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Molecular insights into 4-nitrophenol-induced hepatotoxicity in zebrafish: Transcriptomic, histological and targeted gene expression analyses

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ABSTRACT

Background: 4-Nitrophenol (4-NP) is a prioritized environmental pollutant and its toxicity has been investigated using zebrafish, advocated as an alternative toxicological model. However, molecular information of 4-NP induced hepatotoxicity is still limited. This study aimed to obtain molecular insights into 4-NP-induced hepatotoxicity using zebrafish as a model.
Methods: Adult male zebrafish were exposed to 4-NP for 8, 24, 48 and 96 h. Livers were sampled for microarray experiment, qRT-PCR and various histological analyses.
Results: Transcriptomic analysis revealed that genes associated with oxidative phosphorylation and electron transport chain were significantly up-regulated throughout early and late stages of 4-NP exposure due to oxidative phosphorylation uncoupling by 4-NP. This in turn induced oxidative stress damage and up-regulated pathways associated with tumor suppressors Rb and p53, cell cycle, DNA damage, proteasome degradation and apoptosis. Pathways associated with cell adhesion and morphology were deregulated. Carbohydrate and lipid metabolisms were down-regulated while methionine and aromatic amino acid metabolisms as well as NFKB pathway associated with chronic liver conditions were up-regulated. Up-regulation of NFKB, NFAT and interleukin pathways suggested hepatitis. Histological analyses with specific staining methods and qRT-PCR analysis of selected genes corroborated with the transcriptomic analysis suggesting 4-NP induced liver injury.
Conclusion: Our findings allowed us to propose a plausible model and provide a broader understanding of the molecular events leading to 4-NP induced acute hepatotoxicity for future studies involving other nitrophenol derivatives.
General significance: This is the first transcriptomic report on 4-NP induced hepatotoxicity.

1. Introduction

4-Nitrophenol (para-nitrophenol; 4-NP) is widely used in the manufacture of pesticides, fungicides, paints, dyes, leather preservative and drugs; and it has been found commonly in many industrial wastewaters and diesel exhaust [1,2]. Due to the high-volume production, applications and potential toxicity, 4-NP has been classified as 'organic priority pollutants' [1,2]. Moreover, since 4-NP is an intermediate in the synthesis of acetaminophen which is used as a popular analgesic and antipyretic in many pharmaceutical formulations, the presence of 4-NP as an impurity in pharmaceuticals and its impact on consumer's health is also of concern [3]. 4-NP is known to resist biodegradation and therefore can accumulate and may magnify from lower to higher tropic levels in both aquatic and terrestrial organisms [4–7]. It has been detected in human urine likely due to exposure to organophosphorus pesticides [7,8]. Recently, perhaps of great concern, it has been shown that 4-NP has endocrine-disrupting activities perturbing steroidal hormone signaling pathways in rodent models [9–11]. Although 4-NP has relatively low toxicity, there are still concerns of the increased accumulation of 4-NP in water, soil and air, through the degradation of widely used organophosphorus pesticides and diesel exhaust emissions that could cause adverse effects on wildlife and human health [6,7]. Consequently, there is a concern of 4-NP entering the food chain, since besides surface

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water and sediments, the compound had been detected in vegetables [12] and fish [13].

It has been shown that the biological responses of zebrafish to chemicals, such as small molecules, drugs and environmental toxicants, are similar to that of mammals [14–16]. Therefore, in view of the environmental health concern of 4-NP as well as the advantages and increasing advocacy of using zebrafish as an alternative model for toxicology studies [17–19], the zebrafish has been used to investigate 4-NP metabolism and toxicity. The 96-hour acute toxicity test for 4-NP had been performed in adult zebrafish and the LC_{50} was reported between 10 and 14 mg/L [20,21]. In mammals, 4-NP is metabolized primarily in the liver into conjugated glucuronide and sulfate ester forms before being excreted from the body [1]. 4-NP can be metabolized by hepatic microsomal enzyme systems via glucuronidation and sulfation [22]. In zebrafish, glucuronidation and sulfation of 4-NP into respective glucuronide and sulfate conjugates had been demonstrated [23]. This further suggests some similarity of 4-NP metabolism in zebrafish and mammals. The zebrafish had been used for ultrastructural study of hepatotoxicity effects after a prolonged 2–3 month exposure to 4-NP [20]. It was observed that 25% of the fish treated with 1 and 5 mg/L of 4-NP showed degenerative transformation of the liver tissue, and deformation of the nuclear membrane with partial lysis of the mitochondria could be observed at 5 mg/L [20]. However, mechanistic information on the 4-NP induced hepatoxicity in the zebrafish, as well as in mammals, is still limited at the molecular level. Since the liver is the main organ that performs detoxification processes as well as regulation of metabolic pathways, it is important to further understand the *in vivo* toxic effects induced by 4-NP in the liver.

