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Mutagenicity of PFOA in Mammalian Cells: Role of Mitochondria-Dependent Reactive Oxygen Species

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ABSTRACT: Mutagenicity is often a prerequisite to the development of malignancy. Evidences have shown that exposure to perfluorooctanoic acid (PFOA) results in various cancer inductions. However, whether any mutagenic base exists is still puzzling. In the present study, we exposed exponentially growing A_L cells to PFOA and assayed the cells for survival, mutation induction, and caspase-3/7, -9 activities. Mitochondrial-DNA deficient human-hamster hybrid (ρ^0 A_L) cells and reactive oxygen species (ROS) inhibitor were used to elucidate the possible mechanism. Our results showed that treatment of A_L cells with PFOA for 16 days induced significant mutagenic effects together with the increment of ROS, superoxide anions ($O_2^{\cdot-}$), and nitrogen oxide (NO) levels, while treatment of ρ^0 A_L cells did not have much change. Concurrent treatment of A_L cells with ROS inhibitor significantly decreased the mutagenic potential of PFOA. In addition, caspase activities in A_L cells were increased by PFOA exposure and suppressed by ROS/RNS (reactive oxygen/nitrogen species) inhibitors. Our results suggest that exposure to PFOA lead to mutagenicity induction in A_L cells, and mitochondria-dependent ROS plays an important role in this process. This provides a direct base for PFOA mediated cancer induction.

INTRODUCTION

Perfluorinated fatty acids (PFFAs) have been widely used in a variety of industry and consumer products over the last 50 years.[1] Among all the PFFAs, perfluorooctanoic acid (PFOA, known for its use in DuPont's Teflon products) is one of the most prevalent chemicals, which has been detected in the blood of animals throughout the world including fish, birds, and even the seals of remote arctic regions.[2] In humans, significant levels of PFOA have also been detected in serum samples in USA,[3] China,[4] and Japan.[5] Another characteristic for PFFAs is their persistency. In general, the longer the carbon chain length, the longer they can stay in the body. For example, perfluorobutane sulfonate, which has 4 carbons, has a half-life for elimination in a little over 1 month in humans, while PFOA, so-called C8 compounds, has a half-life of 3.8 years.[6]

Various *in vivo* and *in vitro* models have been used to assess the potential bioeffects of PFOA over the past decades. It was found that exposure to PFOA could arrest cell cycle distribution,[7] alter peroxisomal and MAPK-related signaling pathways,[8] and induce oxidative DNA damage in mammalian cells.[9,10] Regarding the mechanism(s), several reports demonstrated that ROS and mitochondria play important roles.[11,12]

In light of these actions, it has been puzzling that PFOA is only weakly active or, more often, completely inactive in bacterial and mammalian cell mutation assays. For instance, PFOA does not induce bacterial gene mutations in *Salmonella typhimurium-Escherichia coli*/reverse mutation assay with and without mammalian microsomal metabolic activation.[13] Yoshimitsu found that PFOA is not mutagens in the *umu* test.[14] Nor does PFOA induce *hprt* gene mutations in Chinese Hamster Ovary (CHO) cells.[13] The major factors accounting for these could be the nature of mutation assays, cell/tissue specific response, and exposure time. Therefore, a comprehensive study is certainly necessary for evaluating the mutagenic potential of PFOA in mammalian cells.

In this work, we tried to determine whether PFOA are mutagenic to mammalian cells and, if so, what mechanism(s) is involved. We hypothesize that mitochondria and oxidative stress play an important role in these events. To address these issues, human-hamster hybrid (A_L) cells and mitochondria deficient (ρ^0 A_L) cells together with ROS inhibitor are used to elucidate the possible mechanism(s) of PFOA-induced genotoxicity.

