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Review

Toxins and stress in fish: Proteomic analyses and response network

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ABSTRACT

Fish models are increasingly used in toxicological studies in the laboratory as well as in the field. In addition to contributing to the analysis of toxicity mechanisms, one major aim is to select biomarkers from among the metabolic responses to toxic agents observed that could be useful for surveying the aquatic environment. Since proteomics is a developing field in toxicological research, it seems opportune to explore the data obtained using this approach. This article proposes an overview of proteomic studies of fish exposed to environmental stressors comprising a cyanotoxin and the response networks observed. We tend to take a broad view of how proteins communicate and function within the cell, often encompassing large numbers of proteins that operate in pathways.

We start by presenting and discussing the data from four experiments in which the medaka fish was treated under the same conditions with the cyanotoxin, microcystin-LR (MC-LR). Liver proteins were analyzed using two techniques: 2D electrophoresis and LCMSMS.

In the second and main part of our paper, the proteomic data obtained from fish contaminated with chemicals, including those reported above concerning the medaka fish intoxicated with MC-LR, are considered in the round in order to identify fish responses to chemical stress. A tentative general overview of how groups of proteins work together depending on exposure and/or subcellular location is proposed, with the inclusion of MC-LR data obtained in mice for comparison.

1. Introduction

The environment is currently contaminated by myriad chemical and biological pollutants. As a result, organisms are exposed to a diverse array of chemical mixtures which may be simple mixes of just a few identifiable compounds, or may be more complex, containing several hundred related congeners, and/or unrelated compounds. This complexity makes it difficult to characterize the mode or mechanisms of action involved, which are generally based on empirical observations of the toxicities of single chemicals in animal studies. The aquatic environment is a major repository for most of the chemicals generated by human activities. Thus, although estuaries and coastal areas are important sites of living resources, such as fisheries, they are also those most at risk of toxic contamination. It is difficult to assess this risk, partly because of the complexity of the environment, and partly because of the lack of suitable methods. Chemical analysis can be useful in determining body burdens, unless of course the xenobiotic is bio-transformed, but it does not provide any information about its effects (Katagi, 2010). Ecological monitoring of the local aquatic fauna provides information about the disturbance of homeostatic conditions in natural systems, which is mainly based on the use of biomarkers from sentinel aquatic animals. These biomarkers consist of behavioral, histological and biochemical responses, as well as patterns of protein levels (Livingstone, 1993).

Recently, small fish, such as the medaka fish (*Oryzias latipes*), zebrafish (*Danio rerio*), mosquito fish (*Gambusia affinis*), and fathead minnow (*Pimephales promelas*), have been

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among the most commonly-used models for ecotoxicology and biomedical research (Forne et al., 2010). Using a variety of experimental designs, these aquatic models have been exposed to several of the stressors found in aquatic ecosystems, such as micro algal toxins or xenobiotics, in order to elucidate their toxicity characteristics. Our aim was to explore the data obtained using proteomics in order to point out cellular regulatory network set up after environmental stress. The proteins from proteomic studies were listed and grouped according to their biological function, then they were associated within pathways. All the pathways deduced from proteomic studies were superimposed and common pathways were merged. The distinct pathways sharing common proteins were then connected by their edges to establish pathways networks involved in fish responses to environmental stress.

In the *conventional biomarker approach*, most of the biochemical biomarkers selected are quantitatively modified by chemical stress, since they are relatively easy to handle in laboratory or field experiments. For example, enzymatic activities, hormones such as cortisol or growth factor have been assayed in the blood of *Onchorhynchus mykiss* after stressful episodes (Fernandes-de-Castilho et al., 2008; Gravel and Vijayan, 2007; Miller et al., 2007). Components of the energy metabolism, such as lactate hydrogenase, glycogen, glucose, lipid peroxidase, aspartate transmitase, and alanine transmitase, were monitored in fish after exposure to salicylate or cadmium (Almeida et al., 2002; Gravel and Vijayan, 2007). Anti-oxidant proteins, such as catalase, glutathione peroxidase and superoxide dismutase, have also been used as stress-related parameters in fish after selenium exposure, as well as the thyroid hormones, T3 and T4 (Miller et al., 2007). This physiological approach has been criticized as demonstrating physiological changes that are not necessarily associated with toxic events. The same objection can be raised to the metallothioneins, which are closely related to metal pollution, but also to sexual maturity (Olsson et al., 1987). Moreover, only a few studies have focused on biomarkers using Western blotting, probably because of the lack of available specific fish antibodies.

