Regulation of aldosterone secretion by the renin-angiotensin system during sodium restriction in rats

(angiotensin II/adrenal receptors/sodium deficiency/steroidogenesis/converting enzyme inhibitor)

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ABSTRACT The role of angiotensin II as mediator of the aldosterone response to short periods of sodium restriction was studied in rats by administration of a converting enzyme inhibitor to block formation of the octapeptide throughout the duration of decreased sodium intake. In control animals, short-term sodium restriction caused increased levels of adrenal receptors for angiotensin II, with enhancement of early and late steps in aldosterone biosynthesis and elevation of plasma aldosterone concentration. Each of these changes induced by sodium deficiency was abolished during blockade of angiotensin II formation by continuous infusion of the converting enzyme inhibitor, SQ 14,225. The absolute dependence of adrenal glomerulosa cell responses on angiotensin II formation indicates that the renin-angiotensin system is the primary regulator of aldosterone secretion during physiological fluctuations in sodium intake.

Although the importance of the renin-angiotensin system in the control of aldosterone secretion is well established (1–3), the precise role of angiotensin II in mediating the aldosterone response to sodium deficiency has been more difficult to define (4, 5). While it is generally agreed that the presence of the renin-angiotensin system is essential for the normal aldosterone response to sodium deficiency, there has been controversy about the nature and extent of the actions of angiotensin II as a proximate regulator of zona glomerulosa cells (4, 5). Thus, studies in sheep have suggested that factors other than angiotensin II are involved in the control of aldosterone secretion during sodium depletion and repletion (4).

An important question remains about the physiological role of angiotensin II during reduced sodium intake, which is the most frequent stimulus for increased aldosterone secretion under normal conditions in most animal species. Here also a discrepancy has been noted, between the effect of angiotensin II infusions on aldosterone secretion in normal animals and the larger aldosterone responses caused by sodium deficiency (6, 7). However, the sensitivity of the adrenal to angiotensin II in several species increases during sodium restriction (8–12), and this could contribute to the relatively greater aldosterone secretion that accompanies the increased blood concentrations of angiotensin II during sodium deficiency.

The interaction between angiotensin II and altered adrenal sensitivity during the aldosterone response to sodium restriction has been recently analyzed in rats. Initial studies showed that prolonged changes in electrolyte intake were accompanied by altered affinity and concentration of angiotensin II receptors in the rat adrenal (13). In more recent studies during short-term changes in sodium intake, salt restriction for as little as 36 hr increased angiotensin II binding and aldosterone responses in the zona glomerulosa (14). Thus, in rats, sodium restriction led to rapid increases in angiotensin II receptors that accounted for the change in adrenal sensitivity to the octapeptide. Subsequently, infusion of angiotensin II into conscious rats for 1.5–6 days caused an increase in adrenal receptors for angiotensin II and in the aldosterone responses of glomerulosa cells to the octapeptide in vitro (15).

These observations suggested that angiotensin II could serve as the major regulator of aldosterone secretion during physiological fluctuations in dietary electrolyte intake, as well as during diuretic-induced sodium depletion (16). To test this mechanism, we examined the effects upon adrenal function of blocking angiotensin II formation with an inhibitor of angiotensin I converting enzyme in rats during acute sodium restriction. In these experiments, the inhibitor (SQ 14,225, Squibb) was administered by continuous infusion from osmotic minipumps throughout the 4-day period of sodium restriction.

MATERIALS AND METHODS

All experiments were performed in 200- to 250-g male Sprague-Dawley rats (Charles River, Inc., MA) maintained on normal sodium diet for at least 4 days after arrival in the laboratory. Three groups of 15–20 animals were then placed for 4 additional days on normal sodium intake (0.31–0.37% Na+), low sodium diet (0.06–0.1% Na+), and low sodium diet with simultaneous infusion of the converting enzyme inhibitor, SQ 14,225. The inhibitor was administered by continuous infusion from intraperitoneal osmotic minipumps (Alza) at a rate of 1.4 µg/min. In the first of three such experiments, animals on normal and low sodium intake were implanted with empty silastic tubing as controls for the minipump-infused group. In the latter two experiments, no implants were present in the control animals. The effectiveness of the inhibitor in producing blockade of converting enzyme activity was confirmed by measurement of the pressor responses to angiotensin I and angiotensin II in normal and SQ 14,225-infused rats. Blood pressures were measured via an intracarotid catheter and transducer in pentobarbital-anesthetized, pentolinium/atropine-treated, nephrectomized rats (17). The infusion of SQ 14,225 completely blocked the pressor response to angiotensin I, whereas the response to angiotensin II was not affected (Table 1). No effects of the converting enzyme inhibitor at concentrations up to 10−4 M were observed in the binding assay used to measure angiotensin II receptors or in the action of angiotensin II on aldosterone production in isolated adrenal cells (18). Thus, the possibility of interactions between the inhibitor and adrenal receptors during infusion studies could be excluded.

