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IN VIVO REGULATION OF GENE EXPRESSION OF ENZYMES CONTROLLING ALDOSTERONE SYNTHESIS IN RAT ADRENAL

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Summary—We studied the effect of alterations in the intake of sodium and potassium as well as changes in circulating adrenocorticotropin (ACTH) on the expression of the two rate-limiting systems of aldosterone formation in the rat. Low sodium and high potassium intake promoted time-dependent increases in the zona glomerulosa cytochrome *P*450scc (*P*450scc) and cytochrome *P*450c11 (*P*450c11) protein and mRNA levels, but no changes were found in the zona fasciculata-reticularis. In addition, these responses were associated with markedly elevated transcriptional activities. To further define the contribution of *P*450c11 and *P*450c18 (aldosterone synthase) in response to these differing intakes, we evaluated their mRNA levels using gene-specific oligonucleotide probes. *P*450c18 mRNA was restricted to the zona glomerulosa, whereas *P*450c11 mRNA was detected in both zona glomerulosa and zona fasciculata-reticularis. Furthermore, only *P*450c18 mRNA was induced by both low sodium or high potassium intake, as *P*450c11 mRNA levels remained unchanged. Captopril, an inhibitor of angiotensin-I converting enzyme, abolished the enhancing effects of the low sodium regimen on *P*450scc and *P*450c18 mRNA levels. Captopril also suppressed the augmentation of *P*450c18 mRNA observed with potassium supplementation but had no effect on *P*450scc mRNA levels. When the hypocholesterolemic drug 4-aminopyrazolopyrimidine (4-APP) was administered to rats for 3 consecutive days, both the level of plasma ACTH and the adrenal content of mRNA encoding *P*450scc increased 24 h post final injection. The coadministration of dexamethasone with 4-APP prevented these increases. In contrast, the mRNA content of *P*450c11 remained at control levels.

In conclusion, this work demonstrates that variations in the intake of sodium and potassium act on the expression of the CYP11B2 gene, but not on that of the CYP11B1 gene. Moreover angiotensin-II (A-II) is an important factor in this mechanism of action. Both ions also enhance the expression of the CYP11A1 gene. A-II appears to participate in the mechanism of action of the low sodium intake at this level. Another mechanism is postulated for the action of potassium supplementation since captopril did not prevent the increased expression of the CYP11A1 gene. In addition, the fact that 4-APP enhanced the mRNA level of *P*450scc but not that of *P*450c11, also demonstrates different regulation of the *P*450s involved at the early and final steps of aldosterone formation in the rat adrenal zona glomerulosa *in vivo*.

INTRODUCTION

Aldosterone is synthesized in the interrenal tissues of higher vertebrates including reptiles, birds and mammals [1]. This mineralocorticoid is an attribute of terrestrial vertebrates, since it is not present in invertebrates and in fishes. In mammals, aldosterone is synthesized uniquely in the zona glomerulosa of the adrenal cortex which is separate from the zona fasciculata-reticularis and the medulla.

The rat adrenal aldosterone precursor cholesterol originates mainly from plasma lipoproteins, and also from adrenal lipid droplets and from *in situ* synthesis [2]. The enzyme system responsible for the conversion of cholesterol to pregnenolone is a mitochondrial cytochrome *P*450scc (*P*450scc), while pregnenolone is metabolized into progesterone by a microsomal 3β-hydroxysteroid dehydrogenase system (type II) [3]. Progesterone is subsequently hydroxylated at position c21 by a microsomal cytochrome *P*450 (*P*450c21) to yield deoxycorticosterone (DOC). Another mitochondrial cytochrome *P*450 (*P*450c11) has been reported to be capable of catalyzing the last transformation steps of DOC to aldosterone in the bovine adrenal; the same *P*450c11 was also capable of 19-hydroxylation [4, 5]. Two genes CYP11B1 (*P*450c11) and CYP11B2

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(*P*450c18) sharing 93% homology were isolated from human tissue [6–8]. In the rat, two cDNAs have been isolated and their expression products were shown to possess different catalytic activities, 11β-hydroxylase and aldosterone synthase [9–12]. Two genes were also isolated from a rat genomic library, one encoding for *P*450c11 and the other for *P*450c18. These two genes shared 81% homology [13]. Some structural differences in the 5′-flanking sequences of the rat *P*450c11 were described recently [13]. Recent studies have shown that mouse [14] and hamster (Lehoux, unpublished observations) possess multiple genes encoding *P*450c11. For a review of the structure, function and regulation of gene expression of steroidogenic enzymes see Ref. [15].