METHODS AND MATERIALS

Cell Culture. Two human-hamster hybrid cell lines were used in these studies. Normal human-hamster hybrid (A_L) cells contain a standard set of CHO-K1 chromosomes and a single copy of human chromosome 11. Chromosome 11 contains the CD59 gene (also known as M1C1) at 11p13.5, which encodes the CD59 cell-surface antigen marker (formerly known as S1) that renders A_L cells sensitive to killing by the monoclonal antibodies E7.1 in the presence of rabbit serum complement (Merck, Darmstadt, Germany).[15] Antibody specific to the CD59 antigen was produced from hybridoma culture. MtDNA-depleted cells (ρ^0 A_L) were provided by Prof. Tom K. Hei (Columbia University, New York, NY, USA).[16] A_L cells were cultured in Ham's F-12 medium supplemented with 8% heat-inactivated fetal bovine serum (FBS, HyClone, SouthLogan, UT, USA), 25 μg/mL gentamicin, and 2×10^{-4} M glycine. ρ^0 A_L cells were cultured in F12/DMEM (1:1) medium supplemented with 15% heat-inactivated

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FBS, 2.7 g/L glucose, 584 mg/L glutamine, 50 μg/mL uridine, 25 μg/mL gentamicin, and 2×10^{-4} M glycine. All cultures were maintained at 37 °C in 5% CO_2 incubator (humidity 95%).

Exposure to PFOA. PFOA (purity = 99%) was purchased from the AccuStandard, Inc. (New Haven, CT, USA). The stock solution (250 mM) was prepared by dissolving the powder in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA). Working concentrations were prepared by diluting the stock with complete medium and in 200 μM PFOA working solution DMSO concentration was about 0.08%. Exponentially growing A_L cells in 100-mm diameter Petri dishes were treated with 1−200 μM PFOA for 1−16 days. Cells were replated and re-exposed to PFOA every 4 days.

MTT Assay. Cytotoxicity of PFOA was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay.[17] Briefly, after treatment of cells with 1−200 μM PFOA for 1, 4, 8, or 16 days, the medium was removed. MTT stock solution (5 g L^{-1} in PBS) was added to each dish to achieve a finial concentration of 1 g L^{-1}, and the cultures were incubated for another 4 h. The supernatant was removed, and 1 mL of acidic isopropanol was added to dissolve the formazan crystals which were converted by the catalysis of mitochondrial dehydrogenase from a water-soluble tetrazolium salt. The absorbance at 570 nm was determined by a Spetra Max M2 fluorescence reader (Molecular Devices, Sunnyvale, CA, USA).

Mutation Assay. After exposure to PFOA, the cells were subcultured in cell culture dishes for 7 days. This expression period is needed to permit cells to recover from the temporary growth lag from PFOA treatment and to multiply such that the progeny of the mutated cells no longer expresses lethal amounts of the CD59 surface antigen. To determine mutant fractions, 5×10^4 cells were plated into each of six 60-mm dishes in a total of 2 mL of growth medium as described.[18] After incubation for 2 h, the cultures were treated with 0.2% CD59 antiserum and 1.5% (v/v) freshly thawed complement (Merck, Darmstadt, Germany). After continuously incubated for 7 days, the cultures were fixed and stained, and the number of $CD59^-$ mutation colonies was scored. The controls were the sets of dishes containing antiserum alone, complement alone, or neither agent. The mutant fraction was calculated as the number of surviving colonies divided by the total number of cells plated after correction for any nonspecific killing because of complement alone and was expressed as the number of mutants per 10^5 clonogenically viable cells.

ROS, Nitrogen Oxide (NO), and Superoxide Anion ($O_2^{\cdot-}$) Measurement. The fluorescent probe, 2′,7′-dichlorofluorescin (CM-H_2DCFDA, Molecular Probes, Eugene, Oregon, USA) was employed to quantify the level of ROS as described.[19] Cells were inoculated in 96-well plates after PFOA treatment and assayed at indicated time points. The confluent cultures were washed with D-Hank's buffer solution complemented with 1% FBS (FDBS) at 37 °C for 2 min, stained with 2 μM CM-H_2DCFDA for 30 min in incubator, and then washed with cold FDBS twice. The fluorescence was measured in FDBS with Spetra Max M2 fluorescence reader (excitation/emission: 495/515 nm).

The dihydroethidine (Molecular Probes, Eugene, Oregon, USA) was employed to quantify the level of $O_2^{\cdot-}$.[20] Cells were inoculated in 96-well plates after exposure to PFOA. Confluent cultures were washed with D-Hank's buffer solution at 37 °C for 2 min, stained with 10 μM dihydroethidine for 30 min in incubator, and then washed with cold D-Hank's twice. The fluorescence was measured in D-Hank's with Spetra Max M2 fluorescence reader (excitation/emission: 488/610 nm).

