ANTICANCER RESEARCH *39*: 2429-2435 (2019)
doi:10.21873/anticanres.13360

Perfluorooctanoic Acid Enhances Invasion of Follicular Thyroid Carcinoma Cells Through NF-κB and Matrix Metalloproteinase-2 Activation

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Abstract. *Background/Aim: Perfluorooctanoic acid (PFOA) is one of the most common perfluorinated compounds widely used in several applications. Due to its persistence in the environment, PFOA has been associated with various diseases, including cancer. This study explored the effects of PFOA on follicular thyroid carcinoma cells (FTC133). Materials and Methods: Cell invasion, migration, adhesion and activity of matrix metalloproteinase-2 (MMP-2) were investigated using Transwell assays, adhesion assay and gelatin zymography, respectively. The underlying mechanism involved in the effects observed was evaluated by immunoblot analyses. Results: Treatment with PFOA did not affect cell migration, but enhanced cell invasion, adhesion and activity of MMP-2 in FTC133 cells. PFOA selectively enhanced the phosphorylation of nuclear factor kappa B (NF-κB) p65, as well as induced NF-κB nuclear translocation. Treatment with a NF-κB inhibitor (BAY 11-7085) was able to reverse PFOA-induced cell invasiveness. Conclusion: PFOA promotes invasiveness of FTC133 cells mediated through the activation of NF-κB signaling.*

Perfluorooctanoic acid (PFOA) is the main member of a group of perfluorinated compounds, which have recently received increased attention for their potential health consequences in humans. PFOA is a synthetic chemical substance that is composed of an aliphatic eight-carbon backbone with all the hydrogen atoms substituted with fluorine to form strong C-F bonds, and a charged carboxyl group. PFOA is produced in relatively large amounts for commercial purposes and used in several applications such as industrial and consumer processes for stain- and water-resistant coatings, oil-resistant coatings for paper products, firefighting foams, mining and oil well surfactants, as well as in insecticide formulations (1).

The hydrophobic, lipophilic and non-polar properties of PFOA make it more persistent in the environment, becoming a global pollutant of air, water and soil (2, 3). The average daily uptake of PFOA in humans is in the range of 1.1 to 11.6 ng/kg BW/day (4). PFOA is readily absorbed, binds to proteins in blood serum and accumulates mainly in organs such as the liver, kidney, spleen, testicles and brain (2). The renal clearances of PFOA are almost negligible in humans with an estimated half-life in blood serum of 3.8 years (5). The median PFOA levels in sera of the general US population are 4 to 5 ng/ml (approximately 9.6-12.0 nM) (6).

Exposure to PFOA can lead to changes in cancer cell metastasis and thereby contribute to enhanced aggressiveness of cancer. Metastasis is a multi-step process that occurs when tumor cells leave the primary tumor and spread to distant organs (7), and appears to be a major factor leading to death of cancer patients. Exposure to PFOA has been reported to enhance migration and invasion in human Ishikawa endometrial cancer cells through the activation of ERK/MTOR signaling (8). Moreover, PFOA has been shown to promote cancer cell invasion *via* nuclear factor-kappaB (NF-κB) pathway in both breast and colorectal cancer cells (9, 10). Recently, PFOA was detected in several human tissues, including endocrine glands, such as thyroid, pituitary, gonads, and pancreas (11). PFOA has also been detected in surgical thyroid tissue specimens from patients undergoing thyroidectomy for a wide spectrum of thyroid diseases (12). In addition, PFOA has been shown to enter thyroid cells by a gradient-based passive diffusion mechanism (13). Therefore, exposure to PFOA may be a factor that promotes the progression of thyroid cancer.

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Key Words: PFOA, MMP-2, invasion, NF-κB, follicular thyroid cancer.

