

Local aldosterone synthesis in the large intestine of mouse: An *ex vivo* incubation study

Journal of International Medical Research

50(6) 1–18

© The Author(s) 2022

Article reuse guidelines:

sagepub.com/journals-permissions

DOI: 10.1177/03000605221105163

journals.sagepub.com/home/imr



Zan Pang¹ , Hanna Launonen¹,
Riitta Korpela^{1,2} and Heikki Vapaatalo¹

Abstract

Objective: To investigate the regulation of local aldosterone synthesis by physiological stimulants in the murine gut.

Methods: Male mice were fed for 14 days with normal, high (1.6%) or low (0.01%) sodium diets. Tissue liver receptor homolog-1 and aldosterone in the colon and caecum were detected using an enzyme-linked immunosorbent assay (ELISA). Released corticosterone and aldosterone in tissue incubation experiments after stimulation with angiotensin II (Ang II) and dibutryl-cAMP (DBA; the second messenger of adrenocorticotrophic hormone) were assayed using an ELISA. Tissue aldosterone synthase (CYP11B2) protein levels were measured using an ELISA and Western blots.

Results: In incubated colon tissues, aldosterone synthase levels were increased by a low-sodium diet; and by Ang II and DBA in the normal diet group. Release of aldosterone into the incubation buffer was increased from the colon by a low-sodium diet and decreased by a high-sodium diet in parallel with changes in aldosterone synthase levels. In mice fed a normal diet, colon incubation with both Ang II and DBA increased the release of aldosterone as well as its precursor corticosterone.

Conclusion: Local aldosterone synthesis in the large intestine is stimulated by a low-sodium diet, dibutryl-cAMP and Ang II similar to the adrenal glands.

Keywords

Extra-adrenal aldosterone synthesis, intestinal aldosterone, aldosterone synthase CYP11B2, liver receptor homolog-1, *ex vivo*, angiotensin II, cyclic AMP

Date received: 6 December 2021; accepted: 16 May 2022

¹Department of Pharmacology, Faculty of Medicine, University of Helsinki, Helsinki, Finland

²Human Microbiome Research Programme, Faculty of Medicine, University of Helsinki, Helsinki, Finland

Corresponding author:

Heikki Vapaatalo, Department of Pharmacology, Faculty of Medicine, University of Helsinki, P.O. Box 63, Haartmaninkatu 8, 00014, Helsinki, Finland.
E-mail: heikki.vapaatalo@helsinki.fi



Introduction

Aldosterone is the most important mineralocorticoid that is produced in the zona glomerulosa layer of the adrenal gland cortex.¹ It has been shown that many extra-adrenal tissues, including aortic smooth muscle cells in rat blood vessels,^{2,3} neonatal cardiomyocytes in rat heart,⁴ the nucleus of the solitary tract in rat brain,⁵ human skin cells⁶ and the glomeruli and proximal tubules in rat kidney,⁷ are able to synthesize aldosterone locally. Another factor in favour of local synthesis is that the regulation of salt and water balance has been demonstrated to occur not only in the main target, i.e. the distal convoluted tubule of the kidney, but also in different tissues capable of aldosterone synthesis.⁸ The large intestine is also an important regulator of water and electrolyte balance, while mineralocorticoid receptors are also expressed in this organ.⁹ In our previous study, local synthesis of aldosterone was demonstrated in the caecum as well as in the proximal and midsection parts of the colon.¹⁰ Aldosterone exerts important effects in the intestine, such as promoting potassium excretion and sodium absorption.¹¹ The large intestine is able to locally synthesize corticosterone, a precursor of aldosterone, especially in an experimental model of colitis.^{12–14} Thus, there are several lines of evidence to support our previous finding that the intestine is able to produce aldosterone in a parallel way as occurs for corticosterone.

Previous research has demonstrated that components of the renin–angiotensin system are present in the intestine.^{15–17} Angiotensin II (Ang II) is known to be one of the most important factors capable of stimulating the synthesis of aldosterone in the adrenal cortex similarly to the situation when animals are fed a diet with a low sodium content.^{1,18} Moreover, the second messenger of adrenocorticotrophic

hormone (ACTH), cyclic adenosine 3',5'-monophosphate (cAMP) is also a physiological stimulant of aldosterone synthesis.^{18,19}

The aim of this study was to investigate how local aldosterone synthesis in the large intestine is regulated by examining mice consuming diets with different sodium concentrations for 14 days. An *ex vivo* tissue incubation experiment was then undertaken with physiological stimulants in order to determine the mechanism behind this phenomenon.

Materials and methods

Animals and ethical approval

The study was conducted at the Department of Pharmacology, Faculty of Medicine, University of Helsinki, Helsinki, Finland. The animal experiments had ethical approval by the Project Authorization Board of the Regional State Administrative Agency for Southern Finland (no. ESAVI/9377/2019) in accordance with the Finnish Act on Animal Experimentation (no. 62/2006).

Seven-week-old Balb/c male mice (20.7–25.7 g; Envigo, Horst, The Netherlands) were acclimatized for 4 days to a 12 h light/dark cycle at 22°C ± 2°C and relative humidity of 55% ± 15% in individually ventilated cages. The animals were randomly taken from cages after the accommodation period to divide into three feeding groups. Mice in the normal diet group were provided with Teklad Global 18% protein rodent diet with 0.2% of sodium (Harlan, Indianapolis, IN, USA) for 14 days. The low-sodium diet group received a sodium-deficient diet (0.01–0.02% sodium) (Envigo) and the high-sodium diet group was fed with a 4% NaCl diet (Envigo), which contains a 1.6% sodium content. Tap water was given for *ad libitum* drinking. Consumption of food and water as well as

the body weight development of the animals were recorded every 7 days. The behaviour and condition of each animal were monitored every 2 days.

Sample collection and tissue incubation

After 14 days of feeding, the mice were sacrificed by cervical dislocation under isoflurane anaesthesia (Virbac, Carros, France). The animals were bled. The whole caecum along with the proximal and midsection parts of the colon were excised immediately after euthanization. The intestinal sections were opened longitudinally and rinsed with preoxygenated ice-cold Krebs buffer (118.4 mM NaCl, 25 mM NaHCO₃, 11 mM glucose, 1.6 mM CaCl₂ · 2H₂O, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, pH 7.4). Proximal duodenum, the middle section of jejunum and distal ileum as well as the adrenal glands were collected and snap-frozen in liquid nitrogen followed by storage at -80°C before analysis.

Krebs buffer was preoxygenated using carbogen (95% O₂ and 5% CO₂) for 30 min and kept on ice before the start of the incubation. After washing, similar sized samples of caecum and colon were incubated in capped Eppendorf Protein Lobind tubes with gentle agitation at 37°C for 75 min in a volume of 1.0 ml of preoxygenated Krebs buffer without (control group, *n* = 10) or with either 1 μM Ang II (Ang II group, *n* = 6) or 0.1 mM dibutyryl-cAMP (a synthetic derivative of cAMP; DBA group, *n* = 6). The pH of the buffer remained in the range 7.0–7.2 throughout the incubation. The concentrations of Ang II and dibutyryl-cAMP were chosen based on our previous research on the synthesis of corticosterone in tissue incubation studies.^{20,21} The incubation fluid and incubated tissue samples were separately snap-frozen in liquid nitrogen and stored at -80°C prior to the immunological assays.

