Stress-induced regulation of steroidogenic acute regulatory protein expression in head kidney of Gilthead seabream (*Sparus aurata*)

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Abstract

Steroidogenic acute regulatory protein (StAR) transfers cholesterol over the inner mitochondrial membrane. In mammals, StAR controls this rate-limiting step of steroidogenesis, but its expression and regulation has not been well explored in fish. The present work investigates StAR mRNA expression in the head kidney of the gilthead seabream (*Sparus aurata*) under different stressors. We have cloned the StAR cDNA (1461 bp) in seabream (accession number EF640987), which has an open reading frame of 861 nucleotides encoding a polypeptide of 286 aa, and displays high sequence identity with StAR of other fish and mammalian counterparts. Seabream StAR transcripts were found to be expressed exclusively in head kidneys and gonads. In fish under acute stress (chased with a net), plasma cortisol levels peaked within 1 h, were still high after 6 h, and

decreased after 16 h, although no increases in head kidney StAR expression were observed at any time post-stressor. Fish under chronic high-density stress showed cortisol levels 90-fold higher than controls and StAR mRNA levels increased threefold. Lipopolysaccharide (LPS) injection increased head kidney StAR mRNA levels after 6 h, reached a maximum at 12 h, and decreased until 72 h. When the head kidney cells were incubated *in vitro* and treated with ACTH or LPS, ACTH induced an increase in StAR expression as expected, but LPS induced a reduction in StAR expression. In conclusion, StAR expression in seabream head kidneys is highly regulated by different stressors.

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Introduction

In teleost fish, the endocrine response to stress is composed of the adrenergic response and the hypothalamic–pituitary–interrenal (HPI) response (Wendelaar Bonga 1997). The HPI response leads to an increase in glucocorticoid levels, of which cortisol, produced in the interrenal cells of the head kidney, is the most important. Cortisol is synthesized from the precursor cholesterol (Miller 1998), which is first converted to pregnenolone by P450 side chain cleavage (P450SCC), an enzyme located in the inner mitochondrial membrane. The product of this reaction, pregnenolone, is then acted upon by several steroidogenic enzymes to produce 11-deoxycortisol, which in turn is converted to cortisol by 11 β -hydroxylase. The limiting step of steroidogenesis in mammals is the availability of substrate for P450SCC and the steroidogenic acute regulatory (StAR) protein that plays a crucial role in steroidogenesis by transferring hydrophobic cholesterol across the aqueous barrier between the outer and inner mitochondrial membranes (Clark *et al.* 1994). As described in a number of animal models, the adrenocorticotrophic hormone (ACTH), a pituitary peptide, is

the major regulator of the synthesis of glucocorticoids (cortisol or corticosterone; Carsia *et al.* 1984, Netchitailo *et al.* 1984, Klingbeil 1985, Wendelaar Bonga 1997, Ariyoshi *et al.* 1998, Le Roy *et al.* 2000, Carsia & John-Alder 2003). In mammals, ACTH induces a rapid increase in corticosteroid synthesis and a longer term chronic cyclic AMP (cAMP)/protein kinase A (PKA)-based response that affects both the transcription and translation of several genes encoding for steroidogenic enzymes (reviewed by Sewer & Waterman 2003). In addition, in mammals a very rapid increase in both StAR mRNA (Fleury *et al.* 1996, Lehoux *et al.* 1998, Ivell *et al.* 2000, Le Roy *et al.* 2000) and StAR protein (Nishikawa *et al.* 1996; reviewed by Lehoux *et al.* 2003) occurs in response to ACTH. Moreover, plasma cortisol has been found to mirror levels of StAR mRNA (Ariyoshi *et al.* 1998, Le Roy *et al.* 2000) and StAR protein (Liu *et al.* 1996, Nishikawa *et al.* 1996). Transcription of the StAR gene (Clark *et al.* 1995) and activation of StAR protein (Arakane *et al.* 1996) in response to ACTH are mediated via the PKA pathway. In mammalian systems, therefore, StAR gene transcription (Liu *et al.* 1996), StAR protein levels (Sandhoff *et al.* 1998), and steroidogenesis (Sugawara *et al.* 2000) all increase in response

to cAMP, via activation of the PKA intracellular signaling pathway. Current knowledge about StAR in vertebrates is based on mammalian studies, but the protein structure appears to be highly conserved across species (Bauer *et al.* 2000, Kusakabe *et al.* 2002, Goetz *et al.* 2004). StAR cDNA has been previously cloned from rainbow trout, and several regions of the protein showed a high degree of homology with human StAR (Kusakabe *et al.* 2002), especially in the C-terminal region, which contains hydrophobic sites for cholesterol binding (Tsujishita & Hurley 2000). Concerning fish studies, StAR transcripts have been detected in the steroidogenic tissues of rainbow trout, and the levels of StAR transcripts in the head kidney tissue, containing the steroidogenic interrenal cells, have been shown to increase in response to severe acute stress (Kusakabe *et al.* 2002, Geslin & Auperin 2004) or when ACTH was injected in eels (Li *et al.* 2003), suggesting that StAR is an important regulator of corticosteroidogenesis in fish. Despite these results for several fish species, there is relatively little information on StAR gene expression and regulation in perciform fish. As a result, we cloned, sequenced, and quantified StAR mRNA expression in adult gilthead seabream in basal conditions as well as in different stress situations. Moreover, we measured the StAR response to ACTH in an *in vitro* model of the head kidney interrenal cells. Finally, we have tested the effects of bacterial lipopolysaccharide (LPS) both *in vivo* and *in vitro* to check the response of StAR gene expression to an infectious process.

