EFFECTS OF ALDOSTERONE ON STRIATIN AND CAVEOLIN-1 PROTEIN LEVELS IN RAT KIDNEY: ROLE OF MINERALOCORTICOID RECEPTOR

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จุฬาลงกรณ์มหาวิทยาลัย
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ผลของอัลโดสเตอโรนต่อระดับโปรตีนสไตรเอตินและแคฟวีโอลินวันในไตหนูแรท: บทบาทของตัวรับมิเนราโลคอร์ติคอยด์

น.ส.เกวลิน อินทชาติ

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

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Thesis Title	EFFECTS OF ALDOSTERONE ON STRIATIN AND CAVEOLIN-1 PROTEIN LEVELS IN RAT KIDNEY: ROLE OF MINERALOCORTICOID RECEPTOR
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เกวลิน อินทชาติ : ผลของอัลโดสเตอโรนต่อระดับโปรตีนสไตรเอตินและแคฟวีโอลินวันในไตหนูแรท:บทบาทของตัวรับมิเนราโลคอร์ติคอยด์. (
EFFECTS OF ALDOSTERONE ON STRIATIN AND CAVEOLIN-1
PROTEIN LEVELS IN RAT KIDNEY: ROLE OF MINERALOCORTICOID RECEPTOR) อ.ที่ปรึกษาหลัก : รศ. ดร.สมจิตร์ เอี่ยมอ่อง, อ.ที่ปรึกษาร่วม : นพ.กฤษณพงศ์ มโนธรรม

อัลโดสเตอโรน คือ สเตียรอยด์ฮอร์โมน ที่มีบทบาทสำคัญในการรักษาสมดุลของโซเดียม โพแทสเซียม และกรด-ด่าง อัลโดสเตอโรนจะจับกับตัวรับมิเนราโลคอร์ติคอยด์ (เอ็มอาร์) และทำงานผ่านวิถีจีโนมิค หรือ ไม่ผ่านวิถีจีโนมิค พบว่า เอ็มอาร์สามารถจัดวางรูปแบบที่ซับซ้อนกับโมเลกุลต่างๆ รวมถึงสแคฟโฟลดิงโปรตีน เช่น สไตรเอติน และแคฟวีโอลินวัน (แคฟวัน) จากการศึกษาในเซลล์เพาะเลี้ยงพบว่าอัลโดสเตอโรนเพิ่มปริมาณโปรตีนสไตรเอตินและแคฟวัน นอกจากนั้น ทั้งสไตรเอติน และแคฟวันสามารถอยู่ร่วมกับเอ็มอาร์ อย่างไรก็ตามยังไม่มีการศึกษาในสัตว์ทดลองถึงผลการทำงานของอัลโดสเตอโรนต่อระดับโปรตีนสไตรเอตินและแคฟวันในไตหนูแรท หนูแรทสายพันธุ์วิสต้าแบ่งออกเป็น 2 กลุ่มหลักคือ กลุ่ม 30 นาที และกลุ่ม 2 ชั่วโมง โดยที่แต่ละกลุ่มจะแบ่งเป็นอีก 3 กลุ่มย่อย คือ กลุ่มที่ได้รับสารละลายน้ำเกลือ (sham) หรือได้รับอัลโดสเตอโรน (150 µg/kg BW) ฉีดเข้าทางช่องท้อง หรือได้รับอีพลีรีโนน (15 mg/kg BW) ซึ่งเป็นตัวยับยั้งการทำงานของเอ็มอาร์ โดยฉีดเข้าช่องท้องเป็นเวลา 30 นาที ก่อนฉีดอัลโดสเตอโรน ภายหลังการฉีดอัลโดสเตอโรน 30 นาที หรือ 2 ชั่วโมง ทำการตรวจวัดระดับปริมาณโปรตีนสไตรเอตินและแคฟวัน และระดับการแสดงออกของโปรตีนดังกล่าวในเนื้อเยื่อไต โดยวิธีการวิเคราะห์ Western blot และ immunohistochemistry ตามลำดับ และตรวจวัดระดับปริมาณโปรตีนที่อยู่ร่วมกันของ สไตรเอตินกับเอ็มอาร์ แคฟวันกับเอ็มอาร์ และแคฟวันกับสไตรเอตินโดยเทคนิค co-immunoprecipitation และ Western blot ผลการทดลองในกลุ่ม 30 นาที พบว่า อัลโดสเตอโรนเพิ่มปริมาณโปรตีนสไตรเอตินและแคฟวัน เป็น 150% ($p<0.05$) และ 200% ($p<0.001$) ตามลำดับ การให้อีพลีรีโนนไม่มีผลต่อระดับโปรตีนสไตรเอติน ในขณะที่ระดับปริมาณโปรตีนแคฟวันถูกยับยั้งบางส่วนโดยอีพลีรีโนน สำหรับกลุ่ม 2 ชั่วโมง พบว่าปริมาณโปรตีนของสไตรเอตินและแคฟวันมีระดับเท่าเดิมหลังจากได้รับอัลโดสเตอโรน เมื่อให้อีพลีรีโนน พบว่าระดับโปรตีนแคฟวันลดลงอย่างมีนัยสำคัญทางสถิติ อัลโดสเตอโรนกระตุ้นการแสดงออกของโปรตีนสไตรเอตินและแคฟวันในบริเวณคอร์เทกซ์และเมดัลลา การให้อีพลีรีโนนสามารถลดระดับการแสดงออกของสไตรเอตินและแคฟวันในคอร์เทกซ์ สำหรับการอยู่ร่วมกันของโปรตีน ผลการทดลองพบว่ามีโปรตีนสไตรเอตินหรือแคฟวันอยู่ร่วมกันกับเอ็มอาร์ในไตหนูแรท การศึกษานี้เป็นครั้งแรกที่ทำในสัตว์ทดลองที่แสดงถึงข้อมูลจากไตหนูแรท โดยพบว่าอัลโดสเตอโรนมีผลที่แตกต่างในการควบคุมระดับโปรตีนสไตรเอตินและแคฟวัน ซึ่งในการทำงานแบบรวดเร็ว (30 นาที) พบว่าอัลโดสเตอโรนเพิ่มระดับสไตรเอตินโดยไม่ผ่านการทำงานของเอ็มอาร์ แต่เพิ่มระดับแคฟวันได้บางส่วนโดยผ่านของการทำงานของเอ็มอาร์ สำหรับผลระยะยาว (2 ชั่วโมง) พบว่าอัลโดสเตอโรนไม่เปลี่ยนแปลงระดับโปรตีนสไตรเอตินและแคฟวัน การให้ตัวยับยั้งการทำงานของเอ็มอาร์สามารถรบกวนผลของอัลโดสเตอโรนต่อระดับโปรตีนแคฟวัน

สาขาวิชา สรีรวิทยา
ปีการศึกษา 2561

ลายมือชื่อนิสิต .....
ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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KEYWORD: striatin, caveolin-1, mineralocorticoid receptor, rat kidney, aldosterone

Kevalin Inthachart : EFFECTS OF ALDOSTERONE ON STRIATIN AND CAVEOLIN-1 PROTEIN LEVELS IN RAT KIDNEY: ROLE OF MINERALOCORTICOID RECEPTOR. Advisor: Assoc. Prof. Somchit Eiam-Ong, Ph.D. Co-advisor: Krissanapong Manotham, M.D.

The mineralocorticoid hormone aldosterone is a steroid hormone which plays an important role in maintenance of sodium, potassium, and acid-base balance. Aldosterone actions are mediated by mineralocorticoid receptor (MR) that could operate via genomic or nongenomic pathways. It has been shown that MR forms a complex with several molecules including scaffolding proteins, such as striatin and caveolin-1 (cav-1). Previous *in vitro* studies demonstrated that aldosterone increased striatin and cav-1 protein abundance. In addition, both striatin and cav-1 could interact with MR. However, there is no *in vivo* study of aldosterone effects on striatin and cav-1 protein levels in rat kidney. Male Wistar rats were divided into two main groups: 30 minutes and 2 hours. Each main group further divided into 3 subgroups: sham (normal saline solution; ip), aldosterone (Aldo: 150 μg/kg BW) or eplerenone [mineralocorticoid receptor (MR) blocker, 15 mg/kg BW] 30 minutes before aldosterone injection. Thirty minutes or two hours after aldosterone administration, protein abundance and localization of striatin and cav-1 were determined by Western blot analysis and immunohistochemistry, respectively. In addition, the protein interaction of striatin/MR, cav-1/MR, and cav-1/striatin was measured by co-immunoprecipitation and Western blot analysis. The results from 30-minute group showed that aldosterone increased protein abundances of striatin and cav-1 to 150% ($p<0.05$) and 200% ($p<0.001$), respectively. Eplerenone had no significant effect on striatin levels, whereas cav-1 protein was partially blocked by eplerenone. For 2-hour group, protein abundances of striatin and cav-1 were remained after aldosterone injection. Interestingly, eplerenone pretreatment significantly suppressed cav-1 protein levels. Aldosterone stimulated striatin and cav-1 protein immunoreactivity in both cortex and medulla. Eplerenone minimized striatin and cav-1 immunostaining in the cortex. For protein interaction, the data showed that either striatin or cav-1 was able to interact with MR in rat kidney. This is the first *in vivo* study demonstrating that, in rat kidney, aldosterone differently modulates striatin and cav-1 protein levels. In a rapid action, aldosterone increases striatin via MR-independent manner, whereas it partially induces cav-1 through MR-dependent pathway. For a longer effect, aldosterone had no significant alterations on striatin and cav-1 protein levels. Blockage of MR could disturb aldosterone actions on cav-1 protein.

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Field of Study:	Physiology	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....
		Co-advisor's Signature .....

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CHAPTER I
INTRODUCTION

BACKGROUND AND RATIONALE

The mineralocorticoid hormone aldosterone is a steroid hormone produced by the outer zone of adrenal cortex, the zona glomerulosa (Bollag 2014). The main effect of aldosterone is to regulate sodium, potassium, and acid-base balance, principally in the kidney. The classical action of aldosterone is mediated through the cytosolic mineralocorticoid receptor (MR) (Good 2007). The genomic mechanism of aldosterone action involves a binding to MR in cytosol and translocates into the nucleus for gene transcription. The classical genomic action occurs over comparatively longer periods, measured in hours (Rafiq 2011). In addition, aldosterone also acts in the rapid nongenomic mechanism that can mediate by MR or through possibly membrane-associated molecules (Good 2007). However, rapid aldosterone actions occur in a shorter time (≤ 30 minutes) to stimulate a variety of intracellular signal transduction pathways or activation of protein kinases (Boldyreff and Wehling 2003). MR localization is limited to epithelial cells of the kidney, gut, and non-epithelial cells such as neuronal and vascular smooth muscle cells (Tsugita 2009). Lombes et al (1990) examined the localization of MR in the rabbit kidney by immunohistochemical technique. The data showed that MR expression is noted with an intense immunostaining in the connecting tubules, collecting ducts and distal tubules. In addition, MR also expresses in Henle's loops and proximal tubules (Lombès 1990, Salyer 2013).

It has been shown that MR forms a complex with several proteins including striatin and caveolin-1 (cav-1) (Baudrand 2014). Striatin, a scaffolding protein, localizes in the intracellular compartment (Castets 2000). A previous *in vitro* study showed that striatin interacts with MR in mouse aortic endothelial cells, human endothelial

cell (EA.hy926; EaHy), and mouse heart tissue (Pojoga 2012). In addition, both of *in vitro* and *in vivo* studies, the aldosterone increased striatin protein abundance with a peak at 6 hours and returned to baseline by 12 hours. The abundance of striatin protein was blocked by canrenoic acid (MR antagonist) (Pojoga 2012). Recently, the data suggest that striatin is essential in mediating rapid responses of aldosterone (Coutinho 2014). However, it has not been examined the effects of aldosterone, both genomic and nongenomic action, on striatin in the kidney.

For caveolin family, it consists of cav-1, cav-2, and cav-3 (Branza-Nichita 2012). Caveolin contains the scaffolding domain in the part of the oligomerization area that is able to interact with signaling molecules (Gaillard 2001). A Previous study supported the interplay between cav-1 and MR in modulating mechanisms of aldosterone action in mouse heart tissue (Pojoga 2010). In cultured human umbilical vein endothelial cells, aldosterone incubation for 48 hours induced cav-1 protein abundances (Igarashi 2013). By immunoprecipitation and Western blot analysis, the data showed that cav-1 and MR are interacted in the wild type group. The cav-1 knockout mice lost this property (Pojoga 2010). It was demonstrated that striatin directly binds to cav-1 (Gaillard 2001).