The principal function of aldosterone is the maintenance of normal plasma sodium and potassium levels and the volume of extracellular fluid. The regulation of aldosterone synthesis is therefore closely related to the equilibria involving plasma electrolyte homeostasis. It is generally believed that there are two main regulatory sites in the aldosterone biosynthetic pathway, the early rate-limiting step of the conversion of cholesterol to pregnenolone and the final steps involving the transformation of corticosterone to aldosterone. The primary control of the zona glomerulosa is by the renin–angiotensin system, while other regulators include the circulating sodium and potassium levels, adrenocorticotropin (ACTH), atrial natriuretic factor and neural components of the dopaminergic and adrenergic systems.

In this report, we studied the interrelations between the main aldosterone synthesis regulators, the renin–angiotensin system and sodium and potassium ions on the two regulatory systems *P*450scc and *P*450c11 in rat. The differential effect of ACTH on these two systems is also reported.

EFFECTS OF LOW SODIUM AND HIGH POTASSIUM INTAKE ON ALDOSTERONE SYNTHESIS

Low sodium or high potassium intake for 7 days provoked increases in plasma aldosterone and corticosteroid concentrations in rats [16]. These changes occurred rapidly as the increases were apparent after the first day with both intakes, especially for plasma aldosterone. The main effect of these variations in intake was thus an increase in circulating aldosterone level [16]. Such responses have been reported to occur in rats maintained on low sodium or high potassium intake [17–24].

In order to study the contribution of *P*450scc and *P*450c11 to increased plasma aldosterone levels in treated-rats, their content of adrenal protein was measured by Western blot analysis (Fig. 1). Both the low sodium and the high potassium regimen provoked increases in *P*450scc and *P*450c11 protein levels in the zona glomerulosa, indicating that both early and final control sites in the aldosterone biosynthetic pathway were up-regulated by low sodium and high potassium intake. Our data complement works on enzymatic activities reported by other investigators [18, 25, 26], showing that sodium restriction led to increased transformation of cholesterol to pregnenolone as well as of corticosterone to 18-hydroxy-corticosterone and aldosterone. In a previous study [27] we observed an increased transformation of tritiated-cholesterol to corticosterone, 18-hydroxy-corticosterone and aldosterone in homogenates of adrenals from rats maintained on a low sodium intake for 17 days. Similar responses of enhanced activities in the early and final stages of aldosterone synthesis were observed with potassium supplementation [28, 29]. It appears then that the increases in plasma aldosterone levels provoked by sodium restriction or potassium supplementation are associated with increased activity, protein and mRNA levels of *P*450scc and *P*450c11 in the zona glomerulosa.

In contrast, the level of *P*450scc and *P*450c11 proteins was not affected in zona fasciculata-reticularis by low sodium or high potassium intake (Fig. 1). Similarly, no fluctuations in *P*450 activities were detected either in the zona fasciculata-reticularis of sodium-depleted [18, 30], as well as in potassium-supplemented rats [31, 32]. This zone-specific effect is not surprising since aldosterone is synthesized exclusively in the zona glomerulosa and so factors controlling aldosterone synthesis should not influence enzymes of glucocorticoid formation in the adrenal inner zones.

TRANSCRIPTIONAL REGULATION OF *P*450scc AND *P*450c11

The increases in *P*450scc and *P*450c11 protein induced by low sodium or high potassium intake suggest a change in their synthesis,