In this study, we investigated the biological effects of PFOA treatment on the invasiveness of follicular thyroid cancer cells, demonstrating that PFOA contributed to enhanced invasiveness of follicular thyroid carcinoma (FTC133) cells. The role of nuclear factor kappaB (NF-κB) on the PFOA-promoted cancer invasion was also elucidated. This knowledge should improve understanding of the linkage between exposure to an environmental contaminant and thyroid cancer progression.

Materials and Methods

Chemicals. PFOA (purity 95.0-102.0% (Titration)) was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Specific NF-κB inhibitor (BAY 11-7085) was purchased from Calbiochem (San Diego, CA, USA).

Cell culture. Human follicular thyroid carcinoma (FTC133) cell line was kindly provided by Prof. Johan Lillehaug, University of Bergen, Norway. Cells were cultured in a 1:1 mixture of DMEM: Ham's F-12 media (Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic solution, and 2 mM L-glutamine (Gibco). Cells were maintained at 37˚C in 5% CO_2.

Cell invasion and migration assays. Invasion and migration assays were performed, as previously described (14) using Transwell chambers with 8 μm pore size membrane filter (Costar, MA, USA). Briefly, the upper surface of the filter was coated with Matrigel (30 μg of Matrigel/filter) for invasion assays, while uncoated filters were used for migration assays. Chambers were incubated overnight at 37˚C for gelling. FTC133 cells were treated with 1 nM PFOA for 72 h. At the end of the treatment, cells were detached and subjected to the assay. Cell suspensions of FTC133 cells (2.5×10^4 cells/ml) in culture media containing 10% FBS were seeded into the inserts of Transwell chambers (200 μl/insert). The same culture media were added to the lower chambers (500 μl/chamber). The chambers were incubated for 24 h at 37˚C. After incubation, non-invading or non-migrating cells on the upper surface of the filters were removed by wiping, using cotton swabs. The filters were then fixed with 25% methanol and stained with 0.5% (w/v) crystal violet in 25% methanol. The number of invaded or migrated cells was counted for 5 fields/filter, at a magnification of 100× under an inverted microscope (Nikon T5100, Tokyo, Japan).

Cell adhesion assay. FTC133 cells were treated with 1 nM PFOA for 72 h. Cells were detached and seeded onto Matrigel-coated 96-well plates (10 μg Matrigel/well) at a density of 2×10^4 cells/well. Cells were allowed to attach for 1 h at 37˚C before washing with PBS to remove non-attached cells. The number of attached cells was determined by MTT assay, as previously described (14).

Gelatin zymography. MMP-2 and MMP-9 are gelatinase enzymes that are usually secreted into the condition media. FTC133 cells were treated with 1 nM PFOA for 72 h. Cells were washed twice with serum-free media and further incubated in serum-free media for 24 h. Then, the condition media were collected and subjected to gelatin zymography, as previously described (15). Briefly, 1 μg condition media were electrophoresed in 7.5% SDS-polyacrylamide gel containing 1 mg/ml gelatin under non-reducing condition. Gels were rinsed with 2.5% Triton X-100 to remove SDS prior to incubation in a buffer (50 mM Tris-HCL (pH 7), 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.05% NaN_3), for 16 h at 37˚C. Gels were stained with 0.3% Coomassie Brilliant Blue in 30% MeOH and 10% acetic acid, and subsequently destained with 30% MeOH and 10% acetic acid. Gelatinolytic activity of MMP-2 and MMP-9 should be observed as clear bands of digested gelatin on a dark background. The molecular mass of the band at 72 kDa and 92 kDa corresponds to the molecular mass of MMP-2 and MMP-9, respectively.