Plasma aldosterone concentrations were measured by radioimmunoassay after extraction and LH-20 chromatography (19). Plasma renin activity and blood angiotensin II concen-
trations were measured as described (20, 21). Adrenal capsular tissue was homogenized in 20 mM Tris-HCl, pH 7.4/250 mM sucrose/1 mM EDTA/1% bovine serum albumin. The mitochondrial fraction used for aldosterone biosynthetic studies was obtained by centrifugation at 6000 g. The supernate was subsequently centrifuged at 30,000 × g in order to obtain the membrane-rich fraction used in the angiotensin-binding studies.

The conversion of endogenous mitochondrial cholesterol to pregnenolone was measured by incubating aliquots of the mitochondrial suspension at 37° for 15 min in 1 ml of 3 mM Tris-HCl, pH 7.4/20 mM KCl/1 mM EDTA/10 mM sodium malate/10 mM sodium isocitrate/1% bovine serum albumin. Metabolism of pregnenolone during incubation was blocked by the addition of 10 μM cyano k etone (Winthrop 19,578), and pregnenolone formation was measured by direct radioimmunoassay with a highly specific antibody (22). The conversion of corticosterone to aldosterone was measured by incubating aliquots of the mitochondrial suspension with 20 μg of unlabeled corticosterone for 30 min at 37° in the medium described above. The aldosterone formed was measured by radioimmunoassay after extraction with 10 vol of methylene chloride and fractionation by LH-20 Sephadex column chromatography. The content of endogenous cholesterol in the mitochondria was measured by a colorimetric technique with o-phthalaldehyde (23). Binding of 125I-labeled angiotensin II to the 6000–30,000 X g membrane-rich pellet was determined as described for particulate adrenal fractions (24). Association constants and receptor concentration were calculated from equilibrium binding data derived at 20° with monoiodoangiotensin II by computer analysis as described (25).

RESULTS

During sodium restriction, marked and rapid changes occurred in plasma renin activity and in blood angiotensin II and plasma aldosterone concentrations. In addition, the responses to sodium deficiency were significantly altered when animals were treated simultaneously with the converting enzyme inhibitor. Plasma renin activity rose from 4.0 ± 0.6 to 12.5 ± 0.9 ng/ml per hr after 4 days of sodium restriction, and was further increased to 19.5 ± 1.0 ng/ml per hr in sodium-deficient animals infused with SQ 14,225. Significant increases in blood angiotensin II, from the basal value of 9.1 ± 1.4 pg/ml, were observed at 36 hr (to 36.8 ± 6.5 pg/ml) and after 4 days of sodium restriction (to 36.0 ± 6.7 pg/ml). There was a concomitant rise in plasma aldosterone, from the basal value of 12.5 ng/100 ml to 63 ng/100 ml on the fourth day of sodium restriction, and this increase was completely abolished by infusion of the converting enzyme inhibitor (Fig. 1). Thus, the aldosterone response to sodium restriction was completely dependent on formation of angiotensin II and was prevented when formation of the octapeptide was blocked by the converting enzyme inhibitor.

Table 1. Effect of converting enzyme inhibition by SQ 14,225 on pressor responses to angiotensin I and II

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>SQ 14,225-infused</th>
</tr>
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<tbody>
<tr>
<td>Basal</td>
<td>93.3 ± 7.6</td>
<td>95.0 ± 4.9</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>133.5 ± 11.6</td>
<td>138.0 ± 5.1</td>
</tr>
<tr>
<td>Angiotensin I</td>
<td>133.3 ± 11.6</td>
<td>95.0 ± 4.9</td>
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Values are the mean ± SEM of blood pressures (mm Hg) measured in groups of three animals after intravenous injection of 50 ng of each peptide.