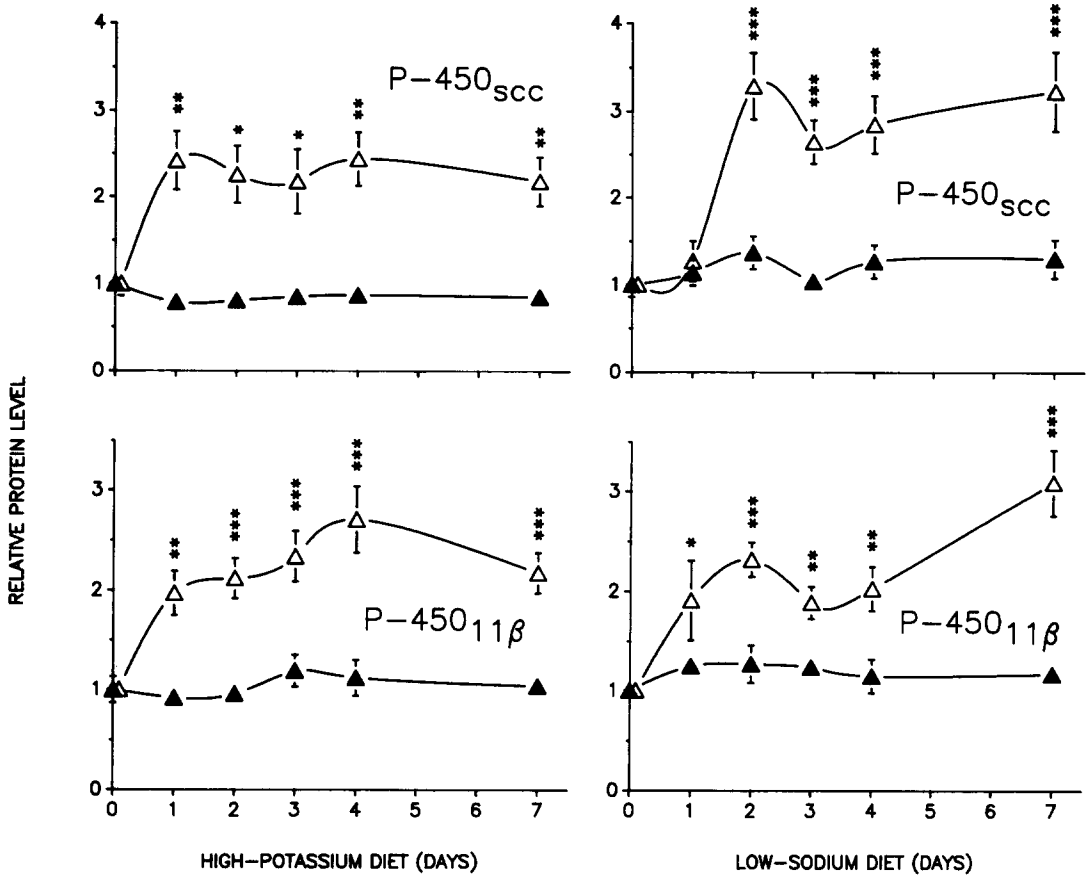

Fig. 1. Profiles of relative protein levels of *P*450scc and *P*450c11 in zona glomerulosa (△) and zona fasciculata-reticularis (▲) of rats maintained on a low sodium or a high potassium intake for indicated times. Mitochondrial lysates were analyzed by Western blot using antibovine *P*450scc and *P*450c11 antibodies as previously described [16, 24]. Values are the mean ± SE of three different experiments. *P* refers to significant differences between experimental and control values for each adrenocortical zone. $*P < 0.05$, $**P < 0.01$, and $***P < 0.005$. Data from [16].

although a change of their half-life cannot yet be ruled out. Therefore, to further add to our understanding, we have utilized our *in vivo* model to measure the levels of the mRNA encoding *P*450scc and *P*450c11. A parallelism was found between the levels of *P*450scc and *P*450c11 mRNA and their respective proteins with either low sodium or high potassium intake, as seen in Fig. 2. Also, a greater response to sodium restriction than to potassium supplementation was observed in the zona glomerulosa when protein (Fig. 1) and mRNA (Fig. 2) induction patterns were compared. Figure 2 shows that the greatest mRNA induction was found for *P*450c11 after 7 days on low sodium intake, this further increased to 8- to 10-fold after 3 weeks [16, 22, 23]. Moreover, the *P*450scc mRNA level increased more rapidly than that of *P*450c11 at the beginning of the study to attain a plateau, whereas that of $P450_{11\beta}$ increased slowly at the beginning but continued to increase thereafter. Finally, the similarities between induction patterns of protein levels and those of their respective mRNA suggest that the increased protein levels were due to the increased mRNA levels.

