Is aldosterone synthesized within the rat brain?

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Submitted 3 August 2004; accepted in final form 8 October 2004

Evidence suggesting that aldosterone synthesis within the brain may be relevant in pathophysiological situations is derived from our studies in the inbred Dahl salt-sensitive (SS) rat. The salt-induced hypertension in SS rats is abrogated by the central infusion of mineralocorticoid receptor antagonists, as well as the amiloride analog benzamil, suggesting that aldosterone is acting in the brain in this form of hypertension (11–13). However, basal plasma levels of aldosterone of the SS rats are normal, much like those of low-renin essential hypertensive patients who respond to anti-mineralocorticoid therapy, for example, spironolactone or triamterene (2). We found that the salt-induced hypertension in the inbred Dahl SS/qr rat was mitigated by the intracerebroventricular infusion of 19-ethyl-11-deoxycorticosterone (19-ethyl-DOC), a suicide inhibitor of aldosterone synthase, at a dose that had no effect when infused systemically (8, 9). We have also found that the intracerebroventricular, but not subcutaneous, infusion of 0.03 µg/h triolostane, a 3β-steroid dehydrogenase antagonist, effectively blocked the increase in systolic blood pressure and reversed the hypertension produced by a high-salt diet in the Dahl SS rat. It is not known what proportion of the aldosterone content of the brain is derived from circulating aldosterone and how much, if any, is synthesized de novo in the brain. The purpose of these studies was to determine whether aldosterone is synthesized in the brain of normotensive rats in vivo and whether this synthesis is regulated by manipulation of dietary sodium.

MATERIALS AND METHODS

Animal subjects. These studies were carried out in young-adult female Wistar rats (Harlan). Rats in a given experiment were of the same age and shipment. Husbandry and all procedures followed the National Research Council’s Guide for the Care and Use of Laboratory Animals and were performed in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility. The animal care and use protocols were approved by the G. V. (Sonny) Montgomery Veterans Affairs Institutional Animal Care and Use Committee. Adrenalectomies were performed through bilateral flank incisions, with animals under isoflurane anesthesia delivered by a standard anesthetic machine with buprenorphine for postoperative analgesia. All adrenalectomized rats were provided 0.9% saline (high salt) to replace sodium losses. Some adrenalectomized animals received corticosterone at 0.83 mg/day or DOC at 0.083 mg/day sc by pellets (IRA, Toledo, OH), aldosterone at 4.8 µg/day sc by miniosmotic pump (Alzet, Cupertino, CA), or DOC at 5 mg sc as an emulsion of 1:1 saline-oil 3 h before euthanasia to provide a large excess of substrate for extra-adrenally expressed 11β-hydroxylase and aldosterone synthase enzymes. Rats were fed ad libitum a standard normal-salt diet (NS; 0.28% NaCl, Teklad), low-salt diet (LS; NaCl <0.02%), or high-salt diet (HS; standard chow + 0.9% saline to
drink). Two to three days before euthanasia, the rats were transported each morning to a quiet room adjacent to the animal surgical suite to acclimatize them to the routine. The rats were mask induced with isoflurane delivered in oxygen with a standard anesthesia machine, blood was drawn from the left ventricle into an EDTA vacutainer, and the still-beating heart was removed, producing immediate exsanguination. Within 5 min of the rat being taken from its cage, the brain was removed and frozen in liquid nitrogen. To assess blood contamination of tissues, hemoglobin was measured in hearts from five rats harvested in this manner, using an ELISA for human hemoglobin (Bethyl Laboratories, Montgomery, TX) and rat hemoglobin to construct the standard curves. Hemoglobin content of hearts was negligible, suggesting minimal contamination of tissues with blood.

**Aldosterone, corticosterone, and DOC assays in plasma.** A volume of 0.5 ml of plasma was extracted in 5 ml of dichloromethane and reconstituted in 200–250 μl of ELISA buffer, and 50 μl were used to measure aldosterone by ELISA with a specific monoclonal antibody (6). The intra- and interassay variability was 10% (1); all samples within an experiment were measured in the same assay. The reconstituted extract was diluted 1:100, and corticosterone was measured by ELISA using a sheep polyclonal antibody (21). Plasma DOC was measured by radioimmunoassay (7) in 0.5 ml of plasma extracted with 7% dichloromethane in hexane (7). All assays were done in triplicate and included blanks; for most experiments, assays were run more than once to assure reproducibility.

