Characterization of a native angiotensin from an anciently diverged serine protease inhibitor in lamprey

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Abstract

Angiotensinogen belongs to family A serine protease inhibitors (SERPIN family) and we have cloned and characterized *SERPIN* genes in two lamprey species, which possess all the properties of angiotensinogen. The putative angiotensinogens in lampreys can be considered as an evolutionary link between SERPIN and other angiotensinogen according to the phylogenetic analyses. The inferred sea lamprey angiotensinogen gene was expressed abundantly in liver and to a lesser extent in other tissues. The predicted lamprey angiotensin I (Ang I) sequence was unique and different from the teleost-type Ang I identified previously by the incubation of lamprey plasma with its kidney extract. Therefore, we characterized and compared the biochemical and physiological properties of this native lamprey Ang II (LpAng II) (EEDYDERPYMQPF) with teleost-type Ang II (NRVYVHPF). Using a newly developed

RIA for LpAng II, plasma levels in Japanese lamprey were measured (157.4 ± 35.2 fmol/ml, $n=6$), but teleost-type Ang II was undetectable. In conscious cannulated lamprey, LpAng II at 100 pmol/kg elicited a transient vasodepressor effect. At doses higher than 300 pmol/kg, a biphasic cardiovascular response with an initial vasodepressor effect followed by a transient rebound vasopressor effect was observed in a dose-dependent manner. However, teleost-type Ang II was not vasoactive up to 1 nmol/kg. In Japanese eel, LpAng II injection up to 3 nmol/kg did not alter the cardiovascular parameters. Our results suggested that the renin–angiotensin system first appeared in cyclostomes, and LpAng II could be important for the regulation of cardiovascular dynamics in lampreys because of its potent and acute vasoactive effect.

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Introduction

The renin–angiotensin system (RAS) is important for the regulation of salt and water balance in vertebrates (Olson 1992, Kobayashi & Takei 1996). Angiotensinogen, the precursor protein of the peptide cascade system, is highly variable among vertebrates, but reasonable homology is observed in angiotensin I (Ang I) sequences located at the N-terminus (Watanabe *et al.* 2009). The presence of the RAS in cyclostomes has been the subject of a long debate (Nishimura *et al.* 1970, Nishimura 1985). Several studies have shown that RAS components including Ang I, Ang II, and angiotensin-converting enzyme-like (ACE-like) activity were present in river lamprey (*Lampetra fluviatilis*) and sea lamprey (*Petromyzon marinus*; Rankin *et al.* 2001, 2004, Cobb *et al.* 2002, Takei *et al.* 2004). However, the majority of ACE-like activity was found in the brain of river lamprey (Cobb *et al.* 2002) instead of the gills as in teleost fishes (Olson 1992). Furthermore, [Asn¹, Val⁵, Thr⁹]-Ang I, which is similar to the Ang Is of other teleosts, was isolated from the incubation of plasma (angiotensinogen source) and kidney extract (renin source) in the river lamprey (Rankin *et al.* 2004) and sea lamprey (Takei *et al.* 2004). However, vasopressor

effect of [Asn¹, Val⁵]-Ang II was extremely low in river lampreys (Rankin *et al.* 2004), and i.p. injection of Ang II (163 nmol/kg) or papaverine, a stimulant of endogenous RAS in various vertebrate species (Tierney *et al.* 1995a), failed to stimulate drinking behavior (Rankin *et al.* 2001). Therefore, it is still not clear whether [Asn¹, Val⁵]-Ang II exerts any physiologically relevant effect in lampreys.

To date, molecular data on the RAS in lamprey are limited. In the sea lamprey genome, we have identified a putative angiotensinogen gene that belongs to family A serine protease inhibitor (SERPIN family), and the putative protein possesses an Ang I that has a sequence that is different from [Asn¹, Val⁵, Thr⁹]-Ang I. The discrepant Ang I sequences from biochemical and molecular data have prompted us to embark on the present investigation. In the present study, we aim to provide evidence to show that the genome-identified angiotensinogen gives rise to a native functional angiotensin in lamprey. Specific RIA was developed to measure the plasma level of the newly identified lamprey Ang II (LpAng II). Cardiovascular functions of LpAng II were examined in conscious lamprey. The present result provides important insights on the evolutionary origin of the RAS and its functional role in vertebrates.

