PROTEOMIC ANALYSIS OF POULTRY LIVER IN REGION EXPOSED TO DIOXIN AT MA DA DISTRICT, DONG NAI PROVINCE, VIETNAM

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Abstract

Agent Orange is the code name for a powerful herbicide and defoliant used by US military in its Herbicidal Warfare program during the Vietnam War from 1961 to 1971. Agent Orange contains TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) that has a harmful influence on people and organisms in the exposed regions, the most serious effects concentrated in the areas, especially in Ma Da, Dong Nai. In this research, we carried out proteomic analysis of poultry liver in region exposed to dioxin (Ma Da, Dong Nai) and Hanoi as control. Two-dimensional electrophoresis (2-DE) was utilized to resolve the protein profile of poultry liver at different regions. Some notably different spots between Ma Da and control gels were selected for protein detection by MALDI-TOF MS PMF. Among identified proteins in Ma Da gels, proteins, which play important roles in growth and development processes, in tumor or cell proliferation suppression and stresses, are noticeable. These are Hox9_Z4, Zinc finger protein, EphB, Tyrosine kinase receptor B, Novel ITAM-containing IgSF receptor 2…Especially heat shock protein 70 kDa, a chaperon protecting organisms against heat stresses or toxic chemicals, was expressed; this protein only obviously expressed in Muscovy duck liver belong to Ma Da.

Introduction

In 1962, the US military initiated use of herbicides (Agent Orange) in Viet Nam for general defoliation and crop destruction through a program codenamed Operation Ranch Hand. Application of herbicides was primarily through cargo aircraft, and ground mechanisms; helicopters were also used in certain areas of the country. More than 72 million litres of herbicide were applied over approximately 10-12% of southern Viet Nam. This figure was recently revised to over 80 million litres. Herbicide applications ceased in 1971. Agent Orange was a mixture of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), which contained dioxin contaminants[1]. According to previous studies, there are large amount of dioxin, which conserved in environment of southern Vietnam. This causes serious effects on people and organisms in the regions exposed to dioxin, for instance, Phu Cat (Binh Dinh Province), Bien Hoa (Dong Nai province), Da Nang, especially, Ma Da (Dong Nai).

Liver, the largest organ in the body, has a number of functions in biosynthesis and metabolism. TCDD accumulates mostly in liver, where it is detoxified, via absorption from intestine and hepatic portal vein. Despite the major affect of TCDD on liver, there are little previous studies illustrating detail mechanism and toxicity of TCDD in animal liver, particularly wild animals. In this research, we perform proteomic analyses on poultry liver in Ma Da (Dong Nai province). Using 2-DE and MALDI-TOF MS, proteins of immune system, growth, and differentiation, chaperon that are involved TCDD toxicities were identified.

Materials and methods

Subjects: Liver tissues of poultry adults: chicken (*Gallus gallus domesticus*) and duck (*Cairina moschata* - Muscovy duck) were collected from both Ma Da and Ha Noi regions on April and November, 2008. Tissues were dissected rapidly on ice, stored at -80°C until analysis.

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Abbreviation: 2-DE, 2-dimensional electrophoresis; MALDI-TOF MS, matrix assisted laser desorption ionization-time of flight mass spectrometry; TCDD: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; Hsp, heat shock protein.

Sample preparation for 2-DE: Tissues (0.1g) were solubilized in PBS buffer 0.05M (pH 7.4), then in lysis buffer containing 7M urea, 2M thiourea, 30mM Tris-base, 4% w/v CHAPS, 65mM DTT, 0.2% Bio-Lyte 3/10 ampholyte (Biorad). The sample was centrifuged at 15000 rpm for 15 minutes at 4^oC. The protein concentration of sample was measured by the modified Bradford method with BSA as a standard and then stored at -20^oC until analysis.

Two- Dimensional Electrophoresis: IPGStrips (7cm, pH 3-10) were rehydrated in 125µL of sample containing 7M urea, 2M thiourea, 30mM Tris-base, 4% w/v CHAPS, 65mM DTT, 0.2% Bio-Lyte 3/10 ampholyte (Biorad) and trace of bromophenol blue. The amount of protein loaded was 169 µg. After rehydration, IEF run was carried out for a total 10000 Vhr using Protean IEF cell system (Biorad). Following IEF separation, the strips were equilibrated in two equilibration solutions for 15 minutes with gentle shaking. The first equilibration solution contained 6M urea, 2%SDS, 50mM Tris–HCl (pH 8.8), 30% glycerol, 1% (w/v) DTT. In the second equilibration solution, DTT of the first equilibration solution was replaced with 2.5% iodoacetamide (IAA). The strip was applied onto 8-16% gradient SDS-PAGE gel. Protein was visualized by Colloidal Coomassie Brilliant Blue G-250 staining method.

