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## EVIDENCE FOR LOCAL ALDOSTERONE SYNTHESIS IN THE LARGE INTESTINE OF THE MOUSE

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Aldosterone, the main physiological mineralocorticoid, regulates sodium and potassium balance in the distal convoluted tubule of the kidney. Aldosterone is synthesized from cholesterol in the adrenal cortex in a sequence of enzymatic steps. Recently however, several tissues or cells *e.g.* brain, heart, blood vessels, kidneys and adipocytes have been shown to possess capability to produce aldosterone locally, and there is some evidence that this occurs also in the intestine. Colon expresses mineralocorticoid receptors and is capable of synthesizing corticosterone, the second last intermediate on the route to aldosterone from cholesterol. Based on such reports and on our preliminary finding, we hypothesized that aldosterone could be synthesized locally in the intestine and therefore we measured the concentration of aldosterone as well as the protein and gene expression of aldosterone synthase (CYP11B2), an enzyme responsible on aldosterone synthesis, from the distal section of the gastrointestinal tract of 10-week-old Balb/c male mice. It is known that sodium deficiency regulates aldosterone synthesis in adrenal glands, therefore we fed the mice with low (0.01%), normal (0.2%) and high-sodium (1.6%) diets for 14 days. Here we report that, aldosterone was detected in colon and cecum samples. Measurable amounts of CYP11B2 protein were detected by Western blot and Elisa analysis from both intestinal tissues. We detected *CYP11B2* gene expression from the large intestine along with immunohistochemical findings of CYP11B2 in colonic wall. Sodium depletion increased the aldosterone concentration in plasma compared to control and high-sodium groups as well as in the intestine compared to mice fed with the high-sodium diet. To summarize, this study further supports the presence of aldosterone and the enzyme needed to produce this mineralocorticoid in the murine large intestine.

**Key words:** *aldosterone, extra-adrenal aldosterone synthesis, intestine, aldosterone synthase, CYP11B2, gene and protein expression, sodium deficiency, local mineralocorticoid production*

### INTRODUCTION

Aldosterone, a steroid hormone, is the primary mineralocorticoid hormone in humans playing a crucial role in sodium, potassium, and water homeostasis through sodium retention and potassium excretion in distal tubules and collecting ducts in renal nephrons (1). Thus, excessive levels of aldosterone and the consequent retention of sodium can evoke an elevation of blood pressure, even hypertension and cardiovascular complications (2-4). Clinical and epidemiological studies have indicated that an increase in the serum aldosterone level is a risk factor for the development of hypertension (5), myocardial infarction (6), renal insufficiency (7) and heart failure (8, 9).

Traditionally, aldosterone has been considered to be a steroid hormone synthesized in and secreted from the *zona glomerulosa* of adrenal cortex (10-12). However, several reports indicate that aldosterone can be synthesized not only in the adrenal glands but also in extra-adrenal tissues (13, 14), including heart (15), blood vessels (10, 16, 17), kidneys (18), brain (19), and adipocytes (20). Furthermore, in the skin, an important physiological and

pathophysiological reservoir for salt (21), the renin-angiotensin-aldosterone system is fully expressed (22). Aldosterone also has various local functions in different tissues, not only in its main target *i.e.* kidney nephrons. Aldosterone exerts effects in the vasculature, *e.g.* inducing inflammation, oxidative stress, stiffening, plaque formation, and endothelial dysfunction in arteries (23, 24). In the brain, aldosterone may induce short-term physiological and behavioral alterations in low-sodium conditions (19, 25). Aldosterone also plays a significant role in the intestine. In distal colon, it promotes sodium absorption into the circulation accompanied by the excretion of potassium (26). This is not unexpected, as intestine is an important organ, involved in the maintenance of fluid and electrolyte balance working in parallel with the kidneys. As far as known, there are no published data on whether aldosterone synthesis actually occurs also locally in the intestine.

Sodium deficiency and angiotensin II (Ang II) are potent stimulants for aldosterone synthesis in the adrenal cortex (12). There is evidence that alterations in the potassium concentration would also affect the extra-adrenal synthesis of aldosterone in

human vascular endothelial cells (27). Previous investigators have shown that the components of renin-angiotensin systems are present in the intestine (28, 29). Experiments on adrenalectomized rats revealed that Ang II promotes sodium and water absorption in jejunum similarly to the function of aldosterone (30). These reports led to our working hypothesis of the possibility of local synthesis of aldosterone in the gastrointestinal tract. The aim of the present study was to find additional evidence for this hypothesis and our preliminary observation (31) by measuring the aldosterone concentration as well as the expression of aldosterone synthase (CYP11B2) and the gene responsible for its expression in murine intestine. The mice were fed with different sodium content diets to assess if a sodium deficient diet functions as a stimulant for the aldosterone synthesis in the intestine.

## MATERIALS AND METHODS

The animal experiments were carried out under the approval of National Animal Experiment Board in Finland (ESAVI/9377/2019) according to the Finnish Act on Animal Experimentation (62/2006).

Seven week-old Balb/c male mice (Envigo, Horst, The Netherlands) were housed in individually ventilated cages under a 12-h light/dark cycle at  $22 \pm 2^\circ\text{C}$ . After a four-day acclimatization period, the animals were randomized to three feeding groups ( $n = 8$  in each). The control group received Teklad Global 18% protein rodent diet with a sodium content of 0.2% (Harlan, Indianapolis, IN, USA), whereas the low-sodium group was fed with a sodium deficient diet (0.01 – 0.02%) (Envigo, Horst, The Netherlands). Mice receiving a high-sodium diet (1.6%) (Envigo, Horst, The Netherlands) were also studied. The foods had an otherwise similar composition. All animals had free access to tap water throughout the study. The consumed amounts of food and water were monitored during the study along with the weights of the animals at the beginning and at the end of the experiment.

After fourteen days of feeding, blood samples were drawn from inferior *vena cava* under isoflurane anesthesia (Virbac, Carros, France) into EDTA-tubes (Kisker, Steinfurt, Germany) and the animals were euthanized by opening of the chest cavity. The cecum, proximal parts and midsection of the colon as well as adrenal glands were collected immediately after euthanasia; after opening of the tissue, the intestines were rinsed gently with ice-cold Krebs buffer to remove the luminal content (118.4 mM NaCl, 25 mM  $\text{NaHCO}_3$ , 11 mM glucose, 1.6 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 4.7 mM KCl, 1.2 mM  $\text{KH}_2\text{PO}_4$ , 1.2 mM  $\text{MgSO}_4$ ). An approximately 5 mm long piece of mid-colon was separated for immunohistochemistry and the rest of the tissues were snap-frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$ . Plasma was separated by centrifuging the samples at  $2000 \times g$  for 15 min at  $4^\circ\text{C}$  followed by storage of the plasma in  $-80^\circ\text{C}$  for further aldosterone analysis.

