

Identification of Angiotensin I in a Cyclostome, *Lampetra fluviatilis*

Authors: Rankin, John Clifford, Watanabe, Takushi X., Nakajima, Kiichiro, Broadhead, Caren, and Takei, Yoshio

Source: Zoological Science, 21(2) : 173-179

Published By: Zoological Society of Japan

URL: <https://doi.org/10.2108/zsj.21.173>

The BioOne Digital Library (<https://bioone.org/>) provides worldwide distribution for more than 580 journals and eBooks from BioOne's community of over 150 nonprofit societies, research institutions, and university presses in the biological, ecological, and environmental sciences. The BioOne Digital Library encompasses the flagship aggregation BioOne Complete (<https://bioone.org/subscribe>), the BioOne Complete Archive (<https://bioone.org/archive>), and the BioOne eBooks program offerings ESA eBook Collection (<https://bioone.org/esa-ebooks>) and CSIRO Publishing BioSelect Collection (<https://bioone.org/csiro-ebooks>).

Your use of this PDF, the BioOne Digital Library, and all posted and associated content indicates your acceptance of BioOne's Terms of Use, available at www.bioone.org/terms-of-use.

Usage of BioOne Digital Library content is strictly limited to personal, educational, and non-commercial use. Commercial inquiries or rights and permissions requests should be directed to the individual publisher as copyright holder.

BioOne is an innovative nonprofit that sees sustainable scholarly publishing as an inherently collaborative enterprise connecting authors, nonprofit publishers, academic institutions, research libraries, and research funders in the common goal of maximizing access to critical research.

Identification of Angiotensin I in a Cyclostome, *Lampetra fluviatilis*

John Clifford Rankin^{1*†}, Takushi X Watanabe², Kiichiro Nakajima²,
Caren Broadhead³ and Yoshio Takei⁴

¹Department of Biology, Odense University, 5230 Odense M, Denmark

²Peptide Institute Inc., Protein Research Foundation, Minoh, Osaka
562-8686, Japan

³Nottingham Clinical Research Ltd., Nottingham NG7 2RH, UK

⁴Ocean Research Institute, the University of Tokyo, Nakano,
Tokyo 164-8639, Japan

ABSTRACT—Angiotensin I (ANG I) was isolated from incubates of plasma and kidney extracts of the river lamprey, *Lampetra fluviatilis*, using eel vasopressor activity as an assay during purification. Its sequence was Asn-Arg-Val-Tyr-Val-His-Pro-Phe-Thr-Leu as determined by the sequence analysis and mass spectrometry. The sequence was confirmed by identity of the elution profile with the synthetic peptide in two different reverse-phase columns of high-performance liquid chromatography. Lamprey ANG I produced dorsal-aortic pressor responses in *L. fluviatilis* but the rise was very small in comparison to that produced by angiotensin II. Angiotensin III produced an even bigger increase. It was not possible to demonstrate a difference in response to Asn¹ (lamprey) ANG I and Asp¹ (human) ANG I. The present study directly demonstrated the presence and biological activity of the renin-angiotensin system in the most primitive extant vertebrates, the cyclostomes. Thus the renin-angiotensin system is a phylogenetically old hormonal system that is present throughout the vertebrates.

Key words: renin-angiotensin system, purification of angiotensin I, lamprey, *Lampetra fluviatilis* (Cyclostomata), vasopressor action

INTRODUCTION

The renin-angiotensin system has long been thought to have evolved for the first time in primitive bony fish (Bentley, 1976). However, angiotensin I (ANG I) has been isolated and sequenced in the elasmobranch fish, *Triakis scyllia* (Takei *et al.*, 1993a), demonstrating directly the presence of the renin-angiotensin system in the cartilaginous fish. The elasmobranch ANG I has a unique sequence with proline residue at position 3 instead of the valine of all other species, and the N-terminal residue is asparagine as in most teleost species. Success in isolating elasmobranch ANG I may have been dependent on the use of the eel as an assay animal, because [Asn¹, Pro³] ANG I has a much higher vasopressor potency in the eel than in the rat, which had

previously been used as an assay animal for isolation of ANG I from non-mammalian vertebrates. Since its native peptide is [Asp¹, Val³] ANG I, non-mammalian ANG I has lower potency than the homologous peptide.

Thanks to the development of separation technology, it is now possible to isolate ANG I from the incubates of small amounts of plasma (source of angiotensinogen) and kidney extracts (source of renin). For instance, only 1 ml of plasma and 0.5 g of kidney are sufficient for isolation of ANG I in the quail (Takei and Hasegawa, 1990). However, several attempts to isolate ANG I from cyclostomes (lamprey and hagfish) have not been successful even though more than 50 ml of plasma was used. This could possibly have been due to cyclostome ANG I having a unique structure with even lower vasopressor potency in the eel than that of elasmobranch ANG I. Another possibility is low angiotensinogen concentration in cyclostome plasma or low renin content in the cyclostome kidney. Whichever the case, the lamprey is preferable for isolation of angiotensin because it has a much larger kidney compared with the hagfish (Fels *et al.*, 1998).

* Corresponding author: Tel. +44-1484-473345;
Fax. +44-1484-472182.
Email: j.rankin@hud.ac.uk

† Present address: Department of Chemistry and Biology Huddersfield University, Huddersfield, HD1 3DH, U.K.

