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Positive feedback of hepatic angiotensinogen expression in silver sea bream (*Sparus sarba*)

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Abstract

The renin–angiotensin system (RAS) is involved in the maintenance of fluid homeostasis in vertebrates. Production of the precursor protein, angiotensinogen, is regulated by other components within the RAS. Angiotensin II (Ang II) stimulates the production and secretion of angiotensinogen in many mammalian models. However, the existence of a similar positive feedback mechanism for angiotensinogen has not been demonstrated for any non-mammalian species. In the present study, we have cloned the angiotensinogen for silver sea bream (*Sparus sarba*) and investigated the role of Ang II on angiotensinogen expression. The nucleotide sequence of angiotensinogen for *S. sarba* only exhibits a fair resemblance to other fish angiotensinogens and shows 76.6% similarity to that of *Takifugu rubripes* and 57.2% similarity to that of *Danio rerio*. Angiotensinogen transcripts have been identified in the brain, liver, kidney, and various parts of the intestine of sea bream, an observation, which probably implies the presence of a local RAS at the tissue level. The liver is probably the major source of angiotensinogen, as it exhibits the highest angiotensinogen transcript abundance among different tissues. Differential angiotensinogen expression was found among different regions of the intestine where the pyloric caeca exhibits the highest expression. Putative Ang I is identified at the N-terminal of the deduced protein with a novel sequence [Asn^1, Ile^5, His^9]-Ang I. Hepatic angiotensinogen expression in sea bream adapted to different salinities remained constant and this is probably due to desensitization of the angiotensin receptors by angiotensin. A positive feedback mechanism of angiotensinogen by Ang II has been demonstrated as exogenous Ang II increased the amount of angiotensinogen transcript in isolated hepatocytes *in vitro*. Blockade of endogenous RAS by the angiotensin converting enzyme (ACE) inhibitor, captopril, significantly lowered the hepatic expression of angiotensinogen *in vivo*. The effect of Ang II stimulation on angiotensinogen expression is more potent in fish than that in mammals. These data suggest that the positive feedback mechanism of angiotensinogen by Ang II has already evolved in teleosts and such mechanism may be involved in the maintenance of angiotensinogen secretion under resting and hypertensive conditions.
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Keywords: Silver sea bream; Renin–angiotensin system; Angiotensinogen cloning; Positive feedback

1. Introduction

Angiotensinogen cDNA was first cloned by Ohkubo et al. (1983) in the rat. After more than 20 years, it is surprising that only a few fish angiotensinogens have been cloned and characterized. The only fish angiotensinogen sequences available in the GenBank include complete sequences for *Danio rerio* (AY049731) and *Takifugu rubripes* (BK001021) and some partial sequences for trout and catfish. Fish angiotensinogens are structurally diverse and this is not surprising since fish is an evolutionarily diverse group of vertebrates. The nucleotide sequences of angiotensinogen molecules of rat and human are 63.6% identical (Kageyama et al., 1984), but the active peptide, Ang II, is highly conserved throughout vertebrate phylogeny (Kobayashi and Takei, 1996). In elasmobranchs [Asn^1, Pro^3, Ile^5]-Ang II is the predominate form (Takei et al., 1993). In teleosts, the most common form is [Asn^1, Val^5]-Ang II and recently Balment et al. (2003) has demonstrated that [Asn^1, Ile^5, Thr^9]-Ang I is the flounder-type Ang I.

The liver, representing the major site for angiotensinogen biosynthesis, lacks the ability to store pre-synthesized protein and primarily translates and secretes synthesized proteins through the constitutive pathway (Brasier and Li, 1996). The extracellular nature of angiotensinogen is particularly relevant to the activity of the RAS, especially in local regulation. Generation of Ang I by renin is usually regarded as the rate-limiting step for the RAS cascade, but this may not be entirely true since the circulating concentration of angiotensinogen may also

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be rate-limiting. Such contention is inferred from the fairly low angiotensinogen level (0.6 μM) when compared with the maximum velocity of Ang I formation within the intravascular space (K_d of renin = 1.0 μM) (Reid et al., 1978). Conditions that alter circulating angiotensinogen may therefore influence RAS status. Transgenic mice that contain multiple copies of angiotensinogen alleles had concurrent increase in blood pressure by 8 mmHg per gene copy (Kim et al., 1995). This provided a strong causal relationship between angiotensinogen genotypes and blood pressure.

In mammals, a positive feedback mechanism is present for regulating angiotensinogen gene expression. In studies on perfused mammalian liver, injection of renin or infusion of Ang II stimulated angiotensinogen release (Nasjletti and Masson, 1973). Ang II is a positive stimulus for the synthesis of angiotensinogen and its effect is not dependent on the stimulation of glucocorticoid secretion (Blair-West et al., 1974; Carretero and Gross, 1967; Herrmann et al., 1980) but the presence of glucocorticoid is necessary to sustain angiotensinogen synthesis (Reid, 1977). Despite the stimulatory effect of Ang II on angiotensinogen expression, such effect has been mostly demonstrated using relatively high concentrations of Ang II (up to mM concentrations) and most of the effective doses employed were not at physiological concentrations (Sernia and Reid, 1980). Such a mechanism could explain the maintenance of angiotensinogen concentration under hemorrhage (Beaty et al., 1976), sodium depletion and dehydration (Gross et al., 1972), when the metabolism of angiotensinogen is elevated. Using nuclei run-on assays, dexamethasone (10 μM) has been shown to stimulate a five-fold increase in angiotensinogen mRNA levels but Ang II treatment did not affect transcription (Klett et al., 1994). Furthermore, in the same study using a cell-free incubation system, a $12K_d$ polysomal protein induced by Ang II treatment significantly increased the half-life of angiotensinogen mRNA, indicating the positive feedback by Ang II was not due to an increase in mRNA transcription, but due to stabilization of angiotensinogen mRNA. However, in a separate study using AT_1-complemented human hepatocytes, Ang II activated transcription of angiotensinogen through the multihormone-inducible enhancer at nucleotide region −615 to −470 (Brasier and Li, 1996). Transcription driven by the multihormone-inducible enhancer occurred over a physiological dose-responsive range producing a statistically significant three-fold increase at 10 mM Ang II. The authors also pointed out that the Ang II-dependent transcriptional activation was only observed in cells cotransfected with Ang II AT_1 receptor expression vector, indicating that AT_1 receptor is essential for the regulation. Furthermore, the fact that suppressed intracellular cAMP stimulated the transcription of angiotensinogen further supported that AT_1 receptor is required for the positive feedback pathway (Klett et al., 1990). Combining these studies, it can be concluded that Ang II dependent positive feedback occurs at both transcriptional and post-transcriptional levels.

