TRα- and TSH-mRNA levels after temporal exposition with methimazole in zebrafish, Danio rerio.

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Introduction

The group of dioxin and dioxin-like substances are highly persistent in the environment. There are evidences from present investigations that a variety of substances are capable of disrupting the endocrine system in the aquatic environment. These substances are called endocrine disruptors. Dioxin and related compounds can act as endocrine disruptors. Aquatic animals like amphibian and fish are especially affected of the impact of these compounds. Investigations concerned so far in particular the domain of reproduction biology and the thyroid axis especially [1-3]. Recent investigations showed that the TRα-mRNA level change after a short temporal expression with T3, methimazole and amiodarone [2,4]. The objective of the project is to identify effects of thyroid endocrine disruptors on the regulation of gene expression of the thyroid receptors TRα, TRβ and thyroid stimulating hormone TSH and associated effects on other system. In preliminary studies the effects of the drug methimazole as model substance on gene expression of TRα and TSH were investigated. Methimazole is an inhibitor of the thyroid peroxidase so that the formation of thyroid hormones is disrupted.

Methods and Materials

Fish culture and drug treatment

Adult zebrafish were maintained as described by Westerfield [5]. The embryos were obtained by natural crosses at 28°C [6]. Newly-hatched zebrafish larvae (48 hpf) were exposed in beakers containing 500 ml embryo medium at 26°C. The larvae were subjected to various concentrations of methimazole (0, 10, 50, 100, 300 µM) for different periods (1, 3, 5, 7 days). For each concentration 100 larvae were sampled. The stock solutions of 10^{-4}M methimazole were prepared freshly for every change of medium. Embryo medium was changed every second day. Samples of larvae were frozen at -80°C until analysis.

Measurement of methimazole

For measurements of methimazole concentrations in embryo medium samples were continuously taken from different time points of treatment (before and after changes of embryo medium and after feeding). Methimazole was measured according to Hollosi using HPLC (unpublished).

Real time RT-PCR

Total RNA was isolated with Trizol®Reagent (Invitrogen) and the first strand cDNA was prepared using the FirstStrand cDNA Synthesis Kit for RT-PCR (AMV; Roche) with Oligo(dT) and 1.0 µg of total RNA. Real time PCR amplifications for TRα (accession U54796), TSH (accession AY135147) and gapdh (accession CA854263) were performed with the LightCycler FastStart DNA Master SYBR Green I Kit (Roche). Gapdh was amplified as internal control. The PCR was run at 95°C (10 min) and for the amplification cycles at 95°C (10 s), 65°C (5 s), 72°C (10 s) using a lightcycler (roche). The level of TSH-mRNA and TRα-mRNA were analysed with the Relative Quantification Software Version 1.0 (roche), normalized as TRα or TSH/gapdh ratios. Primers were as follows: TRα forward 5'-CAA TGT ACC ATT TCG CGT TG-3' and reverse 5'-CTC CTG CTC TGT GTT TTC CA-3'; TSH forward 5'-ATT GTT CAG AGG GGA TGC AC-3' and reverse 5'-CCA ATA TGC TTG GGC GTA GT-3'; gapdh forward 5'-GAA GGT GGC AAA CTG GTC AT-3'and reverse 5'-TTG CAC CAC CCT TAA TGT GA-3'

Results and Discussion

Measurement of methimazole: To control the concentration of methimazole during the expositions samples of embryo medium treated with methimazole were taken (Figure 1). The measurements of the methimazole concentrations in exposition medium demonstrated that all concentrations were consistently lower than the specified values. Possible is that methimazole was bound to a minor extend on glass surfaces or on feed and fish.