**Measurement of aldosterone and corticosterone in the brain.** The brain was weighed, and 5 ml of water were added containing 2,000 counts/min of tritiated aldosterone for estimation of recoveries. It was then homogenized by use of a Polytron and extracted with 25 ml of dichloromethane; the water phase was discarded. To clean the sample, steroids in the dichloromethane extract were adsorbed on to a silica gel column (silica gel grade 62; Sigma-Aldrich, St. Louis, MO) prewashed with 5 ml of dichloromethane and then eluted with 5 ml of dichloromethane containing 7% methanol. The organic extract was evaporated and reconstituted in 250 μl of ELISA buffer (20 mM sodium phosphate, 100 mM NaCl, 0.01% thimerosal, and 0.05% Tween 20). A 50-μl aliquot was used for the estimation of recoveries, and 50 μl were used in triplicate for the aldosterone assay. The sensitivity of the ELISA for aldosterone was 1 pg/well; for corticosterone, it was 10 pg/well. A dilution of 1:100 (vol/vol) was used for measurement of corticosterone in intact rats and 1:10 (vol/vol) for adrenalectomized rats. The dichloromethane was screened and redistilled when necessary to obtain negligible background signals for the steroid ELISAs. Recoveries were measured, and the values were corrected for each tissue sample. Results were expressed as picograms per gram (aldosterone) or nanograms per gram (corticosterone) of tissue and as picograms per milliliter and nanograms per milliliter, respectively, for plasma. No animal in the adrenalectomized groups had significant amounts of plasma; thus none were removed from the study.

Differences between groups were evaluated by ANOVA, followed by Tukey’s contrast where appropriate (STATISTICA 6.0, StatSoft package). Where values in some animals were too low to measure, as in adrenalectomized rats, a nonparametric evaluation using Kruskal Wallis and the Dunn test was used (Mann-Whitney U-test). Results are expressed as means ± SE. Where the differences between the values of the groups were so great that the standard error of one was several orders of magnitude greater than another, logarithmic transformations were done before the ANOVA.

**RESULTS**

Figure 1 shows the effects of different NaCl intakes and adrenalectomy (ADX) on serum and brain aldosterone and corticosterone concentrations of 9-wk-old female Wistar rats (n = 10 for NS and HS; n = 9 for LS, NS, and ADX). The groups were as follows: intact-NS, intact-LS, intact-HS, and ADX-HS. Tissues from all of the rats were harvested on day 15 of the dietary assignment and 7 days after ADX in the ADX-HS group. Dietary salt did not have a predictable or statistically significant effect on plasma corticosterone levels in the intact animals, and plasma corticosterone was detected in one of the ADX-HS rats. Corticosterone contents of the brains of intact-NS, -LS, and -HS rats were not significantly different from each other, and all were significantly greater than those of the ADX-HS brains, four out of nine of which had measurable corticosterone, with a mean of 0.6 ± 0.2 ng/g.

Plasma aldosterone was significantly increased by 15 days of the LS diet compared with the NS diet and was decreased by the HS diet (Fig. 1). Plasma aldosterone was undetectable in all of the ADX rats. Levels of aldosterone in the brains of the adrenalectomized rats were proportional to those in plasma. In contrast to that, in plasma, aldosterone levels in the brains of the ADX rats, although very low, were measurable in five of nine brains (6.4 ± 2.5 pg/g). In a separate analogous experiment, a similar amount of aldosterone (6.9 ± 1.5 pg/g) was measured in the brains of all 11 ADX rats. The aldosterone contents of the brains in the different dietary salt-intake groups were significantly different from each other and those of the ADX rats (P < 0.01).

The effect of ADX and ADX with aldosterone replacement compared with an HS diet is shown in Fig. 2. Twelve-week-old rats (n = 11 for HS and 10 for ADX-HS and ADX-HS + aldosterone) were randomly assigned to three groups. Groups 2 and 3 were adrenalectomized, and group 3 received a miniosmotic pump (Alzet) subcutaneously that was primed to deliver 0.2 μg/h aldosterone on insertion. All rats, including the controls, received an HS diet in the form of 0.9% saline to drink ad libitum thereafter. Tissues were harvested on day 8. Plasma and brain aldosterone contents of the aldosterone replacement-ADX rats were not statistically different from those of the intact rats drinking saline; both were significantly different from the ADX animals. Plasma aldosterone (1 ± 0.8 pg/ml) was measurable in only 2 of 10 ADX rats, while aldosterone was reproducibly measured in all brains of the ADX animals (9.9 ± 1.9 pg/g). Aldosterone concentration in the brain was significantly less in the ADX-HS rats compared with the intact-HS rats (P < 0.0001; ADX-HS + aldosterone, P < 0.001). In addition to negligible tissue hemoglobin content, the consistent detection of aldosterone in the brains, but not plasma, of ADX rats provides further evidence of minimal contamination of tissues with blood.