Despite numerous studies on mammalian models demonstrating the presence of a positive feedback regulation of angiotensinogen by Ang II (Sernia and Reid, 1980), information from non-mammalian vertebrates is lacking and whether this form of regulation exists in early vertebrate lineages is not known. We have previously demonstrated that the status of the RAS in sea bream changes in response to salinity adaptation (Wong et al., 2006), with augmented RAS status being observed in hyperosmotic salinities, but it is not known whether circulating Ang II modulates hepatic angiotensinogen expression. In the present study, we cloned the angiotensinogen from silver sea bream and characterized its deduced protein sequence, and provided evidence to suggest the existence of a positive feedback mechanism for angiotensinogen expression in a teleost.

2. Materials and methods

2.1. Animals

Sparus sarba were purchased from a local fish farm. They were kept in a recirculating seawater system in the Simon F.S. Li Marine Science Laboratory, at the Chinese University of Hong Kong during experiment. Fish were fed daily *ad libitum* using a formulated diet according to Woo and Kelly (1995). Fish were randomly divided into six groups in six individual seawater (33‰) tanks of 1000 l capacity. Salinity in the six experimental tanks was gradually adjusted to 0, 6, 12, 33, 50 and 70‰ by flushing dechlorinated freshwater or double-strength seawater as appropriate. Double-strength seawater was prepared beforehand by evaporation of seawater in a separate tank. The entire flushing process was completed in 1 week. After the final salinities were reached, fish were allowed to acclimate to the final salinities for a further 4 weeks before sampling. During the adaptation period, water temperature was kept at 22–25 °C and fish were exposed to a natural photoperiod (12 h light:12 h dark).

2.2. Total RNA isolation and reverse transcription (RT)

A seawater adapted silver sea bream was killed by spinal transection and liver (∼5 mg) was obtained and homogenized in 0.5 ml tri-reagent (Molecular Research Centre, Cincinnati, OH). Total RNA was extracted according to the manufacturer's protocol. Three micrograms total RNA was mixed with 0.5 μg oligo dT, heated at 72 °C for 10 min and cooled on ice. Reverse transcription (RT) was then carried out in 10 μl reaction mixture containing 1× MMLV buffer, 0.5 mM each dNTPs and 80 U MMLV reverse transcriptase (Promega, Madison, WI) for 2 h at 42 °C. First strand cDNA was diluted to a final volume of 60 μl with sterile water and 3 μl was used as template for subsequent polymerase chain reactions (PCR).

2.3. Amplification of partial fragments of angiotensinogen

Five sense and seven antisense degenerate primers were designed according to the conserved nucleotide regions of angiotensinogen for *D. rerio* (AY049731) and *T. rubripes* (BK001021). The five sense primers used had the following sequences:

- 5′-CAAGCCKCTGSAGACYGTTC-3′ (Ang S2);
- 5′-CCAAYCGWGTSTAYGTSCAYCC-3′ (Ang S3);
- 5′-TGMGSATGTACSAGRCRCTCAG-3′ (Ang S4);
- 5′-CTGGGTYTGMGSATGTACSAG-3′ (Ang S5);
- 5′-TTRAASRYRCTCTTCACTTT-3′ (Ang S6).

The sequences for the seven antisense primers were:

- 5′-TTRAASRYRCTCTTCACTTT-3′ (Ang AS1);
- 5′-TTCTSSAYRAARTTRTTGAC-3′ (Ang AS2);
- 5′-TCTTTTSGKSMMTCRTCAAC-3′ (Ang AS3);
- 5′-ARYAAARGTCCACACRWGGGT-3′ (Ang AS4);
- 5′-GTSYKKARAACTKTGTGTCC-3′ (Ang AS5);
- 5′-GGWGACAGRAGMGTRTTGGTG-3′ (Ang AS6);
- 5′-CTSGTACATSCKCARACCCAG-3′ (Ang AS7).

For degenerate primer amplification, each PCR was carried out in a volume of 25 μl (1× PCR buffer, 0.5 mM each dNTP, 2.5 mM $MgCl_2$, 0.8 mM each primer, 0.5 U of *Taq* polymerase) on an Eppendorf 9600 Thermal Cycler (Eppendorf, Germany) for 36 cycles using a profile of 30 s at 94 °C, 30 s at annealing temperature and 60 s at 72 °C. Each possible combination of primers was subjected to a gradient of annealing temperatures (46.3, 49.2, 54.1, 59.5, 64.1 and 66.5 °C). After 36 cycles, a further 10 min at 72 °C was allowed for final extension. However, after resolving PCR products using agarose gel electrophoresis, it was found that there was considerable non-specific amplification and it was unlikely that suitable fragments could be identified. Considering the expected PCR product from primer Ang S3 and Ang AS1 would cover all the regions of other degenerate primers, the PCR product from Ang S3 and Ang AS1 was diluted 100-fold with PCR grade water and used as the template for series of nested-PCR. A single DNA fragment (430 bp) was obtained using primer Ang S2 and Ang AS4. The amplified cDNA fragment was resolved by agarose gel electrophoresis, isolated, purified by phenol/chloroform extraction and cloned into pBluescript II KS (+) (Stratagene, La Jolla, CA) through T/A cloning. Cloned plasmid DNA was prepared with NucleoSpin Plasmid isolation kit (Macherey-Nagel, Duren, Germany) for sequence analysis. The inserted cDNA was sequenced with BigDye Terminator v3.1 Cycle Sequencing Kit and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