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At present, it is not defined *in vivo* that aldosterone affects on striatin and cav-1 levels in rat kidney. Therefore, the present study aims to investigate aldosterone on striatin and cav-1 protein abundances and localizations in the rat kidney by Western blot analysis and immunohistochemistry, respectively. The role of MR on this regard will be determined. In addition, the protein interaction of striatin/MR, cav-1/MR, and cav-1/striatin will be measured by immunoprecipitation and Western blot analysis.

RESEARCH QUESTIONS

1. Does aldosterone injection change striatin and caveolin-1 levels in rat kidney?
2. Is pretreatment with eplerenone (MR blocker) able to prevent the alteration induced by aldosterone on striatin and caveolin-1 levels in rat kidney?
3. Does aldosterone injection change protein interaction of striatin/MR, caveolin-1/MR, and caveolin-1/striatin in rat kidney?

RESEARCH OBJECTIVES

1. To examine changes of protein abundances and localizations of striatin and caveolin-1 in rat kidney after aldosterone injection.
2. To clarify the role of MR in alternations induced by aldosterone on protein abundances and localizations of striatin and caveolin-1 in rat kidney.
3. To examine protein interactions of striatin/MR, caveolin-1/MR, and caveolin-1/striatin in rat kidney after aldosterone injection.

RESEARCH HYPOTHESIS

After aldosterone injection, protein abundances and localizations of striatin and caveolin-1 in the rat kidney would enhance as compared with NSS-treated group. Pretreatment with eplerenone (MR blocker) would decrease striatin and cav-1 expressions induced by aldosterone.

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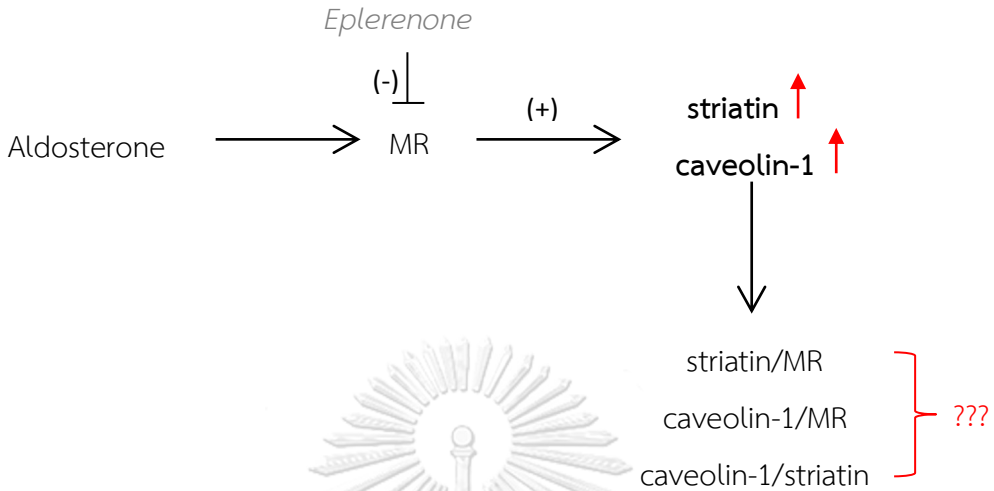
CONCEPTUAL FRAMEWORK

In vivo



ASSUMPTION

All rats used in the experiment will be assessed kidney function to verify that they would have no renal diseases by means of measuring plasma creatinine (Cr) levels (< 1 mg %).

KEY WORDS

aldosterone, striatin, caveolin-1, mineralocorticoid receptor, rat kidney.

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EXPECTED BENEFIT AND APPLICATION

1. This is the first animal study data demonstrating the role of mineralocorticoid receptor in the alterations induced by aldosterone on protein abundances and localizations of striatin and caveolin-1 in rat kidney.
2. This research contributes a fundamental knowledge of effects of aldosterone on scaffolding proteins: striatin and caveolin-1 in rat kidney.
3. This fundamental knowledge will provide documents for further studies in the effects of aldosterone on other membrane receptors.

CHAPTER II
LITERATURE REVIEWS

Aldosterone biosynthesis

Aldosterone, a mineralocorticoid hormone, is one of the steroid hormones which was secreted from the outer layer of the adrenal cortex, the zona glomerulosa (Booth 2002) . The glomerulosa cells synthesize aldosterone at rate of 50 to 200 μg/day, to give plasma levels of 4 to 21 μg/dL (Pearce 2016). Aldosterone substrate is derived from precursor cholesterol by the action of various processing enzyme (Figure 1). The initial enzymatic step in aldosterone biosynthesis is converted cholesterol into pregnenolone (Williams 2005, Hattangady 2012). Pregenolone is released into the cytosol and is converted to progesterone by 3β-hydroxysteroid dehydrogenase type 2 (HSD3B2), which is located on the membrane of the smooth endoplasmic reticulum. Progesterone is 21-hydroxylated by the enzyme CYP21 located on the cytoplasmic surface of the smooth endoplasmic reticulum, producing 11-deoxycorticosterone. CYP11B1 is a mitochondrial enzyme which catalyzes 11-deoxycorticosterone to corticosterone. The terminal two steps in aldosterone biosynthesis are mediated by aldosterone synthase (CYP11B2) which converts corticosterone to 18-hydroxycorticosterone and catalyzes 18-hydroxycorticosterone to aldosterone (Connell and Davies 2005, Williams 2005) (Figure 1).

Aldosterone synthesis is regulated by several factors that have been shown to stimulate or inhibit aldosterone productions (Connell and Davies 2005). The principal regulator of aldosterone synthesis and secretion is angiotensin II (Ang II). In addition, plasma potassium also stimulates aldosterone secretion (Abramowitz 2012). Moreover, other stimuli for aldosterone include adrenocorticotropic hormone (ACTH), low plasma Na^+, endothelin, and serotonin (Thomas and Harvey 2011).

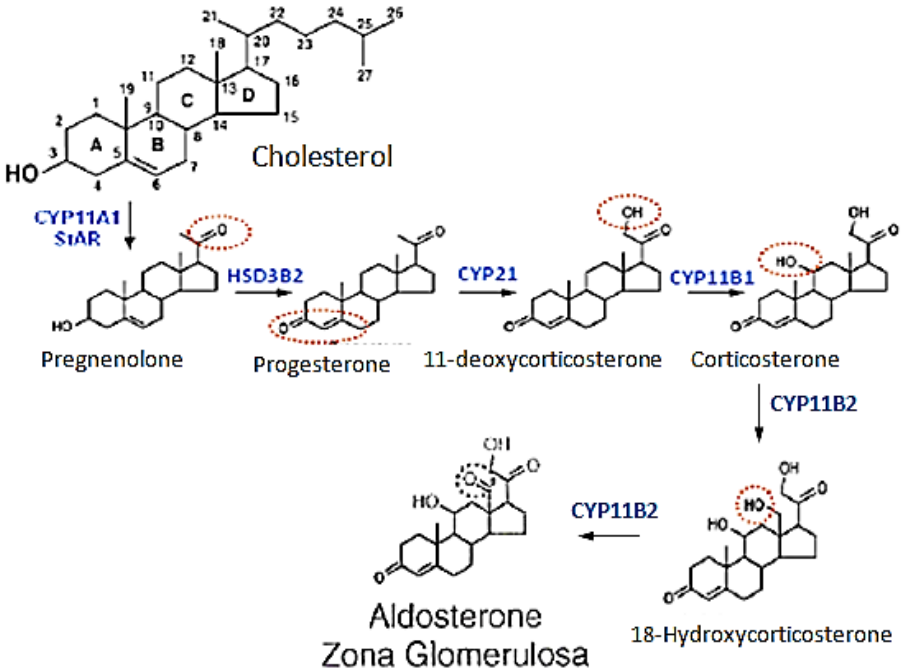

Figure A: Scheme of aldosterone biosynthesis

Mechanism of actions

Steroid hormones modulate many physiological processes. The effects of steroid that are mediated by modulation of gene expression are known to occur with a time lag of hours or even days (Lösel and Wehling 2008). Many studies have identified other responses to steroid that are much more rapid and take place in seconds or minutes (Lösel and Wehling 2008, Thomas and Harvey 2011). These responses follow nongenomic pathways and they are not rare (Lösel and Wehling 2008).

Genomic pathway of aldosterone

According to classically genomic actions, aldosterone enters the cell by passive diffusion through lipid membrane and binds to mineralocorticoid receptor (MR) locating in the cytosol (Figure B) (Good 2007). This aldosterone-MR complex translocates to the nucleus and promotes gene transcription and production of

proteins that modulate the expression and activity of channels and other ion transport proteins (Good 2007). The length time of genomic action is 1-2 hours before obvious change in target cell activity, and the whole pathway is sensitive to particular inhibitors, such as actinomycin D or cycloheximide (Good 2007).

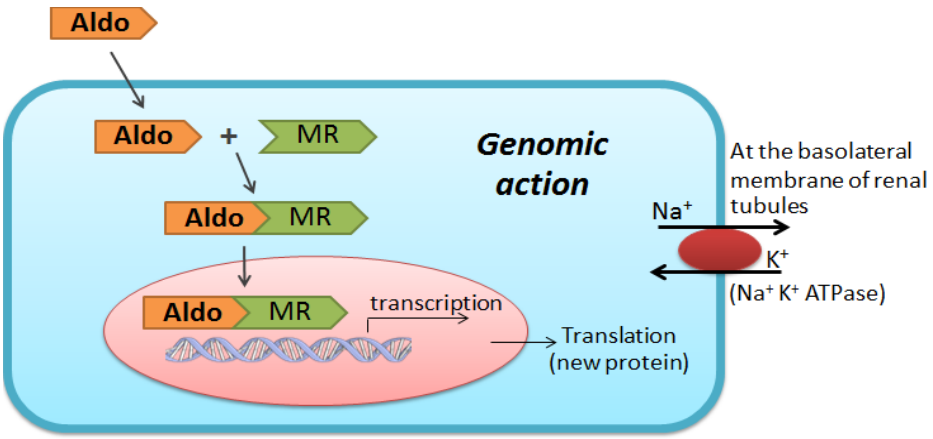

Figure B: Genomic pathway of aldosterone

Nongenomic pathway of aldosterone

In contrast, the rapid effects of aldosterone are referred to as nongenomic actions. A nongenomic action is defined as any action that neither directly nor initially influence gene expression, as does the classical action (Lösel and Wehling 2008). The responses take place in seconds to minutes and are independent on transcription and translation. There is an evidence to support the nongenomic pathway that aldosterone stimulates vacuolar H^+-ATPases in intercalated renal tubule cells within 30 minutes (Winter 2004). In addition, aldosterone nongenomically enhanced protein levels of heat shock protein (Hsp90; **α** and **β**), protein kinase C alpha (PKC**α**), extracellular-signal regulated kinase (ERK) and α_1-Na-K-ATPase in rat kidney (Eiam-Ong 2013, Eiam-Ong 2014). Nongenomic actions of aldosterone involve in the generation of intracellular secondary messengers and various signal transduction cascades (Figure C) (Funder 2005, Lösel and Wehling 2008,

Queisser 2010). These actions have been extensively investigated, mostly in cell culture studies (Lösel 2003, Good 2007, Grossmann and Gekle 2009).