Protein extraction from incubated tissue samples

The caecum, proximal and midsection parts of the colon and the pooled adrenal glands were homogenized separately in a volume of 500 μl of enzyme-linked immunosorbent assay (ELISA) buffer (136 mM NaCl, 8 mM Na₂HPO₄, 2.7 mM KCl, 1.46 mM, KH₂PO₄, 0.001% Tween 20, pH 7.4) with Pierce Protease Inhibitor Mini Tablet (Thermo Fisher Scientific, Waltham, MA, USA) followed by sonication (17 kHz, 10 s). The total protein amount in the tissue was calculated from the results measured by the Pierce BCA Protein assay kit (Thermo Fisher Scientific) as an indication of the amount of tissue that had produced the aldosterone.

Immunochemical assays

Corticosterone, the precursor of aldosterone, was measured from the incubation fluid of the colon and caecum sections using a Corticosterone Parameter Assay kit (KGE009; R&D Systems, Minneapolis, MN USA). The corticosterone level was normalized against total protein in the respective incubated tissue sample.

Aldosterone was measured from the incubation fluid of intestinal sections using an Aldosterone ELISA Kit (501090; Cayman Chemical, Ann Arbor, MI, USA). The aldosterone released into the incubation fluid was normalized according to the total protein amount of the corresponding incubated tissue sample.

Aldosterone synthase (Cytochrome P450 Family 11 Subfamily B Member 2 [CYP11B2]) concentration was measured from the homogenate supernatants of incubated caecum, colon sections and adrenal glands using an ELISA Kit for Aldosterone Synthase (SEA987Mu; Cloud-Clone Corp., Katy, TX, USA). The tissue aldosterone synthase CYP11B2

was normalized against the amount of protein.

Liver receptor homolog-1 (LRH-1), the transcription factor that regulates the expression of steroidogenic enzymes of local corticosterone synthesis, was measured from the nonincubated colon and caecum homogenates using a Mouse Nuclear Receptor Subfamily 5 Group A Member 2 ELISA kit (MBS9717987; MyBioSource, San Diego, CA, USA). The LRH-1 level was normalized according to the protein amount.

In the ELISA assays, the minimum detectable concentrations were 0.028 ng/ml for corticosterone, 0.0156 ng/ml for aldosterone, 0.057 ng/ml for aldosterone synthase and 0.312 ng/ml for LRH-1. Intra- and interassay coefficients of variation were 6.3% and 7.1% for corticosterone, 8.4% and 17.4% for aldosterone, <10% and <12% for aldosterone synthase and <10% and <12% for LRH-1, respectively.

Western blot analysis

After protein extraction from the incubated intestinal samples, the total protein concentrations of homogenate supernatants were diluted to the same protein concentration using ELISA buffer and Laemmli sample buffer (Bio-Rad, Hercules, CA, USA). Samples containing 35 µg protein were pipetted into the wells of Mini-Protean TGX Precast Protein Gels (Bio-Rad) in a random order followed by transfer to nitrocellulose membranes (Bio-Rad). The blockade was performed for 2 h at room temperature using a commercially available buffer (Intercept® [TBS] Blocking Buffer; LI-COR Biosciences, Lincoln, NE, USA). After blockade, the membranes were incubated at 4°C overnight with primary rabbit monoclonal antibody against mouse CYP11B2 (recombinant anti-C11B2/CYP11B2 antibody; Abcam, Cambridge, UK) at a 1:1000 dilution, followed by

washing four times with 1 × TBS (20mM Trizma base and 137mM NaCl, pH 7.6) and incubation for 1 h at room temperature in the presence of IRDye 800CW goat anti-rabbit IgG secondary antibody (1:10000 dilution; LI-COR Biosciences). After incubation, the membranes were washed twice with 1 × TBS-T (20mM Trizma base and 137mM NaCl and 0.05% Tween 20, pH 7.6) and twice with 1 × TBS (pH 7.6). The target bands were scanned using an Odyssey® CLx Imaging System (LI-COR). The membranes were then stripped and the following antibodies were used to determine the loading control: mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:1000 dilution; Cell Signaling Technology, Danvers, MA, USA) and IRDye 800CW goat anti-mouse IgG secondary antibody (1:20000 dilution; LI-COR). The target bands were normalized against GAPDH after detection using an Odyssey® CLx Imaging System.

Statistical analyses

All statistical analyses were performed using IBM SPSS Statistics for Windows, Version 26.0 (IBM Corp., Armonk, NY, USA) using one-way analysis of variance followed by the Least Significant Difference test. The graphs were made using GraphPad Prism 8 (Graphpad Software Inc., San Diego, CA, USA). The data are presented as mean with standard error of the mean (SEM). A *P*-value <0.05 was considered statistically significant.

Results

In order to examine how aldosterone synthesis in the gut is regulated, this study proceeded in a step-by-step manner. After weighing and monitoring the food and drink intake of the study mice, the tests began by examining which sections of the intestine contained more aldosterone and

thus would be used as the study targets. The next step was to conduct a series of assays to follow the aldosterone synthesis pathway from the levels of a transcriptional regulator of steroidogenic enzymes, LRH-1, then to the precursor of aldosterone, corticosterone, followed by the presence of the enzyme, aldosterone synthase, and finally the product, aldosterone.

In order to follow the body weight changes and the consumption of food and water, the mice were monitored every second day to ensure their health and normal behaviour during the 14-day feeding period. The body weight increase of the mice consuming the normal diet was significantly lower compared with the mice fed either the low- or high-sodium diets ($P < 0.05$ for both comparisons) (Table 1). Food intake was significantly higher in the low-sodium diet group compared with the other two groups ($P < 0.05$ for both comparisons); while water consumption was significantly higher in the high-sodium diet group compared with the other two groups ($P < 0.05$ for both comparisons).

Aldosterone was detected in naïve, non-incubated small intestine samples, including the proximal duodenum, the midsection of the jejunum and the distal ileum from mice

in the normal diet group (Figure 1) at a lower level (approximately one-tenth) that measured in the large intestine in our previous study using the same assay method.¹⁰ Therefore, colon and caecum were chosen for further assays. The highest aldosterone

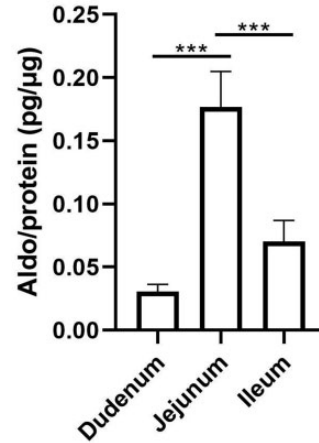


Figure 1. Aldosterone (Aldo) concentration relative to the total protein amount in non-incubated intestinal samples of duodenum, jejunum and ileum of mice fed with a normal diet. Data presented as mean \pm SEM; duodenum and jejunum, $n = 6$; ileum, $n = 5$, *** $P < 0.005$; between-group comparisons were undertaken using one-way analysis of variance followed by the Least Significant Difference test.

Table 1. Total water, food, sodium intake and body weight change in three groups of mice fed diets with different concentrations of sodium during a 14-day study.

Characteristic	Normal diet group $n = 10$	Low-sodium diet group $n = 10$	High-sodium diet group $n = 10$	Statistical analysis ^a
Consumption				
Water, ml	54.4 \pm 1.4	50.5 \pm 2.0	99.4 \pm 1.9	H versus N and L: $P < 0.05$
Food, g	44.7 \pm 0.9	63.1 \pm 1.9	48.4 \pm 1.3	L versus N and H: $P < 0.05$
Sodium, mg	67.0 \pm 1.7	6.3 \pm 0.2	761.2 \pm 21.1	H versus N and L: $P < 0.05$
Body weight				
Change, g	0.6 \pm 0.3	1.8 \pm 0.2	1.7 \pm 0.2	N versus L and H: $P < 0.05$

Data presented a mean \pm SEM.