In order to determine if the enhancing effects of low sodium and high potassium intake on *P*450scc and *P*450c11 mRNA levels were due to *de novo* mRNA synthesis, we performed transcriptional activity assays (run-on). Newly synthesized ^{32}P-transcripts in adrenal zona glomerulosa nuclei of rats fed a low sodium intake or a high potassium intake were enhanced by 6- and 5-fold, respectively, for *P*450scc and by 6.1- and 5.9-fold, respectively, for *P*450c11 compared to controls (unpublished results). These results indicate that increases in *P*450scc and *P*450c11 mRNA levels are more likely to be consequent to an increase in the

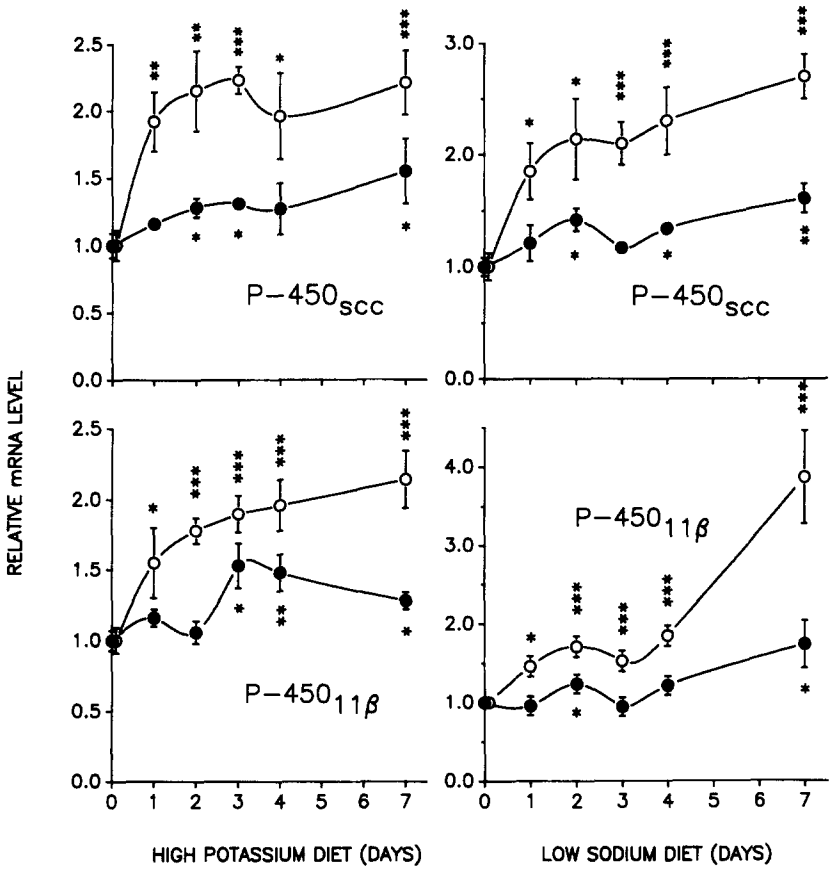

Fig. 2. Effects of low sodium or high potassium intake, as a function of time, on specific mRNA levels of *P*450scc and *P*450c11 in zona glomerulosa (○) and zona fasciculata-reticularis (●). For methodological details see [16]. Blots were hybridized to bovine *P*450scc and mouse *P*450c11 ^{32}P-labeled cDNA probes. Values represent the mean ± SE of densitometric quantitation of signals obtained either by Northern or dot hybridization analyses from three different experiments. $*P < 0.05$, $**P < 0.01$, and $***P < 0.005$. Controls were set arbitrarily at 1.0. Data from [16].

initiation of transcription of their respective genes, rather than to an increased stability.

In the zona fasciculata-reticularis nuclei preparations the quantity of ^{32}P-labeled RNA that hybridized to *P*450scc and *P*450c11 cDNA probes remained near control values. Northern blot analyses of *P*450scc and *P*450c11 (Fig. 2) indicate that the zona fasciculata-reticularis is refractory to changes in sodium or potassium intake, which is in agreement with protein results that we mentioned earlier.

Other authors [33, 34], using an *in vitro* model (bovine adrenal zona fasciculata-reticularis cells in culture) showed that there is a transcriptional regulation of enzymes for optimal steroidogenesis upon ACTH stimulation. Our observations, from an *in vivo* model, allow us to attribute a transcriptional regulation to other stimuli than ACTH, namely to changes in dietary sodium and potassium intake.

