Toxicology Letters 218 (2013) 56–60

Contents lists available at SciVerse ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Perfluoroalkylated compounds induce cell death and formation of reactive oxygen species in cultured cerebellar granule cells

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HIGHLIGHTS

- Perfluorinated alkylated compounds induce cell death of cerebellar granule cells.
- The perfluorinated compounds induce oxidative stress in cerebellar granule cells.
- The effects of the perfluorinated compounds are structure dependent.

ARTICLE INFO

Article history:
Received 30 October 2012
Received in revised form 7 January 2013
Accepted 12 January 2013
Available online 20 January 2013

Keywords:
Perfluoroalkylated compounds (PFC)
PFOS
FTOH
PFOA
PFOSA
Cerebellar granule cells
Reactive oxygen species (ROS)
Cell death

ABSTRACT

The present communication investigates the effects of different perfluoroalkylated compounds (PFCs) on formation of reactive oxygen species (ROS) and cell death in cultured cerebellar granule cells. This allows direct comparison with similar effects found for other environmental contaminants like polychlorinated biphenyls and brominated flame-retardants. The increase in ROS formation and cell death was assayed using the fluorescent probe 2,7-dichlorofluorescin diacetate (DCFH-DA) and the trypan blue exclusion assay. The effects of the PFCs were structure dependent. Cell death was induced at relatively low concentrations by perfluorooctyl sulfonate (PFOS), perfluorooctane sulfonylamide (PFOSA) and the fluorotelomer alcohol 1H, 1H, 2H, 2H-perfluorodecanol (FTOH 8:2) with EC_{50}-values of 62 ± 7.6, 13 ± 1.8 and 15 ± 4.2 μM (mean ± SD) respectively. PFOS, perfluorooctanoic acid (PFOA) and PFOSA induced a concentration dependent increase in ROS formation with EC_{50}-values of 27 ± 9.0, 25 ± 11 and 57 ± 19 μM respectively. Reduced cell viability and ROS formation were observed at concentration level close to what is found in serum of occupationally exposed workers. The effect of PFCs on ROS formation and cell viability was compared with other halogenated compounds and future investigations should emphasize effects of mixtures and how physical chemical properties of the compounds influence their toxicity.

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1. Introduction

Perfluoroalkylated compounds (PFCs) are used in fire-fighting foams, as water and oil repellants in clothes, carpets and leather products and even as surfactants on pharmaceutical tablets (Kissa, 2001). Recent progress in analytical chemistry has shown that the PFCs have become ubiquitously spread and concerns are raised about their persistence in the environment and toxicity (Lau et al., 2007). Several of the PFCs have been used for over 50 years, and the presence of fluorine in human blood was reported as early as 1968 (Traves, 1968). The findings did, however, not receive much attention before accumulation of perfluorooctanoic acid (PFOA) in humans was confirmed in the mid 1990s (Gilliland and Mandel, 1993). The PFCs are extremely persistent to degradation. Their persistence comes from the carbon—fluorine bond, which has very high bond strength and the fluorine atoms act like a stiff armor around the carbon chain, making them practically impossible for microbes to degrade. The extensive use of PFCs over a relatively long period of time and their chemical stability has resulted in substantial increase in environmental concentration of different PFCs in human and wild-life, even in the arctic environment (Smithwick et al., 2005; Bossi et al., 2005; Haukås et al., 2007; Lau et al., 2007; Gilliland and Mandel, 1993, 1996).

The most profound effect of some PFCs in rodent studies is as peroxisome proliferators (Lau et al., 2007). Characteristics for peroxisome proliferators are hepatomegaly, proliferation of smooth endoplasmatic reticulum and peroxisomes in association of enzyme induction, and inhibition of mitochondrial beta-oxidation (Suga, 2004). The amphiphilic nature of these compounds also suggests that they have an effect on membranes. The high mortality of gestationally exposed rat and mice pups (Lau et al., 2003) may suggest an influence on the surface properties of the lungs

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0378-4274/$ – see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.toxlet.2013.01.006

making them less efficient to absorb oxygen. Studies have in addition showed that PFCs affect cell–cell communication, membrane transport and processes of energy generation (Schnellmann and Manning, 1990; Sohlenius et al., 1993; Starkov and Wallace, 2002; Upham et al., 1998) and can even be neurotoxic (reviewed by Mariussen, 2012).