Materials and Methods

cDNA cloning and genome survey of angiotensinogens in vertebrates

Initially, a putative angiotensinogen gene/SERPIN was identified in Sea Lamprey Genome database (contig1818). The contig sequence was subjected to analysis by GENESCAN program to identify putative cDNA. Sea lamprey (*P. marinus*) and Japanese lamprey (*Lethenteron japonicum*) tissues and cDNA were obtained from previous collections of our laboratory (Wong & Takei 2009). Specific primers (SL_AGT_F1: 5'-CTACATG-CAGCCCTTCCATCTAATTC-3' and SL_AGT_R1 5'-GAAGATCACAGCTCTCGTTTTCTCATC-3') were designed from putative coding regions to amplify partial fragments of angiotensinogen in sea lamprey and Japanese lamprey. To clone the full-length cDNA of angiotensinogen in both lamprey species using rapid amplification of cDNA ends, liver cDNA was prepared in each case using a SMART cDNA Library Construction kit (Clontech Laboratories) according to the manufacturer's protocol. PCR was performed using an ABI 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) with Takara ExTaq DNA Polymerase reagents (Takara Bio, Inc., Otsu, Shiga, Japan). PCR products were electrophoresed on 1.2% agarose gels stained by ethidium bromide, excised, extracted using an UltraClean 15 DNA Purification kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA), cloned into pT7blue vector (Novagen, Madison, WI, USA), and sequenced using a BigDye Terminator Cycle sequencing kit and an ABI 3130 DNA sequencer (Applied Biosystems). The full-length angiotensinogen sequence of sea lamprey was aligned with the genomic contig1818 to resolve the exon–intron structure.

Tissue distribution of angiotensinogen mRNA

Total RNA samples were obtained from various tissues including brain, gill, atrium, ventricle, liver, anterior kidney, posterior kidney, anterior intestine, posterior intestine, skeletal muscle, and skin using Isogen RNA extraction system (Nippongene, Toyama, Japan). RNA samples were reverse-transcribed to cDNA using SuperScript III First-Strand Synthesis for RT-PCR (Invitrogen) according to the manufacturer's protocol. Specific primers were designed spanning across introns to prevent amplification of genomic DNA (SL_AGT_Exp_F: 5'-GTCAATGTCATTTATTTCAAAGGATCGTG-3' and SL_AGT_Exp_R: 5'-TATGAGTGATCTTGTCCACGTGTAGCTT-3'). PCR was performed using an ABI 9700 thermal cycler (Applied Biosystems) with Takara ExTaq DNA polymerase reagents (Takara Bio, Inc.), and PCR products were electrophoresed on 1.2% agarose gel stained by ethidium bromide.

Phylogenetic analysis and mature peptide prediction

Extensive genome search was performed to collect most of the available angiotensinogen or putative angiotensinogen

sequences from each species under genome project coverage. Angiotensinogen sequences that are available on the GenBank nucleotide and expressed sequence tag (EST) databases were also collected for phylogenetic reconstruction. Full-length amino acid sequences of various species were aligned using Mega version 4, and a phylogenetic tree was constructed using the neighbor joining method (JTT) and maximum parsimony (Tamura *et al.* 2007). A bootstrap test was performed with 1000 replicates to validate the phylogenetic relationships. To further validate the phylogenetic relationship, the same alignment was analyzed by Bayesian method (Ronquist & Huelsenbeck 2003) to generate an alternative phylogenetic tree for comparison. The signal peptides of angiotensinogens in *P. marinus* and *L. japonicum* were predicted using neural networks and hidden Markov model. Homologous regions of vertebrate Ang II and LpAng II were aligned to identify putative Ang II in lampreys.

Antibody production for LpAng II

LpAng II (EEDYDERPYMQPF) was synthesized (Peptide Institute, Inc., Ibaraki, Osaka, Japan) according to the predicted sequence of Japanese lamprey. LpAng II was N-terminal conjugated to keyhole limpet hemocyanin (KLH) using glutaraldehyde as a catalyst, and the protein complex was used in immunization in rabbits to produce polyclonal antibodies. The rabbits were first immunized with Freud's complete adjuvant followed by two booster injections with Freud's incomplete adjuvant. The interval between each injection was 3 weeks. Titers of antibodies were monitored after each bleed by enzyme immunoassay. The rabbits were killed for final bleed after the third immunization.

Development of a RIA for LpAng II

LpAng II was radiolabeled by ^{125}I using the lactoperoxidase method (Marchalonis 1969). ^{125}I -LpAng II was diluted in RIA buffer containing 0.1% RIA grade BSA to produce 10 000 c.p.m. per assay tube. The titers of the antibodies were determined by serial dilution, and maximum binding was adjusted at 20% of the total binding. Incubation of standard peptides and unknown samples with radiolabeled tracer was carried out at 4 °C for 24 h. Separation of free and bound fractions was achieved by precipitation using a secondary antibody, goat anti-rabbit IgG (Sigma), and 16% polyethylene glycol followed by centrifugation at 3000 g for 1 h at 25 °C and then 30 min at 4 °C. Unbound fractions were removed by aspiration, and specific binding in the pellet was measured using a gamma counter (Perkin Elmer, Waltham, MA, USA). A standard curve ranging from 10 to 100 000 fmol/ml was constructed. Cooperativity of antibody binding was observed at 10–300 fmol/ml region, resulting in a specific binding over maximum binding. Therefore, unknown samples were serially diluted during measurement to delineate between specific displacement and no displacement. Specificity of the LpAng II RIA was verified by measuring the cross reactivity of LpAng II antibody to [Asn¹, Val⁵]-Ang II and [Asn¹, Pro³, Ile⁵]-Ang II.