To analyze the activities of sites in the aldosterone biosynthetic pathway that are known to be influenced by angiotensin II and sodium deficiency, we performed studies on early and late steps in steroid synthesis. As noted above, the activity of the early portion of the biosynthetic pathway in glomerulosa cells was analyzed during sodium deficiency by measuring pregnenolone formation by isolated mitochondria in the presence of cyanoketone (Winthrop 19,578) to prevent further steroid metabolism.

In sodium-restricted rats, the formation of pregnenolone from endogenous cholesterol was significantly increased (P < 0.02) from the control value of 1.6 ± 0.1 to 2.1 ± 0.1 μg/mg of mitochondrial protein per 15 min. This elevation in pregnenolone synthesis was completely abolished in animals infused with SQ 14,225 during sodium restriction, when the rate of pregnenolone formation was 1.5 ± 0.1 μg/mg per 15 min (Fig. 2). To determine whether the increased rate of pregnenolone synthesis during sodium restriction was accompanied by an increase in available cholesterol substrate, we measured the mitochondrial content of cholesterol in the same adrenals. The pattern of changes observed in the total cholesterol content of mitochondria during sodium deficiency was similar to that of pregnenolone, with an increase from 43.3 ± 2.5 μg/mg of protein in the controls to 60.2 ± 4.9 in the sodium-deficient rats that was reduced by infusion of SQ 14,225 to a value of 39.9 ± μg/mg of protein (Fig. 3).

The late portion of the aldosterone biosynthetic pathway was examined by assay of the conversion of unlabeled corticosterone to aldosterone in the glomerulosa mitochondrial fraction, and showed an elevation during sodium restriction, with complete blockade of this response in animals treated with the converting enzyme inhibitor (Fig. 4). The percent of corticosterone converted to aldosterone per mg of mitochondrial protein was 2.9 ± 0.2 in the controls, 5.8 ± 0.3 in the sodium-restricted rats, and 3.2 ± 0.2 when the rats were simultaneously infused with SQ 14,225.
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The extent to which these changes in the steroid biosynthetic pathway and aldosterone production were related to altered cell receptors for angiotensin II was examined by equilibrium binding studies with $^{125}$I-labeled angiotensin II in a particulate fraction of adrenal capsules from groups of normal, sodium-deficient, and SQ 14,225-treated rats. In a typical experiment (Fig. 5), the previously observed rise in angiotensin II receptor sites was evident after 4 days of sodium restriction and was completely abolished by infusion of the converting enzyme inhibitor throughout the period of sodium deficiency. The mean ($\pm$SEM) receptor content in three experiments was significantly increased ($P < 0.02$) from $1097 \pm 83$ to $1723 \pm 166$ fmol/mg of protein, while in the SQ 14,225-treated rats the value was similar to the controls ($1108 \pm 51$). No changes in the angiotensin II receptor affinity were observed between the groups. The $K_a$ values were $1.30 \pm 0.10$, $1.26 \pm 0.04$, and $1.36 \pm 0.10 \times 10^9$ M$^{-1}$ in the controls, sodium-restricted, and sodium-restricted and SQ 14,225-infused animals, respectively.

**DISCUSSION**

These studies were performed to clarify the role of angiotensin II in the acute aldosterone response to sodium restriction, as opposed to the more severe stimulus imposed by sodium depletion. Although there is no dispute about the essential role of the renin-angiotensin system in sustaining the aldosterone response to established sodium deficiency (5), there is debate whether angiotensin acts in long-term sodium depletion as a permissive factor or a primary regulator (26). The acute effects

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**Fig. 2.** Pregnenolone formation by the adrenal glomerulosa mitochondrial fraction obtained from control and sodium-restricted rats with and without infusion of SQ 14,225. Each bar represents the mean $\pm$ SEM of results obtained in three experiments.

**Fig. 3.** Cholesterol content of mitochondrial fractions from adrenal glomerulosa of control and sodium-restricted rats, with and without infusion of SQ 14,225. Each bar represents the mean $\pm$ SEM of results obtained in three experiments.