### *Preparation of the samples for immunological assays*

Proximal and midsection parts of colon (pooled together) and cecum as well as adrenal glands were homogenized in Elisa buffer (136 mM NaCl, 8 mM  $\text{Na}_2\text{HPO}_4$ , 2.7 mM KCl, 1.46 mM  $\text{KH}_2\text{PO}_4$ , 0.001% Tween, pH 7.4) and sonicated (17 kHz, 10 s). The total protein concentration of the supernatant was evaluated with the Pierce™ BCA Protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

### *Aldosterone and CYP11B2 Elisa measurements*

An aldosterone Elisa Kit (Cayman Chemical, Ann Arbor, MI, USA) was used to analyze the plasma samples as well as

different intestinal sections and adrenal glands. The tissue aldosterone concentrations per well were normalized to the relative protein quantity of the sample to indicate the amount of the tissue which had produced the aldosterone. Tissue samples for CYP11B2 Elisa and Western blot measurements were prepared similarly as in the aldosterone measurements; however, Pierce™ protease Inhibitor Mini Tablets inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA) was included in the homogenization. The aldosterone synthase (CYP11B2) concentration was measured from supernatants of homogenized cecum, colon, and adrenal glands using an Elisa Kit for Aldosterone Synthase (Cloud-Clone Corp., Katy, TX, USA).

### *Western blot*

After homogenization of the tissue samples, the supernatants were diluted to the same concentration according to the results of the total protein measurement by using Elisa buffer and Laemmli sample buffer (Bio-Rad, Hercules, CA, USA). A total of  $35 \mu\text{g}/\mu\text{l}$  protein was loaded into each well of commercially available gradients gel (Bio-Rad, Hercules, CA, USA) and moved to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) which were blocked in Intercept (TBS) Blocking Buffer TBS (LI-COR, Lincoln, NE, USA). The membranes were incubated overnight at  $+4^\circ\text{C}$  in the presence of primary recombinant antibody CYP11B2 dilution 1:1000 (ab 167413; Abcam, Cambridge, UK) and loading control GAPDH (Cell Signaling Technology, Danvers, MA, USA) followed by one-hour fluorescein labeled secondary antibody IRDye800CW goat anti-rabbit (LI-COR, Lincoln, NE, USA) or IRDye680LT goat anti-mouse incubation (LI-COR, Lincoln, NE, USA). The detected protein bands were normalized with GAPDH.

### *Immunohistochemistry*

The colon samples were fixed in 10% buffered formaldehyde for 20 h, trimmed for transversal sectioning and embedded in paraffin. Samples of all animals were sectioned at  $4 \mu\text{m}$  and stained with hematoxylin and eosin (H&E) for routine histology as well as being immunohistochemically stained for CYP11B2. All sections ( $n = 36$ ) were evaluated by a veterinary pathologist (J.L.) for CYP11B2 expression and histopathological alterations. In the CYP11B2 immunohistochemistry,  $5 \mu\text{m}$  paraffin sections were subjected to heat-induced antigen retrieval 20 min at  $99^\circ\text{C}$  in 10 mM citrate buffer (pH 6). Nonspecific antibody binding was blocked sequentially with 10% bovine serum albumin (A8022; Sigma-Aldrich, St. Louis, MO, USA) and 10% goat serum (G 9023; Sigma-Aldrich, St. Louis, MO, USA) in phosphate-buffered saline. The primary antibody, also used for Western blot, diluted 1:300, was incubated for 60 min at room temperature. Polymer-linked HRP BrightVision Goat Anti-Rabbit IgG AP and Bright DAB Substrate kits (ImmunoLogic, Duiven, The Netherlands) were used for visualization of the bound antibody. Polyclonal rabbit IgG (ab #37415; Abcam, Cambridge, UK), diluted to the same concentration as the primary antibody, was employed as an isotype control for all samples.

### *RT-qPCR*

RNA was isolated from proximal colon, cecum, and adrenal gland using NucleoSpin RNA kit (Macherey Nagel, Duren, Germany) followed by total RNA concentration and purity assessment with NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA). *CYP11B2* gene expression was measured with Taqman gene expression assay Mm00515624\_m1 (Thermo

Fisher Scientific, Waltham, MA, USA) and amplified using LightCycler® Multiplex RNA Virus Master mix (Roche Diagnostics GmbH, Penzberg, Germany) in the Lightcycler 96 instrument (Roche Diagnostics GmbH, Penzberg, Germany). *CYP11B2* expression was normalized to the expression of the following housekeeping genes:

Rplp0 (F: 5'-TAACCCTGAAGTGCTCGACA-3' and R: 5'-GGTACCCGATCTGCAGACA-3') (32)

Eef2 (F: 5'-TGTCAGTCATCGCCCATGTG-3' and R: 5'-CATCCTTGCGAGTGTCTAGTGA-3') (33)

Actin (F: 5'-CTGAATGGCCAGGTCTGAG-3' and R: 5'-AAGTCAGTGTACAGGCCAGC-3') (34).

Reverse transcription (iScript cDNA Synthesis Kit, Bio-Rad) was completed with the same RNA samples from the target gene measurements and the RT-qPCR run was performed with LightCycler 480 SYBR Green I Master (Roche Diagnostics GmbH, Penzberg, Germany). Housekeeping genes primers were obtained from Sigma-Aldrich (St. Louis, MO, USA). Results were calculated based on the method of Vandesompele *et al.* (35).

#### Data analyses

Data was analyzed in SPSS Statistics version 26 (IBM, Armonk, NY, USA) using one-way ANOVA followed by Tukey's *post-hoc* test. Graphical outputs were created with GraphPad Prism software version 8 (La Jolla, CA, USA). *Table 1* and the data concerning aldosterone positive and negative controls are expressed as mean and standard error of the mean (SEM) and the qPCR data which is presented as the geometrical mean. P values less than 0.05 were regarded as statistically significant.

## RESULTS

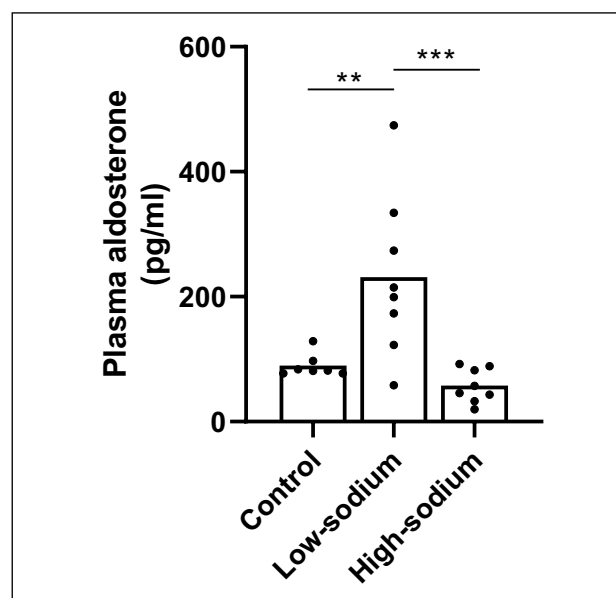
#### Body weight, food and water consumption

The animals were followed each day during the 14 days of feeding by observing their health and behavior in individual cages so that their mean total consumption of food and water could be calculated for the 14 days (*Table 1*). Mice on the high-sodium diet drank more water than the control and the low-sodium groups. Initially, the body weights did not differ between the treatment groups. The mice on both low- and high-sodium diets ate more than the controls and gained more weight during the study.

