in the Lab.

2.3. Isolation of RNA and cDNA synthesis

Lung tissues were processed to extract total RNA by the Trizol method (Ambion, Thermofisher, Waltham, MA, USA) and dissolved in diethyl pyrocarbonate (DEPC) treated water (Invitrogen, Carlsbad, CA, USA). The concentration and purity of RNA were analyzed by Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Then, 1.5 μg of extracted RNA was reverse transcribed to cDNA from each sample using the High-Capacity cDNA synthesis kit (Applied Biosystems, Thermofisher, Waltham, MA, USA). The synthesized cDNA was diluted 25 times in molecular grade water (Corning Mediatech, Inc., Manassas, VA, USA). The final amount of cDNA used as a template was kept at 10 ng/reaction in the qPCR plate.

2.4. Assessing gene expression variation using real-time PCR

To analyze the differential gene expression between the control and PFOA treated mice lungs we used quantitative real-time qPCR (StepOnePlus Real-Time PCR Systems; v 2.0 Applied Biosystems, Waltham, MA, USA). Primers for these genes were designed and ordered using PrimerQuest Tool (Integrative DNA Technologies, Inc., Commercial Park Coralville, IA, USA). Primer annealing temperatures were optimized using gradient PCR. Then, 3 μL of diluted cDNA and 13 μL of Powerup SYBR Green PCR master mix (primers added) (Thermo Fisher Scientific Inc., Waltham, MA, USA) were mixed in each well of the plate. Glyceraldehyde 3-phosphate dehydrogenase (Gapdh), a housekeeping gene was used as an exogenous reference to normalize transcription and the subsequent data were analyzed by the ΔΔCT method.

2.5. DNA bisulfite conversion and CpG pyrosequencing analysis

Genomic DNA from mouse lung samples were extracted per instruction of EpiTest® LyseAll Lysis Kit (QIAGEN, Germantown, MD, USA). Further, extracted DNA was bisulfite converted according to the protocol provided by EpiTest® LyseAll Lysis Kit (QIAGEN). Four sequences containing dense CpG islands within the promotor region of Tmprss2 were selected to investigate the DNA methylation status. Sequences on the antisense strand were examined and respectively provided below: 1. CGACCCTCCTGACATGCTCCAGAGATGTAGCT, 2. CGGGTGGGTTGACCGAGCCACCAGTGACCGCCCC, 3. CGGCCCTGCTG GGGCCCTGGGGACCTTGGCAAGACGGGAATTGTCCGT, 4. AGGTTTG TCCCGATCTTTTCTTTGCGGAAAAACTCTCGGACTCAG. Both tem plate amplification and sequencing primers were acquired from Gene-Globe (QIAGEN). Templates were amplified with bisulfite converted DNA and PyroMark PCR kit (QIAGEN) using hot-start polimerase chain reaction (hsPCR). Amplified templates were processed and isolated with PyroMark Q24 Vacuum Workstation (QIAGEN). CpG methylation assays were performed on amplified templates with PyroMark Q24 Advanced CpG kit per manufacturer’s instruction and adapted methods according
Accumulated PFOA in these groups along with vehicle control is shown in Fig. 1. The mean concentration of PFOA detected was 14.14 ± 2.95 and 36.41 ± 15.09 μg/g of lungs tissue in mice dosed at 5 and 20 mg/kg respectively. PFOA accumulated proportionally with an increase in dosing concentration. High accumulation in lungs suggest high affinity of PFOA for lung tissues and the associated adverse effects.

3.2. Epigenetic changes in lungs

The DNA methylation is one of the key epigenetic mechanisms which regulates gene expression without changing the sequence. DNMT3a and DNMT3b members of methylation enzymes establishes methylation at new CpG sites where as DNMT1 retains the preexisted/inherited methylation [27]. In the current work we examined these 3 functional isoforms of DNMTs upon PFOA exposure.

In mice lungs exposed to PFOA, an overall decrease in expression patterns of Dnms was noted. Dnmt1 and Dnmt3a had lower gene expression at high PFOA concentration (20 mg/kg/day) while the expression of Dnmt3b decreased significantly at 5 mg/kg/day, as shown in Fig. 2A. Overall, a decrease in the expression of these regulatory genes suggests a possible role in decreased methylation in cells from lungs.

Next, we examined the expression of Tets, a class of demethylating enzymes which has a demethylating function contrary to the Dnmts. At the gene expression level there is an overall decrease in mRNA transcription of these demethylation genes. A significant decrease in Tet1 and Tet2 at 5 mg/kg PFOA and at 20 mg/kg in Tet3 gene was noted (Fig. 2B).