The *Omic approaches*, using methods such as genomics and transcriptomics, have made it possible to carry out simultaneous assessments of the expression profiles of numerous genes that respond to a toxic compound within a particular cell type, tissue, or organism (Suter et al., 2004). The data provided by microarrays help us to decipher the signature profile of the modulated genes involved in a stress response (Craig et al., 2009; Iwahashi et al., 2009; Momoda et al., 2007). Thus, signal pathways can be inferred from functional genes. Comparing proteomic data obtained with specific techniques, either 2D-PAGE, DIGE or LCMSMS, with transcriptomic data can sometimes be useful in lower organisms, but is particularly important in higher organisms. In the higher organisms, protein expression is highly regulated, and changes in mRNA and protein levels may not be correlated. The different half-lives of mRNA, as well as protein accumulation, post-translational modifications, and degradation kinetics, all contribute to these differences (Celis et al., 2000). Thus, transcriptomic and proteomic data are complementary.

Toxins induce changes at the level of RNA/protein expression. Decrypting the mechanism of action or molecular targets in environmental relevant organisms *via* omic techniques is an emerging approach. It provides a lot of data, which is still difficult to compare, but that permits promising synthetic overviews of cellular response networks.

The first part of this article deals with the medaka fish model, and proteomic studies of the effects of the cyanotoxin, microcystin-LR (MC-LR). The next section, which constitutes the main part of the article, reports a comprehensive network of proteomic responses deduced from data observed with various different fish and pollutants, including the responses of the exposure of medaka fish to MC-LR, and a toxicological study of MC-LR in mouse liver.

2. Proteomic studies of the effects of the cyanotoxin microcystin-LR on the liver of the medaka fish

Aquatic organisms are exposed to microcystins, the most frequent cyanotoxins, by direct ingestion or through trophic chain. Microcystins are known to be hydrophobic and to have relatively high molecular weights (900–1100Da), and therefore need specific membrane transporters to reach cells such as hepatocytes and renal cells (Xie et al., 2005).Their inhibitory effects on protein phosphatases PP1 and PP2A (Runnegar et al., 1995), ATP synthase (Mikhailov et al., 2003) and aldehyde dehydrogenase (Chen et al., 2006) are dose and time-dependent, and lead to effects including disassembly of the hepatic cytoskeleton, oxidative stress as well as possible DNA damage (Ding et al., 1999; Zegura et al., 2004), apoptosis or necrosis (Malbrouck and Kestemont, 2006).

Medaka fish were treated with microcystin-LR (MC-LR) in four successive experiments in which the proteomic analyses focused on the liver. The first treatment provided the following data (Mezhoud et al., 2008a), and was used to design the protocol for the other three exposures: 1) the cyanotoxin was concentrated in the liver, peaking 3h after the toxin had been added to the medium in which the fish were swimming; 2) in order to obtain rigorous levels of contamination, balneation had to be replaced by gavage with a specified quantity of toxin (5 μg MC-LR in 5 μL water) introduced into the stomach *via* a blunt-tip syringe (Hamilton); 3) in this context of acute toxicity leading to 20% mortality after 2h, metabolic changes could be detected in the livers of the surviving fish by a proteomic approach.

Using this protocol, three experiments were performed, each involving triplicates of 12 fish: 6 treated and 6 controls (which were given 5 μL of pure water under the same conditions). In the first two, 2D electrophoresis was used to analyze the proteome and the phosphoproteome of the cytosolic fraction (Mezhoud et al., 2008b), and of the organelle and membranes fraction (Malécot et al., 2009). In the third experiment, gel-free and iTraq procedures were applied to the entire liver (Malécot et al., 2011).

Many proteins have been shown to be either up- or down-regulated by the treatment compared to controls (Table 1). Clearly the following conclusions can be drawn from the above experiments: 1) as expected, gel-free protein separation with iTraq treatment revealed a higher number of significantly modified proteins; 2) the repeatability of the