INVOLVEMENT OF THE RENIN-ANGIOTENSIN SYSTEM IN THE MECHANISM OF ACTION OF SODIUM RESTRICTION AND POTASSIUM SUPPLEMENTATION

The mechanisms of action of variations in sodium or potassium intake on the function of adrenal zona glomerulosa cells have not yet been elucidated but it is already known that angiotensin-II (A-II), a vasoactive peptide derived from the renin–angiotensin system, is a major stimulant for aldosterone secretion. Other studies have attributed the participation of the renin–angiotensin system in the aldosterone response to low sodium intake [35, 36], and so we evaluated the participation of the renin–angiotensin system in the regulation of *P*450scc and *P*450c11 gene expression during sodium restriction or potassium supplementation. Captopril, an inhibitor of angiotensin-I converting enzyme, was used in combination

with low sodium or high potassium intake. Increases in plasma aldosterone levels otherwise induced by both low sodium and high potassium intake were blocked by captopril, indicating that this drug had inhibited the angiotensin converting enzyme [37]. An increase in plasma renin activity was noted under low sodium intake whereas a decrease occurred under the high potassium intake [37]. Such changes in these plasma parameters following a high potassium intake have been previously reported [16, 34–40]. Under these conditions, captopril prevented the enhancement of *P*450scc and *P*450c11 protein levels in the zona glomerulosa by low sodium intake [37]. Following captopril administration, mRNA profiles obtained by Northern blot analyses were similar to those of their respective proteins for the two cytochromes analyzed (Fig. 3). Our results thus suggest that the renin–angiotensin system is involved in the control of *P*450scc and *P*450c11 gene expression in response to sodium restriction. It further emphasizes that the effect of A-II is directed towards the early and the final limiting steps of aldosterone biosynthesis. The importance of the renin–angiotensin system in the aldosterone response to sodium restriction at the conversion sites of cholesterol to pregnenolone and corticosterone to aldosterone has been well documented [35, 36, 41, 42]. Furthermore, an increased sensitivity of the zona glomerulosa to A-II due to an increased number of A-II receptors has been reported in rats maintained on a low sodium intake [41]. An intraadrenal renin–angiotensin system has recently been described [43–46]; indeed, the presence of each component leading to the formation of A-II has been demonstrated in the adrenal cortex of several species including the rat [43–46]. It was also reported that sodium restriction increased the levels of the mRNA encoding for renin and angiotensinogen in the rat adrenal zona glomerulosa [47–50]. These responses were paralleled by similar increases in aldosterone secretion, thus showing an implication of the renin–angiotensin system in the steroidogenic response to sodium restriction. Our results support these findings and demonstrate that the transcriptional events generated by sodium restriction on steroidogenic gene expression depend upon the formation of A-II. Our results unfortunately do not allow us to discriminate between the utilization of plasma angiotensin or angiotensin formed in the adrenals. In the case of the high potassium diet, the increases in both protein and mRNA levels of *P*450c11 were abolished by captopril [37] (Fig. 3), suggesting that the increase in *P*450c11 synthesis is also controlled by A-II. However, these findings are more consistent with an autocrine production of A-II in the potassium mediation, since plasma renin activity was decreased with high potassium intake [37]. Potassium has also been shown to stimulate the adrenal zona glomerulosa renin–angiotensin system [49–52] leading to the local production of A-II. Our results are also suggestive of a participation of adrenal A-II in the