^aBetween-group comparisons were undertaken using one-way analysis of variance followed by the Least Significant Difference test.

N, normal diet; L, low-sodium diet; H, high-sodium diet.

levels in the small intestine were measured in the jejunum, which were significantly higher than those in the duodenum and ileum ($P < 0.001$ and $P = 0.02$, respectively).

Liver receptor homolog-1, a transcription factor that regulates the steroidogenic enzymes of local steroid synthesis, was detected by immunoassay in non-incubated colon and caecum (Figure 2). In the colon, the LRH-1 protein level was significantly lower in the high-sodium diet group compared with the other two groups ($P = 0.005$ compared with normal diet group; $P = 0.004$ compared with low-sodium diet group). The LRH-1 protein levels in the caecum were lower than those in the colon. Although the mean LRH-1 level in the caecum of the high-sodium diet group was approximately 20% lower than that of the normal diet group, the difference was not significant.

In the incubation experiments, corticosterone, the precursor of aldosterone, was found to be released into the incubation fluid from the colon sections (Figure 3). After the corticosterone levels were

normalized relative to the respective tissue total protein amounts, it was evident that the non-stimulated (control) values ($0.27 \text{ pg}/\mu\text{g}$ protein) in the incubations of the animals consuming the normal diet were over twice as high as those measured from animals on the low- ($0.11 \text{ pg}/\mu\text{g}$ protein) or high-sodium ($0.12 \text{ pg}/\mu\text{g}$ protein) diets. Both Ang II and dibutyryl-cAMP, a synthetic derivative of cAMP, the second messenger of ACTH, increased the mean levels of corticosterone released from the colon in the normal diet group. In the caecum incubation experiments, Ang II as well as dibutyryl-cAMP increased the release of corticosterone during the incubation in samples dissected from mice being fed the normal diet as previously demonstrated (data not shown).

Aldosterone synthase (CYP11B2) protein was detected in the incubated colon tissue using ELISA. Incubation with Ang II or dibutyryl-cAMP significantly upregulated aldosterone synthase CYP11B2 levels compared with the control group in mice

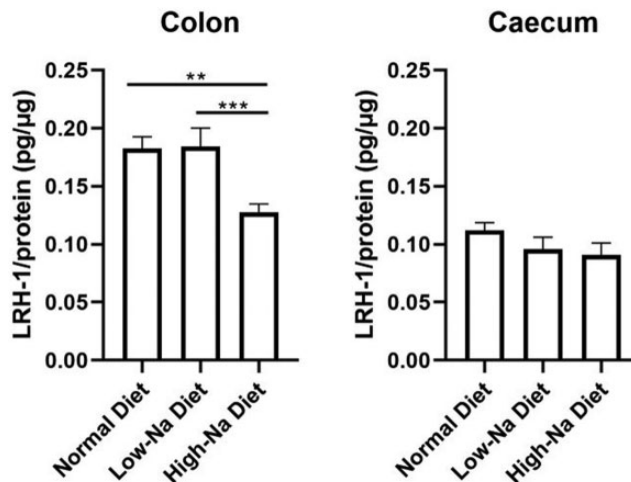


Figure 2. Liver receptor homolog-1 (LRH-1) concentration relative to the total protein amount in non-incubated intestinal tissue samples from three groups of mice fed diets with different protein concentrations of sodium (Na) during a 14-day study. Data presented as mean \pm SEM; colon and caecum, $n = 6$; $**P < 0.01$, $***P < 0.005$; between-group comparisons were undertaken using one-way analysis of variance followed by the Least Significant Difference test.

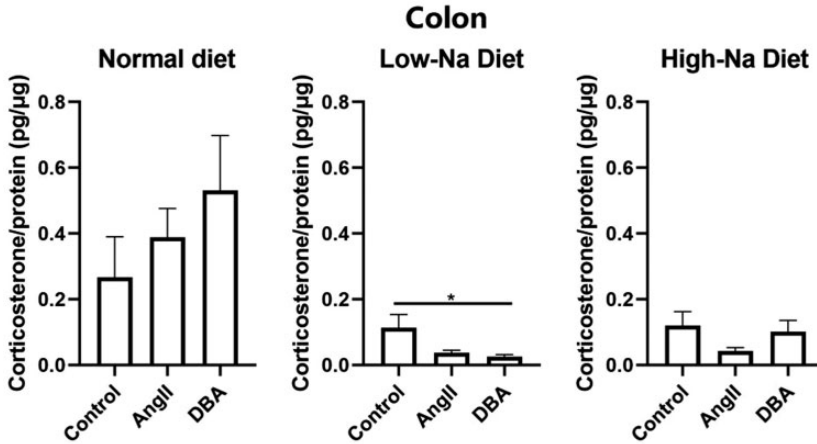


Figure 3. Corticosterone release into the incubation fluid from the colons of three groups of mice fed diets with different concentrations of sodium (Na) during a 14-day study: tissues incubated without (Control) or with either angiotensin II (Ang II; 1 μ M) or dibutyryl-cAMP (DBA; 0.1 mM). The corticosterone concentrations in the incubation fluid were normalized relative to the total protein amount in incubated tissue samples. Data presented as mean \pm SEM; $n = 5-6$ /group; * $P < 0.05$; between-group comparisons were undertaken using one-way analysis of variance followed by the Least Significant Difference test.

fed a normal diet ($P = 0.002$ and $P < 0.001$, respectively), but this was not observed in the other two groups of mice (Figure 4). When the colon samples were subjected to Western blot analysis, the results were similar to those of the ELISA except that in the high-sodium diet group, dibutyryl-cAMP significantly increased the levels of aldosterone synthase CYP11B2 protein compared with the control group ($P = 0.007$ in the normal diet group; $P = 0.028$ in the high-sodium diet group) (Figure 5).

In the incubated caecums from the three groups of mice, the control levels of aldosterone synthase CYP11B2 protein were at least twice as high as the levels in the colon (Figure 4). Neither Ang II nor dibutyryl-cAMP increased the levels of aldosterone synthase CYP11B2 protein in any of the three groups of mice. Consistent with the ELISA results, the Western blot analysis confirmed that incubation with Ang II or dibutyryl-cAMP did not increase the levels of aldosterone synthase CYP11B2 protein

in the caecums from the three groups of mice (Figure 6).