#### Plasma and tissue aldosterone concentrations

Low sodium intake more than doubled the plasma aldosterone levels as compared to the control group, even more when compared to the high sodium animals (*Fig. 1*). It also appeared that the aldosterone concentration was lower in the high-sodium diet mice than in the control group. No statistically significant differences were observed in the plasma sodium concentrations between the feeding groups (data not shown).

Aldosterone was detected from all cecum and colon samples in both tissues being at the same level as animals consuming the normal diet (*Fig. 2*). The sodium deficient diet increased tissue levels of aldosterone in a manner similar to the concentrations in plasma, whereas the high sodium diet lowered these concentrations. No marked differences in the aldosterone concentrations were observed between the two tissues. As a relative control, demonstrating the variation between different sections of the gastrointestinal tract and excluding contamination from blood, we report that duodenum possesses only one fifth of aldosterone compared to cecum ( $27.0 \pm 5.3$  pg/mg protein vs.



*Fig. 1.* Plasma aldosterone concentrations in the three feeding groups (pg/ml). Individual values and arithmetic means,  $n = 7 - 8$ /group, \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

*Table 1.* Total water, food and sodium intake/mouse during the 14-day study and weight of the mice in the beginning and at the end of the feeding period. Mean  $\pm$  SEM,  $n = 8$ /group.

		Control (C)	Low-sodium (L)	High-sodium (H)	Statistics ( $P < 0.05$ )
Consumption	Water (ml)	55.7 $\pm$ 2.0	53.6 $\pm$ 2.4	101.8 $\pm$ 0.5	C vs. H, L vs. H
	Food (g)	36.7 $\pm$ 0.9	56.7 $\pm$ 2.2	46.2 $\pm$ 0.9	C vs. L, C vs. H, L vs. H
	Sodium (mg)	69 $\pm$ 0.2	0.9 $\pm$ 0.04	782 $\pm$ 14.4	C vs. L, C vs. H, L vs. H
Body weights	Beginning (g)	23.4 $\pm$ 0.5	22.9 $\pm$ 0.4	23.1 $\pm$ 0.3	—
	End (g)	23.3 $\pm$ 0.2	24.7 $\pm$ 0.3	24.9 $\pm$ 0.2	C vs. L, C vs. H

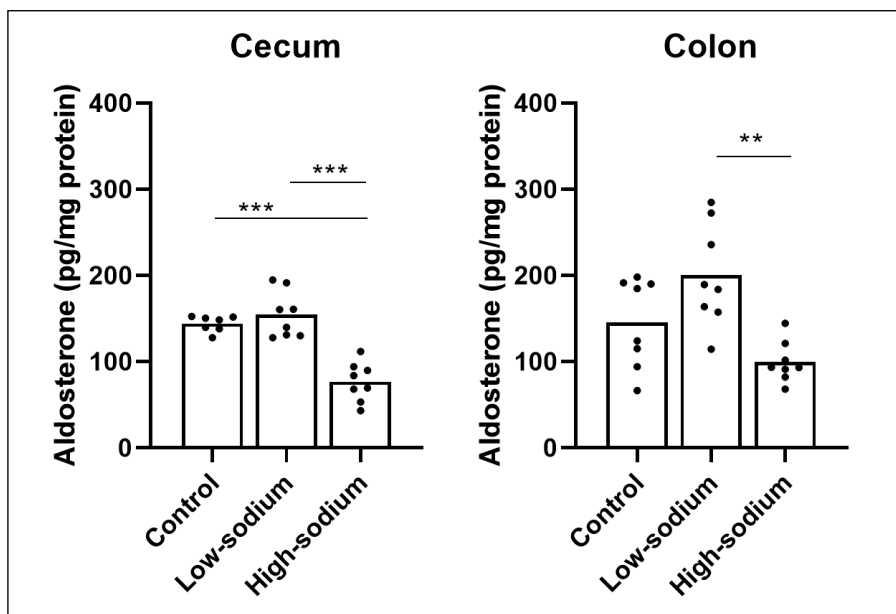


Fig. 2. Concentrations of aldosterone relative to tissue total protein in cecum and in colon (arithmetic mean)  $n = 8/\text{group}$ ,  $**P < 0.01$ ;  $***P < 0.001$ .

