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Species-specific differences in the inhibition of human and zebrafish 11β-hydroxysteroid dehydrogenase 2 by thiram and organotins

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ABSTRACT

Dithiocarbamates and organotins can inhibit enzymes by interacting with functionally essential sulfhydryl groups. Both classes of chemicals were shown to inhibit human 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2), which converts active cortisol into inactive cortisone and has a role in renal and intestinal electrolyte regulation and in the feto-placental barrier to maternal glucocorticoids. In fish, 11β-HSD2 has a dual role by inactivating glucocorticoids and generating the major androgen 11-ketotestosterone. Inhibition of this enzyme may enhance glucocorticoid and diminish androgen effects in fish. Here, we characterized 11β-HSD2 activity of the model species zebrafish. A comparison with human and mouse 11β-HSD2 revealed species-specific substrate preference. Unexpectedly, assessment of the effects of thiram and several organotins on the activity of zebrafish 11β-HSD2 showed weak inhibition by thiram and no inhibition by any of the organotins tested. Sequence comparison revealed the presence of an alanine at position 253 on zebrafish 11β-HSD2, corresponding to cysteine-264 in the substrate-binding pocket of the human enzyme. Substitution of alanine-253 by cysteine resulted in a more than 10-fold increased sensitivity of zebrafish 11β-HSD2 to thiram. Mutating cysteine-264 on human 11β-HSD2 to serine resulted in 100-fold lower inhibitory activity. Our results demonstrate significant species differences in the sensitivity of human and zebrafish 11β-HSD2 to inhibition by thiram and organotins. Site-directed mutagenesis revealed a key role of cysteine-264 in the substrate-binding pocket of human 11β-HSD2 for sensitivity to sulfhydryl modifying agents.

1. Introduction

In humans, 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) essentially catalyzes the conversion of the active glucocorticoid cortisol (corticosterone in rodents) to its inactive form cortisone (11-dehydrocorticosterone in rodents), thereby regulating the access of glucocorticoids to glucocorticoid receptors (GR) and mineralocorticoid receptors (MR), and rendering specificity of MR for aldosterone (Odermatt and Kratschmar, 2012). The consequences of impaired 11β-HSD2 activity on electrolyte balance and blood pressure are manifested in patients with genetic defects and suffering from apparent mineralocorticoid excess, and upon ingestion of large amounts of licorice, which contains the inhibitor glycyrrhetinic acid (Ferrari, 2010). Moreover, in the placenta 11β-HSD2 acts as a protective barrier for the fetus from high maternal cortisol concentrations, and studies in rodents indicated that 11β-HSD2 inhibition during pregnancy causes irreversible changes in fetal development that lead to a higher risk for cardiovascular and metabolic disease (Murphy et al., 2002; Seckl and Holmes, 2007; Shams et al., 1998; Welberg et al., 2005). Thus, besides genetic susceptibility, environmental factors, including the exposure to xenobiotics, need to be considered (Ma et al., 2011; Odermatt and Gumy, 2008; Odermatt et al., 2006).

In contrast to human and other mammalian species, studies addressing the inhibition of 11β-HSD2 by xenobiotics in fish and other aquatic species are missing. Studies on rainbow trout (Kusakabe et al., 2003), Japanese eel (Jiang et al., 2003; Miura et al., 1991) and Nile tilapia (Miura et al., 1991) revealed an important role of 11β-HSD2 in the formation of the main fish androgen 11-ketotestosterone from 11β-hydroxytestosterone. In fish, 11β-HSD2 is highly expressed in the gonads, supporting its role in androgen metabolism. Thus, xenobiotics inhibiting 11β-HSD2 are expected to enhance glucocorticoid effects and suppress androgen action in fish.

Abbreviations: 11β-HSD2, 11β-hydroxysteroid dehydrogenase 2; DBT, dibutyltin; DMSO, dimethylsulfoxide; DMT, dimethyltin; DOT, dioctyltin; DPT, diphenyltin; GR, glucocorticoid receptor; LC–MS, liquid chromatography–mass spectrometry; MR, mineralocorticoid receptor; MRM, multiple-reaction monitoring; NEM, N-ethylmaleimide; TBT, tributyltin; TMT, trimethyltin; TPT, triphenyltin.

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We reported earlier that dithiocarbamates (Atanasov et al., 2003) and organotins (Atanasov et al., 2005), chemicals known to interfere with functionally important sulfhydryl groups, inhibit human 11β-HSD2. Several dithiocarbamates inhibit human 11β-HSD2 in the nanomolar range, i.e. thiram, disulfiram and maneb, and some in the micromolar range, i.e. pyrrolidine dithiocarbamate, diethyldithiocarbamate and zineb. These chemicals are expected to exert additive inhibitory effects on 11β-HSD2 (Atanasov et al., 2003).

