General and Comparative Endocrinology 274 (2019) 87–96

Contents lists available at ScienceDirect

General and Comparative Endocrinology

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

Molecular identification, tissue distribution and functional analysis of somatostatin receptors (SSTRs) in red-spotted grouper (*Epinephelus akaara*)

Yazhou Zhang, Zimu Tang, Weiru Lin, Xi Yuan, Jirong Jia, Caiyun Sun, Wensheng Li*

State Key Laboratory of Biocontrol, Institute of Aquatic Economic Animals and Guangdong Province Key Laboratory for Aquatic Economic Animals, Guangdong Provincial Engineering Technology Research Center for Healthy Breeding of Important Economic Fish, School of Life Sciences, Sun Yat-Sen University, Guangzhou 510275, China

ARTICLE INFO

Keywords:
Somatostatin
SSTR
Red-spotted grouper
Signaling pathways

ABSTRACT

In the present study, four full-length cDNAs of somatostatin receptor (*sstr*) were cloned from the forebrain and pituitary of red-spotted grouper. The four full-length cDNAs were designated 2292, 1522, 1873 and 1789 bp and identified as *sstr1*, *sstr2*, *sstr3*, and *sstr5* by BLAST analysis; the corresponding sizes of the open reading frames (ORFs) were 1155, 1113, 1467 and 1503 bp, which encoding 384, 370, 488 and 500 aa, respectively. The four receptors have seven transmembrane structures and contain the YANSCANPI/VLY sequence, which is the conserved amino acid sequence of the SSTR family. A tissue distribution study showed that the four *sstrs* had different expression patterns, suggesting that they may play different roles in regulating different physiological processes. The four receptors mediate ERK1/2 phosphorylation by SS-14 in HEK293 cells, and SS-14 promotes ATK and ERK1/2 phosphorylation in primary hepatocytes of red-spotted grouper. These results facilitate the study of SSTRs-mediated intracellular signaling pathways.

1. Introduction

Somatostatin (SS) was originally isolated from the sheep's hypothalamus in 1973 (Brazeau et al., 1973). As the study progressed, SS and its receptors were identified in numerous vertebrates, and the entire central nervous system and most peripheral tissues can secrete somatostatin. Only one type of preprosomatostatin (*pss*) was identified in mammals, whereas three types of *pss* cDNAs, named *pssI*, *pssII*, and *pssIII*, were identified in fish (Lin et al., 1999; Feng et al., 2015). Depending on the species, PSSs can be cleaved into different mature peptides, such as SS-14, SS-25, SS-26 and SS-28 (Klein and Sheridan, 2008). To date, studies have shown that the primary structure of SS-14 is highly conserved among different species, suggesting the importance of the structure in vertebrates (Nelson and Sheridan, 2005). In addition, SSTRs were identified as the seven-transmembrane G-protein-coupled receptor superfamily. Five *sstr* subtypes (*sstr1-5*) have been identified in mammals, whereas four *sstr* subtypes (*sstr1*, *sstr2*, *sstr3*, and *sstr5*) were identified in fish (Haiyan et al., 2010; Kumar and Grant, 2010).

The SS system participates in various physiological functions and plays important regulatory roles in both endocrine and exocrine systems. By binding to different receptors, SS inhibits the secretion or production of various hormones, proliferation factors and physiologically active substances (Lahlou et al., 2004; Csaba et al., 2012), such as thyrotropin, prolactin, growth hormone, insulin, glucagon, gastrin, secretin and peptide YY (Ramírez et al., 1997; Chisholm and Greenberg, 2002; Rutter, 2009). In addition, SS plays very important roles in gastrointestinal motility, secretion of digestive juices and regulation of glucose and lipid metabolism (Patel, 1999; Piqueras and Martínez, 2004). In short, SS can be used as a neurohormone, neurotransmitter, neuroregulator or local factor to exert various physiological functions in an autocrine or paracrine manner.

Red-spotted grouper is an economically important marine fish that is widely distributed among the western North Pacific and the southern part of the East China Sea. Given its high economic value and consumer demand, this fish has become an emerging industry in aquaculture (Jiang et al., 2015; Wang et al., 2016a,b). However, high protein requirements for feed and a slow growth rate limit the development of the industry. As SS is the main negative regulator of growth, identification of SSTRs are essential to understand the mode of action of SS Further studies on SS system will provide insight into the physiological significance of SS in teleost.

Abbreviations: SS, somatostatin; SSTR, somatostatin receptor; ORF, open reading frame; ERK, extracellular signal-regulated kinase; AKT, protein kinase B; PSS, preprosomatostatin; RACE, rapid amplification of cdna end; PKC, protein kinase C; JNK, Jun N-terminnal kinase

* Corresponding author at: School of Life Sciences, Sun Yat-Sen University, No. 135 Xingang West Road, Guangzhou 510275, China.
E-mail address: lsslws@mail.sysu.edu.cn (W. Li).

https://doi.org/10.1016/j.ygcen.2019.01.007
Received 18 November 2018; Received in revised form 5 January 2019; Accepted 10 January 2019
Available online 14 January 2019

Fig. 1. The homology analysis of the four SSTRs of red-spotted grouper. Deduced amino acid sequence multiple alignments: the conservative amino acid sequence were marked by stars, and the transmembrane regions were shaded with gray.

