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Activation of the newly discovered cyclostome renin–angiotensin system in the river lamprey *Lampetra fluviatilis*

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Summary

This study describes the first investigations of the physiological signals involved in activating the newly discovered cyclostome renin–angiotensin system (RAS) and its role in the river lamprey *Lampetra fluviatilis*. Experimental manipulation showed that volume depletion (removal of 40% blood volume) rapidly activated the RAS of lampreys acclimated to water at 576 mOsm kg^{-1} (21 p.p.t.), significantly increasing plasma angiotensin concentrations after 30 min and 60 min. In agreement with these results, a rapid change in environmental salinity (758 mOsm kg^{-1} to freshwater (FW) and FW to 605 mOsm kg^{-1}), resulted in a rapid decrease and increase in plasma [angiotensin], respectively. Intraperitoneal (i.p.) injection of FW-acclimated river lampreys with 1% body mass by volume of nominally isosmotic saline (120 mmol l^{-1} NaCl; 233 mOsm kg^{-1}) resulted in a significant decrease in the plasma angiotensin concentration within 15 min. In contrast, i.p. injection of hyperosmotic saline (4 mol l^{-1} NaCl) at 1% body mass by volume, which significantly increased plasma osmolality, had no significant effect on plasma [angiotensin], suggesting that volume/pressure receptors and osmoreceptors interact in regulating the lamprey RAS. These results indicate an important role for volume/pressor receptors, as in teleosts, but with an additional osmoreceptor mechanism, such that circulatory [angiotensin] is determined by interaction of volume/pressure and osmoreceptors and their relative sensitivities. The volume/pressure sensitivity is in keeping with the recent evidence of a vasoconstrictor action of homologous lamprey angiotensin and provides evidence that the fundamental role of the RAS in maintaining volume and pressure is an ancient function conserved over 500 million years of vertebrate evolution.

Key words: renin–angiotensin system, river lamprey, *Lampetra fluviatilis*, plasma angiotensin, salinity adaptation, volume regulation.

Introduction

The renin–angiotensin system (RAS) has been identified in the majority of vertebrate groups, including fish (Kobayashi and Takei, 1996). In the systemic RAS, circulating angiotensinogen, synthesised by the liver, is cleaved by renin released from the kidney to form the inactive decapeptide, angiotensin I (Ang I). Ang I is further cleaved by angiotensin converting enzyme to produce the physiologically active octapeptide, angiotensin II (Ang II) and further cleavage results in formation of angiotensin III (Ang III: fragment 2–8 Ang II) and angiotensin IV (Ang IV: fragment 3–8 Ang II), both of which also show bioactivity, although their function remains to be established in most vertebrates (Kobayashi and Takei, 1996).

In teleost fish there is evidence that the RAS plays important roles in regulating blood pressure, controlling renal and cardiovascular function, stimulating interrenal steroidogenesis and in determining drinking rates (Brown et al., 1980; Arnold-Reed and Balment, 1994; Tierney et al., 1995a,b; Bernier et al., 1999; Bernier and Perry, 1999; Butler and Zhang, 2001).

For decades, the RAS was believed to have first evolved in bony fishes and to be absent from both elasmobranchs and cyclostomes (Nishimura et al., 1970; Nishimura and Ogawa, 1973; Nishimura, 1985; Henderson et al., 1993). However, Ang I has now been isolated and sequenced in the elasmobranch *Triakis scyllia* (Takei et al., 1993) and more recently in two cyclostomes: the sea lamprey *Petromyzon marinus* (Takei et al., 2004) and the river lamprey *Lampetra fluviatilis* (Rankin et al., 2004). The Ang II component in these lamprey species was identified as Asn^{1},Val^{5}-Ang II, as is found in most, but not all teleosts (Hasegawa et al., 1984; Khosla et al., 1985; Conlon et al., 1996; Balment et al., 2003). Recognition of this similarity enabled our first measurements

of circulating angiotensin in cyclostomes, using commercial antisera with high cross-reactivity to Asn^1,Val^5-Ang II and Val^5-Ang III (Rankin et al., 2001). This study showed higher plasma Ang II and Ang III concentrations in river lampreys acclimated to seawater (SW) than in freshwater (FW)-acclimated lampreys (Rankin et al., 2001). From this it can be suggested that for anadromous lamprey species, migrating between rivers and the marine environment during their life cycle (Hardisty, 1979), the RAS could be involved in body fluid homeostasis as has been identified in teleost fish (Olson, 1992; Kobayashi and Takei, 1996).

There are similarities in the processes responsible for regulating body fluid volume and composition of teleosts and lampreys with, for example, hyper-osmoregulation in FW requiring excretion of large amounts of dilute urine to eliminate the high osmotic water influx (Brown et al., 1980; Evans, 1993). Also, in both lampreys and teleosts, hypo-osmoregulation in more saline environments is associated with an osmotic water loss that is balanced by drinking the surrounding water, with fluid absorption in the gut, together with a drastic reduction in urine output and renal adjustments to excrete high concentrations of divalent ions (Logan et al., 1980; Evans, 1993; Rankin, 1997, 2002; Brown and Rankin 1999).

In teleosts and elasmobranchs there is evidence that the RAS is controlled by volume receptors with, for example, release of renin and elevated circulating angiotensin concentrations during hypovolaemia and/or reduction in blood pressure (Nishimura et al., 1979; Bernier et al., 1999; Anderson et al., 2001). However, the physiological signals responsible for activation of the lamprey RAS are as yet unclear. We therefore undertook a range of experimental manipulations of river lampreys followed by measurement of circulating angiotensin levels to investigate the activation of the lamprey RAS and to facilitate the development of hypotheses relating to its physiological role. Specifically, we have examined: (1) whether blood removal activates the lamprey RAS and hence increases circulating angiotensin concentration; for these studies we used lampreys held in a hyperosmotic environment to ensure that volume depletion could not be rapidly compensated for by the natural osmotic influx of water; (2) the impact of extracellular fluid expansion after intraperitoneal (i.p.) injection of a nominally isosmotic saline; (3) whether changes in extracellular fluid osmolality accompanying volume expansion influence the lamprey RAS; (4) how circulating angiotensin concentration is affected after rapid changes in external salinity from FW to a hyperosmotic medium (605 mOsm kg^{-1}) and from a hyperosmotic medium (758 mOsm kg^{-1}) to FW.

