論文の内容の要旨

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論文題目 Studies on the effects of tetrodotoxin on gene expression in pufferfish *Takifugu rubripes*

（テトロドトキシンがトラフグの遺伝子発現に及ぼす影響に関する研究）

Tetrodotoxin (TTX) is a potent blocker of voltage-gated, fast sodium channels. TTX is the toxic principle of pufferfish toxin, also known as fugu toxin. TTX accumulates in most marine pufferfish in a tissue specific way. Previous studies, in which nontoxic cultured pufferfish reared with nontoxic diets became toxic upon ingesting the toxic ovary of wild pufferfish, inferred that pufferfish accumulates TTX by the food chain, which is the widely accepted hypothesis today. However, the exact mechanisms involved in the accumulation of tetrodotoxin in pufferfish, and the role the toxin plays in the pufferfish body, still remain unclear, although various studies tried to shed light on these. Among various possible approaches in which such details can be elucidated, one fundamental way is to obtain more insight about the genes related to the toxicity of pufferfish. Therefore, we set our goal of this study to learn more about biological functions and physiological roles and effects of TTX from a gene expression perspective, using the liver as a central organ of a multitude of biological processes and its vital role in dealing with xenobiotic substances.

To achieve this goal, we identified differentially expressed genes between toxic and nontoxic *T. rubripes* liver tissues in three different approaches that are each more controlled than the previous one and complement each other. Two of these approaches consist of *in-vivo* experiments, of which the first one was performed using tissues of wild specimens of toxic and nontoxic pufferfish, and the second one being a TTX injection experiment in cultured nontoxic pufferfish. The third approach is a well-controlled *ex-vivo* experiment using a liver tissue assay.

By identifying these differentially expressed genes we aim to better understand the role of proteins involved in the pharmacokinetics of TTX in the pufferfish body, such as metabolic enzymes, binding proteins and specific transporters. Furthermore, differences in physiological status of the two groups are further discussed in this dissertation.

Chapter 2 contains both *in-vivo* experiments. 18 wild *T. rubripes* specimens were killed on location and tissue samples of liver were taken and stored in RNA*later* for subsequent total RNA extraction. Remaining liver tissues were analyzed for TTX content using a HPLC system with post column derivatization and fluorescence detection. Separation of TTX from other compounds was achieved based on the principle of ion-pair chromatography using 5 mM heptanesulfonic acid (HSA) as an ion-pair reagent in a mobile phase consisting of

1% (v/v) acetonitrile and 10 mM ammonium formate at pH 5.0. Separated compounds were treated with hot NaOH to facilitate degradation of TTX to detectable fluorescent derivatives. Based on quantitative TTX measurement results and total RNA quality, 4 toxic and 4 nontoxic liver tissue RNA samples were used for subsequent cDNA microarray experiments. The average toxicity of liver samples was 27.8 ± 15.0 μg TTX/g (127 ± 68 MU/g).

Total RNA samples were prepared for microarray analysis by reverse transcription and subsequent labeling with Cy3-labeled CTPs to fluorescent cRNAs. These probes were hybridized to microarrays, which were custom designed from the *T. rubripes* transcriptome database. Expression data of transcripts was digitalized and differential expression between toxic and nontoxic samples was analyzed using different computational approaches. Differential expression analysis of single genes was performed with fold-change analysis using Agilent Technologies' GeneSpring GX software. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database and the Gene Ontology (GO) functional annotation vocabulary were used to gain further insight into underlying biological meaning and functional processes that would otherwise be difficult to extract from the list of single genes. Fisher's Exact test was used on GO data to find significantly enriched annotation terms among the expression data. 4,867 probe sets were found to be up-regulated and 4,535 were down-regulated in toxic liver tissues. Of these, 678 transcripts had a fold-change difference in expression of 2 or larger. 248 transcripts (144 genes) were up-regulated and 430 transcripts (291 genes) down-regulated. Among the up-regulated genes were vitellogenin 6, phospholipase A2 precursor, apolipoproteins A-IV1 and Bb, cytochrome P450 3A4 and glutathione S-transferase mu 3. The list of highly down-regulated gene products contained diacylglycerol kinase eta, transmembrane proteins 165 and 102, peroxisome proliferator-activated receptor gamma (PPAR γ) coactivator 1-beta and NADH dehydrogenase subunits 1, 2 and 5.