To determine whether the rate-limiting factor for aldosterone production in the brains of ADX rats was the lack of precursor steroids, animals received DOC pellets (2.5 mg/30 day = 0.083 mg/day in rats adrenalectomized for 7 days, n = 9). The aldosterone concentrations in the plasma of both groups were undetectable. Aldosterone was measurable in five of nine brains of ADX rats and seven of nine brains of ADX + DOC rats. The mean aldosterone concentration in brains of ADX rats with no treatment was 6.4 ± 2.6 pg/g; that in the brains of ADX + DOC rats was 11.9 ± 3.1 pg/g wet tissue wt. There was no statistical difference between the ADX and ADX + DOC brain aldosterone concentrations in this experiment. In another experiment, rats (n = 8) were adrenalectomized for 48 h, and 5 mg of DOC or vehicle were administered subcutaneously 3 h before tissue collection. Here, plasma DOC was undetectable in the ADX rats and detectable at 21 ng/g in the
DOC replacement rats. Brain aldosterone was 41 ± 23 in the ADX and 54 ± 6 pg/g in the ADX + DOC rats. Aldosterone synthase can also use corticosterone as a substrate. However, provision of exogenous corticosterone as pellets that delivered 0.83 mg/day did not increase the plasma or brain aldosterone concentration in ADX-HS rats.

**DISCUSSION**

Figure 1 represents one of three different experiments in which plasma and brain corticosterone and aldosterone contents were measured in animals with different levels of salt intake. Changes in sodium levels of the diet did not have a significant effect on plasma corticosterone concentration, which was not unexpected, or brain corticosterone concentration. Brain corticosterone levels reflected, but were invariably lower than, those in the plasma, assuming that brain tissue concentrations (measured as ng/g) are comparable to plasma concentrations (expressed as ng/ml). Adrenalectomy resulted in negligible corticosterone levels in plasma and whole brain in all experiments, suggesting that the circulation is the most likely source of brain corticosterone.

Circulating aldosterone levels responded to dietary sodium, as expected, in all experiments: a low-salt diet stimulated and a high-salt diet suppressed production. Brain levels of aldosterone in adrenal-intact rats reflected plasma levels. Adrenalectomy lowered plasma aldosterone to below measurable levels in almost all individuals and to just barely detectable in a very few others. Brain aldosterone levels in the adrenalectomized

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Fig. 1. Top: plasma and brain aldosterone concentrations in rats fed a low-salt (LS), normal-salt (NS), and high-salt diet [HS and adrenalectomy (ADX)-HS] for 15 days (see RESULTS for group descriptions). On day 8, the ADX-HS rats were adrenalectomized. Middle: same data, with the HS and ADX-HS values plotted on an expanded y-axis. Bottom: plasma and brain corticosterone concentrations. Values are means ± SE; n = 10 for NS and HS, and n = 9 for LS, NS, and ADX. Values are compared with NS control (top and bottom) and with HS (middle): *P < 0.01, **P < 0.001, and ***P < 0.0001.
rats were low but consistently and reproducibly measured. These data suggest that aldosterone is produced in the brain, but that most of the aldosterone in the whole brain is derived from the circulation, even in rats whose adrenals are suppressed by a high-salt diet. Because all adrenalectomized animals must be maintained on supplemental NaCl for life, in these experiments (0.9% NaCl ad libitum), the effect of dietary sodium was not tested in the adrenalectomized animal.