Qualitative and quantitative analyses of different angiotensins in lamprey plasma

Upstream migrating adult Japanese lampreys were obtained from local fishermen of Niigata prefecture. All upstream migrating lampreys have regressed intestines and full-grown gonads. They were kept in a freshwater recirculating system maintained at 10 °C. All animal experimentation procedures were approved by the Animal Experiment Committees of the University of Tokyo and performed in accordance with the Manual of Animal Experiments prepared by the Committee. Lampreys were anesthetized using 0.1% ethyl 3-aminobenzoate methanesulfonate (Sigma) neutralized by sodium bicarbonate until ventilation movement ceased. Blood samples were withdrawn by heart puncture into syringes containing an inhibitor cocktail (0.05 M 1,10-phenanthroline, 0.225 M potassium EDTA, and 0.1 TIU aprotinin) that inhibits peptide degradation. Blood samples were centrifuged immediately at 8000 *g* for 5 min, and plasma fractions were frozen at −20 °C until use.

Plasma samples were extracted by equal volumes of acidic acetone (acetone:water:1 M HCl=40:5:1; Brown *et al.* 2005). The partially purified plasma was further resolved by reverse-phase HPLC utilizing an analytical column

(ODS 100 V column, 5 µm, 4.6 mm I.D. × 25.0 cm, Tosoh, Tokyo, Japan). A linear gradient from 15 to 35% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min over 40 min was used for separation, and a single peptide sample could be eluted within 0.4 min under this profile. HPLC fractions were collected at 1 min intervals and dried by lyophilization. The HPLC-resolved fractions were reconstituted in RIA buffer for the measurement of [Asn¹, Val⁵]-Ang II (Tsuchida & Takei 1998) and LpAng II using specific RIAs for each peptide. All measurements were performed in duplicate.

Effects of LpAng II on ventral aortic pressure in lamprey and eel

Japanese lampreys were anesthetized as described above. The ventral aorta was cannulated (SP31 polyethylene tubing, I.D. 0.5 mm, O.D. 0.8 mm, Natsume, Tokyo, Japan) using a procedure modified from the cannulation of eel (Tsuchida & Takei 1998). After operation, the lampreys were allowed to recover from surgical stress in an elongated plastic box with constant supply of aerated freshwater at 10 °C for 24 h before injections.

The ventral aortic cannula was filled with heparinized saline (200 U/ml, 0.9% NaCl) and connected to a DX-300 pressure transducer (Nihon Koden, Tokyo, Japan) coupled to

	↓	
<i>Petromyzon marinus</i>	MKLFILLLAFCAALCSPCVGEEDYDDRPYMQPFHLIPPLSVQATEQPLASNETWDYPE	60
<i>Lethenteron japonicum</i>	MKLFILLL-AFCAALCSPCVGEEDYDERPYMQPFHLIPPLSVQVTEQPLASNETWDYPE	59

<i>Petromyzon marinus</i>	PLAPGQSPAASSEEGSSEEGKDERESHGRGRRGRKDKYKSKTORIASAVNGLGFRLYKQ	120
<i>Lethenteron japonicum</i>	PLAPGQSPAASSEEGSSEEGKDERDPHRDEGKGRKDKKSKTORIASAVNGLGFRLYKQ	119

<i>Petromyzon marinus</i>	VLGGAGPADNIFFSPLSISALGVVAAGANGSTRAELDTALGFKELLHGKKKAKSMKYFA	180
<i>Lethenteron japonicum</i>	VLGGAGPADNIFFSPLSISALGVVTVGANGSTRAELDTALGFKEFLHGKKKAKSMKYFA	179

<i>Petromyzon marinus</i>	RLNSALYRRSAGFELMGKNVVFSSKGLWLYRQFTRTVAHLFKSNVRSVDFGESKEAVELM	240
<i>Lethenteron japonicum</i>	RLNGALYRRSAGFELMGKNVVFSSKGLWLYRQFTRTVAHLFKSNVRSVDFGDSKNAVELM	239
	, **	
<i>Petromyzon marinus</i>	NAYIEKVTSKKFTDVI SDVDTATSLMIVNVIYFKGSWANKFEPDLTKNVRFWVNSSYSMM	300
<i>Lethenteron japonicum</i>	NAHIEKVTSKKFPDVI SDVDTDTSLVIVNVIYFKGSWENKFEPDLTKNVRFWVNSSYSMM	299
	, **	
<i>Petromyzon marinus</i>	VPTMHQRAKLSYAQDRKLSTVIKLPYEGGASMLVIVPHRTEELPKVEESVSQEQLEEWL	360
<i>Lethenteron japonicum</i>	VPTMHQRAKLSYTQDRKLSTVVKLPYEGGASMLVIVPHRTEDLPKVEESVSQEQLEEWL	359

<i>Petromyzon marinus</i>	SLLGPSNHYVQLSLPKFKISVSYDLKAYLSAMGMPSMFSYGADLSRITGMQKLHVDKITH	420
<i>Lethenteron japonicum</i>	SLLGPSNHYVQLSLPKFKISVSYDLKAYLSAMGMSSMFSYGADLSRITGMQKLHVDKITH	419