Image Analysis: Images of the Coomassie Brilliant Blue stained gels were scanned by Xcise System (Shimadzu Biotech) and were analyzed using the Phoretix Software (Shimadzu Biotech). The level of protein expression was determined by normalization volume of the spot. The protein differently expressed with significant difference was selected and identified.

In-gel digestion: Protein spots were excised from gel, destained with 50% (v/v) acetonitrile (AcCN) in ammonium bicarbonate. After dehydrating with acetonitrile and drying in vacuum centrifuge, the gel pieces were incubated with trypsin solution (Sigma Aldrich) overnight at 30^oC. Peptides were extracted using ZipTip C18 (Millipore).

MALDI-TOF MS and Protein identification: The peptide was mixed with alpha-cyano-4-hydroxycinnamic acid matrix solution on a MALDI sample plate and air-dried. Matrix-assisted laser desorption/ionization time of fly mass spectrometry (MALDI-TOF MS) analysis was performed on AXIMA CFR^{PLUS} system (Shimadzu Biotech). For identification of protein, all spectra recorded on tryptic peptides derived from NCBInr databases using the MASCOT search program (www.matrixscience.com).

Results and Discussion

Detection and identification of differentially expressed proteins of chicken and Muscovy duck liver tissues between Ma Da and Hanoi

In 2-DE gel of chicken liver tissue, we only selected 22 notably different spots between Ma Da gel and Ha Noi gel for protein identification, and 15 proteins were identified. In 2-DE gel of duck liver tissue, we only selected 24 notably different spots between Ma Da gel and Ha Noi gel for protein identification, as a result, 17 proteins were identified (fig.1). Among identified proteins in Ma Da gel, proteins, which play important roles in growth and development processes, proteins of immune system and chaperon are noticeable. Because when organisms were exposed to dioxins, they effected on various biological responses including induction not only of xeobiotic metabolizing enzymes, thymus astropy, reproductive disorders, epithelial disorders, hepatotoxicity and cancer but also of teratogenicity and immunotoxicity.

In previous studies, there are little studies that mention of proteins belong to growth and development processes. But in this research, we can see that these proteins up-regulated in Ma Da gels. Hox genes code for homeobox containing transcription factors, which tend to occur in tightly linked clusters in the genome. They are involved in the development of vertebrate body plan characters. Hox9_Z4 is as a transcription factor, which is coded by this gene group[2]. EPTWNT4A NID, AmphiWnt4, proteins that is coded by *Wnt* subfamilies, expressed in these developing vertebrate organs[3]. EphB is a subfamily of the Eph tyrosine kinase (Tk) receptor family. The EphB

receptor subfamily interacts with its ligand family, EphrinB. EphB and their ligands, EphrinB, are associated with different cells. Consequently, EphB:EphrinB associations mediate inter-cellular (cell:cell) communications. These processes are important in cell patterning during embryogenesis; neural processes such as axon guidance, neural crest cell migration, and dendritic spike formation and in the patterning of vascular structure[4]. Semaphorins constitute a large family of secreted and membrane-associated proteins. They bind and signal through receptor complexes consisting of members of the neuropilin and plexin families 1 and can also signal through other protein moieties, including Off-track, integrins. Originally characterized as axonal growth repellants during neural development, these proteins are now implicated in a much wider range of activities, including cell migration, cell death, angiogenesis, and immune system function. Maintenance of expression of semaphorins and their receptors in the adult nervous system, and changes in expression after injury, suggest additional roles for the proteins in plasticity and regeneration[5]. Especially, Ci-FUSE, COS2.3, Zinc finger are proteins taking part in Hedgehog signaling pathway in growing embryo. Recent studies point to the role of hedgehog signaling in regulating adult stem cells involved in maintenance and regeneration of adult tissues. The pathway has also been implicated in the development of some cancers[6]. We suppose that the up-regulated expression of these proteins may contribute to organism teratogenicity or abnormal development.

