THE INVOLVEMENT OF MAPK PATHWAYS AND CELL CYCLE PROTEINS IN PFOS-INDUCED APOPTOSIS OF CEREBELLAR GRANULE CELLS

Lee YJ*, Yang JH

Department of Pharmacology/Toxicology, Catholic University of Daegu, School of Medicine, Daegu, Republic of Korea

Introduction

Perfluorooctane sulfonate (PFOS) is a prominent member of perfluoroalkyl compounds (PFCs), which have been widely used in industrial and many consumer products[1]. As PFOS is very stable and extremely resistant to breakdown, it persists in environment and accumulates in animal tissues[2].

While PFOS is primarily concentrated in the liver and blood, its levels in the brain gradually increase with time after exposure[3]. PFOS is reported to trigger opening of tight junction in brain endothelial cells[4]. Neonatal exposure to PFOS can cause neurotoxic effects in adult mice, manifested as changes in spontaneous behavior and habituation. High dose of PFOS leads to neonatal mortality and neurologic delays. Low dose exposure induces behavioral defects in acetylcholine system, which persists into adulthood life[5]. Accordingly, potential of this compound to elicit developmental neurotoxicity has been a health concern for the recent years.

Apoptosis of neuronal cells is a key element in neurotoxicity. In particular, apoptosis during developmental period of synaptogenesis, known as the brain growth spurt (BSG) period, is a critical event to induce neurobehavioral disturbances expressed either in childhood or with delayed onset in adulthood[6]. Many environmental pollutants are known to induce apoptotic neurodegeneration and closely associated with a variety of neuronal diseases. Recently, PFOS caused apoptosis of cerebellar granule cells (CGC), the most abundant neuronal cells in the mammalian brain[7]. Although the underlying mechanisms responsible for PFOS-induced apoptosis of CGC are largely unknown, studies have suggested that mitogen-activated protein kinases (MAPKs) and cell cycle re-entry are related to apoptosis of postmitotic neurons[8,9]. Thus, the present study examined the effects of PFOS on MAPKs signaling pathways and the levels of cell cycle proteins in CGC in culture to understand the possible mechanism of PFOS-induced neuronal apoptosis.

Materials and Methods

Cerebellar granule cell culture and exposure. Cerebellar granule cells were isolated from 7-day old Sprague-Dawley (SD) rat pups and cultured as described previously[10]. Cells grown on 6-well plates coated with poly-L-lysine and maintain at 37 C in a humidified incubator with 5 % CO2 atmosphere. After 48 h of culture, cells were treated with 5 mM of cytosine arabinoside to prevent growth of non-neuronal cells. Cells were maintained for 7 days in culture (DIV 7) and then were exposed to PFOS (0 - 50 μM) for different time (0 – 24 h).

Whole cell extracts. Cells were lysed on ice in lysis buffer (20 mM HEPES (pH 7.5), 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 2 mM $MgCl_2$, 150 mM NaCl, 10 mM KCl, 1 % NP-40, 1 mM Na_3VO_4, 1 mM DTT, 1 mM

benzamide, 1 mM PMSF, and protease inhibitors). After centrifugation at 16,000 g, the supernatants were used as whole cell extracts.

Western blotting. Western blot analysis was performed as described previously[7]. Whole cell lysates (30 μg) were separated by SDS-PAGE gel and transferred to nitrocellulose membrane by Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA). The membrane was blocked with 5% non-fat dry milk in Tris- buffered saline. The blots were incubated with primary antibodies for phosphor-ERK, phospho-JNK, phospho-p38 MAPK (Cell signaling, Beverly, MA), E2F1 and cyclin E (Santa Cruz Biotechnology, Santa Cruz, CA) and then, reacted with a peroxidase-conjugated secondary antibody. The protein bands were detected by the Super Signal (Pierce, Rockford, IL). The density of respective bands was analyzed by the Chemi-Doc XRS imaging system (Bio-Rad, Hercules, CA). The membranes were reprobed with anti-GAPDH antibody, which was used as loading control.

Caspase-3 activity assay. The caspase-3 activity was measured by using commercially available assay kits (Chemicon, Billerica, MA). Cell lysates were incubated with colorimetrically labeled substrate, Ac-DEVD-pNA and the absorbance was measured using microtiter plate reader at 405 nm (Chemicon, Billerica, MA). The data were represented as fold increase over the control.

Results and Discussion

The perinatal exposure to PFOS induces neurobehavioral effects in rodent model and causes abnormal development of motor neurons in zebrafish embryos[5,11]. The neural apoptosis is the main mechanisms contributing to neurotoxicity. The cerebellar granule cells in PND-14 (day 7 *in vitro*) were exposed to 30 μM PFOS for different time. The caspase-3 activity, a hallmark of apoptosis, increased in time-dependent manner (Fig 1.) in agreement with previous our report[7].