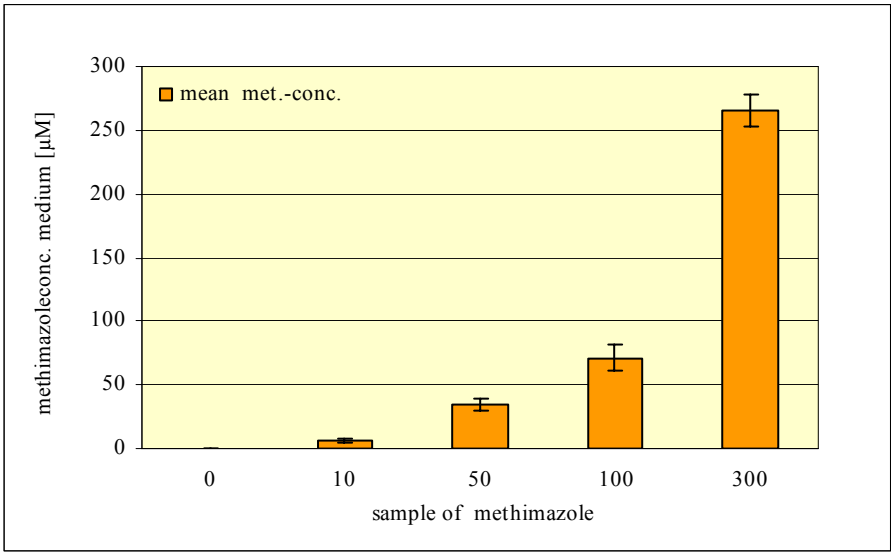

Figure 1
Effective concentrations of methimazole in the embryo medium during the exposition of zebrafish larvae.

Real time RT-PCR: Figure 2 demonstrates that TRα-mRNA was down-regulated compared to control after an exposition with methimazole in accordance to Liu et al. (5). There is a decrease of the level of gene expression from day 1 to day 5 (Figure 2 A, B and C). In Figure 2 D a less strong decrease of gene expression is showed after 7 days treatment. The level of TRα-mRNA expression is similar to the level of gene expression after a treatment of 3 days. It has to be cleared up whether this higher expression level of TRα-mRNA resulted from regulation mechanisms of thyroid-axis. The expression profile of TSH-mRNA after 7 days exposition with methimazole is shown in Figure 3. TSH-mRNA was up-regulated for the methimazole concentrations of 10, 50 and 300 µM. In further expositions of 1, 3, and 5 days it was shown that TSH-mRNA was up-regulated for all expositions except for 100 µM which was not regulated. An exception was exposition day 5, where TSH-mRNA for 100°µM was weakly up-regulated. These results for the gene expression of TSH after treatments with different methimazole concentrations give evidence for different regulation processes depending on exposition time and concentration. Liu et al. (5) described that exposition with T3 or methimazole resulted in alterations of expressed gene levels of TRα and TRβ in only one direction without dose-dependency for various concentrations.