**Fig. 4.** Conversion to aldosterone by the glomerulosa mitochondrial fraction from control and sodium-restricted rats, with and without infusion of SQ 14,225. Each bar represents the mean $\pm$ SEM of results obtained in three experiments.

**Fig. 5.** Binding of $^{125}$I-labeled angiotensin II to adrenal glomerulosa particles from control (■) and sodium-restricted rats with (●) and without (○) infusion of SQ 14,225. Similar results were obtained in each of three experiments.
of angiotensin II infusion on aldosterone secretion are transient in certain species (12, 27), and infusions of the peptide do not quantitatively reproduce the aldosterone response to equivalent periods of sodium deficiency (2, 6, 9, 28). However, it is well known that the action of angiotensin II upon aldosterone secretion is enhanced by sodium deficiency in several species (8-12), and this could account for the apparent discrepancy between the response to angiotensin II infusion and that to sodium restriction or depletion.

We have recently observed that the effects of angiotensin II upon the adrenal glomerulosa cell include changes in the membrane receptors for angiotensin, as well as enhancement of the biosynthetic responses to the polypeptide in \textit{in vitro} (15). After administration of angiotensin II for periods of 36 hr to 6 days, significant rises in receptor sites and blood aldosterone levels were observed, with an increase in the \textit{in vitro} responses of adrenal cells isolated from the zona glomerulosa of the peptide-treated animals (15). Such findings are consistent with earlier observations on the ability of renin or angiotensin II treatment to enhance subsequent aldosterone response to the octapeptide in \textit{in vitro} (28, 29) and could provide an explanation for the increase in adrenal sensitivity that occurs during elevation of blood angiotensin II levels in sodium-deficient animals. In the rat adrenal, sodium restriction also led to significant changes in glomerulosa receptor sites, with an early rise in binding affinity (at 36 hr) and a subsequent rise in receptor content at the fourth day of sodium restriction. These changes were accompanied by corresponding increases in the sensitivity and magnitude of the aldosterone responses to angiotensin II, both in \textit{in vivo} and in isolated cells studied \textit{in vitro} (14).

These findings emphasize the ability of angiotensin II to modulate its receptor sites and steroidogenesis in the zona glomerulosa, as well as to evoke the acute aldosterone responses that accompany short-term elevations of blood angiotensin II. They also raise the possibility that angiotensin II could be responsible for mediating all of the changes seen during sodium deficiency, i.e., the enhanced sensitivity of the adrenal as well as the direct regulation of aldosterone biosynthesis and secretion. It has been noted above that prolonged infusions of angiotensin II in rats and human beings did not quantitatively reproduce the aldosterone response to an equivalent period of sodium restriction. This discrepancy is not large (the difference in response being about 2-fold) and might result from the use of continuous infusion of angiotensin II instead of the normal episodic and circadian secretory pattern that is characteristic of the renin-angiotensin system in normal and sodium-deficient states (30, 31). As in other hormone-regulated cells, maintenance of a high sustained level of angiotensin II may be a less effective stimulus of cell responses than intermittent elevations of the peptide. A significant proportion of the circulating angiotensin II in certain species may be the des-Asp\textsubscript{1}-heptapeptide of angiotensin II, in addition to the [Asp\textsubscript{1}]octapeptide. This situation may in fact apply in the rat, a species in which the heptapeptide has been reported to comprise up to 60% of the circulating angiotensin activity (32). Whichever form of angiotensin II is predominant in the individual species, it is likely that angiotensin II is either the major biologically active peptide or its precursor, and that the effects of blocking converting enzyme activity apply equally well to both of the active angiotensin peptides.