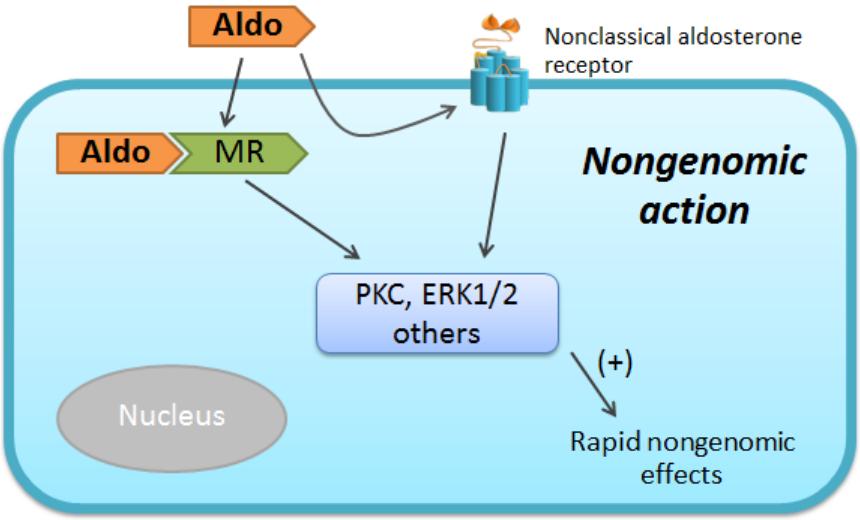

Figure C: Nongenomic pathway of aldosterone

Mineralocorticoid receptors

The structure of mineralocorticoid receptor (MR) protein contains 984 amino acids with a molecular mass of 107 kDa (Pippal and Fuller 2008). MR is the steroid hormone receptor superfamily, along with progesterone, estrogens, androgens, glucocorticoids, and specifically aldosterone (Meinel 2014). MR comprises of three major binding domains: 1) N-terminal domain (NTD), 2) DNA-binding domain (DBD), and 3) C-terminal ligand-binding domain (LBD) (Viengchareun 2007) (Figure D). The LBD domain has multiple functions such as ligand binding, nuclear localization, dimerization, transcriptional coactivators and ligand-dependent transactivation (Yang and Young 2009). For aldosterone, the studies identified that aldosterone binds to MR at the LBD located in the cytosol of target epithelial cells. This binding induces nuclear translocation, gene transactivation, and protein expression (Rogerson 2004). MR is revealed in various tissues that the classic target tissues for aldosterone are the heart, blood vessels, brain, and especially kidney (Ueda and Nagase 2014). The main

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targets of aldosterone action in the kidney are distal convoluted tubules, connecting tubules, and collecting ducts. The principal cells significantly express more MR than the intercalated cells (Thomas and Harvey 2011). MR also expresses in proximal tubules (Salyer 2013). MR is normally activated by aldosterone which increases sodium reabsorption and potassium excretion; therefore it provides maintenances of electrolyte balance and blood pressure (Viengchareun 2007). Furthermore, the activation of MR by aldosterone enhances oxidative stress, inflammation, and vascular dysfunction that lead to human condition, specially renal and cardiovascular diseases. This activation is blocked by specific MR antagonists such as spironolactone and eplerenone (Thomas and Harvey 2011). It has been demonstrated that MR phosphorylation site in LBD on S843 regulates ligand binding and receptor activation. In addition, aldosterone, within minutes, induced MR phosphorylation at the serine and threonine mediated via protein kinase C activation (Viengchareun 2007).

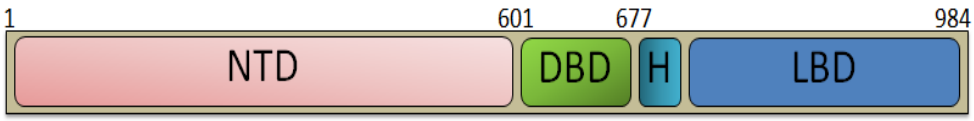

Figure D: Schematic representation of the human MR structure

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Striatin

Striatin, a scaffolding protein, is localized in the intracellular compartment that contains 780 amino acids (Castets 2000). The protein of striatin family consists of striatin, SG2NA, and zinedin containing multiple binding protein-protein association domains including caveolin-binding, coiled-coil, and Ca^{2+}-calmodulin at N-terminus, and WD-repeat domains at C-terminus (Hwang and Pallas 2014) (Figure E). Striatin is found in various tissues including kidney, lung, liver, skeletal, cardiac muscles, and nervous system (Hwang and Pallas 2014). Striatin has been shown associated with the estrogen receptor (ERα) which is a steroid receptor. The N-terminal segment of striatin will interact with the DNA binding domain of ERα in the immortalized human endothelial cell line (EA.hy926) cells. Recent evidence suggested that striatin mediates the nongenomic effect of estrogen. Therefore, Pojoga et al. (2012) hypothesized that striatin may also interact with other steroid hormone receptors, such as MR. It has been demonstrated that striatin interactions with MR in mouse aortic endothelial cells, EA.hy926 cells, and mouse heart tissues (Pojoga 2012).

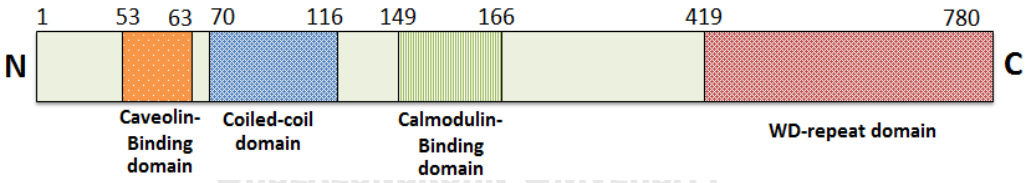

Figure E: Domain structures of striatin family members

Caveolin-1

Caveolin is the one of essential proteins which interacts with MR and striatin. It is the main component of plasma membrane caveolae that influences on receptor activation to signal transduction (Baudrand 2014). Caveolin family consists of caveolin-1 (cav-1), caveolin-2 (cav-2), and caveolin-3 (cav-3) that contain 150-178 amino acids with molecular mass of 22 kDa (Branza-Nichita 2012). Caveolin comprises of three major binding domains: 1) scaffolding domain (CSD), 2) transmembrane domain (TM), and 3) palmitoylated C-terminus (Figure F) (Branza-Nichita 2012). Caveolin can interact directly with various proteins via the CSD in the part of the oligomerization area that is able to interact with specific lipids and various signaling molecules including Ras, serine-threonine kinases, several tyrosine kinases, and G protein-coupled receptors (Gaillard 2001, Reeves 2012). Cav-1 expresses in several tissues, including adipocytes, fibroblasts, and endothelial cells. Cav-2 expression is similar to cav-1, whereas cav-3 expresses in skeletal muscles, smooth muscles and nervous system (Tang 1996, Reeves 2012). However, the role of cav-2 is poorly characterized in signaling regulation while cav-3 has the function likely to cav-1 (Boscher and Nabi 2012). It has been shown that, in the kidney, cav-1 is the main isoform observed in the epithelial cells of the distal convoluted tubules, connecting tubules, and collecting ducts, especially the intercalated cells type A (Breton 1998). Cav-1 is the main scaffolding protein of caveolae plasma membrane which contains N- and C- terminal cytoplasmic tails. Cav-1 is able to hold the signal transducing molecules including Src-family tyrosine kinases, MAPK, and endothelial nitric oxide synthase in an inactive form (Pavlides 2012). Recently, evidence has been showed that Src kinase family is able to phosphorylate cav-1 on tyrosine 14 and interact to the CSD of cav-1 (Kiss 2012). This causes the initial step in caveolar internalization (Kiss 2012). The scaffolding domain of cav-1 mediates protein-protein interactions

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and alters signal transduction pathway (Thompson 2010). In addition, it has been documented that cav-1 interacts with striatin and MR (Gaillard 2001, Coutinho 2014).

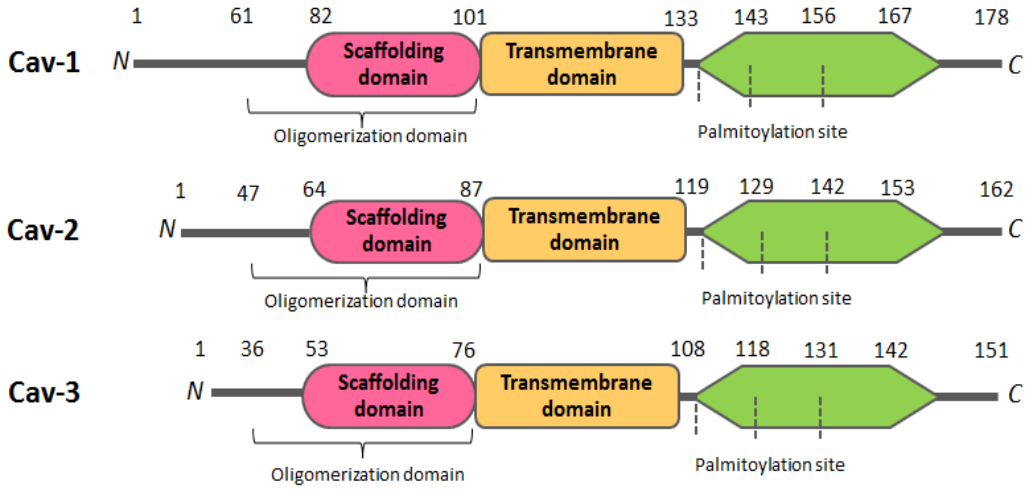

Figure F: Schematic presentation of the structure of the caveolin family

Aldosterone — striatin — cav-1

According to the action of aldosterone on striatin, Pojoga et al. (2012) investigated the effects of aldosterone on striatin protein abundance both of *in vitro* and *in vivo* studies. The results showed that aldosterone increases striatin levels with a peak at 6 hours and returned to baseline by 12 hours in human endothelial cell (EA.hy926; EaHy). In addition, this study confirmed that aldosterone (10 nmol/L, 5 hours) increases striatin protein abundance in a MR-dependent manner. For *in vivo* study, the heart tissues were obtained from mice at 0, 1, 2, or 3 hours after intraperitoneal administration of aldosterone. Aldosterone treatment increased striatin protein abundances with a peak at 2 hours in mouse heart as compared with sham group (Pojoga 2012). In cultured human umbilical vein endothelial cells, aldosterone incubation for 48 hours induced cav-1 protein abundances (Igarashi 2013). There are no available data showing the nongenomic action of aldosterone on cav-1 protein expression *in vivo*.

At present, no data simultaneously demonstrate the effects of aldosterone on striatin and cav-1 in the rat kidney. Therefore, the present study aimed to investigate the effects of aldosterone on striatin and cav-1 protein abundances and localizations in rat kidney by Western blot analysis and immunohistochemistry, respectively. In addition, it was not clearly defined that the triple complex between striatin, cav-1, and MR is expressed in the kidney. These interactions were determined by immunoprecipitation and Western blot analysis.

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CHAPTER III
MATERIALS AND METHODS

Animal preparation

Male Wistar rats weighing 200-240 g (National Center of Scientific Use of Animals, Mahidol University, Nakornpathom, Thailand) were given conventional housing and diet. At the end of each experiment, the rats were terminated with an overdose of thiopental by intraperitoneal (ip.) injection. All animal protocols were approved by Animal care and use committee of Faculty of Medicine, Chulalongkorn University, and performed following the guideline of experimental animals announced by the National Research Council of Thailand.

Chemicals

Chemical agents

Aldosterone, eplerenone (Ep), dimenthyl sulfoxide (DMSO), protease inhibitor cocktail, 3,3'-diaminobenzidine (DAB) solution, Tris base, sodium dodecyl sulfate, 3-aminopropyltriethyloxy-silane, paraffin powder embedding medium, and Bradford protein assay kit were purchased from Sigma, MO, USA. Thiopental was obtained from Jagsonapal Pharmaceuticals Ltd., Haryana, India. Methanol, acetic acid, absolute ethanol, 95% ethanol, xylene, sodium chloride (NaCl), potassium chloride (KCl), di-sodium hydrogen phosphate (Na_2HPO_4), potassium di-hydrogen phosphate (KH_2PO_4), sodium phosphate monobasic monohydrate ($NaH_2PO_4H_2O$), 6% hydrogen peroxide, Tween-20, sucrose, and 40% formaldehyde were purchased from Merck, NJ, USA. Avidin-biotin peroxidase complex (ABC) solution, Prestained protein molecular marker were purchased from New England Biolabs, MA, USA. SuperSignal® West Pico Chemiluminescent, Laemmli sample buffer, ammonium persulfate, 40% acrylamide/Bis, Glycine, TEMED, filter paper and nitrocellulose membrane were purchased from Pierce, IL, USA.

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Antibodies

Monoclonal antibodies against striatin (Catalog No. sc-136084), monoclonal antibody against caveolin-1 (Catalog No. sc-53564), or against β-actin (Catalog No. sc-47778) were purchased from Santa Cruz Biotechnology, Inc., TX, USA. Monoclonal antibody against MR (Catalog No. ab2774) was purchased from Abcam, Cambridge, UK.

