TIPS, TRICKS & TECHNIQUES

A Simple Experiment Demonstrating Hormonal Control of Cutaneous Drinking in Toads

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ABSTRACT

Simple teaching experiments demonstrating endocrinological concepts are difficult to come by. I discuss a simple experiment demonstrating control of cutaneous drinking by the hormone angiotensin II in terrestrial toads, designed after methods published in the primary literature and presented herein to make this exercise more widely accessible to ABT *readers. This experiment is notable in that it (1) permits students to quantify both a physiological and a behavioral response to exogenous hormone administration; (2) can be implemented in relatively small class sizes; (3) does not require animal euthanasia; and (4) can be accomplished in one class period. Furthermore, data collection can be easily carried out by students using common laboratory supplies, and analysis and interpretation of the collected data are straightforward. Finally, in demonstrating the function of the highly conserved renin-angiotensin-aldosterone system, this hands-on experiment has obvious clinical connections relevant to human medicine (e.g., blood-pressure regulation and treatment of hypertension).*

Key Words: *Angiotensin II; blood pressure; endocrinology; hormone; osmoregulation; toad.*

Introduction

The peptide hormone angiotensin II (AII) is well known for its role in plasma-volume and blood-pressure regulation in vertebrates, in part due to its effects as both a dipsogen (i.e., promoter of thirst) and a vasoconstrictor of arterioles. AII is generated as one of three major hormone products of the renin-angiotensin-aldosterone system (RAAS; Figure 1 illustrates the details of the RAAS in mammals, including humans). As a dipsogen, AII stimulates oral drinking in some fishes and in most terrestrial vertebrates (i.e., reptiles, birds, and mammals; Fitzsimons, 1998). Amphibians, however, are a major exception in that, rather than drinking water orally, they absorb water across their skin (a process termed *cutaneous drinking*; Bentley & Yorio, 1979). The majority of water absorbed via cutaneous drinking in anurans (frogs and toads) occurs across a highly vascularized region of the ventral skin called the *pelvic patch*, located primarily in the "seat" area between the hindlimbs. AII is now known to stimulate cutaneous drinking in anurans by inducing the *water absorption response*, a stereotyped behavior in which the pelvic patch is pressed against a moist substrate while abducting the hindlimbs, thus increasing the surface area of apposed ventral skin engaged in water absorption (see photographs and behavioral descriptions in Stille, 1958; Brekke et al., 1991; Hillyard et al., 1998, 2007). The mechanism of action for AII in anurans (Figure 2) is hypothesized to involve direct stimulation of water-absorption behavior (mediated via hypothalamic AII receptors; Propper et al., 1995; Hillyard et al., 1998; Johnson et al., 2010) and an increase in cutaneous blood flow to the pelvic patch (Slivkoff & Warburton, 2001), as well as the possible indirect effect of AII in stimulating release of the neuropeptide arginine vasotocin (AVT; the antidiuretic hormone found in non-mammalian vertebrates; Bentley, 2002) from the posterior pituitary. In anurans, AVT induces insertion of aquaporins into the epithelium of the ventral and hindlimb skin, urinary bladder, and collecting duct of the renal tubule, thus enhancing passive water absorption, storage, and reabsorption in response to dehydration (Hasegawa et al., 2003; Willumsen et al., 2007; Ogushi et al., 2010).

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The goal of this experiment is to compare water absorption rate and time spent in the water absorption response (WR) between control and AII-treated toads provided with voluntary access to a water-saturated gauze substrate. I modeled the protocol presented herein after a seminal study by Hoff and Hillyard (1991), as well as after some subsequent related experiments (e.g., Goldstein et al., 2003). Interested readers are also referred to the wonderful review of the subject by