3.3. Ace2 and Tmprss2 genes mRNA level increased with PFOA

ACE2 is the key membrane protein which has been implicated in different physiological and pathophysiological conditions of the human and other mammals. TMPRSS2, a member of serine protease family which fuse with the v-ets erythroblastosis virus E26 oncogene homolog (ERG) is reported to be dominant in the pathophysiology of prostate cancer [50]. In this study we analyzed the differential expression of these genes in PFOA treated and untreated mice. There is a dose dependent increase in the gene expression level of Ace2 and Tmprss2 genes in the lungs of these mice exposed to PFOA. Ace2 mRNA level significantly increased at 20 mg/kg/day whereas Tmprss2 expression was significantly enhanced at both the lower and higher PFOA dosing levels (Fig. 3).

3.4. Alteration in the Tmprss2 gene promotor methylation level by pyrosequencing

Since Tmprss2 gene expression increased significantly in both low and high PFOA dosing, we tested the CpG methylation alterations in the promotor region of this gene and targeted 20 different CpG sites which clustered in 4 sequences. In 20 mg/kg/day PFOA treatment group, 6 out of 20 different CpG sites were significantly hypomethylated whereas 2 sites were hypermethylated (Fig. 4). In the 5 mg/kg/day treatment group, 3 sites were noted to have significant hypomethylation pattern and 1 site exhibited increased methylation as shown in Fig. 4. Overall significant hypomethylation pattern of target CpG sites in promotor region support the upregulation of Tmprss2 in qPCR analysis.

4. Discussion

PFOA, the ubiquitous synthetic chemical contaminant in the environment enters the drinking water and food chain, to which human and other organisms are exposed frequently. Although PFOA is banned in US now, due to its abundant use in the past, high bioaccumulation, high stability, and long half-life the toxicant is bound to exist in the environment and water sources for a long period [1–3]. Few studies have reported on the toxic effects of PFOA in the lungs and no report exists on
the alteration of epigenetic modulators and key epithelial cell receptors in lungs upon PFOA exposure. In the current work we evaluated accumulation of PFOA in lung and explored its effects on key epigenetic regulators and key membrane SARS-CoV-2 receptors.

4.1. PFOA exposure by oral route accumulate in lungs

Lung is one of the primary organs exposed to environmental contaminants directly through inhalation and indirectly through oral ingestion [51]. PFOA in drinking water and food is absorbed by the body from gut and distributed to different body organs. In our experiment, the orally administered PFOA accumulates in proportion with dosing concentration in lungs. Variation observed in PFOA accumulated in 20 mg/kg group is possibly due to the individual animal physiology, metabolism, and elimination capability. Prior studies have shown such a large variation in the accumulation of these PFAS compounds in different tissues, specifically in high treatment groups [6,19]. An animal model study showed that lung has the 3rd highest PFOA accumulation after liver and kidney in rats exposed to PFOA [19]. Autopsies of organisms from humans showed that PFAS accumulation in the lungs is the highest, amounting to 29.2 ng/g of tissue weight [52]. Our current accumulation data support the previous reported level of PFOA in lungs. The high concentration of these contaminants can lead to pulmonary toxicity leading to other complications of the immune functions requiring further inquiry on the accumulation and role of PFOA specifically in lungs.

4.2. PFOA alters DNA methylation regulating enzymes in lungs

Epigenetic mechanisms regulate the development and differentiation of organs and their proper function by controlling gene expression without mutating the original DNA sequence [53]. To explore epigenetic mechanisms that result in gene expression alterations in lung tissues exposed to PFOA, we evaluated gene expression alterations in DNA methylation regulators. Epigenetic alterations regulate development of lungs and physiological functioning, leading to abnormal conditions that could potentially lead to disease [54]. Epigenetic toxicity due to PFOA is a less explored area specifically in tissues of the lungs where PFOA has the highest accumulation. Our recent studies have already reported on the alteration of epigenetic regulators such as DNMTs, TETs and Histone deacetylase enzymes in different mice organs and human cell lines [23–26]. Our current findings show significant alteration in the DNMTs enzymes expressing genes. A significant decrease in trend was observed in the genes responsible for these DNA methylating and demethylating enzymes in both low and high PFOA dosing circumstances. Pro-metastatic oncogene synuclein-γ was abnormally upregulated due to the downregulated DNMT3B in cigarette smoke exposed lung cancer cells based on in vitro studies [55]. DNMTs alteration has been reported in several tumorigenesis conditions delineating their role in the initiation and inhibition of cancer [56].