Materials and methods

Experimental animals

Adult river lampreys *Lampetra fluviatilis* L. were caught in eel traps in Ringkøbing Fjord (ca. 400–500 mOsm kg^{-1}) on the west coast of Jutland, Denmark, during September and October, at the start of their migration into FW (several months prior to spawning in the following spring). During this migration, river lampreys metamorphose from a silver marine form through intermediates to a yellow form (Rankin, 1996, 1997). River lampreys caught in Ringkøbing Fjord (ca. 400–500 mOsm kg^{-1}) in the autumn are predominantly silver forms, with many individuals having gastrointestinal tracts full of blood and still capable of hypo-osmoregulating in SW for long periods of time after capture (Rankin, 1997). Captured river lampreys were transported on ice to the Aquatic Biology Research Centre, Kerteminde, Denmark and kept in either aerated FW (15 mOsm kg^{-1}; 0.5 p.p.t.) or gradually acclimated to Kerteminde SW (576 mOsm kg^{-1}; 21 p.p.t.) in a closed and filtered aquarium system using Great Belt surface SW. Some lampreys were acclimated to a higher salinity of 758 mOsm kg^{-1} (higher salinity water than 26 p.p.t. cannot normally be obtained in Kerteminde). Experimental studies were carried out during October and November and all lampreys were held at 10°C under a 12 h:12 h light:dark cycle with reduced light achieved by shading most of the tank surface with a lid. Tank water osmolality (vapour pressure osmometry: model 5520, Westcor Inc., Logan, UT, USA or freezing point depression: Osmomat model 030, Gonotech Gmbtt, Berlin, Germany), salinity and temperature (model 30M/50FT, YSI Inc., Yellow Springs, OH, USA) were monitored daily. River lampreys were held in these conditions for 3–5 weeks prior to experimental manipulation.

Anaesthesia and blood sampling

River lampreys were anaesthetised by immersion in MS222 (3-amino-benzoic acid ester methanesulfonate salt; Sigma, Poole, UK; 0.065 g l^{-1}, as previously, Brown and Rankin, 1999). Once anaesthetised, lampreys were weighed and placed on their backs in individual Perspex troughs with their heads immersed in aerated water containing anaesthetic and maintained at 10°C; the rest of the body was covered with damp tissue. Respiratory movements were monitored, and anaesthesia adjusted to maintain strong ventilation and stable blood pressure and renal function (McVicar and Rankin, 1983; Brown and Rankin, 1999; Rankin et al., 2004). Blood samples were collected by needle puncture of the caudal vein. Blood for measurement of circulating angiotensin concentrations was collected into 100 µl of inhibitor solution (0.225 mol l^{-1} EDTA, 50 Kallikrein IU aprotinin and 0.05 mol l^{-1} 1,10-phenanthroline; Sigma, UK) in chilled syringes containing air-dried ammonium heparin (Sigma, UK). Samples were centrifuged (20 000 ***g***, 2 min at 4°C, Microcentrifuge, Ole Dich, Hvidovre, Denmark), plasma removed and transferred into a fresh tube, and tubes frozen in liquid nitrogen. Haematocrit (with inhibitor dilution) was also determined in heparinised microhaematocrit tubes (Mikro 12-24 Hettich Zentrifugen, Berlin, Germany; 13 000 ***g***, 2 min) to enable correction for the dilution of measured plasma angiotensin concentration by the inhibitor solution; the dilution factor was determined from haematocrit and total mass of blood sample. Immediately after collecting the first blood sample, a further

small sample (0.2 ml blood) was collected into a 1 ml syringe coated with ammonium heparin (Sigma, UK). This sample was used for determination of haematocrit (without inhibitor) as before, and plasma osmolality (model 5520 Vapor Pressure Osmometer, Westcor Inc.). Plasma samples for angiotensin analysis were held at –80°C until transport on dry-ice to the University of Exeter, UK, where they were held at –80°C until extraction and radioimmunoassay of angiotensins.

Experimental series

Blood volume depletion

River lampreys (42–91 g; *N*=30) acclimated to Kerteminde SW (576 mOsm kg^{-1}; 21 p.p.t.) were anaesthetised in MS222 as described above and an initial blood sample of 3.2% of body mass (1.3–2.9 ml, calculated to reduce blood volume by 40%) was collected from the caudal vein for analysis of plasma angiotensin and determination of sample haematocrit (with inhibitor). A further 0.2 ml was taken immediately for measurement of blood haematocrit (without inhibitor) and plasma osmolality. For each lamprey, blood samples (approximately 1 ml) were subsequently collected into inhibitor at 30 min (*N*=10), 60 min (*N*=11) or 90 min (*N*=7) after the initial blood volume depletion (one time point per lamprey) and used to measure plasma angiotensin and haematocrit (in presence of inhibitor). Immediately after the allocated sampling point a further 0.2 ml blood sample was collected to determine haematocrit (without inhibitor) and plasma osmolality.

Isosmotic and hyperosmotic injections

In order to determine the effects of volume expansion on the RAS, with and without salt loading, FW-acclimated river lampreys were lightly anaesthetised and injected i.p. with 1% body mass by volume of either nominally isosmotic saline or hyperosmotic saline, with control, non-injected lampreys run in parallel.

FW-acclimated river lampreys (43–101 g) were lightly anaesthetised as outlined above and weighed. Experimental lampreys were i.p.-injected at 1% by volume of body mass with either nominally isosmotic saline (120 mmol l^{-1} NaCl; 233 mOsm kg^{-1}) or hyperosmotic saline (4 mol l^{-1} NaCl). Blood samples were collected from each lamprey as described earlier for determination of plasma angiotensin, blood haematocrit and plasma osmolality. Samples were collected from separate lampreys 15 min after i.p. injection of hyperosmotic saline (*N*=8) or isosmotic saline (*N*=5) and 30 min after i.p. injection of hyperosmotic saline or isosmotic saline (*N*=5 in each group). Control non-injected lampreys from the same stock tanks were held under light anaesthesia until removal of a single blood sample after 15 min (*N*=8) or 30 min (*N*=5).

