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Phosphoproteome analysis reveals an important role for glycogen synthase kinase-3 in perfluorododecanoic acid-induced rat liver toxicity

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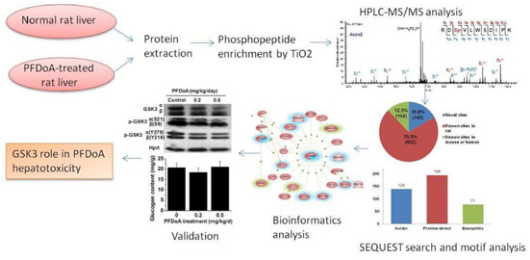
HIGHLIGHTS

- A total of 1443 unique phosphopeptides from 769 phosphoproteins were identified.
- A total of 143 unique phosphorylation sites were considered to be novel.
- Chronic PFDoA exposure inhibited insulin signal pathway.
- Inhibition of GSK3 might contribute to increase lipid levels in PFDoA treated liver.

GRAPHICAL ABSTRACT



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ABSTRACT

Perfluorododecanoic acid (PFDoA) is a member of the perfluoroalkyl acid (PFAA) family and has broad applications and a wide distribution in the environment. Here, we used TiO_2-based phosphopeptide enrichment coupled with LC–MS/MS analysis to identify phosphopeptides in rat livers that were influenced by PFDoA treatment. We identified a total of 1443 unique phosphopeptides from among 769 phosphoproteins identified in normal and PFDoA-treated rat livers, 849 unique phosphorylation sites were also identified. Of these sites, 143 were considered to be novel phosphorylation sites. Many phosphoproteins were found to be associated with hepatic injuries and diseases, such as hepatotoxicity, regeneration, fatty liver, neoplasms and carcinoma. Furthermore, 25 of the identified phosphoproteins were found to be related to glycogen synthase kinase-3 (GSK3), either directly or indirectly. Western blot and qPCR results suggested that chronic PFDoA exposure inhibited insulin signal pathways and that inhibition of GSK3 might contribute to the observed increases of lipid levels in the liver.

1. Introduction

Perfluoroalkyl acids (PFAAs) including perfluorooctanoic acid (PFOA, C8), perfluorooctansulfonate (PFOS, C8) and perfluorododecanoic acid (PFDoA, C12) are a class of highly stable compounds used widely in commercial and industrial applications as surfactants, lubricants, paints, polishes, paper and textile coatings, food packaging and fire-retardant foams (Kissa, 2001). They have strong C—F bonds which enable them to resist hydrolysis, photolysis, biodegradation and metabolism, leading to their wide global distribution (Kissa, 2001; Pan et al., 2011; Zhang et al., 2010), bioaccumulation (Houde et al., 2006; Martin et al., 2004; Pan et al., 2011) and various toxicities (Lau et al., 2004, 2007). Several studies addressing PFAAs with C7—C10 indicated that PFAAs with longer carbon chains were eliminated more slowly and thus, were more bioaccumulative and toxic than those with a shorter carbon chain (Kudo et al., 2006; Ohmori et al., 2003). Accordingly, PFDoA, with twelve carbon atoms, is likely to be more hazardous

Abbreviations: PFDoA, perfluorododecanoic acid; GSK3, glycogen synthase kinase-3; SREBP1c, sterol regulatory element binding protein 1c; TG, triglyceride; TC, total cholesterol.

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to the environment and human health than PFOA and PFOS. PFDoA has been identified in water, soil, wildlife and humans (Guruge et al., 2005; Liu et al., 2011; Olsen et al., 2011), but it has not been subjected to toxicological analyses despite the high accumulated environmental concentrations detected. For example, the highest concentration of PFDoA observed in beluga livers from Arctic locations was 9.89 ng/g (Reiner et al., 2011). Unlike most other persistent organic pollutants (POPs), which accumulate in lipid-rich tissues, PFAAs can bind to blood and liver proteins and accumulate mainly in the liver, kidneys and bile secretions (Jones et al., 2003). PFAAs increase the liver-to-body weight ratio, lipid levels, hepatocellular hypertrophy, and peroxisome proliferation and induce liver tumors in hepatocytes in laboratory animals (Kennedy et al., 2004). Although many studies have attempted to explore the mechanisms underlying PFAA hepatotoxicity (Rosen et al., 2010; Shi et al., 2009; Wei et al., 2008), few studies have addressed the effect of PFAAs on post-translational regulation.