The activation of caspase-3 is regulated by various signaling pathways including MAPKs. The major subfamilies of MAPKs are extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK, which have been known to involved in both survival and apoptosis of CGC depending on stimuli[8,12]. The treatment with PFOS increased the activation of ERK at 5 min, which maintained up to 6 h. However, PFOS did not changed the activation of either JNK or p38 MAPK, suggesting that PFOS selectively activated ERK in CGC (Fig 2A). The PFOS-induced caspase-3 activity was significantly blocked by pretreatment with a selective inhibitor of ERK pathway, PD98059, whereas the inhibitions of JNK and p38 MAPK by SP600125 and SB203580 had no effects. These results indicated that PFOS-activated ERK in CGC is proapoptotic.

The apoptosis of postmitotic neuron is also mediated by cell cycle regulators under stress situation, where the transcription factor E2F-1 and cyclins involved in G_0/G_1 phase, namely cyclin D1 and cyclin E are known to be important modulators[8,13,14]. E2F-1 is regulated by interaction with its binding partner retinoblastoma protein (Rb). Upon phosphorylation of Rb by upstream kinases including cyclin-dependent kinases (CDKs) and p38 MAPK, E2F-1 is released from Rb and induces Bax and caspase activation resulting in neuronal apoptosis[15]. Of relevance, the increased activity and/or expression of cyclin D1-CDK4/6 complex, a well known upstream

regulator of Rb has been observed in neuronal apoptosis induced by serum/potassium (S/K) deprivation[8]. Similarly, cyclin E-CDK2 has been also involved in neuronal apoptosis and the protein level of cyclin E and its apoptotic role are regulated in ERK-dependent manner[16]. Western blot analysis revealed that PFOS induced the expression of cyclin E protein but not E2F-1 protein (Fig 3). Our results suggest that PFOS-induced increased expression of cyclin E may be one of downstream target of ERK activation, resulting in apoptosis of CGC. Although the increased level of E2F-1 protein has been observed in apoptosis of CGC induced by certain stimuli, the increased DNA binding activity of E2F-1 without change in its total protein level in neuronal apoptosis has been also reported[17]. Further studies are warranted to clarify the role of cell-cycle progression and its relation to MAPK signaling pathway in PFOS-induced apoptosis of CGC.

Acknowledgment

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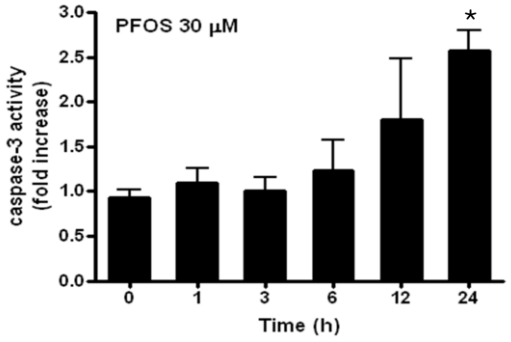
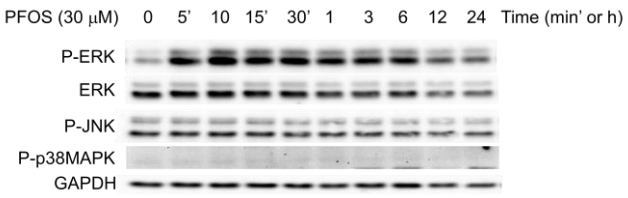


Figure 1. The effects of PFOS on apoptosis of CGC.

(A)



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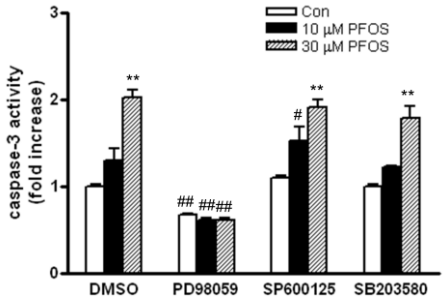

Figure 2. The effects of PFOS on MAPKs activation (A) and roles of MAPKs in PFOS-induced apoptosis of CGC (B).

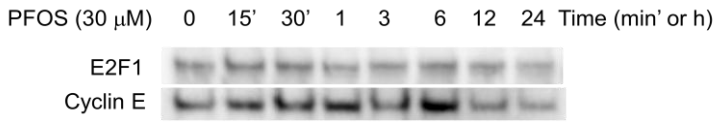

Figure 3. The effects of PFOS on cell cycle proteins.