Protein extraction and immunoblot analysis. Effects of PFOA treatment on the expression levels of signaling proteins were analyzed by immunoblot analysis, as previously described (14). FTC133 cells were treated with 1 nM PFOA for 72 h and then proteins were extracted. In order to determine the effect of the NF-κB inhibitor, cells were treated with 1 μM BAY 11-7085 for 1 h prior to PFOA treatment. Cells were lysed in RIPA lysis buffer containing protease inhibitor cocktail and phosphatase inhibitors (1 mM Na_3VO_4, 10 mM NaF, and 20 mM β-glycerophosphate). Cell lysates were sonicated and cell debris removed by centrifugation at 12,000 × *g* for 5 min, at 4˚C. The supernatants of cell lysates were collected and mixed with 5× sample loading buffer (0.5 mM Tris-HCl pH 6.8, 20% glycerol, 10% SDS, 0.1% bromophenol blue, and 5.0% β-mercaptoethanol), followed by denaturation by boiling for 5 min and storage at –80˚C. Total protein concentration of each cell lysate was measured by using Bradford protein assay (Bio-Rad, CA, USA).

Proteins (15 μg) in the cell lysate were resolved in 10% SDS-PAGE at 10 mA/gel for approximately 60 min, and blotted onto PVDF membranes at 100 V, 4˚C for 60 min. Then, the membranes were washed with Tris-buffered saline, pH 7.6, containing 0.1% (v/v) Tween-20 (TBS/T). Membranes were blocked with 3% bovine serum albumin (BSA) in TBS/T for 1 h at room temperature, and subsequently incubated with specific rabbit primary antibodies against total Akt (1:3,000), phospho-Akt (S473) (1:1,000), total Erk1/2 (1:2,000), phospho-Erk1/2 (T202/Y204) (1:2,000), total STAT3 (1:1,000), phospho-STAT3 (Y705) (1:1,000), total NF-κB p65 (1:1,000) and phospho-NF-κB p65 (S536) (1:1,000) (Cell Signaling Technology, MA, USA) overnight at 4˚C. The membranes were then washed with TBS/T, followed by incubation with horseradish peroxidase-linked anti-rabbit secondary antibody (Cell Signaling Technology) for 1 h at room temperature. Membranes were washed three times with TBS/T and then incubated with Western Bright ECL reagents (Advansta, CA, USA) for 5 min. Bands of specific proteins were visualized by using ImageQuant LAS 4000 mini (GE Healthcare, WI, USA). Band intensity of the phosphorylated form of each protein was normalized to that of the total protein.

NF-κB nuclear translocation assay. FTC133 cells were seeded onto 96-well plates and treated with 1 nM PFOA for 72 h. Cells were washed with phosphate buffered saline (PBS), fixed by immersion at room temperature with 4% paraformaldehyde for 10 min at 4˚C, and permeabilized with blocking buffer (3% BSA, 0.2% Triton-X100 in PBS) for 45 min at 4˚C. Cells were then washed with PBS and incubated with primary monoclonal rabbit anti-NF-κB p65 in blocking buffer overnight at 4˚C, followed by incubation with anti-rabbit secondary Alexa Fluor(R) 488 (Cell Signaling Technology) and Hoechst cell nuclear counter staining in blocking buffer for 45 min at room temperature. Subsequently, cells were washed with blocking buffer, followed by washing with PBS. Plates were imaged

at 20x magnification on ImageXpress Micro XLS High Content Screening System (Molecular Devices, CA, USA) using FITC and DAPI channels.

Statistical analysis. Data were calculated from at least three independent experiments and presented as mean±SD. Differences between treated and untreated control cells were analyzed by using Student's *t*-test in Graph-Pad Prism 5 software (GraphPad Software, Inc., CA, USA). Data were considered significantly different when *p*-values were less than 0.05.