2. Materials methods

2.1. Animals

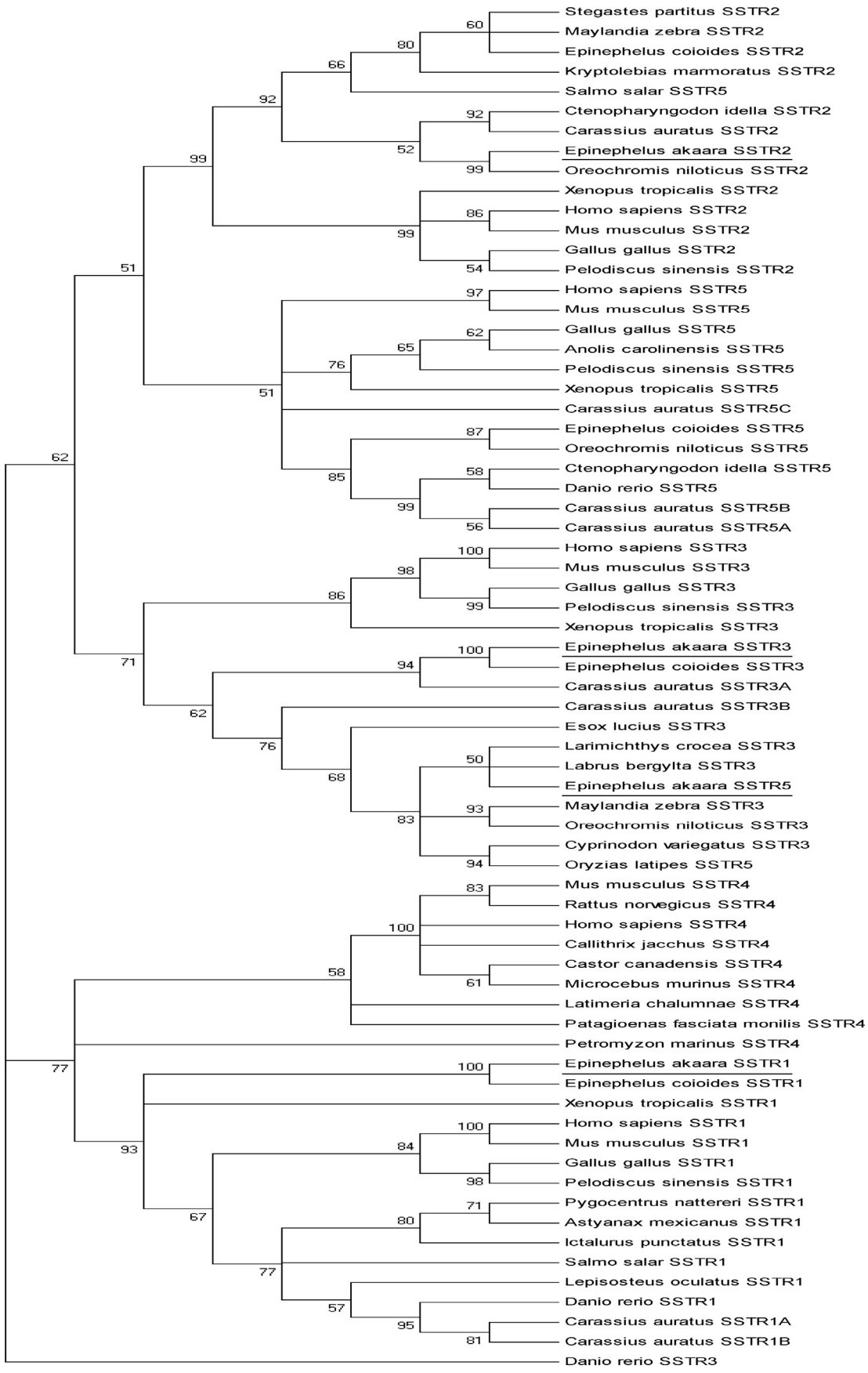
Red-spotted grouper (*Epinephelus akaara*) is a species of the Actinopterygii, Perciformes, Serranidae, *Epinephelus*, and obtained from the Huangsha Aquatic Products Wholesale Market in Guangzhou, China. Mature female fish with a body weight of 450–550 g were used for cloning, tissues distribution studies and *in vitro* experiments. All fish were domesticated to indoor tanks with circular seawater for one week under a cyclic light-dark period (12 h:12 h). During this time, the fish were fed once daily. Fish were anesthetized with MS222 and decapitated. All the samples were stored at −80 °C before total RNA extraction. The Animal Care Committee of Sun Yat-Sen University approved all of the animal experiments.

2.2. Preparation of cloning cDNA templates

Total RNA was extracted using TRIzol reagent (Invitrogen, USA) from tissues. First stand cDNA templates for cloning *sstrs* from red-spotted grouper forebrain and pituitary were synthesized using the M-MLV kit (Thermo Fisher Scientific, US). Oligo (dT) and AUAP primers were used as the reverse transcription of the templates for cloning fragment cDNAs and 3′-RACE, respectively. The first strand cDNA templates were gel-separated and purified. Poly C was added to the 3′-end to generate the cloning template for 5′RACE.

2.3. Molecular cloning and sequence analysis of sstrs

Specific primers were designed based on the conserved sequence of the vertebrate SSTR family and the red-spotted grouper transcriptome, and 3/5′ RACE primers were designed based on partial fragment sequences (Supplemental Table 1). We used ORF Finder in NCBI (http://www.ncbi.nlm.nih.gov/grof/orfig.cgi) to predict the ORF. To confirm



(caption on next page)

Fig. 2. Phylogenetic tree based on amino acid sequence alignment for SSTRs in vertebrates. It was constructed by MEGA5.0 using neighbor-joining method. *Epinephelus akaara* SSTR1: MK153162; *Epinephelus akaara* SSTR2: MK153163; *Epinephelus akaara* SSTR3: MK153164; *Epinephelus akaara* SSTR5: MK153165; *Homo sapiens* SSTR1: NM_001049; *Homo sapiens* SSTR2: AAF42809; *Homo sapiens* SSTR3: AAA60592; *Homo sapiens* SSTR4: NM_001052.2; *Homo sapiens* SSTR5: BAA04107; *Mus musculus* SSTR1: AAA58255; *Mus musculus* SSTR2: AAA58256; *Mus musculus* SSTR3: AAA40144; *Mus musculus* SSTR4: NM_009219.3; *Mus musculus* SSTR5: AAC53353; *Gallus gallus* SSTR1: NP_001106638; *Gallus gallus* SSTR2: NM_001030345; *Gallus gallus* SSTR3: NM_001024583; *Gallus gallus* SSTR5: NM_001024834; *Pelodiscus sinensis* SSTR1: XP_006117770.1; *Pelodiscus sinensis* SSTR2: XP_006119128.1; *Pelodiscus sinensis* SSTR3: XP_006118974.1; *Pelodiscus sinensis* SSTR5: XP_006115585.1; *Xenopus tropicalis* SSTR1: XP_004917297.1; *Xenopus tropicalis* SSTR2: XP_004916398.1; *Xenopus tropicalis* SSTR3: XP_004913945.1; *Xenopus tropicalis* SSTR5: XP_002932329.2; *Carassius auratus* SSTR1A: AAF08613.1; *Carassius auratus* SSTR1B: AAF08614.1; *Carassius auratus* SSTR2: AAF98367.1; *Carassius auratus* SSTR3A: AAM82355.1; *Carassius auratus* SSTR3B: XP_026094152.1; *Carassius auratus* SSTR5A: AAM18805.1; *Carassius auratus* SSTR5B: AAN76495.1; *Carassius auratus* SSTR5C: AAP68899.1; *Epinephelus coioides* SSTR1: ACR19335.1; *Epinephelus coioides* SSTR2: ACI12981.1; *Epinephelus coioides* SSTR3: ACI12982.1; *Epinephelus coioides* SSTR5: ACI12983.1; *Danio rerio* SSTR1: XP_696666.1; *Danio rerio* SSTR3: XP_021321995.1; *Danio rerio* SSTR5: XP_688757.1; *Oreochromis niloticus* SSTR2: XP_003438730.1; *Oreochromis niloticus* SSTR3: XP_019215874.1; *Oreochromis niloticus* SSTR5: XP_003452896.1; *Salmo salar* SSTR1: XP014050052.1; *Salmo salar* SSTR5: XP014013880.1; *Ctenopharyngodon idella* SSTR2: ACB69421; *Ctenopharyngodon idella* SSTR5: ACB69422; *Stegastes partitus* SSTR2: XP_008289295.1; *Maylandia zebra* SSTR2: XP_004559458.1; *Maylandia zebra* SSTR3: XP_014266869.2; *Kryptolebias marmoratus* SSTR2: XP_017271719.1; *Esox Lucius* SSTR3: XP_010863618.1; *Larimichthys crocea* SSTR3: XP_019109341.1; *Labrus bergylta* SSTR3: XP_020499706.1; *Cyprinodon variegatus* SSTR3: XP_015229492.1; *Pygocentrus nattereri* SSTR1: XP_017555306.1; *Astyanax mexicanus* SSTR1: XP_007251951.1; *Ictalurus punctatus* SSTR1: AHH37375.1; *Lepisosteus oculatus* SSTR1: XP_006632214.1; *Anolis carolinensis* SSTR4 XP_003224807.1; *Oryzias latipes* SSTR5: XP_004071203.1; *Rattus norvegicus* SSTR4: EDL95104.1; *Callithrix jacchus* SSTR4: XP_002747551.3; *Castor Canadensis* SSTR4: XP_020034060.1; *Microcebus murinus* SSTR4: XP_012594662.1; *Latimeria chalumnae* SSTR4: XP_012594662.1; *Patagionias fasciata monilis* SSTR4: OPJ58974.1; *Petromyzon marinus* SSTR4: ALF99964.1.