SARS-CoV-2 infection downregulated the expression levels of DNM1T, DNMT3A, and DNMT3B in lung epithelial cells [57]. We further noticed a significant decrease in the mRNA level of Tets expressing genes in the PFOA dosed mice groups. TETs enzymes are reported in several carcinomas and their lower expression is considered a hallmark of gastric, lung, prostate, liver and breast tumors [58]. TET1 enzyme downregulation has been recently reported in the promotion and occurrence of hepatocellular and bladder carcinoma [59,60]. There is a similar decrease in the expression levels of these epigenetic genes based on our previous PFOA treatment studies which suggests possible downstream effects in pathways and mechanisms of these altered epigenetic modulators [25,26].

We observed that PFOA has downregulated expression of both Dnmts and Tets genes. This collaborative up or down regulation is possible and has already been observed in some diseased conditions and developmental process. For example, in hepatoblastoma patients DNMTs and TET3 genes are upregulated compared to normal liver but downstream effects are only observed for high level of TETs enzymes [61]. Another recent mouse model study focused on the hypothalamus development which showed highest expression of all Tets and Dnmts genes irrespective of their methylation and demethylation activities [62].

4.3. PFOA upregulates expression levels of Ace2 and Tmprss2

ACE2 and TMPRSS2 are the key epithelial membrane proteins which are more frequently reported in different diseased conditions instead of
their normal physiological functions. These genes are coregulated [63] and are highly expressed in testis, prostate, aerodigestive tract and cardiovascular epithelial cells [64]. Our evaluation of Ace2 and Tmprss2 gene expression in mice lungs tissue showed significant change in expression among PFOA treated groups. Tmprss2 expression level increased proportionally upon lower and higher exposure of PFOA. Recent reports showed upregulation of ACE2 and TMPRSS2 genes in smokers in comparison to non-smokers in both human and rodent models [35, 65, 66]. These genes were also upregulated in murine lungs with environmental pollutant: particulate matter which is a mixture of different ions, elemental and organic carbon, metals, and polycyclic aromatic hydrocarbons [67]. Our findings are in line with the previous reports and suggests a possible effect of the external environment on these genes.

These coregulated receptors in epithelial membrane paves the way for different viral infections, specifically the SARS-CoV-2. Spike protein of SARS-CoV-2 is recognized by the host and the virus binds to ACE2 receptors in human. Then TMPRSS2 protein cleaves the spike protein and the viral envelop fuses with host membrane and invades the cell [35]. Individuals with preexisting debilitating health conditions for example obesity, diabetes, cardiovascular implications, hypertension, chronic lung, liver, and kidney diseases are more prone to SARS-CoV-2 infection and comorbidities due to their higher ACE2 expression [68]. In contrast children and infants with very low pulmonary ACE2 expression are almost not affected by this viral infection [69, 70]. This indicates that ACE2 expression is directly proportional to the SARS-CoV-2 infection and fatalities. Overexpression of these genes with PFOA accumulation in the lungs increases their susceptibility to SARS-CoV-2 and potentially other viral infections from the SARS family.

Further evaluation of the promotor region of Tmprss2 gene showed differential CpG methylation pattern at almost 33 % of our target sites in PFOA treated versus control group. Higher level of DNMT1 is linked to hyper-methylation and downregulation of TMRPSS2 gene in androgen receptor negative prostate cancer cells [71]. Dnmt1 is downregulated in our case which supports the link between this epigenetic regulator and Tmprss2 methylation pattern and expression. 30 % of our target CpG sites in the promotor region of Tmprss2 had significantly reduced methylation after the highest PFOA dosing. Ace2 and Tmprss2 genes have been reported to be epigenetically modulated by DNA methylation patterns in the promotor regions [42, 43]. In our current work although the downregulation of Dnmts support the upregulation data for the Ace2 and Tmprss2 genes there could be several other responsible factors involved and the need for further studies to explore the exact mechanism is imperative.

In conclusion, PFOA has significant effect on DNA methylation regulators and key membrane proteins Ace2 and Tmprss2. Alteration in the expression profiles of the target genes is potentially due to the higher amount of PFOA accumulation in these lung tissues. There is a need for future research to categorically assess these initial findings at the protein level by knockout models. Current work provides a baseline to evaluate other PFAS compounds and environmental toxicants and their effects on our health and our vulnerabilities to viral infections, specifically SARS-CoV-2 amid exposure to these contaminants. Future large scale epidemiological studies should be designed to further evaluate the possible association between PFAS accumulation in vulnerable populations and SARS-CoV-2 infectiousness and fatalities.

Data availability

Not applicable.

Availability of data and materials

Not Applicable.
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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.

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