Since hagfish plasma contains similar Na^+ and Cl^- concentrations to seawater (Rankin, 1997), hagfish renin may have low activity under the current condition for incubation of plasma and kidney extract with cation-exchange resin at low ion concentrations (Boucher *et al.*, 1967). Furthermore, ANG may play a more significant role in the euryhaline lamprey, as it is known that the renin-angiotensin system plays a central role in water and electrolyte regulation in all vertebrate and invertebrate species (Makara and Prior, 1985; Salzet *et al.*, 1995; see Kobayashi and Takei, 1996).

In the present study, we collected more than 100 ml of plasma and 50 g of kidney tissue from the river lamprey, *L. fluviatilis*, and ANG I was isolated from incubates of the two using the eel as an assay animal. The sequence analysis showed that the lamprey has [Asn¹, Val³] ANG I as in teleost fish. The lamprey ANG I was synthesized and its vasopressor activity was compared in the lamprey with that of human ANG I and with lamprey angiotensin II (ANG II) and angiotensin III (ANG III).

MATERIALS AND METHODS

Animals

River lampreys (*L. fluviatilis*) of either sex weighing 56.8 ± 13.9 g (mean \pm SD, $n=271$) were caught over a period of several years (prior to their autumnal spawning migrations to freshwater) in eel traps in the brackish water Ringkøbing Fjord, on the west coast of Denmark and transported to aquaria at Odense University. They were acclimated to either freshwater (FW; these provided ca. 25% of the plasma and kidneys) or seawater (SW; from 18 to 35ppt), as it was not known if salinity affected the activity of the renin-angiotensin system. They were anaesthetised in 0.1 g/l MS222 (3-aminobenzoic acid ester methanesulphonate salt: Sigma, UK) and blood was collected by puncture of the caudal vessels into chilled heparinised (ammonium heparin: Leo Pharmaceutical Products, Denmark) syringes containing 15% ammonium EDTA (Sigma: 10 μ l/ml blood). After centrifugation, the plasma was collected and frozen in liquid nitrogen. Next, the kidneys were removed, together with the attached urinary ducts, frozen in liquid nitrogen and then stored at -80°C . Frozen samples were shipped to the Ocean Research Institute, University of Tokyo on dry ice for ANG I production and purification.

For blood pressure measurements lampreys of either sex weighing 60.0 ± 10.3 g ($n=29$) were collected towards the ends of 1999 and 2000 from Ringkøbing Fjord, acclimated to freshwater or, in a few cases seawater, and studied during the early months of the following years.

Angiotensin I production

Kidney tissues (50.5 g) from freshwater and seawater fish were thawed, minced with a razor blade and homogenized in the same volume of 5.8 mM disodium EDTA in a polytron (Kinematica, Germany) at dial 8 for 1 min. The homogenate was centrifuged at $18,000 \times g$ for 30 min at 4°C . The supernatant was acidified to pH 3.0 with 1 M HCl and left for 1 hr. The precipitate was removed by centrifugation at $20,000 \times g$ for 30 min. The supernatant was neutralized to pH 7.4 with 1 M NaOH and centrifuged again as above. The supernatant (kidney extracts) and plasma (125 ml) were dialyzed against 5.8 mM EDTA for 24 hr at 4°C . The precipitate was removed by centrifugation as above, and the dialyzed kidney extract (40 ml) and plasma (118 ml) were incubated together with 1.2 ml of 2.5% phenylmethylsulphonyl fluoride and 30 ml of Dowex

50W-X2 resin (pH 7.4, NH_4^+ form) for 6 hr at 20°C under gentle agitation. The incubate was poured onto 10 ml of Dowex 50W-X2 resin (pH 6.0, NH_4^+ form) and the resin was washed with 400 ml each of 0.5 M NH_4OAc (pH 6.0), 10% AcOH and water in this order. The materials adsorbed to the resin were eluted with 400 ml each of 0.1 M diethylamine and 0.5 M NH_4OH . The eluates were evaporated at 40°C at reduced pressure, reconstituted in 10% CH_3CN in 0.1% trifluoroacetic acid (TFA). Bioassay of 1/10 of the eluates showed vasopressor activity only in the diethylamine fraction. The materials were partially purified by Sep-Pak cartridge (Waters, Milford, MA, USA) before chromatography.

Angiotensin I purification

The materials eluted from the Sep-Pak cartridge with 45% CH_3CN in 0.1% TFA were then subjected to cation-exchange high-performance liquid chromatography (HPLC) on an SP-2SW column (4.6×250 mm, Tosoh, Tokyo, Japan) with a linear gradient NH_4OAc concentrations from 10 mM to 1 M in 10% CH_3CN for 120 min at a flow rate of 1 ml/min. The fraction exhibiting vasopressor activity in the eel (see below) was then subjected to gel-permeation HPLC using Superdex Peptide HR 10/30 column (Bed volume=24 ml; Pharmacia Biotech., Tokyo, Japan). The column was eluted with 30% CH_3CN in 0.1% trifluoroacetic acid (TFA) at 0.25 ml/min. The bioactive fraction was further subjected to reverse-phase HPLC on an ODS-120T column (4.6×250 mm, Tosoh) and eluted with a linear gradient of CH_3CN concentrations from 10% to 60% in 0.1% TFA for 60 min at 1 ml/min. The fraction was collected by each peak reflection at 220 nm and assayed. The vasopressor material was finally purified by reverse-phase HPLC on a 219T54 phenyl column (4.6×250 mm, Vydac, Hesperia, CA, USA) with a linear gradient of CH_3CN from 15% to 35% in 0.1% TFA for 40 min at 1 ml/min.