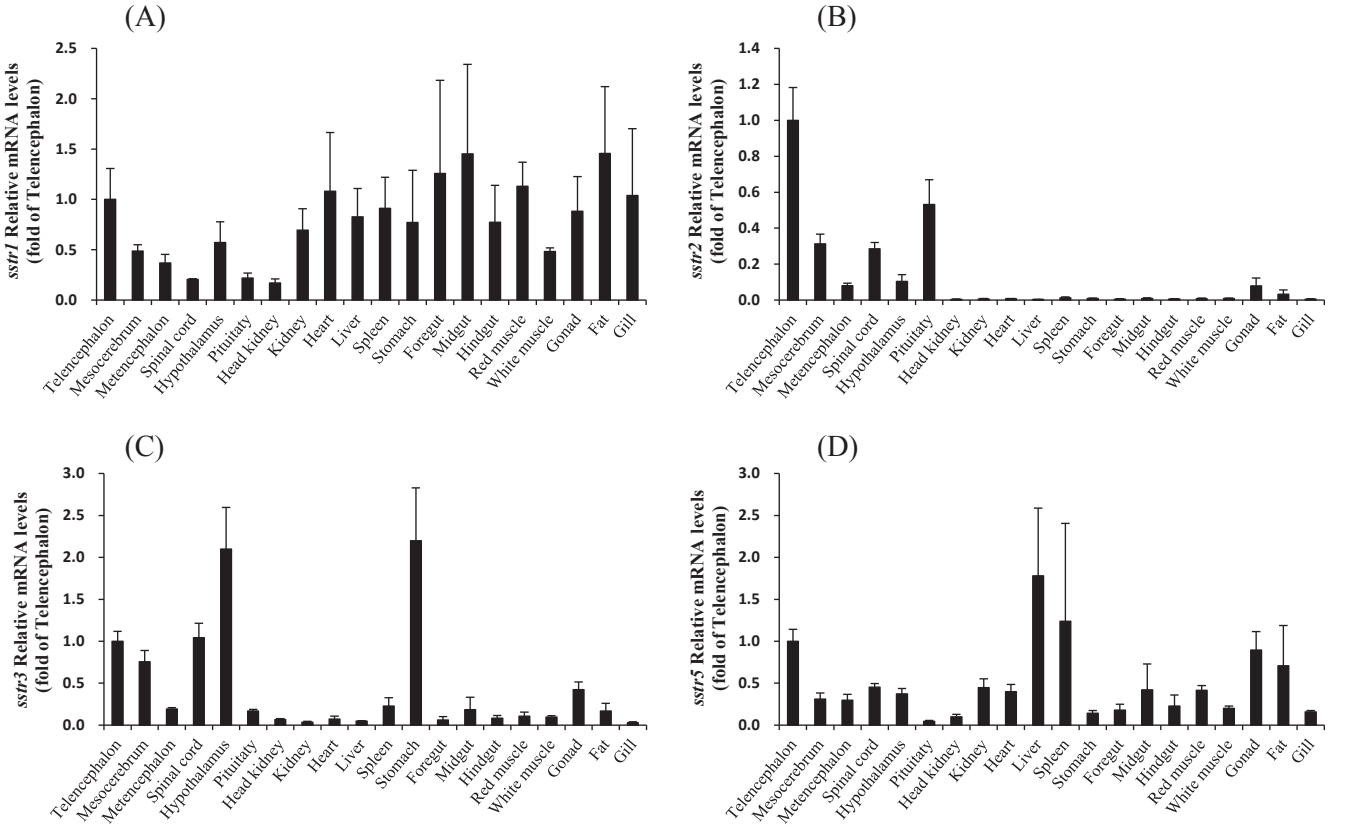


Fig. 3. The mRNA expressions of red-spotted grouper somatostatin receptor type one (A), two (B), three (C), and five (D) in different tissues. The mRNA levels were quantified by real-time PCR and normalized against 18S gene transcripts. The values represent the fold of telencephalon. Data were represented as mea ± S.E.M. (n = 3).

the ORF, sequences were used to perform PCR. All PCR programs utilized the following program: 95 °C for 5 min; 94 °C for 30 s; 50–62 °C for 30 s and 72 °C for 30–90 s for 35 cycles; 72 °C for 5–10 min. All PCR products were purified using the E.Z.N.A Gal Extraction Kit (OMEGA; Bio-tek) and then ligated to the PCR2.1 vector for DNA sequencing. Seven transmembrane domains were predicted using TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The glycosylation sites and phosphorylation sites of the four SSTRs were predicted using NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) and NetPhos3.1 Server (score > 0.7) (http://www.cbs.dtu.dk/services/NetPhos/), respectively. Similarity analysis of SSTRs amino acid sequences was performed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). The phylogenetic tree was constructed using MEGA5.0 via the neighbor-joining method.

2.4. Tissue distribution of red-spotted grouper sstr mRNAs

All experiment procedures were performed according the standard procedures established in our lab (Wang et al., 2016a,b). In brief, total RNA was extracted from tissues using Trizol (Omega, US) and digested with DNase I (New England, USA). cDNA was generated by M-MLV Reverse Transcriptase (Thermo Fisher Scientific, US). *sstr* mRNA expression levels were determined with real-time quantitative PCR using an SYBR Premix Ex Taq (TOYOBO, Japan). The 18S gene was used as