Materials and Methods

Animals

Sexually immature gilthead seabream (*Sparus aurata*), with an average weight of 80 g, were obtained from a fish farm (Masnou, Barcelona, Spain) and maintained in a semi-closed seawater flow circuit with water at a temperature of 17 °C and a salinity of 37 ‰ under a 12 h light:12 h darkness cycle and a density of 7 kg/m³. Blood was sampled from the caudal vein using heparinized syringes in <1 min/individual and <5 min/treatment group. Plasma was obtained by centrifugation and stored at -80 °C until analysis by RIA. To collect the seabream tissues, adult fish were killed by an excess of anesthesia (2-phenoxyethanol, (1:1000 v/v; Sigma-Aldrich, #P1126)), which is the standard method to kill fish, and the tissues were removed and immediately frozen in liquid nitrogen until analysis.

Cloning and sequencing of gilthead seabream StAR

A cDNA clone with high homology to StAR was identified in a collection of expressed sequence tags obtained from a gilthead seabream cDNA library made in λ ZAP Express III (Stratagene, Cedar Creek, TX, USA) with RNA pooled from several relevant immune tissues. This library was enriched for the sequences up-regulated after immune stimulation, and processed as described by our group (Castellana *et al.* personal communication). Two clones corresponding to the seabream StAR cDNA

were identified, and each one was sequenced a minimum of four times in both directions using BigDye Terminator (Applied Biosystems, Foster City, CA, USA) on an ABI 3730 automated sequencer (Applied Biosystems). The full-length sequence of the StAR cDNA was obtained by overlapping the two sets of partial sequences from each clone. The sequence was confirmed by performing RT-PCR to amplify the open reading frame (ORF).

Sequence analysis

Sequence data were compiled from the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA), and sequences were analyzed for similarity with other known sequences using BLAST within the ExPASy Proteomics Tools server. Alignments were performed using the Bioedit Sequence Alignment Editor (Hall 1999), which uses the CLUSTAL W algorithm (Thompson *et al.* 1994). The assembled full-length cDNA sequence was entered in GenBank with accession number EF640987. The pI and molecular mass of seabream StAR protein were predicted using the ExPASy Proteomics Tools server of the Swiss Institute of Bioinformatics (SIB; <http://www.expasy.ch/tools/>; Gasteiger *et al.* 2003).

Analysis of StAR tissue expression

For tissue expression analysis, total RNA was isolated from a pool of tissues using Tri Reagent (Molecular Research Center Inc., Cincinnati, OH, USA) following the manufacturer's instructions. Total RNA (5 µg) was reverse transcribed with Superscript III reverse transcriptase (Invitrogen SA), according to the manufacturer's protocol. The PCRs were performed using *Taq* DNA polymerase (Biotools, Madrid, Spain) with 2 mM MgCl₂, 800 nM primer final concentration, and 1 µl reverse-transcribed tissue RNA. The cycling conditions were as follows: 94 °C for 5 min followed by 35 cycles of 94 °C for 40 s, 59 °C for 50 s, and 72 °C for 50 s. The final step was an extension at 72 °C for 10 min. The reactions were run on 1% agarose gel and stained with ethidium bromide. Parallel RT-PCRs were carried out with specific primers against a conserved region of 18S (primers 18S forward and 18S reverse) as a control (Table 1).

Experimental procedure

We designed an acute stress, chronic stress, and experimental infection in gilthead seabream. For the acute stress experiment, fish were chased with a net in the tank for 5 min, and sampled 1, 6, and 16 h after the stress (*n*=8 for each time point). Fish were killed by an excess of anesthesia (2-phenoxyethanol), and tissues and blood were obtained as described earlier. Undisturbed animals not subjected to stress were used as a control (*n*=8).

For the chronic stress experiments, fish (*n*=16) were kept for 15 days at a high density (50 kg/m³) in the tank. After that period, fish were killed by an excess of anesthesia, tissues were quickly removed and immediately frozen and blood obtained as indicated above. Undisturbed animals were used as controls (*n*=16).