The 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM diacetate, Molecular Probes, Eugene, Oregon, USA) was employed to quantify the level of NO.[21] Cells were inoculated in 96-well plates after treated with PFOA. Confluent cultures were washed with tyrode solution at 37 °C for 2 min, stained with 5 μM DAF-FM diacetate for 30 min in incubator, and then washed with cold-tyrode solution twice. The fluorescence was measured in tyrode solution with Spetra Max M2 fluorescence reader (excitation/emission: 495/515 nm).

Caspase Assays. Caspase-3/7 and caspase-9 activities were measured using a Caspase-Glo assay kit (Promega, Madison, WI, USA).[22] Briefly, the proluminescent substrate containing the amino acid sequence Asp-Glu-Val-Asp (DEVD) and Leu-Glu-His-Asp (LEHD) are cleaved by caspase-3/7 and caspase-9, respectively. After caspase cleavage, a substrate for luciferase (aminoluciferin) is released, which results in the luciferase reaction and the production of luminescent signal. After PFOA treatment, cells were mixed with CellTiter-Glo reagent and incubated for 60 min at ambient temperature to stabilize the luminescence signal. The luminescence was quantified by a GloMax 20/20 Luminometer (Promega, Madison, WI, USA).

Effects of ROS/RNS Quenchers on Mutagenicity and Caspase-3/7 Activities of PFOA. To demonstrate the involvement of ROS in the mutagenicity induced by PFOA, exponentially growing A_L cells were exposed to PFOA for 16 days with or without concurrent treatment with DMSO. After treatment, cells were plated to determine mutant fractions as described above. To evaluate the role of ROS/RNS in PFOA-mediated caspase-3/7 activities, exponentially growing A_L cells were exposed to PFOA for 1 day with or without concurrent treatment with 0.5% DMSO/0.2 mM NG-methyl-L-arginine (L-NMMA, a nonselective inhibitor of NOS, Sigma-Aldrich, St. Louis, MO, USA) respectively. After treatment, cells were plated to determine caspase-3/7 activities as described above.

Statistical Analysis. All data were pooled from at least three independent experiments. The results were presented as means ± SD. Significance between controls and exposed cells were assessed using one-way analysis of variance (ANOVA) with the Post hoc LSD test. A P-value of < 0.05 between groups was considered significant.

RESULTS

Exposure to PFOA Decreased the Viabilities of A_L cells. A_L cells were exposed to graded concentrations of PFOA ranging from 1 to 200 μM PFOA for different time points. As shown in Figure 1, the viabilities of A_L cells were reduced to 82.85 ± 4.67 and 79.04 ± 3.52% after treatment with 100 and 200 μM PFOA for 1 day, respectively. As the exposure time increased to 4 days, the viabilities were reduced to 74.52 ± 5.96 and 71.57 ± 3.96%. There were no further decreases of cell viabilities with the longer exposure time points (8 days or 16 days). These results imply that exposure to PFOA induced a significant decease of the viability in A_L cells.

Exposure to PFOA Increased the Mutation Frequencies at CD59 Gene Loci of A_L but Not ρ^0 A_L Cells. A_L cells were exposed to graded concentrations of PFOA ranging from 1 to 200 μM PFOA for different time points. The average mutation backgrounds of A_L cells at CD59 locus used in these experiments were about 100 (74−156) mutants per 10^5 survivors. As shown in Figure 2A, there were no distinct mutation inductions when cells were treated with 1−200 μM PFOA for 1, 4, or 8 days.

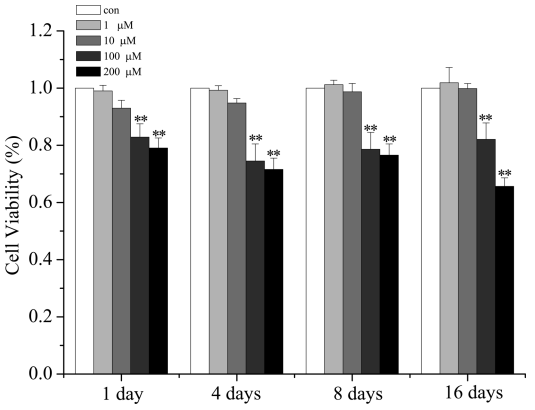

Figure 1. Effects of PFOA on cellular viability. Exponentially growing A_L cells were exposed to graded concentrations of PFOA for 1, 4, 8, or 16 days. Data were pooled from at least three independent experiments and analyzed with one-way ANOVA. The symbol (**) indicated $p < 0.01$. Error bars indicate SD.