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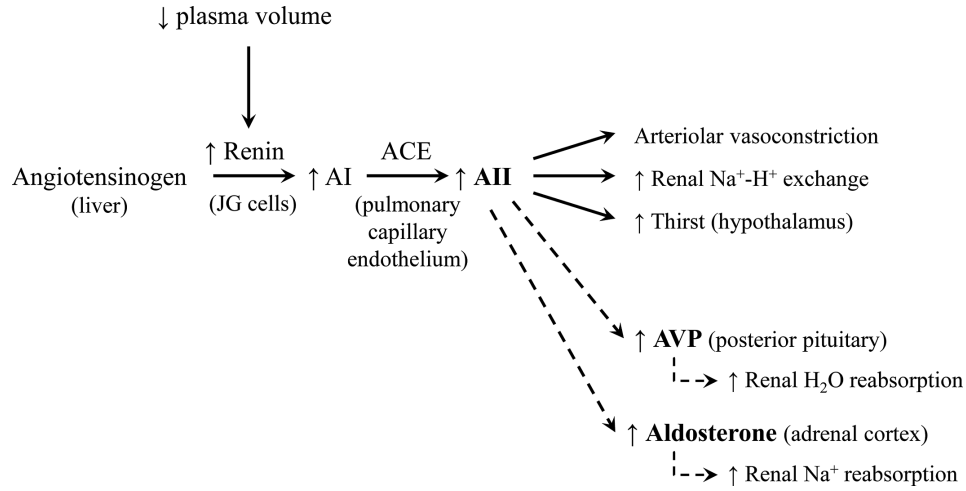

Figure 1. The renin-angiotensin-aldosterone system (RAAS) in mammals. The RAAS is initiated in response to low plasma volume, resulting in the formation of three major products: angiotensin II (AII), arginine vasopressin (AVP, the antidiuretic hormone found in mammals), and aldosterone. Angiotensin II is produced via a two-step pathway catalyzed by the enzymes renin (released by juxtaglomerular [JG] cells of the kidney) and angiotensin converting enzyme (ACE). Low renal and systemic blood pressures serve as the primary stimuli for renin release, thus initiating the RAAS. The indicated effector responses collectively raise plasma volume and blood pressure. Indirect effects of AII are represented with dashed arrows. AI = angiotensin I. (After Boron & Boulpaep, 2012; Hill et al., 2012; Costanzo, 2014.)

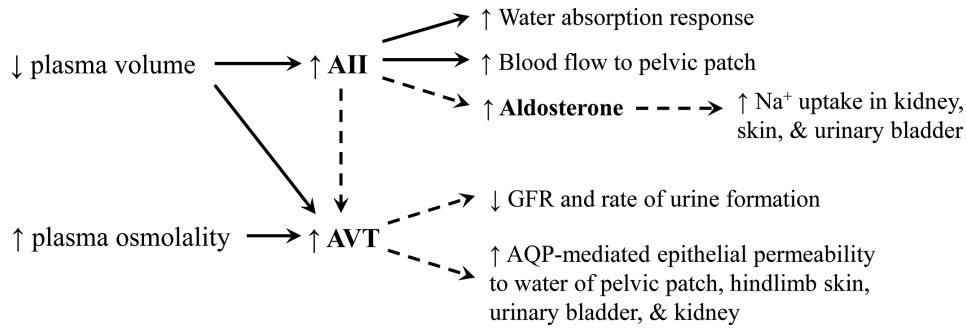

Figure 2. Possible mechanisms of angiotensin II (AII) action in anurans. The enzymatic pathway resulting in production of AII (not shown) is the same as for mammals (see Figure 1). Responses initiated by AII, arginine vasotocin (AVT), and aldosterone serve to restore water balance by promoting increased water absorption and retention (thus offsetting initial water losses due to dehydration). GFR = glomerular filtration rate (i.e., the rate at which primary urine is formed via filtration of blood plasma within the kidneys); AQP = aquaporin. (After Hillman et al., 2009.)

Hillyard et al. (1998). Although this exercise is intended for an undergraduate-level vertebrate or human physiology course in which students are exposed to endocrinology and/or osmoregulation in lecture, the experiment is likely simple enough in concept and practice to be performed by high school students as well.