Reversible protein phosphorylation is the most common type of PTM and is involved in almost all cellular and developmental processes. This reversible type of phosphorylation is mediated by the opposite actions of large families of protein kinases and phosphatases (Zolnierowicz and Bollen, 2000). Many diseases such as cancer, auto-immune diseases, metabolic disorders and pathogenic infections are associated with protein kinase-mediated signaling pathways (Gatzka and Walsh, 2007; Hanahan and Weinberg, 2000; Taniguchi et al., 2006). Therefore, large-scale analysis of differentially regulated phosphorylation events associated with different biological conditions can be used to identify and understand complex signaling networks. Mass spectrometry (MS) has become a powerful tool for the global identification of phosphorylated proteins and the characterization of phosphorylation sites due to its high level of sensitivity and accuracy. In addition, various phosphopeptide enrichment methods such as the use of titanium dioxide (TiO_2) (Larsen et al., 2005), IMAC (Ficarro et al., 2002), strong cation exchange (SCX) chromatography (Beausoleil et al., 2004), and other methods (Kweon and Hakansson, 2006; Rush et al., 2005), have been developed recently and applied to obtain large phosphorylation profiles in many biological systems. However, due to the complexity of the tissue samples, the selection of ions for MS/MS fragmentation varied between analyses. This led to less reproducibility of the phosphopeptides identified in two independent analyses for a given sample mixture (Moser and White, 2006). Thus, the label-free quantification of protein phosphorylation in tissue extracts remained challenging and lacked standard methods.

In this study, we performed a phosphoproteomic analysis of rat liver both with and without PFDoA treatment employing TiO_2-based phosphopeptide enrichment followed by LC–MS/MS analysis. We did not quantify the change of phosphorylation level of certain proteins in different PFDoA treatments through counting phosphopeptide numbers in this study. Moreover, we performed several different bioinformatics analyses to find the possible proteins and biological processes affected by PFDoA. Then, qPCR and western blot analysis was used to verify the transcriptional regulation and phosphorylation levels of a number of signaling molecules in response to PFDoA. Our work provides further insight into the molecular mechanisms underlying PFDoA hepatotoxicity and the possibility of discovering new biomarkers based on exposure to PFDoA.

2. Materials and methods

2.1. Animal treatment

Male Sprague-Dawley rats (230–240 g) were obtained from Weitong Lihua Experimentary Animal Central (Beijing, China). After one week of adaptation, the rats were separated into three groups. The treatment rats were given doses of 0.2 and 0.5 mg of PFDoA (Sigma–Aldrich, dissolved in 0.2% Tween-20) per kg of body weight/d *via* oral gavage for 110 consecutive days. Control animals were fed with only 0.2% Tween-20 (vehicle) in a similar manner. At the end of the experiment, six rats from each group were euthanized by decapitation. The liver was immediately removed, washed with PBS and divided into small aliquots. One portion was fixed in 10% neutral buffered formalin for histological examination, while the others were flash frozen in liquid nitrogen and stored at −80 °C.

2.2. Sample preparation, in-solution tryptic digestion and phosphopeptide enrichment

The total protein was extracted from the rat liver samples and the details given in supplemental data. Tryptic digestion was carried out according to a previously described method with some modifications (Dai et al., 2007). A phosphopeptide enrichment procedure described previously (Wu et al., 2007) was used in this study with some modification.