Results

Effects of PFOA treatment on metastatic processes of follicular thyroid cancer cells. In order to investigate the ability of FTC133 cells to metastasize, cells were treated with 1 nM of PFOA for 72 h, followed by cell invasion, cell migration, and cell adhesion assays, as well as gelatin zymography of MMP-2 and MMP-9. PFOA enhanced the invasiveness of FTC133 cells by up to 145%, when compared to that of the untreated control. However, the ability of FTC133 cells to migrate did not significantly change after treatment with PFOA. Results from cell adhesion assay showed that PFOA significantly increased the adhesion of FTC133 cells by up to 118%, when compared to the untreated control cells. Since the process of cell invasion involves degradation of extracellular matrix (ECM), we explored the effects of PFOA on MMP-2 and MMP-9 activities, using gelatin zymography assay. The results using 1 μg of loaded protein showed clear bands at the molecular weight of 66 kDa corresponding to active MMP-2. After 72 h treatment with PFOA, MMP-2 activity was significantly increased up to 140%, which suggested its involvement in the promotion of cancer cell invasion (Figure 1). However, MMP-9 activity was not detected at 92 kDa and barely detectable even at a higher protein concentration (data not shown).

Effects of PFOA on invasion-related signaling pathways in FTC133 cells. Immunoblotting studies were used to investigate several intracellular signaling pathways that may be responsible for cancer cell invasion, including those involving protein kinase B (Akt), extracellular signal-regulated kinases (ERK), activator of transcription 3 (STAT3) and nuclear factor kappa B (NF-κB) signaling, as well as the role of PFOA in enhancing FTC133 cell invasion. Protein lysates were extracted from FTC133 cells after treatment with 1 nM PFOA and subjected to western blotting to measure the levels of expression of each signaling protein. Alterations in the expression of signaling proteins were detected after 72 h incubation (Figure 2A). Results showed that treatment with PFOA significantly increased the levels of phosphorylation of NF-κB p65, without affecting phosphorylation of Akt, ERK, and STAT3 (Figure 2B).

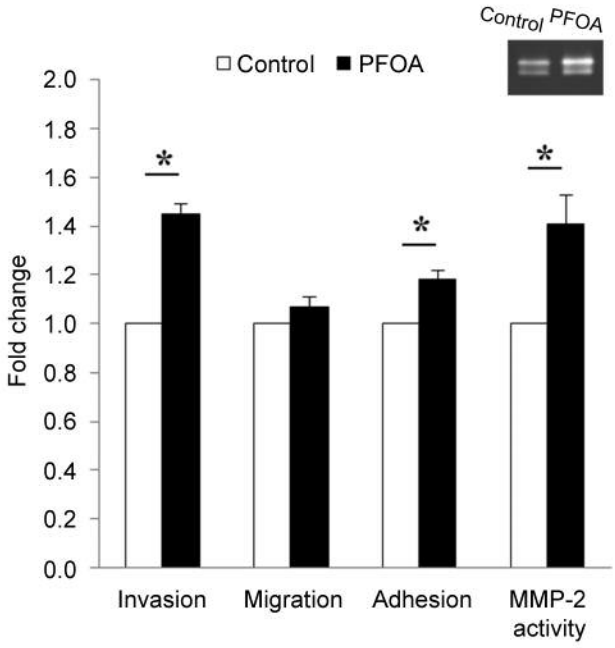

Figure 1. *Effects of PFOA on the metastasis of FTC133 cells. Cells treated with 1 nM PFOA or without PFOA for 72 h were assayed for cell migration, cell adhesion and cell invasion capability, as well as gelatin zymography of MMP-2. Inset shows the zymogram of MMP-2 activity upon treatment. Data are presented as mean±SD in fold change of three independent experiments. *p<0.05.*

Effects of PFOA on nuclear translocation of NF-κB in FTC133 cells. Translocation of NF-κB is a critical step in the transcriptional activation of specific target genes. Upon cell stimulation, the nuclear localization signal of NF-κB is exposed and the protein translocates into the nucleus, where it activates transcription factors and induces specific gene expression. In order to investigate the effects of PFOA on the activation of NF-κB signaling, which may be involved in cancer cell invasion induced by PFOA, immunofluorescence studies were performed to confirm whether PFOA promotes NF-κB nuclear translocation. Cellular nuclei were counterstained with Hoechst (shown in blue), whereas NF-κB p65 was stained green. Images of FTC133 cells showed that upon treatment, NF-κB p65 appeared to be distributed in the nucleus (Figure 3).

