Comparative Biochemistry and Physiology, Part A 157 (2010) 377–381

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

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

Plasma and brain angiotensin concentrations associated with water response behavior in the desert anuran, *Scaphiopus couchii* under natural conditions in the field

William E. Johnson [a,*], Stanley D. Hillyard [b], Catherine R. Propper [a]

[a] Department of Biological Sciences, Northern Arizona University, Flagstaff, AZ, USA
[b] School of Dental Medicine, University of Nevada at Las Vegas, Las Vegas, NV, USA

ARTICLE INFO

Article history:
Received 2 July 2010
Received in revised form 3 August 2010
Accepted 4 August 2010
Available online 12 August 2010

Keywords:
Angiotensin
Thirst
Hypothalamus
Amphibian
Anura

ABSTRACT

Terrestrial amphibians obtain water by absorption across a specialized region of the ventral skin and exhibit a behavior, the water absorption response (WR) to place that region in contact with moist surfaces. Spadefoot toads (*Scaphiopus couchii*) spend dry months of the year in burrows, then emerge during brief periods of summer rainfall and seek water sources for rehydration and reproduction. We tested the hypothesis that these toads have changes in plasma and/or central angiotensin concentrations that are associated with seasonal emergence and WR behavior. Immunoreactive concentrations of combined angiotensin II and III (ir-ANG) were measured in plasma samples and microdissected regions of brain tissue taken from toads moving across the road or toads showing WR behavior in shallow puddles on the road. Plasma ir-ANG concentrations were not significantly different between these groups, but were significantly higher in the periventricular region of the hypothalamus in toads showing WR behavior. Concentrations in other brain regions, while highly variable among individuals, were not different between groups. Within the context of the natural history of a specialized desert toad, these results support the hypothesis that ir-ANG is associated with WR behavior in spadefoot toads in a manner analogous to oral drinking exhibited by other vertebrate clades.

1. Introduction

Angiotensin II (A-II) has been proposed to regulate thirst responses in vertebrates by two mechanisms. First, circulating A-II may act on receptors in circumventricular regions of the brain. Second, this peptide is produced in central regions where it may act as a neurotransmitter coordinating osmotic and hormonal information (Lind et al. 1985; Phillips, 1987; Unger et al. 1988; Jhamandas et al. 1989: Li and Ferguson, 1993). These proposed mechanisms are based on experiments in which A-II injected either intraperitoneally (ip) or intracerebroventricularly (icv) can induce thirst-related behavior (i.e. oral drinking) in mammals (Epstein et al. 1970), birds (Wada et al. 1975; Snapir et al. 1976; Takei, 1977), reptiles (Fitzsimons and Kaufman, 1977), and fish (Perrott et al. 1992). In addition, increases in serum osmolality and plasma sodium concentrations that result from dehydration and plasma volume depletion are associated with elevated plasma A-II levels in mammals (Mann et al. 1980; Yamaguchi, 1981) and birds (Gray and Erasmus, 1988). Finally, elevated plasma A-II concentrations are associated with increased thirst-related behavior in mammals (Johnson et al. 1984 and fish (Carrick and Balment, 1983; Tierney et al. 1995).

Unlike other vertebrate classes, amphibians obtain water almost exclusively by absorption across their skin (reviewed by Hillyard et al. 1998). In our previous studies with desert anurans, including the spadefoot toad, *Scaphiopus couchii*, either ip or icv administration of A-II induced water absorption response (WR) behavior, in which a specialized area of the ventral skin is pressed to a moist surface (Brekke et al., 1991; Hoff and Hillyard, 1991; Propper and Johnson, 1994; Propper et al. 1995). These findings supported the hypothesis that A-II is involved in the induction of thirst-related behavior in anurans as has been found for oral drinking in other vertebrates (Viborg and Rosenkilde, 2001; Goldstein et al. 2003). However, in laboratory studies, dehydration to 85% of the standard weight (SW; weight of a hydrated toad with an empty urinary bladder, Ruibal, 1962) induced an increase in plasma osmolality and WR behavior in *S. couchii* without a concomitant increase in plasma or brain immunoreactive angiotensin (ir-ANG) concentrations (Johnson and Propper, 2000). Furthermore, neither intracellular nor extracellular dehydration, produced by injection of hypertonic NaCl or volume depletion respectively, was able to induce changes in plasma ir-ANG (Mayer and Propper, 2000) even though these forms of dehydration were