GO analysis allowed for functional classification of differentially expressed transcripts in the three categories Biological Process, Molecular Function and Cellular Compartment. "Ion binding", "hydrolase activity" and "oxidoreductase activity" were the highest represented terms in the Molecular Function category. Many functional terms that are localized to mitochondria were found to be significantly enriched, as well as immune system and lipid binding and transport related processes. Purine and thiamine metabolism related pathways were among the highest represented KEGG pathways. These results suggest that natural occurring long-term exposure to TTX changes pufferfish physiology and metabolism on a gene expression level by modifying immune system and lipid related processes.

The second *in-vivo* experiment was performed under more controlled conditions by injecting TTX into the caudal muscle of cultured specimens of *T. rubripes* and investigating differential gene expression after 5 days. The 10 fish used in this study were 18 months old and had a body weight of approximately 1 kg. TTX was dissolved in modified Hank's balanced salt solution buffer and 0.25 mg TTX/kg body weight was injected into 5 of the fish. On the fifth day after administration, liver samples were prepared as previously described for subsequent quantitative TTX determination and for microarray analysis, with the 5 samples from TTX-injected

fish as a test group, and 5 samples from buffer-only injected fish as a control group. After 5 days, the TTX content in livers was 28 ± 13% of the original dose.

In microarray analysis, probe sets with a p-value of 0.05 or smaller and a fold-change difference in expression of 2.0 or higher were selected as differentially expressed transcripts. 490 transcripts (466 genes) passed this filter. Of these, 63 transcripts (40 genes) were up-regulated and 427 transcripts (426 genes) down-regulated. The list of up-regulated genes contained chymotrypsin-like elastase family member 2a, transmembrane protein 168, Rho GTPase-activating protein 29, calcium channel alpha-1 subunit homolog and nicotinic acetylcholine receptor alpha 9d subunit. Among the down-regulated genes, nuclear receptor coactivator 2, transcription factor SOX-5, hepatocyte nuclear factor 3-gamma and plasma membrane calcium-transporting ATPase 3 were highly differentially expressed, among others. Functional classification according to GO terminology did not differ much from the one in liver samples of wild *T. rubripes*, with the difference that among Molecular Function terms after "ion binding", "signal transducer activity" and "transferase activity" were the most highly represented terms, and "oxidoreductase activity" was the least represented one, suggesting a higher prevalence of signal transduction related differentially expressed genes (DEGs). According to Fisher's Exact test, none of the differentially expressed probe sets are significantly enriched in a particular functional group. KEGG pathway analysis results are similar to those of genes in wild liver, with purine and thiamine metabolism related pathways being highly represented among the tested genes. Since thiamine is necessary for biosynthesis of neurotransmitters like acetylcholine and plays an important role in neural health, a difference in its metabolism between toxic and nontoxic pufferfish is likely a response to TTX neurotoxicity. "T cell receptor signaling pathway" related transcripts were also enriched and suggest an effect of TTX on expression of immune system related genes.

Quantitative real-time PCR was used to measure the expression of one of the highest differentially expressed transcripts (ENSTRUT00000045544) in toxic and nontoxic liver tissues. The measured fold-change difference in expression of 27.22 ± 4.18 showed good correlation with the 37.6-fold difference measured by microarray analysis, and further validates the microarray obtained results.