<i>Petromyzon marinus</i>	KSVLHVNEEGTEAKAETVVGIMPI SMPPTVTVDPRPFVVL IYDEKTRAVIFMGRVADPKQ	479
<i>Lethenteron japonicum</i>	KSVLHVNEEGTEAKAETVVGIMPI SMPPTVTVDPRPFVVL IYDEKTRAVIFMGRVADPKQ	478

Figure 1 Amino acid sequence comparison between the angiotensinogens of sea lamprey (*Petromyzon marinus*) and Japanese lamprey (*Lethenteron japonicum*). Putative lamprey Ang I sequences are boxed. Predicted signal peptide cleavage site is indicated by an arrow. Putative N-glycosylation sites are underlined, and the shaded region represents feature motif of SERPIN.

Species names	Ang I	Ang I nucleotides	
<i>Oncorhynchus mykiss</i>	NRVYVHPFNL	AACCGTGTATATGTCACCCGTTTAACTC	Teleost
<i>Rhabdosargus sarba</i>	NRVYIHPFHL	AACCGGTCTACATTACCCCTTCCATCTC	
<i>Callorhynchus milii</i>	NRPHIHPFLI	AACCGACCCACATTATCCTTTCTCCTC	
<i>Triakis scyllium</i>	NRPYIHPFOL	AACCGGCCCTACATTACCCCTTCTCTTG	Chondrichthyan
<i>Dasyatis akajei</i>	DRPYIHPFFL	GACCGGCCCTACATCCACCATTTTTTTTA	
<i>Leucoraja erinacea</i>	YRPYIHPFSL	TACCGGCCCTACATCCACCATTTAGTCTG	
<i>Petromyzon marinus</i>	EEDYDDRPYMQPFHL	GAAGAGGACTACGACGATCGGCCCTACATGCAGCCCTTCCATCTA	Lamprey
<i>Lethenteron japonicum</i>	EEDYDERPYMQPFHL	GAAGAGGACTACGACGAGCGGCCCTACATGCAGCCCTTCCATCTA	
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Figure 2 Alignments of several Ang Is of different vertebrate representatives at the amino acid and nucleotide levels. Difference in corresponding amino acid sequence in lamprey Ang I can be attributed to the single-base changes at the nucleotide level. Shaded regions in amino acid and nucleotide alignment indicate a conserved codon usage among lamprey and chondrichthyan species.

a monitoring system (MTS00658, Medical Try System, Tokyo, Japan) via a three-way stopcock for the measurement of blood pressure and heart rate. The same cannula was used for drug administration and blood pressure measurement. Cultured Japanese eels were cannulated for measurement of ventral aortic pressure using the same procedure as for lamprey. LpAng II and [Asn¹, Val⁵]-Ang II were diluted in saline (0.9% NaCl), and bolus injections containing various

doses of the peptides were administered in 50 µl followed by a flush of 50 µl saline.

Statistical analysis

Changes in average blood pressure and pulse pressure of the lamprey and eel after injections were analyzed by two-way ANOVA followed by Bonferroni test.

Figure 3 Exon–intron structures of angiotensinogens (AGT) of sea lamprey and other vertebrate representatives. The gene structure of lamprey was reconstructed by aligning cloned cDNA against the genomic contig. Angiotensinogen gene structures of *Homo sapiens* (NG_008836), *Gallus gallus* (XM_419584), *Anolis carolinensis* (Scaffold_200), *Xenopus tropicalis* (Scaffold_JG14), and *Danio rerio* (BC095585) were obtained or predicted from GenBank or Ensembl genome browser. Putative lamprey angiotensin I (LpAng I) and other vertebrate Ang I, indicated by the filled boxes, are located at the homologous position of exon 1.

Figure 4 Distribution of angiotensinogen (AGT) mRNAs in various tissues of sea lamprey. GAPDH was used for internal control. B, brain; G, gill; A, atrium; V, ventricle; L, liver; K, kidney; AK, anterior kidney; AI, anterior intestine; PI, posterior intestine; T, testis; M, skeletal muscle and SK, skin.