* Corresponding author. Department of Molecular Pharmacology and Physiology, College of Medicine, University of South Florida, 12901 Bruce B. Downs Blvd., MDC 8, Tampa, FL 33612-4799, USA. Tel.: +1 813 396 9963; fax: +1 813 974 3079.
E-mail address: wjohnso3@health.usf.edu (W.E. Johnson).

1095-6433/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpa.2010.08.009

sufficient to induce WR behavior (Taylor et al. 1999). Thus, in laboratory conditions, increased WR behavior in dehydrated *S. couchii* is not correlated with increases in endogenous ir-ANG.

S. couchii are only active above ground during brief periods of summer monsoon rainfall. They remain in burrows for the remainder of the year and may experience considerable increases in plasma osmolality during these periods of aestivation (McClanahan, Jr., 1967; Shoemaker et al. 1969). With the first monsoon rains, emergent toads seek out water accumulating in ephemeral ponds and initiate breeding activity. While in transit to the ponds, toads can be observed in WR behavior, presumably to restore body fluid homeostasis (Johnson, Propper and Hillyard, pers. obs.). Newly emergent toads have higher plasma osmolalities than toads captured several days later after they have had opportunities for rehydration (Johnson et al. 1995). Increased osmolality and associated endocrine signals may coincide with environmental cues to act as a signal for emergence and for rapid rehydration for *S. couchii* (Dimmitt and Ruibal, 1980; Hillyard, 1999). We tested the hypothesis that WR behavior exhibited by newly emerged *S. couchii* is associated with increased plasma osmolality and that elevated plasma and brain A-II concentrations are associated with WR behavior exhibited under natural conditions.

2. Materials and methods

2.1. Animal capture and tissue collection

A total of 18 male *S. couchii* were collected from roadways in and around Buenos Aires National Wildlife Refuge in Pima County, AZ following the onset of summer monsoon rainfall. Toads were collected while in one of two behaviors: WR behavior on roadways on the first two nights ($N=7$), active on the roadway but not in WR behavior on the second and third nights ($N=11$). A maximum of 10 min was allowed from capture to sample collection. All animals were collected according to state of Arizona and Federal regulations, and all animal treatment procedures were approved by the Northern Arizona University IUCAC in accordance with NIH Guide for the Care and Use of Laboratory Animals. Toads were euthanized by direct decapitation to avoid possible central nervous effects of MS 222 as noted by Hedrick (2003) in the bullfrog brainstem.

Trunk blood was collected into a 1 mL heparin treated syringe, and then transferred to a 1.5 mL centrifuge tube containing 7.5 μL of a peptidase inhibitor cocktail (4 μg/mL enalpralat maleate (Sigma), 440 μg/mL 1,10 phenanthroline (Sigma), 40 μg/mL APMSF (Sigma), 1 μg/mL pepstatin (Sigma), 0.1%-mercaptoethanol (Sigma), and 6.25 mM EDTA (Johnson and Propper, 2000). The blood was immediately centrifuged at 9000×*g* (Tomy-Seiko HF-120 microcentrifuge) for 2 min. The plasma was pipetted into a cryogenic storage vial and stored in liquid nitrogen. Plasma osmolality was measured from 10 μL samples on a Wescor 5500 osmometer. In a pilot study with *Bufo cognatus* plasma, the addition of the peptidase inhibitor had no effect on osmolality (Johnson, unpublished data).

Brains were dissected and placed in Tissue Tek O.C.T. compound then frozen in a foil boat that rested on top of liquid nitrogen. After the brain-O.C.T. block was frozen, it was placed in a cryogenic storage tube and stored in liquid nitrogen until transfer to a -80 °C freezer at the laboratory. Brains were frozen to cryostat chucks with Tissue Tek O.C.T. compound then sectioned in 200 μm thick transverse sections in a Leica cryostat kept at -17 °C. Sections were frozen to microscope slides and stored at -80 °C in an airtight slide box until microdissection. Eight areas of the brain were identified using descriptions from Northcutt and Royce (1975) and Neary and Northcutt (1983). The micropunch technique was utilized to remove all brain areas (Palkovitz and Brownstein, 1982; Zoeller and Moore, 1985; Johnson and Propper, 2000). Brain sample preparation was previously described in Johnson and Propper (2000).

