IJHSR
ijhighschoolresearch.org
RESEARCH ARTICLE

Vacuolation: A Novel Biomarker of Environmental Pollutant PFOA Induced Human Renal Proximal Tubular Cell Injury

Sankeeth Udayakumar

Daegu International School, 22 Palgong-ro 50-gil, Dong-gu, Daegu 41021, Republic of Korea; ussankeeth@gmail.com
Mentor: Elumalai Suma

ABSTRACT: Endocrine-disrupting chemicals (EDCs) such as per- and poly-fluoroalkyl substances (PFAS) pose a documented adverse impact on humans and wildlife because of their global distribution. PFAS have received much attention as a possible cause of metabolic disorders, including adverse effects on kidney function. Because of its durability, bio-accumulative potential, and connection to the pathophysiology of metabolic diseases, Perfluorooctanoic acid (PFOA) has received the most attention among PFAS. There is much concern that PFOA exposure may present overwhelming adverse effects on kidney function, and there are only a few outcomes based on the biological effects of this compound in the human kidney cells. This study finds that kidney tubular cell vacuolation and Neutrophil gelatinase-associated lipocalin (NGAL) are acute PFOA-induced kidney tissue injury biomarkers. Chronic exposure of human kidney tubular cells (HK-2) to PFOA stimulated NGAL induction and tubular cell vacuole formation. Upregulated NGAL and vacuole formation led to elevated cleaved caspase-3 activation with decreased human tubular cell viability. Intriguingly, enhanced NGAL and vacuole formation in PFOA-treated cells was reduced with N-acetyl cysteine, a Food and Drug Administration-approved antioxidant. Indeed, N-acetyl cysteine treatment significantly reduced PFOA-induced cleaved caspase-3 activation and enhanced human tubular cell viability.

KEYWORDS: Biomedical and Health Sciences, Pathophysiology, Perfluorooctanoic Acid, Neutrophil Gelatinase-Associated Lipocalin, Vacuolation.

Introduction

Per- and poly-fluoroalkyl substances, widely distributed man-made persistent chemicals used in various consumer and industrial processes, negatively affect human health.[1] PFOA postulates a wide range of toxic effects on the kidney, liver, endocrine function, immune system, and nervous system, but knowledge about PFOA's potential toxicity on human kidney tubular cells is limited and needs a focus of attention. The metabolically active renal tubules are in charge of absorbing or eliminating various chemicals. Several enzymes that are abundantly present in the lysosomes inside or on the brush borders of tubular epithelial cells promote the transport of these substances.[2] Research on epidemiology has demonstrated that PFOA is linked with an elevated risk of renal cell carcinoma in humans at varying levels of chronic exposure.[3,4] There are various ways that humans can be exposed to PFOA. Most humans are exposed to PFOA through their food and water. PFAS can contaminate groundwater, a vital drinking water supply, through various channels, including contaminated soil, river water infiltration, and industrial leaks. An industrial plant in South Korea's Nakdong River Basin experienced a PFHxS leakage incident in 2018. Following the event, higher PFAS concentrations (mainly short-chain PFAS, especially PFHxS) were discovered in tap water in the Nakdong River Basin region.[5,6] However, persistent exposure to comparatively low levels of PFOA will result in a physical load and raise the possibility of adverse health effects. As the primary organ for eliminating the body's metabolic wastes, the kidney is thought to be a target for PFOA, and the body's delayed renal clearance of PFOA contributes to its biological accumulation in the human body. We are unsure how the environmental pollutant PFOA affects renal tubules and eventually develops kidney injury. Therefore, early predictive biomarkers are urgently needed, and early management dramatically improves the prognosis of PFOA-induced kidney injury. Through this study, we aim to identify the early predictive protein and morphological biomarkers of PFOA-induced kidney injury.