Results

Cloning, tissue expression, and phylogenetic analysis of lamprey angiotensinogen

The alignment of sea lamprey and Japanese lamprey angiotensinogen is shown in Fig. 1. Both amino acid sequences showed typical angiotensinogen characteristics, including a putative Ang I sequence after the signal peptide, glycosylation sites for addition of sugar chain, and a SERPIN-specific motif at the C-terminal region. The amino acid identities of sea lamprey angiotensinogen to those of elephant fish (*Callorhynchus milii*) and zebrafish (*Danio rerio*) were only 24 and 26% respectively. However, the putative Ang I sequences of both lamprey species showed a moderate homology to other vertebrate representatives at the amino acid and nucleotide levels (Fig. 2). Among elasmobranchs and lamprey, a codon usage bias was observed in the nucleotide region encoding [Arg²] and [Pro³] of elasmobranch Ang Is. Despite high degeneracy of codon usage in [Arg] and [Pro], identical nucleotide sequence was found between lamprey and elasmobranchs. Although considerable amino acid substitutions are present among the Ang I sequences, the nucleotide alignment showed that all the corresponding substitutions can be attributed to single base changes. The gene structure of lamprey angiotensinogen is highly comparable to that of other vertebrate representatives from fish to mammal (Fig. 3), including those of chondrichthyans (Watanabe *et al.* 2009). Lamprey angiotensinogen was expressed in various tissues including brain, atrium, kidney, intestine, and muscle, and its expression in liver was relatively higher than other tissues (Fig. 4). The phylogenetic tree (Fig. 5) showed that lamprey angiotensinogens were closely related to SERPIN A family and chondrichthyan angiotensinogens. The phylogenetic trees inferred by neighbor joining, maximum parsimony, and Bayesian methods had the same topology, and therefore only the former was shown.

Characterization of different Ang II forms in lamprey plasma

The polyclonal antibodies raised against LpAng II exhibit positive cooperativity binding as shown in the standard curve (Fig. 6). The specific binding, ranging from 10 to 300 fmol/ml, was slightly higher than that of maximum binding. In practice, unknown samples were serially diluted

for measurement to validate specific bindings. The LpAng II assay is highly specific, with only 0.14 and 0.53% cross reactivities to teleost [Asn¹, Val⁵]-Ang II and elasmobranch [Asn¹, Pro³, Ile⁵]-Ang II respectively. Intra-assay and inter-assay coefficients of variations were 4.4 and 12.3% respectively ($n=8$). The LpAng II assay has a detection range of 10–3000 fmol/ml.

A pooled plasma sample from three individuals was shown to exhibit positive cooperative characteristics, and the curve derived from serial diluted plasma was parallel to the standard curve of LpAng II (Fig. 6). Average plasma levels of LpAng II were 157.4 ± 35.2 fmol/ml ($n=6$), but [Asn¹, Val⁵]-Ang II was undetectable in the same plasma samples (Fig. 7). In the

Figure 5 Phylogenetic tree of vertebrate angiotensinogen depicted by the neighbor joining method. Numbers on the branches are the bootstrap values of 1000 replicates. Scale bar represents 20% amino acid substitution. *Danio* SERPIN A was used as an outgroup. GenBank accession numbers or genome contig numbers are as follows: *Tursiops* (Scaffold_106652), *Bos* (NM_001114082), *Canis* (XM_536348), *Myotis* (Scaffold_12156), *Equus* (XM_001496084), *Oryctolagus* (GeneScaffold_2959), *Rattus* (BC078741), *Cavia* (Scaffold_16), *Mecaca* (Chromosome 1), *Pongo* (Chromosome 1), *Homo* (NM_0000029), *Gorilla* (AF188488), *Echinops* (GeneScaffold_3833), *Monodelphis* (Chromosome 2), *Xenopus* (Scaffold_JG14), *Anolis* (Scaffold_200), *Gallus* (XM_419584), *Meleagris* (Chromosome 2), *Taeniopygia* (Chromosome 3), *Anas* (Scaffold_759), *Danio* (BC095585), *Takifugu* (BK001021), *Plecoglossus* (AM921805), *Salmo* (NM_001140446), *Oryzias* (Chromosome 15), *Rhabdosargus* (DQ136311), *Gasterosteus* (GroupVI), *Callorhynchus* (AB433535), *Triakis* (AB433533), *Dasyatis* (AB433534), *Leucoraja* (AB433536), *Petromyzon* (GU566000), *Lethenteron* (GU391575), *Lampetra* (FM954978), and *Danio* SERPIN A (BC075902).

Figure 6 A standard curve of the RIA of lamprey Ang II (LpAng II). The RIA exhibits cooperativity characteristics resulting in increased specific binding at particular concentration. Serially diluted lamprey plasma samples pooled from three individuals show parallelism to the standard curve. Upper scale shows the dilution factors of the pooled plasma sample.

same sets of plasma HPLC fractions, immunoreactive LpAng II was detected at the elution position identical to that of synthetic standard. The LpAng II RIA did not detect any extra peaks in the HPLC-resolved plasma fractions, indicating a high specificity of the developed assay as well as adequate protection of sample from peptide degradation.