Table 1 Sequences of primers used in gene expression analysis

	DNA sequence	Direction	Size of amplicon (bp)
Primer			
StAR	5'-AGTAACTGGATCAGCCAAGTCC-3'	Forward	440
StAR	5'-CCACCACATTACCAGGAGTCTCT-3'	Reverse	440
StAR real time	5'-GCTGGATCCCAAGACAATC-3'	Forward	175
StAR real time	5'-CTTGCTCTCCTTCGACTGCT-3'	Reverse	175
r18S	5'-CGAGCAATAACACGTCTGTG-3'	Forward	211
r18S	5'-GGGCAGGGACTTAATCAA-3'	Reverse	211

To simulate stress by infection, a concentration of 8 mg/kg of bacterial LPS was injected intraperitoneally. Fish ($n=5$) were killed by an excess of anesthesia (2-phenoxyethanol) at different times (6, 12, 24, 72 h) and tissues were quickly removed and immediately frozen. Blood was sampled from the caudal vein using heparinized syringes; plasma was obtained by centrifugation and stored at -80°C until analysis by RIA. Animals injected with saline were used as controls ($n=5$).

Preparation of head kidney cells and in vitro assays

Gilthead seabream head kidney cells were isolated and cultured by adapting the protocol previously described for trout macrophages (Mackenzie *et al.* 2003). Head kidneys were dissected from seabream killed by overanesthetization in 2-phenoxyethanol and placed in PBS. Tissues were finely minced with sterile scalpel blades, passed through a pipette to disperse into small aggregates, and filtered through a cell strainer (100 μM , Falcon #352360). The resulting homogenate was allowed to settle via sedimentation on culture plates and kept at 17°C , 5% CO_2 with Dulbecco's Modified Eagle Medium (Life Technologies, #11971-025) containing high glucose, 10% heat-inactivated fetal calf serum (Gibco, #16140-071), and penicillin (100 U/ml)/streptomycin (100 $\mu\text{g/ml}$; Life Technologies).

Following isolation, cells were left undisturbed for 3 h to stabilize the cortisol levels as described previously (Rotllant *et al.* 2001). After this period, the head kidney cells were incubated with medium with or without ACTH (150 ng/ml) for 1, 2, 6, and 18 h. In order to check the specificity of ACTH effects on StAR gene stimulation, in another experiment we incubated the head kidney preparations with ACTH and ACTH plus 1 μM corticotropin-inhibiting peptide (CIP; Sigma-Aldrich, #A1527), an ACTH antagonist that binds to ACTH receptor without activating its signal transduction, and therefore blocking ACTH effects.

In another set of experiments, cells were incubated in medium with or without LPS (10 $\mu\text{g/ml}$) for 1, 2, 6, and 16 h. Each time condition was replicated six times on a six-well culture plate. After incubation, medium was removed, pooled for each condition, and frozen immediately for cortisol determination, and total RNA was isolated with TRI reagent following the manufacturer's instructions and pooled.

Quantitative real-time PCR

The cDNAs from the seabream tissue and head kidney preparations were used for quantitative PCR analysis using SYBR Green PCR Supermix (Bio-Rad). Results were evaluated with the ICYCLER IQ real-time detection system software (Bio-Rad). The sequences of the primers used in gene expression analysis are presented in Table 1. The total volume (20 μl) of every reaction contained 500 nM of each amplification primer, 10 μl 2 $\times$ SYBR Green PCR Mix, and 5 μl of a 1:100 dilution of cDNA (1:1000 for 18S determination). Products were amplified in an ICycler IQ Real Time PCR Detection System (Bio-Rad Laboratories). Controls lacking cDNA and controls containing RNA were included. The real-time analysis consisted of 1 cycle of 95°C for 5 min, 40 cycles of 95°C for 10 s and 60°C for 30 s, 1 cycle of 95°C for 1 min, 1 cycle of 55°C for 1 min, and a melting curve of 81 cycles (from 55°C to 95°C). All samples were run in triplicate and fluorescence was measured at the end of every extension step. Threshold cycle (C_t) values for each sample were expressed as 'fold differences', calculated relative to untreated controls and normalized to endogenous control 18S rRNA.

Cortisol measurements

For cortisol measurements, the samples were kept frozen at -20°C until assays could be performed. Cortisol levels were measured both in plasma samples from adult fish and the culture media of head kidney cells by an RIA method (RIA) according to Rotllant *et al.* (2001). The antibody used for the assay was purchased from Biolink, SL (Costa Mesa, CA, USA) in a final dilution of 1:6000. Antibody cross-reactivity with cortisol is 100%. The radioactivity was quantified using a liquid scintillation counter.

Statistical analysis

For gene expression quantification by real-time PCR on *in vitro* assays, total RNA was pooled from six different wells for each treatment and analyzed in triplicate. Statistical significance was analyzed by one-way ANOVA followed by Tukey's *post hoc* test, using the software package SPSS (Chicago, IL, USA) for Windows. Differences were considered significant when $P<0.05$. All cortisol measurements were performed

using medium of six wells per experiment on *in vitro* experiments and eight animals per condition on *in vivo* assays, and its statistical significance was analyzed by one-way ANOVA followed by Fisher's LSD *post hoc* test. Differences were considered significant when $P < 0.05$.

