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Testicular phosphoproteome in perfluorododecanoic acid-exposed rats

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HIGHLIGHTS

- A total of 4077 unique phosphopeptides from 1777 proteins were identified.
- 937 unique phosphorylation sites were considered to be novel in testicular proteins.
- MAPK pathway and CDC2 protein phosphorylation are critical for PFDoA toxicity.

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ABSTRACT

Perfluorododecanoic acid (PFDoA) is a common environmental pollutant, which has been detected in human sera and has adverse effects on testicular function in animal models. Exploring phosphorylation events in testes helps elucidate the specific phosphorylation signals involved in testicular toxicity of PFDoA. Combining efficient prefractionation of tryptic peptide mixtures using self-packed reversed phase C18 columns with TiO_2 and IMAC phosphopeptide enrichment techniques followed by 2D-LC–MS/MS, we identified 4077 unique phosphopeptides from 1777 proteins with a false discovery rate below 1.0% in the testes of rats exposed to PFDoA for 110 days. In addition, 937 novel phosphorylation sites were discovered in testicular proteins. Hundreds of phosphorylated proteins identified might be involved in spermatogenesis and sperm function. With increasing PFDoA dosage, the number of casein kinase 2 kinase-modified peptides significantly increased. Pathway analysis suggested that the mitogen-activated protein kinase pathway and cell division cycle protein 2 (CDC2) may have contributed to sperm activity and testicular function. By *in vitro* experiments, CDC2 phosphorylation activity was found to be likely involved in PFDoA-induced toxicity in Leydig cells. This study provides the first examination of the whole proteins' phosphorylation profile in rat testis and suggests that the MAPK pathway and CDC2 protein phosphorylation are critical for PFDoA testicular toxicity.

1. Introduction

Male testes are responsible for spermatogenesis, which is enabled by testosterone produced in the Leydig cells. Testicular functions are regulated by complex signals at the genetic and protein levels, such as the post-translational modification of proteins. Phosphorylation by protein kinases is the most widespread post-translational modification used in signal transduction and regulates many biological processes (Urner and Sakkas, 2003). Global identification of *in vivo* phosphorylation is essential for a thorough and therapeutically applicable understanding of cellular functions during physiological and pathological states. Phosphoproteomics aims to map the phosphorylation status of proteomes in a high-throughput manner, discover new phosphorylation sites, and ultimately improve understanding of biological function regulation mediated by phosphorylation (Hojlund et al., 2009). Several protein kinases are involved in testicular functions such as steroidogenesis and spermatogenesis. The cAMP-PKA pathway and protein phosphorylation perform crucial roles in steroid hormone synthesis in the testis (Andric et al., 2007; Evaul and Hammes, 2008). For example, PKA-induced phosphorylation of

Abbreviations: PFAAs, perfluoroalkyl acids; PFDoA, perfluorododecanoic acid; TiO_2, titanium dioxide; IMAC, immobilized metal affinity chromatography; MAPK, mitogen-activated protein kinase; TSSK, testis-specific serine/threonine kinases; ODF, outer dense fiber protein; CDC2, cell division cycle protein 2.

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steroidogenic acute regulatory protein (StAR) is essential for producing progesterone and testosterone in Leydig cells. Sperm phosphoproteomics studies have provided important insights into sperm activity and related diseases (Ficarro et al., 2003). Although many studies have been published on phosphorylation events in testes, detailed mechanisms of protein phosphorylation and the related significance to testicular function are poorly understood.