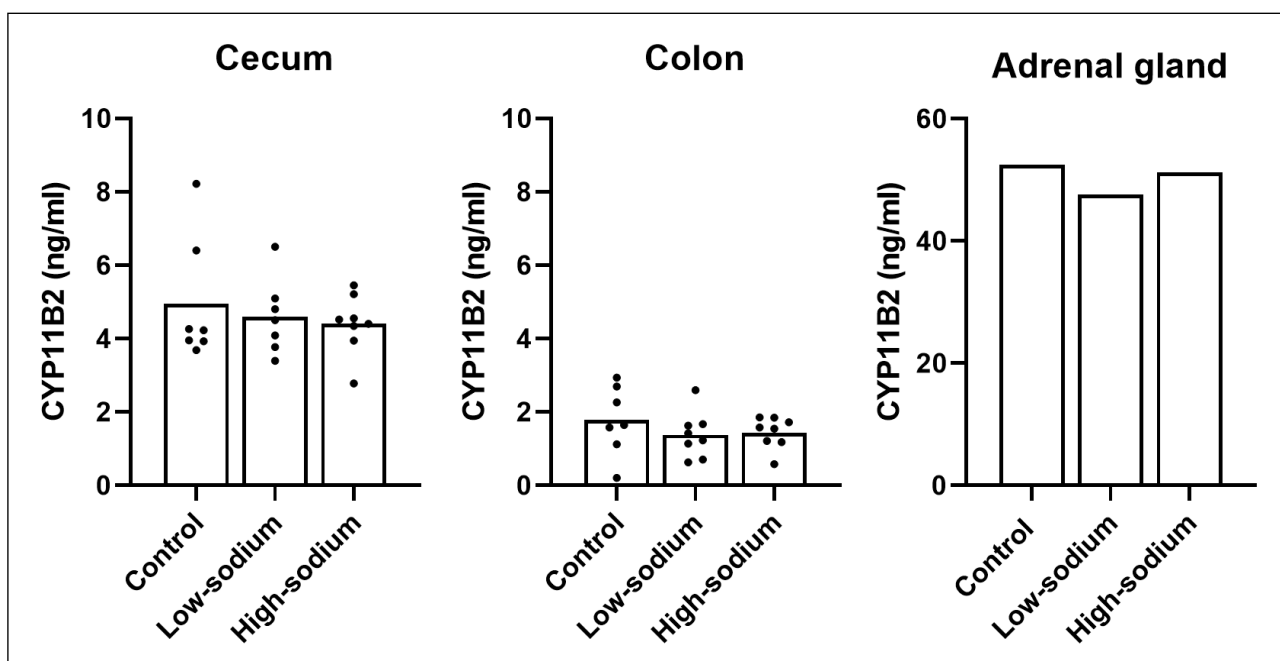


Fig. 3. CYP11B2 protein concentrations (ng/ml) in homogenized cecum, colon (arithmetic mean), and adrenal gland supernatants assessed with an Elisa assay. Samples were diluted to the same total protein concentration before the analysis. Pooled adrenal glands from six mice were used as the positive control.  $n = 7 - 8/\text{colon}$  and  $\text{cecum}$  groups,  $n = 1/\text{adrenal gland pool}$ .

$144.1 \pm 3.0$  pg/mg protein) (Pang *et al.*, unpublished data). In contrast, the pool of all adrenal gland samples used as positive control, harbored 2.7 times higher concentration of aldosterone than cecum in mice fed with low-sodium diet, when related to the tissue protein ( $410.2$  pg/mg protein vs.  $154.6 \pm 8.5$  pg/mg protein).

#### Aldosterone synthase (CYP11B2)

The expression of the aldosterone-generating enzyme CYP11B2 was identified by an Elisa assay from the homogenized tissue supernatants. This indicated that the concentrations of the enzyme protein were in the colon homogenate less than half of those in the cecum tissue. The

dietary sodium concentration did not influence the enzyme levels in comparison to the control diet (Fig. 3). The supernatants of pooled adrenal gland homogenate were used as the positive control in the Elisa assay and showed 10 to 20 times higher concentrations than in the intestine tissue.

Western blotting (Fig. 4), using ab 167413 (validation data available upon request from Abcam (Cambridge, UK)), confirmed the expression of the CYP11B2 protein both in cecum and colon indicating that there were approximately similar levels of the protein, when related to the marker GADPH and without any effects of the different diets. The positive control, adrenal glands showed clearly higher intensity of the bands when compared to those of intestine.

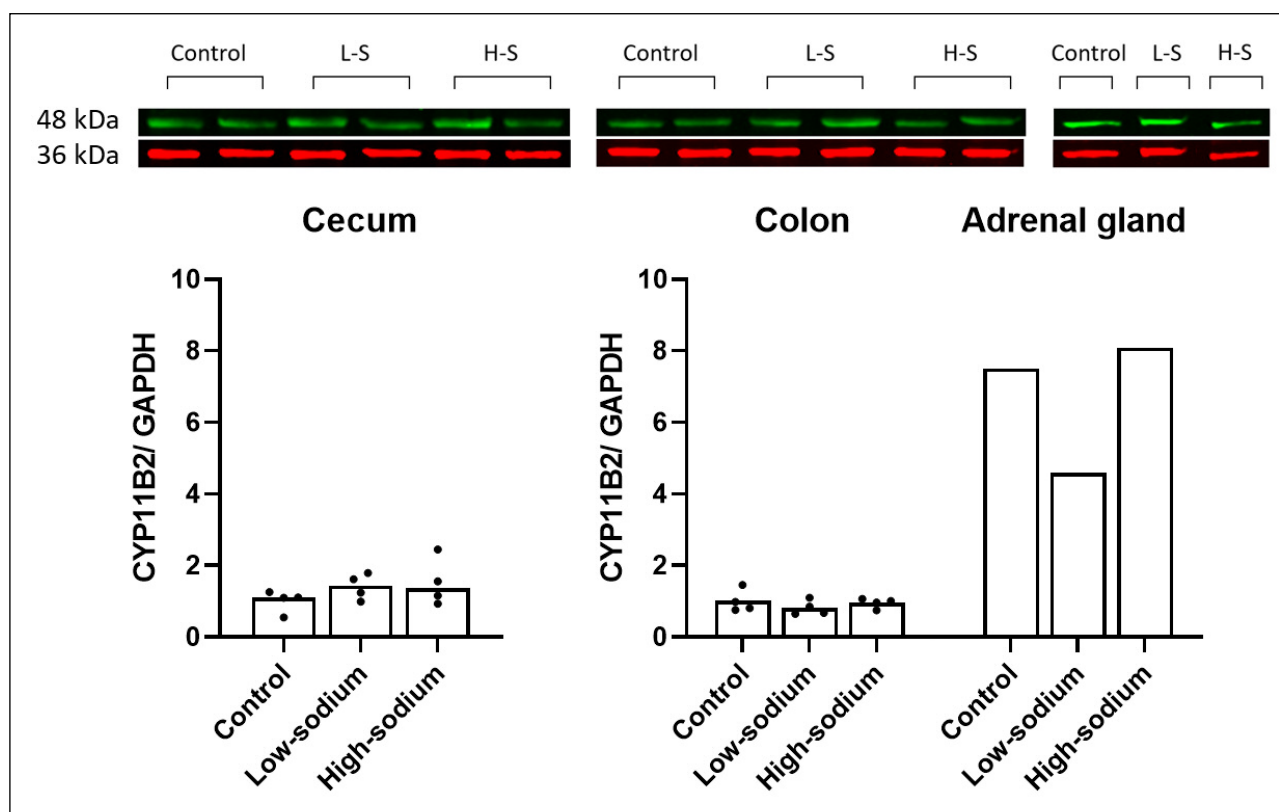


Fig. 4. CYP11B2 protein levels measured from cecum, colon (arithmetic mean), and pooled adrenal gland tissue lysates. Two representative bands from each intestinal group as well as the single band from the pooled adrenal gland sample from six mice are presented above. L-S, low sodium diet; H-S, high sodium diet;  $n = 4$ /cecum,  $n = 4$ /colon and  $n = 1$ /adrenal gland pool.

#### Immunohistochemistry

Colon immunohistochemistry (IHC) revealed consistent light CYP11B2 reactivity in the surface epithelium (Fig. 5A) and abundant reactivity in the smooth muscle cells of the *muscularis externa* (Fig. 5C) across all diets. Focal unspecific reactivity was present in the apical parts of the crypt epithelial cells and in some goblet cells as well as in macrophages in *lamina propria* (Fig. 5B and 5D). The H&E-stained sections displayed no histological differences between the diets.