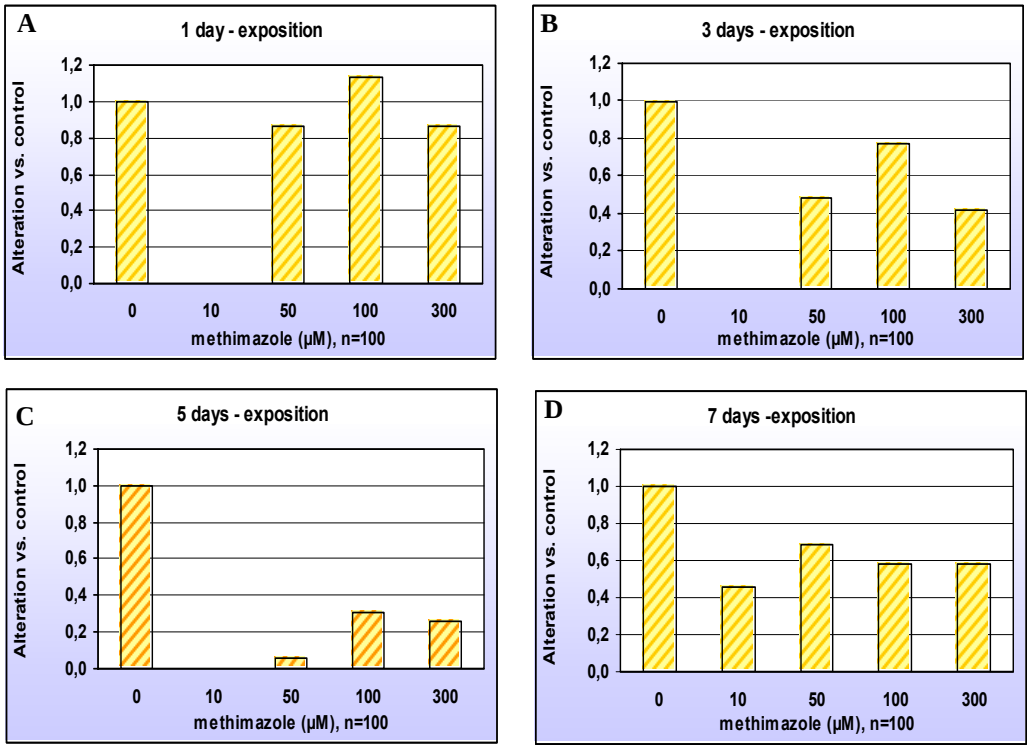

Figure 2
Effects of expression of zebrafish larvae TRα-mRNA after *in vivo* exposition with methimazole. Newly-hatched larvae were exposed with methimazole (0, 10, 50, 100 and 300µM) for 1 (A), 3 (B), 5 (C) and 7 days (D). For each treatment, 100 larvae were harvested for RT-PCR. As internal control gapdh-mRNA was amplified.

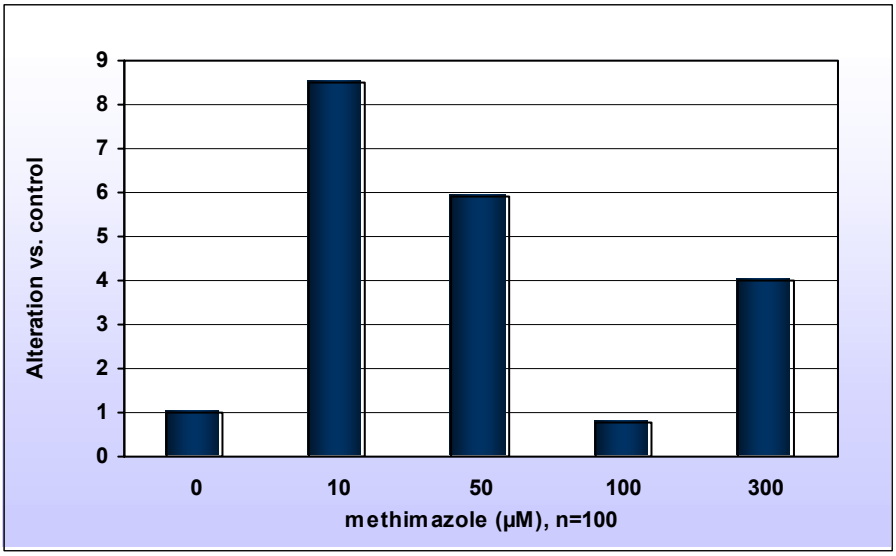

Figure 3
Effects of expression of zebrafish larvae TSH-mRNA after *in vivo* exposition with methimazole. Newly-hatched larvae were exposed with methimazole (0, 10, 50, 100 and 300µM) for 7 days. For each treatment, 100 larvae were harvested for RT-PCR. As internal control gapdh-mRNA was amplified.

Conclusion

Objective of the project is to identify the effects of thyroid endocrine disruptors on the regulation of different genes like TRα, TRβ and TSH. Furthermore associated genetic effects on other hormone systems have to be revealed. Enlarged and detailed investigations are still under progress in order to confirm these first results and to establish correlations between concentration and exposition time. Similar investigations are also planned with T3.

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