Reactive oxygen (ROS) and nitrogen species (RNS) have been implicated as an important causative factor in the mechanism of action of several environmental contaminants (Ali et al., 1992; Mariussen et al., 2002; Myhre et al., 2001, 2000; Reistad et al., 2007; Schulz et al., 1995; Voie et al., 1998). Oxidative stress has been implicated in the development of several diseases such as cancer, immune deficiencies and neurological sufferings, such as Parkinson's. Our group has previously shown that polychlorinated biphenyls and brominated flame retardants reduce cell viability and induce oxidative stress in cerebellar granule cell. This is a convenient in vitro model to study effects of environmental contaminants and indicate a toxic potential of the substance of concern in mammalian cell. In this communication we have studied the effect of four different PFCs on cell viability and ROS formation in cultured cerebellar granule cells in order to compare the toxic potential with that of PCBs and BFRs. The PFCs included perfluorooctanesulfonic acid (PFOS), which e.g. is used in a wide range of industrial applications such as fire fighting foams and surface coating on carpets; perfluorooctanesulfonamide (PFOSA), which is the precursor for most industrial perfluorooctanesulfonyl derivatives; fluorotelomer alcohol (FTOH 8:2), which e.g. is used as fire fighting foams and as grease resistant coatings on carpets and textiles; and perfluorooctanoic acid (PFOA), which e.g. is used in the manufacturing polytetrafluoroethylene (Kissa, 2001).

2. Materials and methods

2.1. Chemicals

The suppliers of the chemicals used for cell cultivation, monitoring of oxidative stress and cell viability are described in detail in Reistad et al. (2006,2007). The test substances perfluorooctyl sulfonate (PFOS, purity >98%) and perfluorooctanoic acid (PFOA, approximately purity 95%) were from Fluka Chemie AG, Switzerland. Perfluorooctane sulfonylamide (PFOSA, approximately purity of 97%) and 1H, 1H, 2H, 2H-perfluorodecanol 8:2 (FTOH, approximately purity of 97%) were from Abcr GmbH & Co. KG, Germany. Stock solutions were prepared by dissolving the compounds in DMSO. The final DMSO concentration was always less than 1%.

2.2. Preparation of cerebellar granule cells

Primary cultured neurons from rat cerebellum were isolated mainly as described by Schousboe et al. (1989) and Reistad et al. (2007). The cerebella from 6- to 8-day-old pups (from Møllegaard Laboratories, Denmark) were dissected under sterile conditions and the brain tissue was then mechanically dissected from the meninges, trypsinated and chopped in buffered solution. The cells were seeded (10^6/ml) in basal Eagle's medium (containing 10% heat inactivated fetal bovine serum, 25 mM KCl, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin) and plated into 50 mm cell culture dishes that had been coated previously with poly-L-lysine (10 μg/ml). After 16–22 h cytosine β-D-arabinofuranoside was added (final concentration 2.5 μg/ml) to prevent growth of glial cells. The cells were grown for 7–9 days (37 °C with 5% CO_2) before experiments were performed in vitro.

2.3. Assay for measuring cell viability of cerebellar granule cells

The growth media (BME with fetal bovine serum) was removed from the plated granule cells and replaced by 4 ml prewarmed culture media without fetal bovine serum containing fluorinated compounds. Thereafter, the granule cells were incubated for 24 h prior to determining neuron survival by using the trypan blue exclusion assay. The cells were incubated with 100 μl of 1% trypan blue mixture at 37 °C for 3 min followed by counting of the relative number of dead cells (blue) by using light microscopy. Failure to exclude trypan blue is associated with necrotic cell death because of loss in the plasma membrane integrity. The effect on cell death is presented as the mean (±SD) of 5 separate experiments assayed in duplicate.