The levels of aldosterone synthase CYP11B2 protein in the non-incubated adrenal glands from the three groups of mice were measured using Western blot analysis (Figure 7). The low-sodium diet decreased the amount of aldosterone synthase CYP11B2, which was similar to our previous study with the same experimental setup.¹⁰

In the non-stimulated colon, the low-sodium diet significantly increased the levels of aldosterone synthase CYP11B2 protein compared with the normal diet ($P < 0.001$) (Figure 8). The high-sodium diet did not decrease the levels aldosterone synthase CYP11B2 protein as predicted, but increased the levels significantly compared with the normal diet ($P = 0.004$). In the non-stimulated caecum, the levels of aldosterone synthase CYP11B2 protein were approximately double those detected in the colon, which was consistent with

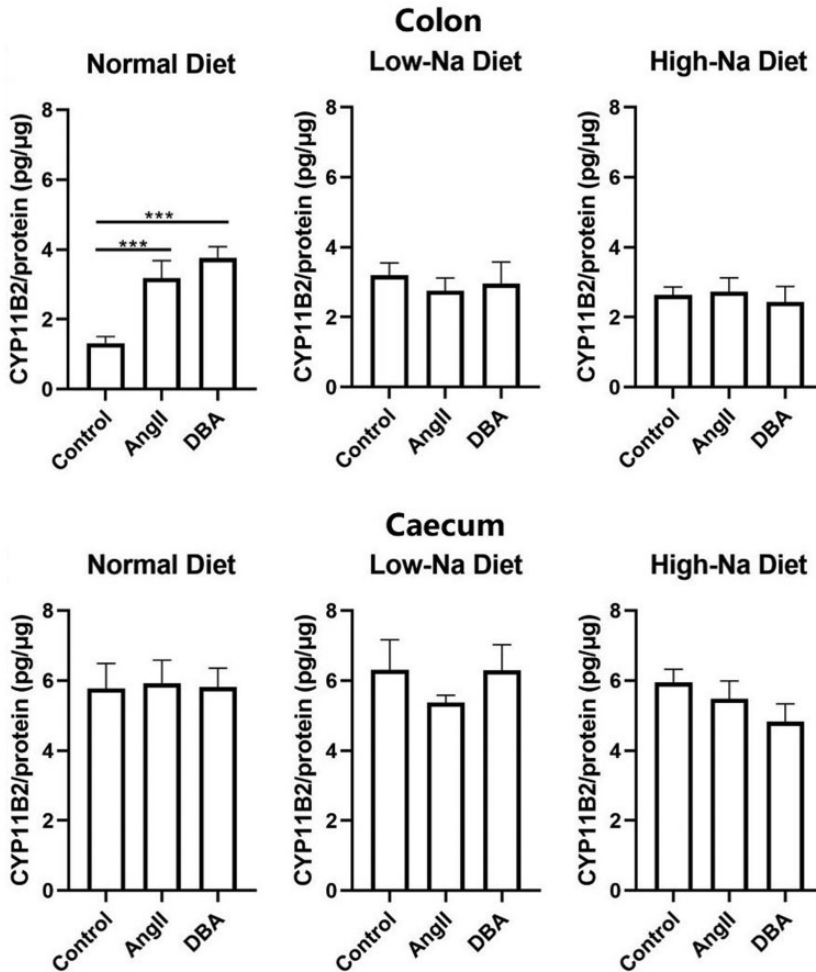


Figure 4. Aldosterone synthase (CYP11B2) protein levels were measured using enzyme-linked immunosorbent assay and normalized relative to the total protein amount in the incubated colon and caecum tissue from three groups of mice fed diets with different concentrations of sodium (Na) during a 14-day study: tissues incubated without (Control) or with either angiotensin II (Ang II; 1 μ M) or dibutyryl-cAMP (DBA; 0.1 mM). Data presented as mean \pm SEM; $n = 5-6$ /group; *** $P < 0.005$; between-group comparisons were undertaken using one-way analysis of variance followed by the Least Significant Difference test.

the amounts of aldosterone released into the incubate. Neither a low-sodium nor a high-sodium diet exerted any significant effect on the levels aldosterone synthase CYP11B2 protein.

In the colon incubation experiments (Figure 9), the control level of aldosterone in the incubate was elevated in the

low-sodium group compared with the control levels in the other two groups of mice. Both Ang II and dibutyryl-cAMP increased the release of aldosterone when the animals were fed with a normal diet, but not significantly in the low-sodium diet group. Treatment with dibutyryl-cAMP, but not Ang II, stimulated the release of

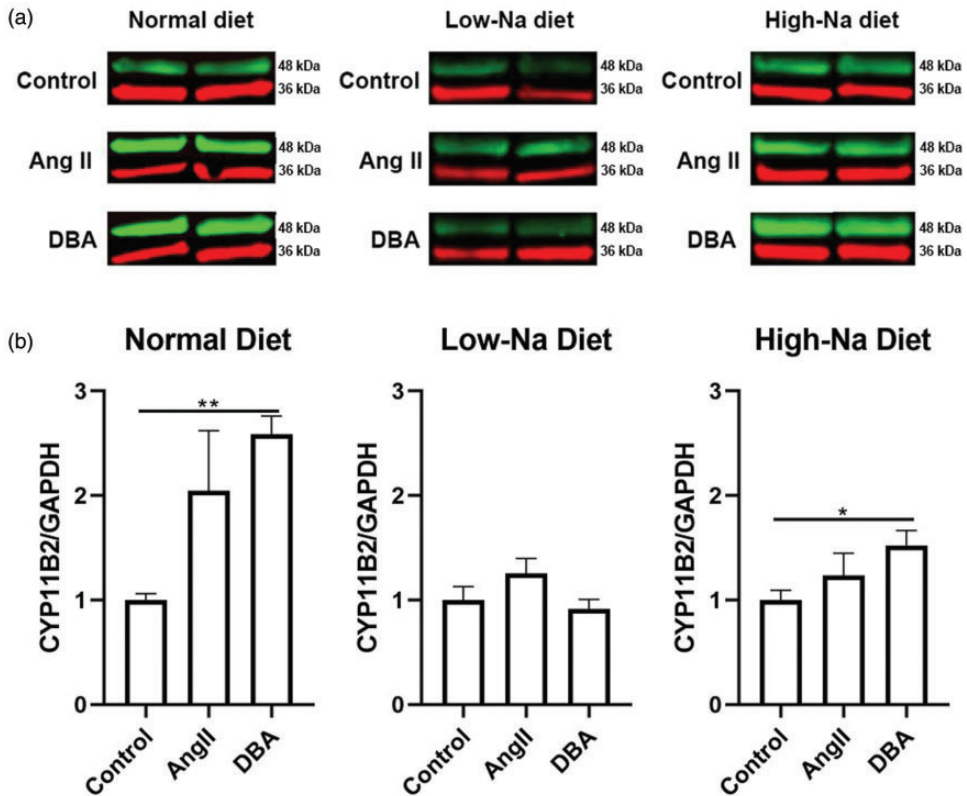


Figure 5. Aldosterone synthase (CYP11B2) protein levels were measured using Western blot analysis in the incubated colon tissue from three groups of mice fed diets with different concentrations of sodium (Na) during a 14-day study: tissues incubated without (Control) or with either angiotensin II (Ang II; 1 μ M) or dibutyryl-cAMP (DBA; 0.1 mM). (a) Bands of CYP11B2 (green) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (red) from the colon. GAPDH was utilized as a loading control. (b) Effects of Ang II and DBA in colon when the control group columns without stimulants were set as 1. Data presented as mean \pm SEM; $n = 5-6$ /group; * $P < 0.05$, ** $P < 0.01$; between-group comparisons were undertaken using one-way analysis of variance followed by the Least Significant Difference test. The colour version of this figure is available at: <http://imr.sagepub.com>.

aldosterone in the high-sodium diet group. In the caecum incubation experiments (Figure 9), the control levels of aldosterone were higher than those measured in the corresponding colon incubates. The lowest control level of aldosterone was measured in the caecum incubation in the high-sodium diet group, being approximately 50% of that detected in the normal diet group. Dibutyryl-cAMP significantly increased the release of aldosterone in

both the low- ($P = 0.021$ compared with the control group) and high-sodium groups ($P < 0.001$ compared with the Ang II group), but not in the normal diet group.