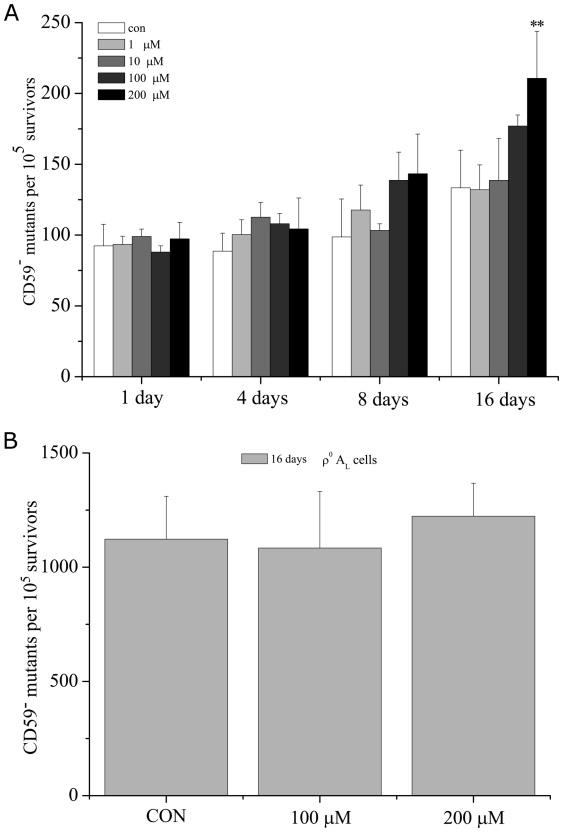

Figure 2. Effects of PFOA on induction of $CD59^-$ mutants per 10^5 survivors in A_L (A) and ρ^0 A_L (B) cells. Cells were exposed to graded concentrations of PFOA for 1, 4, 8, or 16 days. Data were pooled from at least three independent experiments and analyzed with one-way ANOVA. The symbol (**) indicated $p < 0.01$. Error bars indicate SD.

However, the mutation fraction was significantly increased after treatment of cells with 200 μM PFOA for 16 days. On the other hand, ρ^0 A_L cells did not have much change to the mutagenic challenge of PFOA (Figure 2B, $p > 0.05$).

PFOA Treatment Increased Intracellular ROS, NO, and $O_2^{\cdot-}$ Production in A_L Cells. ROS have been demonstrated to play