Involvement of NF-κB on cell invasion. Inhibition of phosphorylated NF-κB signaling protein by BAY 11-7085 was employed to confirm the role of NF-κB in promoting the invasive ability of FTC133 cells. Treatment of FTC133 cells with 1 μM BAY 11-7085 did not affect cell viability (data not shown). However, it significantly reduced the levels of phosphorylated NF-κB p65 compared to those without BAY 11-7085 (Figure 4A). In addition, after treatment with 1 μM

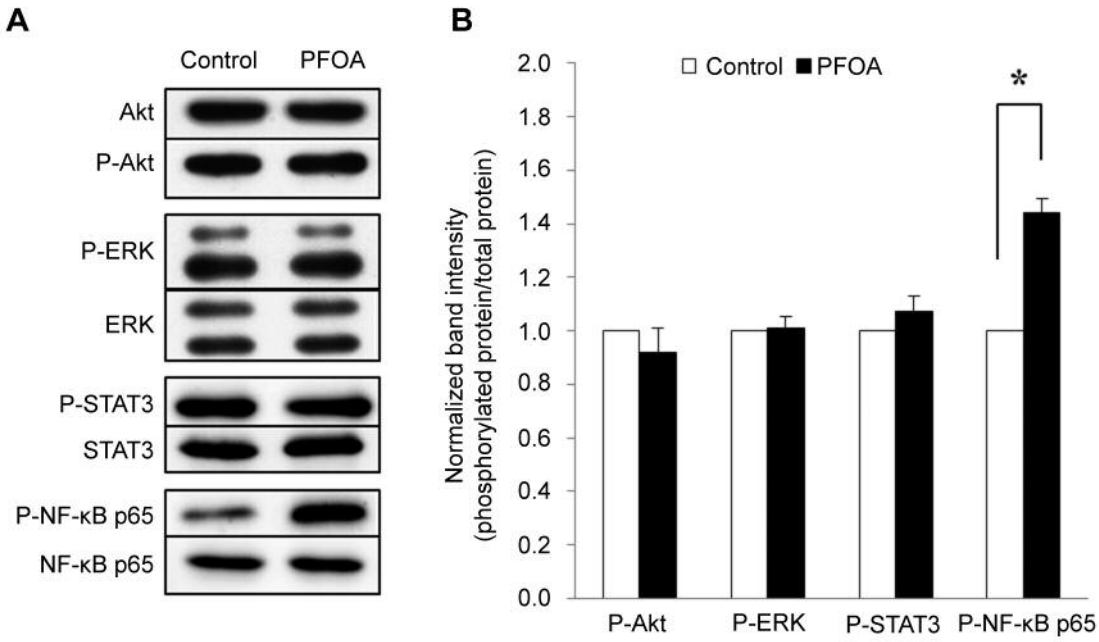

Figure 2. *Effects of PFOA treatment on the expression of signaling proteins in FTC133 cells. Cells were incubated with 1 nM PFOA or without PFOA for 72 h, and then expression of proteins involved in signal transduction was analyzed by immunoblotting. A: Protein expression detected by immunoblot analysis. B: Normalization of band intensity of phosphorylated protein to that of the corresponding total protein. Data are presented as mean±SD of three independent experiments. *p<0.05.*

BAY 11-7085 followed by treatment with 1 nM PFOA, the levels of phosphorylated NF-κB p65 expression were decreased by about 75%. Treatment with BAY 11-7085 alone decreased also basal levels of phosphorylated NF-κB p65 by about 50%. This indicates that NF-κB inhibitor (BAY 11-7085) could partially inhibit NF-κB p65 phosphorylation, even in the presence of PFOA (Figure 4B).