Acute changes in external salinity

The aquarium system used allowed changes in environmental salinity to be rapidly achieved, avoiding the stress of removing the lampreys from the water. This allowed investigations to determine how the lamprey RAS is affected during the initial period after rapid shifts in external salinity. In the first experiment, the initial FW (14 mOsm kg^{-1}) was altered to a hyperosmotic medium (605 mOsm kg^{-1}); in a second experiment hyperosmotic Kerteminde SW (758 mOsm kg^{-1}) was rapidly replaced by FW to reach 22 mOsm kg^{-1}.

For the experiment involving transfer from FW to a hyperosmotic environment, river lampreys (45–87 g) were held in a flow-through FW system (approx. 500 litres) for 2 weeks. At the start of the experiment (time zero), the tank FW volume was reduced to a depth of 10 cm (approx. 80 litres) and switched to a closed system with filtration for the 24 h prior to the start of the experiment. An initial group of lampreys (*N*=8) was removed, anaesthetised and blood samples collected. Kerteminde SW (salinity 25 p.p.t.) was pumped into the tank to increase the salinity from 0.5 p.p.t. to 20 p.p.t. within 6 min and achieve a peak salinity of 21 p.p.t. (605 mOsm kg^{-1}) within 20 min. Sub-groups of river lampreys (*N*=8 per group) were blood sampled at 1, 2, 4, 8 or 24 h from the start of SW addition in order to determine plasma osmolality, haematocrit and plasma angiotensin concentration, as described in the blood volume depletion experiments.

For the experiment involving transfer from a hyperosmotic environment to FW, river lampreys (44–87 g) were held in a closed and filtered system of aerated Kerteminde SW (26 p.p.t., 758 mOsm kg^{-1} at 10°C) for 3 weeks. 24 h before starting the experiment, the SW was reduced to a depth of approximately 10 cm. At the start of the experiment a group of lampreys (*N*=10) was removed from the experimental tank, anaesthetised, and blood samples collected as in other experiments. Salinity was lowered by rapid addition of FW to reach 1 p.p.t. (23 mOsm kg^{-1}) within 30 min and 0.6 p.p.t. at 1 h. Sub-groups of lampreys (*N*=8 per group) were blood sampled at 2 h, 4 h, 8 h and 24 h after the start of FW addition.

Extraction and radioimmunoassay of plasma angiotensin

Plasma angiotensin was extracted according to the method described by Bernier et al. (1999). Briefly, 100 µl samples of river lamprey plasma held on ice at 4°C were each mixed with 100 µl acidic acetone (acetone:water:1 mol l^{-1} HCl, ratio 40:5:1) and vortexed vigorously for 1 min. The mixture was centrifuged at 10 000 ***g*** for 10 min at 4°C. The supernatant was collected and the pellet re-solubilised and re-extracted, as before. Supernatants were combined, freeze dried under vacuum at –45°C (Edwards EF4 Modulyo freeze dryer, Edwards High Vacuum, Crawley, UK) and stored at –80°C until radioimmunoassay (RIA).

Prior to RIA, the extracted residues were resuspended in phosphate-buffered saline (PBS, 400 µl at 0.01 mol l^{-1}, pH 7.4, containing 0.25% (w/v) bovine serum albumin fraction V RIA grade and 0.25% (v/v) Triton X-100; Sigma, UK). Triplicate samples of these plasma extracts and [Asn^1,Val^5]-Ang II standards (100 µl) were incubated overnight at 4°C with 100 µl (~8000 c.p.m.) ^{125}I-[Ile^5]-Ang II (74 Tbq $mmol^{-1}$, ~2000 Ci $mmol^{-1}$; Amersham, UK) and 100 µl of angiotensin

antiserum (100 000× diluted in PBS). The heterologous antiserum was initially raised against mammalian [Asp^1,Ile^5]-Ang II (Yamaguchi, 1981). Serially diluted extracted lamprey plasma ran parallel to the standard curve of [Asn^1,Val^5]-Ang II, the native Ang II sequence in lampreys (Fig. 1). The antiserum used shows <0.5% cross-reactivity with Ang I from mammals, teleosts and elasmobranchs but high cross-reactivity with Ang II from mammals (100%), teleosts (63–85%), elasmobranchs (74%) and lampreys (63%) (Bernier et al., 1999; Gary Anderson, Gatty Marine Laboratory, University of St Andrews, UK, personal communication). However, the antiserum, in common with most commercial Ang II antisera, also shows high cross-reactivity (~90%) with mammalian Ang III and Ang IV (G. Anderson, personal communication). Therefore, our RIA measurements of angiotensin levels would incorporate Ang II, Ang III and Ang IV.

The following day, separation of free and bound angiotensins was achieved by addition of a 100 µl of a solid phase second antibody-coated cellulose suspension (anti-rabbit IgG serum; Sac-Cel IDS, Boldon, UK) and incubation at room temperature for 30 min. Distilled water (1 ml) was then added and tubes centrifuged (2500 ***g***, 4 min, 4°C). Supernatants were aspirated and radioactivity of the pellets containing bound Ang II was determined (Packard Cobra Auto-gamma, B5002, Reading, UK). The angiotensin content of samples of river lamprey plasma was determined using a software package (RIASMART, Biosoft, Cambridge, UK). Final values of plasma angiotensin concentration were corrected for the calculated dilution of plasma samples by the inhibitor mix. This dilution was determined by calculation of the plasma volume in each sample based on the measured haematocrit and the gravimetric determination of the blood volume collected.

Estimated recovery of angiotensin through the extraction procedure and RIA was 78.5±5.7% for [Asn^1,Val^5]-Ang II and 105.7±3.7% for [Val^5]-Ang III (N=10 in both cases). Intra-assay variability was 10.1% and 7.2% for Ang II at 9.8 pmol l^{-1} and 78.1 pmol l^{-1}, respectively, and 7.3% and 10.6% for Ang III at identical concentrations (N=10 in all cases). The minimum detectable level of plasma angiotensin concentration was 11.7 pmol l^{-1}.