Results

StAR cloning and sequencing

A gilthead seabream (*S. aurata*) cDNA library made in λ ZAP Express III (Stratagene) from several relevant immune sources was used to clone the steroidogenic acute regulatory protein, StAR (GenBank accession number EF640987). The 1461 bp StAR full cDNA contains a 123 bp 5' UTR, an 861 bp ORF and a 477 bp 3' UTR.

Deduced amino acid sequence analysis

The *S. aurata* StAR gene encodes a 286 amino acid protein, which has a predicted molecular mass of 32 kDa and a pI of 9. Amino acid analysis was performed to determine the relatedness of gilthead seabream StAR to StARs from other fish (Fig. 1). At the amino acid level, seabream StAR shows the highest sequence identity to StAR protein from black bass (94%), whereas identity with rainbow trout (87%) is lower. On the other hand, seabream StAR shows about 69% identity with other vertebrate StARs, such as birds and amphibians, and a 66% sequence identity with human StAR.

StAR expression and regulation by stress

Primer sequences used in the PCR studies are shown in Table 1. Using RT-PCR, we found the StAR gene to be expressed in head kidneys and gonads. The basal expression of StAR was higher in head kidney than in gonads, and the rest of tissues showed no StAR gene expression (Fig. 2). In addition, we found no expression in muscle samples (data not shown).

Under acute stress (5 min chase with a net in the tank), plasma cortisol reached a maximum peak 1 h post-stressor, and levels were about 160 times higher than those in control, undisturbed animals (229.64 ± 43.18 nM versus 1.36 ± 0.64 nM respectively). Cortisol remained at very high levels 6 h after the acute stress (262.86 ± 105.31 nM) and after 16 h they had largely decreased, although they were still significantly higher than controls (27.48 ± 7.26 nM). We found no increase in StAR levels (measured by real-time quantitative PCR) after 1, 6, or 16 h of acute stress (Fig. 3).

Under chronic stress (15 days in overcrowded conditions), we found that stressed fish showed a significant increase (about 90 times) in plasma cortisol levels compared with control animals (129.33 ± 24.66 nM versus 1.48 ± 0.72 nM respectively). StAR mRNA levels in head kidneys from stressed animals were significantly higher (about 2.5-fold) than those of undisturbed animals (Fig. 4).

In fish injected with bacterial LPS at a dose of 8 mg/kg to simulate an acute stress by infection, real-time quantitative PCR showed that StAR mRNA levels in head kidneys were slightly increased 6 h after the injection (1.36-fold), and reached maximum levels at 12 h (4.46-fold). After that, StAR mRNA levels decreased progressively, although after 72 h, they were still clearly higher than the levels of saline-injected animals (3.31-fold; Fig. 5). Plasma cortisol levels followed the same pattern already described by our group in control and LPS-injected seabream (Acerete *et al.* 2007), peaking at 6 h post-injection and decreasing progressively with time (at 12 and 24 h) to the levels of control animals after 72 h.

StAR expression and regulation on *in vitro* head kidney preparations

When the seabream head kidney cells were incubated with 150 ng/ml ACTH, cortisol levels in the incubation media dramatically rose after 1 h (5553% of control). After 6 h, cortisol levels were lower, although still higher than control levels (628% of control; Fig. 6B). The StAR mRNA levels measured by quantitative real-time PCR increased in ACTH-stimulated cells at 1 h (about threefold the control levels). However, after 6 h of incubation, StAR mRNA levels were lower compared with controls (Fig. 6A).

In addition, CIP, the ACTH receptor antagonist, in a concentration of 1 μ M, blocked effectively the ACTH-induced stimulation of StAR gene expression when co-incubated with ACTH, but it did not affect the StAR basal expression in the head kidney preparations when incubated alone (Fig. 7).

On the other hand, when the head kidney preparations were incubated with bacterial LPS (10 μ g/ml), cortisol levels in the incubation media decreased at all times tested; after 1 h of incubation, cortisol levels decreased to 20.37% of control levels in LPS-incubated cells, and a similar situation was found after 16 h (reduction to 48.49% of control; Fig. 8B). The expression levels of StAR decreased although not significantly after 1 h of incubation, whereas after 16 h there was a significant reduction compared with controls (79% reduction; Fig. 8A).

Discussion

In this study, the complete sequence of a StAR cDNA (with a length of 1461 bp) has been obtained from a seabream cDNA library made from several relevant immune tissues. The *S. aurata* StAR protein is composed of 286 amino acid residues, a size that is identical or similar in length to those of teleosts and mammals identified so far. The seabream peptide displays a high identity to that of other fish and mammalian StAR proteins. When compared with the StAR protein of other vertebrate species, the putative phosphorylation sites by PKA (RRSS; reviewed by Strauss *et al.* 1999) are conserved in the seabream StAR protein. In the N-terminal 25 residues, the seabream StAR protein displays 100% identity to that of