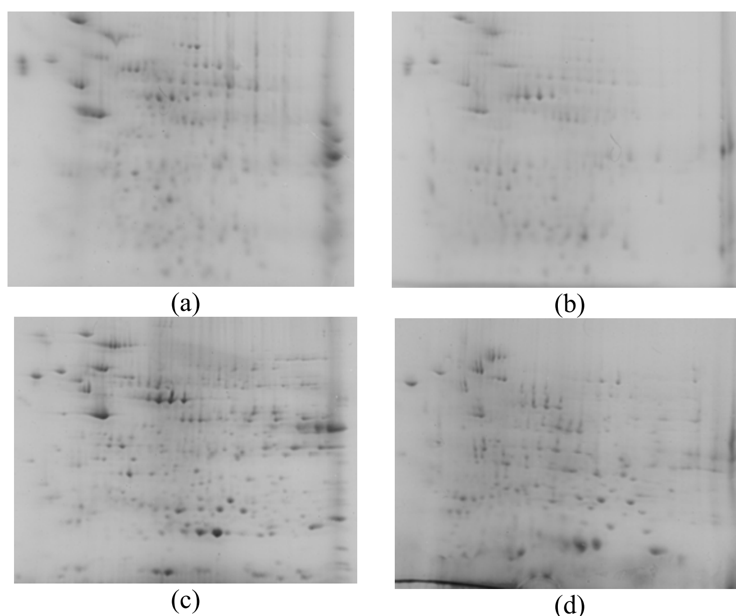

Fig. 1. 2-DE patterns of proteins from chicken (a,b) and Muscovy duck (c,d) liver tissues at Ha Noi control region (a,c) and Ma Da, Dong Nai (b,d). Total proteins were separated by 2-D electrophoresis and CBB G-250 stained

Novel ITAM-containing IgSF receptor 2, Tyrosine kinase receptor B, MHC class I antigen are proteins that join in Tyrosine kinase receptor - dependent cell proliferating signal transduction pathway. The immunoglobulin superfamily (IgSF) is a glycoprotein group on cell surface; for example, Sirpα1 consists of a single transmembrane region or a large extracellular region with 3 immunoglobulin-like domains : a N-terminal V-set domain and two C1-set domains, which are closely related to Ag receptors Ig, TCR and MHC, a proline-rich region, and a single hydrophobic transmembrane region, and an intracellular region containing 2 immunoreceptor tyrosine-based inhibitory motifs (ITIMs). The activity of the protein is dependent on phosphorylation of tyrosine residues in the ITIM and recruitment of Src homology 2(SH2)domain-containing phosphatases like the protein-tyrosine phosphatases SHP-1 and SHP-2. Hence the protein negatively regulates cell proliferation of oncogene products induced by growth factor depending on protein-tyrosine kinase receptor and recruits protein-tyrosine phosphatases containing cytoplasmic SH2 domain when phosphorylation of the tyrosine in the ITIM. ITIMs negatively regulate cell activation containing immunoreceptor tyrosine-based activated motifs (ITAMs). As an activated substrate of receptor tyrosine kinase (RTK), Sirpα1 negatively regulates RTK-dependent cell proliferating signal transduction pathway, and its overexpression of decreases response to RTK ligand (EGF, PDGF,insulin). This signal transduction pathway plays an important role in tumor suppression, especially in proliferation of hepatocellular carcinoma[7]. Overexpression of Novel ITAM-containing IgSF receptor 2, Tyrosine kinase receptor B, MHC class I antigen may implicate that

organism has reactions against certain factors developing tumor or hepatocellular proliferation in environmental that influence studied organisms.

Up-regulated heat shock 70kDa protein is a chaperon. In cooperation with other chaperon, Hsp 70 stabilizes protein against aggregation and mediates the folding of newly translated polypeptides in the cytosols as well as within organelles. Hsp 70kDa protein is indicative of previously unknown mechanism by which immune modulators stimulate the release of intracellular Hsp70, which is known to bear tumor suppressing in early hepatocellular carcinoma[8,9]. Like transferins, Hsp 70 stimulates B-cell proliferation[10].

Heat-shock proteins influence on suppressing stress-induced apoptosis. Hsp70 and Hsp27 prevent cytochrome *c* (cyt. *c*) release from mitochondria. Apoptosome formation is blocked by Hsp27 binding to cytochrome *c* and by Hsp90 and Hsp70 binding to Apaf-1. Hsp27 can prevent apoptosis downstream of caspase-3 (casp.3) activation by interacting with caspase-3. Hsp70 also prevents apoptosis downstream of caspase-3 activation by an unknown mechanism. Hsp70 prevents release of AIF (Apoptosis-Inducing Factor) from mitochondria and also prevents the nuclear import of released AIF. Thus, the appearance of Hsp 70 indicates that there are adverse effects of protein-damaging stresses. On the other hand, overexpression of Hsp70 can protect cells from the cell death which strongly occurred[11].

In conclusion, the up-regulated proteins in Ma Da gels, which are relative to the growth and development, the tumor or cell proliferation suppression and stresses, implicate that there are many abnormal signals in metabolism of studied organism in Ma Da, Dong Nai. These disorders may be caused by heat shock, deceases, UV,…But they may be caused by toxic chemicals which excessively exist in environment; it's necessary to particularly care for agent orange sprayed in Southern Vietnam from 1961 to 1971. Because such a huge number of herbicide was used in Operation Rand Hand, Agent Orange was still saved in environment, effecting seriously organisms that live in the region exposed to toxic chemicals.

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