The present observations have extended the analysis of angiotensin's role in sodium deficiency by blocking formation of the peptide in sodium-deprived rats. Infusion of the converting enzyme inhibitor can lead to increased plasma bradykinin levels, as well as the anticipated decrease in angiotensin II formation (33). However, no effects of bradykinin upon angiotensin-stimulated aldosterone production have been detected in isolated adrenal glomerulosa cells (unpublished observations) and there is no reason to believe that actions of bradykinin are involved in the present study. The results have clearly shown that all aspects of the adrenal response to sodium restriction could be inhibited during blockade of angiotensin II production. The mitochondrial accumulation of cholesterol and its conversion to pregnenolone, as well as the conversion of corticosterone to aldosterone, were unchanged during sodium restriction in animals given the converting enzyme inhibitor, whereas each of these steps was increased in the untreated sodium-deficient control rats. Also, the induction of angiotensin II receptors during sodium restriction was completely abolished by administration of the inhibitor. The absence of these characteristic adrenal responses during inhibition of converting enzyme activity provides an unequivocal demonstration of the central role of the renin-angiotensin system in mediating the adrenal response to sodium restriction.

This observation is of particular significance in the physiological control of aldosterone secretion, a process which has often been studied in animals (usually dogs or sheep) subjected to quite severe sodium depletion or long-term sodium restriction. It is clear that a distinction should be made between sodium depletion, induced by diuretic treatment or salivary loss, and sodium restriction imposed by reduced sodium intake. The former case is accompanied by sodium loss and extracellular fluid volume changes, and the stimulus to aldosterone secretion is more intense and probably also more complex. During sodium restriction, the degree of sodium loss and fluid volume change is much less, particularly during the first few days of sodium deprivation. Even after periods of sodium restriction for up to 6 weeks in rats, no changes in plasma sodium and potassium concentration are detectable (15).

For these reasons, we studied the short-term response to low-sodium intake in rats as a model for the physiological regulation of aldosterone secretion during fluctuations in dietary salt content. Recent studies in sodium-depleted dogs showed that the aldosterone response to acute, diuretic-induced sodium loss could be abolished by treatment with an angiotensin II antagonist (the Sar\textsuperscript{1}, Ala\textsuperscript{8} derivative). Also, plasma aldosterone levels in chronically sodium-depleted dogs were markedly reduced by administration of the nonapeptide converting enzyme inhibitor (SQ 20,881), though not to completely normal levels (16). These, and numerous earlier studies (3-5, 34-36), have emphasized the importance of renin and angiotensin II in the control of aldosterone secretion during sodium deficiency. Despite this, a controversy had remained about the precise role of angiotensin II, regarded by some as a primary regulator of the aldosterone response (5) and by others as a permissive factor in aldosterone secretion (26). These discrepant views have arisen in part from the complexity of aldosterone regulation in long-term sodium deficiency and in animals subjected to various forms of sodium depletion, sometimes with accompanying changes in other regulating factors such as blood volume and plasma potassium concentration. Also, different animal models have been used, and results derived under anesthesia have frequently differed from those obtained in conscious animals (13, 37, 38).

Until recently, the rat was not regarded as a satisfactory animal model for studies on the control of aldosterone secretion, largely due to initial misconceptions about the responsiveness of the rat adrenal to angiotensin II. However, several earlier reports had indicated that angiotensin II would indeed stimulate aldosterone secretion by the rat adrenal (9, 39), and more recent
studies have shown that the zona glomerulosa cells of the rat adrenal are highly sensitive to angiotensin II in vivo and in vitro (14, 18, 36, 38, 40). In the present study performed on conscious rats, it has been possible to analyze the role of angiotensin II during simple dietary restriction of sodium intake, to provide the most physiologically appropriate conditions for examination of this control mechanism. Our results have indicated that angiotensin II is the mediator of the physiological response in aldosterone secretion during the onset of sodium deficiency.

The actions of angiotensin II upon the zona glomerulosa are complex, and include a triphasic effect as well as an acute stimulating action on aldosterone secretion. The trophic actions of angiotensin II include effects on angiotensin II receptors and enzymes in the steroid biosynthetic sequence, as well as stimulation of glomerulosa cell differentiation and multiplication. Both of these actions, by leading to changes in acute steroidogenic capacity and long-term glomerulosa cell mass, contribute to the enhanced sensitivity of the adrenal seen during sodium deficiency (3, 14) and angiotensin II infusion (10, 15). The multiple actions of angiotensin II on glomerulosa cell structure and function clearly increase the complexity of analyzing aldosterone regulation in various animal models. However, during short periods of sodium deficiency, as demonstrated by the present studies, there remains little reason to doubt that angiotensin II functions as the major physiological regulator of aldosterone secretion.

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