Experiment protocol

Rats were divided into three main groups (n=12/group) as follows:

Sham: Rats were received normal saline solution (NSS) (0.5 mL/kg BW) by intraperitoneal injection (i.p.) for 30 minutes (n=6) or 2 hours (n=6) (Pojoga 2012, Eiam-Ong 2013).

Aldo: Rats were received aldosterone 150 μg/kg BW; diluted in NSS; i.p. for 30 minutes (n=6) or 2 hours (n=6) (Sinphitukkul 2011, Pojoga 2012, Eiam-Ong 2013).

Ep.+Aldo: Rats were received eplerenone (mineralocorticoid receptor blocker) 15 mg/kg BW; diluted in DMSO; i.p. 30 minutes before aldosterone injection (30 minutes or 2 hours) (Cook 2003, Sinphitukkula 2019).

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The number of sample size is calculated by G*Power program analysis. G*Power is a statistical software used for the sample size calculation (Kim and Seo 2013). For sample size solving, the program should be set to the F family of test, to a one-way ANOVA, and to the 'A Priori' power analysis necessary to identify sample size (Figure G). The results showed that the total sample size of 36 is obtained from the achievement of 80% power with the probability of rejecting the null hypothesis when it is false. The analysis uses a cutoff for statistical significance of 0.05 and an effect size is expected to be 0.7. The number of group is 6 and the sample size is 6 per group (Figure G). The G*Power program is available at http://www.gpower.hhu.de/

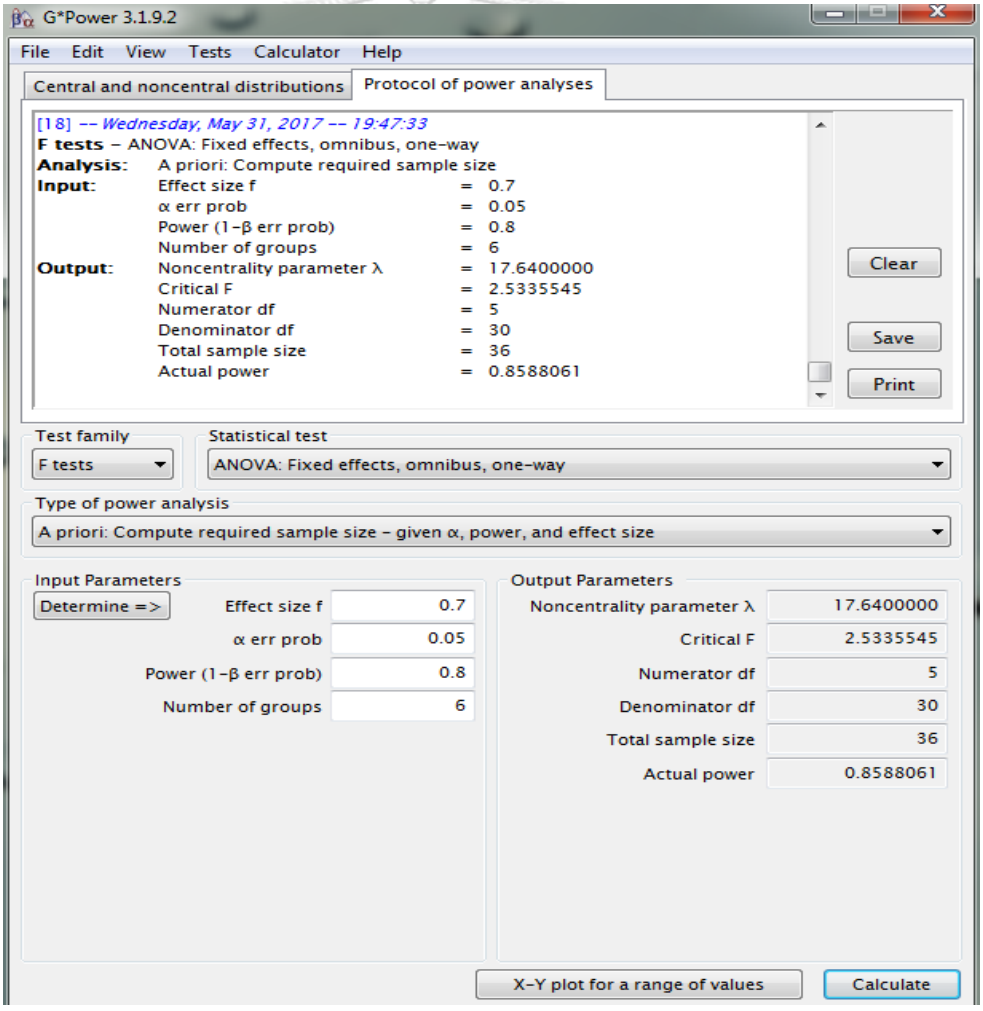

Figure G: The sample size analysis from G Power program

Surgical operation

On the date of the experiment, after a 30-minute and 2-hour injection period of NSS or aldosterone, the rats were anesthetized with thiopental (100 mg/kg BW, i.p.) and the abdomen were opened via midline incision. The kidneys were removed, and a half of each kidney was fixed in liquid nitrogen and then stored at -80°C until use for measurement of striatin and cav-1 protein abundances by Western blot and protein interactions by co-immunoprecipitation analysis. In addition, the other half of renal tissue was fixed in 10% paraformaldehyde overnight, subjected to tissue processing (dehydration, clearing and infiltration) by automatic tissue processor (Shandon Citadel 2000, Thermo Scientific, PA, USA) and embedded in paraffin for localization of striatin and cav-1 proteins by immunohistochemistry (Eiam-Ong 2017).

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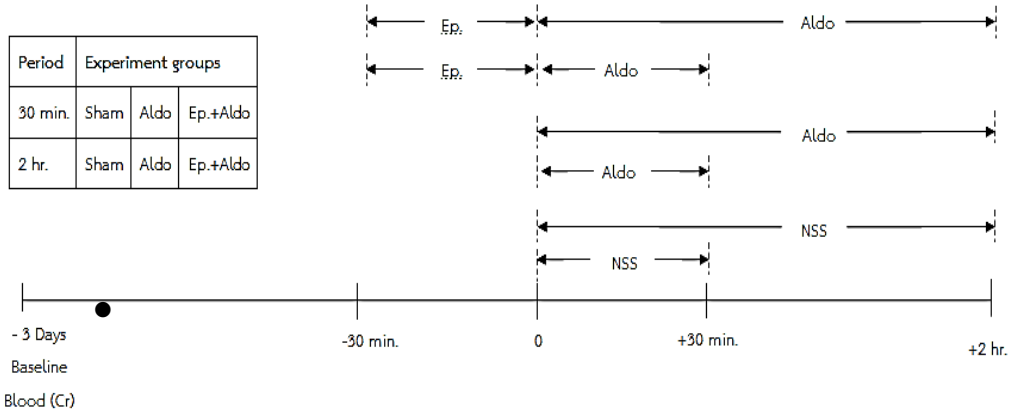
Experimental design

Period	Experiment groups		
30 min.	Sham	Aldo	Ep.+Aldo
2 hr.	Sham	Aldo	Ep.+Aldo



- Experiment due date: anesthetize with thiopental (100 mg/kg BW, i.p.)
- Renal tissues

: - Western blot

- protein abundances for striatin and cav-1

: - Immunoprecipitation + Western blot

- interaction of proteins for striatin/MR, cav-1/MR, and striatin/cav-1

: - Immunohistochemistry

- localizations and distributions for striatin and cav-1

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DETERMINATION OF PROTEIN CONCENTRATIONS BY BRADFORD METHOD

Protein extraction

The frozen kidney from cortex or medulla region was homogenized on ice with a homogenizer (IKA, T25 Basic, Selangor, Malaysia) in homogenizing buffer [20 mMTris-Hcl; pH 7.5, 2mM $MgCl_2$, 0.2 M sucrose, and 5% (v/v) protease inhibitor cocktail (Sigma)]. The homogenated tissue was centrifuged at 4,000×g (Biofuge PrimoR, Heracus, Germany) for 10 minutes at 4°C. The supernatant was collected as homogenated samples. For plasma membrane preparation, the homogenated sample was centrifuged at 17,000×g for 20 minutes at 4°C. Then the supernatant was discarded. The resulting pellets was resuspended in homogenizing buffer containing 2.5 mM $MgCl_2$ and 2.5 mM $CaCl_2$. Total protein concentrations of homogenated samples and membrane fractions were measured with Bradford protein assay kit (Sigma) (Sheikh 1982, Kruger 2009).

Protein assay

***Principle*:** Bradford protein assay is a simple and widely used for determining the protein quantitation (Bradford 1976). The method is based on the observation that the binding of the dye Brilliant Blue to protein. The absorbance maximum for the Brilliant Blue G shifts from 465 nm to 595 nm when binding to protein occurs by spectrophotometer (SP 3000 Plus, Optima, Tokyo, Japan). The protocol is performed by the sequential step as follow:

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Preparation of bovine serum albumin (BSA) standards

Five standard dilutions in duplicate (0, 50, 100, 200, and 500 µg/mL) are preferred by dissolving the stock standard BSA solution (2 mg/mL) as indicated in Table A. Prepare double distilled deionized water 180, 450, 250, 250, and 200 µL into tubes No. 1-5, respectively. Then add BSA stock (2 mg/mL) 60 µL into tube No.1, and 50 µL into tube No.2, mix each tube thoroughly. Then, add 250 µL of solution from

tube No.2 into tube No.3 and mix well. Finally, add 250 µL of solution from tube No.3 into tube No.4, then mix each tube thoroughly. There will be sufficient volume of each diluted standard for two replications.

Table A: Preparation of BSA standards

Tube Number	BSA (µg/mL)	BSA (µL)	DDW (µL)	Total volume (µL)
1	500	60	180	240
2	200	50	450	500
3	100	250	250	500
4	50	250	250	500
5	0	-	200	200

Note: DDW = Double distilled deionized water

Procedure of quantitate total protein

1. Add 30 µL each of standard solution and unknown protein sample replication into an appropriately labeled test tube.
2. Add 900 µL Bradford reagent to each tube and mix well. Incubate each tube at room temperature for exactly 20 minutes.
3. With the spectrophotometer set to 595 nm. Subsequently, measure and record the absorbance of all samples.
4. Plot standard curve from the absorbance of standard samples and determine protein concentration of unknown samples.

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DETERMINATION OF PROTEIN ABUNDANCES BY WESTERN BLOT ANALYSIS

Principle: Western blot also called immunoblotting is significant technique used for measured and detected abundance of protein using antibodies. The process concerns the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to separate various proteins from the gel and the transferred onto the nitrocellulose membrane. The membrane binds and immobilizes the proteins in the same pattern as in the original gel. The membrane is then incubated with a solution containing antibodies specific protein of interest. The unbound antibodies is removed thus only the bound antibodies to the protein of interest (Mahmood and Yang 2012). The antibodies bound to the membrane are detected by any of a varitety of techniques, usually involving treatment with a secondary antibody.

Separation of Protein by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Principle: The most commonly used method for qualitative analysis of a protein mixture is SDS-PAGE by using the buffer system of Laemmli (1970). The Laemmli sample buffer used polyacrylamide gels comprising a stacking gel component that helps focus the proteins into sharp bands at the beginning of the electrophoretic run. The resolving gel component that separate protein based on molecular size after sample proteins are solubilized by boiling at 65°C, 10 minutes in the presence of anionic detergent and 2-Mercaptoethanol (2-ME). The 2-ME functions as reducing agents to remove secondary and tertiary structure and thus allows separation of proteins by their molecular weight.