Testicular diseases and abnormal reproduction are serious issues in the general population. Numerous studies have suggested that testes are potential target organs for various environmental pollutants such as man-made perfluoroalkyl acids (PFAAs), which are of global concern due to their widespread application in cosmetics, lubricants, fire retardants, and insecticides (Kennedy et al., 2004). These chemicals consist of a series of compounds with different carbon chain lengths, including perfluorododecanoic acid (PFDoA, C12), perfluorodecanoic acid (PFDA, C10), and perfluorooctanoic acid (PFOA, C8). Because of the high energy C—F bond, PFAAs are difficult to degrade and therefore persist in water, soil, wildlife, and humans (Kennedy et al., 2004; Tao et al., 2008; Van de Vijver et al., 2007). For example, PFDoA has been detected in human breast milk and sera and in the livers of harbor porpoises, with the highest detected concentrations reaching 9.74 pg/ml, 0.022 ng/ml, and 9.5 ng/g (wet weight), respectively (Kennedy et al., 2004; Tao et al., 2008; Guruge et al., 2005). Animal experiments have demonstrated that PFDoA is the most toxic among the 8–12 carbon chain PFAAs (Kennedy et al., 2004) and its potential risk to the environment and human populations has been raised among environmental agencies and toxicologists. In our previous studies, we observed that chronic PFDoA exposure disrupted testicular steroid production and testicular structure in rats (Shi et al., 2009). In addition, our proteomic study on PFDoA-treated rat testes showed that the toxic effects of PFDoA were related to oxidative stress and mitochondrial disruption in testes (Shi et al., 2010a). However, the detailed molecular mechanism by which PFDoA leads to testicular toxicity in rats remains unclear.

Combining efficient prefractionation of tryptic peptide mixtures using self-packed reversed phase C18 columns with titanium dioxide (TiO_2) and immobilized metal affinity chromatography (IMAC) phosphopeptide enrichment techniques, along with two-dimensional liquid chromatography tandem mass spectrometry (2D-LC MS/MS), we analyzed the phosphoproteome of normal rat testes and testes after 110 days of PFDoA exposure. We aimed to clarify global phosphorylation profiles and explore the key phosphorylation events in normal testicular function. Comparing the testicular phosphorylation profiles between normal and PFDoA-treated rats will help determine the molecular mechanism of the toxic action of PFDoA in testes at the protein phosphorylation level.

2. Materials and methods

2.1. Animal treatment

Male Sprague-Dawley rats (40–50 g) were obtained from the Weitong Lihua Experimenter Animal Center, Beijing, China. Animals were randomly classified by body weight into treatment and control groups (six rats per group), and were individually housed and maintained in a mass air-displacement room with a 12-h light-dark cycle at 20–26 °C and a relative humidity of 50–70%. Animals had access to food and water *ad libitum*. The PFDoA (95% purity; Sigma–Aldrich, St. Louis, MO) was dissolved in 0.2% Tween-20. The treated rats were daily gavaged for 110 days with PFDoA doses of 0.02, 0.2, or 0.5 mg/kg/d. The control rats were similarly treated with 0.2% Tween-20 only. At the end of the experimental period, all rats were euthanized by decapitation. Testes were immediately isolated and frozen in liquid nitrogen and stored at −80 °C for further analysis.

2.2. Protein extract, tryptic digestion and peptide prefractionation

Details of protein extract and tryptic digestion are given in supporting information. The peptide prefractionation has been described previously (Hou et al., 2010). Briefly, an individual sample was loaded sequentially onto three self-packed C18 columns (40–60 μm, 120-Å pore size, SunChrom, Friedrichsdorf, Germany). The final fractions eluted from the three columns using the same elution buffer were combined, divided into two equal parts, and dried with a speed vac. One dried sample was used for subsequent TiO_2 enrichment, and the other was used for IMAC enrichment.

2.3. Phosphopeptide enrichment with TiO_2 and IMAC and 2D-nano LC–MS/MS analysis

Phosphopeptide enrichment was carried out using TiO_2 and IMAC with slight modifications and details of them are given in supporting information (Wu et al., 2007). Finally, all fractions from the same treatment were combined, dried, and stored at −80 °C until 2D-LC–MS/MS analysis.

Analysis was performed as previously described (Hou et al., 2010; Cui et al., 2009). Details of analysis are given in supporting information. The application of mass spectrometer scan functions and HPLC solvent gradients were controlled by an XCalibur data system (Thermo Fisher Scientific). The MS analysis was performed twice for each set of pooled samples.