In the colon incubation experiments, consistent with its precursor corticosterone, aldosterone release was detected in all three groups of mice (Figure 10). The highest level of aldosterone released into the incubation fluid was observed in the low-sodium diet group, which was significantly

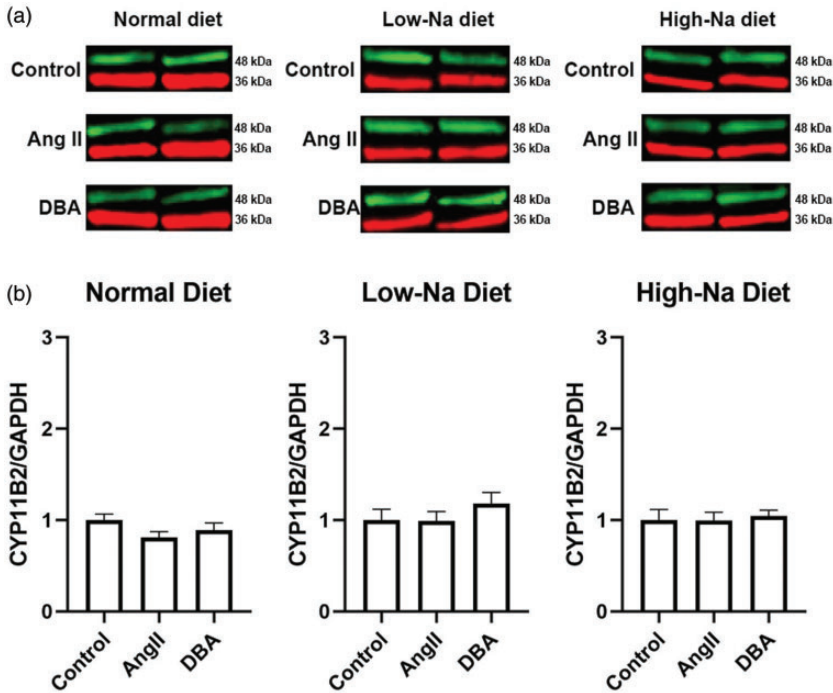


Figure 6. Aldosterone synthase (CYP11B2) protein levels were measured using Western blot analysis in the incubated caecum tissue from three groups of mice fed diets with different concentrations of sodium (Na) during a 14-day study: tissues incubated without (Control) or with either angiotensin II (Ang II; $1 \mu\text{M}$) or dibutyl-*l*-cAMP (DBA; 0.1 mM). (a) Bands of CYP11B2 (green) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (red) from the caecum. GAPDH was utilized as a loading control. (b) Effects of Ang II and DBA in caecum when the control group columns without stimulants were set as 1. Data presented as mean \pm SEM; $n = 5\text{--}6/\text{group}$; no significant between-group differences. The colour version of this figure is available at: <http://imr.sagepub.com>.

higher than in the normal and high-sodium diet groups ($P = 0.028$ and $P = 0.040$, respectively). In the caecum incubation experiments, the release of aldosterone into the incubation fluid was nearly three-fold higher than that detected in the colon incubation experiments in the normal diet group; and the levels were also higher in the groups fed either with the low- or high-sodium diets. When these findings for the levels of aldosterone released into the incubation fluid were compared with the results of the aldosterone synthase CYP11B2 protein levels (Figure 8), low- and high-sodium diets displayed a similar pattern in the colon.

Discussion

The aim of the present study was to investigate how the local synthesis of aldosterone in intestinal tissues detected in our previous studies is regulated.^{10,22} Mice were fed two different diets (low-sodium and high-sodium) or a normal control diet for 14 days to elucidate whether the sodium intake would influence the production of intestinal aldosterone similar to that known to occur in the adrenal glands. Based on a pilot experiment on the entire small intestine from the duodenum to the ileum and our previous work on the large intestine (colon and caecum) using the same

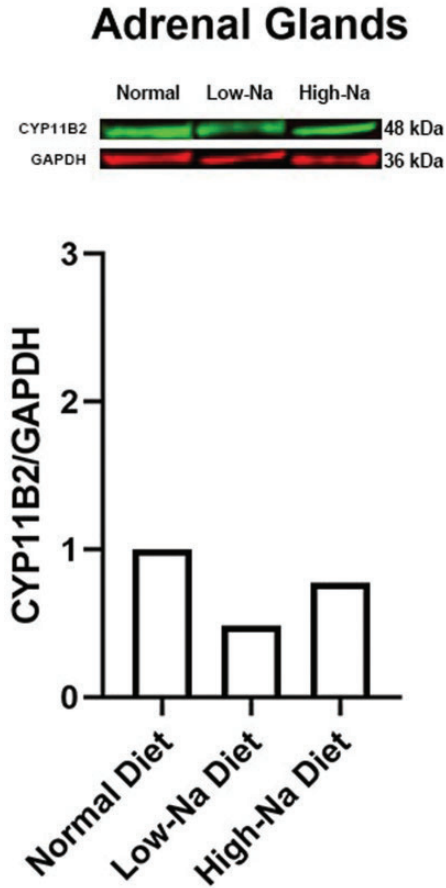


Figure 7. Aldosterone synthase (CYP11B2) protein levels were measured using Western blot analysis in the non-incubated adrenal glands from three groups of mice fed diets with different concentrations of sodium (Na) during a 14-day study. Bands of CYP11B2 (green) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (red) from the adrenal glands. GAPDH was utilized as a loading control. The normal diet control group column was set as 1. The adrenal glands from six mice from each group were pooled as one single sample ($n = 1$). The colour version of this figure is available at: <http://imr.sagepub.com>.

murine model,¹⁰ the colon and caecum were chosen for further incubation experiments since they contained higher tissue aldosterone concentrations than the small intestine. An *ex vivo* incubation of the colon

and caecum was undertaken to test whether two physiological activators, Ang II and dibutyryl-cAMP, the second messenger of ACTH, would be able to induce aldosterone release. The levels of the aldosterone synthase (CYP11B2) protein, which is the enzyme responsible for the formation of aldosterone from its precursor corticosterone, was verified using two different methods, ELISA and Western blot analysis. Furthermore, a putative transcription factor, LRH-1, which regulates the steroidogenic enzymes, was found to be expressed in the intestinal tissues. Corticosterone, the precursor of aldosterone synthesis, was also shown to be released in incubation experiments. This current study has detected most of the components of the pathway leading to the synthesis of aldosterone locally. When combined with our recent findings on the expression of the *CYP11B2* gene,¹⁰ these new results provide supportive evidence that there is local synthesis of aldosterone in intestinal tissues.