The binding efficiency of the SDS is generally one SDS molecule for every two amino acid residues. The negative charged conferred by SDS to polypeptide chains is proportional to their length, resulting in a constant charge to mass ratio. Then

proteins move through a polyacrylamide gel matrix toward the anode. The polyacrylamide gel is cast as a separating gel topped by a stacking gel and secured in an electrophoresis apparatus. In addition, the proteins could be separated by SDS-PAGE electrophoresis according to their molecular weight and the relative molecular mass can be estimated. Purity is determined by the presence of a band associated with the desired protein and the absence of bands associated with contaminating proteins. The SDS gel is comprised of a main separating gel and a stacking gel. The proteins, which have been mixed with a loading buffer, contaminating on ionizable tracking dye bromophenol blue, are loaded into wells formed in the stacking gel. A current is passed through the gels and the proteins migrate through the stacking gel and are concentrated into a solid band at the separating gel. When the proteins enter into the separating gel, the negatively charged protein-SDS complexes migrate toward the anode. Their migration in the gel relative to each other is the same based on their uniform negative charge. Separation, therefore, occurs as a result of the molecular sieving properties of the gel. The larger the protein, the more its mobility is retarded by the frictional resistance of the gel the smaller the molecule the further its mobility in the gel. The bromophenol blue is completely unretarded in the gel due to its small size relative to proteins and it is thus used to monitor the progress of the electrophoresis. The current is turned off once the tracking dye has migrated to bottom of the gel. The experimental protocol is performed by the sequential steps as follow:

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Preparation of slab gel

The casting clamps were used to mount the outer and inner glass plates facing together. For separating gel, 10% acrylamide was carefully filled into the space of sandwich plates from bottom to top with no air bubbles. The height of the gel was adjusted by the comb, approximately 1 cm below the bottom

edge. The top layer was filled with 1 ml of distilled water. Wait for 45-50 minutes at room temperature until the gel turning solidified. After polymerization, the water was drained off and excess liquid was removed with a piece of Whatman 3 MM paper. The selected comb (number of wells, thickness same as a spacers) was gently inserted, then 4% acrylamide solution was filled into the space for making the stacking gel. Insert the comb, ensuring that there are no air bubbles. The gel was allowed to polymerize for 30 minutes at room temperature.

Preparation of sample

During polymerizing the stacking gel, equal amounts of total protein from each sample were mixed with sample buffer. The amount of total protein used for striatin, cav-1, and MR was 100 µg.

Elecrophoresis

After polymerization was complete, the comb was gently removed. The wells were filled with running buffer (Appendix). Pour the running buffer into the chamber. Remove any air bubbles trapped at the bottom of the wells. Each protein sample was loaded into the wells with a pipette using gel loading tips. The molecular weight markers (New England Biolabs, USA) were also loaded. Then, the running buffer was poured into the lower chamber. The electrophoresis apparatus was attached to an electric power supply (PowerPac™ HC, Bio-Rad, USA) and turned on at 120 volt. Small bubbles should start to be produced and rise off the electrode wire at the bottom of the lower chamber. If no bubbles appear then there is an electrical circuit problem. The gel was run until the dye front reaches the bottom of gel. The power supply was turned off. Then, the gel from the glass plate was removed and placed into the transfer buffer.

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Protein transfer

Cut the nitrocellulose membrane and two sheets of absorbent filter paper to the same size of the gel. The gel was equilibrated and the membrane, filter papers, and fiber pads were soaked in the transfer buffer for 10 minutes. The transfer cassette was assembled by lying the black side down, then plate one fiber pad, two sheet of filter paper, gel, membrane, two sheet of filter paper, and one fiber pad. Use a glass tube which gently rolls air bubbles out. Close the cassettes firmly and put the complete transfer cassette into the transfer tank containing transfer buffer by facing the membrane side to the positive electrode (anode, red electrode) and transferred for 90 minutes at 110 volt. After transfer, the membrane was removed from the cassette and stained it with Ponceau S solution to check the transfer quality.

Blocking

The membrane was incubated in blocking solution (5% non fat dry milk in Tris buffer solution (TBS-T) (TBS + 0.1 % Tween) for 3 hours at room temperature will gentle rotation on platform shaker (Stuart Orbital Shaker S01, Manchester, UK). This process will reduce the background of non-specific binding site with irrelevant protein. After blocking, the membrane was washed for 10 minutes two times by TBS-T washing buffer.

Detection of bound antibody

Incubate membrane primary against to detect striatin, cav-1, and MR in concentrations of 1:500 dilution in 10 mL of blocking solution, and β-actin in concentrations of 1:2000 dilution in 10 mL of TBS-T with gentle agitation overnight at 4°C. After incubation, the primary antibody solution was discarded. The membrane was washed with 30 mL of TBS-T for 10 minutes three times. The secondary antibody was prepared in 5% non fat dry milk TBS-T. The goat anti mouse IgG conjugated HRP

antibody at 1:3000 dilution was used for striatin, cav-1, MR and β-actin. The membrane was incubated in the secondary antibody for 1 hour at room temperature on a platform shaker. After that, the blotting membrane was washed with TBS-T for 10 minutes three times on a platform shaker. Finally, it was washed with TBS for 5 minutes one time on a platform shaker.

Protein detection and image analysis

The blot membrane was placed on a cleaned glass plate. The detection reagent (Clarity™ Western ECL Blotting Substrates, Bio-Rad, USA) was prepared by mixing substrate kit components in a 1:1 ratio, and then overlay the reagent directly on the membrane surface carrying the protein. After incubation one minute at room temperature, the excess reagent was drained off, and wrapped by a piece of saran wrap. The protein was documented by using a Molecular imager ChemiDoc XRS system (Bio-Rad). Each intensity band was quantified by High Resolution UV and White Light Gel Doc System linked to a computer analysis system (Quantity One Version 4.2; Bio-Rad). The intensity ratio of each studied protein to β-actin was calculated.

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ISOLATION OF NATIVE PROTEIN COMPLEXES BY CO-IMMUNOPRECIPITATION

Principle: Co-immunoprecipitation (co-IP) is a common approach to study protein:protein interactions that uses an antibody to immunoprecipitate the antigen (bait protein) and co-immunoprecipitate any interacting proteins (prey proteins). Isolation of native protein complexes from a tissue lysate or other complexes mixture by directly immobilizing purified antibodies onto resin matrix supporter. Next, nonspecific protein bindings are washed and then antigen-specific antibody was eluted. Eluted sample was recovered by Laemmli sample buffer and analyzed by

SDS-PAGE. Finally, protein interaction was determined by Western blot analysis with specific antibody to interacting protein.

Procedure for co-IP by following the manufacturer's instruction

A. Antibody immobilization using striatin or cav-1 antibody

1. Equilibrate the AminoLink Plus Coupling Resin and reagents to room temperature.
2. Prepare 2 mL of 1X Coupling Buffer for each co-IP reaction by diluting the 20X Coupling Buffer with ultrapure water.
3. Gently swirl the bottle of AminoLink Plus Coupling Resin to obtain an even suspension. Using a wide-bore or cut pipette tip, add 50 µL of the resin slurry into a Pierce Spin Column. Place column into a microcentrifuge tube and centrifuge at 1000×g for 1 minute. Discard the flow-through.
4. Wash resin twice by adding 200 µL of 1X Coupling Buffer, centrifuge at 1000×g for 1 minute and discard the flow-through.
5. Gently tap the bottom of the spin column on a paper towel to remove any excess liquid and insert the bottom plug.
6. Prepare 10 µg of monoclonal anti striatin (6: sc-136084, Santa Cruz Biotechnology) or cav-1 antibody (7C8: sc-54564, Santa Cruz Biotechnology) for coupling by adjusting the volume to 200 µL, using sufficient ultrapure water and 20X Coupling Buffer to produce 1X Coupling Buffer. For example add 10 µL of 20X Coupling Buffer, 180 µL of ultrapure water and 10 µL of antibody at 1µg/1µL. Add the ultrapure water, 20X Coupling Buffer and affinity-purified antibody directly to the resin in the spin column.

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7. In a fume hood, add 3 µL of the Sodium Cyanoborohydride Solution for every 200 µL of reaction volume.
8. Attach the screw cap to the column and incubate on a rotator or mixer at room temperature for 90 minutes, ensuring that the slurry remains suspended during incubation.
9. Remove and retain the bottom plug and loosen the screw cap. Place the spin column into a collection tube and centrifuge at 1000×g for 1 minute. Save the flow-through to verify antibody coupling.
10. Remove the screw cap, add 200 µL of 1X Coupling Buffer, centrifuge at 1000×g for 1 minute and discard the flow-through. Repeat this step once.
11. Add 200 µL of Quenching Buffer to the column, centrifuge at 1000×g for 1 minute and discard the flow-through.
12. Tap the bottom of the column on a paper towel to remove excess liquid and insert the bottom plug. Add 200 µL of Quenching Buffer to the resin.
13. In a fume hood, add 3 µL of Sodium Cyanoborohydride Solution and attach the screw cap. Incubate for 15 minutes with gentle shaking or end-over-end mixing.
14. Remove plug and loosen the screw cap. Place spin column in a collection tube, centrifuge at 1000×g for 1 minute and discard the flow-through.
15. Remove screw cap, wash the resin twice with 200 µL of 1X Coupling Buffer, centrifuging at 1000×g for 1 minute after each wash.
16. Wash the resin six times with 150 µL of Wash Solution, centrifuging after each wash.

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B. Pre-clear lysate using the Control Agarose Resin

1. For 1 mg of lysate (plasma membrane protein), add 80 µL of the Control Agarose Resin slurry (40 µL of settled resin) into a spin column.
2. Centrifuge column at 1000×g for 1 minute to remove storage buffer.
3. Add 100 µL of 1X Coupling Buffer to the column, centrifuge at 1000×g for 1 minute and discard the flow-through.
4. Add 1 mg of plasma membrane protein sample to the column containing the resin and incubate at 4ºC for 45 minutes with gentle end-over-end mixing.
5. Centrifuge column at 1000×g for 1 minute. Discard the column containing the resin and save the flow-through, which was added to the immobilized antibody for the co-IP.

C. Co-IP

Perform all co-IP steps at 4ºC.

1. Prepare 1 mg of plasma membrane protein sample by diluting with 200 µL IP Lysis/Wash Buffer.
2. Wash the resin twice by adding 200 µL of IP Lysis/Wash Buffer to the spin column containing the antibody-coupled resin, centrifuge at 1000×g for 1 minute and discard the flow-through.
3. Gently tap the bottom of the spin column on a paper towel to remove excess liquid and insert the bottom plug.
4. Add the diluted plasma membrane protein into appropriate resin. Attach cap and incubate with gentle mixing or rocking for overnight at 4ºC.

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5. Remove the bottom plug, loosen the screw cap and place the column in a collection tube. Centrifuge the spin columns at 1000×g for 1 minute. Save the flow-through for future analysis.
6. Remove the screw cap, place the column into a new tube, add 200 µL of IP Lysis/Wash Buffer and centrifuge at 1000×g for 1 minute.
7. Wash the sample two more times with 200 µL IP Lysis/Wash Buffer and centrifuge at 1000×g for 1 minute after each wash.

D. Elution of Co-IP

1. Place the spin column into a new collection tube. Add 10 µL of Elution Buffer and centrifuge at 1000×g for 1 minute.
2. Keep the column in the tube and add 10 µL of Elution Buffer. Incubate for 5 minutes at room temperature. The column does not need to be closed or mixed. Repeat this step once.
3. Centrifuge the tube at 1000×g for 1 minute and collect the flow-through. Analyze the flow-through for protein. Perform additional elutions. Approximately 30 µL of eluted protein sample contains all precipitated proteins which can interact with striatin or cav-1. Total protein concentration was measured with Bradford protein assay reagent (Pierce) as former described. Protein concentration in each sample was in range of 5-10 µg/µL.

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Procedure of Western blotting for striatin/MR, cav-1/MR, or cav-1/straitin detection

1. In order to examine striatin/MR, cav-1/MR, or cav-1/striatin in eluted protein samples, 180 µg of each eluted sample was mixed with Laemmli sample buffer and loaded on 10% SDS-PAGE in each lane.
2. Protein sample in SDS-PAGE were transferred into a nitrocellulose membrane.
3. To identify interacting proteins, the membrane was probe with monoclonal anti striatin (6: sc-136084, Santa Cruz Biotechnology), cav-1 antibody (7C8: sc-54564, Santa Cruz Biotechnology) or MR (H10E4C9F: sc-53000, Santa Cruz Biotechnology) at 4ºC overnight.
4. The membranes were washed three times for 10 minutes wash with 15 mL of TBS-T.
5. The membranes were incubated with respective peroxidase-linked secondary antibody (1:3000) in 10 mL of TBS-T with gentle agitation for 1 hour at room temperature.
6. The membranes were washed three times for 10 minutes each with 15 mL of TBS-T.
7. After washing, the immunoreactive proteins were detected by an enhanced chemiluminescence detection system (SuperSignal West Pico kit, Pierce, USA) and documented by using a Molecular imager ChemiDoc XRS system (Bio-Rad, Laboratories).
8. The intensity bands were quantified by High Resolution UV and White Light Gel Doc System linked to a computer analysis system (Quantity One Version 4.2; Bio-Rad, Laboratories).

