ERK AND AMPK ARE REGULATED BY NMDA RECEPTOR IN NEUROINAL CELLS EXPOSED TO PFHxS

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Introduction

Environmental pollutant is considered one of the important etiologies of neurodegenerative diseases. Increasing number of studies suggest that neuronal exposure to environmental toxicants in early life increases the risk of neurodegenerative diseases[1]. Perfluoroalkyl compounds (PFCs) have been widely used in industrial and many consumer products since 1950s. Due to their extreme stability, PFCs persist in environment and accumulate in human via food web[2]. Perfluorohexanesulfonate (PFHxS), one of the major PFCs, has been detected in serum from general population as well as in umbilical cord and breast milk[3-5]. Recent animal study has shown that a single neonatal exposure to PFHxS caused behavioral and cognitive disturbance in adult mice[6]. We have also previously shown that PFHxS induced neuronal cell apoptosis, indicating the potential neurotoxic effect of PFHxS[7]. However, studies on its neuronal effects are limited and not much is known about the underlying mechanisms.

The overactivation of N-methyl-D-aspartic acid (NMDA) receptor, an ionotropic glutamate receptor has been suggested to play an important role in neuronal cell death associated with neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease[8,9]. The activation of NMDA receptor induces Ca^{2+} influx into cells, which has been shown to be involved in several signaling pathways responsible for excitotoxic neuronal death[10,11]. We previously demonstrated that neuronal cell apoptosis induced by PFHxS involved NMDA receptor-mediated ERK pathway[12]. In addition, the excess intracellular Ca^{2+} leads to disturbance of ion homeostasis which decreases the level of intracellular ATP. AMP-activated protein kinase (AMPK) is activated in response to ATP depletion and is implicated in a series of catabolic pathways to restore cellular energy level, showing its neuroprotective effect[13]. In addition, AMPK has been also shown to be involved in neuronal apoptosis[14-15]. Therefore, AMPK plays both pro-apoptotic and anti-apoptotic roles depending on types of stimuli and cell.

In the present study, we have examined the role of AMPK in PFHxS induced neuronal apoptosis and the involvement of NMDA receptor-mediated Ca^{2+} influx in AMPK regulation using a rat pheochromocytoma cell line PC12 cells.

Materials and Methods

PC12 cell culture and neuronal differentiation. Cells were grown in RPMI 1640 Medium supplemented with

10% horse serum (HS), 5% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin. For neuronal differentiation, cell culture medium was changed to RPMI 1640 containing 1 % HS, 5 % FBS and 1 % penicillin/streptomycin. Then, cells were treated with NGF (100 ng/ml) for 5~6 days, and then used for experiment.

Western blotting. Western blot analysis was performed as described previously[7].

Calcium detection. Cells were lysed by homogenization and intracellular calcium concentration was measured by using commercially available assay kits (Abcam, Cambridge, UK).

Caspase-3 activity assay. The caspase-3 activity was measured with colorimetrically labeled substrate, Ac-DEVD-pNA by using commercially available assay kits (Chemicon, Billerica, MA, USA) as described previously[12].

Results and Discussion

The influx of calcium ($[Ca^{2+}]_i$) plays a pivotal role in neuronal excitotoxic injury induced by NMDA receptor overactivation. The increased intracellular calcium ($[Ca^{2+}]_i$) has shown to activate several signaling pathways involved in neuronal cell death. We first examined the effect of PFHxS on calcium influx. PFHxS rapidly increased the level of $[Ca^{2+}]_i$. The level of $[Ca^{2+}]_i$ reached the maximum increase at 1 h and then gradually decreased (Fig 1A). The increase in $[Ca^{2+}]_i$ was almost completely blocked by NMDA receptor antagonist, MK801 and Ca^{2+} channel blockers, diltiazem (DTZ) and nifedipine (NFP) (Fig 1B). This shows that PFHxS increased $[Ca^{2+}]_i$ through NMDA receptor and calcium channel. The increased caspase-3 activity induced by PFHxS was significantly reduced by MK801, DTZ and NFP (Fig 1C), indicating that intracellular calcium influx by NMDA receptor activation induced neuronal cell apoptosis.