2.4. Assay for measuring reactive oxygen species (ROS)

Formation of ROS was measured by using the fluorescent probe DCFH-DA as previously described in detail (Myhre et al., 2000; Reistad et al., 2007). The cells were preincubated with DCFH-DA (5 μM). The medium with DCFH-DA was then replaced with 1.5 ml incubation medium (HBSS with 20 mM Hepes and 10 mM glucose) containing the test compound. After 15 min the cells were harvested and three aliquots of 250 μl were taken from each cell culture dish and placed in the wells of a multiwell plate (microtiter plate, 96 wells). Fluorescence was recorded in a Perkin Elmer LS50B luminescence spectrometer (excitation wavelength 485 nm, emission wavelength 530 nm) at 37 °C for 180 min. Results are presented as mean ± SD, and each experiment was performed in duplicates. DMSO, which was used for dilution of the test compounds, had no significant effect on ROS formation (98 ± 7% of controls, mean ± SD).

2.5. Data analysis

Statistics (descriptive statistics, one-way or two-way ANOVA), non-linear regression analysis (Hill-function) for the calculation of EC_{50} values (concentration causing 50% cell death or ROS formation) and mathematical calculations were computed in GraphPad Prism 5 or Excel 2007.

3. Results and discussion

PFOS, PFOSA and FTOH 8:2 induced a concentration dependent increase in cell death (Fig. 1). The EC_{50} (concentration causing 50% cell death) values for PFOS, PFOSA and FTOH 8:2 were 62 ± 7.6, 13 ± 1.8 and 15 ± 4.2 μM (mean ± SD), respectively. The PFOA did not cause any significant increase in cell death, compared to the control (Fig. 1). Slotkin et al. (2008) measured cell viability showing, similar to our study, that PFOSA was the most potent in reducing the cell viability of PC12 cells as measured with trypan blue. PC12 is a cell line derived from a pheochromocytoma of the rat adrenal medulla. In their study, also PFOS induced cell death, whereas, as observed in our study, PFOA did not induce loss of cell viability. Slotkin et al. (2008) observed loss of cell viability after exposing the cell to 250 μM. The higher concentrations used in their study may be due to use of serum in the incubation media. PFCs have high affinity to proteins (Vanden Heuvel et al., 1992; Ylinen and Auriola, 1990; Ylinen et al., 1989), which may reduce the amount reaching the actual target. The sensitivity of the two different cell types may also differ. Of interest is to compare the effect of the PFCs with the effect of other halogenated compounds. The brominated flame retardants TBBP-A, pentabromodiphenylether (PBDE) and hexabromocyclododecane (HBCD) showed EC_{50} values from 3 to 7 μM (Reistad et al. 2006), the PCB mixtures Aroclor 1242

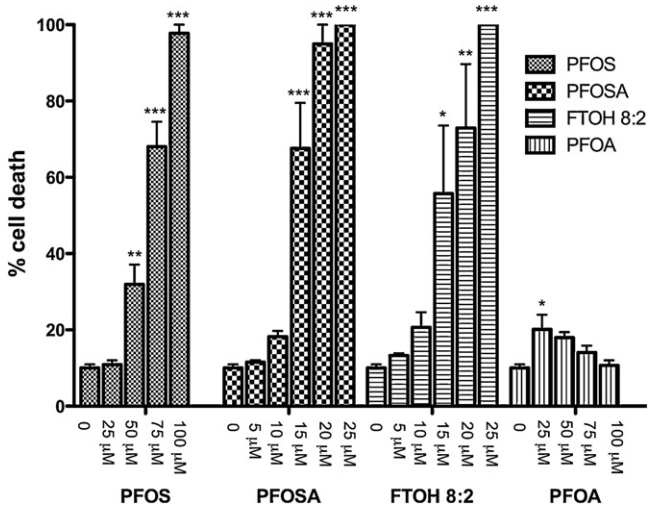

Fig. 1. Death of cerebellar granule cells after exposure to different concentrations of the fluorinated compounds PFOS, PFOA, PFOSA and FTOH. The cells were incubated for 24 h, and the EC_{50} values were estimated to 61 ± 7.6, 13 ± 1.8 and 15 ± 4.2 μM for PFOS, PFOSA and FTOH 8:2, respectively. Values are presented as percentage cell death (mean ± SD) from five experiments in duplicate. One-way ANOVA followed by Bonferroni's post hoc test was performed to indicate statistical significant differences between exposure groups and DMSO control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

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