2.4. 5′-RACE and 3′-RACE for sea bream angiotensinogen

Gene-specific sense and antisense primers for angiotensinogen were designed based on the sequencing information obtained previously. The reaction was carried out using the SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA). The first-strand cDNAs from sea bream liver were made according to the manufacturer protocol. The specific primers used for 5′-RACE and 3′-RACE had the following sequences: 5′-CTCAGCAGCAAGCAAAGCAGCAACAC-3′ (sense) and 5′-ACACACAGTCCTCTCGGTGGGTGCCAC-3′ (antisense). Amplification of 5′- and 3′-ends was carried out in 30 μl reaction mixture containing 1× advantage 2 buffer, 0.5 mM each dNTP, 0.4 mM gene specific primer, 1 mM Universal Primer Mix, 0.5 U of Advantage 2 Polymerase Mix (Clontech). A step down profile was used on the Eppendorf 9600 Thermal Cycler for 35 cycles with the profile of 30 s at 94 °C, 30 s at annealing temperature (72 °C × 5 cycles, 70 °C × 5 cycles and 68 °C × 25 cycles) and 3 min at 72 °C for extension. After 35 cycles, a further 10 min at 72 °C was allowed for final extension of DNA fragments. The amplified cDNA ends were cloned and sequenced as described previously. After the primary full-length angiotensinogen sequence was obtained, specific sense and antisense primers were designed at 5′- and 3′-ends and they were used to amplify the major cDNA fragments from the liver RT products. The sense and antisense primers sequences for sequence integrity confirmation are 5′-GGTGCCACCAAATCTAGGTTAATGAC-3′ and 5′-AATCTTGGTTACTGTACCTACTCTACTG-3′ respectively, which are located at 5′- and 3′-end regions. The PCR reactions were carried out in a 25 μl reaction mixture containing 1× *Pfu* buffer, 0.5 mM each dNTP, 0.2 mM gene specific primers, and 0.5 U of *Pfu* Polymerase (Promega). PCR product at 2500 bp was purified as previously described and the purified PCR product was sequenced using a direct sequencing profile with BigDye Terminator v3.1 Cycle Sequencing Kit and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

2.5. Validation of semi-quantitative RT-PCR for sea bream angiotensinogen

The RT-PCR assays used to analyze the expression of the target gene, angiotensinogen, and the housekeeping gene, β-actin, were first validated by optimizing the number of cycles used for each gene in the assay. RT products from sea bream liver were used as templates for PCR as described previously. The primers used for β-actin were adopted according to Deane et al. (2002) and had the following sequences: 5′-TCACCAACTGGGATGACATG-3′ (sense) and 5′-ATCCACATCTGCTGGAAGGT-3′ (antisense). Primers used for angiotensinogen were designed from sequence information obtained as outlined in the previous section and had the following sequences: 5′-AAGCTTGTTCCAGCAACTACTG-3′ (sense) and 5′-CCAGCTACCTTGGAAGTTGAAG-3′ (antisense). PCR was performed in 1× PCR buffer, 0.5 mM each dNTP, 2.5 mM $MgCl_2$, 0.2 mM of each of primer, 0.5 U of *Taq* polymerase in a total volume of 25 μl for various cycles with the profile of 30 s at 94 °C, 30 s at 62 °C and 60 s at 72 °C. The PCR products were electrophoresed on a 1.3% agarose gel and stained with ethidium bromide. After gel electrophoresis, the band intensities were quantified and analyzed with the Gel-Doc 1000 system and Molecular Analyst Software (Bio-Rad). The number of cycles that gave half-maximal amplification would be used for semi-quantitative PCR assay for each gene. To further validate the RT-PCR assays, PCR was performed on serially diluted templates of known amounts with the optimized PCR profile.

2.6. Hepatic angiotensinogen expression

Liver tissue was collected from sea bream adapted to different salinities and stored at −80 °C. In order to assess the differential distribution of angiotensinogen in other body tissues, samples of brain, blood, gill, liver, kidney, stomach, pyloric caeca, ileum and rectum were also collected and frozen at −80 °C. Total RNA was extracted from each liver sample and RT was performed using the protocol described previously. In tissue distribution experiment, RT (−ve) samples were prepared with the same amount of RNA but without MMLV reverse transcriptase. The same amount of template was used in amplification of angiotensinogen and β-actin using the validated profile. PCR products of angiotensinogen and β-actin were electrophoresed on the same agarose gel and quantified. Expression of hepatic angiotensinogen was expressed as a ratio of intensity between angiotensinogen and β-actin.

Hepatocytes were obtained from sea bream liver according to the protocol of Seddon and Prosser (1999). Sea bream was anaesthetized using MS-222 (1:10,000). A ventral incision was made to expose the liver and heart. A sharpened PE-50 cannula was inserted into the sinus venosus and tied tightly with silk suture. Retrograde perfusion of liver was performed using a peristaltic pump at a constant flow rate of 2 ml/min. The composition of the perfusion buffer was 2 mM EDTA, 110 mM NaCl, 4 mM KCl and 25 mM $NaHCO_3$. When the liver was completely softened, it was removed to a Petri dish containing 5 ml wash buffer (110 mM NaCl, 4 mM KCl and 2.5 mM $CaCl_2$). The liver was then diced using razor blades and diced tissue was passed through a 40 μm cell-collecting sieve with up to 50 ml wash buffer. The cell suspension was centrifuged for 4 min at $30 \times g$, and the pellet was resuspended in Leibovitz-15 (L-15) medium (GIBCO) buffered with 25 mM HEPES. The centrifugation was repeated three times with L-15 medium and the final pellet was resuspended in 30 ml L-15 medium. Viability of the isolated hepatocytes was determined by exclusion of 0.5% trypan blue (DeRenzis and Schechtmann, 1973).

Hepatocytes with viability over 95% were seeded to a 24-well culture plate with 2×10^5 cells/well in 1 ml L-15 medium. The hepatocytes were incubated with 0, 0.1, 1, 10, 100 and 1000 nM [Asn^1, Val^5]-Ang II (Sigma) at 25 °C, in triplicate, for 4 h. The hepatocytes were then harvested and homogenized in Trireagent for total RNA extraction. Three micrograms of total RNA was used in each RT reaction as described previously. In another experiment, three groups of seawater-adapted sea bream ($n = 6$) were each subjected to two intraperitoneal injections. The saline-injected group received 0.8% NaCl at 0 and 6 h. The second group was injected with 0.8% NaCl at 0 h, followed by captopril (100 μg/100 g) at 6 h. The third group was injected with captopril (100 μg/100 g) at 0 and 6 h. All injection volumes were kept at 0.1 ml/100 g body weight. All fish were killed 12 h after the first injection and liver samples were obtained for quantification of angiotensinogen transcripts using the developed RT-PCR assay.

In another experiment where the effect of hemorrhage was investigated, 1 ml of blood was withdrawn from the caudal vessels of individual seawater-adapted sea bream (200–250 g) using a syringe. Fish were killed after 2 h and liver tissues were obtained for angiotensinogen assay. Intact seawater-adapted fish were used as control.

2.7. Statistical analysis

All data are expressed as mean ± S.E.M. Values from different groups were subjected to one-way analysis of variance (ANOVA) followed by Tukey's test ($p < 0.05$) to delineate significant difference.