#### CYP11B2 gene expression

The *CYP11B2* gene was expressed both in the cecum and colon tissues presented as NRQ values of messenger RNA (Fig. 6). The geometric means did not clearly differ between the two tissues nor did the different diets influence these levels. The adrenal glands expressed very high levels compared to the intestine samples and here also the low sodium diet increased by many-fold the expression. Due to the small size of the adrenal tissue, the total extracted RNA amount of the pooled positive control samples in the RT-qPCR experiments was one fourth of that in the intestinal samples.

#### DISCUSSION

This study had two aims; first, to find further evidence for our recent preliminary observation that murine intestinal tissue expresses aldosterone (31). Secondly, to evaluate whether a dietary sodium deficiency in a prolonged feeding experiment

could stimulate intestinal aldosterone synthesis similarly to the situation in adrenal glands as reflected in plasma aldosterone concentrations. Here we show that aldosterone was detected in mouse large intestine and that dietary sodium depletion increased its concentration in plasma as well as in the large intestine in comparison to the dietary high-sodium group. Furthermore, the presence of CYP11B2 protein, measured with both an immunological method and Western blotting as well as *CYP11B2* gene expression were confirmed in the cecum and colon samples. These results, along with the preliminary CYP11B2 immunohistochemical finding in the surface epithelium and *muscularis externa* of the colon, indicate that large intestine possesses capability to produce aldosterone locally.

A sodium deficient diet increased the plasma aldosterone levels as well as *CYP11B2* mRNA expression in the adrenal glands as reported in the literature (36, 37). The plasma or serum levels of aldosterone measured using Elisa in recent publications (38, 39) agree well with our measurements. Our results indicate that sodium depletion stimulates aldosterone synthesis not only in the adrenal glands but also in the intestine. Extra-adrenal stimulation of aldosterone synthesis by increasing the potassium concentration has been previously shown in human vascular endothelial cell culture (27). Our finding is also in line with reports from investigations in brain and kidney samples, where a dietary sodium deficiency elevated murine *CYP11B2* mRNA levels, indicating that sodium could regulate aldosterone production in extra-adrenal tissues (19, 40). In the present study, a sodium deficient diet, however, did not influence *CYP11B2* gene expression or the protein level of CYP11B2 in the intestine to any significant extent. This could be explained by the different tissues examined in previous studies and the duration of the feeding.



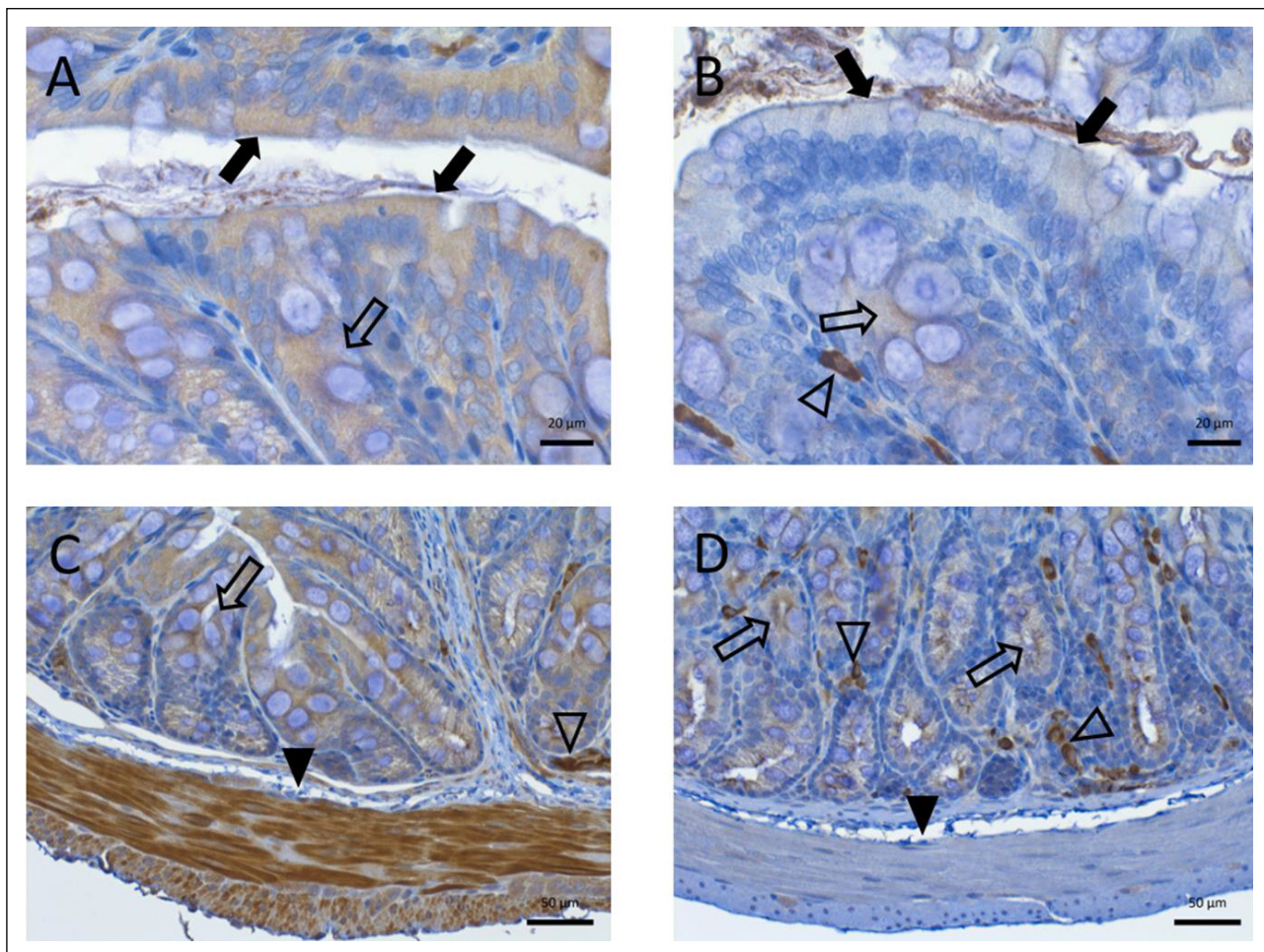


Fig. 5. Representative immunohistochemical staining of CYP11B2 in the colon wall. Figs. A and C (on the left) exhibit CYP11B2 staining, and B and D (on the right) show negative isotype controls. Figures A and B show superficial mucosa and figures C and D, basal mucosa, submucosa, and muscularis. The surface epithelial cells (black arrows) exhibit modest staining; muscularis externa smooth muscle cells (black arrowheads) display strong staining. Apical parts of the crypt epithelial cells and goblet cells (open arrows) show focal unspecific staining; lamina propria macrophages (open arrowheads) display strong unspecific reactivity. Objective magnification is 40  $\times$  in figs. A and B, and 20  $\times$  in C and D.

