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Research paper

Proteomic analysis for early neurodegenerative biomarker detection in an animal model

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ABSTRACT

The exposure to xenobiotics in the early stages of life represents the most important component in the etiology of many neurodegenerative disorders.

Proteomic analysis of plasma and brain samples from early life treated animal model was performed in order to identify early biomarkers of neurodegeneration. Two-dimensional gel electrophoresis followed by liquid chromatography-tandem mass spectrometry identified four proteins in the plasma of adolescent rats that deviated from the control group. Low expression levels of transthyretin and plasma transferrin, and the absence of long-chain fatty acid transport 1 were measured. On the other hand, the same proteomic approach was done on striatum of an adult rat model of neurodegeneration. Mitochondrial aspartate aminotransferase and voltage-dependent anion channel were under expressed, while mitochondrial malate dehydrogenase, myelin basic protein and ubiquitin-60S ribosomal protein L40 were absent in striatum of animal model compared to control group. Data show that early biomarkers for the diagnosis of neurodegeneration can be obtained by proteomic analysis, starting from adolescent age and the results highlight the time frame for the onset of neurodegeneration due to early exposure to xenobiotics.

1. Introduction

The neonatal period is an important phase of life in which environmental factors (i.e. xenobiotics, nutrition), may promote the development of diseases in adulthood [1–3]. The exposure to xenobiotics in the early stages of life, represents the most important component in the etiology of many neurodegenerative disorders, cancer, cardiovascular and metabolic diseases in adulthood [4–6]. These findings are supported by evidences which show that the main mechanism leading to long-term diseases is associated with epigenetic modifications that influence gene expression in a tissue specific manner, leading to non-Mendelian variations that might also account for transgenerational effects on progenies [7–9]. Researchers have demonstrated that because of cell programming in a tissue specific manner, early life represents a “window” of wide plasticity for epigenetic modifications that can influence gene expression later in life [1–3,10]. This finding might be usefully employed to monitor the progression of neurodegeneration starting from its early phases and to identify premature biomarkers.

Crucial to this experimental landscape has been the development of an animal model of neurodegeneration with the features of Parkinson's–like disease, where the typical behavioral changes and dopaminergic damage (i.e. decrease of dopamine level, Nurr1 down-regulation, alpha-synuclein up-regulation and aggregation, oxidative stress etc.), were measured in brain and plasma of adult rats after 15 days of early life exposure to the xenobiotic permethrin [11–15]. The advantage of using this animal model instead of blood samples from early-stage Parkinson's patients, can be explained

List of abbreviations: 2DE, two-dimensional gel electrophoresis; AATM, Mitochondrial aspartate aminotransferase; MDHM, Mitochondrial malate dehydrogenase; VDAC, Voltage-dependent anion channel; MBP, Myelin basic protein; URP, Ubiquitin-60S ribosomal protein L40; TTR, transthyretin; PTF, plasma transferrin; FATP1, long-chain fatty acid transport 1.

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considering that our research was aimed to identify the mechanisms associated with the development of idiopathic Parkinson's disease following exposure to a common pesticide, permethrin, during early life. The use of an animal model, living under controlled conditions (i.e. same diet and environmental conditions) has the advantage to screen biomarkers in different organs in order to evaluate the complexity of the disorder induced only by the toxicant, without the variability associated with life style as in humans. The identification of these changes represents a useful necessary step to identify an appropriate preventive therapy or an approach to counterbalance the progression of neuronal damage.

Proteomic analysis of plasma could represent an useful tool to screen early for progressive neurodegeneration that is presently diagnosed mainly by clinical features when loss of neurons have already occurred. It would be more important for patients to evaluate diagnostic biomarkers in an early phase in order to facilitate neuroprotective therapies. With this in mind, we analyzed plasma from an adolescent and adult rat model of neurodegeneration in an effort to identify possible predictive biomarkers, using proteomic analysis based on two-dimensional gel electrophoresis (2DE) followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to sequence protein signals. The same proteomic approach was utilized in the analysis of striatum of early life treated rats at adult age with the aim to better characterize those proteins that are involved in neurodegenerative diseases.

2. Methods

All reagents were of analytical grade and were obtained from Sigma Chemical Co. (USA). Technical grade (75:25, trans:cis; 94% purity) 3-phenoxybenzyl-(1R,S)-cis,trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate, Permethrin was a generous gift by Dr. A. Stefanini of ACTIVA, Milan, Italy.