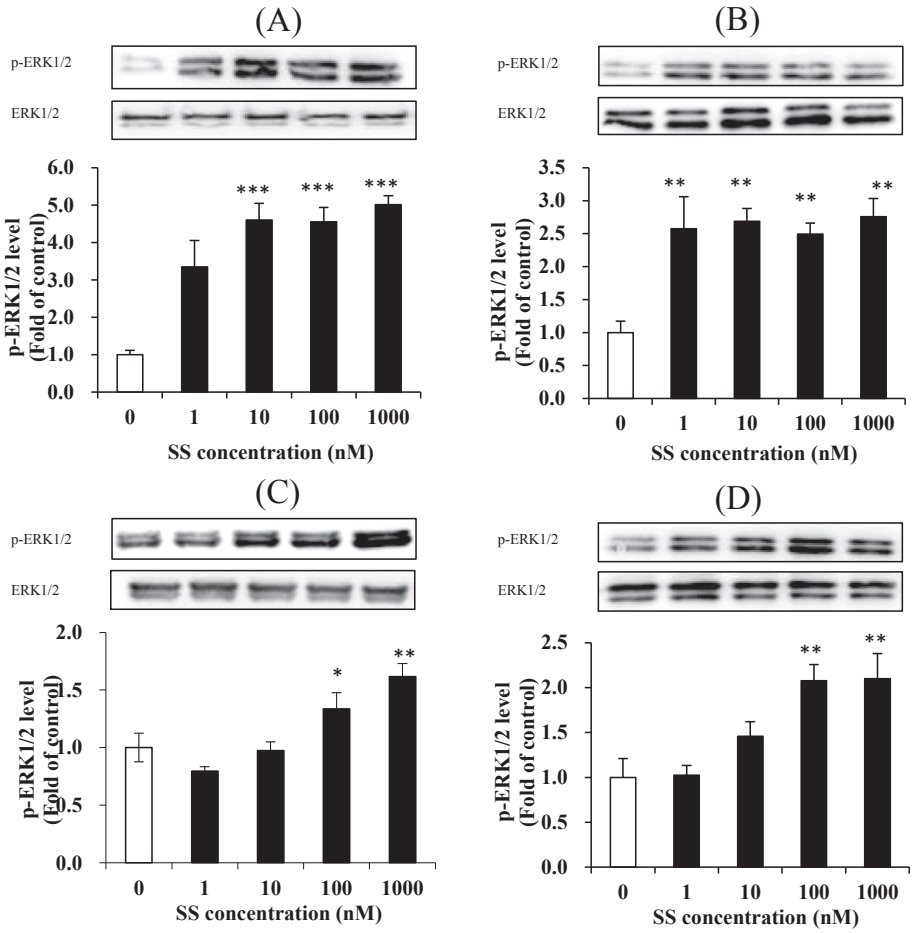


Fig. 4. Dose-response and time-course studies of SS on levels of ERK1/2 phosphorylation via red-spotted grouper SSTRs. The dose-response studies is expressed as A, B, C, and D corresponding to SSTR1, SSTR2, SSTR3, and SSTR5, respectively. The time-course studies is expressed as E, F, G, and H corresponding to SSTR1, SSTR2, SSTR3, and SSTR5, respectively. The significant differences were estimated using one-way ANOVA followed by Fisher's least significance difference (LSD) test (*P < 0.05, **P < 0.01, ***P < 0.001), n = 3–4.

the internal control, and its expression remained stable throughout the study. The relative gene expression levels were calculated using the comparative Ct method and compared with 18S gene levels (Schmittgen and Livak, 2008). The primer sequences used in the study are described in Supplemental Table 1.

2.5. HEK293 cells culture and treatments

Dispersed HEK293 cells were cultured in poly-L-lysine-coated 24-well culture plates (Corning, NY, USA) at a density of 5×10^4 cells/well for 48 h. Cells were transfected with 250 ng endotoxin-free pcDNA3.1(−)-*sstr1/sstr2/sstr3/sstr5* or pcDNA3.1(−) using Lipofectamine 3000 (Life Technologies, USA). After 36 h, cells were preincubated with fresh DMEM without FBS for 1 h. After pre-incubatation for 1 h, (1) the cells were incubated with 1–1000 nM of SS for 5 min; (2) the cells were incubated with 100 nM of SS for 0, 5, 10, 15, 30 and 60 min; (3) the cells were pretreated with 2 μM U0126 for 30 min before exposure to SS for 5 min or no exposure. Then, the DMEM was removed. The cells were lysed with RTPA (Beyotime, Haimen, China) and centrifuged at 12,000× g for 15 min.

2.6. Hepatocyte isolation, culture and treatments

The method of hepatocyte isolation and culture was previously described (Wang et al., 2016a,b). Briefly, primary hepatocytes were digested from liver fragments using Collagenase IV/DNase II and seeded in 24-well culture plates (coating with rat tail collagen). After overnight incubation, the medium was gently replaced with fresh L15 medium and the cell culture was incubated for 1 h before adding SS-14 (10, 100, 1000 nM) for 5, 10, 15, 30 and 60 min. At the end of the culture, the cells were lysed with RIPA (Beyotime, China), and protein was used to western blot analysis.

2.7. Western blot analysis

Western blot analysis was executed as previously described (Wang et al., 2014). In brief, 5 μg of total protein was separated by SDS-polyacrylamide gel and transferred to a 0.45 μm PVDF membrane. After blocking with 5% (w/v) skimmed milk powder, the membrane was incubated with antibodies (Cell signal technology, USA) diluted 1000-fold. On the next day, the membranes were incubated with goat anti-rabbit IgG antibody conjugated with HRP (Boster, Wuhan, China) diluted 4000-fold. Finally, an ECL detection kit (Amersham, USA) was used to present the final results. The protein bands were quantified by densitometry software (ImageJ 1.48v, NIH, USA).

2.8. Statistical analysis

Data were analyzed using an independent -samples T-test or one-way analysis of variance (ANOVA) followed by Fisher's least significance difference (LSD) test using SPSS18 software. Differences were considered significant when a probability of less than 0.05 ($P < 0.05$) was observed. All data were presented as the mean ± SEM.

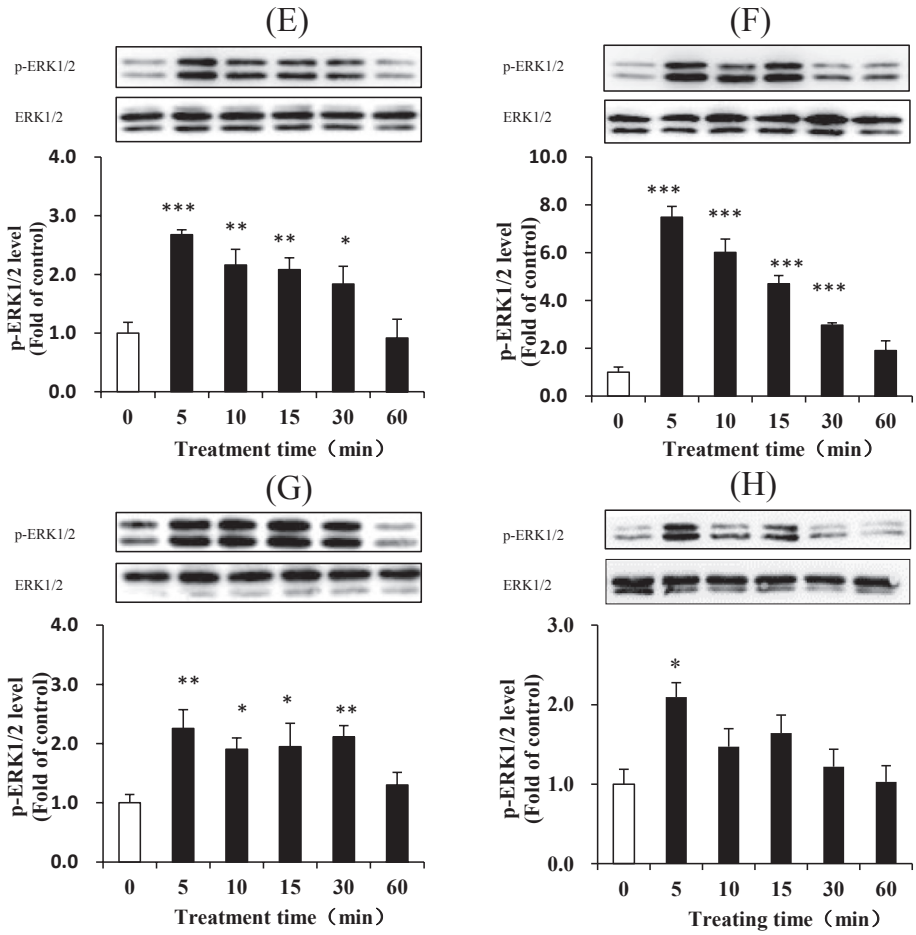


Fig. 4. (*continued*)

3. Results

3.1. Molecular cloning and sequences analysis of sstrs

The four *sstrs* cDNA (*sstr1*, *sstr2*, *sstr3* and *sstr5*) were cloned using RT-PCR and 3′/5′RACE. Their sizes were 2292, 1522, 1873 and 1789 bp, respectively. The ORFs of these receptors are 1155 (Supplemental Fig. S1A), 1113 (Supplemental Fig. S1B), 1467 (Supplemental Fig. S1C) and 1503 bp (Supplemental Fig. S1D), encoding 384, 370, 488 and 500 aa, respectively. SSTRs exhibited high amino acid identity with other vertebrates. SSTR1, SSTR2, SSTR3 and SSTR5 shared 64.30–98.70%, 68.51–96.22%, 50.77–98.36% and 46.80–59.38% similarity to other known vertebrates SSTRs, respectively (Supplemental Table 2). The homology of the deduced amino acid was 42.94% between SSTR1 and SSTR2, 45.90% between SSTR1 and SSTR3, 45.77% between SSTR1 and SSTR5, 49.86% between SSTR2 and SSTR3, 52.05% between SSTR2 and SSTR5, and 55.77% between SSTR3 and SSTR5 (Fig. 1, Supplemental Table 3).

