General and Comparative Endocrinology 176 (2012) 252–258

Contents lists available at SciVerse ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

High levels of corticosterone, and gene expression of *star*, *cyp17a2*, *hsd3b*, *cyp21*, *hsd11b2* during acute stress in common carp with interrenal hyperplasia

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ARTICLE INFO

Article history:
Received 25 May 2011
Revised 28 November 2011
Available online 7 February 2012

Keywords:
Common carp
Corticosterone
Cortisol
Star
Steroidogenic enzymes
17α-Hydroxylase deficiency
Congenital adrenal hyperplasia (CAH)

ABSTRACT

We investigated the acute stress response in a common carp strain (E5) with interrenal hyperplasia due to 17α-hydroxylase deficiency, and in an isogenic standard (STD) carp strain. Cortisol, corticosterone and the head kidney-somatic index were measured during and after a 3 h net confinement stress. *Star*, *cyp17a2*, *hsd3b*, *cyp21*, *hsd11b2* mRNA levels were measured in head kidneys using real-time qPCR. The results show very high corticosterone levels and enlargement of the head kidney in E5 fish. This is the first report in a teleost fish showing a significant increase of corticosterone levels in response to stress due to interrenal hyperplasia. The high levels of corticosterone in E5 suggest that corticosterone is not converted to aldosterone in common carp. *star* and *hsd3b* mRNA levels were significantly higher in E5 compared to STD fish, while *cyp17a2* levels were significantly lower in E5. In contrast to E5, *star levels* did not change during stress and recovery in STD, suggesting that the enzyme is regulated in a different manner in E5 and STD fish. In E5, the levels of cyp17a2 dropped below control values after 20 min stress. These findings strongly suggest that *cyp17a2* is impaired at (post)-transcriptional level. As a consequence the accumulated precursor (pregnenolone) is not converted to cortisol, but to corticosterone. In contrast to STD, significant levels of cortisol could not be detected in E5. Finally, *hsd11b2* mRNA levels were significantly lower in E5 compared to STD, and did not change during stress and recovery. These results support the idea that *hsd11b2* is involved in the conversion of physiologically active cortisol to inactive cortisone, as reported earlier for STD carp. In conclusion our results show high levels of corticosterone in E5 and differences in *star* and mRNA levels of steroidogenic genes between E5 and STD carp during net confinement stress.

1. Introduction

Congenital adrenal hyperplasia (CAH) is a group of disorders in the biosynthesis of cortisol caused by an enzymatic deficiency in the conversion of cholesterol to cortisol and has been first identified in humans [16,27,29]. Impaired function of these enzymes results in low production of cortisol and overproduction of androgens and has hyperplasia as a consequence due to increased compensatory ACTH-production. In women, androgen overproduction can cause masculinization, hirsutism, infertility and hypertension [44,10].

In fish, the head kidney is the functional homologue of the mammalian adrenal. In 2005, we described the first case of interrenal hyperplasia in fish, an isogenic line of common carp (E5), having the classic symptoms of congenital adrenal hyperplasia: enlarged head kidney, a low production of cortisol in response to ACTH stimulation and female to male sex reversal [34,35]. Experiments with E5 head kidney homogenates showed that conversion of pregnenolone to 17a-hydroxypregnenolone was significantly lower in E5 compared to a standard isogenic male strain of carp, suggesting that interrenal hyperplasia and low cortisol production in response to stress are caused by a dysfunction of the enzyme 17α-hydroxylase/17, 20-lyase (CYP17) [35].

In fish, cortisol is the main glucocorticoid. It is synthesized by the interrenal cells of the head kidney. Its primary function is in osmoregulation and glucose metabolism, while it also affects the immune response [28,42]. Cortisol secretion is regulated through the hypothalamus by CRF (corticotropin-releasing hormone) and

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doi:10.1016/j.ygcen.2012.01.023

the pituitary through ACTH (adrenocorticotrophic hormone). ACTH and CRF are inhibited by high cortisol levels in a negative feedback loop [4,37,30]. Cortisol is synthesized from its precursor, cholesterol, by side chain cleavage of the $P450_{scc}$ complex, located in the inner mitochondrial membrane. Pregnenolone, the product of this cleavage, then undergoes a series of isomerisations and hydroxylations by 17α-hydroxylase (CYP17A2), 3β-hydroxysteroid dehydrogenase (HSD3B), 21-hydroxylase (CYP21) and 11β-hydroxylase (HSD11B1) to produce cortisol (Fig. 1). The rate-limiting step of steroidogenesis is the availability of cholesterol for the $P450_{scc}$ complex and the steroidogenic acute regulatory protein (STAR); the latter plays a crucial role in steroidogenesis by transferring hydrophobic cholesterol across the aqueous barrier between the outer and inner mitochondrial membrane. In mammals both STAR protein and mRNA increase rapidly in response to ACTH [9,13,22,21,43]. In teleost fish different results have been obtained depending on the stressor applied. *star* transcript levels in head kidney increased in response to severe acute stress, in several species. The transcript levels increased after endocrine disruption in rainbow trout, lipopolysaccharide injection in gilthead seabream, capture and anesthesia in rainbow trout and a vortex stressor in zebrafish [1,8,14,15,19]. Moreover, injection of ACTH in eels and chronic manipulation of ACTH levels in Chinook salmon resulted in an increase of *star* mRNA levels [23,25]. However, in net confinement studies, *star* mRNA levels were not correlated to changes in cortisol levels [8,15,20,26]. For *hsd3b* there is only limited information available. *hsd3b* mRNA levels did not change during net confinement stress in rainbow trout and common carp [8,26] and in ACTH treated chinook salmon [25]. The enzyme CYP17 plays a key role in steroid hormone synthesis. In head kidney of fish CYP17 is involved in biosynthesis of cortisol and of sex steroids. CYP17a2 (CYP17A2), which only possess the 17α-hydroxylase activity, catalyzes the 17α-hydroxylase activity required for the conversion of pregnenolone to 17α-hydroxypregnenolone, a precursor of cortisol [47,46]. In common carp, *cyp17a2* mRNA levels only increased during the recovery period following net confinement, suggesting regulation at the level of pre-existing proteins.