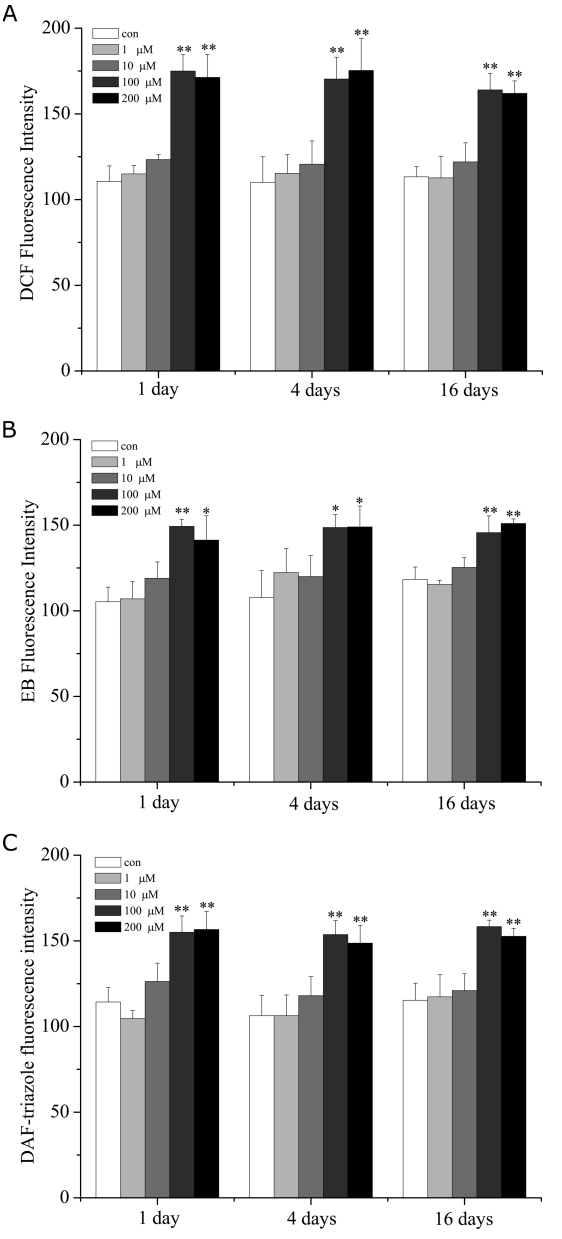

Figure 3. Intracellular ROS (A), $O_2^{\cdot-}$ (B), and NO (C) production induced by graded concentrations of PFOA for 1, 4, or 16 days in A_L cells. DCF: dichlorofluorescein, the fluorescent product of CM-H_2DCFDA when reacted with ROS. Ethidium bromide (EB): the fluorescent product of dihydroethidine when reacted with $O_2^{\cdot-}$. DAF-triazole: the fluorescent product of DAF-FM when reacted with NO. Data were pooled from at least three independent experiments and analyzed with one-way ANOVA. The symbol (*) and (**) indicated $p < 0.05$ and $p < 0.01$, respectively. Error bars indicate SD.

an important role in PFOA-related cellular dysfunction. As shown in Figure 3A, after treatment with 100 μM PFOA for 1 day, ROS level was significantly increased 1.6 times in the exposure group compared with that in the control. However, there were no further increases in ROS levels with PFOA concentration >100 μM or with longer exposure time. When treated with 100 μM PFOA for 1 day, $O_2^{\cdot-}$ and NO concentrations were significantly increased in the exposure groups compared with their corresponding controls (Figure 3B and C). Similarly, there were no further increases in $O_2^{\cdot-}$ and NO levels

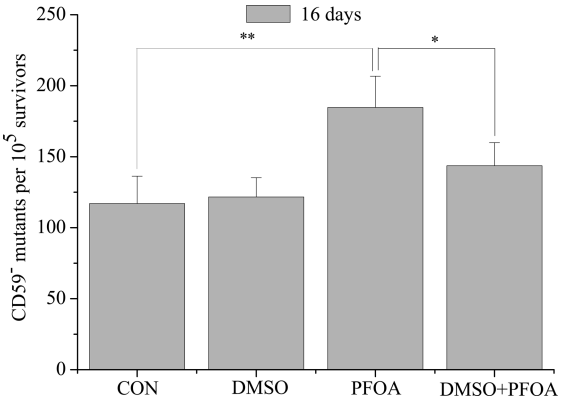

Figure 4. Effect of 0.5% DMSO on mutagenicity of A_L cells exposed to 200 μM PFOA for 16 day. Data were pooled from at least three independent experiments and analyzed with one-way ANOVA. The symbol (*) and (**) indicated $p < 0.05$ and $p < 0.01$, respectively. Error bars indicate SD.

with PFOA concentration >100 μM or with longer exposure time.

ROS Inhibitor Suppressed the Mutagenic Effect of PFOA. DMSO as an ROS inhibitor was used to further assess the mechanism underlying PFOA-induced mutagenicity. It was found that 0.5% DMSO significantly reduced the mutation yield at CD59 locus by 200 μM PFOA from 185 ± 22 to 144 ± 16 ($p < 0.05$, Figure 4). The concentration of DMSO used had been tested to be free of cytotoxicity and mutagenicity (data not shown).