○ Methods

Instructor Preparation

Small, terrestrial, bufonid toads in the genus *Anaxyrus* (formerly *Bufo*) are seasonally available from Carolina Biological Supply and Ward's Science (e.g., American Toads [*A. americanus*] and Southern Toads [*A. terrestris*]). Toads can be maintained at room temperature in small groups (two or three individuals) in 38 L (10 gallon) glass aquaria with moist moss and/or soil substrate deep enough to permit burrowing. Water should be available ad libitum in a large glass dish, and crickets should be offered as food two or three times weekly. Toads should be fasted for one to two days before use.

Just prior to experimentation, toads are hydrated by soaking them in distilled water (2 cm depth) for two hours. This ensures that all toads are properly hydrated before initiating the experiment (so that control toads do not have elevated levels of endogenous AII). After the hydration period, the toads' urinary bladders are drained by inserting a polished glass microhematocrit tube into the cloaca and gently applying pressure to the abdomen (previous work indicates that toads spend more time in WR, absorb greater amounts of water, and are more responsive to AII treatment if their

bladders are empty; Tran et al., 1992). When performing this procedure, I hold the toad in one hand with its ventral surface facing up, situating my thumb and index finger on each side of the abdomen just anterior to the hindlimbs. The microhematocrit tube is then inserted a short distance (~1 cm or less) into the cloaca, so that the cloaca is held open as the thumb and index finger are gently depressed to expel urine from the bladder. In many toads, the procedure is often facilitated by voluntary urination during manipulation.

After voiding the urinary bladder, toads are injected with equal volumes of angiotensin II or control solutions. AII-treated toads are injected intraperitoneally with 0.1 mL of 2 mg/kg [Val^5]-angiotensin II acetate salt hydrate (Sigma-Aldrich: A2900-5MG) dissolved in amphibian Ringer's solution (6.50 g/L NaCl, 0.14 g/L KCl, 0.20 g/L $NaHCO_3$, 0.12 g/L $CaCl_2$). Control toads are injected intraperitoneally with 0.1 mL of amphibian Ringer's solution only.

Overview of Student Experiment

Following injection, each toad is immediately given to a pair of students. The toads are then carefully blotted with a KimWipe, and initial body masses (±0.01 g) are recorded on a digital balance. After recording initial mass, each toad is placed individually in a ventilated small plastic container (floor dimensions approximately 13 cm × 24.5 cm) containing a water-saturated gauze pad substrate (I saturate 10.1 cm × 10.1 cm Johnson & Johnson large gauze pads with 50 mL distilled water). I have had success in providing toads with either a partial or a complete saturated gauze substrate (one or two gauze pads covering the container floor, respectively), but I recommend the latter to prevent the likelihood that a toad never encounters the substrate during a trial. The toads are allowed to adjust to container conditions for 30 minutes, after which time body mass is recorded at 10-minute intervals over a total observation period of 60–90 minutes (subject to time available during the lab period). Students are instructed to note the toad's location in the container before removing it for weighing, and to return it to the same exact location after recording its mass. Throughout the trial (including the initial 30 minutes), time spent in the WR posture is measured with a stopwatch and recorded concurrently with body-mass measurements. To make behavioral assessment as objective as possible for students, a toad is scored as in the WR posture whenever it is seated with any aspect of the ventral surface in contact with the gauze substrate (rather than distinguishing between "seat patch down" and full WR posture; Hillyard et al., 2007). As part of the introduction to the lab, students are shown photographs from the primary literature (Hillyard et al., 1998, 2007) to emphasize the difference between a toad's normal resting posture (ventral surface elevated with only feet touching the substrate) and WR posture; such differences in posture are readily diagnosed by students. Students work in pairs during data collection, such that one student monitors toad posture and works the stopwatch, while the other is tasked with obtaining and recording mass measurements. If a toad urinates or defecates while in the test container or during handling, data collected during the corresponding time interval are omitted.