Seabream	MLPATFKLCAGISYRHRNMNTGLRKNAMVAIHHELNRLA--GPSPSNWIS	48
Rainbow trout	MLPATFKLCAGISYRHRNMNTGLRKNAMVAIHHELNMILA--GPNPSSWIS	48
Atlantic Cod	MLPATFKLCAGISYRHRNMNTGLRKNALAAIHHELNRRA--GPSSGNWIN	48
Japanese eel	MLPATFKLCAGISYRHRNMNTGLRKTAMVAINHLSRLS--GPGSGWIS	48
Zebrafish	MLPATFKLCAGISYRHRNMNTGLRKNAMIAIHHELNKLK--GPGASTWIN	48
Black bass	MLPATFKLCAGISYRHRNMNTGLRKNAMVAIHHELNRLA--GPGPSNWIN	48
Chicken	MLPATFKLCAISYQHLRNVTGLRRQAATAISQELSKLSRSTGPSTWIS	50
Rat	MLLATFKLCAGSSYRHRNMKGLRHQAVLAIGQELNRRALGDPSPG-WMG	49
Human	MLLATFKLCAGSSYRHRNMKGLRQQAATAISQELNRRALGGPTPSTWIN	50
Seabream	QVRRRSSLLSSRIREVEGYSEAEMSYVKQGEDALQKAISILSEQDGWTIE	98
Rainbow trout	HVRRRSSLLSSRIEEEGYNEAEVSYVKQGEALQKSIISILGDQDGWTTE	98
Atlantic Cod	QVRRRSSFLSSRIEEAEEYSPEEVTVVRQGESALQEAISILGEQEGWTV	98
Japanese eel	QVRRRSSLLSSRIEEK-AFSETDMSYIKQGEALQKSIISILSDQDGWQTE	97
Zebrafish	HIRRRSSLLSSPIAEET-YSEADQCYVQQQGEALQKSIISILEDQDGWQTE	97
Black bass	QVRRRSSLLSSRIEEEGYSDEEMSYVKQGEDALQKAISILSDQDGWTTE	98
Chicken	QVRRRSSLLSSRLEEK-PFSEMEMSYIKQGEALQKSIISILGDQEGWKTE	99
Rat	QVRRRSSLLGSQLEATL-YSDQELSYIQQGEAMQKALGILNNQEGWKKE	98
Human	QVRRRSSLLGSRLLEETL-YSDQELAYLQQGEAMQKALGILSNQEGWKKE	99
Seabream	TVAANGDKVLSKTLDPDVGKVFKEVLMLEQHPDNLVEELVGNMEQMGDWNP	148
Rainbow trout	IIAANGDKVLSKVLDPVGKVFKEVLLDQSRDNLVVELVGNMEQMGDWNP	148
Atlantic Cod	TVASNGDRVLSKVVAGVGKVFKEVVVDQPLETLYGELVGNMEQMGDWNP	148
Japanese eel	IIVAANGDKVLSKVLDPDGKVFKEVLLDQRPDNLVYELVDNMEQMGEWNP	148
Zebrafish	IESINGEKVMSKVLPGIGKVFKEVLTLEQQTGDLDELVDNMEQMGEWNP	147
Black bass	IIVAANGDKVLSKMLPDIGKVFKEVLMLEQRPDNLVYELVDNMEQMGEWNP	147
Chicken	TVADNGDKVLSKVLDPVGKVFRLVVDQPLDAVSYELVDNMEQMGDWNP	149
Rat	SQQENGDEVLSKVVPVGKVFRLVLLDQPMRLYEELVDRMEAMGEWNP	148
Human	SQQDNGDKVMSKVVPDVGKVFRLVVDQPMERLYEELVERMEAMGEWNP	149
Seabream	NVKQVKILQKIGQDTMVTHEVSAETPGNVGPRDFVSVRCAKRRGSTCFL	198
Rainbow trout	NVKEVKILQKIGQETMVTHEVSGPTPGNVGPRDFVSVRCAKRRGSTCFL	198
Atlantic Cod	NVKQVKILQKIGADTMVTHEVSGDTPGNVGPDPDFVSVRCAKRRGSTCFL	198
Japanese eel	NVKQIKILQKIGQDTMVTHEMAAETPGNVGPRDFVSVRCAKRRGSTCFL	197
Zebrafish	NVKQVKILQKIGQETMITHEISAETPGNVGPRDFVSVRCAKRRGSTCFL	197
Black bass	NVKQVKILQKIGQDTMVTHEVSAETPGNVGPRDFVSVRCAKRRGSTCFL	197
Chicken	SVKEVKILQKIGQDTLITHTAAAPPNGIVGPRDFVSVRCSRRRGSTCFL	199
Rat	NVKEIKVLKIKGKDTVITHELAAAAGNLVGPDPDFVSVRCKRRGSTCFL	198
Human	NVKEIKVLQKIGKDTFITHELAAAAGNLVGPDPDFVSVRCAKRRGSTCFL	199
	* *	
Seabream	AGMSTQHPKMPEQKGVVRAENGPTCIVMKPCAEDPNKTKFTWLLNIDLKG	248
Rainbow trout	AGMSTQHPMTPEQKGVVRAENGPTCIVMRPSADDPNKTFTWLLSIDLKG	248
Atlantic Cod	AGKSTQHPGMPEQKGVVRAENGPTCIVLKPDAADPNRTKFTWLLSIDLKG	248
Japanese eel	AGMSTHYAGMPEQKGVVRAENGPTCIVMRPSAEDPDKTFTWLLSIDLKG	248
Zebrafish	AGMSTQHPGMPEQKGVVRAENGPTCIVMRPSADDPNKTFTWLLSIDLKG	247
Black bass	AGMSTQHPKMPEQKGVVRAENGPTCIVMKPCVEDPNKTKFTWLLNIDLKG	247
Chicken	AGMSTTHGAMPEQQGFIRAENGPTCMVLRPLAGSPSKTKLTWLLSIDLKG	249
Rat	AGMATHFGEMPEQSGVIRAENGPTCMVLRPLAGSPSKTKLTWLLSIDLKG	248
Human	AGMDTDFGNMPEQKGVIRAENGPTCMVLRPLAGSPSKTKLTWLLSIDLKG	249
	* ** *	
Seabream	WIPKTIINRVLSQTQVDFANHLRQRMANNVSM-EMAHAC	286
Rainbow trout	WIPKTIINKVLSQTQVDFANHLRQRMADNSVSMEMAAAC	287
Atlantic Cod	WIPKTIINRVLSQTQVDFANHLRQRMASN-VSLEMAPAC	286
Japanese eel	WIPKTIINRVLSQTQVDFAQHLRQMAAG-YSMEAAQAC	285
Zebrafish	WIPKTVINRVLSQTQVDFVNHRLDRMASGG-GIDAAIAC	286
Black bass	WIPKTIINKVLSQTQVDFANHLRQRMANN-VSMEMAHAC	286
Chicken	WLPKTIINQVLSQTQVDFAKHLRQRMARAAGC-----	281
Rat	WLPKTIINQVLSQTQIEFASHLRKRLESPAS-E-AQC-	284
Human	WLPKSIINQVLSQTQVDFANHLRKRLESPAS-E-ARC-	285
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Figure 1 Alignment of the deduced amino acid sequence of seabream StAR with those of other vertebrates analyzed by Clustal W multiple alignment. The sequences were obtained from the GenBank database: gilthead seabream (EF640987), rainbow trout (BAB18779), Atlantic cod (AAP44111), Japanese eel (BAC66210), zebrafish (AAH75967), black bass (AAZ92554), chicken (AAZ92554), rat (AAH88859), and human (AAB88174). Amino acid numbers are shown on the right. Amino acid residues that are identical in all StAR proteins are shown with gray background. Gaps in the amino acid sequences are indicated with a dash (-). The N-terminal 25 residues are indicated by a solid horizontal line. Two potential sites of protein kinase A-mediated serine phosphorylation, which may be important to the regulation of StAR activity, are boxed (Arakane *et al.* 1997). Amino acid residues that disrupt StAR function when mutated are indicated by asterisks (Stocco 2002). The single letter code is used for the amino acids.