Immunohistochemical detection of the CYP11B2 protein in the epithelial layer of the colon further suggests possible role of locally synthesized aldosterone in the transfer of electrolytes and water in the large intestine, since it is known that in addition to the kidneys, large intestine is an important water and electrolyte balancing organ (41, 42). Intriguingly, in the immunohistochemical evaluation, the *muscularis externa* layer exhibited considerably higher CYP11B2 reactivity than the surface epithelial cells. We have no definitive explanation for this preliminary finding, except perhaps the rapid renewal of the intestinal epithelium, and therefore loss of the enzyme. The expression of the enzyme in the epithelium resembles findings in the vascular wall, where the endothelium is responsible for aldosterone production (27).

Production and release of aldosterone has been shown (Pang *et al.*, unpublished data) in large intestine incubation *ex vivo*, as well as the synthesis of another mineralocorticoid receptor stimulating steroid, corticosterone, which was also released into the incubation fluid (43). Mineralocorticoid receptors have been identified in the distal colon of the rat and reported to participate in the active transport of both sodium and potassium (44). The subtypes of the mineralocorticoid receptors are different in colon and renal tubules (45). Furthermore, their selectivity and

sensitivity to aldosterone and the temporal reactivity to the stimulation by the hormone vary from minutes (non-genomic receptors) to hours (genomic receptors) in the cardiovascular system (46). Therefore, local mineralocorticoid hormone production might be needed to stimulate both types of receptors. Our hypothesis is that locally in the large intestine, synthesized aldosterone acts paracellulally in its site of formation and only in very small amounts, if any, are released into circulation from healthy intestine. To further evaluate the physiological and pathophysiological relevance of local intestinal aldosterone synthesis, aldosterone synthase knockout animals and/or intestinal inflammatory models could be used.

In the present study, the proximal part of gastrointestinal tract, duodenum, was used as a relative control to show how aldosterone production varies markedly in the gut. One could also attempt to locate a tissue that does not produce aldosterone locally and that could be used as a negative control. This, however, was not the aim of this study and not a viable option as so many tissues have been shown to possess the capability to synthesize aldosterone (10, 15-20).

No marked differences were observed in aldosterone concentrations between cecum and colon, but cecum harbored

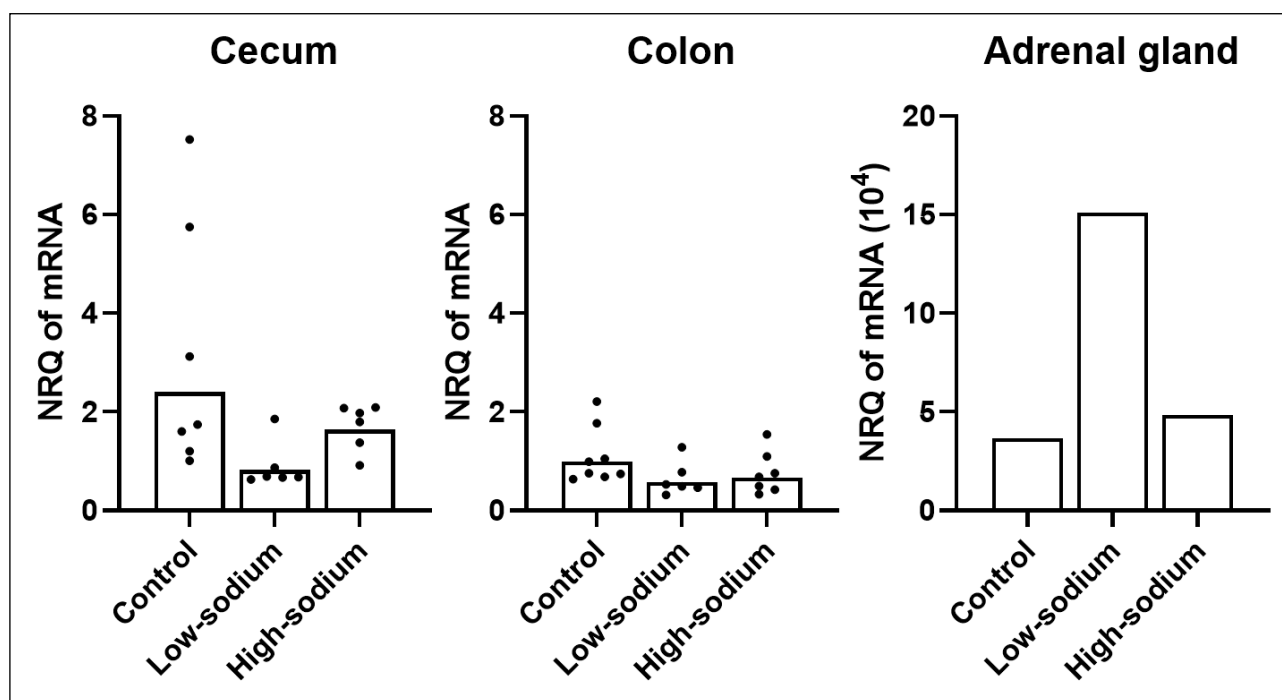


Fig. 6. *CYP11B2* gene expression in the intestinal tissues. Pooled adrenal glands from two mice/ group were used as the positive control. Data is expressed as geometric mean. NRQ, normalized relative quantity.

more than double the amount of *CYP11B2* protein than colon. Even though cecum and colon are parts of the large intestine, they differ anatomically and physiologically as well as exhibiting different microbial contents (47-49). These differences could be responsible for the observed differences between these two sections of the large intestine. In the preparation of the tissues, the consistency of the fecal content in cecum seemed to be softer and more fluid-like than in colon.

Both aldosterone and aldosterone synthesis measurements are based on immunological assays which are widely used and accepted. When tissues from living animals are obtained for *ex vivo* experiments and tissue concentrations of physiological agents are determined, the question of blood contamination may arise. However, calculation based on plasma aldosterone concentration, size of the large intestine and the sections used, total plasma volume of the mice and thus possible amount of plasma in the gut capillaries, indicate that this error is negligible. We have also shown in incubation of sections of large intestine that physiological stimulants Ang II and dibutyl-cAMP increase the synthesis and release of aldosterone into the incubation fluid indicating that the measured aldosterone concentrations are not stationary but vary as expected (Pang *et al.* unpublished data). Furthermore, our hypothesis is that locally synthesized aldosterone remains in the tissue, acting with a paracrine way and is not released in plasma. This could explain an apparent discrepancy between our results and those from an elegant experimental setup on rat Langendorff heart perfusion (50), opposing cardiac aldosterone synthesis locally, because in perfusion fluid aldosterone concentrations were only at the detection limit. Similar perfusion experiments using intestine would be valuable.