Liver receptor homolog-1, the putative transcription factor that regulates the steroidogenic enzymes of local steroid synthesis,²³ was detected in the large intestine in the current study. LRH-1 is a factor that is involved in the regulation of several metabolic pathways. For example, in mice, LRH-1 cooperates with liver X receptor (LXR) to regulate cholesterol metabolism.²⁴ LXR controls the levels of steroidogenic acute regulatory protein leading to the accumulation of free cholesterol in mouse adrenal glands.²⁵ Furthermore, LRH-1 is known to regulate the intestinal levels of steroidogenic enzymes and glucocorticoid synthesis in response to immunological stress.²³ In the current colon experiments, a high-sodium diet significantly decreased the levels of LRH-1 and also reduced corticosterone release into the incubation fluid. These current findings suggest that the synthesis of steroids in the colon

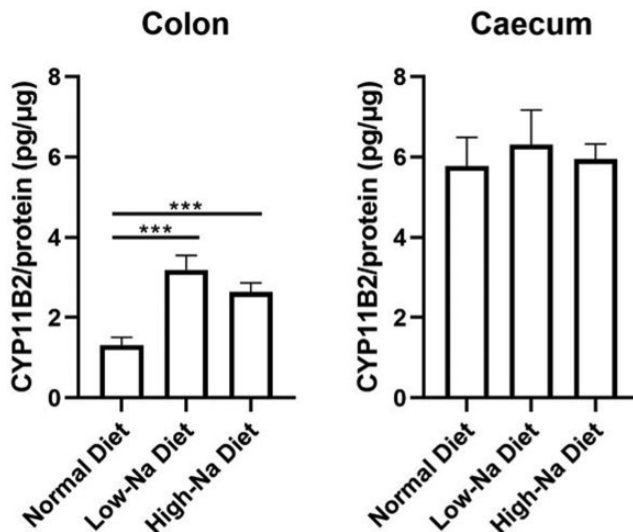


Figure 8. Aldosterone synthase (CYP11B2) protein levels were measured using enzyme-linked immunosorbent assay and normalized relative to the total protein amount in the incubated non-stimulated colon and caecum tissue from three groups of mice fed diets with different concentrations of sodium (Na) during a 14-day study. Data presented as mean \pm SEM; $n = 5-6$ /group; *** $P < 0.005$; between-group comparisons were undertaken using one-way analysis of variance followed by the Least Significant Difference test.

might be a consequence of the downregulated LRH-1.

Corticosterone, the precursor of aldosterone, was released into the incubation fluid of the large intestine samples in the current study, suggesting the local synthesis of this steroid. This finding is in agreement with those from other extra-adrenal tissues, such as skin,²⁶ salivary glands²⁷ and the intestines.^{20,28} As cAMP, the second messenger of ACTH, is one of the most crucial regulatory signals in steroidogenesis,²⁹ the current study demonstrated that dibutyryl-cAMP (a synthetic cAMP analogue) increased corticosterone release in the incubation of colon samples from mice fed a normal diet. In addition, Ang II stimulated corticosterone synthesis in the colon of mice consuming a normal diet, in accordance with our previous report.²⁰ Feeding a high-sodium diet reduced the control non-stimulated corticosterone levels, which was

consistent with the LRH-1 result. Furthermore, both low- and high sodium-diets abrogated the stimulatory effect of Ang II and dibutyryl-cAMP on corticosterone levels in the colon in the current study. These findings are evidence of a central role for LRH-1 in glucocorticoid synthesis in the gut.^{12,23,30}

Concerning the effects of the addition of the stimulants Ang II and dibutyryl-cAMP to the incubations of the caecum and colon in the current study, both agents stimulated aldosterone production and release in a similar manner to that observed in the adrenal glands.³¹ The changes in the aldosterone synthesis pathway from levels of the aldosterone synthase (CYP11B2) protein to the final product paralleled the changes observed for corticosterone. Previously, it has been reported that ACTH and Ang II could induce the conversion of corticosterone to aldosterone in rat adrenal cells

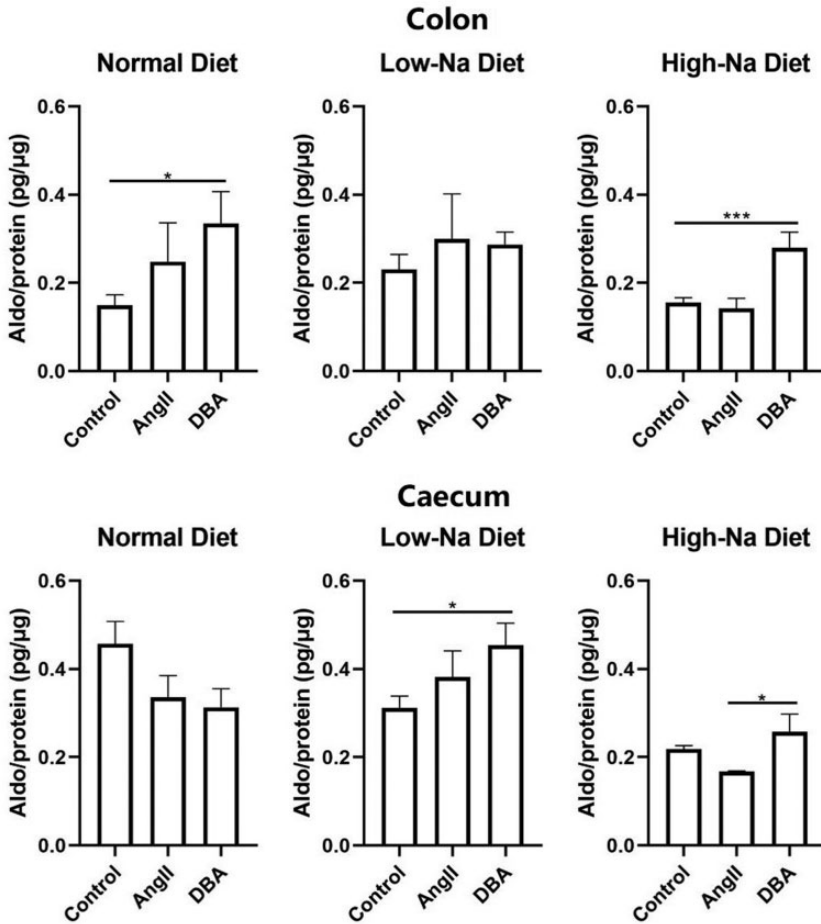


Figure 9. Aldosterone levels released into the incubation fluid were measured using enzyme-linked immunosorbent assay and normalized relative to the total protein amount in the incubated colon and caecum tissue from three groups of mice fed diets with different concentrations of sodium (Na) during a 14-day study: tissues incubated without (Control) or with either angiotensin II (Ang II; $1 \mu\text{M}$) or dibutyryl-cAMP (DBA; 0.1 mM). Data presented as mean \pm SEM; $n = 8-10/\text{control}$ group, $n = 4-6/\text{Ang II}$ and DBA groups; * $P < 0.05$; *** $P < 0.005$; between-group comparisons were undertaken using one-way analysis of variance followed by the Least Significant Difference test.

in vitro.³² The effect of Ang II on aldosterone release in the colon of the animals on a normal diet in the current study suggested that Ang II is not only an important stimulus of aldosterone in the adrenal gland,^{33,34} blood vessels³⁵ and kidney,⁷ but it also acts to promote the synthesis of aldosterone in the colon. In the colon, dibutyryl-cAMP stimulated aldosterone synthesis in

the current study, which resembles the close correlation between the levels of cAMP and the steroid hormone production rate in adrenal cortical cells.³⁶ However, in the caecum of mice fed a normal diet, the levels of aldosterone synthase (CYP11B2) protein and aldosterone release were not upregulated by Ang II or dibutyryl-cAMP, indicating that these two large