2.3. LC–MS/MS analysis on LTQ-Orbitrap

All phosphopeptides were analyzed using a nanoscale HPLC–MS system. A Surveyor liquid chromatography system was coupled online to an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with a nanoelectrospray interface operated in positive ion mode. Separation was performed on a C18 reverse phase column (75 μm × 15 cm, Column Technology Inc., CA). The flow rate was 140 μL/min prior to splitting and 200 nL/min after splitting. Peptides were eluted using a linear gradient with the percentage mobile phase B (0.1% FA in ACN) increasing from 2 to 35% in 120 min. The spray voltage was set to 1.85 kV, and the temperature of the heated capillary was 160 °C. The normalized collision energy was 35.0. The mass spectrometer was set to perform a full MS scan, followed by ten MS/MS scans on the ten most intense ions from the MS spectrum, with the following Dynamic Exclusion settings: repeat count 2, repeat duration 0.5 min, and exclusion duration at 90 s. The resolving power of the Orbitrap mass analyzer was set at 100,000 ($m/\Delta m$ 50% at m/z 400) for the precursor ion scans.

2.4. Database searching, data filtering and site localization

The derived mass spectrometry datasets were converted to generic format (*.dta) files using Bioworks 3.2. These files were then searched against the real and reverse IPI rat protein sequence database (version 3.28) using the SEQUEST searching program. Trypsin was designated as the protease, with two missed cleavage sites being allowed. The search parameters included: carboxyamidomethylation modification of cysteine as a fixed modification, phosphorylationon serine, threonine, and tyrosine as dynamic modifications and oxidation on methionine as a variable modification. Up to six phosphorylation sites were allowed per peptide. Previously published filter parameters were set for phosphopeptide identification (Wu et al., 2007), with a ΔCn score of at least 0.1 required, regardless of charge state, and a false discovery rate (FDR) of ≤1%. The FDR was calculated based on the following formula: % fal = $n_{rev}/(n_{rev} + n_{real})$, where n_{rev} is the number of peptide hits matched to a "reverse" protein and n_{real} is the number of peptide hits matched to a "real" protein. In addition, every spectrum of the phosphopeptides was manually checked to ensure that the fragment ion peaks have a high signal-to-noise ratio. Precursor ion mass tolerances were 10 ppm and a fragment ion mass tolerance was 0.8 Da. The two mass spectrometry datasets for the same treatment were combined for future phosphoprotein group identification.

For phosphorylation site localization, each SEQUEST-identified phosphorylation site from the peptides in Table S1 was subjected to analysis using ArNone software (Jiang et al., 2010). Sites with Ascore values above 19 were considered to be localized with near certainty, while those with scores between 13 and 19 were considered to be localized with high certainty. Unique novel sites were confirmed by searching the combined phosphorylation sites (Ascore value above 19) in all groups inside the rat database on the UniProt (http://www.uniprot.org) and PhosphoSite (http://www.phosphosite.org) website and manually excluding sites homologous to both humans and mice.

For protein identification, we used the IPI database, which offers complete non-redundant datasets built from the Swiss-Prot, TrEMBL, Ensembl and RefSeq databases. A protein group was removed if all identified peptides assigned to that protein group were also assigned to another protein group. To extract a single protein member from a protein group, we chose the protein with the highest sequence coverage.

2.5. Data analysis

Specific motifs were obtained from the combined datasets for all treatments using the Motif-x algorithm (http://motif-x.med.harvard.edu) (Schwartz and Gygi, 2005). All single phosphorylation and extendible sites (non-N/C-terminal peptides) with an Ascore ≥ 19 were used for motif analysis. NetworKIN-2.0 (Linding et al., 2007) was used to predict probable kinase families for the identified phosphorylation sites. Because *Rattus norvegicus* was not included in the NetworKIN database, human taxonomy was chosen as an alternative, as it is generally assumed that kinase substrates are highly conserved between humans and rats. Kinase–substrate