Treatment with PFOA Had No Effect on Intracellular ROS and $O_2^{\cdot-}$ Production in ρ^0 A_L Cells. ρ^0 A_L cells were treated with 100 and 200 μM PFOA for 1, 4, or 16 days and then intracellular ROS and $O_2^{\cdot-}$ production were measured. As shown in Figure 5A-B, there were no significant difference for intracellular ROS and $O_2^{\cdot-}$ production between the exposure groups and their controls. These data suggested that PFOA-induced ROS might be derived from mitochondria.

Caspase Activities Were Increased by PFOA Exposure in A_L Cells and Suppressed by ROS/RNS Inhibitor. To assess whether PFOA could induce apoptotic pathway, caspase-3/7 and caspase-9 activities were examined. As shown in Figure 6A-B, after treatment with PFOA for 1 day, caspase-3/7 and caspase-9 activities were significantly increased 1.31 and 1.29 times, respectively, in the 200 μM exposure groups compared with their controls. There were no further increases in caspase-9 and caspase-3/7 activities in prolonged exposure time.

Furthermore, 0.5% DMSO/0.2 mM L-NMMA dramatically suppressed the PFOA-mediated caspase-3/7 activation induced by 200 μM PFOA for 1 day from 40895 ± 1139/40397 ± 2356 RLU to 35549 ± 2490/35153 ± 2199 RLU ($p < 0.05$, Figure 7). Concentrations of DMSO and L-NMMA used here were tested without cytotoxicity (data not shown). These results indicated PFOA induction of caspase-3/7 and caspase-9 were ROS/RNS mediated.

■ DISCUSSION

Epidemiological data gathered for the past decades showed that PFOA was a potent carcinogen[23] and could induce DNA damage in mammalian cells.[9,10] However, their mutagenic effects were still puzzling. Yoshimitsu found that 0−1000 μM PFOA were not mutagenic in the *umu* test after 5 h exposure.[14] In mammalian cells, mutagenicity study at the *hprt* locus showed that

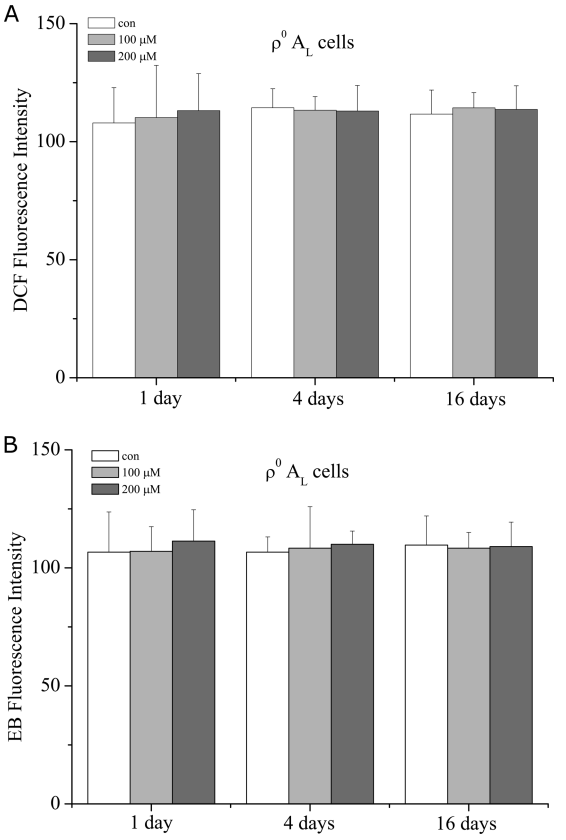

Figure 5. Intracellular ROS (A) and $O_2^{\cdot-}$ (B) production induced by graded concentrations of PFOA for 1, 4, or 16 days in ρ^0 A_L cells. Data were pooled from at least three independent experiments and analyzed with one-way ANOVA. Error bars indicate SD.