Changes in *hsd11b2* transcripts during stress were observed in rainbow trout, common carp and zebrafish [14,17,26]. Although the specific role of the HSD11B2 enzyme in teleost fishes is not known, it has been suggested that this enzyme may be involved in regulating the balance of active versus inactive cortisol [26]. Moreover, an increase in *hsd11b2* transcript levels during the stress response could reduce cortisol signaling at the level of the interrenal cells [14]. Earlier studies in coho salmon already suggested that cortisol may exert ultra-short-loop feedback directly at the level of interrenal cells [6]. Further studies are needed to confirm the role of HSD11B2 in short-loop-feedback.

The E5 carp strain with interrenal hyperplasia provides a unique model to study stress-induced regulation of key steroidogenic enzymes. The low production of cortisol in E5 during the acute stress response may result in negative feedback inhibition and continuous activation of key steroidogenic enzymes. The purpose of this study was to compare the stress response during net confinement and recovery in a normal isogenic male strain (STD) with E5 carp by measuring cortisol production and mRNA levels of *star*, *cyp17a2*, *hsd3b*, cyp21 and *hsd11b*.

2. Materials and method

2.1. Animal production and net confinement procedure

All procedures are approved by the Wageningen University committee on approval in animal experimentation. Isogenic male carp (hereafter named STD) were produced by the conventional breeding of an E4E5 (XX) female with a R3R8 (YY) male. Fish of the E5 homozygous mutant strain were reproduced by androgenesis. The E5 strain is homozygous XX but reverses from female to male during early sexual differentiation. The cause of this sex reversal is not known but has been attributed to the action of a recessive mutation in a putative sex determining gene (details on all strains in Komen and Thorgaard [18]). Fish production was done as described previously [5]. Fish larvae were fed freshly hatched *Artemia* nauplii for the first 21 days and with pelleted food (Provimi, Rotterdam) thereafter according to standard procedures, with a photoperiod of 14 h light/10 h dark [40]. Six weeks prior to the experiment, 40 fish of each strain were randomly sampled and divided in two groups of 20 fish which were stocked in two 140 L glass rectangular tanks and fed 1.5% body weight per day. All rearing tanks were connected to a recirculation system equipped with a bio-filtration unit and UV treatment. NH_4^+ and NO_2 levels never exceeded 1 ppm, pH was kept at 6.5 and oxygen levels fluctuated between 8 ppm in the morning and 5 ppm in the afternoon

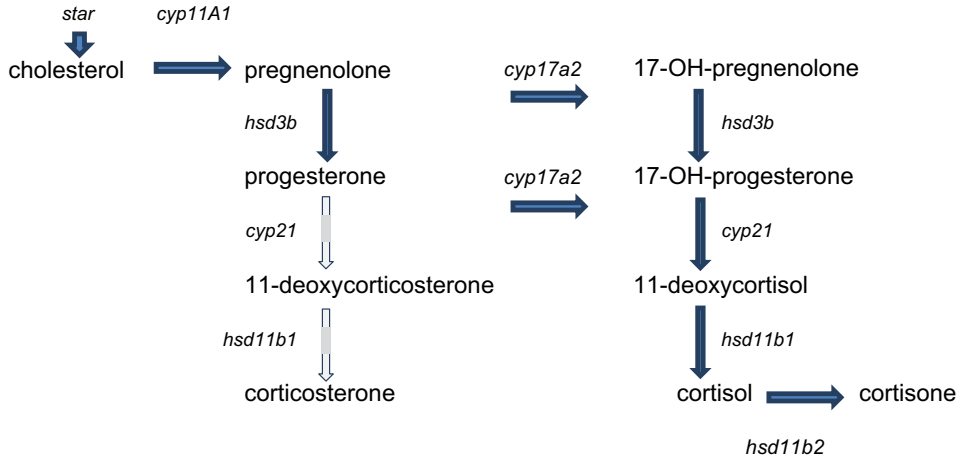

Fig. 1. Schematic representation of genes involved in the synthesis/regulation of cortisol and corticosterone. The genes *star*, *cyp11A1*, *cyp17a2*, *hsd3b*, *cyp21*, *hsd11b1* and *hsd11b2*, respectively encode the proteins, steroidogenic acute regulatory protein (STAR), cytochrome P450 (P45Oscc), steroid 17α-hydroxylase (CYP17a2), 3-beta hydroxy-delta-5-steroid dehydrogenase (HSD3B), steroid 21-hydroxylase (CYP21), 11-beta hydroxysteroid dehydrogenase, type 1 (HSD11B1) and 11-beta hydroxysteroid dehydrogenase, type 2 (HSD11B2). The black arrows indicate that the activity of the enzymes in the pathway has been confirmed in head kidneys of teleost fish. The grey arrows indicate that the activity is not confirmed.