Methods

HK-2 cells, which are an immortalized human proximal tubular cell line [7] were purchased from ATCC and maintained in a humidified atmosphere containing 5% CO2 in Roswell Park Memorial Institute (RPMI)-1640 (Life Technologies, Inc., Grand Island, NY) medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin. PFOA was purchased from Sigma-Aldrich (St. Louis, MO, USA). HK-2 cells were exposed to PFOA (50 & 100 μM) for 24 hours. HK-2 cells were exposed to N-acetyl cysteine (200 μM) for 2 hours and then to PFOA (50 μM) for 36 hours. The morphology (vacuolation) of HK-2 cells was captured using an inverted microscope after 24 hours. The percentage of viable cells was determined using the Cell Counting Kit-8 (CCK-8; Dojindo Lab., Kumamoto, Japan).

NGAL Immunofluorescence Analysis:

HK-2 cells were exposed to PFOA (50 & 100 μM) for 12 hours. Then, they were fixed in 2% paraformaldehyde for 15

DOI: 10.36838/v7i4.9

minutes and permeabilized with 0.2% Triton X-100 for 15 minutes at room temperature. The NGAL primary antibody (Abcam—ab125075) was applied overnight and incubated for 1 hour with the secondary antibody Alexa-Fluor488 (Invitrogen, Eugene, OR, USA). NGAL images were captured using a fluorescence microscope.

Western Blotting:

HK-2 cells were exposed to PFOA (50 μM) for the indicated periods, and after that, cell lysates were prepared using a lysis buffer containing protease and phosphatase inhibitors. HK-2 cell protein lysates were resolved by running the samples on a NuPAGE 4%-12% Bis-Tris gel (Invitrogen) and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). After blocking, membranes were incubated at 4 ˚C with the following primary antibodies: NGAL (Abcam, Cambridge, UK), Cleaved caspase 3 (Cell Signaling Technology, USA), and ACTIN (Abcam, Cambridge, UK). Membranes were then washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. Immunoreactive proteins were detected using ECL reagents (ECL Plus; Amersham, GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK).

Result and Discussion

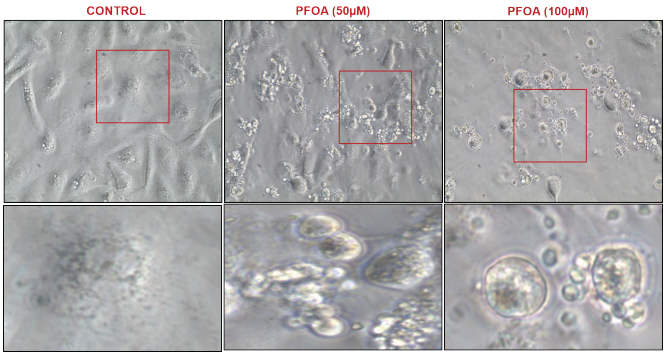

Figure 1: Exposure of HK-2 cells with PFOA (50 & 100 μM) for 24 hours resulted in changes in cell morphology, which include cell rounding-up, which is also followed by cell shrinkage beginning at the periphery and accompanied by membrane blebbing and the formation of cells with numerous vacuoles.

We started by determining whether long-term PFOA exposure affects the proximal tubular cell morphology of the human kidney. Exposure of HK-2 cells with PFOA (50 & 100 μM) for 24 hours resulted in changes in cell morphology, which include cell rounding-up, which is also followed by cell shrinkage beginning at the periphery and accompanied by membrane blebbing and the formation of cells with numerous vacuoles as depicted in Figure 1. Moreover, higher PFOA concentrations cause more pronounced morphological alterations, dramatically reducing the number of cells with severe constrictions, as shown in Figure 1. Cells often adjust their volume due to water moving into or out of the cell in response to changes in the osmotic equilibrium. Cell swelling results from an increase in intracellular osmolarity or a decrease in extracellular osmolarity, whereas cell shrinkage results from a reduction of intracellular osmolarity or an increase in extracellular osmolarity. When cells invade an anisotonic extracellular environment or when intracellular osmolyte concentrations change due to the uptake or release of ions or nutrients or metabolic changes, such as the formation or degradation of macromolecules, these changes occur physiologically.[8] However, lipophilic substances with weakly basic amines are the most commonly known inducers of temporary vacuolization.[9,10] Intriguingly, PFOA is not a lipophilic chemical, and more research is necessary to determine how PFOA causes vacuolation. Importantly, acute kidney injury (AKI), which causes a sudden reduction in renal function, is mainly caused by injury to the tubules, which are responsible for the reabsorption and secretion of several solutes. The data from the present work demonstrate for the first time that PFOA alters the organization of human renal tubular cells characterized by visible cytoplasmic vacuoles.