PFOA is either inactive or weakly active.[13] The failure of PFOA to induce gene mutations in mammalian cells has been taken as evidence that a nongenotoxic pathway through peroxisomal proliferation is responsible for PFOA induced toxicity.[24] In the present study, however, we showed clearly that PFOA was mutagenic to endogenous genes in mammalian cells. Such inconsistency could be due to the nature of mutation assays, cell/tissue specific response, and exposure time. The human−hamster hybrid (A_L) cell, which contains a full set of hamster chromosomes and a single copy of human chromosome 11, is sensitive in detecting mutagens such as ionizing radiation [25] and heavy metals.[18] Because only a small segment of the human chromosome (11p15.5) is required for the viability of A_L cells, mutations or deletions out of this range in size up to 140 Mbp of DNA will not affect the viabilities of the cells.[18] It was found that there is a 50-times increase in mutations at the CD59 locus when compared with the *hprt* locus in crocidolite-treated A_L cells.[26] Another factor that caused failure to detect mutants in mammalian cells in previous literatures might be due to insufficient treatment time. In our study, mutagenicity could only be detected after long-term exposure of PFOA (200 μM PFOA for 16 days).

Multiple studies demonstrated that ROS was critical for PFOA-induced dysfunction.[12] ROS are transient species due to their high chemical reactivity and can react with DNA, proteins, carbohydrates, and lipids in a destructive manner.[27] Nitric oxide (NO), generated from arginine by the activation of nitric oxide synthase (NOS), is a major signaling molecule in the immune, cardiovascular, and nervous systems.[28] In our study, the levels of ROS as well as NO and $O_2^{\cdot-}$ were significantly increased in A_L

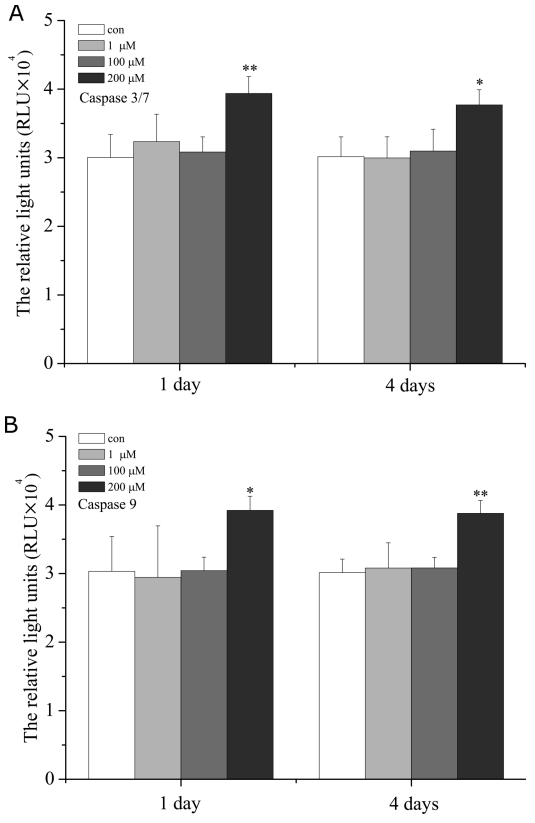

Figure 6. Effects of PFOA on caspase-3/7 (A) and caspase-9 (B) activities. Cells were exposed to graded concentrations of PFOA for 1 or 4 days. The relative light units of *y*-axis represent the caspase activity. Data were pooled from at least three independent experiments and analyzed with one-way ANOVA. The symbol (*) and (**) indicated $p < 0.05$ and $p < 0.01$, respectively. Error bars indicate SD.

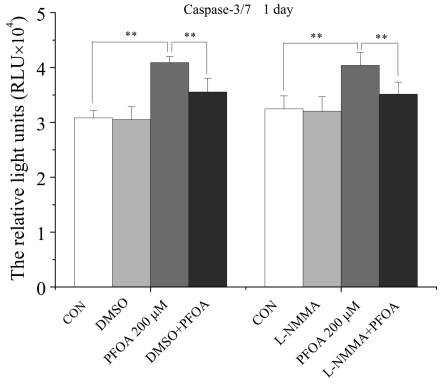

Figure 7. Effects of 0.5% DMSO and 0.2 mM L-NMMA on caspase-3/7 activities in A_L cells exposed to PFOA at 200 μM for 1 day. The relative light units of *y*-axis represent the caspase activity. Data were pooled from at least three independent experiments and analyzed with one-way ANOVA. The symbol (**) indicated $p < 0.01$. Error bars indicate SD.