2.1. Animals

Male and female Wistar rats aged about 90 days weighing 250–270 g were obtained from Charles River (Calco, LC, Italy). The animals were housed in plastic (Makrolon) cages (five rats/cage) in a temperature controlled room (21 ± 5 °C) and maintained on a laboratory diet with water ad libitum. The light/dark cycle was from 7 a.m. to 7 p.m. Animal use in this study complied with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Rat pups born in our laboratory from primiparous dams were used in the study. The parturition day was set as Post Natal Day 0 (PND0). On PND1, all litters were examined externally for the presence of gross abnormalities, sexed, weighed and the female pups were excluded from this study. Two male pups were assigned to each dam until weaning (PND21). No cross-fostering was employed. At 2 days of age, litters were randomly assigned to two experimental groups (n = 28 rats totally: 14 sacrificed at PND80 and 14 sacrificed at PND 240).

2.2. Treatment

Permethrin was dissolved in corn oil and administered orally by an intragastric tube (4 ml/kg) at a dose of 1/50 of LD_{50} corresponding to 34.05 mg/kg body weight (Agency for Toxic Substance and Disease Registry, 2005). The dosage was chosen by considering that the "no observed adverse effect level" (NOAEL) for permethrin is 25 mg/kg body weight. The compounds were administered once a day in the morning from PND6 to PND21. Control rats were treated with vehicle (corn oil 4 ml/kg) on a similar schedule. The volume of the compound administered was adjusted daily based on body weight measured during the dosing period. On PND21, the pups were weaned and the littermates were housed together. At adolescent (PND80, n = 14 rats) and adult (PND 240, n = 14 rats) age, 7 rats from each group (7 permethrin treated and 7 control rats) were sacrificed by exposure to CO_2. Blood was obtained by cardiac puncture and the plasma was isolated from blood by centrifugation at 1000 *g* for 15 min at 4 °C. The supernatant was collected, aliquoted, and stored at−80 °C until analysis. Striatum from each rat was isolated and immediately placed in liquid nitrogen and stored at −80°.

2.3. Two-dimensional electrophoresis

Before 2DE, plasma samples were processed as follows: an amount of plasma containing 200 μg of proteins, determined by Bradford assay [16], obtained from rats treated with permethrin, or from controls (untreated rats) was cleaned up with a 2-D Clean-Up Kit (GE-Healthcare Life Sciences, Uppsala, Sweden), according to the manufacturer's instructions and then dissolved in a 350 μl rehydration solution containing: 8 M urea; 2% (w/v) 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS); 65 mM dithiothreitol (DTT); 0.001% (w/v) bromophenol blue; 0.5% (v/v) IPG buffer, pH range 4–7.

Furthermore, from treated and control adult rats, samples of striatum were analyzed by 2DE. In this case, the samples were treated as follows: striatum from treated and control rats was weighed, chopped and resuspended in a lysis buffer (15 mM Tris/HCl pH 8.0 containing: 7 M urea; 2 M thiourea; 2% CHAPS; 2% TritonX-100; 0.5 mM ethylenediamminotetraacetic acid (EDTA); 1% (v/v) IPG buffer, pH range 3–10; 65 mM DTT) and homogenized by sonication with a MSE sonicator (MSE, London, UK) for 9 times (10 s per time) maintaining the samples in ice. The homogenized samples were centrifuged at 27000 *g* for 20 min and on the supernatant the protein content was determined by Bradford assay. For 2DE analysis 0.4 mg of total protein were mixed with 350 μl of rehydration buffer containing the IPG buffer, pH range 3–10.

Isoelectric focusing (IEF) was performed using a pre-cast-immobilized pH gradient gel strip ImmobilineDryStrip, (IPG-strip, length 18 cm) with a linear pH gradient range of 4–7 for plasma samples and a pH gradient range of 3–10 for striatum samples. The IPG-strips were placed on IPGphor-isoelectric focusing cell (GE-Healthcare) and were rehydrated for 12 h at 20 °C without voltage. The focusing was then performed at 20 °C in 3 steps: first step, voltage of 500 V for 1 h; second step, voltage of 1000 V for 1 h; third step, voltage of 8000 V for 4 h. The current limit per IPG-strip was 50 mA. After IEF, IPG strips were equilibrated for 15 min in the equilibration buffer (50 mM Tris–HCl, pH 8.8; 6 M urea; 30% glycerol; 2% SDS; 65 mM DTT and a few grains of bromophenol blue) and loaded on a 13% SDS-PAGE, using a Protean II apparatus (Bio-Rad, Hercules, CA, USA). The gels (180 × 200 × 1.5 mm) were run at 30 mA per gel for 6–7 h. Proteins were stained for 1 h with 0.1% Coomassie Brilliant Blue R250. The gels were de-stained until the protein spots became evident and the gel background transparent.

2.4. Protein visualization and image analysis

After staining, the gels were scanned at 600 dpi resolution, and the gel images were analyzed using PDquest software (Version 7.1.1; Bio-Rad Laboratories) according to the protocols provided by the manufacturer in order to define spot-intensity calibration, spot detection, calculation of molecular mass and pI. For computational purposes, the gels were cropped to frame the same cluster of spots and subsequently a set of spot generation conditions such as faintest spot, smallest spot, size of the largest spot, and a selected