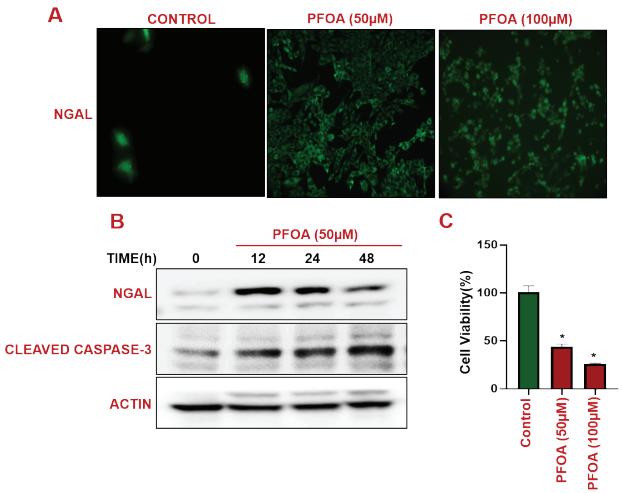

Figure 2: (A). Exposure of HK-2 cells with PFOA (50 & 100 μM) for 24 hours resulted in increased NGAL expression analyzed by the fluorescence microscope. (B). Exposure of HK-2 cells with PFOA (50 μM) for the indicated periods increased NGAL expression, followed by apoptotic marker-cleaved caspase-3 protein induction. (C). Exposure of HK-2 cells with PFOA (50 & 100 μM) for 48 hours induces cell viability loss over time compared to control cells. The statistically significant differences were found using the student's t-test.

NGAL was first discovered using gene arrays in ischemic kidneys and then using immunoblots in hospitalized patients.[11,12] To determine whether the NGAL protein fulfills the requirements for a biomarker, we measured the expression of NGAL in PFOA-exposed human kidney tubular cells. As shown in Figure 2A, Comparing HK-2 cells to control cells using a fluorescence microscope, PFOA treatment increased NGAL expression in a dose-dependent manner. In addition, Western blot images were captured in PFOA-treated cells to measure NGAL protein levels. Significantly, PFOA treatment raised the amounts of the NGAL protein, and the apoptotic marker cleaved caspase-3 protein in the HK-2 cells over time compared to control cells, as illustrated in Figure 2B. Additionally, the activation of the cleaved caspase-3 protein caused by PFOA exposure results in a loss of HK-2 cell viability (Figure 2C). The increase in NGAL production and release from tubular cells in response to harmful stimuli of various kinds may have a self-defensive intent.[13]

Proteomic investigations confirmed that ischemic and nephrotoxic acute kidney injury caused the NGAL protein to

be produced in the kidneys. The urine concentration increased severalfold shortly after the insult (within hours). Under typical circumstances, the proximal tubules almost entirely re-absorb filtered NGAL via megalin-cubulin receptor-mediated endocytosis, resulting in negligible urine NGAL levels.[14,15] Therefore, given the significance of NGAL and vacuolation for apoptosis, it is logical to assume that PFOA regulates these processes to induce kidney tubular cell injury.