2.4. MS data processing

Raw MS data were processed using Bioworks 3.3 (Thermo Fisher Scientific Inc.) with the following parameters: mass range, 450–5500; precursor tolerance, 1.4 amu; ion counts, 10; intensity threshold, 1000 (absolute). Bioworks-generated peak lists (.dta files) were searched against the rat International Protein Index (IPI) database version 3.51 (40,288 protein entries) and its random sequences with SEQUEST v.28 (rev. 12) (Eng et al., 1994). Parameters of the database search were as follows: full tryptic specificity, 2.0 Da for a precursor ion mass tolerance, 0.8 Da for fragment ion mass tolerance, Cys carboxyamidomethylation (57.02 Da) as fixed modification, two missed cleavage sites, Ser, Thr, and Tyr residue phosphorylation (79.96 Da), and Met residue oxidation (15.99 Da) as the dynamic modifications. Each peptide was allowed three phosphorylation sites.

Phosphopeptide hits were filtered by Rsp, Sf, DeltaCn', and Xcorr' scores (Hou et al., 2010). The Rsp is the preliminary score rank for each peptide based on the SEQUEST algorithm. In our study, we chose Rsp ≥ 2. The Sf score (0–1.0) for each phosphopeptide created by the Bioworks program was calculated using a neural network algorithm that incorporated the Xcorr, DeltaCn, Sp, RSp, peptide mass, charge state, and the number of matched peptides for the search. The XCorr' was calculated using the following formula: $XCorr' = \ln(XCorr)/\ln(L)$. In this formula, L stands for peptide length. The DeltaCn' was defined as the normalized difference between the XCorr values of the top hit and the next hit with a different amino acid sequence, calculated with an in-house Perl program. The DeltaCn' of each peptide should be ≥0.1; for peptides with a charge of +2, the Xcorr' value should be ≥0.25, and for peptides with a charge of >+3, the Xcorr' value should be ≥0.35. The false-discovery rate (FDR) was calculated as the number of decoy-hits divided by the number of target-hits, with the threshold of the Sf value (not less than 0.4) adjusted to fit a final FDR of ≤0.01. After filtering, the lowest Xcorr values in the dataset were 1.565, 2.024, 2.022, and 2.056 for groups one to four, respectively.

The ambiguity scores (Ascore) of each phosphorylation site were calculated using software developed by Beausoleil et al. (2006). Only phosphorylated sites with Ascores above 19 were considered reliable ($p \leq 0.01$).

2.5. Bioinformatics analysis

Specific motifs were obtained from the data set with the Motif-X algorithm (Schwartz and Gygi, 2005). All single phosphorylation sites with an Ascore value above 19 were used for motif analysis. NetworKIN-2.0 was used to predict probable kinase families and identify phosphorylation sites (Linding et al., 2008). All phosphorylated sites with an Ascore value ≥19 were utilized for this analysis, kinase-substrate relationships with a NetworKIN score above 1.0 and a String score above 0.6 were regarded as significant. When one specific phosphorylation site was predicted to be potentially recognized by multiple kinases, the one with the highest NetworKIN Score was retained.

Gene ontology data analysis was carried out with Cytoscape and Plugin BiNGO (Maere et al., 2005). We compared the annotations of phosphorylated proteins with those of the entire *Rattus norvegicus* proteome. The hypergeometric statistical test and the multiple-test Benjamini and Hochberg FDR correction were adopted to derive over-represented functions. The level of significance was set as $p < 0.05$.

Analysis of the relationship between identified phosphorylated proteins in rat testes was performed with text-mining Pathway Studio™ (v7.0) software (Ariadne Genomics, Inc., Rockville, MD), which uses a database assembled from scientific abstracts and a manually created dictionary of synonyms to recognize biological terms. The phosphorylated proteins were converted to their corresponding gene IDs and imported into Pathway Studio software; each identified relationship was confirmed manually with the relevant PubMed/Medline hyperlinked texts.

For novel phosphorylated sites analysis, the phosphorylated peptides (Ascore value ≥19) were first searched against rat data from UniProt to filter the known sites (http://www.uniprot.org). The filtered peptides were then manually compared to mouse and human phosphorylated sites in phosphoSitePlus (http://www.phosphosite.org) to obtain the novel sites.