Dithiocarbamates, including thiram (tetramethylthiuram disulfide), are widely used as fungicides on seeds and as foliar fungicides on turf, vegetables and fruits (Vettorazzi et al., 1995). The pesticides ferbam and ziram are environmentally degraded to thiram. Furthermore, thiram is used as an accelerator and vulcanization agent in the rubber industry. Gupta et al. have shown that the half-life of thiram under controlled laboratory conditions is longer than that of other carbamates and ranges from 5 to 12 days in water, depending on multiple parameters (Gupta et al., 2012). The extensive use of thiram, the fact that thiram is a degradation product of other pesticides, the possible persistence in the environment, and additive inhibitory effects of mixtures of dithiocarbamates, led us to investigate whether thiram might inhibit 11β-HSD2 of the aquatic model organism zebrafish (*danio rerio*).

Cadmium, that also may affect the function of sulfhydryl groups on proteins, has been found to decrease 11β-HSD2 activity in cultured primary human trophoblast cells and in cultured human choriocarcinoma JEG-3 cells, whereby it remained unclear whether the reduced activity was due to direct inhibition of 11β-HSD2 or reduced expression (Ronco et al., 2010; Yang et al., 2006). A very high environmental enrichment factor has been reported for cadmium (Shi et al., 2012).

Moreover, we tested whether organotins might inhibit zebrafish 11β-HSD2. Organotins are, even after the worldwide ban of TBT, readily detected in water ecosystems (Castro et al., 2012). They accumulate in sediments and show high bioaccumulation in various aquatic species with concentrations up to 53 μg/g in Cobia (Rachycentron canadum) (Jadhav et al., 2011; Kannan et al., 1995; Liu et al., 2006). Previously, we found that the organotins dibutyltin (DBT), tributyltin (TBT), diphenyltin (DPT) and triphenyltin (TPT) inhibit human 11β-HSD2, that they show additive inhibitory effects, and that mutant C264S was less sensitive to inhibition by TBT (Atanasov et al., 2005), suggesting reversible sulfhydryl modification as inhibitory mechanism.

In the present study, we assessed the effects of organotins, thiram, cadmium and the sulfhydryl modifying reference compound N-ethylmaleimide (NEM) on zebrafish 11β-HSD2 activity and compared the effects with those on the human enzyme. Finally, we performed site-directed mutagenesis to explain differential effects on human and zebrafish 11β-HSD2 by the xenobiotics investigated.

2. Materials and methods

2.1. Materials

Cadmium chloride was purchased from Merck KGaA (Darmstadt, Germany), [1,2,6,7-^{3}H]-cortisol from Amersham Pharmacia (Piscataway, NJ, USA), unlabeled steroids from Steraloids (Newport, RI), and all other chemicals and cell culture medium from Sigma–Aldrich Chemie GmbH (Buchs, Switzerland). The solvents were of analytical and high performance liquid chromatography grade and the reagents of the highest grade available. Cadmium chloride, thiram and organotins were dissolved in dimethyl sulfoxide (DMSO) and stored as 20 mM stock solution at −20 °C. N-ethylmaleimide (NEM) was dissolved in ethanol and stored as 20 mM stock solution at −20 °C.

2.2. Construction of expression plasmids and site-directed mutagenesis

Expression plasmids for human wild-type 11β-HSD2 and mutant C264S have been described earlier (Atanasov et al., 2005; Odermatt et al., 1999). A full length zebrafish (*danio rerio*) cDNA clone was purchased from ImaGenes GmbH, RZPD, Berlin, Germany. The cDNA was amplified by PCR using an oligonucleotide at the start codon to introduce a BamHI endonuclease restriction site and a Kozak consensus sequence (5′-CATAAGCTTCCGCCATGTCTATTTTTGTTGGTGGAGCAG-3′) and an oligonucleotide at the stop codon either to add an XbaI endonuclease restriction site (5′-ACCTCGAGCTAATCAATACACTTTGTGAAGTTGC-3′) or to attach a FLAG-epitope followed by the stop codon and an XbaI endonuclease restriction site (5′-ACCTCGAGTCACTTGTCATCGTCGTCCTTGTAGTCCATAGAACCATCAATACACTTTGTGAAGTTGCTG-3′). The PCR product was inserted into the BamHI–XbaI sites of the pcDNA3.1 vector. Site-directed mutagenesis to construct mutant A253C was performed as described earlier (Atanasov et al., 2005). The selected clones used in this study were sequence verified. Protein expression and enzyme activity was assessed in transiently transfected HEK-293 cells. Protein expression of zebrafish wild-type 11β-HSD2 and mutant A253C was verified by Western blotting (Fig. S1), as described for human 11β-HSD2 wild-type and mutant C264S (Atanasov et al., 2005). Briefly, proteins were separated by sodium dodecyl sulfate gel electrophoresis and transferred on a polyvinyl difluoride membrane. The FLAG-tagged 11β-HSD2 was detected by mouse M2 antibody from Sigma–Aldrich Chemie GmbH. Actin was detected by rabbit anti-actin IgG from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated secondary antibodies were used to visualize the bands with Immobilon Western Chemiluminescent HRP substrate from Millipore Corporation (Billerica, MA, USA). Untagged and C-terminally FLAG-epitope tagged proteins showed comparable activities as seen before for human 11β-HSD2 expression constructs (Odermatt et al., 1999).