A phylogenetic tree of the SSTRs was created using MEGA5 to illustrate the phylogenetic relationships of red-spotted grouper SSTRs with other known vertebrates (Fig. 2). The cloned red-spotted grouper SSTR1, SSTR2 and SSTR3 were grouped with their counterparts of other vertebrates, whereas SSTR5 exhibited the closest relationship to SSTR3 of other known vertebrates.

The amino acid sequences of the red-spotted grouper SSTRs consist of five parts: seven conserved transmembrane regions, three extracellular loops, four intracellular loops, N-terminal region and C-terminal segment. In total 7, 10, 16 and 16 potential kinase specific phosphorylation sites are located in the intracellular and C-terminal regions of SSTR1, SSTR2, SSTR3 and SSTR5 proteins, respectively, as predicted by NetPhos. Furthermore, 2, 4, 3 and 3 N-glycosylation sites were present in SSTR1, SSTR2, SSTR3 and SSTR5 proteins, respectively, as predicted by NetNGlyc (Supplemental Fig. S1).

3.2. Tissues distribution of the four sstr genes in red-spotted grouper

To examine the expression pattern of *sstrs* in different tissues of red-spotted grouper, real-time PCR was performed using cDNA prepared from telencephalon, mesocerebrum, metencephalon, spinal cord, hypothalamus, pituitary, head kidney, kidney, heart, liver, spleen, stomach, foregut, midgut, hindgut, red muscle, white muscle, gonad, fat and gill. As presented in Fig. 3, *sstr1* (Fig. 3A) and *sstr5* (Fig. 3D) gene expression could be detected in all tissues, whereas *sstr2* (Fig. 3B) and *sstr3* (Fig. 3C) mRNA levels were increased in the central nervous system compared with peripheral tissues with the exception of the stomach. The highest levels of *sstr1* mRNA were detected in fat, and low levels were detected in head kidney. The highest levels of *sstr2* mRNA were detected in telencephalon, followed by pituitary and mesocerebrum. The highest levels of *sstr3* expression were found in the stomach, and abundant expression was detected in the hypothalamus, telencephalon and head kidney. The highest *sstr5* gene expression was found in liver followed by spleen and telencephalon, and low expression was detected in pituitary and head kidney.

3.3. Functional confirmation of SSTRs

To demonstrate whether the four SSTRs we cloned have biological functions, we examined levels of ERK1/2 phosphorylation mediated by SSTRs after transfection of pcDNA3.1(−)-*sstr1/2/3/5*. First, we performed dose-response and time-course experiments. Here, 100 nM SS-

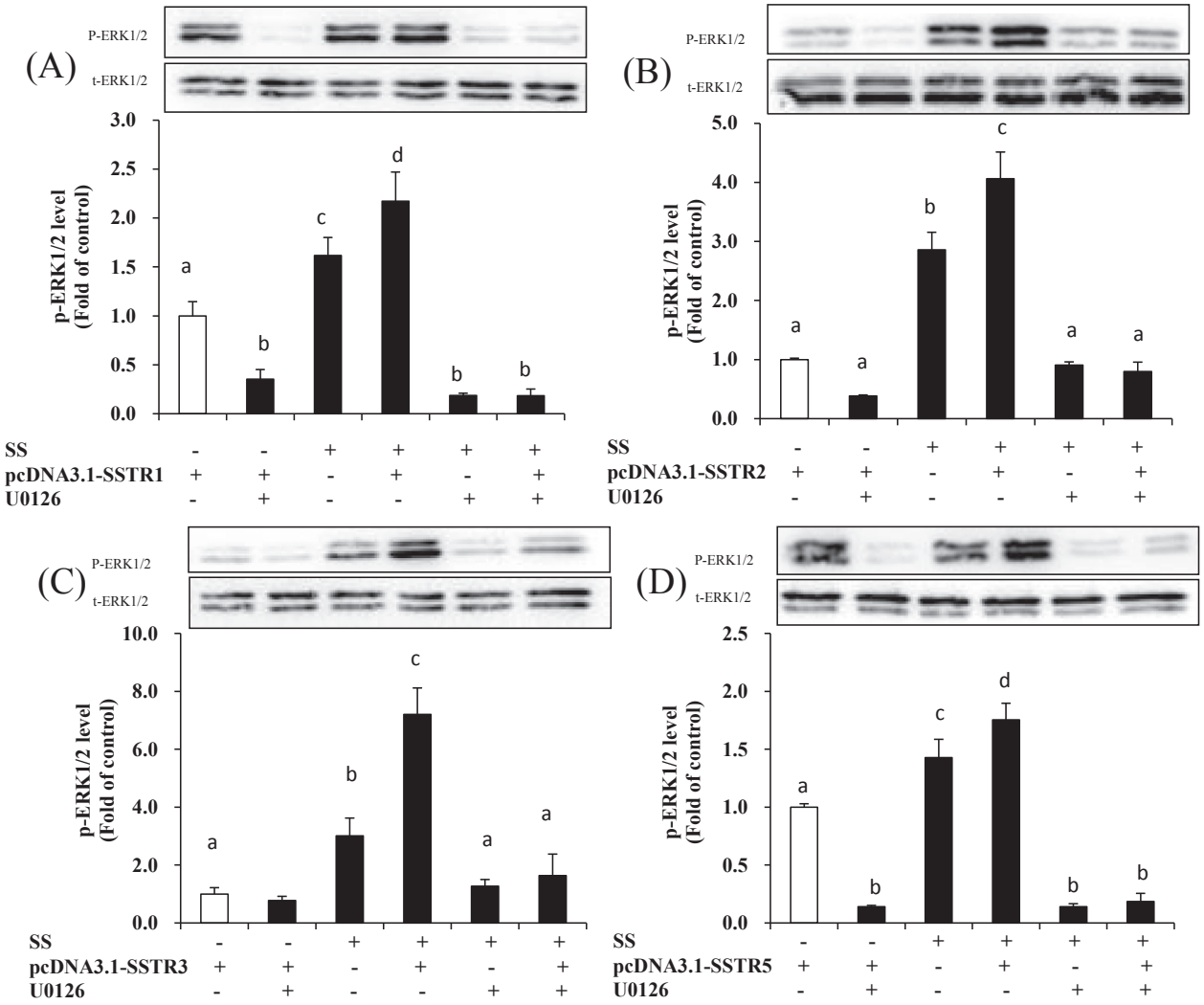


Fig. 5. Effect of SS-14 and U0126 on levels of ERK1/2 phosphorylation via red-spotted grouper somatostatin receptor type one (A), two (B), three (C), and five (D). There is a significant difference between different lowercase letters on the column ($P < 0.05$), n = 3–4.