cells when treated with 100 μM PFOA for either 1, 4, or 16 days. When intracellular generation of ROS was inhibited with DMSO, the mutagenicity of PFOA in mammalian cells was significantly suppressed. These results suggested strongly that ROS plays a role in mediating the mutagenic activities of PFOA. Beckman et al. found that the toxicity of NO is linked to its ability to combine with $O_2^{\cdot-}$ to form peroxynitrite ($ONOO^-$).[29] As both of NO and $O_2^{\cdot-}$ concentrations were increased after exposure to PFOA, they might further react and form peroxynitrite ion ($ONOO^-$), which has a much longer half-life compared with hydroxyl radicals and is considered to be a more reactive species that cause DNA damage directly. It should be mentioned that compared to mutation induction, which occurred in 16 days under 200 μM PFOA treatment, ROS induction was observed much earlier (1 day) and at relatively lower concentration of PFOA (100 μM, Figure 3A). Eriksen et al. also found that exposure of human HepG2 cells to PFOA for 3 h could increase ROS production but not generate DNA damage.[30] This might be due to that though ROS could induce DNA damage, DNA repair systems such as the base excision repair (BER), the nucleotide excision repair (NER), etc. was initialized at the beginning of the treatment.[31] As exposure time (16 days) and concentration (200 μM) increased, the accumulative oxidative stress might decrease the DNA repair capacity and thus mutagenicity was induced. Recently, peroxisome proliferator-activated receptor-alpha (PPARα) was found to play a critical role in PFOA-induced cellular toxicity such as lipid homeostasis and carcinogenesis.[32] PPARα is one of the nuclear hormone receptor super family of ligand-activated transcription factors. These transcription factors could alter target gene expression in response to endogenous and exogenous ligands.[33] Thus, some potential relationships between ROS and PPARα may exist. PFOA might activate PPARα, which is the principle mediator of peroxisome proliferation, and such peroxisome proliferation could increase the cellular oxidative stress,[34] which in turn cause DNA damage.

As ROS play an important role in mediating the genotoxic effects of PFOA, the question to be addressed is the origin of these oxyradical species. Mitochondria, the metabolic center of a cell, are intimately involved in the production of ROS and is a potentially susceptible target of environmental mutagens/carcinogens such as ionizing radiation[19] and heavy metals.[16] Some studies also found that mitochondrial dysfunction were related to PFOA induced toxicity.[12,35] Our data showed that $CD59^-$ mutation yield did not change in mtDNA-depleted cells after long-term exposure to PFOA and PFOA stimulated cellular ROS and $O_2^{\cdot-}$ levels in A_L cells but not in ρ^0 A_L cells, indicating the critical role of mitochondria in the mutagenic process. Mitochondria contain their own extranuclear DNA (mtDNA), which is very important for various functions, especially for electron transport chain (ETC).[36] Studies have demonstrated that ETC was one of the major cellular generators of ROS, such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl free radical (.OH).[37] As 13 proteins of ETC were coded by the mtDNA [36] and loss of mtDNA would cause the dysfunction of ETC, it can be postulated that mitochondria were possibly involved in the mutagenicity of PFOA in mammalian cells and PFOA-induced ROS might be derived from ETC.

Apoptosis is recognized as the safeguard mechanism for preventing the process toward mutation.[38] In mammals, caspase-3/7 have been identified as key executors of apoptosis.[39] We found here that activities of caspase-3/7 and caspase-9 both increased after exposure to 200 μM PFOA for 1 day and could be dramatically suppressed with ROS/RNS inhibitors. Similar findings were reported in human HepG2 cells and tilapia hepatocytes cells, of which showed apoptosis after 24 h exposure.[11,35] We hypothesize that two events might be involved in PFOA-induced cellular dysfunction. The earlier event was the generation of ROS leading to the activation of initiator caspase-9. Caspase-9 in turn cleaved

and activated caspase-3/7, inducing the apoptotic pathway. The later event was that as the exposure time (16 days) increased, the accumulative oxidative stress might decrease the DNA repair capacity and thus mutagenicity was induced.[31,40]

In summary, the present study provided direct evidence to show that exposure to PFOA could induce mutagenicity in mammalian cells and mitochondria-dependent ROS played a very important role in such a process. Since PFOA have been ubiquitously detected in the environment as well as in human serum, a better understanding of the mutagenic/carcinogenic mechanisms of PFOA should provide a basis for better interventional approach both in treatment and prevention.

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