AMPK known as one of the downstream signal molecule of $[Ca^{2+}]_i$ in neuronal excitotoxic pathway has shown to be involved in both cell survival and death. To examine the role of AMPK in PFHxS-induced neuronal apoptosis, AMPK activation was measured. PFHxS increased the phosphorylation of AMPK which was inhibited by compound C, AMPK inhibitor (Fig 3). The increased caspase-3 activity by PFHxS was significantly reduced by compound C (Fig 1C). This result was confirmed by using AMPK siRNA. AMPK siRNA reduced the AMPK protein level as well as AMPK phosphorylation. The phosphorylation of acetyl-CoA-carboxylase (ACC), a downstream target molecule of AMPK was also prevented without changing its protein level (Fig 2A). Similar to the effect of compound C, AMPK siRNA significantly reduced PFHxS-induced caspase-3 activity (Fig 2B). This shows that PFHxS increased the activation of AMPK which plays a pro-apoptotic role. The phosphorylation of AMPK and ACC was substantially reduced by MK801, DTZ and NFP (Fig 3). However, compound C had no inhibitory effect on PFHxS induced the increase in $[Ca^{2+}]_i$. (Fig 1B). This suggests that the activation of AMPK is regulated by NMDA receptor activation and subsequent increase in the influx of $[Ca^{2+}]_i$.

Previously, we have shown that PFHxS-induced neuronal apoptosis was mediated by NMDA receptor-regulated ERK pathway. To identify whether there is cross talk between AMPK and ERK pathways, the phosphorylation of ERK and AMPK was measured in the presence of compound C and PD98059 (PD), an ERK

inhibitor. The activation of ERK induced by PFHxS was inhibited by MK801, DTZ and NFD but not by compound C (data no shonwn). Likewise, PD had no effect on the activation of AMPK (Fig 3). These results suggest that ERK and AMPK are regulated by NMDA receptor in different signaling pathways although both ERK and AMPK are downstream molecules of NMDA receptor and intracellular calcium.

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References

1. Fox DA, Grandjean P, de Groot D, Paule MG. (2012); *Neurotoxicology* 33(4):810-816.
2. Lau C, Anitole K, Hodes C, Lai D, Pfahles-Hutchens A, Seed J. (2007); *Toxicol Sci*. 99:366-394.
3. Lee YJ, Kim MK, Bae J, Yang JH. (2013); *Chemosphere* 90:1603-1609.
4. Kato K, Wong LY, Jia LT, Kuklenyik Z, Calafat AM. (2011); *Environ Sci Technol*. 45:8037-8045.
5. Kärrman A, Ericson I, van Bavel B, Darnerud PO, Aune M, Glynn A, Lignell S, Lindström G. (2007); *Environ Health Perspect*. 115:226-230.
6. Viberg H, Lee I, Eriksson P. (2013); *Toxicology* 304:185-191.
7. Lee YJ, Choi SY, Yang JH.(2014); *Chemosphere* 94:121-127.
8. Wu YN, Johnson SW**.** (2011)**;** *Neuroscience* 195:138-**1**44.
9. Liu Z, Lv C, Zhao W, Song Y, Pei D, Xu T. (2012); *Neurochem Res* 37(7):1420-1427.
10. Choi DW (1987)**;** *J Neurosci* 7:357-368.
11. Manev H, Favaron M, Guidotti A, Costa E. (1989); *Mol Pharmacol* 36 (1):106–112.
12. Lee YJ, Choi SY, Yang JH.(2014)**;** *Sci Total Environ* 491-492:227-234.
13. Weisová P[1], Anilkumar U, Ryan C, Concannon CG, Prehn JH, Ward MW. (2012); *Biochim Biophys Acta* 1817(5):744-53.
14. Concannon CG, Tuffy LP, Weisová P, Bonner HP, Dávila D, Bonner C, Devocelle MC, Strasser A, Ward MW, Prehn JH. (2010); *J Cell Biol* 189(1):83-94.
15. Rousset CI[1], Leiper FC, Kichev A, Gressens P, Carling D, Hagberg H, Thornton C.(2015); *J Neurochem* 133(2):242-252.