The present study was carried out in healthy animals fed with different sodium level diets to stimulate (sodium deficient) or to suppress (high sodium) the synthesis of aldosterone. The different diets caused the expected changes in plasma, cecum and colon aldosterone concentrations. However, neither the

aldosterone synthase (*CYP11B2*) concentrations nor the gene (*CYP11B2*) expression changed accordingly. Thus, we have no explanation at which level or by which mechanisms the local aldosterone synthesis in the large intestine is regulated. One possible mechanism might be, instead of the amount of the critical enzyme, influence on its activity. Different pathological situations like stress, diabetes and inflammatory conditions (18, 51, 52) elevate *CYP11B2* gene expression locally in rat heart, kidney and peripheral sensory neurons. As aldosterone production and *CYP11B2* expression are also elevated in heart failure patients (53, 54), it would be important to continue these experiments in pathophysiological conditions of large intestine using animal models.

In conclusion, as far as we are aware, this is the first time that the whole chain for local aldosterone synthesis from the *CYP11B2* gene to the end product has been identified as being expressed in murine intestinal tract. The findings agree with our hypothesis that the distal section of gastrointestinal tract - cecum and colon, potentially can produce aldosterone locally, which might have a physiological function in water absorption, sodium retention and potassium excretion. However, some questions remain to be clarified; mainly, the physiological, and pathophysiological relevance of the aldosterone synthesis in large intestine as well as its regulatory mechanisms.

*Authors' contribution:* H. Launonen and Z. Pang contributed equally to this work.

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## REFERENCES

- Nanba K, Vaidya A, Rainey WE. Aging and adrenal aldosterone production. *Hypertension* 2018; 71: 218-223.
- Milliez P, Girerd X, Plouin P-F, Blacher J, Safar ME, Mourad J-J. Evidence for an increased rate of cardiovascular events in patients with primary aldosteronism. *J Am Coll Cardiol* 2005; 45: 1243-1248.
- Catena C, Colussi G, Nardalini E, et al. Cardiovascular outcomes in patients with primary aldosteronism after treatment. *Arch Intern Med* 2008; 168: 80-85.
- Mulatero P, Monticone S, Bertello C, et al. Long-term cardio- and cerebrovascular events in patients with primary aldosteronism. *J Clin Endocrinol Metab* 2013; 98: 4826-4833.
- Vasan RS, Evans JC, Larson MG, et al. Serum aldosterone and the incidence of hypertension in nonhypertensive persons. *N Engl J Med* 2004; 351: 33-41.
- Janower S, Collet J-P, Benoliel J-J, et al. High plasma aldosterone levels on admission are associated with death in patients presenting with acute ST-elevation myocardial infarction. *MT Cardio* 2007; 3: 48-50.
- Tylicki L, Larczynski W, Rutkowski B. Renal protective effects of the renin-angiotensin-aldosterone system blockade: from evidence-based approach to perspectives. *Kidney Blood Press Res* 2005; 28: 230-242.
- Guder G, Bauersachs J, Frantz, et al. Complementary and incremental mortality risk prediction by cortisol and aldosterone in chronic heart failure. *Circulation* 2007; 115: 1754-1761.
- Tsutamoto T, Sakai H, Tanaka T, et al. Comparison of active renin concentration and plasma renin activity as a prognostic predictor in patients with heart failure. *Circ J* 2007; 71: 915-921.
- Te Riet L, van Esch JH, Roks AJ, van den Meiracker AH, Danser AH. Hypertension: renin-angiotensin-aldosterone system alterations. *Circ Res* 2015; 116: 960-975.
- Nakamura Y, Yamazaki Y, Konosu-Fukaya S, Ise K, Satoh F, Sasano H. Aldosterone biosynthesis in the human adrenal cortex and associated disorders. *J Steroid Biochem Mol Biol* 2015; 153: 57-62.
- Williams GH. Aldosterone biosynthesis, regulation, and classical mechanism of action. *Heart Fail Rev* 2005; 10: 7-13.
- Freel EM, Bernhardt M, Ingram R, et al. Endogenous corticosteroid biosynthesis in subjects after bilateral adrenalectomy. *Clin Endocrinol* 2007; 66: 659-665.
- Takeda Y, Yoneda T, Demura M, Furukawa K, Miyamori I, Mabuchi H. Effects of high sodium intake on cardiovascular aldosterone synthesis in stroke-prone spontaneously hypertensive rats. *J Hypertens* 2001; 19: 635-639.
- Fujisaki M, Nagoshi T, Nishikawa T, Date T, Yoshimura M. Rapid induction of aldosterone synthesis in cultured neonatal rat cardiomyocytes under high glucose conditions. *Biomed Res Int* 2013; 2013: 161396. doi: 10.1155/2013/161396
- Takeda Y, Miyamori I, Yoneda T, et al. Production of aldosterone in isolated rat blood vessels. *Hypertension* 1995; 25: 170-173.
- Henrion D, Benessiano J, Le'vy 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.
- Xue C, Siragy HM. Local renal aldosterone system and its regulation by salt, diabetes, and angiotensin II type 1 receptor. *Hypertension* 2005; 46: 584-590.
- Geerling JC, Loewy AD. Aldosterone in the brain. *Am J Physiol Renal Physiol* 2009; 297: F559-F576.
- Briones AM, Nguyen Dinh Cat A, Callera GE, et al. Adipocytes produce aldosterone through calcineurin dependent signaling pathways: implications in diabetes mellitus-associated obesity and vascular dysfunction. *Hypertension* 2012; 59: 1069-1078.
- Selvarajah V, Connolly K, McEniery C, Wilkinson I. Skin sodium and hypertension: a paradigm shift? *Curr Hypertens Rep* 2018; 20: 94. doi: 10.1007/s11906-018-0892-9
- Aleksiejczuk M, Gromotowicz-Poplawska A, Marcinczyk N, Przyłipiak A, Chabielska E. The expression of the renin-angiotensin-aldosterone system in the skin and its effects on skin physiology and pathophysiology. *J Physiol Pharmacol* 2019; 70: 325-336.
- Chrissobolis S. Vascular consequences of aldosterone excess and mineralocorticoid receptor antagonism. *Curr Hypertens Rev* 2017; 13: 45-56.
- Pantelidis P, Sideris M, Viigimaa M, et al. The mechanisms of actions of aldosterone and its antagonists in cardiovascular disease. *Curr Pharm Des* 2019; 24: 5491-5499.
- Connell JMC, Davies E. The new biology of aldosterone. *J Endocrinol* 2005; 186: 1-20.
- Harvey BJ, Alzamora R, Stubbs AK, Irnaten M, McEneaney V, Thomas W. Rapid responses to aldosterone in the kidney and colon. *J Steroid Biochem Mol Biol* 2008; 108: 310-317.
- Takeda Y, Miyamori I, Yoneda T, et al. Regulation of aldosterone synthase in human vascular endothelial cells by angiotensin II and adrenocorticotropin. *J Clin Endocrinol Metab* 1996; 81: 2797-2800.
- Zamolodchikova TS, Shoibonov BB, Tolpygo SM. Local renin-angiotensin system of small intestine. *Eksp Klin Gastroenterol* 2016; 12: 97-104.
- Mastropaolo M, Zizzo MG, Auteri M, et al. Activation of angiotensin II type 1 receptors and contractile activity in human sigmoid colon in vitro. *Acta Physiol* 2015; 215: 37-45.
- Levens NR, Peach MJ, Carey RM. Interactions between angiotensin peptides and the sympathetic nervous system mediating intestinal sodium and water absorption in the rat. *J Clin Invest* 1981; 67: 1197-1207.
- Varmavuori L, Launonen H, Korpela R, Vapaatalo H. Detection of immunoreactive aldosterone in murine gastrointestinal tract. *J Physiol Pharmacol* 2020; 71: 597-601.
- Salmenkari H, Pasanen L, Linden J, Korpela R, Vapaatalo H. Beneficial anti-inflammatory effect of angiotensin-converting enzyme inhibitor and angiotensin receptor blocker in the treatment of dextran sulfate sodium-induced colitis in mice. *J Physiol Pharmacol* 2018; 69: 561-572.
- Eissa N, Hussein H, Wang H, Rabbi MF, Bernstein CN, Ghia JE. Stability of reference genes for messenger RNA quantification by real-time PCR in mouse dextran sodium sulfate experimental colitis. *PLoS One* 2016; 11: e0156289. doi: 10.1371/journal.pone.0156289
- Salmenkari H, Holappa M, Forsgard RA, Korpela R, Vapaatalo H. Orally administered angiotensin-converting enzyme-inhibitors captopril and isoleucine-proline-proline have distinct effects on local renin-angiotensin system and corticosterone synthesis in dextran sulfate sodium-induced colitis in mice. *J Physiol Pharmacol* 2017; 68: 355-362.
- Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002; 3: research0034. doi: 10.1186/gb-2002-3-7-research0034
- Jurgens G, Graudal NA. Effects of low sodium diet versus high sodium diet on blood pressure, renin, aldosterone,