Figure 2 Pattern of tissue expression of StAR in gilthead seabream. Tissues from adult seabream were analyzed by RT-PCR as described in the text for StAR gene expression. H, heart; B, brain; L, liver; G, gills; Go, gonads; HK, head kidney; S, spleen. 18S was used to show equivalency in the amount of cDNA of the sample. Results are representative of three independent experiments.

trout, 80% to chicken, and 84% to rat and human. This domain is composed of basic and hydrophobic amino acids characteristic of mitochondrial targeting sequences (Kallen *et al.* 1998). On the other hand, in the C-terminal domain we also found conservation of amino acid residues believed to be important for the steroidogenic function of StAR (Watari *et al.* 1997) and those contributing to the hydrophobic tunnel structure for lipid transport (Tsujishita & Hurley 2000), as well as the amino acid residues critical to StAR function and regulation (e.g., the serine phosphorylation motif at Ser195). This indicates that the primary structure of the StAR protein is highly conserved throughout vertebrates.

We have found StAR mRNA to be expressed in the head kidney and gonads. This finding is in agreement with previous data in several fish species where StAR is mainly expressed in the steroidogenic tissues (Bauer *et al.* 2000, Li *et al.* 2003, Goetz *et al.* 2004), although it is in contraposition to the results obtained in other species, where StAR mRNA expression has been described in extragonadal and extra-adrenocortical tissues, such as the brain and heart of the freshwater stingray (Nunez *et al.* 2005); the kidney of Atlantic croacker (Nunez & Evans 2007); the intestine, pyloric ceca, spleen, and kidney of trout (Kusakabe *et al.* 2002); human fetal and adult kidney (Sugawara *et al.* 1995); and several areas of the human brain (King *et al.* 2002). Therefore, StAR tissue distribution can be slightly different according to the species, and its role in these other tissues remains to be clarified.

Figure 3 The effects of acute stress on StAR expression in *Sparus aurata*. Fish were subjected to an acute stress as described in the text, and head kidneys were removed after 1, 6, and 16 h. Control fish remained undisturbed. StAR levels in head kidneys were analyzed by quantitative real-time PCR and normalized to 18S rRNA. Cortisol levels in plasma were also analyzed. *n* = 8 for each condition. Different letters indicate significant differences (*P* < 0.05).