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EXAMINATION OF LOCALIZATION AND DISTRIBUTION OF STUDIED PROTEINS

Principle: Immunohistochemistry (IHC) is an important technique for the study of protein and other macromolecules in tissue and cell. The sample is detected to show label localization and distribution of protein in tissue section. Immunohistochemical staining is used antibodies to recognize the target protein. The antibodies will bind only to the protein of interest in the tissue section. After the antibody-antigen interaction, it is visualized by using either chromogenic detection, in which an enzyme conjugated to the antibody, cleaves a substrate to produce a colored precipitate at the location of the protein, and can be visualized using microscopy.

Immunohistochemistry for striatin and cav-1

Paraffin-embedded kidney sections were cut at 4-μm thickness in a serial section of three for examination of striatin and cav-1. Tissues sections were mounted on 3-aminopropyltriethyloxy-saline-coated slides (Sigma). The slides were deparaffinized in xylene and alcohol, with endogeneous peroxidase activity being quenched in 3% hydrogen peroxide for 10 minutes. The non-specific binding of antibody were blocked by incubating tissue sections with 5% normal horse serum (Vector, USA) in phosphate buffer solution (PBS-T) (PBS+0.1% Tween) for 30 minutes at room temperature. Then, the section were incubated in 100 μl primary antibody against striatin (1:1000) or cav-1 (1:1000) for over one hour at room temperature.

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The sections were then rinsed 3 × 10 minutes with PBS-T and incubated with 100 μL biotinylated goat anti-mouse-rabbit immunoglobin (Vector, USA) diluted 1:400 in PBS-T for 60 minutes at room temperature. After incubation, tissues sections were rinsed 2 ×10 minutes with PBS-T and the 1 × 10 minutes in PBS. The tissue sections were reacted with ABC-streptavidin horseradish peroxidase complex (Vector, USA) for 60 minutes at room temperature. The sections were then

rinsed 2 × 10 minutes in PBS and 10 minutes in 0.05 M Tris-HCl buffer (pH 7.6). The sections were reacted for peroxiadative activity in 3, 3'-diaminobenzidine (DAB) solution (Sigma, USA) for 10 minutes. Then, the sections were washed 2 × 5 minutes with distilled water, counterstained with haematoxylin (CV Laboratories, Thailand) and coverslipped with permount.

Areas of staining were indentified and semi-quantitative scored by three pathologists in a blinded manner. The intensity of staining was scored from 0 to 4 (0 = no staining, 1 = trace, 2 = weak, 3 = moderate, 4=strong) (Fujigaki 2007, Sinphitukkul 2011, Eiam-Ong 2013).

Statistical analyses

Results of renal striatin and cav-1 protein abundances were expressed as mean ± SD. Statistical differences between the groups were assessed by one-way ANOVA (analysis of variance) with post-hoc comparison by Tukey's test where appropriate. A p value < 0.05 was considered statistically significant. Statistical tests will be analyzed using SPSS program version 16.0 (SPSS Inc., Chicago, IL, USA). The median staining intensity (score) of renal striatin and cav-1 will be presented as previously described (Eiam-Ong 2017).

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CHAPTER IV
RESULTS

Effect of aldosterone on protein abundance of striatin and caveolin-1

By Western blot analysis (Figure 1A), the protein levels of striatin (110 kDa) and cav-1 (22 kDa) were assessed. The results from 30 minute-group showed that aldosterone significantly elevated protein abundances of striatin and cav-1 from sham (100%) to 149 $\pm$ 9% ($p<0.05$) and 211 $\pm$ 10% ($p<0.001$), respectively. Eplerenone had no significant effect on striatin levels induced by aldosterone (140 $\pm$ 7%, $p<0.05$ vs. sham, $p = 1.00$ vs. Aldo). The abundance of cav-1 protein induced by aldosterone was partially blocked to 149 $\pm$ 8% ($p<0.05$ vs. sham, and vs. Aldo).

For 2 hour-group (Figure 1B), aldosterone had no significant changes on protein abundance of striatin (sham = 100%; Aldo = 97 $\pm$ 2%, $p = 1.00$) and cav-1 (sham = 100%; Aldo = 109 $\pm$ 1%, $p = 0.2$). Interestingly, pretreatment with eplerenone significantly suppressed cav-1 protein levels (68 $\pm$ 3%, $p<0.001$ vs. sham, and vs. Aldo), whereas striatin levels maintained (92 $\pm$ 4%, $p = 0.5$ vs. sham, and vs. Aldo).

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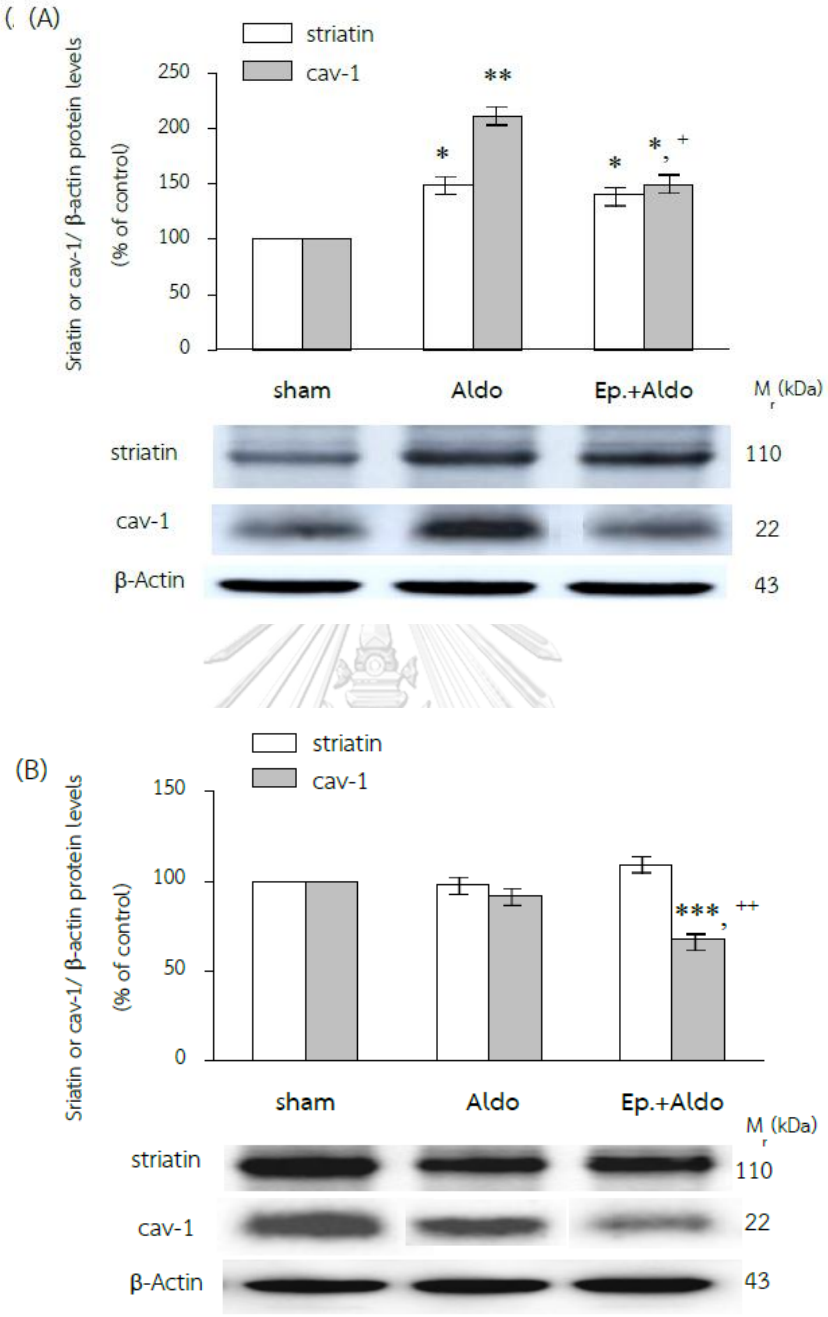

Figure 1. Western blot analysis of renal striatin and cav-1 protein abundance in sham, Aldo, and Ep.+Aldo groups. Histogram bars show densitometric analyses ratios of striatin and cav-1 to β-actin intensity, and the representative immunoblot photographs are present from 30-minute (A) and 2-hour (B) groups, respectively. Data are means±SD of 6 independent experiments. *p< 0.05, **p<0.01 vs. sham, $^{+}$p<0.05 vs. Aldo, ***p<0.001 vs. sham, $^{++}$p<0.001 vs. Aldo

Effect of aldosterone on protein localization of striatin

For 30-minute group, the protein localization of striatin in the cortex in sham group is demonstrated in Figure 2B and Table 1. The immunoreactivity was trace in the glomerulus (GL), proximal convoluted tubule (PCT), distal convoluted tubule (DCT), and peritubular capillary (Pcap). The staining score was weak the cortical collecting duct (CCD). Aldosterone increased the intensity score in the GL, CCD, and Pcap to moderate level (Figure 2C), whereas the immunoreactivity in the PCT and DCT was weak. Eplerenone could minimize the intensity score induced by aldosterone only in the GL and CCD (Figure 2D).

In the outer medulla (OM), aldosterone elevated the intensity scores from 3 to 4 in the vasa recta (VR) (Figure 2G and Table 1). The staining in the thick ascending limb of the loop of Henle (TALH), medullary CD (MCD), and thin limb of LH (tLH) did not change. Surprisingly, staining intensity scores in all studied areas were increased although pretreatment with eplerenone was applied (Figure 2H).

In the inner medulla (IM), immunoreactivity was enhanced by aldosterone to strong levels in the MCD and VR, whereas the intensity score in the tLH increased to be moderate (Figure 2K). Eplerenone had no inhibitory effect on immunoreactivity induced by aldosterone in all studies areas (Figure 2L).

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For 2 hour-group, in the cortex, the striatin protein staining score was 1 in the GL, PCT, DCT, and Pcap whereas the score was 2 in the CCD of sham group (Figure 3B and Table1). After aldosterone injection, the staining showed weak levels in GL and Pcap, whereas the immunoreactivity in the CCD was trace. The staining in PCT and DCT did not change (Figure 3C). However, pretreatment with eplerenone enhanced the intensity score in GL, Pcap, and CCD. Staining intensity scores in PCT and DCT remained (Figure 3D).

In the OM, aldosterone induced striatin protein levels in all studied areas (Figure 3G). Interestingly, eplerenone decreased striatin intensity scores only in the VR and tLH (Figure 3H).

In the IM, aldosterone increased staining intensity score from 2 to 3 in the MCD, VR, and tLH (Figure 3J, 3K). Immunoreactivity was declined by eplerenone treatment (Figure 3L).

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Table 1. Median staining intensity (score) of renal striatin protein localization

	Median staining intensity (score)					
	30 minutes			2 hours		
	sham	Aldo	Ep.+Aldo	sham	Aldo	Ep.+Aldo
Cortex						
GL	1	3	2	1	2	4
PCT	1	2	2	1	1	1
DCT	1	2	2	1	1	1
CCD	2	3	2	2	1	3
Pcap	1	3	3	1	2	3
Outer medulla						
TALH	2	2	3	1	2	2
MCD	2	2	4	1	2	2
VR	3	4	4	2	4	3
tLH	3	3	4	2	3	2
Inner medulla						
MCD	3	4	4	2	3	1
VR	3	4	4	2	3	2
tLH	2	3	4	2	3	2

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Staining intensity: 0 = negative, no reactivity; 1 = trace, faint or pale brown staining with less membrane reactivity; 2 = weak, light brown staining with incomplete membrane reactivity; 3 = moderate, shaded of brown staining of intermediate darkness with usually almost complete membrane reactivity; 4 = strong, dark brown to black staining with usually complete membrane pattern, producing a thick outline of the cell (Eiam-Ong 2017).

PCT = proximal convoluted tubule; DCT = distal convoluted tubule; CCD = cortical collecting duct; Pcap = peritubular capillary; TALH = thick ascending limb of the loop of Henle; MCD = medullary collecting duct; VR = vasa recta; tLH = thin limb of the loop of Henle (n = 5/ group).