Statistical analyses

All data are presented as means or percentages of means ± standard error (S.E.M.). All statistical analyses used SPSS version 10.0 for Windows. All data were initially tested by the Kolmogorov–Smirnov test to determine whether they were normally distributed. When data were normally distributed or normality was achieved by transformations, further analyses used analysis of variance (ANOVA), followed by *post-hoc* multiple comparison tests (Tukey's HSD when data showed homogeneous variances after Levene's tests, and Games Howell when variances differed significantly). When transformations failed to achieve normality, data were analysed by a non-parametric ANOVA for independent samples and Mann–Whitney U-tests. Plasma angiotensin data after transfer of lampreys from FW to higher environmental salinities showed significant differences between groups (ANOVA) that could not be located by *post-hoc* multiple comparison tests; data were therefore analysed by linear contrasts comparing angiotensin concentrations at each time point after exposure to the higher environmental salinity with angiotensin concentrations in FW-acclimated lampreys at time 0 h. Significant differences between pre-blood volume depletion and post-blood volume depletion data for plasma angiotensins, osmolarity and haematocrit were established using paired t-tests. Statistical differences between means were considered significant at P<0.05.

Results

Blood volume depletion

The removal of an estimated 40% of the blood volume of river lampreys acclimated to 576 mOsm kg^{-1} (21 p.p.t.) resulted in a significant decrease in haematocrit (Fig. 2A). Blood volume depletion did not significantly affect the plasma osmolality of these lampreys (Fig. 2B).

A time course investigation of the changes in plasma angiotensin concentration after blood volume depletion showed that the plasma concentration of angiotensin had more than doubled after 30 min (Fig. 2C; P<0.01). Plasma angiotensin concentration remained significantly elevated in lampreys sampled 60 min after blood volume depletion (P<0.001) with a 62% increase compared to basal levels (Fig. 2C). After 90 min, there was no significant difference in plasma angiotensin concentration compared to that of basal samples taken to achieve the imposed blood volume depletion (Fig. 2C).

Isosmotic and hyperosmotic saline injection

The haematocrit of non-injected lampreys remained stable under the light anaesthesia (Fig. 3A). Injection with either of the saline solutions resulted in a pronounced reduction in haematocrit with the mean values in non-injected controls of

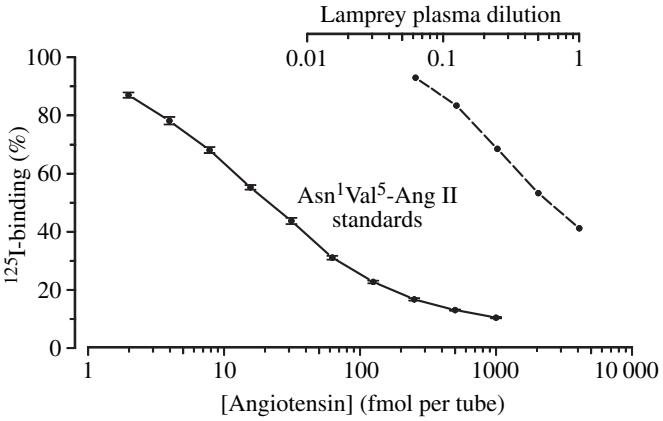

Fig. 1. Parallelism of the [Asn^1,Val^5]-Ang II radioimmunoassay standard curve (shown as means ± S.E.M., N=6) and serial dilution of an extract of pooled plasma from the river lamprey *Lampetra fluviatilis*. For details of the assay, see Materials and methods.

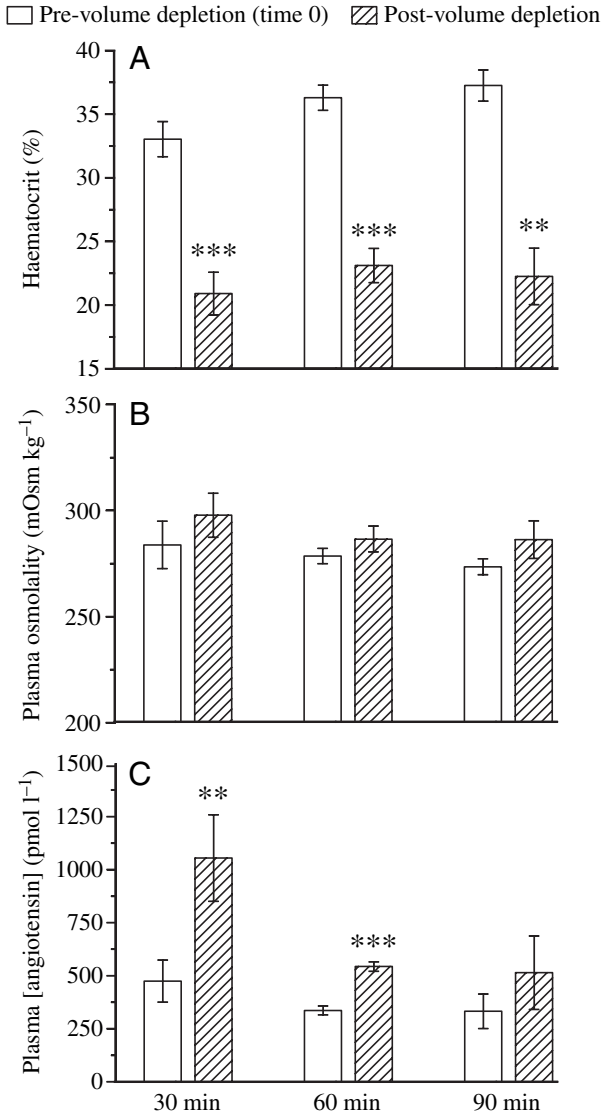

Fig. 2. Blood volume depletion in river lampreys acclimated to Kerteminde seawater at 576 mOsm kg^{-1} (21 p.p.t.). The first blood sample (pre-volume depletion; open bars) was taken to achieve a 40% decrease in blood volume and a second sample (post-volume depletion; hatched bars) was collected after 30 min (N=10), 60 min (N=11) or 90 min (N=7). Data for (A) haematocrit (%), (B) plasma osmolality (mOsm kg^{-1}) and (C) plasma angiotensin concentrations (pmol l^{-1}) are shown (**P<0.01; ***P<0.001, paired t-tests).