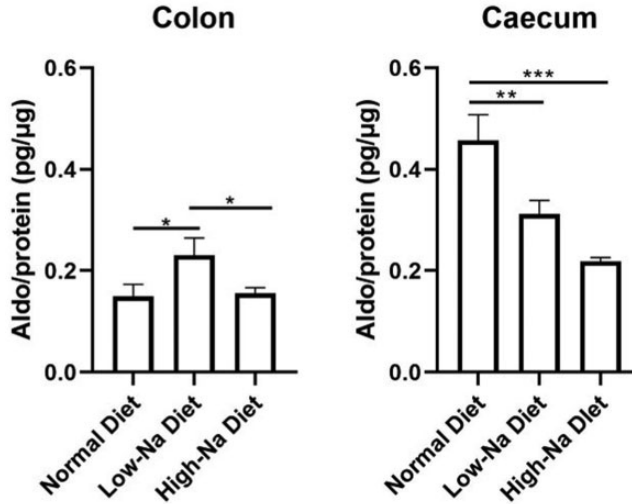


Figure 10. Aldosterone levels released into the incubation fluid were measured using enzyme-linked immunosorbent assay and normalized relative to the total protein amount in the incubated non-stimulated colon and caecum tissues from three groups of mice fed diets with different concentrations of sodium (Na) during a 14-day study. Data presented as mean \pm SEM; $n = 8-10$ /group; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$; between-group comparisons were undertaken using one-way analysis of variance followed by the Least Significant Difference test.

intestinal tissues respond differently to these stimulants. It remains to be clarified whether this is due to different physiological functions of these two parts of the large intestine. In the mouse, the caecum acts as a microbial fermentation organ. However, in addition to water absorption, the proximal colon also transports a mixture of bacteria and mucus back to the caecum.³⁷ It can be speculated that the difference between these two parts of the large intestine is also related to the different gut physiology in rodents. The caecum can be considered to act as a type of 'sac' to retain the mass of digested food but with slower peristaltic movements than the colon, therefore it has less and thinner smooth muscle as reflected in the lower protein amount of its smooth muscle.³⁸ This fact should, but could not, be taken into consideration, because the current measurements were normalized to the tissue protein content. The difference in tissue protein content might partly

explain the higher CYP11B2 level and elevated aldosterone release from the caecum compared with the colon in the current experiments. In the caecum specimens from the low-sodium diet group, although the levels of aldosterone synthase (CYP11B2) protein did not change, the aldosterone level was increased by dibutyl-cAMP stimulation. This might be due to the induction of aldosterone synthase (CYP11B2) activity as this is known to be the case for many CYP enzymes instead of the upregulation of the enzyme protein level.³⁹

In order to study the effects of different diets on aldosterone synthase (CYP11B2) protein levels and aldosterone release, a prolonged reduction in the sodium intake was used to stimulate intestinal aldosterone production as this is known to occur in the adrenal glands.⁴⁰ In the colon, a low-sodium intake increased aldosterone synthase (CYP11B2) protein levels followed

by increased aldosterone release into the incubation fluid, which was similar to the effects of the two stimulants, Ang II and dibutyryl-cAMP. In the rat adrenal gland, a low sodium intake has been reported to enhance the expression of the *CYP11B2* gene⁴¹ and even small changes in the sodium content of the perfusate exerted a powerful effect on Ang II- or potassium-stimulated aldosterone secretion in adrenal glands.⁴⁰ It is recognized that Ang II is the most important physiological stimulus of aldosterone release in the adrenal gland.^{33,34} Thus, it is not unreasonable to speculate that the induction of this pathway in response to the sodium content in the diet would also affect the aldosterone level in colonic tissue as reflected by the current finding that Ang II was able to control the levels of aldosterone, similar to the situation in the adrenal glands.

Some methodological issues in this current study should be taken into consideration. Corticosterone and aldosterone were measured using ELISA kits so both specificity and cross-reactivity are of importance. The manufacturers provided information that the aldosterone ELISA kit displays around 0.1% cross-reactivity with the non-target compound, corticosterone; and the corticosterone kit had 0.5% cross-reactivity with other related molecules. However, due to the extent of the hormone levels measured, the impact of these cross-reactions was considered to be negligible. Contamination from blood in the capillaries of the tissues was theoretically possible during the measurements of the hormone levels, so the animals were bled before sample preparation and the samples were rinsed carefully. Thus, only very small amounts (a few microliters) of blood and a few picograms of aldosterone could have remained in the colon. In our previous study, plasma aldosterone concentrations were between 100–200 pg/ml depending on the sodium concentration in the diet.¹⁰

Furthermore, only a section of the whole tissue was incubated and the measurements were made from the incubation fluid. Thus, the possibility that the measured aldosterone could have originated from the peripheral circulation was very unlikely. The antibody used for detecting the aldosterone synthase CYP11B2 protein in the Western blot analysis was that reported to possess the highest specificity according to the manufacturer's literature.

This current study demonstrated that aldosterone can be detected in the murine small intestine (i.e. duodenum, jejunum and ileum) but at lower levels than those reported previously in the large intestine.²² In the colon and caecum, the steroidogenic enzyme regulator LRH-1 and the key enzyme aldosterone synthase CYP11B2 were detected and corticosterone, the precursor of aldosterone, was released in the *ex vivo* experiment along with the release of aldosterone itself. These observations indicate that the whole chain of crucial events needed for local aldosterone synthesis exists in the murine large intestine. In the colon of mice consuming a normal diet, two physiological stimulants, Ang II and cAMP (dibutyryl-cAMP), were able to upregulate upstream components in the pathway leading to aldosterone synthesis, including corticosterone and the key enzyme aldosterone synthase CYP11B2. In the caecum, addition of cAMP to the incubation medium as well as feeding the mice with a low-sodium diet for 14 days increased the synthesis of aldosterone. These findings with the stimulants were further supported by parallel increased aldosterone synthase protein and elevated release of aldosterone in the colon incubation experiments of the normal diet group.

In conclusion, this current study demonstrated that Ang II, cAMP and sodium-deficient feeding were able to stimulate aldosterone release and increase aldosterone synthase protein levels in a mouse

colon *ex vivo* preparation. In the caecum, high-sodium intake decreased aldosterone release. These current findings suggest that aldosterone synthesis in the colon could be regulated by the same stimulatory factors that are known to be present in the adrenal cortex.

Acknowledgement

We acknowledge the excellent work of Dr Ewen MacDonald in checking the language and grammar.


Declaration of conflicting interest

The authors declare that there are no conflicts of interest.

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by grants from Finska Läkaresällskapet, Einar och Karin Stroems Stiftelse, Finland (H.L. and H.V.). Zan Pang was supported by an EDUFI Fellowship from the Finnish National Agency for Education. Open access was funded by Helsinki University Library.