The primary aim of this study was to obtain molecular insights into 4-NP-induced hepatotoxicity using zebrafish as a model. DNA microarray was used to investigate the hepatic transcriptomic changes induced by 4-NP. The transcriptomic approach is applied in toxicology to determine the relative changes in gene expression levels induced by exposure to toxicant(s). In zebrafish, transcriptomic technology has been applied to capture differential gene expression profiles induced by specific toxicants [16,24]. Moreover, toxicant-induced global gene expression profiles of zebrafish have been demonstrated to be useful for identifying biomarkers of effects and deregulated signaling pathways, as well as for inferring possible affected biological functions, perturbed physiological systems and increased health-risks for mechanistic and predictive toxicology [16,24–28]. For example, it has been shown that genes that are predictive of prenatal arsenic exposure in humans were also identified as zebrafish orthologs in arsenic-treated zebrafish embryos using gene expression profiling [29]. To our knowledge, there has been no publication on the global gene expression profiling study to investigate the transcriptomic changes induced by 4-NP. Therefore, this is the first transcriptomic report on 4-NP induced hepatotoxicity. Furthermore, we corroborated our transcriptomic analysis with various histological analyses to further understand the toxicity mechanism of 4-NP. We also conducted gene-targeted validation using real-time PCR performed on an independent batch of fish samples treated with different concentrations of 4-NP, and these validated genes were used to further support the transcriptomic analysis of 4-NP induced hepatotoxicity. Taken together, our findings allow us to propose a plausible integrated model depicting 4-NP induced hepatotoxicity and provide a broader understanding of the molecular events leading to acute hepatotoxicity for further studies.

2. Experimental procedures

2.1. 4-NP exposure and fish sampling

Four main experiments were performed independently in this study, *i.e.* preliminary acute toxicity test, microarray experiment, histological analyses, and real-time PCR targeted gene expression validation. To determine suitable sub-lethal and low lethal concentrations for the experiment, male adult zebrafish were treated with 4-NP (Sigma-Aldrich, USA) at nominal concentrations of 1, 5 and 7 mg/L for 96 h at 27 ± 2 °C in a static condition. Control group was maintained in water as 4-NP is soluble in water at the concentration used and no organic vehicle was used. Fresh chemical and water were renewed daily. For the microarray experiment, a new batch of male adult fish were exposed to 4-NP at a low-lethal concentration of 7 mg/L for 96 h with a respective control group maintained in a similar condition as described above. Triplicate pooled liver samples, where each replicate was pooled from four individual livers, were obtained for each sampling time-point at 8, 24, 48 and 96 h treatment for the microarray experiments. For histology and real-time PCR targeted gene expression validation experiments, another new batch of male adult fish were exposed to vehicle 0.5 and 5 mg/L of 4-NP for 96 h maintained with a control group under a similar condition as above. Livers from five fish were sampled individually for targeted gene expression validation experiment at 96 h. All liver samples were snap-frozen in liquid nitrogen and stored at −80 °C for total RNA extraction for microarray and targeted gene expression validation experiments. For histological analysis, livers from four fish of each concentration of the 4-NP treated group and the control group were fixed in Bouin's solution for hematoxylin and eosin (H & E) staining, another four livers from each group were fixed in formalin solution (10%, Neutral Buffered, Sigma-Aldrich) for immunohistochemical and periodic acid-Schiff (PAS) staining, and another four livers from each group were freshly frozen for Oil-Red O staining.

2.2. Total RNA extraction

Total RNA was extracted from the fish liver samples using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. The integrity of RNA samples was verified by gel electrophoresis and by UV spectrophotometer (Nanodrop 2000; Thermo Scientific, USA). The concentrations were also determined by UV spectrophotometer.

2.3. Microarray hybridization

The arrays contained 16.4 K oligonucleotide probes. The probes were resuspended in 3× SSC at 20 μM concentration and spotted onto in-house poly-L-lysine-coated microscope slides using a custom-built DNA microarrayer in GIS. A two-color microarray hybridization format between test *versus* common reference, and control *versus* common reference was used. Common reference RNA for microarray hybridization was obtained by pooling total RNA extracted from untreated whole adult male and female fish. Both experimental (test and control) samples and common reference RNAs were reverse-transcribed and labeled with fluorescent dyes Cy-5 and Cy-3 (Amersham, USA), respectively. After hybridization at 42 °C for 16 h in hybridization chambers (Gene Machines, USA), the microarray slides were washed in a series of washing solutions (2× SSC with 0.1% SDS; 1× SSC with 0.1% SDS; 0.2× SSC and 0.05× SSC; 30 s each), dried using low-speed centrifugation and scanned for fluorescence detection using the GenePix 4000B microarray scanner (Axon Instruments, USA). Further detailed protocol for the microarray hybridization and data acquisition had been previously described [30,31]. The microarray data had been submitted to the Gene Expression Omnibus (GEO) with accession number GSE30058.

2.4. Microarray data processing and transcriptome analysis

The raw microarray data was normalized using Lowess method in the R package (http://www.braju.com/R/) followed by Student's *t*-test. To increase statistical power, triplicate arrays of 8 and 24 h were pooled and analyzed together as 'early responsive stage' whereas arrays of 48 and 96 h were pooled and analyzed together as 'late responsive stage'. Although the resolution time/kinetics is reduced into two phases, this will help to capture genes that are showing a similar expression trend, hence more consistent and robust, by considering two sampling time