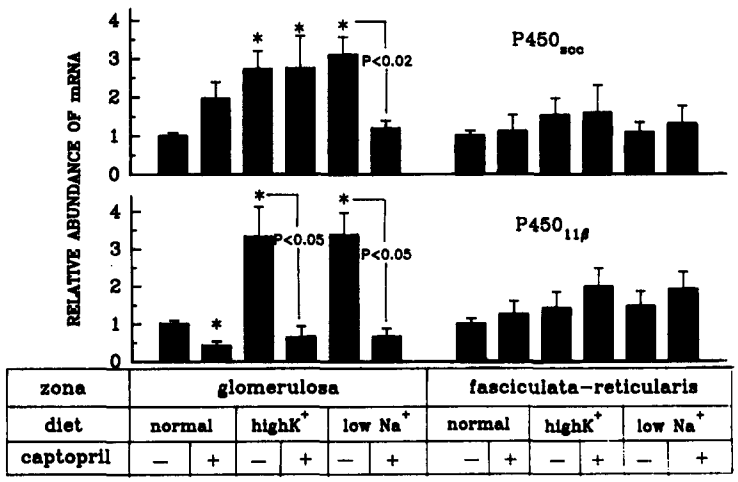

Fig. 3. Profiles of relative mRNA levels of *P*450scc (upper panel) and *P*450c11 (lower panel) in adrenal zona glomerulosa and zona fasciculata-reticularis of control and rats maintained on a low sodium (low Na^+) or a high potassium (high K^+) intake for 7 days. Bars represent the mean ± SE of three different experiments and are derived from densitometric analyses of hybridization signals obtained by Northern blot. Control values were set arbitrarily at 1.0. $^*P < 0.05$ vs control values. Significant differences for the same diet with (+) or without (−) captopril are also shown. From [37].

aldosterone response to potassium. Thus, regulation of the final steps of aldosterone biosynthesis is under the control of the renin–angiotensin system with either low sodium or high potassium intake, and we believe that the local production of A-II to be physiologically more important than circulating A-II in the adrenal response.

In contrast to our observations on the control of *P*450c11, we found no inhibitory effects of captopril on *P*450scc expression in the adrenal zona glomerulosa of rats maintained on high potassium intake [37] (Fig. 3). This suggests that the regulation of the expression of the early step of steroidogenesis by potassium is not mediated by A-II and implies the existence of other control mechanisms. It is well known that potassium acts directly upon adrenal zona glomerulosa cells. Kojima *et al.* [53] showed that potassium caused plasmic membrane depolarization and the opening of voltage-dependent calcium channels. According to Ganguly *et al.* [54] this intracellular calcium augmentation would lead to increased steroidogenesis via a calmodulin-dependent process [54]. Kojima *et al.* [53] suggested instead an increase of the second messenger cAMP in the response of zona glomerulosa cells to potassium. This is supported by Hyatt *et al.* [55] who showed a parallelism between extracellular potassium concentration and cAMP production in isolated rat adrenal zona glomerulosa cells. Although the exact mechanism of the effect of potassium on zona glomerulosa *P*450scc is not clearly identified, it is well known that *P*450scc expression is sensible to variations in cAMP (for review see Ref. [33]), and that its regulation is associated to responsive elements in the gene promoter region [56–60].

Thus, this work suggests that the response to variations in sodium and potassium by *P*450c11, catalyzing final steps in the pathway of aldosterone synthesis is dependent on the presence of A-II. However, the early rate-limiting step in steroidogenesis, catalyzed by *P*450scc seems to be modulated by various factors, including A-II.

No changes occurred in the zona fasciculata-reticularis under such experimental conditions [37] (Fig. 3), the adrenal zona fasciculata-reticularis does not appear to possess a functional renin–angiotensin system. Indeed, a low sodium intake had no effect on the level of renin and its mRNA [38, 50], nor on A-II production in those cells [61]. Furthermore, an absence of response of the renin–angiotensin system was reported in cases of potassium supplementation [49, 50]. All these observations confirm our results concerning the insensitivity of the zona fasciculata-reticularis to variations in the levels of sodium and potassium.

11β-HYDROXYLASE AND ALDOSTERONE SYNTHASE

As previously mentioned, two distinct forms of cytochrome *P*450c11 designated aldosterone synthase (*P*450c18) and 11β-hydroxylase (*P*450c11), have recently been identified in rat adrenals. In this study we used gene-specific oligonucleotide probes (a 20-mer spanning positions 863–882 of the encoding region of the rat *P*450c11 [9] and a 35-mer corresponding to positions 857–891 of the rat *P*450c18 [10]) to define more precisely the effects of alterations of intake of sodium or potassium on the accumulation of the mRNAs encoding these two forms. The transcripts detected by the