3. Results

3.1. Cloning and sequence characterization of sea bream angiotensinogen

In the first round PCR, all combinations of primers were used to amplify angiotensinogen fragments but none of them gave products of suitable sizes. Therefore, a second round (nested) of PCR was performed using 100-fold diluted PCR product from primers Ang S3 and Ang AS1, which should cover all other primers within the targeted region. The second round PCR yielded a PCR product of 430 bp using primers Ang S4 and Ang AS2. Alignment of nucleotides showed that the fragment contained an angiotensinogen-like protein, which suggested that a correct clone has been found (primary sequence not shown). Specific primers were designed to amplify the 5′- and 3′-ends of angiotensinogen cDNA in sea bream. The sizes of PCR products of 5′- and 3′-RACE are 1100 and 1600 bp, respectively. The purified PCR products were cloned and sequenced, and nucleotide alignment suggested that the fragments were part of sea bream angiotensinogen.

The primary full-length sequence for angiotensinogen was obtained by joining the overlapping regions of 5′- and 3′-RACE products. The integrity of the cDNA of angiotensinogen was verified by amplification from the liver RT-product using specific primers at 5′- and 3′-regions. The sequence information was confirmed using a direct sequencing profile, which may lower the chance of incorrect sequence information due to cloning of mutated sequences onto the vector. Full-length sea bream angiotensinogen contains 2590 bp with an open reading frame of 1392 bp coding for 463 amino acids. The full-length nucleotide and deduced amino acid sequences (deposited with GenBank under accession no. DQ136311) are given in Fig. 1. From the sequence information, it can be deduced that sea bream produces putative angiotensinogen that cleaves [Asn^1, Ile^5, His^9]-Ang I (NRVYIHPFHL) from the N-terminus of the protein. Subsequent cleavage of [Asn^1, Ile^5, His^9]-Ang I by angiotensin converting enzyme (ACE) gives [Asn^1, Ile^5]-Ang II, which has the same structure as flounder Ang II (Balment et al., 2003). Nucleotide sequence of sea bream angiotensinogen has 76.6% and 57.2% similarity to those of *T. rubripes* and *D. rerio*, respectively. The deduced protein sequence is 72.7% and 52.4% similar to those of *T. rubripes* and *D. rerio*, respectively. Based on angiotensinogen sequences, the phylogenetic relationship between representatives from different vertebrate lineages was evaluated and from the constructed phylogenetic tree, it appears that sea bream angiotensinogen is most similar to that of puffer fish *T. rubripes* (Fig. 2).

3.2. Tissue distribution of angiotensinogen

An optimized RT-PCR profile (32 cycles) was used to perform PCR assays on different tissue samples reverse transcribed with (RT +ve) or without (RT −ve) MMLV reverse transcriptase. The results are shown in Fig. 3 and the signal for angiotensinogen was found to be present in a number of tissues including brain, liver, kidney and intestine. The signal was found to be the highest in liver, which is the major organ for angiotensinogen production but no signal was detected in blood and gill in the present assay. Differential angiotensinogen expression was found among different regions of the intestine with the pyloric caeca exhibiting the highest expression. Moreover, no PCR product was observed for any of the RT −ve control, indicating that the two sets of primers used were unable to recognize genomic DNA, and therefore allowing specific amplification of cDNA in the semi-quantitative RT-PCR assay.

3.3. Validation of semi-quantitative RT-PCR for angiotensinogen

The optimal cycle number giving half-maximal amplification was estimated by examining PCR kinetics. A typical sigmoid curve was obtained when the intensity of the PCR product on the agarose gel was plotted against the number of increasing cycles. The sigmoid curves for amplifying angiotensinogen and β-actin are given in Fig. 4. The optimized cycle number for hepatic angiotensinogen and β-actin were 27 and 25 cycles, respectively. With the optimized cycle numbers, the PCR on serially diluted templates of angiotensinogen and β-actin produced a linear relationship at a range of 10^3–10^6 fg plasmid DNA template (Fig. 5). These results suggested that the semi-quantitative RT-PCR assay could be applied to a wide range of input molecules and was suitable for expression studies.

3.4. Expression of hepatic angiotensinogen

Using the RT-PCR assay developed, the expression levels of hepatic angiotensinogen were quantified in sea bream adapted to different salinities (Fig. 6). The angiotensinogen expression normalized to β-actin expression showed no significant difference among groups. Isolated hepatocytes incubated with different concentrations of Ang II showed a significant increase in angiotensinogen transcripts (Fig. 7). Conversely, captopril blockade in intact sea bream for 12 h caused a significant decrease in angiotensinogen transcription (Fig. 8). The hepatic angiotensinogen expression in sea bream after hemorrhage remained unchanged when compared with that of the control group (Fig. 9).

4. Discussion

The present study is the first study on sea bream angiotensinogen to date. From the nucleotide sequence analysis, sea bream angiotensinogen has a relatively higher homology to that of the puffer *T. rubripes* (76.6%) but a lower homology to that of the zebrafish *D. rerio* (57.2%). Genetic distances also revealed that sea bream is more related to *T. rubripes*. The heterogeneity of angiotensinogen is comparatively high, indicating that the protein itself was subjected to extensive divergence in evolution, even within the teleostean lineage (Fig. 2). The sea bream angiotensinogen nucleotide sequence encodes 463 amino acids with a N-terminal hydrophobic leucine-rich signal peptide (22 amino acids). Five possible glycosylation sites were identified on the putative protein. Immediately after the signal peptide, a

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TTACACACAGTCCTCTCGGTCGGTGCCACCAAATCTAGGTTAATGACAATAACTTATAATTAATCAAGCAAAATATTAATTCTAACTTTT   90

ATAATCAACTCAACGCGTGTGTAAAAGTTATACTGACTCATTCATGGATTTATTTGTACAGTTATTACAACATTAATACGGCCAAACAGT  180

GAGTGAACGCTTTTGTCTTCTTGTATTTTGACAATTTGCGCATGTGAATTTGCTCAACCGCCTGAGCACGCAACCAGAGAGCTGCCTTTT  270

TTAACAATATCCATCTTGTTTAAGTCCGTGAACACAAACAAATTCACACACACAGACGCACATAGATGCGTCTTCATTATCTATGCGACC  360

CCTGTTGCGGAGGGTAATGCCCTGCTCTTATTCATCTGGCCAGCAGAGAGAGAGGTAACACAAAGAGCAATCAATCCATCACTGACATGC  450

CGCTCTATGATCCAAGGATGGTTCCTGGTTCCTGCTCAGGCATTGTGCTGGCAGCGTAACCAGATCCAGCCAGATTTCTGTCAAAAGCCA  540

CACTAATACTAACGTACCATTCACAGCCCGATACTCAGACTCAAGAGCCTGTTGATCACTTGGCTTACTCATCATGAACTAAAAATATCT  630

GTCTCTCTCCTCAGAAGAAATCGTTTTAAGGTGACTATCAACAAAAGGTAAAAATGCAGATCCTGCGGTCGCCTCTCCTTGCCCTCCTAC  720
                                                      M  Q  I  L  R  S  P  L  L  A  L  L