Next, we performed Transwell invasion assay to verify the involvement of NF-κB in cell invasion by treatment with BAY 11-7085. Our results indicate that the invasiveness of FTC133 cells, induced by PFOA, is significantly reduced to that of untreated control or cells treated only with BAY 11-7085 (Figure 4C). This suggests that PFOA activates FTC133 cell invasion through increased NF-κB activation, which correlates with the increased expression of NF-κB with increased cell invasiveness.

Discussion

PFOA is a ubiquitous compound that persists in the environment. Many studies have reported that PFOA can modulate cancer cell metastasis and thereby contribute to the enhanced aggressiveness of cancers (16). However, the effects and molecular consequences of PFOA exposure on thyroid cancer have not been elucidated. Thus, in this study, we investigated the effects of PFOA on follicular thyroid cancer cell metastasis and the underlying mechanism of PFOA-induced invasiveness.

Cell invasion is an indispensable event for metastasis, which allows tumor cells to move through the ECM barrier (7). PFOA treatment at various concentrations (10^{-9}-10^{-4} M) did not affect FTC133 cell viability (data not shown), consistent with previous reports in various cancer cells (9). However, we showed that treatment with a low concentration of PFOA (1 nM) had the ability to significantly enhance the invasiveness of FTC133 cells. This concentration is relevant as it is in the range of actual concentrations of PFOA found in human sera (6). These results indicate that PFOA showed effects on thyroid cancer invasiveness even at a low concentration.

However, cell invasion requires three major processes, namely cell migration, modulation of cell adhesion and proteolysis of ECM. Cell migration, assessed using a Transwell chamber with uncoated filter in the absence of Matrigel, indicated that PFOA did not show any significant effect on the migration of FTC133 cells. This indicates that PFOA has no effect on the mobility of the invaded cells. Since cancers undergo repetitive attachment-detachment to ECM during the invasion process, the effect of PFOA on FTC133 cell adhesion was determined. Our results showed that PFOA significantly increased cell adhesion to ECM, suggesting that PFOA increased the strength of attachment of thyroid cancer cells.

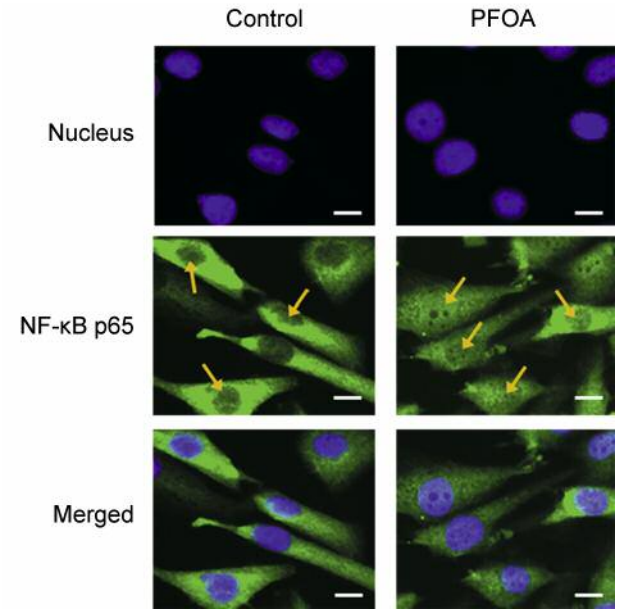

Figure 3. *Effects of PFOA treatment on NF-κB nuclear translocation. Representative immunofluorescence images at 20x magnification of FTC133 cells upon treatment with 1 nM PFOA for 72 h. NF-κB p65 (Alexa Fluor(R) 488-labeled, green) was distributed in the nucleus (Hoechst stain, blue) upon treatment, as labeled by solid yellow arrows. White bars represent length of 10 μm.*