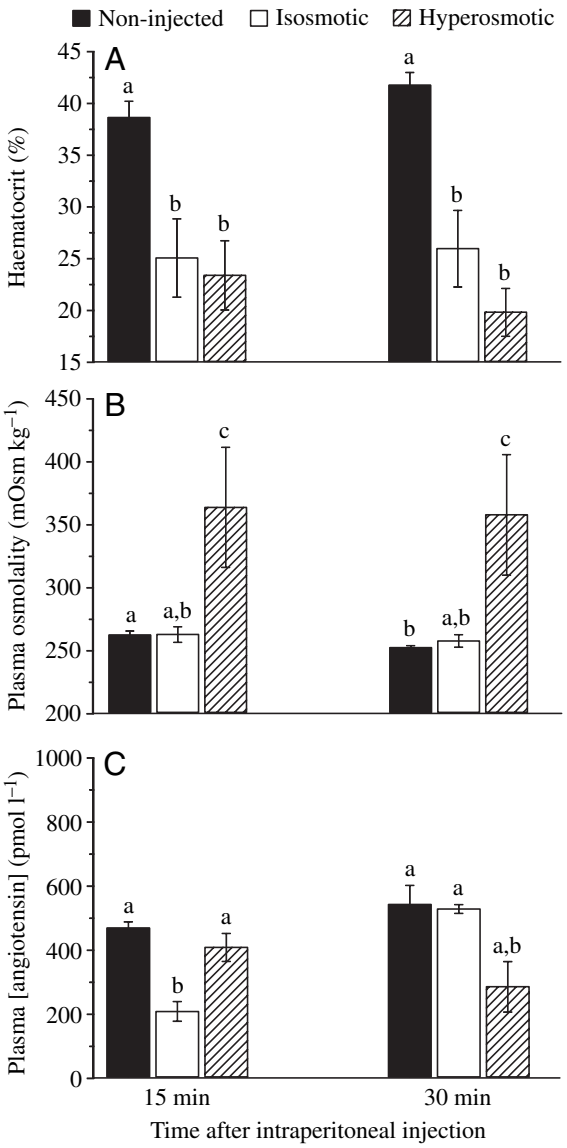

Fig. 3. (A) Haematocrit (%), (B) plasma osmolality (mOsm kg^{-1}) and (C) plasma angiotensin concentrations (pmol l^{-1}) of river lampreys acclimated to freshwater at 15 mOsm kg^{-1}. Control lampreys (non-injected; black bars) were held under light anaesthesia for 15 min (N=8) and 30 min (N=4) in the absence of further manipulation. Experimental lampreys were blood sampled at either 15 min or 30 min after an i.p. injection of 1% body mass with either isosmotic saline (white bars; 120 mmol l^{-1} NaCl; 233 mOsm kg^{-1}; N=5 at each time point) or hyperosmotic saline (cross-hatched bars; 4 mol l^{-1} NaCl; N=5 at 30 min; N=8 at 30 min). Different letters above error bars signify groups that differ significantly (ANOVA and *post-hoc* multiple comparison tests).

38.7% and 41.8% at the two time points declining to mean values of 20–26% in the four injected groups (Fig. 3A). The reduction in haematocrit was similar after injection of iso-osmotic and hyperosmotic saline and not significantly affected over the two sampling time points.

Injection of hyperosmotic saline significantly increased plasma osmolality (Fig. 3B). The nominally isosmotic saline (233 mOsm kg^{-1}) proved to be slightly hypo-osmotic in these lampreys, but not sufficiently so as to result in any change in plasma osmolality (Fig. 3B). The two experimental groups showed no differences over the two time points, but non-injected lampreys showed a slight (3.8%) decrease in plasma osmolality between the 15 min and 30 min sampling points.

Injection with the nominally isoosmotic saline resulted in a significant depression in the concentration of plasma angiotensin at 15 min (P<0.01; Fig. 3C), but plasma angiotensin concentration of lampreys injected with hyperosmotic saline was not significantly different from that

of the non-injected controls both after 15 min and 30 min (Fig. 3C).

Acute changes in external salinity

The rapid increase in environmental salinity, rising from 14 to 605 mOsm kg^{-1} within 20 min, resulted in a significant increase in plasma osmolality within 1 h (Fig. 4A). Blood haematocrit was not significantly altered by exposure to high salinities (Fig. 4B). Plasma angiotensin concentrations followed a visibly similar pattern to the rising plasma osmolality (Fig. 4C) but there was a large variability in plasma angiotensin concentration at all time points, particularly at time 0 h. Statistical analysis by ANOVA indicated a significant change after exposure to higher environmental salinity, but multiple comparison procedures did not locate a significant difference between groups. However, linear contrasts of angiotensin concentrations after exposure to higher salinity, compared to control values in FW-acclimated lampreys, indicated a significant rise after 4 h (P=0.022), 8 h (P=0.005) and 24 h (P=0.004).

A rapid decrease in environmental salinity, from 758 to 22 mOsm kg^{-1}, significantly lowered the blood haematocrit and plasma osmolality in river lampreys within 4 h (Fig. 5A,B; P<0.05 and P<0.001, respectively). Plasma osmolality remained depressed at 8 h and 24 h but blood haematocrit was restored. Plasma angiotensin concentrations dropped steadily after the acute change in environmental salinity, but only reached a significantly lower value after 24 h (Fig. 5C).

Discussion

Our studies were aimed principally at investigating the signals involved in regulating the recently discovered lamprey RAS. Our recent studies of river lampreys showed almost a doubling of plasma angiotensin concentration in river lampreys acclimated to SW of 28 p.p.t. salinity (845 mOsm kg^{-1}) compared to the plasma angiotensin concentration of lampreys acclimated to FW (Rankin et al., 2001). In the present studies, however, although the mean plasma concentration of immunoreactive angiotensin was slightly higher in lampreys acclimated to SW of 758 mOsm kg^{-1} than in FW-acclimated lampreys, the difference was not statistically significant. This seems likely to reflect at least partially the lower osmotic challenge faced by the lampreys in the present studies, but differences in assay techniques between the two studies also complicate direct comparisons. Our previous analyses included HPLC separation of Ang II, III and IV, prior to RIA, but in the present study the large number of samples precluded routine separation. Although the antisera used in present and previous studies differed, this is of less importance as both showed high cross-reactivity with Ang II, Ang III and Ang IV and <0.5% cross-reactivity with Ang I. Thus, in each case RIA would have measured the major active angiotensin fragments (Butler and Oudit, 1995; Kobayashi and Takei, 1996). Our previous HPLC separation resulted in a combined (Ang II plus Ang III) value of ~200 pmol l^{-1} in FW-acclimated lampreys and indicated that plasma Ang III was present at similar concentrations to plasma Ang II (Rankin et al., 2001). This contrasts with the apparently low level of Ang III found after RIA of HPLC-separated trout plasma (Bernier et al., 1999), using the same antibody that we employed in the present studies. Given that in the present studies we measured concentrations of approximately 400–500 pmol l^{-1} total immunoreactive angiotensin in plasma samples collected from FW-acclimated lampreys, it is probable that a significant amount of Ang IV is also present in lamprey plasma. Light anaesthesia for short periods (up to 30 min) has no apparent effect on plasma angiotensin levels (see Fig. 3C).