TCTGCTGCTACCTCTCAGCAAGCCAAGCAAACCGCGTCTACATTCACCCCTTCCATCTCTTTGCCGCTGAGAATGTCAGCTGTGAGACCC  810
L  C  C  Y  L  S  A  S  Q  A  N  R  V  Y  I  H  P  F  H  L  F  A  A  E  N  V  S  C  E  T

TTCAGGCCCAGATGTCCAAGCCTCTGGAGACACTTCCAGTGGCCCCCCTTGATATTGAAGTCCTGACGCCAGACAGCAGGGACCCGTCGA  900
L  Q  A  Q  M  S  K  P  L  E  T  L  P  V  A  P  L  D  I  E  V  L  T  P  D  S  R  D  P  S

AGCTGGACGCACAGAAGCAGAACGTCACAGAGAGGACGGCGGTTCTGGCAGAGCTGTTGAACTCTCTGGGCTTCAGGATGTACAAGGCGC  990
K  L  D  A  Q  K  Q  N  V  T  E  R  T  A  V  L  A  E  L  L  N  S  L  G  F  R  M  Y  K  A

TCAGCAGCAAGCAGCAAAGCAGCAACACCCTCCTGTCCCCGGTCAACACCTACGGATCCCTCTTCACCTTCTACCTTGGAGCCTCCAAGA 1080
L  S  S  K  Q  Q  S  S  N  T  L  L  S  P  V  N  T  Y  G  S  L  F  T  F  Y  L  G  A  S  K

AGACAGCAAGCTTGTTCCAGCAACTACTGGGCCTGAGCAGTGGCACCGACCGAGAGGACTGTGTGTCCTTAGTGGATGGACACAAGGTTC 1170
K  T  A  S  L  F  Q  Q  L  L  G  L  S  S  G  T  D  R  E  D  C  V  S  L  V  D  G  H  K  V

TCAAGACCCTGCAGAGCATTAACTCTCTGGTGGACGACGGGCCGAAAGATGAAATCACTACACATGTGTGGACCTTCGCTCGTCACGGTG 1260
L  K  T  L  Q  S  I  N  S  L  V  D  D  G  P  K  D  E  I  T  T  H  V  W  T  F  A  R  H  G

CTCAGCTATCAGAGGATTTTATTCAAGGCACGCAAGACTTTTCGGACACATCGTTCATCCGTGGCGTGGACTTCACCAAACCTCAGGAGG 1350
A  Q  L  S  E  D  F  I  Q  G  T  Q  D  F  S  D  T  S  F  I  R  G  V  D  F  T  K  P  Q  E

CCGAGCAGCTGGTGAACAGCTTCGTGGAGAAGACGTCGGAAGGGAAGGTGAAGAGCGTCTTCAAGGATCTGAACGCCACCAGCGACCTTC 1440
A  E  Q  L  V  N  S  F  V  E  K  T  S  E  G  K  V  K  S  V  F  K  D  L  N  A  T  S  D  L

TGTTTCTTACCTCCTTCAACTTCCAAGGTAGCTGGAGGACAGCTTTCCAGCCAGAGGAGACCTCCTATCAAGAATTTCATGTGGACGAAA 1530
L  F  L  T  S  F  N  F  Q  G  S  W  R  T  A  F  Q  P  E  E  T  S  Y  Q  E  F  H  V  D  E

CAACCACAGTGATGGCTCCACTGATGACCCACACGGGTCGGTACCACTACCTGAATGATAAGGTACGGCGGTGCACAGTTGTGAAGCTGT 1620
T  T  T  V  M  A  P  L  M  T  H  T  G  R  Y  H  Y  L  N  D  K  V  R  R  C  T  V  V  K  L

CTCTGAGCAAACGCTCCTACATGCTGCTGGTCCTGCCTCATGAAGGGGCTAACCTCCACGACATAGAGTCTAAGCTGGGCACTGCTGTCA 1710
S  L  S  K  R  S  Y  M  L  L  V  L  P  H  E  G  A  N  L  H  D  I  E  S  K  L  G  T  A  V

TGTCTGACTGGCACCAGAACCTCCAGGAAGGTCTGTTGGAGCTGTCCCTCCCGAAGTTCTCCATGTCTTCCGCGACTGACATGCGCGACC 1800
M  S  D  W  H  Q  N  L  Q  E  G  L  L  E  L  S  L  P  K  F  S  M  S  S  A  T  D  M  R  D

TGCTGACCAACATTAGTCCAGAACTCGAGGCCAAACTGTTGGGCTCCGAGGCTGAGTTCAGCCAACTCAGCAACACCCAACCCTTCGCTA 1890
L  L  T  N  I  S  P  E  L  E  A  K  L  L  G  S  E  A  E  F  S  Q  L  S  N  T  Q  P  F  A

TAGATAAGGCGGTCAACAAGGTGATGTTTGAAATGTCTGAGGAAGGAGCAGAACCTCAGGACAAGATCCAGGAGGCAGGCATCCCTCTGA 1980
I  D  K  A  V  N  K  V  M  F  E  M  S  E  E  G  A  E  P  Q  D  K  I  Q  E  A  G  I  P  L

AACTCTCCATCAACAGGCCGTTCTTCTTTTCTGTCATAGAGGGGGATTCGAACGCCATCCTCATGCTGGGAAAGATCACCAACCCTACAC 2070
K  L  S  I  N  R  P  F  F  F  S  V  I  E  G  D  S  N  A  I  L  M  L  G  K  I  T  N  P  T

TCTGAAGTAACACAGTGCCCTAAAAACCAACAAAAACCTCTGACATGAAAAACAGCCAACACACGAGACCCATTTCTTTACAGTGTTCTT 2160
L

CATCGCCTACAGCTCCTGCAGTTGCTGAAATCAATCATGCGACAGATGCCTCATTTAGACCCAGCTGAAATAATTTCCACATGCAGTATT 2250

CGAGGAACGAAGCACAACCTCGCTCACAGAGGATCTATCGATATTACGCAGTGTTGCAGAAACAGTATTTATGAAAAACCTGTTACTTTA 2340

AGCTGCGTTGTTTTCAAGCAGCCACTTAAATAAATCCTAATTTGTTTTGTTTTTTTCTCCTGTAAAATTTATTATAGCAGAGTTCTGTAC 2430

AGTATTTTTCAGTAGAGTAGGTACAGTAACCAAGATTCTCTACGATCAATGACTGACGACGTTGTATATGTAACTAAGAGTTTATTTTTG 2520

TTTTGTTGCGATTGTGATTTCGTAATAAACCATTTGTACCCAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 2590
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Fig. 1. Full-length nucleotide sequence and deduced amino acid sequence of silver sea bream angiotensinogen. The putative Ang I sequence is boxed. Five possible glycosylation sites are underlined. Start and stop codons are double-underlined. Arrows with single arrow head enclose the confirmed sequence region using direct sequencing profile. Dashed underlined sequence under the single-headed arrow indicates the region where the specific primers were designed. Arrows with double arrow heads enclose the region amplified by the primers used in RT-PCR assay and the dashed underlined sequence under the double-headed arrow indicates the region where the specific primers were designed. Leucine-rich hydrophobic signal peptide precedes Ang I at the N-terminal. Poly-A tail is located at the 3′-end, indicating full-length sequence has been found.