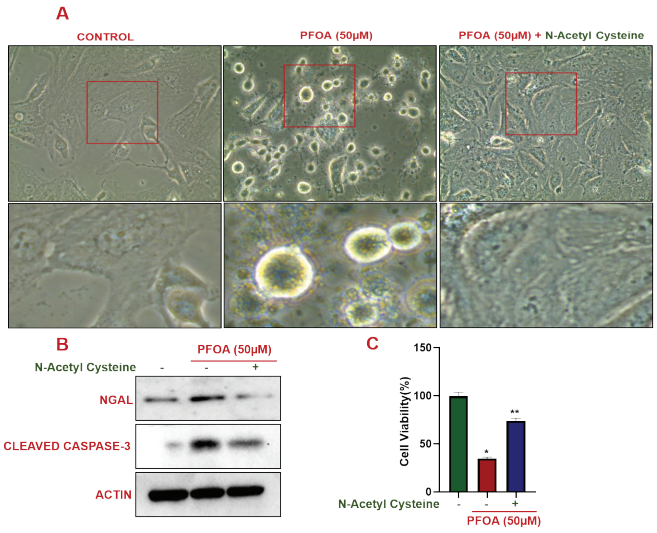

Figure 3: (A). Exposure of HK-2 cells with N-acetylcysteine (NAC) for 2 hours and then with PFOA (50 μM) for 24 hours resulted in changes in cell morphology and the inhibition of vacuole formation. (B-C). Exposure of HK-2 cells with N-acetylcysteine (NAC) for 2 hours and then with PFOA (50 μM) for 36 hours resulted in the prevention of NGAL and caspase-3 induction and the inhibition of cell viability loss.

N-acetyl cysteine (NAC) is a well-known antioxidant that can pass across membranes and alter oxidative processes' balance by reducing ROS production and boosting antioxidant defenses. In clinical research as well as experimental cell and animal biology, it is arguably the most commonly employed "antioxidant." Traditionally, NAC is assumed to function as a precursor for glutathione production, a scavenger of reactive oxygen species, and/or a reductant of disulfide bonds.[16] We hypothesized that endogenously produced oxidative stress is responsible for tubular cell vacuolation, and their inhibition might improve the function of tubular cells against PFOA. N-acetyl cysteine treatment prominently repressed PFOA-induced intracellular vacuole formation, paralleled by the inhibition of NGAL expression and the cleaved caspase-3 level, as depicted in Figure 3A-B. Subsequent analyses revealed that NAC treatment prominently repressed PFOA-mediated HK-2 cell viability loss (Figure 3C). We outline how NAC reacts to PFOA circumstances by increasing antioxidant defense, which regulates vacuolation and NGAL activation. We proposed early kidney damage markers in response to PFOA in the kidney tubular cells, and there seems to be a need for more research on PFOA's effect on kidney injury.

Conclusion

Over the past decade, studies in animals and humans have suggested that long-term exposure to poly-fluoroalkyl substances, like PFOA, from the environment, contributes to the damage of human kidney tubular cells, leading to metabolic diseases like acute and chronic kidney injury. Conversely, we demonstrated for the first time that the rise in NGAL protein and vacuolation could serve as a sensitive marker for identifying PFOA-induced kidney tubular cell damage. As a proof-of-concept, our work supports further investigation into protein biomarkers and other putative markers in PFOA-induced kidney tubular cell damage. In the future, we want to look into the molecular mechanism through which kidney tubular cells exposed to PFOA experience a rise in NGAL and vacuolation and suppression of their natural antioxidant system.

Acknowledgments

I would like to thank Prof Kyu-Chang Won at the Department of Internal Medicine, Yeungnam University Hospital, for providing the lab space and materials, and my mentors, Dr. Suma Elumalai and Dr. Udayakumar Karunakaran, for their valuable support, advice, and guidance on this research.