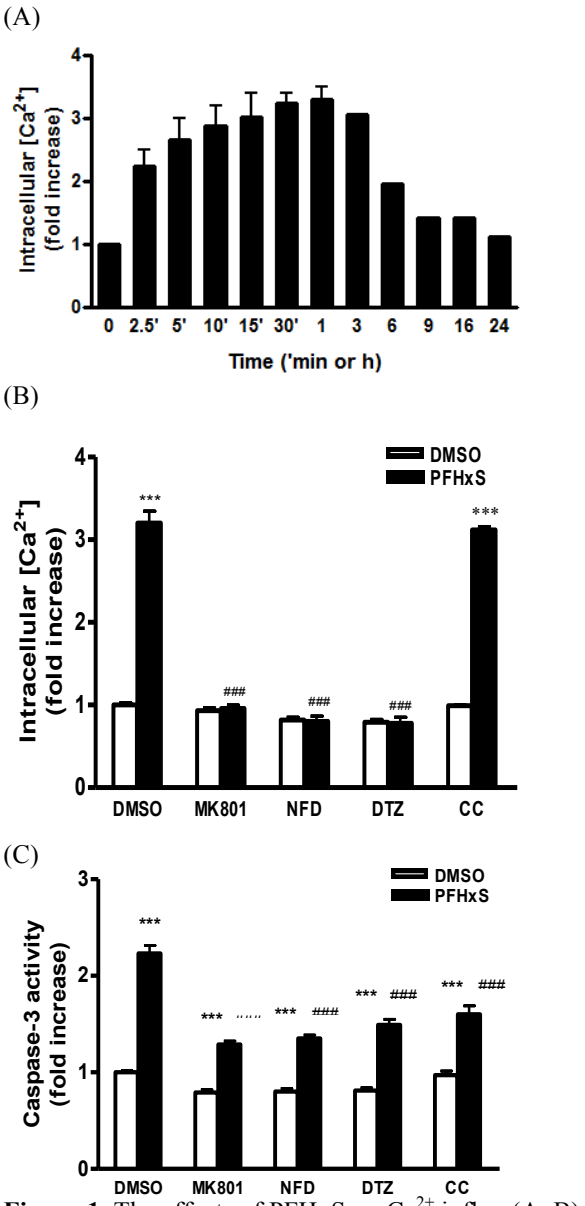

Figure 1. The effects of PFHxS on Ca^{2+} influx (A, B) and role of $[Ca^{2+}]_i$ PFHxS-induced apoptosis (C).

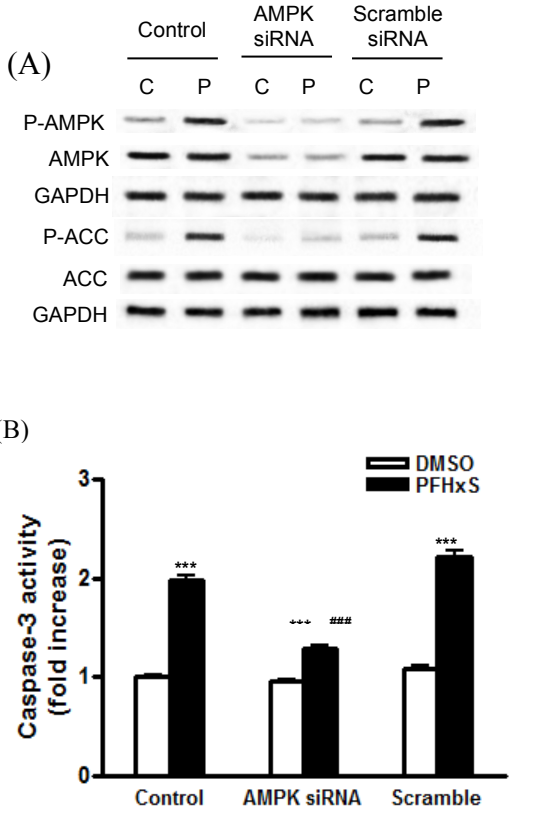

Figure 2. The effects of AMPK siRNA on the phosphorylation of AMPK and ACC (A) and PFHxS-induced apoptosis (B).

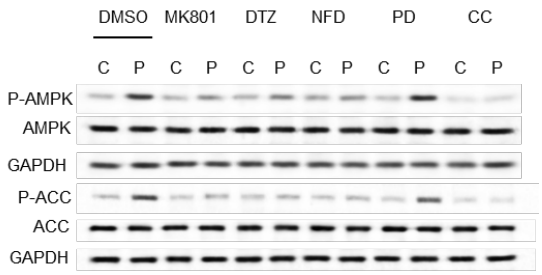

Figure 3. The effects of NMDA receptor antagonist, MK801 and Ca^{2+} channel blockers, diltiazem (DTZ) and nifedipine (NFD) in PFHxS-induced AMPK activation.