In previous studies, transfer of river lampreys from FW to 503 mOsm kg^{-1} suggested a possible link between the circulating Ang II concentration and rising plasma osmolality (Rankin et al., 2001). The present studies therefore examined

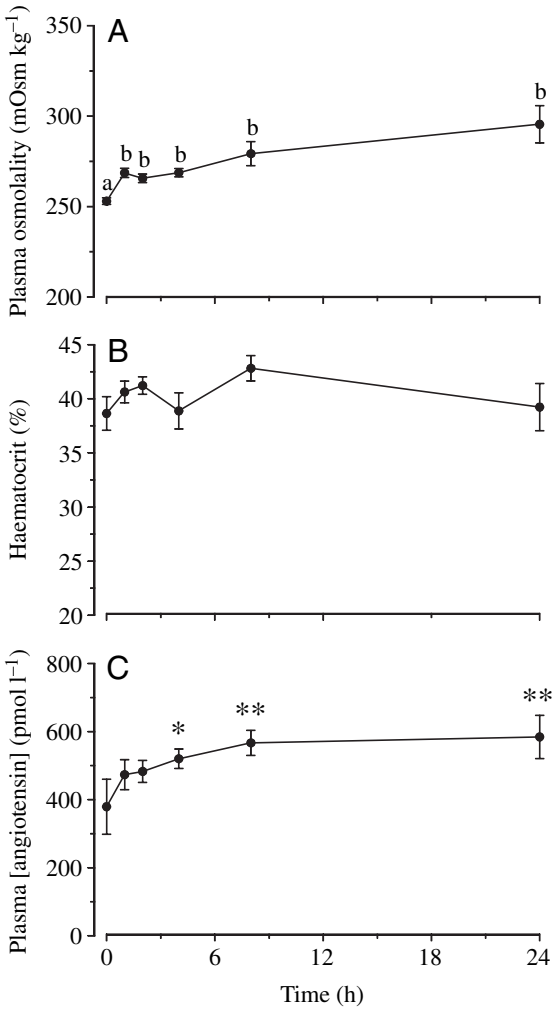

Fig. 4. Effects of a rapid increase in environmental salinity (FW to 21 p.p.t.; 605 mOsm kg^{-1}) on (A) plasma osmolality (mOsm kg^{-1}), (B) blood haematocrit (%) and (C) plasma angiotensin concentration (pmol l^{-1}) of river lampreys (N=8 at each time point: 0 h, 2 h, 4 h, 8 h and 24 h after transfer). Groups with different letters differ significantly (ANOVA and *post-hoc* multiple comparison tests). Asterisks signify groups that differ significantly from time 0 h (*P<0.05, **P<0.01, ANOVA followed by linear contrast analyses).

more fully the changes in plasma osmolality and plasma angiotensin concentrations after rapidly increasing external salinity from FW to 605 mOsm kg^{-1} and also after the reverse manoeuvre, rapidly decreasing salinity from 758 mOsm kg^{-1} to FW. Lampreys exposed to these rapid changes in environmental salinity showed good osmoregulation over the next 24 h, as shown previously (Rankin, 2002). After an acute rise in external salinity, there was no significant change in blood haematocrit, indicating limited, if any, volume depletion, but there was a small though significant rise in plasma osmolality that was associated with a similar pattern of change in plasma angiotensin concentration, with a significant rise after 4 h, 8 h and 24 h suggested by the linear contrast analyses of data at each time point compared to the initial level in FW. In agreement with these trends, transfer from a hyperosmotic saline environment to FW, resulted in a steady decline in plasma angiotensin concentration alongside declining plasma osmolality. However, our data indicate that relationships are complex since the largest (and significant) decline in plasma angiotensin concentration occurred between 8 h and 24 h when plasma osmolality was stable.

Movements between different environmental salinities are highly likely to affect extracellular fluid volume as well as plasma osmolality, as in teleosts (Olson, 1992). We therefore examined the impact of both hypovolaemia and volume expansion. A severe haemorrhage of 40% of a nominal blood volume of 8% of body mass was chosen to test for stimulation of the RAS in SW-acclimated lampreys. Another lamprey species (*Petromyzon marinus*) was estimated to have a blood volume of 8.5% of body mass, based on the volume of distribution of Evan's Blue (Thorson, 1959). This technique is inaccurate in teleost fish (Olson, 1992), but in two *Petromyzon marinus* obtained from Ringkøbing Fjord, Denmark, the more accurate technique involving re-injection of red blood cells labelled with ^{51}Cr (Olson, 1992), gave values of 8.3% and 8.1% of body mass (J. C. Rankin, unpublished observations).

SW-acclimated lampreys are faced with the need to continually drink their environmental medium in order to regulate body fluid volume, but anaesthesia has been found to block drinking (Rankin, 1997). Therefore, in the hypovolaemia experiments the lampreys would not have been able to increase drinking in order to restore blood volume. Based on expected drinking rates in SW-acclimated lampreys, an inhibition of drinking would have resulted in a further volume loss of 4.8 ml h^{-1} kg^{-1} body mass that could not be absorbed from the gut to replace branchial and renal losses (Rankin, 2002). Within the 90 min experimental period after the imposed blood volume depletion this would have resulted in a further 4.5% hypovolaemia.