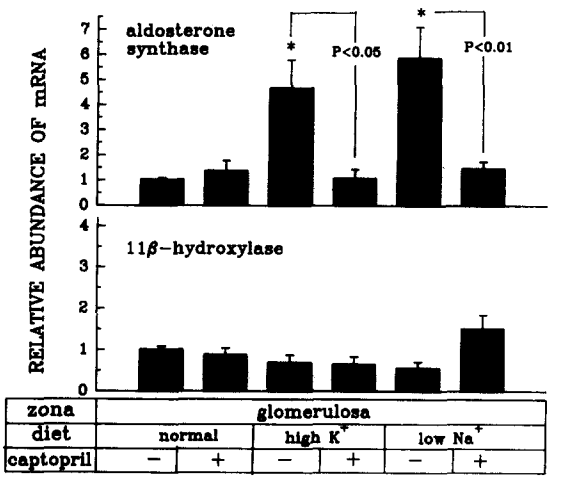

Fig. 4. Effects of low sodium and high potassium intake and captopril administration on expression of *P*450c11 related forms. Rats were maintained for 1 week either on sodium-restricted (low Na^+), high-potassium (high K^+), or standard diet (normal), without (−) or with (+) captopril. Total RNA samples (10 μg) from zona glomerulosa were subjected to agarose gel electrophoresis, blotted onto GeneScreenPlus(TM), and hybridized to [γ-^{32}P]dATP-labeled oligonucleotide probes specific to either rat aldosterone synthase or to 11β-hydroxylase as indicated. Data shown are expressed as relative abundance of messenger RNAs encoding related forms in adrenal zona glomerulosa. Bars represent the mean ± SE from three separate experiments and are derived from densitometric analysis of hybridization signals. Control values were set arbitrarily at 1.0. Statistical significance was determined by Student's *t*-test where $*P < 0.05$ vs control values. Significant differences obtained for the same intake without (−) or with (+) captopril are also shown [62].

two probes were of similar size (2.7 kb), but differed in their zonal distribution: *P*450c18 mRNA was detected only in zona glomerulosa, whereas *P*450c11 was expressed in both the zona glomerulosa and in the zona fasciculata-reticularis [62]. Rats maintained for 1 week on low sodium or high potassium intake have increased *P*450c18 mRNA levels by approx. 6- and 5-fold as seen in Fig. 4. These effects were suppressed by treatment with captopril. These results further substantiate the report of Shikata *et al.* [63] showing by immunoblotting technique that the zona glomerulosa mitochondria from rats fed on a low sodium-normal potassium diet or a low sodium-high potassium diet for 4–10 days contained significantly higher amount of *P*450c18 than rats fed on a normal diet. In contrast, the various manipulations of intake failed to significantly affect *P*450c11 expression in either zones. [62].

Recently the technique for *in situ* hybridization and RNAse protection assays were used to obtain anatomical differentiation of the two compartments of the rat adrenal cortex [64]. From these experiments, the authors concluded that the two *P*450 homologs differ in their zonal distribution and regulation by sodium. The use of a probe that recognized both gene products, however, precluded definitive conclusions about their zonal distribution of expression. Our results with gene-specific probes clearly establish that the *P*450c18 mRNA is expressed only in the zona glomerulosa where it is specifically induced by changes in sodium or potassium. The lack of concomitant induction of *P*450c11 mRNA indicates a high degree of specificity in the expression of *P*450 isoforms in the zona glomerulosa. This specific response of aldosterone synthase is associated with changes in plasma aldosterone levels, which suggests that this induction is an important part of the homeostatic response to changes in the concentration of circulating cations. Curnow *et al.* [65] reported that cultured human zona glomerulosa cells in the presence of A-II increased the levels of *P*450c18 and *P*450c11 mRNA. In contrast, Malee and Mellon [64] reported a decreased expression of *P*450c11 in rats on a low sodium intake which is known to activate the renin–angiotensin system. We found no significant changes in *P*450c11 mRNA levels following sodium restriction or potassium supplementation. These results indicate that humans and rats may differ in the regulation of *P*450c11. Treatment with the angiotensin converting enzyme inhibitor suppressed the induction of *P*450c18 mRNA in response to either sodium restriction or potassium supplementation (Fig. 4). This confirms the results with the mouse probe (Fig. 3) which recognized both forms of *P*450, indicating that A-II is an important component of the mechanism of action of these ions in enhancing plasma aldosterone levels.