Cortex

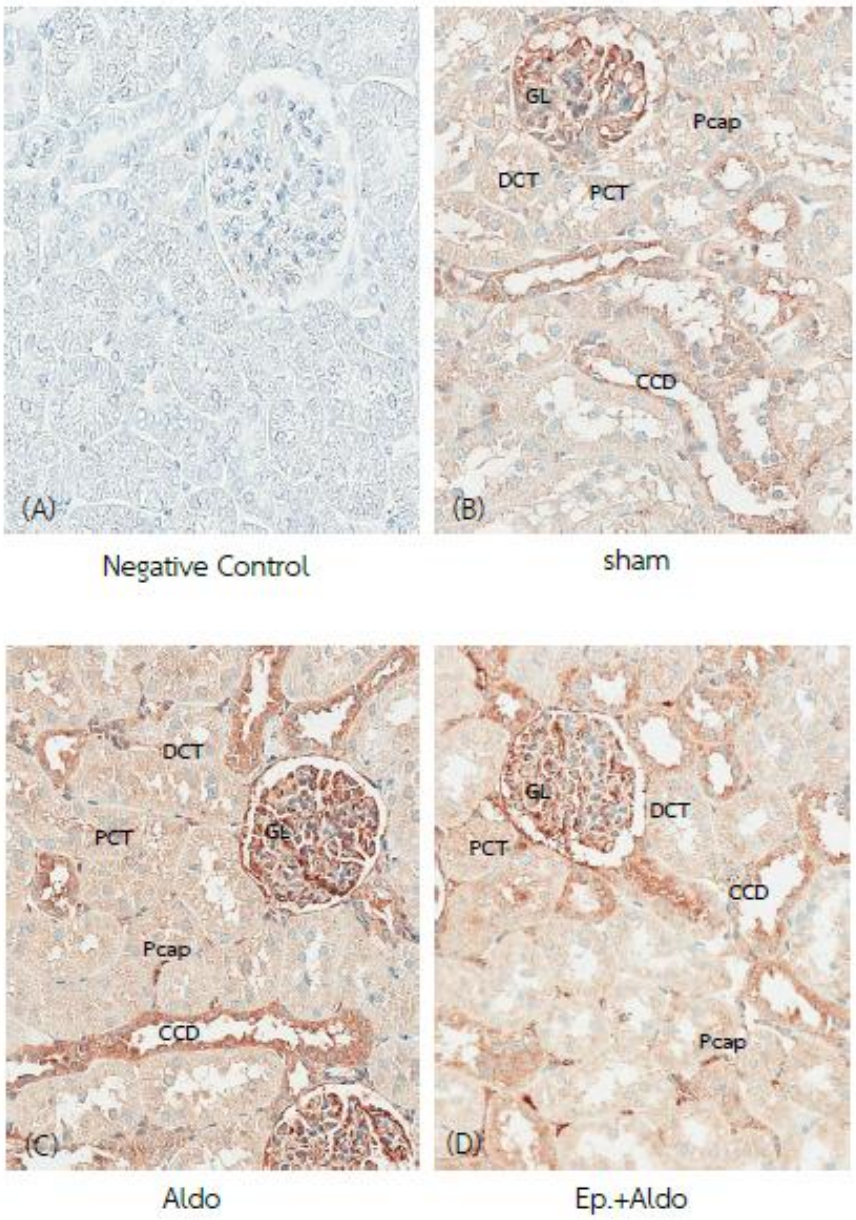

Figure 2: Effects of aldosterone (30 min.) on renal striatin protein localization. Representative immunohistochemical staining micrographs of renal striatin protein in the cortex (A-D), from sham (B), Aldo (C), and Ep.+Aldo (D) (*n* = 5/group). Negative controls: A, Original magnification: A-D, x400.

Outer medulla

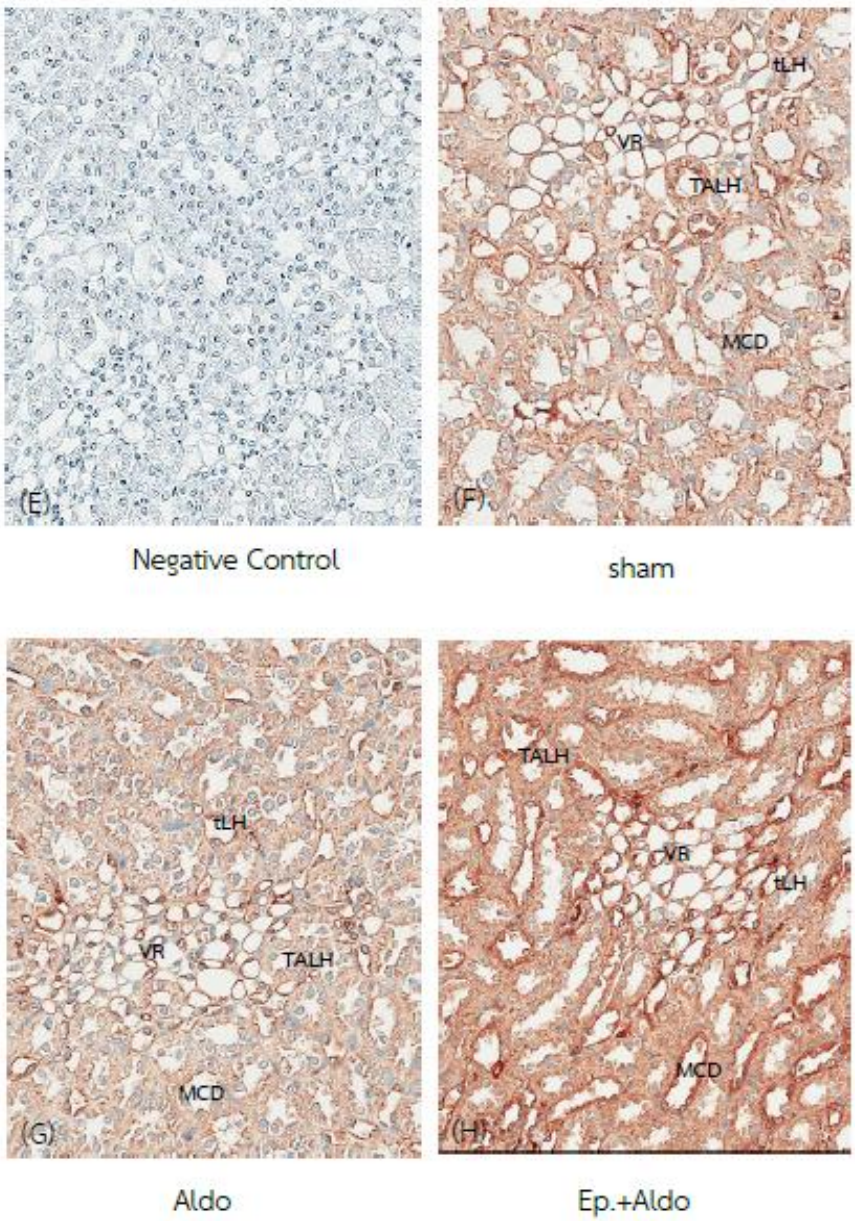

Figure 2. (cont.) Effects of aldosterone (30 minutes) on renal striatin protein localization. Representative immunohistochemical staining micrographs of renal striatin protein in the outer medulla (E-H), from sham (F), Aldo (G), and Ep.+Aldo (H) (n = 5/group). Negative controls: E, Original magnification: E-H, x200.

Inner medulla

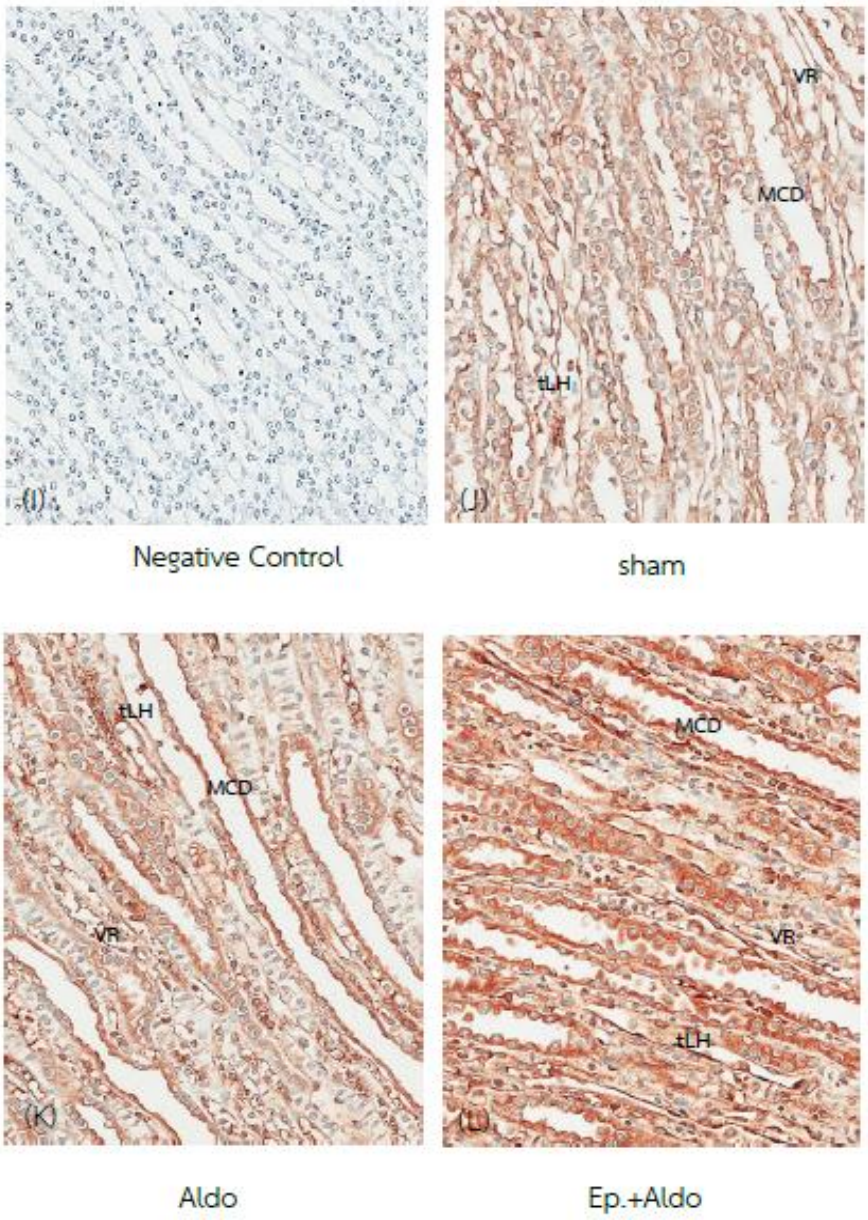

Figure 2. (cont.) Effects of aldosterone (30 minutes) on renal striatin protein localization. Representative immunohistochemical staining micrographs of renal striatin protein in the inner medulla (I-L), from sham (J), Aldo (K), and Ep.+Aldo (L) (n = 5/group). Negative controls: I, Original magnification: I-L, x200.