Cardiovascular effects of different Ang II forms in lamprey and eel

The average ventral aortic pressure of Japanese lamprey was 19.4 ± 1.3 mmHg, which was similar to the reported values in other lamprey species (Donald 1998, Farrel 2007). LpAng II elicited a dose-dependent, biphasic response in ventral aortic pressure in Japanese lamprey (Fig. 8). Bolus injection of LpAng II induced a transient decrease in blood pressure followed by a rebound increase in blood pressure. The original blood pressure tracing showed that the initial vasodepressor response occurred within 1–2 min, and the rebound increase started 2–3 min after the injection. Furthermore, the vasodepressor effect was more potent, as significant pressure drop was observed with a dose of 100 pmol/kg but the rebound increase was only significant with higher doses starting from 300 pmol/kg when compared with the resting levels. Despite a prominent change in blood pressure, the heart rate was not altered during the biphasic response. After the rebound increase in blood pressure, there was a significant elevation in the pulse pressure at doses starting from 300 pmol/kg. We did not include the cardiovascular data of doses higher than 1000 pmol/kg because temporal cardiac arrest was sometimes observed, and then the lamprey started undulating violently within the plastic box when higher doses of LpAng II were administered. On the other hand, $[\text{Asn}^1, \text{Val}^5]$ -Ang II did not change aortic pressure of lamprey at a series of doses up to 1 nmol/kg (Fig. 9).

To show the species specificity of LpAng II, Japanese eel was used to study the possible effects of LpAng II in teleost. In cannulated eel, LpAng II did not produce any vasoactive response at all doses up to 3 nmol/kg (Fig. 10). Homologous $[\text{Asn}^1, \text{Val}^5]$ -Ang II injection in eels at 1000 pmol/kg induced a profound increase in ventral aortic pressure, serving as a positive control for the responsiveness.

Discussion

In previous studies to demonstrate the presence of RAS in lamprey, plasma and kidney extract of river and sea lamprey were incubated, and using an eel vasopressor bioassay, $[\text{Asn}^1, \text{Val}^5, \text{Thr}^9]$ -Ang I was isolated and identified as the homologous Ang I in lampreys (Rankin *et al.* 2004, Takei *et al.* 2004). However, we recently cloned the putative angiotensinogen genes in sea lamprey and Japanese lamprey respectively according to the genome information. Angiotensinogen belongs to the member 8 of SERPIN family A, and the putative lamprey angiotensinogen possesses a

Figure 7 Immunoreactive Ang II and LpAng II in lamprey plasma measured by respective RIAs after reverse-phase HPLC fractionation. Plasma samples from six individuals were examined. Arrows indicate the elution positions of synthetic $[\text{Asn}^1, \text{Val}^5]$ -Ang II, $[\text{Asn}^1, \text{Ile}^5]$ -Ang II, and LpAng II.

Figure 8 Dose-related effect of bolus injection of LpAng II on ventral aortic pressure (BP; B), pulse pressure (PP; C), and heart rate (HR; D) in Japanese lamprey. A shows an example of the original tracing of ventral aortic pressure and heart rate recording. Numbers in parentheses indicate the number of observations in each treatment. Values are shown as mean $\pm$ S.E.M. Significant differences are denoted by asterisks (two-way ANOVA, Bonferroni test, *** $P < 0.001$).

SERPIN-specific motif at the C-terminal region as well as three putative *N*-glycosylation sites, which are common characteristics of angiotensinogen in vertebrates (Irving *et al.* 2007). In addition, the exon-intron structure of the lamprey angiotensinogen was highly comparable to that of other vertebrate species. The putative LpAng I sequences exhibit

moderate homology to those of other Ang Is (Fig. 1), but the sequences were different from that previously isolated using biochemical method. The codon usage bias for [Arg²] and [Pro³] in elasmobranch Ang Is was also observed in lamprey, indicating that the Ang Is of these lineages may have high evolutionary constraint in such motif. Phylogenetic analysis further revealed that the lamprey angiotensinogens were closely related to SERPIN A family, but to a lesser extent to the angiotensinogens of the chondrichthyan lineage. Although the position of lamprey angiotensinogens in the phylogeny was closer to SERPIN A than other angiotensinogens, the lamprey angiotensinogens could be considered as an evolutionary link between the two sister groups, suggesting that lamprey may possess the earliest angiotensinogen in extant vertebrates. It would be interesting to examine whether hagfish possess similar angiotensinogen to that of lamprey.

Analysis of tissue distribution of lamprey angiotensinogen mRNA revealed its expression in various types of tissues including brain, heart, liver, kidney, intestine, and skeletal muscle, with relatively higher expression in the liver. The angiotensinogen expression pattern in lamprey was similar to that of other fish species such as seabream (Wong *et al.* 2007), ayu (Chen *et al.* 2008), and dogfish (Watanabe *et al.* 2009). The liver is the major site of angiotensinogen production in most vertebrate species, though other tissues such as kidney and brain also produce angiotensinogen as a local RAS for supplying Ang II in a paracrine manner (Paul *et al.* 2006). The widespread distribution of angiotensinogen gene expression, as observed in lamprey and other fishes, implies that circulating angiotensinogen could be generated by various organs in early diverged vertebrates and that the liver subsequently became the major organ for angiotensinogen production.