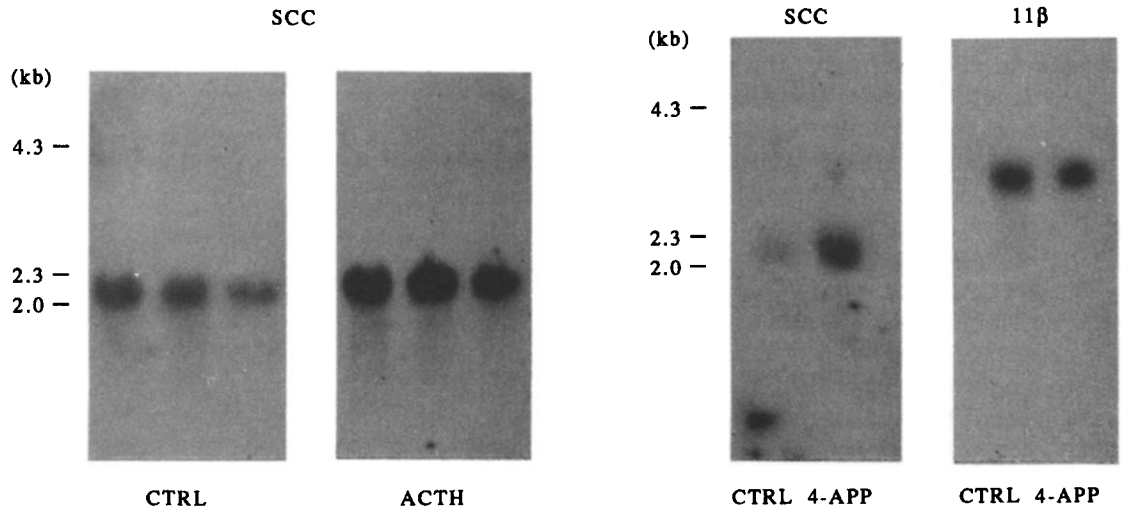

Fig. 5. Effect of ACTH and 4-APP on *P*450scc and *P*450c11 mRNA levels in rat adrenal. Rats were daily administered adrenocorticotropin (ACTH Synacthen depot, 60 μg/100 g body weight) or 4-APP (5 mg/100 g body weight) for 3 days. Controls (CTRL) received the vehicle only. Northern blot analyses were performed using either the bovine cytochrome *P*450scc or the mouse cytochrome *P*450 ^{32}P-labeled cDNA.

EFFECT OF ACTH

We used another model to compare the regulation of gene expression of *P*450scc and *P*450c11. When rats were given ACTH for 3 consecutive days, the adrenal *P*450scc mRNA level was increased compared to rats injected with the vehicle only (Fig. 5, left panel). We previously reported that when rats had daily administration of 4-aminopyrazolopyrimidine (4-APP) for 3 consecutive days, their plasma ACTH level significantly increased at 24 h post final injection, whereas the coadministration of dexamethasone with 4-APP eliminated this effect [66]. We thus used this model to study the effect of endogenously formed ACTH on the mRNAs encoding *P*450scc and *P*450c11. Similarly to changes observed in plasma ACTH, the adrenal content of the mRNA encoding *P*450scc was increased 24 h post last 4-APP treatment (Fig. 5, right panel). This enhancing effect was abolished by the coadministration of dexamethasone (results not shown). In contrast to *P*450scc, the mRNA level of adrenal *P*450c11 was the same as the control. These results show that these two cytochromes respond differently to long term ACTH administration.

CONCLUSION

Our results clearly show that both low sodium and high potassium intake are factors which control the expression of the early and final steps of aldosterone synthesis in the rat adrenal. While *P*450c11 mRNA was found in both adrenocortical zones, *P*450c18 mRNA was located uniquely in the zona glomerulosa, and only this latter form was altered by changes in sodium and potassium intake. The renin–angiotensin system plays a key role in the action of sodium restriction, since captopril blocked the enhancing effect of a low sodium intake on the levels of *P*450c18 and *P*450scc mRNA. The fact that plasma renin activity was decreased by a high potassium intake and that the enhancing effect of potassium on *P*450c18 mRNA levels was abolished by captopril, suggests a possible involvement of the *in situ* adrenal renin–angiotensin system in the control by potassium of the final steps of aldosterone biosynthesis. In contrast, as captopril did not counteract the enhancing effect of a high potassium intake on *P*450scc mRNA levels, it is concluded that other factors than the renin–angiotensin system are involved in the expression of *P*450scc by potassium stimulation. Finally, results obtained with the 4-APP model also indicate that rat adrenal *P*450scc and *P*450c11 are regulated differently.

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