In Chapter 3, TTX accumulation and differential gene expression in *T. rubripes* liver slices of an *ex-vivo* tissue experiment are investigated. The liver of one female specimen of cultured *T. rubripes* was dissected, perforated with sterile perfusion buffer and then cut into 1 mm thick slices of 8 mm diameter, which were incubated at 20°C on a 24-well culture plate in Leibovitz's L-15 medium containing antibiotics. After 24h pre-incubation, TTX was added to a final medium concentration of 50 μM and 200 μM to some of the slices. After 1, 5, and 24 hours incubation, all liver discs of the respective experimental group were harvested and cut into 3 parts, one for cell viability analysis, one for quantitative TTX analysis, and one for total RNA extraction and gene expression analysis. Cell viability was confirmed with alamarBlue cell viability reagent and TTX levels were measured with the HPLC-FLD system as described previously. TTX quantification demonstrates that the liver tissue slices actively took up TTX from the medium with a linear correlation between uptake amount and medium

concentration at a certain time. After 24 hours, liver slices accumulated TTX at a concentration that is more than 4 times higher than surrounding medium concentration.

Total RNA was extracted from liver tissues and mRNA was isolated. In order to prepare a whole transcriptome library for next-generation sequencing, mRNA was fragmented, ligated with an adapter and subjected to reverse transcription. Barcodes were attached to thus obtained cDNAs, and 9 each of the barcoded libraries pooled and sequenced using the IonProton™ sequencer and Ion PI™ sequencing chip. Sequence data was subjected to quality control and mapped against the Ensembl release 76 (October 2014) of the *T. rubripes* reference genome. RNA-Seq analysis was performed and normalized samples investigated for differential gene expression using the "Exact Test" for two group comparisons, as implemented in CLC Genomics Workbench Ver. 7. Samples with an absolute fold-change difference of 1.5 or higher and a p-value smaller than 0.01 were used in functional analysis with GO and KEGG, which was performed as described. Lists of significant DEGs were chosen for single gene analysis if they passed an FDR-corrected cut-off filter of 0.05. The number of DEGs increased with time and TTX dose and ranged from 182 in 1h 50 μM samples to 391 in samples incubated with 200 μM TTX for 24h. 13 DEGs showed a TTX dose-dependent effect in gene expression, among them ATP synthase, glutaredoxin, cytochrome c oxidase subunit VIIc and mitochondrial tRNAs. GO analysis showed only little difference in the functional groups that are expressed in each experimental group, suggesting little time-dependent differences in the functional quality of gene expression in reaction to TTX exposure. Most of the GO-terms that were found to be significantly enriched among DEGs are related to mitochondria. KEGG analysis results are similar, with oxidative phosphorylation related pathways being highly represented by DEGs. A closer investigation in the single-genes list identifies many down-regulated DEGs involved in mitochondria function, such as ATPases, cytochrome c oxidase subunits VIIa and VIIc, ubiquinol-cytochrome c reductase complex III subunit IV and many others. It is likely that the observed effects on gene expression related to ATP production in mitochondria and of cytochrome oxidases is directly connected to effects of TTX acting on voltage-dependent Na^+ channels in hepatocytes, with possible implications for energy metabolism and other Ca^{2+} signaling dependent processes. These results also suggest that although *T. rubripes* is widely immune to the toxic effects of TTX, TTX is still able to modify various physiological processes in the pufferfish body.

Lastly, a separate virtual experiment was set up to analyze the expression of Pufferfish Saxitoxin and Tetrodotoxin Binding Protein (PSTBP) homologs Tr1 and Tr3. Although previous studies found Tr1 and Tr3 to be only constitutively expressed, and no toxin-triggered change in expression was observed, we demonstrate in our study the TTX dose- and time-dependent up-regulation of Tr3 and simultaneous down-regulation of Tr1, while expression in control group remains unchanged. This result suggests a direct or indirect effect of TTX on the expression of these genes, which is likely to affect TTX accumulation and distribution in the pufferfish body by facilitating uptake into tissue.