References

1. Lind L, Zethelius B, Salihovic S, van Bavel B, Lind PM. Circulating levels of perfluoroalkyl substances and prevalent diabetes in the elderly. *Diabetologia*. 2014; 57(3):473-479. doi: 10.1007/s00125-013-3126-3.
2. Ho KM, Morgan DJR. The Proximal Tubule as the Pathogenic and Therapeutic Target in Acute Kidney Injury. *Nephron*. 2022; 146(5):494-502. doi: 10.1159/000522341.
3. Winquist A, Hodge JM, Diver WR, et al. Case-Cohort Study of the Association between PFAS and Selected Cancers among Participants in the American Cancer Society's Cancer Prevention Study II LifeLink Cohort. *Environ Health Perspect*. 2023; 131(12):127007. doi: 10.1289/EHP13174.
4. Rhee J, Chang VC, Cheng I, et al. Serum concentrations of per- and polyfluoroalkyl substances and risk of renal cell carcinoma in the Multiethnic Cohort Study. *Environ Int*. 2023; 180:108197. doi:10.1016/j.envint.2023.108197.
5. Park H, Choo G, Kim H, Oh JE. Evaluation of the current contamination status of PFASs and OPFRs in South Korean tap water associated with its origin. *Sci Total Environ*. 2018; 634:1505-1512. doi:10.1016/j.scitotenv.2018.04.068.
6. Yong ZY, Kim KY, Oh JE. The occurrence and distributions of per- and polyfluoroalkyl substances (PFAS) in groundwater after a PFAS leakage incident in 2018. *Environ Pollut*. 2021; 268(Pt B):115395. doi:10.1016/j.envpol.2020.115395.
7. Ryan MJ, Johnson G, Kirk J, Fuerstenberg SM, Zager RA, Torok-Storb B. HK-2: an immortalized proximal tubule epithelial cell line from normal adult human kidney. *Kidney Int*. 1994; 45(1):48-57. doi:10.1038/ki.1994.6.
8. Okada Y. Ion channels and transporters involved in cell volume regulation and sensor mechanisms. *Cell Biochem Biophys*. 2004; 41(2):233-258. doi: 10.1385/CBB: 41:2:233.
9. Ohkuma S, Poole B. Cytoplasmic vacuolation of mouse peritoneal macrophages and the uptake into lysosomes of weakly basic substances. *J Cell Biol*. 1981;90(3):656-664. doi:10.1083/jcb.90.3.656.
10. Morissette G, Moreau E, C-Gaudreault R, Marceau F. Massive cell vacuolization induced by organic amines such as procainamide. *J Pharmacol Exp Ther*. 2004; 310(1):395-406. doi:10.1124/jpet.104.066084.

DOI: 10.36838/v7i4.9

11. Supavekin S, Zhang W, Kucherlapati R, Kaskel FJ, Moore LC, Devarajan P. Differential gene expression following early renal ischemia/reperfusion. *Kidney Int*. 2003; 63(5):1714-1724. doi:10.1046/j.1523-1755.2003.00928.x.
12. Mori K, Lee HT, Rapoport D, et al. Endocytic delivery of lipocalin-siderophore-iron complex rescues the kidney from ischemia-reperfusion injury. *J Clin Invest*. 2005; 115(3):610-621. doi: 10.1172/JCI23056.
13. Bolignano D, Donato V, Coppolino G, et al. Neutrophil gelatinase-associated lipocalin (NGAL) as a marker of kidney damage. *Am J Kidney Dis*. 2008; 52(3):595-605. doi:10.1053/j.ajkd.2008.01.020.
14. Mishra J, Mori K, Ma Q, Kelly C, Barasch J, Devarajan P. Neutrophil gelatinase-associated lipocalin: a novel early urinary biomarker for cisplatin nephrotoxicity. *Am J Nephrol*. 2004; 24(3):307-315. doi: 10.1159/000078452.
15. Hvidberg V, Jacobsen C, Strong RK, Cowland JB, Moestrup SK, Borregaard N. The endocytic receptor megalin binds the iron transporting neutrophil-gelatinase-associated lipocalin with high affinity and mediates its cellular uptake. *FEBS Lett*. 2005; 579(3):773-777. doi:10.1016/j.febslet.2004.12.031.
16. Hernández-Cruz EY, Aparicio-Trejo OE, Hammami FA, et al. N-acetylcysteine in Kidney Disease: Molecular Mechanisms, Pharmacokinetics, and Clinical Effectiveness. Kidney Int Rep. 2024; 9(10):2883-2903. Published 2024 Jul 20. doi:10.1016/j.ekir.2024.07.020

Author

Sankeeth Udayakumar, a junior at Daegu International School in Daegu, South Korea, is enthusiastic and has a keen interest in learning biology, especially in biomedical science. He aspires to achieve a career path in the medical field and public health and desires to pursue his goals in the future