Figure 4 Effects of chronic stress on StAR expression in *Sparus aurata*. Fish were maintained in a high-density confinement (50 kg/m³) for 15 days. StAR levels in head kidneys were analyzed by quantitative real-time PCR and normalized to 18S rRNA. Cortisol levels in plasma were also analyzed. *n* = 16 for each condition. Different letters indicate significant differences (*P* < 0.05).

We applied two different types of stressors (acute stress caused by chasing the fish with a net and a chronic stress by overcrowding) that caused a significant increase in the plasma cortisol levels of stressed fish compared with undisturbed fish. In the case of the acute stress, this increase was rapid and fish reached the highest cortisol levels between 1 and 6 h. After that period, cortisol levels decreased, although they were still higher than controls 16 h after the stressor. This kinetic profile is in accordance with the previously published data on salmonid fish (Pickering *et al.* 1982, Patino *et al.* 1987, Geslin & Auperin 2004) and white sucker (Bandeian & Leatherland 1997). StAR levels in our experiment did not increase significantly at any time post-stressor, a result also obtained by Geslin & Auperin (2004) in rainbow trout, who found no increase in StAR expression in the head kidney of fish chased with a net for 5 min. By contrast, these authors found that a stronger acute stress induced by anesthesia provoked a higher increase (30-fold) of cortisol levels in plasma and an increase in StAR mRNA levels in head kidney, a finding that led the authors to suggest that high levels of cortisol

Figure 5 Effects of LPS injection on head kidney StAR expression. A dose of 8 mg/kg LPS was injected as described in the text, and StAR expression levels in head kidneys were analyzed by quantitative real-time PCR. Expression levels of StAR were normalized to 18S rRNA. Different letters indicate significant differences (*P* < 0.05).

Figure 6 *In vitro* effects of ACTH on StAR expression in head kidney. Cell suspensions were performed as described in the Materials and Methods section and incubated with 150 ng/ml ACTH. StAR mRNA levels were analyzed by quantitative real-time PCR and normalized to 18S rRNA (A). Cortisol levels of culture medium in response to ACTH are also represented (B). Different letters indicate significant differences ($P < 0.05$).

after stress require an activation of StAR gene, although lower levels do not. However, in the present experiment, plasma cortisol levels in stressed fish increased 160-fold, which is a very high value (about five times higher than in trout; Geslin & Auperin 2004), yet the StAR mRNA levels did not increase significantly over controls. This suggests that, although seabream is highly responsive to acute stress (as shown by cortisol levels), the StAR expression in head kidney could be regulated differently and the rise in plasma cortisol levels may be due to a different mechanism rather than increased gene transcription. For example, it is well known in mammals that StAR protein could be regulated post-transcriptionally (Fleury *et al.* 1998), translationally (reviewed by Stocco 1999), and/or post-translationally via serine phosphorylation by a PKA, which also results in the activation of StAR protein (Lehoux *et al.* 1998, Miller & Strauss 1999, Artemenko *et al.* 2001).

When we exposed seabream to high density for 2 weeks, the cortisol levels in the plasma increased dramatically (threefold), a result that agrees with those obtained by other authors for many other fish species, since a prolonged elevation of plasma cortisol levels is frequently used as a marker for exposure to a chronic stressor, with the magnitude of the cortisol response generally reflecting the severity of the stressor (reviewed by Wendelaar Bonga 1997). In addition, we found that StAR mRNA levels in the head kidney of chronically stressed animals were increased threefold over controls. Interestingly, when we analyzed StAR gene expression on an individual basis, we found

Figure 7 CIP (1 μ M) effectively blocks the ACTH-stimulated increase in StAR expression without affecting the basal levels in head kidney *in vitro* preparations. Cell suspensions were performed as described in the Materials and Methods section and incubated with 150 ng/ml ACTH, ACTH plus 1 μ M CIP, or 1 μ M CIP alone. StAR mRNA levels were analyzed by RT-PCR (A) and quantitative real-time PCR (B) and normalized to 18S rRNA. Different letters indicate significant differences ($P < 0.05$).

Figure 8 *In vitro* effects of LPS on StAR expression in head kidney. Cell suspensions were performed as described in the Materials and Methods section and incubated with 10 μ g/ml LPS. StAR mRNA levels were analyzed by quantitative real-time PCR and normalized to 18S rRNA (A). Cortisol levels of culture medium in response to LPS are also represented (B). Different letters indicate significant differences ($P < 0.05$).

that some of them had much higher StAR mRNA levels and cortisol plasma levels than others (data not shown), which may correlate with the already demonstrated fact that socially subordinated individuals in small groups of fish show higher circulating cortisol levels, and therefore more stress than others (Winberg & Lepage 1998, Hoglund *et al.* 2000). This may be an interesting implication of StAR as a marker of fish behavior and stress. Further studies will be undertaken in this area.