Hypovolaemia, induced by removal of an estimated 40% of the blood volume, in river lampreys held in hyperosmotic media, would potentially cause an immediate drop in blood pressure, as in teleost fish (e.g. Nishimura et al., 1979). This could cause a decrease in kidney perfusion pressure, releasing renin. However, as yet, there is no evidence for renin-releasing granular epithelioid cells in the lamprey kidney or

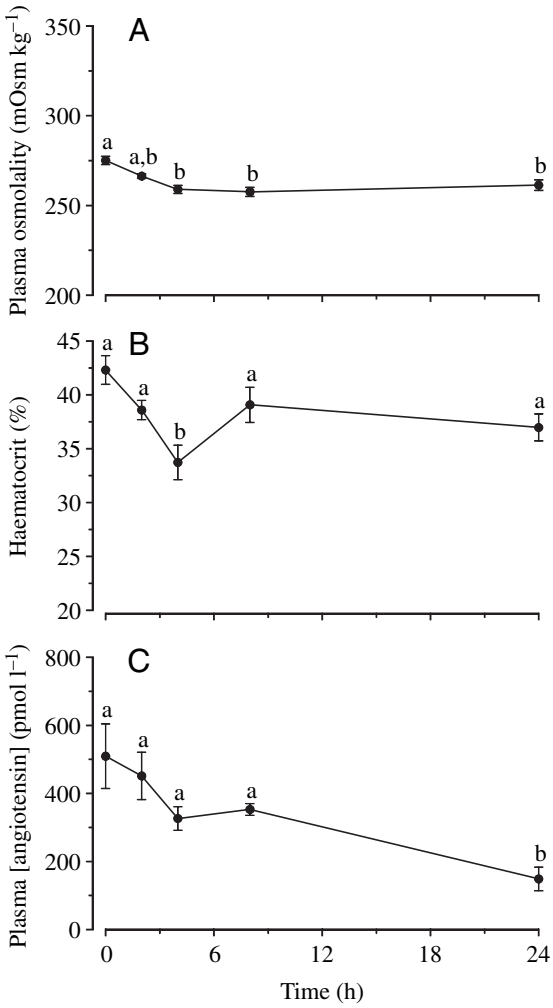

Fig. 5. Effects of a rapid decrease in environmental salinity (from 26 p.p.t., 758 mOsm kg^{-1} to FW, 22 mOsm kg^{-1}) on river lampreys. Blood samples were collected from a group of lampreys prior to transfer and further groups at 2 h, 4 h, 8 h and 24 h after transfer. Data for (A) plasma osmolality (mOsm kg^{-1}; N=8 at each time point), (B) haematocrit (%; N=10 at 0h, 8 at all other time points) and (C) plasma angiotensin concentration (pmol l^{-1}; N=9 at 0 h, N=8 at 2 h and 24 h, N=7 at 4 h and 8 h) are shown). Groups with different letters above error bars differed significantly (ANOVA and *post-hoc* multiple comparison tests).

measurement of plasma renin concentration in cyclostomes (Henderson et al., 1993; Kobayashi and Takei, 1996). Nevertheless, the more than twofold increase in plasma angiotensin concentration observed 30 min after hypovolaemia suggests that pressure/volume receptors play an important role in activating the lamprey RAS, as has been reported in both teleosts and elasmobranchs (Bailey and Randall, 1981; Nishimura et al., 1979; Nishimura and Bailey, 1982; Galli and Phillips, 1996; Bernier et al., 1999). Hypotension following haemorrhage will result in fluid influx to the blood from the interstitial fluid due to the oncotic pressure of plasma proteins. In lampreys, which lack a spleen (and a secondary circulation, as is found in teleost fish), reductions in haematocrit may

quantitatively reflect such haemodilution. If so, the resultant falls in haematocrit (by 36.7%, 36.3% and 40.3% after 30, 60 and 90 min, respectively) indicate that the intention of reducing blood volume by 40%, had indeed been achieved. The initial transient reduction in blood volume (prior to compensatory fluid influx) can be predicted to have triggered a rapid activation of the RAS and plasma angiotensin concentration could well have peaked before the 30 min sample was taken.

The demonstration of pressure/volume-sensitive regulation in the lamprey RAS is perhaps indicative of the most ancient role of the RAS in blood pressure regulation, which has remained a fundamental feature throughout vertebrate evolution. The most immediate effect of elevated levels of circulating angiotensin after blood volume depletion is likely to be vasoconstriction, which would serve to restore blood pressure. The mean circulating concentration of angiotensin that we measured at 30 min after hypovolaemia was ca. 1 nmol l^{-1} (1054.5±203.5 pmol l^{-1}, N=10). Injection of 1 nmol kg^{-1} body mass angiotensin II produced a significant (6 mmHg) increase in dorsal aortic blood pressure in the river lamprey (Rankin et al., 2004). Although this would have rapidly produced a concentration of about 12 nmol l^{-1} if distributed only in the blood volume, pressure effects were observed with lower doses and initial angiotensin concentrations following blood volume depletion could well have been much higher than 1 nmol l^{-1}.

In the present study, plasma angiotensin concentrations recovered within ~1.5 h of blood volume depletion. Our measurements of the declining concentrations of plasma angiotensin after an infusion of Ang II have shown that the half-life of angiotensin in lampreys is approximately 16 min (J. A. Brown, S. C. Frankling, C. S. Cobb and J. C. Rankin, unpublished data) and thus if RAS activation after volume depletion is a short-lived event, basal levels could be achieved within 90 min of volume depletion. Pressure effects would be very rapidly achieved (Rankin et al., 2004) and a restoration of blood pressure would be likely to inhibit further angiotensin formation. A further part of the recovery, at least in FW-acclimated lampreys, could involve a reduction in urine flow rates initiated by angiotensin, as has been shown to occur in teleosts (Brown and Balment, 1997; Brown et al., 2000). Studies are required to describe fully the renal actions of the lamprey RAS, but Asn^1,Val^5-Ang II at 10^{-10} or 10^{-9} mol min^{-1} kg^{-1} body mass has been shown to reduce urine flow rates of FW-acclimated *Lampetra fluviatilis* and *Petromyzon marinus* (J. A. Brown, C. S. Cobb and J. C. Rankin, unpublished data). However, in the blood volume depletion experiments, lampreys were acclimated to hyperosmotic SW and urine flows would already have been minimal (McVicar and Rankin, 1983; Brown and Rankin, 1999).