putative sea bream Ang I was identified with an entirely novel sequence [Asn^1, Ile^5, His^9]-Ang I. Compared with other known Ang I sequences in fish species, it is most similar to the flounder-type Ang I except at position 9, which is known to be highly variable among different species (Takei et al., 2004).

In the experiment to determine tissue distribution, mRNA signals for angiotensinogen were detected in various osmoregulatory tissues including kidney and intestine. The highest signal was detected in the liver, the primary organ that produces circulating angiotensinogen. Angiotensinogen expression was not

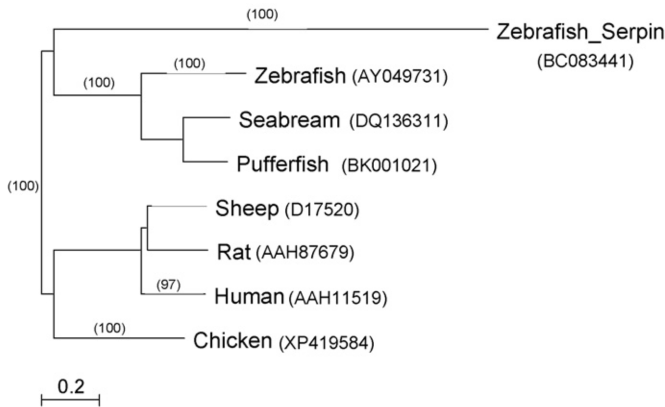

Fig. 2. Phylogenetic relationship of angiotensinogen proteins using neighbor joining. Different branch lengths on the tree indicate the proportional genetic distance between different representatives of vertebrate lineages. Numbers in the brackets on the tree indicate the bootstrap values between groups with 1000 replicates. The codes in the brackets beside the common names represent the GenBank accession numbers. Zebrafish serine protease inhibitor (Zebrafish_Serpin) is included in the phylogenetic tree to serve as an outgroup to indicate the origin of the tree.

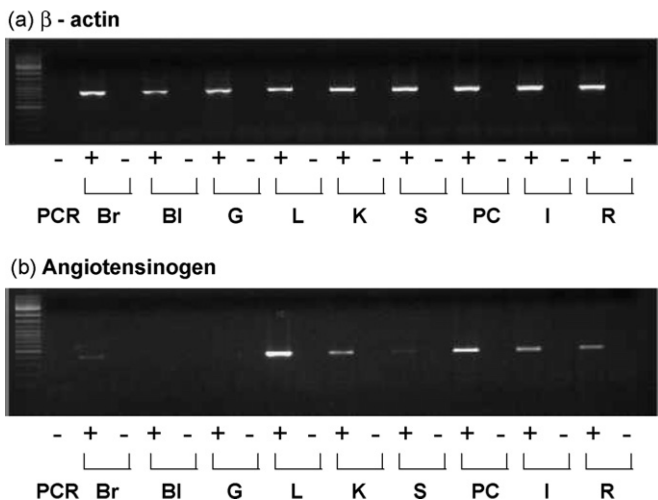

Fig. 3. Tissue distribution of: (a) β-actin and (b) angiotensinogen mRNA. PCR water was used as cDNA template for PCR −ve. RT +ve and RT −ve were prepared with and without MMLV reverse transcriptase. Br = brain; Bl = blood; G = gill; L = liver; K = kidney; S = stomach; PC = pyloric caeca; I = ileum; R = rectum.

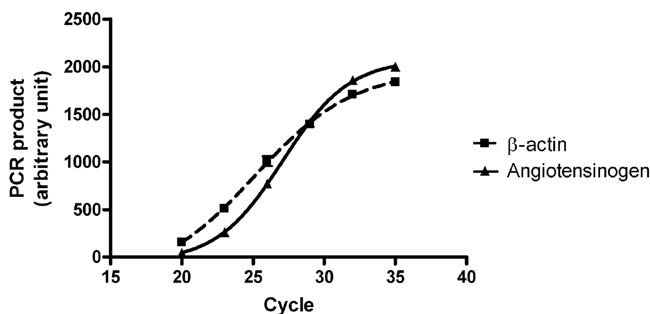

Fig. 4. Optimization of cycle numbers of semi-quantitative RT-PCR for angiotensinogen and β-actin in silver sea bream. A pooled liver RT product was used as template in the PCR reaction. The cycle numbers that gave half-maximal intensity were 27 and 25 for angiotensinogen and β-actin, respectively.

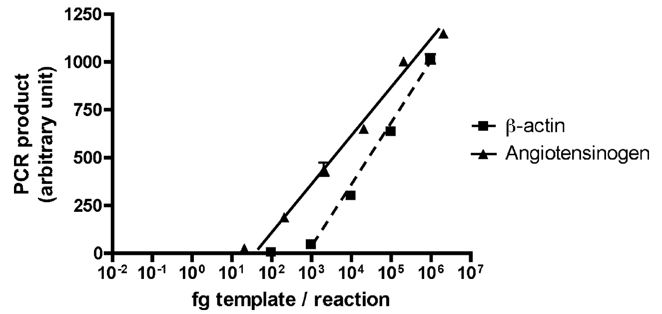

Fig. 5. RT-PCR primer efficiencies of angiotensinogen and β-actin in silver sea bream. The cycle numbers used were optimized previously, which were 27 and 25 for angiotensinogen and β-actin, respectively. Plasmid DNA of angiotensinogen and β-actin were used as template in the PCR reaction.