14 treatment for 5 min achieved the best results (Fig. 4). Finally, to further study SSTR-mediated ERK1/2 phosphorylation, we employed a specific ERK1/2 inhibitor U0126. Phosphorylation levels of ERK1/2 in all four pcDNA3.1(−)-*sstr* groups were significantly increased compared with the pcDNA3.1(−) group after SS treatment. Phosphorylation was blocked by U0126 (Fig. 5), suggesting that four *sstrs* cDNA of red-spotted grouper express biologically active receptor proteins.

3.4. Effect of SS-14 on AKT and ERK1/2 phosphorylation in primary hepatocytes

To explain the effects of SS-14 on the signaling pathway in primary hepatocytes of red-spotted grouper, we performed a time-course study of SS-14 to select a suitable incubation period. As shown in Fig. 6A, ERK1/2 phosphorylation levels reached a maximum at 5 min upon SS-14 treatment and subsequently decreased. SS-14 activated AKT phosphorylation at 5 min, reaching maximum levels at 15 min (Fig. 6C). However, SS-14 did not affect the phosphorylation levels of PKC (Fig. 6B) and JNK (Fig. 6D).

We performed a dose-response study of SS-14 to select a proper treatment dose. As shown in Fig. 6E, SS-14 promoted ERK1/2 phosphorylation in a dose-dependent manner. Remarkably, all concentrations of SS-14 significantly increased the ERK1/2 phosphorylation level compared with the control group. Moreover, only 1000 nM of SS-14 obviously stimulated AKT phosphorylation (Fig. 6G). However, PKC (Fig. 6F) and JNK (Fig. 6H) phosphorylation levels were not affected by all SS-14 concentrations. Thus, the submaximal dose of 100 nM SS-14 was chosen for the subsequent experiments on ERK1/2, and the maximal dose of 1000 nM SS-14 was chosen for AKT experiments.

To determine the effect of SS-14 on ERK1/2 and AKT signaling pathways, we used the specific ERK1/2 inhibitor U0126 and the AKT inhibitor MK-2206. As shown in Fig. 6I–J, SS-14 induced ERK1/2 and AKT phosphorylation, and this effect was blocked by U0126 and MK-2206, respectively.

4. Discussion

In the present study, we cloned four *sstrs* gene from the telencephalon or pituitary of red-spotted grouper. Phylogenetic analysis of SSTRs was performed based on their structural characteristics. The four different *sstrs* cDNA isolated from the red-spotted grouper were named *sstr1*, *sstr2*, *sstr3* and *sstr5*. Several similar features common to other known vertebrate SSTRs were observed in these four SSTRs, and they contained the SSTR family signature sequence YANSCANPI/VLY (Reisine and Bell, 1995; Patel and Srikant, 1997). These characteristic amino acid motifs may play an important role in conferring functional activity (Reisine and Bell, 1995). N-linked glycosylation sites were predicted in the N-terminal domain of all the four receptors. These N-

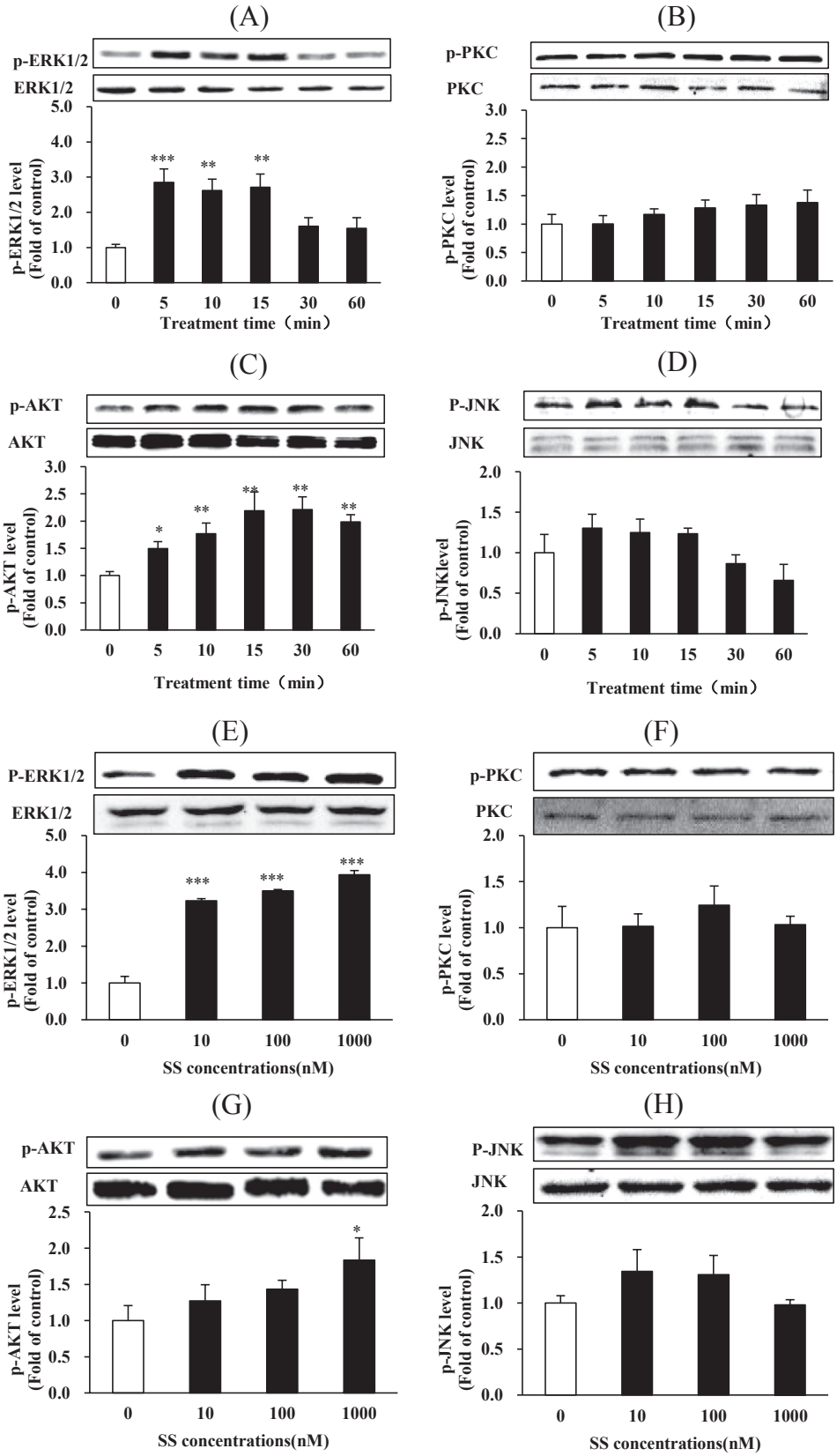


Fig. 6. Effect of SS-14 on intracellular signal mediating in primary hepatocytes of red-spotted grouper. Time-course studies of SS-14 on ERK1/2, PKC, AKT and JNK phosphorylation levels were expressed as A, B, C, and D, respectively; dose-response studies of SS-14 on ERK1/2, PKC, AKT and JNK phosphorylation levels were expressed as E, F, G, and H, respectively; asterisks indicate significant differences between treatment and control groups (*, $P < 0.05$;**, $P < 0.01$; ***, $P < 0.001$), n = 3–4. Effect of inhibitors on phosphorylation of AKT (I) and ERK1/2 (J), there is a significant difference between different lowercase letters on the column ($P < 0.05$), n = 3–4.