In order to facilitate invasion, cancer cells produce enzymes to degrade or remodel the ECM barriers. The major components of ECM include fibrous proteins, such as type IV collagens and elastins, adhesive glycoproteins such as laminin and fibronectin, and proteoglycans (17). One group of enzymes playing an important role in ECM-degradation is the MMPs. Among these, MMP-2 (72 kDa gelatinase) and MMP-9 (92 kDa gelatinase), which can efficiently degrade native type IV collagen, are well known to play critical roles in cancer progression and their expression levels were shown to increase with increasing malignancy potential of ovarian tumors (18, 19). Since it has been reported that MMP-2/-9 correlate with the invasion capability of cancer cells (20), the effects of PFOA on the enzymatic activity of MMP-2/-9 in FTC133 cells were determined using gelatin zymography. Our results revealed that PFOA could enhance the activity of only MMP-2 in FTC133 cells, as shown by clear bands at the molecular weight of 66 kDa for active MMP-2, without detecting MMP-9 activity. This could be attributed to the low levels of MMP-9 secreted from thyroid cancer cells. We can, therefore, infer that PFOA-enhanced invasion is due to the increased activity of MMP-2.

It has been reported that expression of MMP-2/-9 is associated with the expression of NF-κB, an important transcription factor that controls several cellular responses including inflammation, invasion and cell survival (21). NF-κB