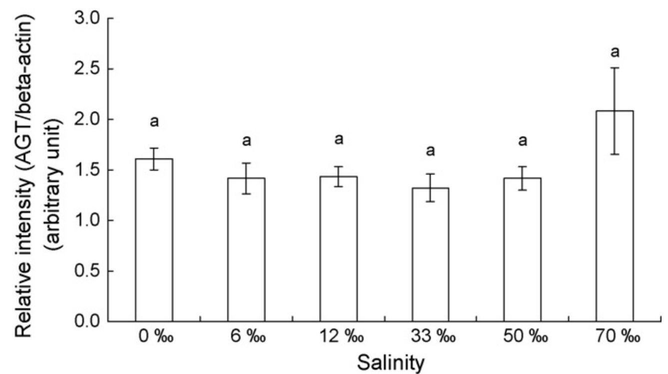

Fig. 6. Hepatic angiotensinogen mRNA expression of silver sea bream adapted to different salinities. Angiotensinogen (AGT) expression was expressed as relative intensity of PCR product of angiotensinogen per β-actin in semi-quantitative RT-PCR assay ($n = 6$). Values were not significantly different among groups after one-way ANOVA, and Tukey's test ($p > 0.05$).

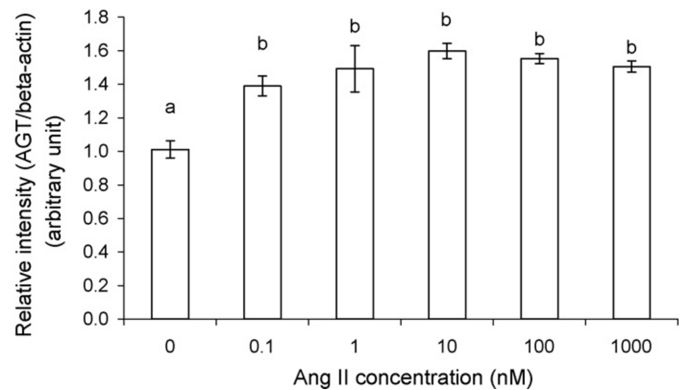

Fig. 7. Angiotensinogen (AGT) mRNA expression of isolated hepatocytes incubated with 0, 0.1, 1, 10, 100 and 1000 nM Ang II for 4 h. Angiotensinogen expression was expressed as relative intensity of PCR product of angiotensinogen per β-actin in semi-quantitative RT-PCR assay ($n = 3$). Groups with different letters are significantly different (one-way ANOVA followed by Tukey's test, $p < 0.05$).

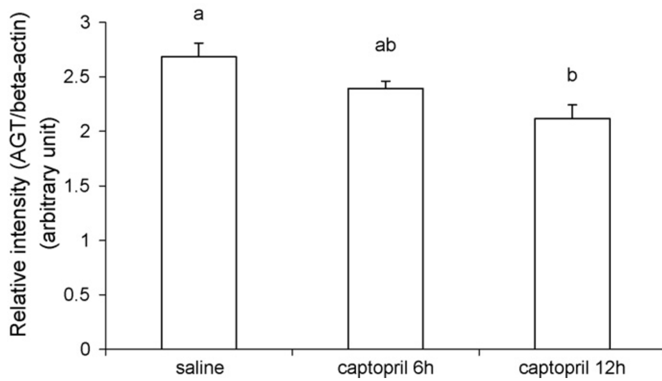

Fig. 8. Angiotensinogen (AGT) expression of liver of seawater silver sea bream injected with saline (0.8% NaCl) and captopril (100 μg/100 g) ($n = 6$). Angiotensinogen expression was expressed as relative intensity of PCR product of angiotensinogen per β-actin in semi-quantitative RT-PCR assay. Groups with different letters are significantly different (one-way ANOVA followed by Tukey's test, $p < 0.05$).

detected in the blood and gills after 32 cycles of RT-PCR and this suggests that the presence of systemic angiotensinogen is the result of secretion by other organs. The existence of a local RAS is suggested in sea bream because angiotensinogen, at different expression levels, were found in organs such as kidney and brain which have complete local regulation and expression (Brown et al., 2000; Lancien et al., 2004). Angiotensinogen was also detected in the intestine, and interestingly at different expression levels in different regions. A low expression level was found in stomach, moderate expression in ileum and rectum, and a comparatively high expression in the pyloric caeca. The presence of a RAS component in the intestine of fish is somehow unexpected and the function of the expressed angiotensinogen in different regions of the intestine is still unknown and as such, local regulation of intestinal RAS is an open area for further investigation.

Hepatic angiotensinogen expression in fish adapted to different salinities did not differ from each other, suggesting that the expression was already in excess of the requirement for secretion to maintain normal levels of circulating angiotensinogen.

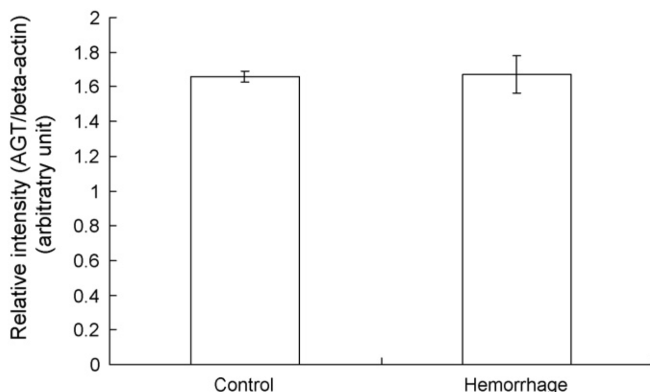

Fig. 9. Angiotensinogen (AGT) expression in liver of seawater silver sea bream after removal of 1 ml of blood (hemorrhage). Angiotensinogen expression was expressed as relative intensity of PCR product of angiotensinogen per β-actin in semi-quantitative RT-PCR assay. The control and hemorrhage groups were not statistically differed from each other.

Such a contention is further supported by the fact that the liver exhibited the highest expression of angiotensinogen among different tissues of the body. The high level of angiotensinogen expression could be associated with the maintenance of systemic angiotensinogen level and that Ang II levels may not depend on the concentration of precursor protein, but depends on renin secretion. Higher circulating levels of Ang II was found among hyperosmotic-adapted sea bream (Wong et al., 2006), but such pattern was not concurrent with the high substrate availability, possibly suggesting difference in amounts of circulating renin. However, our results point to the existence of a positive feedback control for angiotensinogen in sea bream liver since *in vitro* incubation of hepatocytes with exogenous Ang II increased angiotensinogen expression significantly. The increment was not drastic (1.5-fold in 4 h) but was similar to the levels of stimulation observed in isolated rat hepatocytes (1.4-fold in 3 h) (Klett et al., 1990) and intact rat liver (2.0-fold in 3 days) treated with Ang II (Schunkert et al., 1992). As suggested by Sernia and Reid (1980), the positive feedback of angiotensinogen by Ang II only plays a minor role in hepatic angiotensinogen secretion as the stimulation only occurred at relatively high levels of Ang II (100 nM). Nevertheless, we found that Ang II was effective in stimulating angiotensinogen expression in isolated sea bream hepatocytes at concentrations as low as 0.1 nM (Fig. 7), and this concentration was considerably lower than those reported in other studies on mammalian models. This may indicate that such positive feedback might be more potent in non-mammalian vertebrates. In a hepatocyte culture where *in vivo* Ang II stimulation has been removed, transcription of angiotensinogen can still be detected by the existing RT-PCR assay. When synthetic Ang II was added to the culture, angiotensinogen expression was stimulated at low but physiological doses (0.1 nM). Considering that the circulating levels of Ang II in sea bream adapted to different salinities ranged from 0.6 to 6.0 nM (Wong et al., 2006), the hepatocytes *in vivo* may actually experience maximal Ang II stimulation at all times. Therefore it is quite reasonable to note that sea bream adapted to different salinities possess similar hepatic angiotensinogen expression.