In the present study, the RIA developed for LpAng II exhibited cooperativity characteristics. As shown in Fig. 6, the specific bindings of samples and standards were up to 20% higher than those of maximum binding (B_0) ranged between 10 and 300 fmol/ml, which is a rare but well-documented phenomenon called cooperative immunoassay (Ehrlich *et al.* 1982). In cooperative RIA, the specific binding may exceed the maximum binding because of the formation of an ultra-stable antigen-antibody complex that probably exists as a tetramer or circular complex (Moyle *et al.* 1983a,b). Cooperativity is commonly observed when two or more monoclonal antibodies, which possess binding sites for different epitopes that are close to each other on the antigen, are mixed in the immunoassay (Ehrlich *et al.* 1982). It is uncommon for a polyclonal antibody to exhibit cooperativity. LpAng II has two consecutive hydrophilic amino acid sequences, [Glu¹-Glu²-Asp³] and [Asp⁵-Glu⁶-Arg⁷], which are separated by a [Tyr⁴] residue. The two hydrophilic groups are predicted to be highly antigenic (Hopp & Woods 1981); thus, antibodies may recognize these epitopes separately. Because of the closely linked antigenic epitopes in LpAng II, the polyclonal antibodies may possess cooperative characteristics similar to those of a monoclonal antibody mixture

Figure 9 Dose-related effect of bolus injection of [Asn¹, Val⁵]-Ang II on ventral aortic pressure (BP; B) and pulse pressure (PP; C) in Japanese lamprey. A shows an example of the original tracing of ventral aortic pressure and heart rate (HR) recording. Numbers in parentheses indicate the number of observations in each treatment. Values are shown in mean $\pm$ S.E.M.

(Ehrlich & Moyle 1984). Furthermore, cooperativity increased the specificity and sensitivity of the RIA, as non-specific bindings are less likely to possess such cooperativity characteristics (Cheong *et al.* 1990). The RIA developed for LpAng II was highly specific, as the cross reactivities to other fish-type Ang IIs were low ($<1\%$). Using the newly developed RIA, plasma LpAng II concentration measured in Japanese lamprey (157.4 ± 35.2 fmol/ml) is similar to the Ang II levels reported in other fishes (Tierney *et al.* 1995b, Tsuchida & Takei 1998).

It was previously found that [Asn¹, Val⁵]-Ang II could be circulating in river lamprey at a range between 200 and 1000 fmol/ml (Brown *et al.* 2005). In the present study, however, [Asn¹, Val⁵]-Ang II was not detected by our RIA system (detection limit 5 fmol/ml) in the plasma of Japanese lamprey caught during upstream migration (Fig. 7). Here, we hypothesize that the [Asn¹, Val⁵]-Ang II found in lamprey plasma is not the native lamprey form, but could be related to

a blood-feeding diet, as mature lampreys are ectoparasites of teleosts. The Japanese lamprey ceases feeding, and the intestine has regressed before the upstream migration begins, but the river lamprey used in previous studies was reported to have a functional intestine capable of acclimation to half-strength seawater (Brown *et al.* 2005). We have performed a parallel study to show that teleost-type angiotensin components were present in the buccal gland secretion of the lamprey, which could be a possible source of teleost-type angiotensin (MKS Wong, SA Sower, D Berlinsky & Y Takei, in preparation).

To further investigate the inconsistency between LpAng II and [Asn¹, Val⁵]-Ang II, the vasoactive properties of these peptides in Japanese lamprey and eel were examined. LpAng II produced a dose-dependent, biphasic response in the ventral aortic pressure of lamprey. Upon the bolus injection of LpAng II, ventral aortic blood pressure decreased immediately, followed by a rebound increase in blood pressure. An increase in pulse pressure was observed during the rebound phase, which could be due to an increase in cardiac output and/or systemic resistance. The biphasic response resembled the action of homologous Ang II in fowl (Nishimura *et al.* 1982) but not those of other mammals and fishes (Khosla 1985). It was shown that the vasodepressor action of Ang II in bird was endothelium-dependent, nitric oxide-independent, and signaling through prostaglandin (Nakamura *et al.* 1982). The vasopressor effect of Ang II in fowl is mediated through the α -adrenergic pathway and is independent of the vasodepressor effect (Nishimura *et al.* 1994). However, information on lamprey cardiovascular dynamics and regulation is limited (Farrel 2007). The blood vessels in fish are rarely innervated, and the autonomic nervous system is poorly developed (Donald 1998). The subendothelial layer in lamprey cardiocytes is not innervated but contains catecholamine granules, resulting in a humoral basis of adrenergic stimulation in lamprey (Augustinsson *et al.* 1956, Falck *et al.* 1966). Vagal stimulation in lamprey accelerates heart rate through nicotinic receptors, but cardiac vagal stimulation typically induces a decrease in heart rate through muscarinic receptors in other vertebrates. Stimulation of β -adrenergic receptors also increased heart rate and pulse pressure in lamprey (Lignon 1979) via the modulation of intracellular calcium availability (Shiels *et al.* 2002, Vornanen *et al.* 2002). In isolated lamprey aortic ring constricted by endothelin, and prostaglandin but not nitric oxide produced significant vasodilation (Evans & Harrie 2001). Based on available information, direct comparison between the angiotensin effect in bird and lamprey is inappropriate because of the intrinsic differences of their cardiovascular systems. Unlike the Ang II-induced non-adrenaline effect in bird, the rebound increase could be a secondary response to the acute vasodepressor effect. Upon injection of LpAng II at doses higher than 1000 pmol/kg, lampreys started undulation movements, which could be a response to increase the venous return to restore the drop in blood pressure. The restoration of blood pressure is unlikely