We have been able to demonstrate strong *in vitro* effects of ACTH on mRNA levels of StAR in seabream head kidney. In the time-course experiment with seabream head kidney preparations, we found an important increase in StAR mRNA levels 1 h after treatment with 150 ng/ml ACTH, and no effect thereafter. As expected, cortisol levels were also elevated after 1 h and returned to normal levels after 18 h of incubation with ACTH (data not shown). ACTH stimulated the StAR gene expression in the seabream head kidney preparations by binding to its specific membrane receptors, as demonstrated when 1 µM CIP, an antagonist of the ACTH receptor (Li *et al.* 1978), effectively blocked the ACTH-stimulated StAR expression in our *in vitro* model, without affecting the basal levels of StAR expression. The rapid increase in cortisol levels, which we observed after ACTH treatment, is in agreement with several previous studies conducted under various experimental conditions (Donaldson 1981, Nichols & Weisbart 1984, Girard *et al.* 1998, Pottinger & Carrick 2001, Hagen *et al.* 2006). It also agrees with the previous data found in the same species, *S. aurata*, in our laboratory (Rotllant *et al.* 2001, Ganga *et al.* 2006) showing that ACTH caused a rapid increase in cortisol levels in superfused head kidney tissue. In addition, our results on StAR expression correlate with data from mammals showing that acute *in vivo* or *in vitro* treatment with ACTH leads to a rapid and significant increase (between 1 and 3 h after the treatment) in StAR mRNA in the adrenals (Ariyoshi *et al.* 1998, Fleury *et al.* 1998, Lehoux *et al.* 1998, Ivell *et al.* 2000, Le Roy *et al.* 2000). The results are also similar to other fish species, such as eel, where Li *et al.* (2003) found that ACTH injection increased StAR mRNA levels 1.5 h post-injection, and in trout, where Aluru *et al.* (2005) reported a 170% increase in interrenal StAR transcripts in response to 0.5 IU/ml ACTH after 3 h of incubation. Interestingly, recently, Hagen *et al.* (2006) found no rapid stimulation of StAR mRNA levels in the rainbow trout head kidney interrenal cells. They reported only a late effect after 18 h, although they used the same ACTH concentration (150 ng/ml) which they found produced the strongest peak in cortisol production.

LPS injection is the most frequently used acute stress model to mimic the fish response to an immune stressor (Balm *et al.* 1995, Balm 1997, Mackenzie *et al.* 2005). When we injected seabream with 8 mg/kg i.p. of bacterial endotoxin to induce infection, it provoked a marked rise in plasma cortisol levels similar to those previously described (Balm *et al.* 1995, Holland *et al.* 2002) and to the results previously reported (Acerete *et al.* 2007). This produced a cortisol peak after 6 h (threefold the controls) and a progressive decrease in cortisol until 72 h, which are similar to the results reported in rats (Grinevich *et al.* 2001) where a single

LPS injection stimulated plasma cortisol levels. LPS injection also increased StAR gene expression in our *in vivo* seabream head kidney model and, together, these results confirm the previously described important role of immunoendocrine interactions in the head kidney of fish (Schreck & Bradford 1990, Weyts *et al.* 1999).

In contrast with the stimulatory effects of LPS *in vivo*, we found inhibitory effects of LPS (10 µg/ml) on *in vitro* cortisol production in a head kidney preparation. This is in agreement with the previous studies where LPS was not able to increase cortisol production in head kidneys (Balm *et al.* 1995). These authors reported that LPS (50 µg/ml) decreased the ACTH-stimulated cortisol production without being toxic to the head kidney preparations, an inhibitory effect that we observed as well. In addition, LPS incubation caused a decrease in StAR mRNA levels in the head kidney preparations, coincident with the reduction in cortisol levels that we observed. Therefore, it appears that the effects of LPS treatment may differ significantly from *in vitro* or *in vivo* treatments. Whereas *in vivo*, an experimental infection invokes a systemic reaction in which not only immune responses are generated but also neural and endocrine signals, both from pituitary sources and interrenal tissue; LPS administered *in vitro* may interfere with the local cortisol production or secretion mechanisms. One may speculate that *in vivo* either pituitary signals prevail in the interrenal tissue in the presence of the LPS or that circulating immune signals such as cytokines resulting from an experimental infection do not significantly affect the function of the HPI axis. Therefore, more work is in progress regarding these effects.

In conclusion, we have isolated a cDNA from seabream that encodes a protein very similar in form and function to StAR in other vertebrates. It is expressed in head kidney and gonads, and while fish under acute stress showed increased cortisol levels but no changes in StAR mRNA expression, a chronic stress provoked a marked increase in StAR expression and cortisol levels. Moreover, ACTH caused a rapid increase in cortisol and StAR mRNA levels in the head kidney cells *in vitro*, whereas LPS infection stimulated StAR expression *in vivo* but was inhibitory *in vitro*. All these data together show that seabream StAR expression and cortisol production in interrenal cells can be highly affected by different stress conditions (acute/chronic as well as stress by infection) and provides new data on the important relationship between the immune and endocrine systems in fish.

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