At the conclusion of a trial, water absorption rate (grams per hour) is calculated as follows:

$$\frac{\text{Final body mass} - \text{Initial body mass}}{\text{Total minutes elapsed}} \times 60$$

If the toads vary appreciably in body size, it may be reasonable to instead express water absorption rate as a percentage of each toad's initial mass (e.g., percent initial body mass per hour). Because total elapsed time might vary among student groups, time spent in the WR posture is expressed in relation to the length of the sampling period:

$$\frac{\text{Elapsed minutes in WR}}{\text{Total minutes elapsed}} \times 100\%$$

○ Results & Discussion

Sample data are provided in Figure 3. Note the obvious difference between treatment groups, even without a very large sample size: AII-treated toads absorb water at a faster rate and spend more time in the water absorption response than control toads. If statistical analysis is desired, data can be analyzed by students with a simple t-test.

Although this experiment has proved quite effective, some caveats apply. Variability among toads in activity level can be problematic, so data for some individuals in either treatment may need to

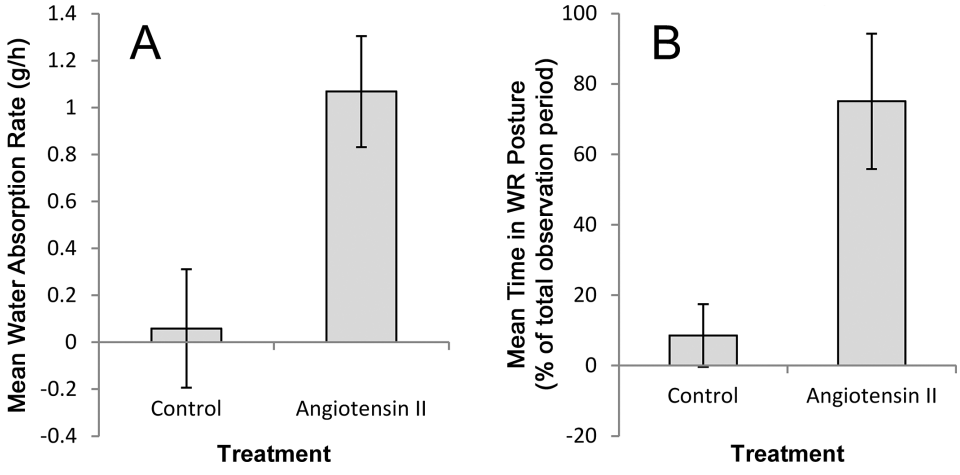

Figure 3. (**A**) Mean water absorption rate and (**B**) mean percentage of time spent in the water absorption response (WR) posture for control (n = 6 trials) and angiotensin II-treated (n = 8 trials) toads. Data are combined from two laboratory sessions using a total 9 individual toads. Values are means ± 2 SE.

be entirely omitted if a particular toad is continuously active. For that reason, pooling data among lab sections may be beneficial. Furthermore, investigators have noted interspecific differences among toads in willingness to engage in WR behavior on an artificial substrate or in an unfamiliar environment (Hillyard et al., 1998), which should be considered if the instructor intends to use an anuran species that has not previously been a subject of study. Likewise, latency (i.e., the delay between AII administration and initiation of the water absorption response) may vary among species but has infrequently been reported in the primary literature (Viborg & Rosenkilde, 2001); although latency may be on the order of 60 minutes in some species, AII treatment effects can be observed in as little as 30 minutes. Thus, the observation period of 60–90 minutes recommended here should be adequate for most species. Sensitivity to AII also appears to vary among toad species, with minimum effective doses ranging from 0.01 to 1 mg/kg among species (Hillyard et al., 1998). The 2 mg/kg dosage I use is at the high end of dosages employed in the primary literature, and should be sufficient for most species.

The experiment presented here is meant to be simple but can be modified to meet the goals of the instructor. For example, if resources are not limited, additional components might include use of multiple AII dosages to examine dose–response relationships, application of an AII + saralasin (AII receptor antagonist) treatment (Hoff & Hillyard, 1991), variation in toad hydration state, variation in osmotic concentration of the substrate, or interspecific comparisons among anurans from different habitats (e.g., terrestrial vs. aquatic or semiaquatic species).

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