Sequence determination

Five percent of the purified material (ca. 6 pmol) was subjected to a KOMPACT MALDI III mass spectrometer (Shimadzu, Tokyo, Japan) to determine its molecular mass. Half of the remainder was used for amino acid sequencing in a gas-phase protein sequencer (Model 476A, Applied Biosystems, San Francisco, CA, USA). The peptide with deduced amino acid sequence was then synthesized in a peptide synthesizer (Model 430A, Applied Biosystems). The identity of the purified peptide with the synthetic peptide was examined using the remaining half with a ODS-120T and 219T54 phenyl columns with a linear gradient of 15% to 35% CH_3CN in 0.1% TFA for 40 min.

Bioassay

The presence of ANG I in each fraction during the course of purification was assayed by vasopressor activity in the eel. Cultured immature eels, *Anguilla japonica* (ca. 200 g) were acclimated to the laboratory freshwater tank at 18°C for more than one week before use. Eels were anaesthetized in 0.1% (w/v) of tricaine methanesulphonate (Sigma), and catheters were inserted into the ventral and dorsal aortas as described elsewhere (Takei, 1988). The catheter in the ventral aorta was connected with a pressure transducer (P23XL-1, Ohmeda Medical Devices Division Inc., Madison, WI, USA), which was then connected to a carrier amplifier (Type 3126, Yokogawa Electric Works Ltd., Tokyo, Japan) and a pen recorder (R-03A, Rikadenki Co. Ltd., Tokyo, Japan). After 24 hr of recovery, the sample or standard (eel ANG I) dissolved in 0.9% saline containing 0.01% Triton X-100 (Nakarai, Kyoto, Japan) were injected into the dorsal aorta in a volume of 50 μ l followed by a flush of the catheter with 50 μ l saline. The dead volume of the catheter was 10 μ l.

Physiological studies

Lampreys were anaesthetised in 0.1 g/l MS222 and placed ventral side up in a V-shaped trough connected to a box filled with

aerated 0.065 g/l MS222 in which the head and gill pouch openings were left hanging. The trough was lined with a paper towel dipping into the anaesthetic solution to ensure that the skin remained moist. The anaesthetic concentration was adjusted if necessary to maintain strong respiratory movements. A 3–4 cm mid-ventral incision was made posterior to the liver and anterior to the kidneys and gonad tissue was removed to expose the ventral aorta and posterior cardinal veins. Electrocautery (Thackray, UK) was used to minimise blood loss. The dorsal aorta was cannulated with polythene tubing (0.58 mm internal diameter; Portex, UK) tipped with the metal part of a 23G syringe needle bent in two places so that the distal end lay free in the aorta whilst the proximal end lay on the body surface. The tubing, which contained 0.6% NaCl (lamprey saline) containing 2000 IU heparin (Leo Pharmaceutical products, Denmark) was connected to a pressure transducer (Druck model PDCR75 connected to an SE Labs. model SE905 transducer/converter). 0.1 ml of the heparinised saline was injected slowly into the artery. It was sometimes necessary to repeat this injection during an experiment as blood tended to clot in the needle, leading to a decrease in the amplitude of the pressure pulse. The output was recorded on a pen recorder. The transducer was periodically calibrated using a water manometer. The maximum increase in mean blood pressure (calculated as diastolic + 1/3 pulse pressure, measured in cm water) was used to quantify the hormone effects. River lampreys caught in the autumn at the start of their upstream migration slowly mature

over the winter to spawn and die in the spring (Larsen and Dufour, 1993). In view of the profound physiological changes during this period, control blood pressures were measured at monthly intervals.

Hormone and control injections were made into one or other of the posterior cardinal veins. Initially this was done by inserting a bent 23G needle attached to a 1 ml syringe directly into the vein. Usually the vein sealed as the needle was withdrawn, but in some cases the negative pressure in the vein (McVicar and Rankin, 1985) resulted in a small air bubble being sucked in. This sometimes caused disruption to, or cessation of, the heart beat and termination of the experiment. An alternative procedure was to inject via a short length of polythene tubing (0.5 mm i.d.; Portex) tipped by a bent 25G needle which remained in the vein throughout the experiment. Lamprey ANG I (synthesised as described above), the corresponding ANG II (Asn¹ Val⁵; Sigma) and ANG III (Val⁴; Sigma) and human ANG I (Sigma) were dissolved in distilled water and immediately frozen in small aliquots. Solutions for injection were made by serial dilution in lamprey saline. Solutions of 100 µl saline /100 g body weight were made up to contain the calculated doses. Thus 65 µl aliquots were injected into a 65 g lamprey. When injection was made via a cannula, it was cut to the exact length to contain the injected volume. When the cannula was inserted it contained the first dose to be injected. Injection consisted of flushing this out of the cannula with the same volume of saline, followed by injection of