2.3. Cell culture

Human embryonic kidney cells (HEK-293) were cultivated in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose (D5796 Sigma–Aldrich), 10% fetal bovine serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 1× MEM non-essential amino acids and 10 mM HEPES buffer, pH 7.4. Cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere.

Zebrafish embryonic fibroblast cells ZF-4 (kindly provided by Dr. Jerzy Adamski, Helmholtz Zentrum, Munich, Germany) were cultivated in DMEM:F12 (D8437 Sigma–Aldrich), supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 0.1 mg/mL streptomycin. These cells were maintained at 28 °C in a humidified 5% CO_2 atmosphere.

2.4. Transient transfection and harvesting of cells

HEK-293 cells were transiently transfected with plasmids for human wild-type 11β-HSD2 (Odermatt et al., 1999) or mutant C264S (Atanasov et al., 2005) using the calcium phosphate precipitation method. Transfection efficiency was approximately 20%. Zebrafish wild-type 11β-HSD2 and mutant A253C were transfected into ZF-4 cells using Fugene HD according to the manufacturer's protocol (Roche Applied Science, Rotkreuz, Switzerland). Transfection efficiency was approximately 25%. After 48 h transfected cells were detached, centrifuged and cell pellets (5 pellets/10 cm^2 dish) shock frozen on dry ice and stored at −80 °C until further use.

2.5. Determination of recombinant human, mouse and zebrafish 11β-HSD2 activities by liquid chromatography–tandem mass spectrometry (LC–MS)

Reactions were performed for 10 min at 37 °C in a total volume of 500 μL containing lysates of HEK-293 cells expressing human, mouse or zebrafish 11β-HSD2 in buffer TS2 (100 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM $MgCl_2$, 250 mM sucrose, 20 mM Tris–HCl, pH 7.4), supplemented with 500 μM NAD^+ and the corresponding substrate (2 nM–2 μM final concentration). Internal standard (100 nM deuterized d8-corticosterone) was added, followed by extraction with 1 mL ethyl acetate. The organic phase was transferred to a new tube, evaporated to dryness and reconstituted in 100 μL of methanol containing 0.1% formic acid.

Steroids were resolved on an Atlantis T3 (3 μm, 2.1 mm × 150 mm) column (Waters, Milford, MA) at 30 °C using an Agilent model 1200 Infinity Series chromatograph (Agilent Technologies, Basel, Switzerland). The mobile phase consisted of water and acetonitrile (95:5) containing 0.1% formic acid (solvent A), and water and acetonitrile (5:95) containing 0.1% formic acid (solvent B) at a total flow rate of 0.4 mL/min. A linear gradient was used starting from 30% solvent B to 70% solvent B from 0 to 13 min, followed by 95% solvent B for 2 min, and re-equilibration with 30% solvent B. A built-in switching valve was used to direct the LC flow to an Agilent 6410 triple quadrupole MS (controlled by Mass Hunter workstation software version B.01.04). The injection volume of each sample was 5 μL. The MS was operated in atmospheric pressure electrospray positive ionization mode, with nebulizer pressure and nebulizer gas flow rate of 45 psi and 10 L/min, respectively, a source temperature of 350 °C and capillary and cone voltage of 4000 V and 190 V, respectively.

The six steroids were analyzed using multiple-reaction monitoring (MRM). Metabolites were identified by comparing their retention time and mass to charge ratio (m/z) with those of authentic standards. The transitions, collision energy and retention time were m/z 363/121, 25 V, 11.4 min for cortisol; m/z 361/163, 20 V, 11.6 min for cortisone; m/z 347/121, 40 V, 13.4 min for corticosterone; m/z 355/125, 28 V, 13.4 min for d8-corticosterone; m/z 345/121, 40 V, 12.9 min for