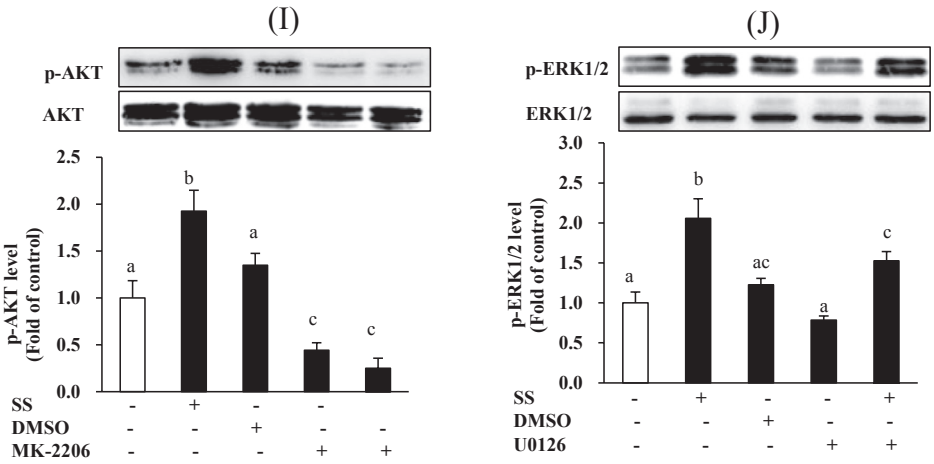


Fig. 6. (*continued*)

linked glycosylation sites are crucial in ligand binding (Ferreira et al., 2018). Rats lacking two glycosylation sites in SSTR3 exhibited high affinity, and nonglycosylated SSTRs in the rat central nervous system may play a role in altering neural signaling and reducing cellular responses (Nehring et al., 2000). The third intracellular loop and C-terminal potential phosphorylation sites of SSTRs are important for the phosphorylation, internalization and desensitization (Liu et al., 2005; Jacobs and Schulz, 2008). The transmission efficiency of somatostatin receptor signaling is tightly regulated by the synergistic effect of phosphorylation and dephosphorylation (Schulz et al., 2014). Some potential kinase-specific phosphorylation sites were also identified in the third intracellular loop and C-terminal of the red-spotted grouper SSTRs. These results suggested that this region might be involved in the regulation of signal pathways within the cell.

The amino acid sequence homology analysis of the four cloned SSTRs showed that grouper SSTR1 and SSTR2 exhibited relatively high similarity with those SSTRs from other known vertebrates, while SSTR5 exhibited low similarity with those from other known vertebrates. SSTR3 also exhibited low similarity with those SSTRs of other known vertebrates with the exception of high similarity to *Epinephelus coioides*. Previous studies have reported that SSTR2 was more conserved in vertebrates than were the other three subtypes of SSTRs (Haiyan et al., 2010). Only 42.94–55.77% similarity was noted among the four red-spotted grouper SSTRs, and high similarity was noted for the same subtypes among the various species. Phylogenetic relationships between red-spotted grouper SSTRs and other known SSTR family members in vertebrates are analyzed. The phylogenetic tree was divided into two main branches (SSTR1/4 and SSTR 2/3/5). SSTR1 appears to be a branch of SSTR2, while SSTR2 and SSTR5 are separated from SSTR3. The phylogenetic relationships suggested that the polygenes of SSTRs originate from a series of gene duplication events in the evolution of vertebrates. This event was considered to be the genetic basis for the origin of multiple gene families (Soukup, 1970). In some ray-finned fish, multiple subtypes of SSTRs may be repeated in the third round of genome production. A unique third genome duplication occurred in some fish (Meyer and Van de Peer, 2005), which may be the reason some fish species contain multiple subtypes of SSTRs.

Four red-spotted grouper *sstrs* exhibited different expression patterns. Different expression characteristics suggest that these *sstrs* mediate different physiological functions. *sstr1* is mainly expressed in brain, stomach, pancreas and kidney, whereas *sstr2* is highly expressed in brain, muscle and liver (Slagter and Sheridan, 2004; Kittilson et al., 2011). Glucose-dependent increases in *sstr2* mRNA levels in rainbow trout suggest that *sstr2* may be involved in regulating glucose metabolism (Kittilson et al., 2011). Our results demonstrate that *sstr1* was widely expressed in red-spotted grouper, and the expression levels in each tissue were very similar. These results suggest that *sstr1* may mediate a wide range of physiological functions in red-spotted grouper. A recent study found that *sstr2* might exert its pro-apoptotic function in rat neurons through inhibiting AKT activity following intracerebral hemorrhage (Tan et al., 2018). *sstr2* is the most widely distributed somatostatin receptor subtype in tumors (Liu et al., 2005). In our study, *sstr2* of red-spotted grouper was mainly distributed in the central nervous system and pituitary. This expression pattern may be related to its function, suggesting that *sstr2* may be involved in regulating the central nervous system and mediating apoptosis. *sstr3* was first identified in nonmammals, specifically electric fish (Zupanc et al., 1999). In our experiments, *sstr3* was not as highly expressed in the pituitary as noted other species. In addition to increased expression in the central nervous system, *sstr3* exhibits the highest expression in the stomach. These results suggest that *sstr3* may be involved in the regulation of food intake or metabolism. Goldfish *sstr5* contains three subtypes, namely, *sstr5a*, *sstr5b* and *sstr5c*, and all of these subtypesare highly expressed in the pituitary (Lin and Peter, 2003). In addition to pituitary and small intestine, extremely low levels of *sstr5* mRNA were detected in all tissues in orange-spotted grouper (Haiyan et al., 2010). Surprisingly, our results demonstrated that *sstr5* exhibited the lowest expression in the pituitary of all tissues tested. Instead, the highest expression of *sstr5* was observed in the liver. *sstr5* is widely expressed, indicating its participation in various physiological functions. Overall, all four receptors had different expression patterns in red-spotted grouper, suggesting that they may play different roles in regulating different physiological processes.

To date, the intricate web of intracellular signal mediating the action of SS system remains far from being fully understood. ERK is an important cell signaling molecule that exerts biological functions when activated by phosphorylation (Amin et al., 2003). In the primary hepatocytes of rainbow trout, SS-14 inhibit the expression of growth hormone receptor and insulin-like growth factor by activating ERK and PI3K signaling pathways (Hanson et al., 2010). The four *sstrs* were transfected into HEK293 cells, separately, and treatment with SS-14 increased ERK1/2 phosphorylation levels significantly compared with the nontransfected group. This stimulation could be inhibited by U0126, suggesting that the four *sstrs* cloned from red-spotted grouper exhibited normal physiological function. Further research found that SS-14 dose-dependently induces ERK1/2 and ATK phosphorylation in primary hepatocytes of red-spotted grouper, and this activation is blocked by the specific inhibitors U0126 and MK-2206. This finding suggested that SS-14 binds to SSTRs to induce ERK1/2 and AKT pathway in hepatocytes of red-spotted grouper. Overall, this research aiming to elucidate the signaling pathways mediating the actions of SSTRs could also contribute to understanding the functional divergence

of the SS system in teleosts.