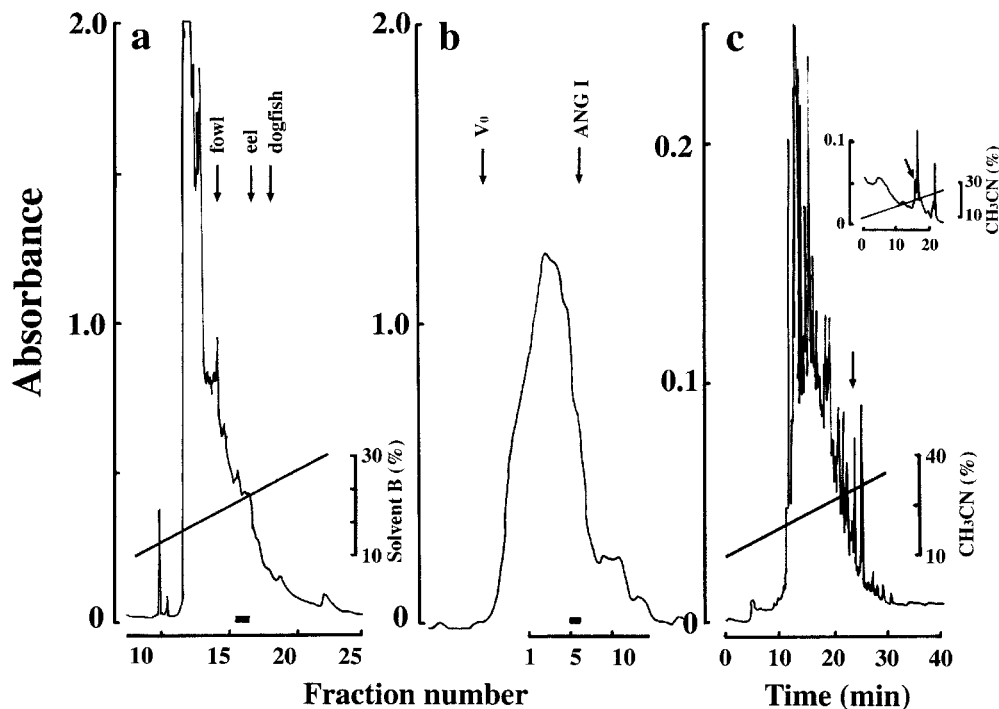


Fig. 1. Chromatograms for purification of lamprey angiotensin I. (a) Cation-exchange HPLC (SP-2SW, 4.6×250 mm, Tosoh) of the fraction eluted with diethylamine from Dowex 50W-X2 resin and further purified by a Sep-Pak cartridge. The oblique line shows linear gradient from solvent A (10% CH₃CN in 10 mM NH₄OAc) to solvent B (10% CH₃CN in 1 M NH₄OAc) in 120 min at 1 ml/min. Fractions were collected every 2.5 min. Only the marked fraction exhibited vasopressor activity in the eel. Arrows show elution positions of fowl, eel, and dogfish ANG I. For each sequence, see Fig. 2. (b) Gel-permeation HPLC (Superdex Peptide HR10/30, bed volume=24 ml, Pharmacia Biotech.) of the fraction 16 of cation-exchange HPLC. Sample was eluted with 30% CH₃CN in 0.1% TFA for 100 min at 0.25 ml/min. Fractions were collected every 2 min. Only marked fractions showed vasopressor activity. V₀, void volume; ANG I, elution position of eel ANG I. (c) Reverse-phase HPLC (ODS-120T, 4.6×250 mm, Tosoh) of the fraction 6 of gel-permeation HPLC. The oblique line shows a linear gradient from 10% to 60% CH₃CN in 0.1% TFA for 60 min at 1 ml/min. Fractions were collected by each peak. Only the peak marked by an arrow exhibited vasopressor activity. Inset: Reverse-phase HPLC (219T54 phenyl, 4.6×250 mm, Vydac) of the fraction marked by an arrow in panel (c). The oblique line shows a linear gradient from 15% to 35% CH₃CN in 0.1% TFA for 40 min at 1 ml/min. Fractions were collected by each peak. The minor peak marked by an arrow exhibited vasopressor activity and was subjected to sequence analysis.

the next dose to just fill the cannula. Care was taken to clamp the cannula whilst the syringe was being changed to avoid inspiration of air. Different lampreys were used for replications of a particular hormone at a particular dose. Dose response curves were constructed based on the maximum change in mean blood pressure (in mmHg) recorded.

Statistics

Monthly variations in blood pressure were analysed by one way ANOVA followed by the Scheffe test. The Wilcoxon signed-ranks test was used to test for the lowest doses giving a pressor response significantly different from zero (the response to control injections). The differences between the responses to the maximum ANG I (lamprey and human) and ANG II dose used (10^{-7} mol kg $^{-1}$) were analysed by one way ANOVA followed by the Bonferroni multiple comparisons test. In all cases the level of probability considered significant was $p < 0.05$.

RESULTS

Biochemical studies

An ANG I-like vasopressor material was produced after incubation of lamprey plasma with the kidney extracts. Since the eluate from the 50W-X2 resin with diethylamine contained large amounts of proteins and other substances, it was subjected to a Sep-Pak cartridge before HPLC purification. After cation-exchange HPLC with a SP-2SW column, vasopressor materials were eluted at the position of eel [Asn1] ANG I (Fig. 1a). The gel-permeation HPLC showed

that the materials had a molecular mass similar to eel ANG I (Fig. 1b). The subsequent two steps of reverse-phase HPLC could isolate the vasopressor material to a single minor peak (Fig. 1c). The final yield was 130 pmol as determined by comparing the absorbance at 220 nm with that of eel ANG I. The vasopressor activity in the eel also gave a similar value as compared to the activity of eel ANG I. Amino acid sequencing revealed that the purified peptide had the sequence of H-Asn-Arg-Val-Tyr-Val-His-Pro-Phe-Thr-Leu-OH (Fig. 2). Thus the lamprey has the sequence identical to lungfish ANG I (Joss *et al.*, 1999). The measured molecular mass of the purified peptide ($MH^+ = 1244$) was similar to the theoretical value (1245.44). Furthermore, the purified peptide co-migrated with synthetic lamprey ANG I in two different HPLC systems.