2.2. Radioimmunoassay of angiotensin

Plasma procedures were carried out on ice (4 °C). Unextracted plasma was used because of small volumes of plasma from some animals (150-500 μL). Mann et al. (1980) used unextracted plasma when measuring dipsogenic (thirst-inducing) levels of A-II in rats, and Linjen et al. (1978) found significant correlation between extracted and unextracted angiotensin II concentrations. Cross reactivity was measured for angiotensin I, angiotensin III (A-III), and arginine vasotocin. Since angiotensin III cross-reacted 100% with the antiserum (Johnson and Propper, 2000), results are reported as immunoreactive angiotensin (ir-ANG) concentrations. Standards and parallelism for plasma and brain samples were determined previously in experimental methods described in Johnson and Propper (2000).

Sixty microliters of unextracted plasma was brought up to 100 μL with PE-gel buffer (10 mM potassium phosphate, 1 mM EDTA, 0.1% gelatin, pH 7.3). Standard curves were obtained by adding 100 μL of ^{5}Val-A-II (0.25-1000 pg; BACHEM) in assay buffer, 100 μL of the antiserum 1:75,000 in assay buffer (Peninsula IHC-7002), and 50 μL of 7000-8000 cpm of labeled tracer (3-[^{125}I]-(*iodotyrosyl*^{4}) angiotensin II (5-leucine), Amersham UK). All standards were run in duplicates. Tubes were incubated 18 h at 4 °C. One milliliter of dextran-coated charcoal (1 g NORIT-A charcoal with 0.02 g Dextran (Sigma) in 100 mL PE-Gel assay buffer) was pipetted into each tube and tubes were immediately centrifuged at 900×*g* for 10 min. Supernatants were counted on a Beckman 5500B gamma counter. The minimum detection limit ranged from 0.39 to 1.2 pg. The intraassay coefficient of variation was 9.0 ± 1.3%, and the interassay coefficient of variation was 21 ± 0.8 % for 2 pg and 20 pg replicates on five assays.

Lyophilized brain homogenates were reconstituted in 100 μL PE-gel assay buffer. Standard curves were obtained by adding 100 μL of assay buffer with standard ^{5}Val-A-II (Bachem, 0.25-1000 pg), 100 μL of the antiserum diluted 1:75,000 (Peninsula IHC-7002), and 50 μL of 7000-8000 cpm of labeled tracer (3-[^{125}-I]-(*iodotyrosyl*^{4}) angiotesin II (5-leucine), Amersham UK. The minimum detection limit ranged from 0.39 to 1.2 pg. The intraassay coefficient of variation was 7.6%, and the interassay coefficient of variation was 12.5%.

2.3. Statistical analysis

Plasma osmolality concentrations were analyzed by Student's *t*-test. Differences in plasma ir-ANG concentrations between groups were analyzed by Students *t*-test on natural log transformed data. Because transformations did not remove the inequality of variances between treatments, differences in brain angiotensin concentrations between active and WR behavior toads were analyzed using the Wilcoxon–Mann–Whitney test. Spearman rank order was used to conduct correlation analysis. The power-efficiency of the nonparametric tests used in this study approaches 96% in moderate sized samples, thus they are an excellent alternative to the parametric *t*-test when the assumptions associated with the t-test cannot be fulfilled (Siegel & Castellan, 1988).

3. Results

3.1. Plasma angiotensin and osmolality

Active toads had significantly higher plasma osmolalities than the WR toads (Fig. 1, $p=0.04$). The plasma ir-ANG concentrations were not significantly different between the two groups (Fig. 2; $p=0.07$); however, there was a negative correlation between plasma osmolality and ir-ANG concentrations (Fig. 3; $r_s=-0.52$, $p=0.04$).