5. Conclusion

In the present study, the full-length cDNA of four *sstrs* were characterized in red-spotted grouper, and they had different expression patterns. In addition, these SSTRs mediated the regulation of intracellular signaling pathways. To the best of our knowledge, minimal research has been conducted on the emerging aquaculture species of red-spotted grouper. This study provides novel information on its growth mechanism.

Declaration of interest

The authors declare that they have no conflict of interest.

Funding

This work was supported by the National Science Foundation of China (31472259 and 31272639), National Key R&D Program of China 2018YFD0900101, the Guangdong Provincial Science and Technology Program (2015A020216006), Guangdong Oceanic and Fishery Program (A201601C02), Science and Technology Planning Project of Guangzhou (No. 201607020014), Modern Agriculture Talents Support Program (2016–2020) and the Fundamental Research Funds for the Central Universities (17lgjc18) to Dr Wensheng Li.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.01.007.

References

Amin, M.A., et al., 2003. Migration inhibitory factor mediates angiogenesis via mitogen-activated protein kinase and phosphatidylinositol kinase. Circ. Res. 93 (4), 321–329.
Brazeau, P., et al., 1973. Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone. Science 179 (4068), 77–79.
Chisholm, C., Greenberg, G.R., 2002. Somatostatin-28 regulates GLP-1 secretion via somatostatin receptor subtype 5 in rat intestinal cultures. Am. J. Physiol. Endocrinol. Metab. 283 (2), E311.
Csaba, Z., et al., 2012. Molecular mechanisms of somatostatin receptor trafficking. J. Mol. Endocrinol. 48 (1), 1–12.
Feng, X., et al., 2015. Molecular characterization and expression of three pre-prosomatostatin genes and their association with growth in common carp (Cyprinus carpio). Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 182, 37–46.
Ferreira, I.G., et al., 2018. Glycosylation as a main regulator of growth and death factor receptors signaling. 19(2).
Haiyan, D., et al., 2010. Comparative analyses of sequence structure, evolution, and expression of four somatostatin receptors in orange-spotted grouper (Epinephelus coioides). Mol. Cell. Endocrinol. 323 (2), 125–136.
Hanson, A., et al., 2010. The ERK and PI3K signaling pathways mediate inhibition of insulin-like growth factor-1 receptor mRNA expression by somatostatin. Mol. Cell. Endocrinol. 315 (1–2), 57–62.
Jacobs, S., Schulz, S., 2008. Intracellular trafficking of somatostatin receptors. Mol. Cell. Endocrinol. 286 (1–2), 58–62.
Jiang, Y.-D., et al., 2015. Effect of dietary lipid level on growth performance, feed utilization and body composition by juvenile red spotted grouper (Epinephelus akaara). Aquacult. Int. 23 (1), 99–110.
Kittilson, J.D., et al., 2011. Isolation, characterization, and distribution of somatostatin receptor subtype 2 (SSTR 2) mRNA in rainbow trout (Oncorhynchus mykiss), and regulation of its expression by glucose. Comp. Biochem. Physiol. A: Mol. Integr. Physiol. 160 (2), 237–244.
Klein, S.E., Sheridan, M.A., 2008. Somatostatin signaling and the regulation of growth and metabolism in fish. Mol. Cell. Endocrinol. 286 (1–2), 148–154.
Kumar, U., Grant, M., 2010. Somatostatin and somatostatin receptors. Results Probl. Cell Differ. 50, 137–184.
Lahlou, H., et al., 2004. Molecular signaling of somatostatin receptors. Ann. N. Y. Acad. Sci. 1014 (1), 121–131.
Lin, X., et al., 1999. Expression of three distinct somatostatin messenger ribonucleic acids (mRNAs) in goldfish brain: characterization of the complementary deoxyribonucleic acids, distribution and seasonal variation of the mRNAs, and action of a somatostatin-14 variant. Endocrinology 140 (5), 2089–2099.
Lin, X., Peter, R.E., 2003. Somatostatin-like receptors in goldfish: cloning of four new receptors. Peptides 24 (1), 53–63.
Liu, Q., et al., 2005. Receptor signaling and endocytosis are differentially regulated by somatostatin analogs. Mol. Pharmacol. 68 (1), 90–101.
Meyer, A., Van de Peer, Y., 2005. From 2R to 3R: evidence for a fish-specific genome duplication (FSGD). BioEssays 27 (9), 937–945.
Nehring, R.B., et al., 2000. Glycosylation affects agonist binding and signal transduction of the rat somatostatin receptor subtype 3. J. Physiol. Paris 94 (3–4), 185–192.
Nelson, L.E., Sheridan, M.A., 2005. Regulation of somatostatins and their receptors in fish. Gen. Comp. Endocrinol. 142 (1–2), 117–133.
Patel, Y.C., 1999. Somatostatin and its receptor family. Front. Neuroendocrinol. 20 (3), 157–198.
Patel, Y.C., Srikant, C.B., 1997. Somatostatin receptors. Trends Endocrinol. Metab. 8 (10), 398–405.
Piqueras, L., Martínez, V., 2004. Role of somatostatin receptors on gastric acid secretion in wild-type and somatostatin receptor type 2 knockout mice. Naunyn-Schmiedeberg's Arch. Pharmacol. 370 (6), 510–520.
Ramírez, J.L., et al., 1997. Somatostatin plays a dual, stimulatory/inhibitory role in the control of growth hormone secretion by two somatotrope subpopulations from porcine pituitary. J. Neuroendocrinol. 9 (11), 841–848.
Reisine, T., Bell, G.I., 1995. Molecular biology of somatostatin receptors. Endocr. Rev. 16 (4), 427–442.
Rutter, G.A., 2009. Regulating glucagon secretion: somatostatin in the spotlight. Diabetes 58 (2), 299–301.
Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C(T) method. Nat. Protoc. 3 (6), 1101–1108.
Schulz, S., et al., 2014. Fine-tuning somatostatin receptor signalling by agonist-selective phosphorylation and dephosphorylation: IUPHAR Review 5. Br. J. Pharmacol. 171 (7), 1591–1599.
Slagter, B.J., Sheridan, M.A., 2004. Differential expression of two somatostatin receptor subtype 1 mRNAs in rainbow trout (Oncorhynchus mykiss). J. Mol. Endocrinol. 32 (1), 165–177.
Soukup, S.W., 1970. Evolution by gene duplication. S. Ohno. Springer-Verlag, New York. 1970. 160 pp. Teratology 9 (2), 250–251.
Tan, X., et al., 2018. SSTR2 associated with neuronal apoptosis after intracerebral hemorrhage in adult rats. Neurol. Res. 40 (3), 221–230.
Wang, B., et al., 2014. Differential involvement of signaling pathways in the regulation of growth hormone release by somatostatin and growth hormone-releasing hormone in orange-spotted grouper (*Epinephelus coioides*). Mol. Cell. Endocrinol. 382 (2), 851–859.
Wang, B., et al., 2016a. In vitro effects of somatostatin on the growth hormone-insulin-like growth factor axis in orange-spotted grouper (Epinephelus coioides). Gen. Comp. Endocrinol. 237, 1–9.
Wang, J., et al., 2016b. Dietary protein requirement of juvenile red spotted grouper (*Epinephelus akaara*). Aquaculture 450, 289–294.
Zupanc, G.K., et al., 1999. Molecular cloning and pharmacological characterization of a somatostatin receptor subtype in the gymnotiform fish Apteronotus albifrons. Gen. Comp. Endocrinol. 115 (3), 333–345.