Physiological studies

Control dorsal aortic blood pressures in the lampreys were measured each month from October (start of upstream migration) to April (full maturation). There were no significant changes in the months October to February inclusive (combined mean \pm SEM: 25.8 \pm 0.9 mm Hg, $n = 35$). In March pressure fell to 19.2 \pm 1.6 mm Hg ($n = 4$) but the difference was not statistically significant. In April it fell to 13.7 \pm 1.5 mm Hg ($n = 12$) and this was significantly different ($p < 0.05$, ANOVA followed by Scheffe test) to each of the October to

lamprey	Asn-Arg-Val-Tyr-Val-His-Pro-Phe-Thr-Leu
rat	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu
ox	Asp-Arg-Val-Tyr-Val-His-Pro-Phe-His-Leu
fowl	Asp-Arg-Val-Tyr-Val-His-Pro-Phe-Ser-Leu
turtle	Asp-Arg-Val-Tyr-Val-His-Pro-Phe-His-Leu
alligator	Asp-Arg-Val-Tyr-Val-His-Pro-Phe-Ala-Leu
bullfrog	Asp-Arg-Val-Tyr-Val-His-Pro-Phe-Asn-Leu
lungfish	Asn-Arg-Val-Tyr-Val-His-Pro-Phe-Thr-Leu
goosefish	Asn-Arg-Val-Tyr-Val-His-Pro-Phe-His-Leu
salmon	Asn-Arg-Val-Tyr-Val-His-Pro-Phe-Asn-Leu
eel	Asn-Arg-Val-Tyr-Val-His-Pro-Phe-Gly-Leu
bowfin	Asp-Arg-Val-Tyr-Val-His-Pro-Phe-Asn-Leu
dogfish	Asn-Arg-Pro-Tyr-Ile-His-Pro-Phe-Gln-Leu

Fig. 2. Amino acid sequences of angiotensin I in vertebrates. Amino acid residues different from those of lamprey ANG I are shaded. The lamprey sequence is identical to that of lungfish. The rat sequence is identical to those of man, sheep, horse, dog and pig. The fowl sequence is identical to that of quail. For references, see Kobayashi and Takei (1996).

February values.

Control injections had no effect on blood pressure. The mean response to lamprey ANG II was significantly different from zero at 10^{-9} mol kg $^{-1}$ but not at lower doses, whereas for lamprey ANG II only 10^{-7} mol kg $^{-1}$ produced effects significantly different to zero ($p < 0.05$; Wilcoxon signed-ranks test). High doses (10^{-7} mol kg $^{-1}$) of lamprey ANG I initiated significant pressor effects lasting about 15 minutes (following a 1–2 min latent period). Much lower doses (10^{-9} mol kg $^{-1}$) of lamprey ANG II produced much shorter (<2 min) pressor pulses with approximately the same maximum (Fig. 3a, which also illustrates the lack of effect of control saline injection). In this experiment a cannula was used for injection: the lack of effect of saline showed that the previous dose had been washed in and that the next dose had remained within the cannula. Human ANG I had a very similar effect to lamprey ANG I. The pressor responses to ANG I and ANG II were dose-related (Fig. 4). The latter produced a maximum effect at 10^{-8} mol kg $^{-1}$ whereas the curve for ANG I did not appear to have reached a maximum at the

highest dose used, 10^{-7} mol kg $^{-1}$. At 10^{-7} mol kg $^{-1}$ ANG II produced a significantly ($p < 0.05$, Bonferroni test) greater elevation of mean blood pressure than lamprey or human ANG I. There was no statistically significant difference between the responses to lamprey and human ANG I at 10^{-7} mol kg $^{-1}$.

Injections of 10^{-8} mol kg $^{-1}$ ANG III in two lampreys produced much greater pressor responses (increases of 13.9 and 11.2 mm Hg respectively) than ANG II. A copy of the recording of the smaller of the two ANG III responses is shown together with a response to 10^{-8} mol kg $^{-1}$ ANG II injected just before in the same lamprey (Fig. 3b and c). Recovery from these high doses took about 10 min; the recorder was slowed down to include the recovery periods in the figure. In these examples there was no change in heart rate following injection; for example with ANG II there were 76 beats in the 1 min before injection, 38 beats in the ensuing 30 sec of increasing pressure and 76 beats in the first min of the plateau.