In addition to examining the effects of blood volume depletion, we explored the effect of i.p. injection of nominally isosmotic saline on plasma angiotensin concentrations with the aim of inducing extracellular fluid expansion, with non-injected FW lampreys as controls. Anaesthesia in the control lampreys resulted in a slight reduction in plasma osmolality between the 15 and 30 min sample times, perhaps reflecting some water retention. This could be the result of a slight decrease in blood pressure that would influence individual nephron filtration rates (McVicar and Rankin, 1985) and hence reduce urine flow (Brown and Rankin, 1999). This argument is questionable, however, as lampreys given approximately isosmotic saline (actually slightly hyposmotic), showed no evidence of any change in plasma osmolality. The i.p. injection of isosmotic saline resulted in a significant decline in plasma angiotensins compared to the level in non-injected FW-acclimated river lampreys at 15 min after the injection, and supports our hypothesis that volume/pressure receptors are important in regulating the lamprey RAS. Similar findings were reported in the Australian lungfish *Neoceratodus forsteri*, in which plasma renin activity was significantly lower after injection of isosmotic saline, although plasma angiotensin concentration was not measured in this study (Blair-West et al., 1977). Our results suggest that the i.p. injection of approximately isosmotic saline led to expansion of the extracellular fluid volume, lowering blood haematocrit by 35% (at 15 min), and hence inhibited the RAS, whereas stimulation of the lamprey RAS occurred after volume depletion.

Although a significant reduction in plasma angiotensin concentration was induced by i.p. injection of nominally isosmotic saline, this did not occur after injection of lampreys with a similar volume of hyperosmotic saline. It is arguable that i.p. injection of hyperosmotic saline might stimulate initial withdrawal of extracellular fluid into the peritoneal cavity and that reduced plasma volume may account for the absence of any reduction in plasma angiotensin concentration. However, there was no evidence of any increase in haematocrit that would have accompanied fluid redistribution into the peritoneal cavity. After both isosmotic and hyperosmotic saline injections, haematocrit was dramatically reduced compared to the haematocrit of non-injected lampreys and did not differ significantly between lampreys injected with isosmotic saline and hyperosmotic saline. Therefore, it would appear that both groups of lampreys were exposed to a volume expansion. However, declining plasma angiotensin concentration was not seen in lampreys i.p.-injected with hyperosmotic saline. The injection of hyperosmotic saline significantly raised plasma osmolality within 15 min and this suggests that osmoreceptors may either directly activate the lamprey RAS, or inhibit the impact of volume/pressure receptors. These results are in agreement with the longer-term increase in plasma angiotensin concentration that accompanied the rise in plasma osmolality after exposure to increased environmental salinity.

In mammals, renin has been suggested to be the major rate-limiting component of the RAS and a complex array of mechanisms involving renal nerve stimulation, baroreceptors and the macula densa interact to control renin release (Kobayashi and Takei, 1996; Nishimura, 2004; Peti-Peterdi et al., 2004). In non-mammalian vertebrates, the control of renin release is still poorly understood (Henderson et al., 1993; Kobayashi and Takei, 1996; Nishimura, 2004) and few studies

have investigated the impact of extracellular osmolality on renin release. In the fowl, infusion of hypertonic saline into the kidney (*via* the renal portal system) and intravenous injection of hypertonic saline depress plasma renin release and plasma angiotensin, respectively (Nishimura and Bailey, 1982; Kobayashi and Takei, 1996). This inhibition of renin release by high osmolality agrees with the results in mammalian studies, suggesting an inverse relationship between plasma sodium/osmolality and the number of renin cells, renin release and plasma renin activity (Kobayashi and Takei, 1996; Peti-Peterdi et al., 2004). Our results show an opposite pattern in lampreys, since high plasma osmolalities were associated with high plasma angiotensin concentrations in both experiments involving changes in external osmolality. Furthermore, while plasma angiotensin concentration declined after injection of lampreys with nominally isosmotic saline, similar changes did not occur after the hyperosmotic saline injection. Stimulation of the RAS after injection of hyperosmotic saline was also reported for the eel (reviewed by Kobayashi and Takei, 1996), so this may be a feature of lower vertebrates. However, we cannot assume that renin was released in either the study of eels or in the present study of lampreys, since plasma renin activity was not determined in either study. Even if plasma renin activity was known, this may not be the only rate-limiting step in the lamprey RAS. In mammals, secretion of angiotensinogen has a rate-limiting effect on maximal formation of plasma Ang I and ultimately, the production of Ang II (Klett and Granger, 2001). Furthermore, our recent studies of the rainbow trout exposed to an osmotic stress have shown increased hepatic angiotensinogen mRNA (R. K. Paley, J. G. Aust, S. J. Aves and J. A. Brown, unpublished observations; Aust, 2002). This suggests that regulation of hepatic angiotensinogen secretion plays a significant role in producing the reported elevations in circulating angiotensin levels and adaptation of teleost fish to hyperosmotic media. However, as yet we have no information on the regulation of lamprey angiotensinogen secretion.

In conclusion, our results indicate that volume receptors exert control on the lamprey RAS, but these receptors may be modulated by sodium/chloride/osmo-sensitive receptors and circulating angiotensin levels will be determined by the interaction of the putative volume and osmo/salt receptors and their relative sensitivities. However, the predictable changes in plasma volume and electrolytes when anadromous lampreys migrate between FW and SW would act as complementary signals in activating the RAS in hyperosmotic environments and inhibition of the RAS in FW. While feeding on fish during the marine phase of their life cycle, lampreys may face imposed changes on extracellular fluid volume and osmolarity that impact on the functioning of the RAS. For example, river lampreys caught in Ringkøbing Fjord, Denmark, just as they begin their upstream migration, have been found with intestines distended with blood representing up to 17% of their body mass (Rankin, 1997). This gut distension when feeding on teleosts may result in a rapid isosmotic volume load, a potential inhibitory signal to the RAS. In contrast, sea lampreys (*Petromyzon marinus*) may meet simultaneous volume and hyperosmotic challenges when feeding on marine sharks that have body fluids roughly isosmotic to seawater (Wilkie et al., 2004). Although undoubtedly difficult to achieve, investigations exploring the potential regulatory role of endocrine systems such as the RAS during the parasitic feeding of lampreys, would be extremely valuable.

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