Figure 10 Dose-related effect of bolus injection of LpAng II on ventral aortic pressure (BP) in Japanese eel. Homologous [Asn¹, Val⁵]-Ang II injection was used as a positive control to confirm the responsiveness. Upper panel shows examples of the original tracing of ventral aortic pressure and heart rate (HR) recording. Numbers in parentheses indicate the number of observations in each treatment. Values are shown in mean $\pm$ S.E.M. Significant differences are denoted by asterisks (two-way ANOVA, Bonferroni test, *** $P < 0.001$).

related to β -adrenergic stimulation, as the heart rate remained unchanged. To date, the cardiovascular dynamics in lamprey is not well understood, possibly due to the lack of homologous hormones. We demonstrated that LpAng II could be a potent cardiovascular regulator, but further work is required to investigate its signaling pathways and physiological functions. Isolated blood vessel studies should be performed to elucidate the signaling pathways of LpAng II. Furthermore, possible involvement of LpAng II in kidney functions should be investigated, as Ang II is highly involved in the regulation of glomerular and tubular functions in other vertebrates (Cobb *et al.* 1999).

It was previously shown that [Asn¹, Val⁵]-Ang II was a vasopressor in river lamprey, but the minimum effective dose was extraordinarily high (100 000 pmol/kg) and [Asn¹, Val⁵, Thr⁹]-Ang I was without effect even at the same dose (Rankin *et al.* 2004). The minimum effective dose of LpAng II in Japanese lamprey is 3 orders smaller than that of [Asn¹, Val⁵]-Ang II in river lamprey. In the present study, various doses of [Asn¹, Val⁵]-Ang II up to 1 nmol/kg did not produce any

observable changes in the blood pressure of Japanese lamprey. Combining the observation that [Asn¹, Val⁵]-Ang II was undetectable in the plasma, it seems that [Asn¹, Val⁵]-Ang II is not a native hormone in lamprey.

To investigate the possible interspecific effect of LpAng II, cannulated Japanese eel was used for comparison. The eel responded to [Asn¹, Val⁵]-Ang II injection with a typical vasopressor response but not to LpAng II. According to the three-dimensional CPH modeling of LpAng II and [Asn¹, Val⁵]-Ang II (Nielsen *et al.* 2010), their molecular configurations are highly different due to the extra amino acids in the N-terminus as well as due to the substitution in the aligned region (See Supplementary Figures 1 and 2, see section on supplementary data given at the end of this article). The preliminary modeling showed that [Asn¹, Val⁵]-Ang II, similar to human Ang II, has a linear structure with a hydrophobic pocket formed by the [Tyr⁴] and [Pro⁷] (Tzakos *et al.* 2003). On the other hand, the predicted structure of LpAng II is helical shape and shared little structural resemblance to [Asn¹, Val⁵]-Ang II three dimensionally. Deletion of the first five amino acids at the N-terminus of LpAng II greatly affected the three-dimensional structure and abolished the vasoactive properties in lamprey (data not shown). Therefore, it is not surprising that LpAng II did not activate the angiotensin receptors in the eel and thus was not detected by the eel vasopressor bioassay in previous isolation studies (Rankin *et al.* 2004, Takei *et al.* 2004).

In conclusion, we discovered and characterized the native angiotensinogen in lamprey, which suggested that the RAS first evolved in the cyclostomes. The circulating form and level of LpAng II were determined by a newly developed RIA in combination with HPLC fractionation. In Japanese lamprey, LpAng II elicited a biphasic vasoactive response, but [Asn¹, Val⁵]-Ang II was undetectable and not vasoactive. The species-specific action of LpAng II in lamprey and eel showed that the RAS has undergone independent paths in vertebrate evolution. Whether LpAng II may signal through the AT1 and AT2 receptors as in other vertebrates is open to question for future research. Questions do remain on the source and function of [Asn¹, Val⁵, Thr⁹]-Ang I identified by incubation of plasma and kidney extract in previous studies (Rankin *et al.* 2004, Takei *et al.* 2004). Fishes are capable of absorbing intact proteins through the gut directly into the blood stream (Georgopoulou *et al.* 1988). Active feeding river lamprey used in the previous studies may have absorbed the host angiotensinogen and renin into their blood stream, and therefore subsequent incubation of plasma with kidney extract may have generated the host-type Ang I. Alternatively, lamprey has a long history of parasitism on fishes (Gess *et al.* 2006); horizontal gene transfer or selective gene exploitation may have introduced some teleost genes into the lamprey genome (Salzet *et al.* 2000, Yu *et al.* 2008). We look forward to the completion of the sea lamprey genome project, so that more information can be put together to analyze the possibility of horizontal gene transfer between lamprey and fishes.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-10-0422>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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