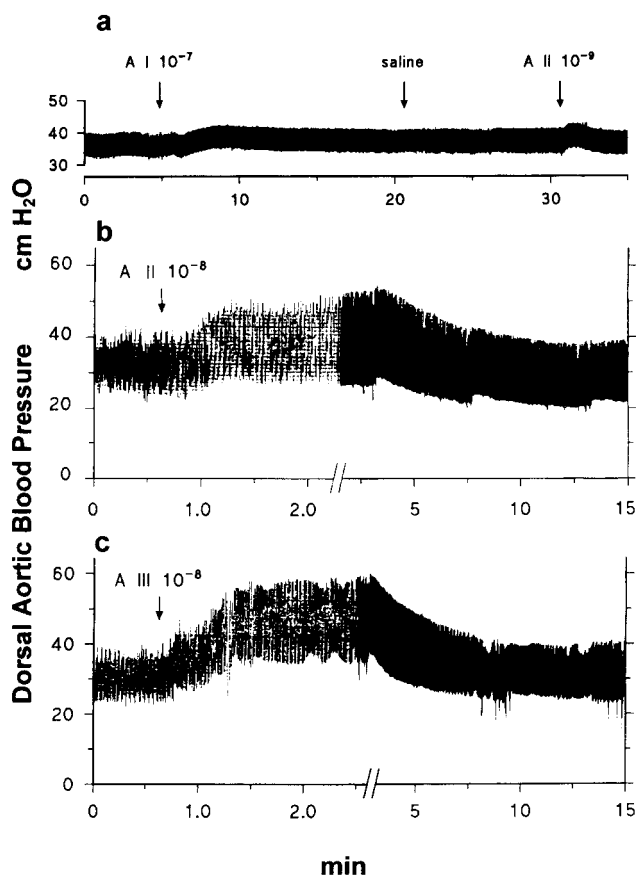


Fig. 3. Recordings of dorsal aortic blood pressure in anaesthetised river lampreys. a) Effects of sequential injection of lamprey ANG I (A I; 10^{-7} mol kg $^{-1}$), lamprey saline and lamprey ANG II (A II; 10^{-9} mol kg $^{-1}$) through a cannula into a posterior cardinal vein of an anaesthetised lamprey. b) Effect of lamprey ANG II (A II; 10^{-8} mol kg $^{-1}$) injected into the posterior cardinal vein of an anaesthetised lamprey. c) Effect of lamprey ANG III (A III; 10^{-8} mol kg $^{-1}$) injected into the posterior cardinal vein of an anaesthetised lamprey.

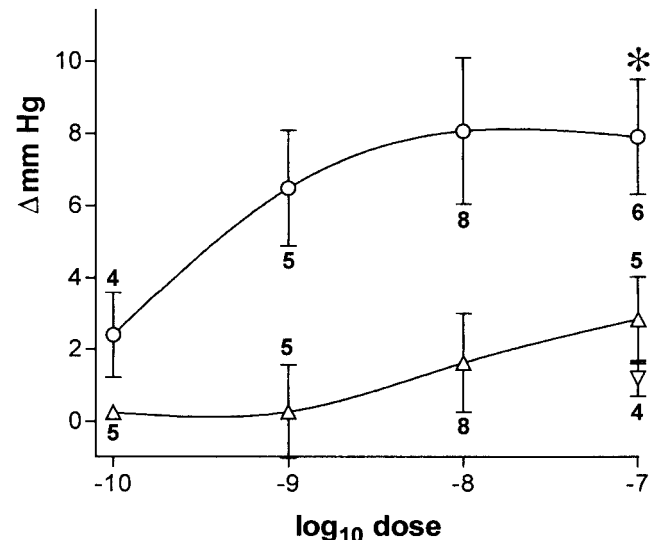


Fig. 4. Dose-response curves for the pressor effects of lamprey ANG II (circles), lamprey ANG I (triangles) and human ANG I (inverted triangles) in the anaesthetised lamprey. Error bars represent standard errors of means. Numbers are number of observations. * = significantly different from both ANG I values ($p < 0.05$; One way ANOVA followed by Bonferroni test)

DISCUSSION

The present study clearly demonstrated the presence of the renin-angiotensin system in the most primitive extant vertebrate Class, Cyclostomata, by identifying ANG I in the lamprey, *L. fluviatilis*. Therefore, it is obvious that the renin-angiotensin system is a phylogenetically ancient hormonal system that may be present in all vertebrate species. Attempts in another, but very distantly related (Janvier, 1999) cyclostome species, the hagfish, have been unsuccessful, perhaps because of scantiness of renal tissues (Fels *et al.*, 1998). The phylogenetical ancientness of the

renin-angiotensin system is supported by the fact that ANG II molecules have been identified in two species of leech (Laurent *et al.*, 1995; Salzet *et al.*, 1995), and that angiotensin converting enzyme (ACE) has been detected in the fruit fly (Cornell *et al.*, 1995).

It is generally accepted that most tetrapod species, from amphibians to mammals, have [Asp¹, Val³] ANG I while most teleost fishes have [Asn¹, Val³] ANG I (Fig. 2). An exception in fish is the bowfin, a holostean fish, in which [Asp¹, Val³] ANG I was identified (Takei *et al.*, 1998). In the lungfish that is related to the tetrapod lineage, however, teleost type [Asn¹, Val³] ANG I was isolated (Joss *et al.*, 1999). [Asn¹] ANG I can be separable from [Asp¹] ANG I by ion-exchange HPLC, but no vasopressor activity was detected at its elution position even though the sensitivity of the assay is very high (2 pmol/eel). Elasmobranch ANG has a unique proline residue at position 3 (Takei *et al.*, 1993a). Thus, lamprey ANG I is more similar to the teleost type than the elasmobranch type. The 9th amino acid residue is highly variable among species and thus, coincidentally, lamprey ANG I was identical to lungfish ANG I.