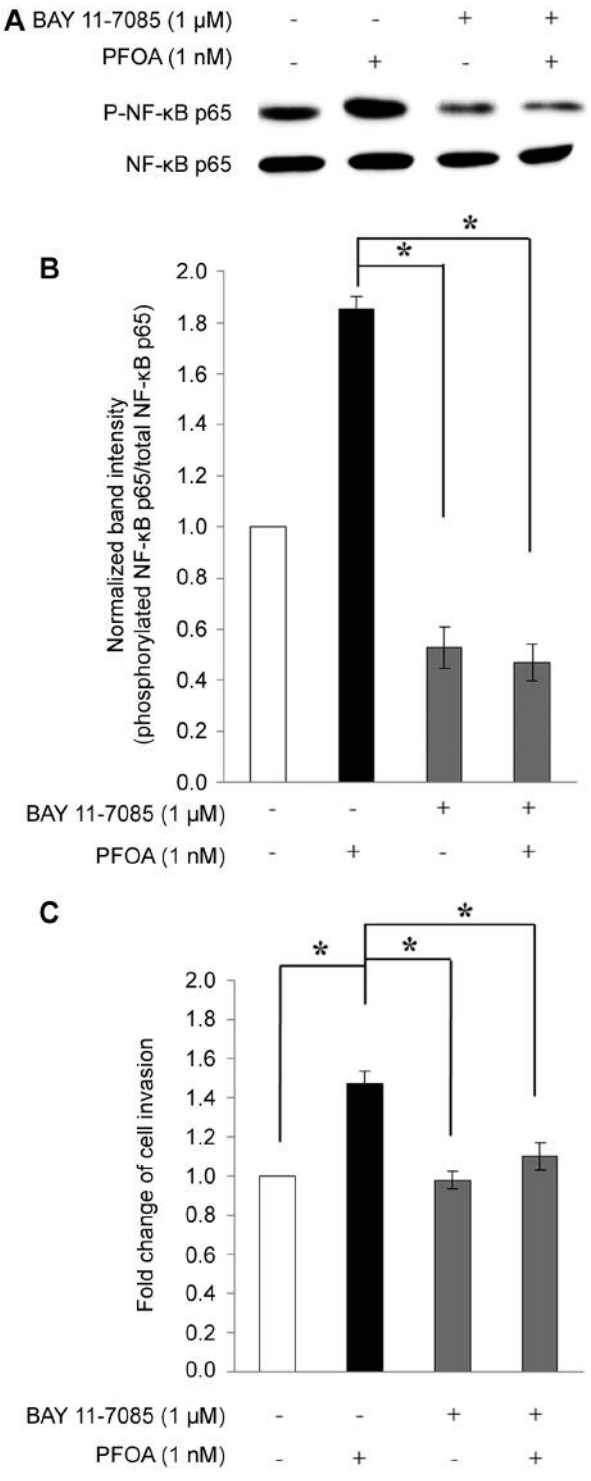

Figure 4. *Involvement of NF-κB in PFOA-enhanced invasiveness of FTC133 cells. Cells were treated with 1 μM BAY 11-7085, an inhibitor of NF-κB, for 1 h, followed by treatment with 1 nM PFOA for a total of 72 h. A: Protein expression detected by immunoblot analysis. B: Normalization of band intensity of phosphorylated protein to that of the corresponding total protein. C: Effect of BAY 11-7085 on PFOA-enhanced invasiveness of FTC133 cells. Results are expressed as mean±S.D. from three independent experiments. *p<0.05.*

can be activated by pro-inflammatory cytokines, viral products, bacterial toxins and other stimuli (exogenous compounds, UV light, γ-radiation) (22). Once NF-κB becomes activated, it translocates from the cytoplasm to the nucleus where it binds target genes and initiates transcription. Previous studies have indicated that NF-κB activation contributes to the enhanced invasiveness of breast and colorectal cancer cells by regulating MMP-2 and MMP-9 expression (9, 10). Thus, we studied the effect of PFOA treatment on the activation of NF-κB signaling in FTC133 cells, and found that the enhancement in the PFOA-induced FTC133 cell invasion was accompanied by significantly increased phosphorylation of NF-κB, when compared to that of untreated control cells. Moreover, immunofluorescence assay showed that PFOA treatment promoted nuclear translocation of NF-κB. Our results demonstrated that PFOA induced NF-κB activation and translocation into the nucleus, which may contribute to the PFOA-enhanced invasiveness of FTC133 cells.

To further demonstrate this causative relationship, we studied the effect of BAY 11-7085 on the phosphorylation of NF-κB. BAY 11-7085 is an irreversible inhibitor of IκBα phosphorylation that is generally used to block gene expression regulated through the classical pathway of NF-κB activation, such as inflammation (23). Inhibition of NF-κB/relA through the suppression of IkBα phosphorylation, leading to a reduction in the formation of active NF-κB, has been shown to reduce tumor invasion and metastasis (24). Our results showed that treatment of FTC133 cells with BAY 11-7085 inhibited the phosphorylation of NF-κB that was induced by PFOA treatment. As a result, NF-κB activation and increased cell invasion were not observed in the presence of BAY 11-7085, suggesting that NF-κB is involved in the enhanced invasiveness of FTC133 cells upon treatment with PFOA.

In conclusion, we demonstrated that PFOA can significantly promote the invasion of follicular thyroid cancer cells by inducing NF-κB activation and nuclear translocation. In addition, PFOA could stimulate the activity of MMP-2. Indeed, NF-κB activation contributed to the enhanced invasiveness of follicular thyroid cancer cells upon treatment with PFOA. These findings emphasize the toxic effects of PFOA even at a low concentration, which may lead to further investigations towards novel prevention and/or treatment strategies against the progression of follicular thyroid cancer.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

P.S. performed all experiments and wrote the manuscript. K.L. contributed to data analysis and interpretation. J.S. provided critical revision of the manuscript. N.M.P. designed the experiments, implemented the research, wrote the manuscript and supervised this study. All Authors read and approved the final manuscript.

Acknowledgements

Funding was from the Chulabhorn Research Institute and Chulabhorn Graduate Institute. The Authors would like to thank to Dr. Benjaporn Homkajorn Songvasin from the Research Laboratory of Pharmacology, Chulabhorn Research Institute, for her assistance on the use of the fluorescence microscope.

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Received March 22, 2019
Revised April 10, 2019
Accepted April 11, 2019