11-DOC is the primary substrate for 11β-hydroxylase and aldosterone synthase, the last enzymes in the synthesis of corticosterone and aldosterone, respectively. While all of the enzymes required to make DOC from cholesterol have been documented in the brain, most of the neurosteroids are derived from pregnenolone and progesterone, leading to very little synthesis of DOC (22). If substrate were the limiting factor, the synthesis of DOC (22) would have increased aldosterone synthesis in the brain, just as the systemic renin-angiotensin system is the primary regulator of aldosterone production by the adrenal. Ye et al. (28) have reported that the systemic infusion of angiotensin II had no effect on aldosterone synthase mRNA. However, aldosterone synthase mRNA is expressed in very small quantities in the brain and may have been below the limits of detection in the relatively large areas of the brain assayed. This may explain the extensive background signal and large variations in the real-time RT-PCR assays presented (28). In addition, angiotensin II administered peripherally may not have access to areas of the brain in which aldosterone synthase is active (28).

Steroids are lipophilic and able to cross the blood-brain barrier; therefore, it is not surprising that most of the aldosterone and corticosterone present in the normal brain is derived from the circulation. Adrenalectomy causes a large and protracted increase in ACTH production; however, corticosterone levels in both the plasma and brain of adrenalectomized rats were below or just at the limits of detection.

The relevance of aldosterone production in the brain of healthy individuals is still not known. Extra-adrenal production of aldosterone apparently occurs and is physiologically important in the rat cochlea (17). MR are expressed in large numbers in the cochlea, and mineralocorticoids are important for endolymph homeostasis. However, adrenalectomy has little effect on the ionic composition of endolymph or hearing ability. Le Cain et al. (17) demonstrated both mRNA and protein for all of the enzymes required for the synthesis of aldosterone from cholesterol by RT-PCR, in situ hybridization, and immunohistochemistry in the cochlea but were unable to demonstrate the expression of 11β-hydroxylase. The authors speculate that locally produced aldosterone acts in a paracrine fashion through the classic MR and/or through a nongenomic mechanism on membrane receptors or ionic exchangers (17).

Paracrine or autocrine access of aldosterone to MR of the brain has important implications. The MR is not intrinsically selective for aldosterone over the natural glucocorticoids, corticosterone and cortisol. Because these circulate at 100- to 1,000-fold the amounts that aldosterone does, the MR is occupied by glucocorticoids in the absence of a mechanism that gives aldosterone competitive advantage. In the kidney, the 11β-hydroxysteroid dehydrogenase 2 enzyme (11-HSD2) confers extrinsic selectivity to the MR by converting corticosterone to the inactive 11-dehydrocorticosterone (and cortisol to cortisone). 11-HSD1 is bidirectional but is primarily responsible for conversion of the 11-dehydro-metabolites of corticosterone and cortisol to their active products. There are distinct regional differences in the expression of the 11-HSD isozymes in the brain (24); however, the 11-HSD isozymes in most neurons are thought to modulate glucocorticoid binding to the MR and glucocorticoid receptors rather than limit access exclusively to aldosterone (25). Hippocampal MR are occupied by glucocorticoids at low physiological levels (16) and require glucocorticoids as ligands for neuronal homeostasis (3). The
mechanism(s) for MR selectivity for aldosterone in the circumventricular organs is still not clear but may involve more than protection of the MR by colocalization with 11-HSD2, as expression of this enzyme decreases dramatically in the brain at birth (14). Local production of aldosterone would confer stoichiometric advantage over corticosterone binding to the MR. Ye et al. (28) reported that the expression of aldosterone synthase mRNA in the hippocampus and cerebellum increased with sodium restriction, as it does in the adrenal gland; however, as mentioned above, aldosterone synthase mRNA was not altered by chronic sodium loading or angiotensin II administration, conditions that decrease or increase, respectively, aldosterone synthase in the adrenal gland (28).

In summary, most of the aldosterone in the brain comes from the circulation, although synthesis of small amounts of aldosterone in the brain is demonstrated in the adrenalectomized rat. These studies did not address the regulation of aldosterone synthesis in the brain by alterations in sodium intake, because the amount of aldosterone produced by the adrenal masks production by the brain, and adrenalectomized animals require salt supplementation. Dysregulation of aldosterone synthesis within the brain appears to have a pathophysiological role in the hypertension of the Dahl salt-sensitive rat. Although the relevance of aldosterone synthesis in the brains of healthy animals is not yet known, studies of the extra-adrenal aldosterone synthesis and action within the cochlea demonstrate its importance and independence from adrenally produced aldosterone.

GRANTS

These studies were supported by medical research funds from the Department of Veteran Affairs and National Heart, Lung, and Blood Institute Grants HL-27737, HL-27255, and HL-75321.

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