In the rat, the vasopressor activity of [Asp¹] ANG I is higher than [Asn¹] ANG I (Watanabe *et al.*, 1977). Thus, it is likely that the initial attempt to detect ANG activity in the lamprey was not successful because of the use of rat as an assay animal (Nishimura *et al.*, 1970). In the eel, however, [Asn¹] ANG I has higher vasopressor potency than [Asp¹] ANG I (Takei, 1987), indicating the coevolution of the ANG molecule and its receptor during vertebrate phylogeny. It seems that one of the clues for the success in isolating lamprey ANG I may be the use of eel as an assay animal, since lamprey ANG has asparagine at position 1. However, the present study showed that ANG I production after incubation of plasma and kidney extract was much smaller in the lamprey than in other tetrapod and piscine species; in fact, relative yield of ANG I per milliliter of plasma is ca 1 pmol/ml in the lamprey compared to 222 pmol/ml in the alligator (Takei *et al.*, 1993b). This may be due to a low concentration of angiotensinogen in the lamprey plasma or low renin content in the lamprey kidney.

Few measurements have been reported of blood pressure in lampreys, so measurements of dorsal aortic blood pressure were made in control freshwater-acclimated anaesthetised animals throughout the "lamprey season". For most of the winter values were similar to the 21.6±2.0 mm Hg reported by McVicar and Rankin (1985) for *L. fluviatilis* and also to the "between 25 and 44 cm H₂O" reported for *Entosphenus tridentatus* by Johansen *et al.* (1973). The angiotensin responses were investigated between January and April. Unfortunately this was a period of falling blood pressures (and potentially also of falling responsiveness to angiotensins, although no evidence of this was seen). The aim was to see if angiotensins were vasopressor. This was achieved, but a full investigation of the role of the RAS in blood pressure regulation in lampreys is still awaited.

Lamprey ANG I had a very small, but prolonged, pres-

or action in the river lamprey. In theory, quantification using the integrated pressor response would have been preferable. In practice it was very difficult to determine when the pressure had returned to normal, as slow baseline drift was not uncommon (probably on account of the difficulty of maintaining a precise degree of anaesthesia). In contrast, pressure elevations produced by ANG II injection were much greater and of shorter duration. It would appear that conversion of ANG I to ANG II was slow following intravenous injection. If lampreys were like teleosts, the ANG I would be expected to be converted to ANG II on passage through the gills (Olson *et al.*, 1989) before reaching the peripheral circulation. However, in the river lamprey angiotensin converting enzyme-like activity is very low in the gills (Cobb *et al.*, 2002). Slow conversion and mixing in the blood stream would result in a much lower initial ANG II concentration reaching the receptors mediating peripheral vasoconstriction.

The contrast between the ANG I and ANG II responses is evident from their dose-response curves (Fig. 4). The ANG II response at 10⁻⁹ mol kg⁻¹ was significantly different from zero (control injections always produced zero responses) but for lamprey ANG I, 10⁻⁷ mol kg⁻¹ was required to produce a significant elevation in blood pressure. It is also evident that the highest dose of ANG I used (10⁻⁷ mol kg⁻¹) was insufficient to produce a maximal response, whereas the ANG II curve had levelled off by 10⁻⁸ mol kg⁻¹. The half maximal pressor dose for ANG II (ca. 2.10⁻¹⁰ mol kg⁻¹) would have produced a plasma concentration of approximately 5 nM if mixed in the entire plasma volume. This compares to plasma concentrations of around 0.2 and 0.4 nM for ANG II+ANG III in river lampreys acclimated to FW and SW respectively (Rankin *et al.*, 2001).

Human ANG I at 10⁻⁷ mol kg⁻¹ produced a smaller mean pressor response than the same dose of lamprey ANG I but, although 10⁻⁷ mol kg⁻¹ ANG II produced a significantly greater response than either lamprey or human ANG I, the difference between the ANG I responses was not significant. This contrasts with greater response to endogenous ANG I in the eel. In view of the difficulty of quantifying such small responses and the possible rate-limiting effects of conversion to ANG II, it was not possible to draw any conclusions regarding the relative potency of Asp¹ and Asn¹ angiotensins in the lamprey.

ANG III is pressor in the eel, increasing cardiac output without affecting heart rate but the increase in mean dorsal aortic pressure produced by iv injection of 150 ng kg⁻¹ was significantly less than that produced by the same dose of eel ANG I (Butler and Oudit, 1995). The mean pressor response to injections of 10⁻⁸ mol kg⁻¹ ANG III in two lampreys was considerably greater than the mean increase produced by the same dose of ANG II. The end of availability of lampreys meant that it was not possible to obtain data for a dose-response curve, but recordings of ANGII and ANG III injected consecutively in the same lamprey clearly illustrate the difference. This meagre evidence is presented to sug-

gest that further work on ANG III actions in lower vertebrates would be of some interest. ANG II levels have often been measured using antibodies which cross-react with ANG III. In *Lampetra fluviatilis*, where ANG II and ANG III were separated by HPLC before assay, ANG III plasma concentrations were comparable to those of ANG II (Rankin *et al.*, 2001). The possibility that ANG III plays an important role as an active hormone in lower vertebrates should be considered.

ACKNOWLEDGEMENTS

This work was supported in part by Grant-in-Aid for Specially Promoted Grant (01902008) and for Creative Basic Research (12NP0201) from the Ministry of Education, Science, Sports and Culture of Japan, for Scientific Research (13304063) from the Japan Society for the Promotion of Science, by grants from the Danish Natural Sciences Research Council and from the UK Natural Environment Research Council (GR3/12190).