ORCID iD

Zan Pang  <https://orcid.org/0000-0001-6386-2490>

References

- Williams GH. Aldosterone biosynthesis, regulation, and classical mechanism of action. *Heart Fail Rev* 2005; 10: 7–13.
- Henrion D, Benessiano J and Lévy BI. In vitro modulation of a resistance artery diameter by the tissue renin-angiotensin system of a large donor artery. *Circ Res* 1997; 80: 189–195.
- Matsuzawa Y, Suematsu S, Saito J, et al. Vascular aldosterone production at the pre-diabetic stage of young Otsuka Long-Evans Tokushima Fatty (OLETF) rats, compared with Long-Evans Tokushima Otsuka (LETO) rats. *Molecules* 2013; 18: 15636–15647.
- Fujisaki M, Nagoshi T, Nishikawa T, et al. Rapid induction of aldosterone synthesis in cultured neonatal rat cardiomyocytes under high glucose conditions. *Biomed Res Int* 2013; 2013: 161396.
- Geerling JC and Loewy AD. Aldosterone in the brain. *Am J Physiol Renal Physiol* 2009; 297: F559–F576.
- Nehme A, Cerutti C, Dhaouadi N, et al. Atlas of tissue renin-angiotensin-aldosterone system in human: A transcriptomic meta-analysis. *Sci Rep* 2015; 5: 10035.
- Xue C and Siragy HM. Local renal aldosterone system and its regulation by salt, diabetes, and angiotensin II type 1 receptor. *Hypertension* 2005; 46: 584–590.
- Schiffrin EL. Effects of aldosterone on the vasculature. *Hypertension* 2006; 47: 312–318.
- Turnamian SG and Binder HJ. Regulation of active sodium and potassium transport in the distal colon of the rat. Role of the aldosterone and glucocorticoid receptors. *J Clin Invest* 1989; 84: 1924–1929.
- Launonen H, Pang Z, Linden J, et al. Evidence for local aldosterone synthesis in the large intestine of the mouse. *J Physiol Pharmacol* 2021; 72. DOI: 10.26402/jpp.2021.5.15.
- Harvey BJ, Alzamora R, Stubbs AK, et al. Rapid responses to aldosterone in the kidney and colon. *J Steroid Biochem Mol Biol* 2008; 108: 310–317.
- Bouguen G, Langlois A, Djouina M, et al. Intestinal steroidogenesis controls PPAR γ expression in the colon and is impaired during ulcerative colitis. *Gut* 2015; 64: 901–910.
- Salmenkari H. Renin-angiotensin system in intestinal inflammation—experimental studies with therapeutic interventions. PhD Thesis, University of Helsinki, Finland, <https://helda.helsinki.fi/bitstream/handle/10138/306844/Renin-an.pdf?sequence=2&isAllowed=y> (2019, accessed 15 November 2019).
- Coste A, Dubuquoy L, Barnouin R, et al. LRH-1-mediated glucocorticoid synthesis in enterocytes protects against inflammatory

- bowel disease. *Proc Natl Acad Sci U S A* 2007; 104: 13098–13103.
15. Mastropaolo M, Zizzo MG, Auteri M, et al. Activation of angiotensin II type I receptors and contractile activity in human sigmoid colon in vitro. *Acta Physiol (Oxf)* 2015; 215: 37–45.
 16. Garg M, Angus PW, Burrell LM, et al. Review article: the pathophysiological roles of the renin–angiotensin system in the gastrointestinal tract. *Aliment Pharmacol Ther* 2012; 35: 414–428.
 17. Spak E, Hallersund P, Edebo A, et al. The human duodenal mucosa harbors all components for a local renin angiotensin system. *Clin Sci (Lond)* 2019; 133: 971–982.
 18. Hattangady NG, Olala LO, Bollag WB, et al. Acute and chronic regulation of aldosterone production. *Mol Cell Endocrinol* 2012; 350: 151–162.
 19. Elliott ME, Farese RV and Goodfriend TL. Effects of angiotensin II and dibutyryl cyclic adenosine monophosphate on phosphatidylinositol metabolism, 45Ca^{2+} fluxes, and aldosterone synthesis in bovine adrenal glomerulosa cells. *Life Sci* 1983; 33: 1771–1778.
 20. Salmenkari H, Issakainen T, Vapaatalo H, et al. Local corticosterone production and angiotensin-I converting enzyme shedding in a mouse model of intestinal inflammation. *World J Gastroenterol* 2015; 21: 10072–10079.
 21. Vapaatalo H, Bieck P and Westermann E. Actions of various cyclic nucleotides, nucleosides and purine bases on the synthesis of corticosterone in vitro. *Naunyn Schmiedebergs Arch Pharmacol* 1972; 275: 435–443.
 22. Varmavuori L, Launonen H, Korpela R, et al. Detection of immunoreactive aldosterone in murine gastrointestinal tract. *J Physiol Pharmacol* 2020; 71: 597–601.
 23. Mueller M, Cima I, Noti M, et al. The nuclear receptor LRH-1 critically regulates extra-adrenal glucocorticoid synthesis in the intestine. *J Exp Med* 2006; 203: 2057–2062.
 24. Lee YK and Moore DD. Liver receptor homolog-1, an emerging metabolic modulator. *Front Biosci* 2008; 13: 5950–5958.
 25. Cummins CL, Volle DH, Zhang Y, et al. Liver X receptors regulate adrenal cholesterol balance. *J Clin Invest* 2006; 116: 1902–1912.
 26. Slominski AT, Manna PR and Tuckey RC. On the role of skin in the regulation of local and systemic steroidogenic activities. *Steroids* 2015; 103: 72–88.
 27. Ieko T, Sasaki H, Maeda N, et al. Analysis of Corticosterone and Testosterone Synthesis in Rat Salivary Gland Homogenates. *Front Endocrinol (Lausanne)* 2019; 10: 479.
 28. Kostadinova F, Schwaderer J, Sebeo V, et al. Why does the gut synthesize glucocorticoids? *Ann Med* 2014; 46: 490–497.
 29. Tsai LC and Beavo JA. The roles of cyclic nucleotide phosphodiesterases (PDEs) in steroidogenesis. *Curr Opin Pharmacol* 2011; 11: 670–675.
 30. Mueller M, Atanasov A, Cima I, et al. Differential regulation of glucocorticoid synthesis in murine intestinal epithelial versus adrenocortical cell lines. *Endocrinology* 2007; 148: 1445–1453.
 31. Gambaryan S, Butt E, Tas P, et al. Regulation of aldosterone production from zona glomerulosa cells by ANG II and cAMP: evidence for PKA-independent activation of CaMK by cAMP. *Am J Physiol Endocrinol Metab* 2006; 290: E423–E433.
 32. Haning R, Tait SA and Tait JF. In vitro effects of ACTH, angiotensins, serotonin and potassium on steroid output and conversion of corticosterone to aldosterone by isolated adrenal cells. *Endocrinology* 1970; 87: 1147–1167.
 33. Mulrow PJ. Angiotensin II and aldosterone regulation. *Regul Pept* 1999; 80: 27–32.
 34. Pratt JH. Role of angiotensin II in potassium-mediated stimulation of aldosterone secretion in the dog. *J Clin Invest* 1982; 70: 667–672.
 35. Mazak I, Fiebler A, Muller DN, et al. Aldosterone potentiates angiotensin II-induced signaling in vascular smooth muscle cells. *Circulation* 2004; 109: 2792–2800.
 36. Kojima I, Kojima K and Rasmussen H. Role of calcium and cAMP in the action of adrenocorticotropin on aldosterone secretion. *J Biol Chem* 1985; 260: 4248–4256.
 37. Hugenholtz F and de Vos WM. Mouse models for human intestinal microbiota

- research: a critical evaluation. *Cell Mol Life Sci* 2018; 75: 149–160.
38. Hebel R and Stromberg MW. *Anatomy and embryology of the laboratory rat*. 1st ed. Wörthsee: BioMed Verlag, 1986, p.50.
 39. Hakkola J, Hukkanen J, Turpeinen M, et al. Inhibition and induction of CYP enzymes in humans: an update. *Arch Toxicol* 2020; 94: 3671–3722.
 40. Schneider EG, Taylor RE Jr, Radke KJ, et al. Effect of sodium concentration on aldosterone secretion by isolated perfused canine adrenal glands. *Endocrinology* 1984; 115: 2195–2204.
 41. Takeda Y, Demura M, Wang F, et al. Epigenetic regulation of aldosterone synthase gene by sodium and angiotensin II. *J Am Heart Assoc* 2018; 7: e008281.