- catecholamines, cholesterol, and triglyceride. *Cochrane Database Syst Rev* 2004; (1):CD004022. doi: 10.1002/14651858.CD004022.pub2
37. Kakiki M, Morohashi K-I, Nomura M, Omura T, Horie T. Regulation of aldosterone synthase cytochrome P450 (CYP11B2) and 11 beta-hydroxylase cytochrome P450 (CYP11B1) expression in rat adrenal zona glomerulosa cells by low sodium diet and angiotensin II receptor antagonists. *Biol Pharm Bull* 1997; 20: 962-968.
  38. Schewe J, Seidel E, Forslund S, *et al.* Elevated aldosterone and blood pressure in a mouse model of familial hyperaldosteronism with CLC-2 mutation. *Nat Commun* 2019; 10: 5155. doi: 10.1038/s41467-019-13033-4
  39. Gu H, Ma Z, Wang J, *et al.* Salt-dependent blood pressure in human aldosterone synthase-transgenic mice. *Sci Rep* 2017; 7: 492. doi: 10.1038/s41598-017-00461-9
  40. Ye P, Kenyon CJ, MacKenzie SM, *et al.* Regulation of aldosterone synthase gene expression in the rat adrenal gland and central nervous system by sodium and angiotensin II. *Endocrinology* 2003; 144: 3321-3328.
  41. Shields R, Mulholland AT, Elmslie RG. Action of aldosterone upon the intestinal transport of potassium, sodium, and water. *Gut* 1966; 7: 686-696.
  42. Ward JBJ, Keely SJ, Keely SJ. Oxygen in the regulation of intestinal epithelial transport. *J Physiol* 2014; 592: 2473-2489.
  43. Salmenkari H, Issakainen T, Vapaatalo H, Korpela R. Local corticosterone production and angiotensin-I converting enzyme shedding in a mouse model of intestinal inflammation. *World J Gastroenterol* 2015; 21: 10072-10079.
  44. Turnamian SG, 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.
  45. Nakamura T, Kurihara I, Kobayashi S, *et al.* Intestinal mineralocorticoid receptor contributes to epithelial sodium channel-mediated intestinal sodium absorption and blood pressure regulation. *J Am Heart Assoc* 2018; 7: e008259. doi: 10.1161/JAHA.117.008259
  46. Sztachman D, Czarzasta K, Cudnoch-Jedrzejewska A, Szczepanska-Sadowska E, Zera T. Aldosterone and mineralocorticoid receptors in regulation of the cardiovascular system and pathological remodelling of the heart and arteries. *J Physiol Pharmacol* 2018; 69: 829-845.
  47. Gawenis LR, Bradford EM, Alper SL, Prasad V, Shull GE. AE2 Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger is required for normal cAMP-stimulated anion secretion in murine proximal colon. *Am J Physiol Gastrointest Liver Physiol* 2010; 298: G493-G503.
  48. Knapp BK, Bauer LL, Swanson KS, Tappenden KA, Fahey GC Jr, de Godoy MR. Soluble fiber dextrin and soluble corn fiber supplementation modify indices of health in cecum and colon of Sprague-Dawley rats. *Nutrients* 2013; 5: 396-410.
  49. Nascimento-Goncalves E, Mendes BAL, Silva-Reis R, Faustino-Rocha AI, Gama A, Oliveira PA. Animal models of colorectal cancer: from spontaneous to genetically engineered models and their applications. *Vet Sci* 2021; 8: 59. doi: 10.3390/vetsci8040059
  50. Chai W, Garrelds IM, de Vries R, Danser JA. Cardioprotective effects of eplerenone in the rat heart: interaction with locally synthesized or blood-derived aldosterone? *Hypertension* 2006; 47: 665-670.
  51. Bose HS, Whittall RM, Marshall B, *et al.* A novel mitochondrial complex of aldosterone synthase, steroidogenic acute regulatory protein, and tom22 synthesizes aldosterone in the rat heart. *J Pharmacol Exp Ther* 2021; 377: 108-120.
  52. Mohamed DM, Shaqura M, Li X, *et al.* Aldosterone synthase in peripheral sensory neurons contributes to mechanical hypersensitivity during local inflammation in rats. *Anesthesiology* 2020; 132: 867-880.
  53. Mizuno Y, Yoshimura M, Yasue H, *et al.* Aldosterone production is activated in failing ventricle in humans. *Circulation* 2001; 103: 72-77.
  54. Satoh M, Nakamura M, Saitoh H, *et al.* Aldosterone synthase (CYP11B2) expression and myocardial fibrosis in the failing human heart. *Clin Sci* 2002; 102: 381-386.

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