REFERENCES

- Bentley PJ (1976) Comparative Vertebrate Endocrinology, Academic Press, San Diego
- Boucher R, Menard J, Genest J (1967) A micromethod for measurement of renin in the plasma and kidney of rats. *Can J Physiol Pharmacol* 45: 881–890
- Butler DG, Oudit, GY (1995) Angiotensin-I- and -II-mediated cardiovascular responses in the freshwater North American eel, *Anguilla rostrata*: effect of Phe⁸ deletion. *Gen Comp Endocrinol* 97: 259–269
- Cobb CS, Frankling SC, Rankin JC, Brown JA (2002) Angiotensin converting enzyme-like activity in tissues from the river lamprey or lampern, *Lampetra fluviatilis*, acclimated to freshwater and seawater. *Gen Comp Endocrinol* 127: 8–15
- Cornell MJ, Williams TA, Lamango NS, Coates D, Corvol P, Soubrier F, Hoheisel J, Lehrach H, Isaac RE (1995) Cloning and expression of an evolutionary conserved single-domain angiotensin converting enzyme from *Drosophila melanogaster*. *J Biol Chem* 270: 13613–13616
- Fels LM, Kastner S, Stolte H (1998) The hagfish kidney as a model to study renal physiology and toxicology. In “The Biology of Hagfish” Ed by JM Jorgensen, JP Lomholt, RE Weber, H Malte, Chapman & Hall, London, pp 347–363
- Janvier P (1999) Catching the first fish. *Nature* 402: 21–22
- Johansen K, Lefant C, Hanson, D (1973) Gas exchange in the lamprey, *Entosphenus tridentatus*. *Comp Biochem Physiol* 44A: 107–109
- Joss JMP, Itahara Y, Watanabe TX, Nakajima K, Takei Y (1999) Teleost-type angiotensin is present in Australian lungfish, *Neoceratodus forsteri*. *Gen Comp Endocrinol* 114: 206–212
- Kobayashi H, Takei Y (1996) The renin-angiotensin system. A comparative approach. In “The Renin-Angiotensin System” Ed by H Kobayashi, Y Takei, Springer International, Berlin, pp 77–79
- Larsen LO, Dufour S (1993) Growth, reproduction and death in lampreys and eels. In “Fish Ecophysiology” Ed by JC Rankin, FB Jensen, Chapman and Hall, London, pp 72–104
- Laurent V, Bulet P, Salzert, M (1995) A comparison of the leech *Theromyzon tessulatum* angiotensin I-like molecule with forms of vertebrate angiotensinogens: a hormonal system conserved in the course of evolution. *Neuroscience Letters* 190: 175–178
- McVicar AJ, Rankin, JC (1985) Dynamics of glomerular filtration in the river lamprey, *Lampetra fluviatilis* L. *Am J Physiol* 249: F132–F138
- Makara ME, Prior DJ (1985) Angiotensin II can initiate contact-rehydration in terrestrial slugs. *J Exp Biol* 119: 385–388
- Nishimura H, Oguri M, Ogawa M, Sokabe H, Imai M (1970) Absence of renin in kidneys of elasmobranchs and cyclostomes. *Am J Physiol* 218: 911–915
- Olson KR, Lipke DW, Kullman D, Evans AP, Ryan JW (1989) Localisation of angiotensin-converting enzyme in the trout gill. *J Exp Zool* 250: 109–115
- Rankin JC (1997) Osmotic and ionic regulation in cyclostomes. In “Ionic Regulation in Animals”, Ed by N Hazon, FB Eddy, G Flik, Springer, Berlin, pp 50–69
- Rankin JC, Cobb CS, Frankling SC, Brown JA (2001) Circulating angiotensins in the river lamprey, *Lampetra fluviatilis*, acclimated to freshwater and seawater: possible involvement in the regulation of drinking. *Comp Biochem Physiol* 129B: 311–318
- Salzet M, Bulet P, Watzet C, Verger-Bocquet M, Malecha J (1995) Structural characterization of a diuretic peptide from the central nervous system of the leech *Erpobdella octoculata*. *J Biol Chem* 270: 1575–1582
- Takei Y (1987) In vivo characterization of angiotensin II receptors and converting enzymes in the quail and eel. *Zool Sci* 4: 1088
- Takei Y (1988) Changes in blood volume after alteration of hydromineral balance in conscious eels, *Anguilla japonica*. *Comp Biochem Physiol* 91A: 293–297
- Takei Y, Hasegawa Y (1990) Vasopressor and depressor effects of native angiotensins and inhibition of these effects in the Japanese quail. *Gen Comp Endocrinol* 79: 12–22
- Takei Y, Hasegawa Y, Watanabe TX, Nakajima K, Hazon N (1993a) A novel angiotensin I isolated from an elasmobranch fish. *J Endocrinol* 139: 281–285
- Takei Y, Silldorff EP, Hasegawa Y, Watanabe TX, Nakajima K, Stephens GA, Sakakibara S (1993b) New angiotensin I isolated from a reptile, *Alligator mississippiensis*. *Gen Comp Endocrinol* 90: 214–219
- Takei Y, Itahara Y, Butler DG, Watanabe TX, Oudit GY (1998) Tetrapod-type angiotensin is present in a holostean fish, *Amia calva*. *Gen Comp Endocrinol* 110: 140–146
- Watanabe TX, Sokabe H, Honda I, Sakakibara S, Nakayama T, Nakajima T (1977) Specific pressor activity and stability of synthetic angiotensins. *Jpn J Pharmacology* 27: 137–144

(Received September 12, 2003 / Accepted October 24, 2003)