Captopril inhibition significantly lowered the expression of hepatic angiotensinogen of intact sea bream. Such dosage of captopril used has been previously shown to lower circulating Ang II dramatically from 1.2 to 0.1 nM (Wong et al., 2006). Since the half-life for captopril is relatively short (1–3 h oral administration) (Levy et al., 1991), we therefore extended the blockade using two injections to ensure that the ACE inhibitory effect was continuous throughout the experiment. Such treatment may prevent the sudden increase in plasma Ang II arising from the conversion of accumulated Ang I pool following ACE inhibition. The inhibition of angiotensinogen transcription by captopril *in vivo* was relatively small (∼20% at 12 h), which may be due to the fact that positive feedback in sea bream functions at low physiological levels (e.g. 0.1 nM) while residual Ang II or angiotensin metabolites in the circulation may provide a considerable level of stimulation. The small level of inhibition may also indicate that such form of positive feedback was a minor regulatory element that provides some “fine tuning” of substrate availability in the RAS. In sea bream, blood volume is

approximately 2% of the body weight (Wong et al., 2006), and 1 ml blood withdrawal from a 200–250 g fish may translate to a 25% blood volume loss. Such hemorrhage may significantly activate systemic RAS, which cause some 10-fold increase in circulating Ang II (Wong, Takei and Woo, unpublished data). In the present study, the angiotensinogen expression of fish subjected to hemorrhage remained at the same level as the control group, without any significant increase. Such observation implies that further increases in circulating Ang II *in vivo* cannot lead to further increase in hepatic angiotensinogen expression, indicating that this form of positive feedback is limited by saturated expression.

Although the stimulatory effect of Ang II on angiotensinogen expression was relatively small at the transcriptional level, such regulation was found to be a key factor in determining the status of RAS. In adrenalectomized rats that possessed high renin activity in the plasma, Dup 753 (losartan) further increased plasma renin activity but the plasma Ang II concentration remained unchanged because of the limited plasma angiotensinogen (Iwao et al., 1991). Therefore, it was suggested that an elevation in plasma angiotensinogen concentration was pre-requisite for the increase in plasma Ang II regardless of high or low levels of plasma renin activity. Under conditions of rapid conversion of angiotensinogen to Ang II, it is important to increase the production of substrate to keep pace with its consumption. In situations like hemorrhage, where a stimulation of angiotensinogen has been shown to be an essential element that allowed renin to mount an efficient response, Ang II was shown to be a regulator for angiotensinogen production (Beaty et al., 1976). However, no such effect of hemorrhage on angiotensinogen expression was found for the sea bream.

In catfish hepatocytes, the predominant Ang II receptors are pharmacologically distinct from mammalian AT_1 and AT_2 receptors and the affinity (K_d) of catfish hepatocyte Ang II receptor to [Asn^1, Val^5]Ang II is 2.7 nM, with a maximum binding capacity (B_{max}) of 185 fmol/mg protein (Oliveres-Reyes et al., 1997). In the rat liver plasma membrane, the K_d for AT_1 receptor was found to be 3.7 nM and the B_{max} was 1143.7 fmol/mg protein, which meant a lower affinity but higher binding capacity when compared with catfish hepatocytes (Del Carmen Caro et al., 1998). However, in the rat liver, distinct nuclear receptors for Ang II exist ($K_d = 1.4$ nM), and the affinity of which is higher than that found on plasma membrane (Tang et al., 1992). More importantly, the B_{max} for the nuclear receptors is extremely low (10 fmol/mg protein), representing only 460 binding sites per nucleus. Such receptor subtype has a molecular weight of 66 kD, which is the same as that of an angiotensin-binding protein previously found in rabbit liver homogenate (Sen et al., 1984). The nuclear receptor for Ang II is not membrane bound and possesses different binding characteristics when compared to the plasma membrane type (Tang et al., 1992). The nuclear receptor may displace bound Ang II at 10^{-9} M concentration in isolated nuclei of rat (Booz et al., 1992). In another study, hepatic nuclei exhibit a single high affinity ($K_d = 0.4$ nM) Ang II-specific binding site, which is associated with elevated RNA transcription (Eggena et al., 1993). Angiotensinogen transcription was increased by 2.5-fold when the hepatic nuclei were treated with Ang II. Therefore, the transcriptional control of angiotensinogen may be associated with the high affinity, but low capacity nuclear receptor subtype. There were also studies showing Ang II can be internalized (Jimenez et al., 1999) and intracellular Ang II directly regulates gene expression (Cook et al., 2001). In the present study, we demonstrated that hepatic angiotensinogen transcription can be stimulated by Ang II *in vitro* at a concentration as low as 0.1 nM. Although there is no evidence showing the presence of a nuclear Ang receptor in sea bream, further investigation may discover similar receptor complexity as observed in mammalian studies.

In conclusion, the present study not only cloned the first sea bream angiotensinogen, but also provided data to suggest that the RAS may play differential roles in various organs locally. It was also demonstrated that hepatic angiotensinogen expression exhibited no significant difference among sea bream chronically adapted to different salinities and it is possibly due to desensitization of hepatocytes *in vivo*. Our results also provided the first piece of evidence to suggest the existence of positive feedback control of angiotensinogen expression in a non-mammalian vertebrate. Though the level of increment or suppression is considerably low, the Ang II-dependent regulation of angiotensinogen expression in sea bream is highly comparable to that found in mammalian models. This indicates that such regulatory mechanism is an ancient function that has appeared early in vertebrate evolution. As such, more comparative studies on different vertebrate groups are needed to elucidate the functional significance of such form of positive regulation throughout phylogeny.

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