Cortex

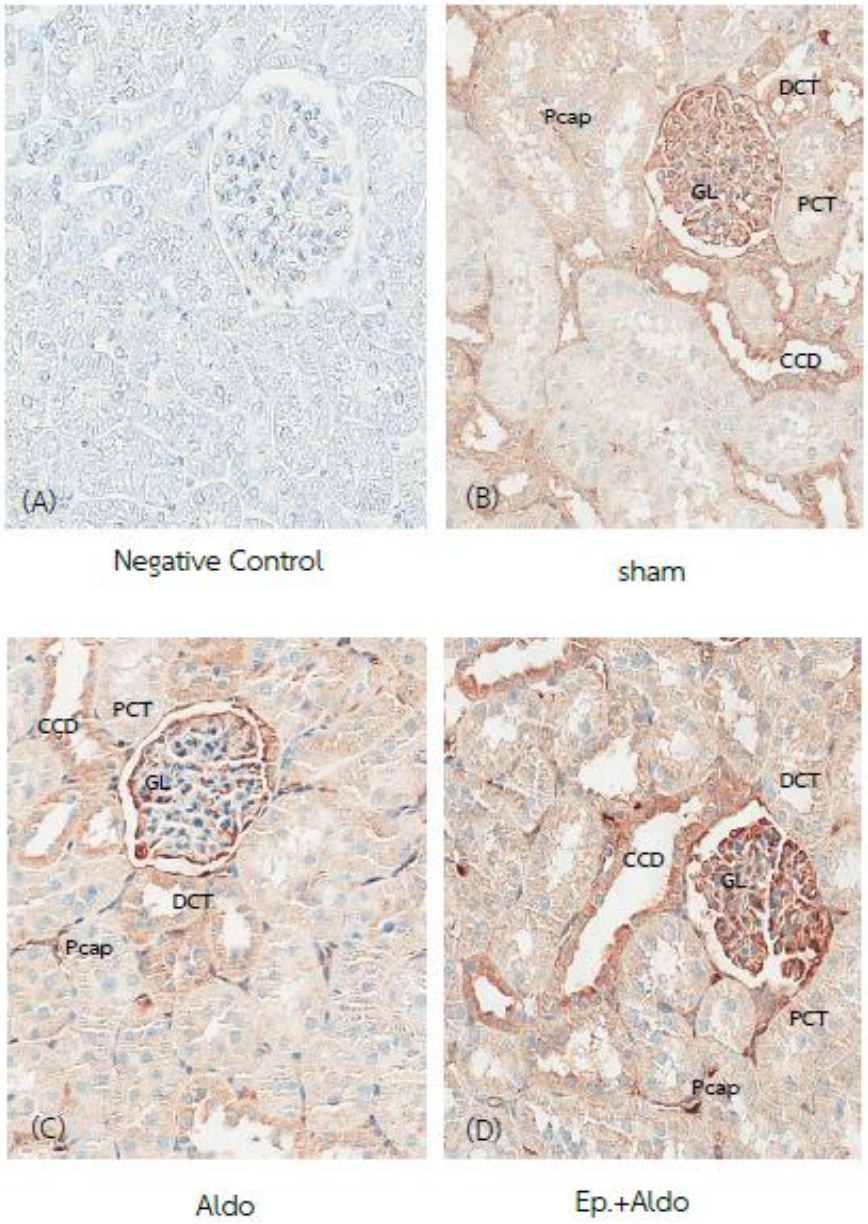

Figure 3. Effects of aldosterone (2 hours) on renal striatin protein localization. Representative immunohistochemical staining micrographs of renal striatin protein in the cortex (A-D), from sham (B), Aldo (C), and Ep.+Aldo (D) (n = 5/group). Negative controls: A, Original magnification: A-D, x400.

Outer medulla

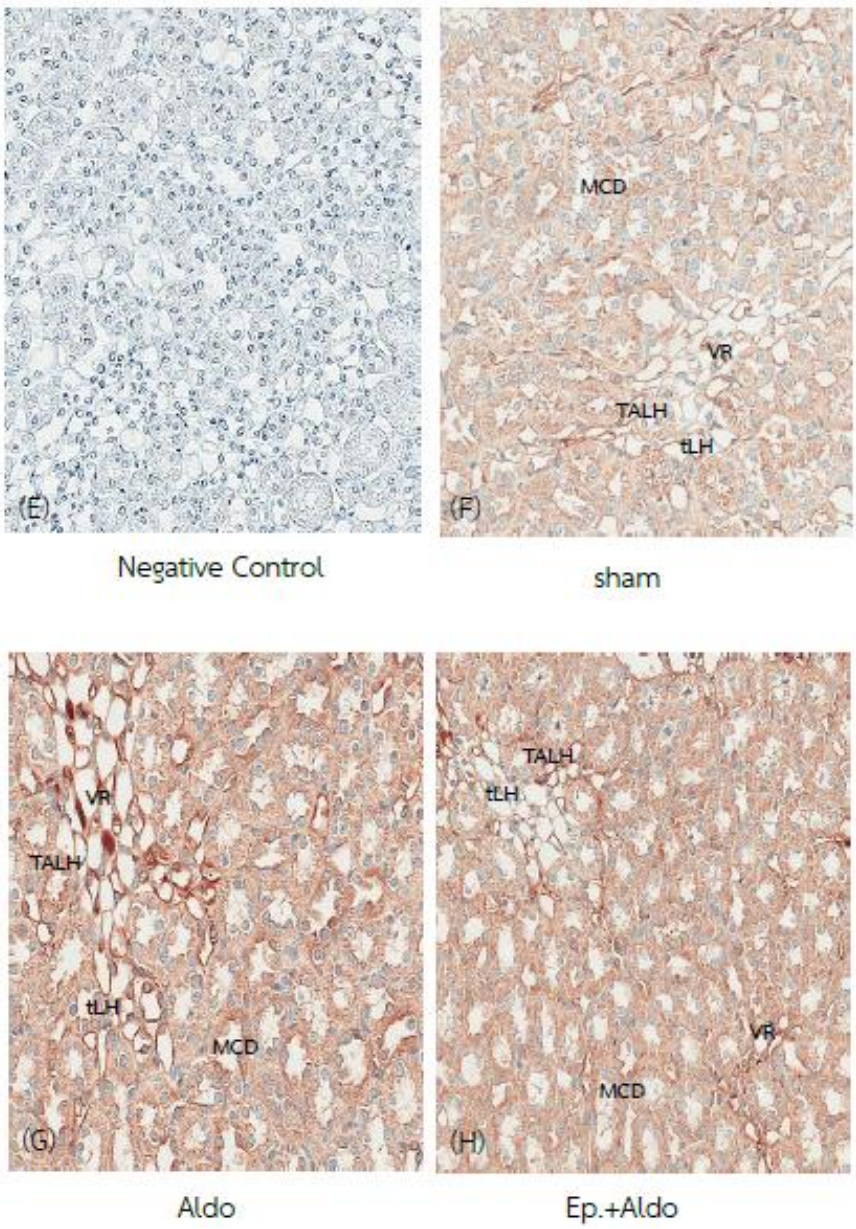

Figure 3. (cont.) Effects of aldosterone (2 hours) on renal striatin protein localization. Representative immunohistochemical staining micrographs of renal striatin protein in the outer medulla (E-H), from sham (F), Aldo (G), and Ep.+Aldo (H) (n = 5/group). Negative controls: E, Original magnification: E-H, x200.

Inner medulla

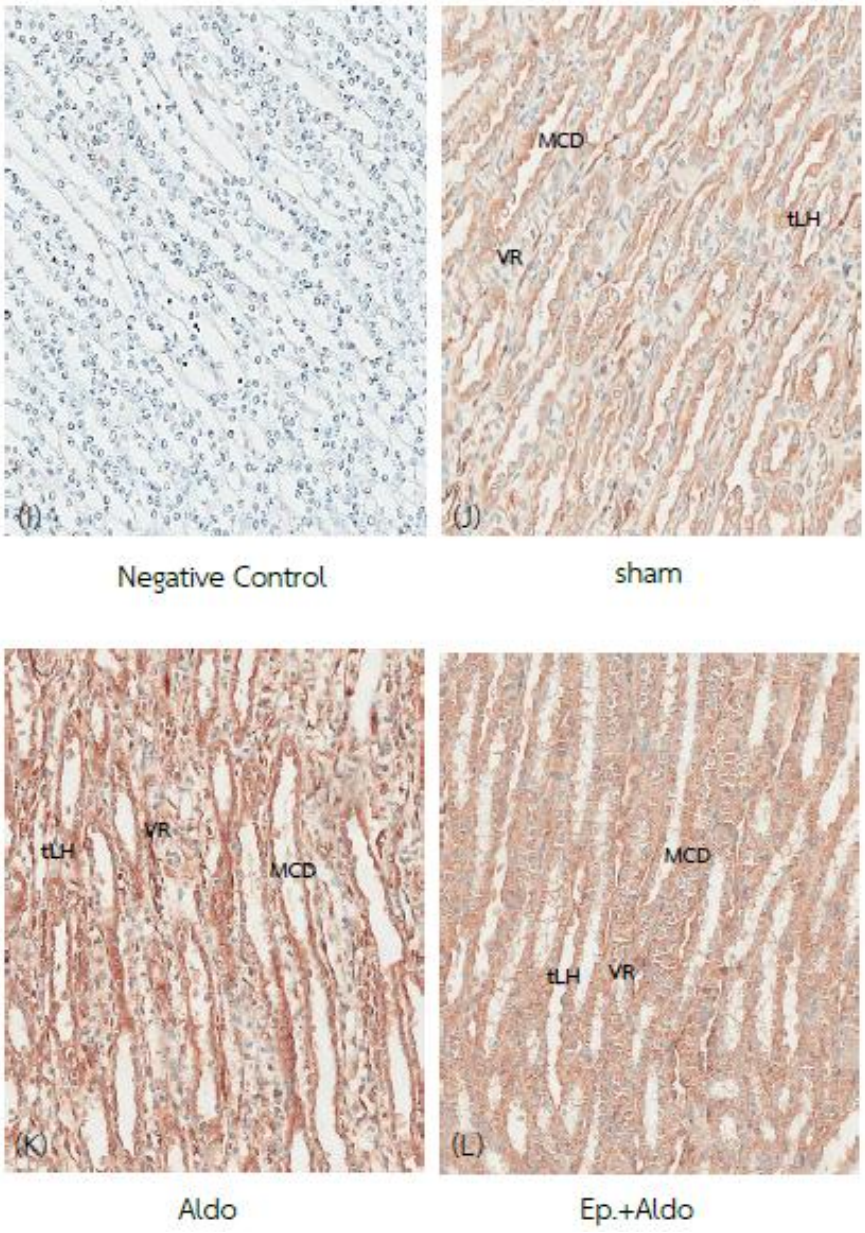

Figure 3. (cont.) Effects of aldosterone (2 hours) on renal striatin protein localization. Representative immunohistochemical staining micrographs of renal striatin protein in the inner medulla (I-L), from sham (J), Aldo (K), and Ep.+Aldo (L) (n = 5/group). Negative controls: I, Original magnification: I-L, x200.

Effect of aldosterone on protein localization of caveolin-1

For 30-minute group, the protein localization of cav-1 in the cortex in the sham group is shown in Figure 4B and Table 2. The immunoreactivity was trace in the GL and moderate in the Pcap, but no staining was noted in the PCT, DCT, and CCD. Aldosterone increased immunoreactivity in the glomerulus and Pcap to be strong, whereas immunostaining in the PCT, DCT, and CCD was trace level (Figure 4C). Eplerenone reduced the staining score only in the glomerulus to be moderate (Figure 3D).

In the OM, aldosterone elevated the intensity score to be weak in the TALH, MCD, and tLH (Figure 4G). These changes could be inhibited by pretreatment with eplerenone (Figure 4H). The intensity score in the VR remained strong level in all groups (Figure 4G, 4H). In the IM, aldosterone increased the immunoreactivity to be strong level in the VR but decreased to be trace level in the tLH (Figure 4K). Eplerenone had no inhibitory effect on immunoreactivity induced by aldosterone in the VR (Figure 4L). By contrast, staining intensity score in the tLH was increased although pretreatment with eplerenone was provided (Figure 4L). The intensity score in the MCD remained trace level in all groups (Figure 4K, 4L).

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For 2-hour group, the cav-1 protein staining in the sham group was 2 in the glomerulus and was 3 in the Pcap. There was no staining in PCT, DCT, and CCD (Figure 5B and Table 2). Aldosterone increased the intensity scores in all studied areas (Figure 5C). Eplerenone normalized intensity scores to similar levels as sham group (Figure 5D).

In the OM, the sham group showed trace staining in TALH, MCD and tLH, whereas the score was strong in the VR (Figure 5F). Aldosterone had no effect in the TALH, VR and tLH but slightly suppressed the immunoreactivity in the MCD (Figure 5G). In the IM, aldosterone increased immunostaining in the MCD and VR (Figure 5K). Eplerenone diminished the score in the MCD but had no effect on immunoreactivity

levels induced by aldosterone in VR (Figure 5L). No siginificant alteration of immunoreactivity in the tLH was noted. The scores remained at trace level (Figure 5L).

Table 2. Median staining intensity (score) of renal caveolin-1 protein localization

	Median staining intensity (score)					
	30 minutes			2 hours		
	sham	Aldo	Ep.+Aldo	sham	Aldo	Ep.+Aldo
Cortex						
GL	1	4	3	2	3	2
PCT	0	1	1	0	1	0
DCT	0	1	1	0	1	0
CCD	0	1	1	0	1	0
Pcap	3	4	4	3	4	3
Outer medulla						
TALH	1	2	1	1	1	0
MCD	1	2	1	1	0	0
VR	4	4	4	4	4	3
tLH	0	2	1	1	1	1
Inner medulla						
MCD	1	1	1	0	1	0
VR	2	4	4	2	3	3
tLH	2	1	2	1	1	1

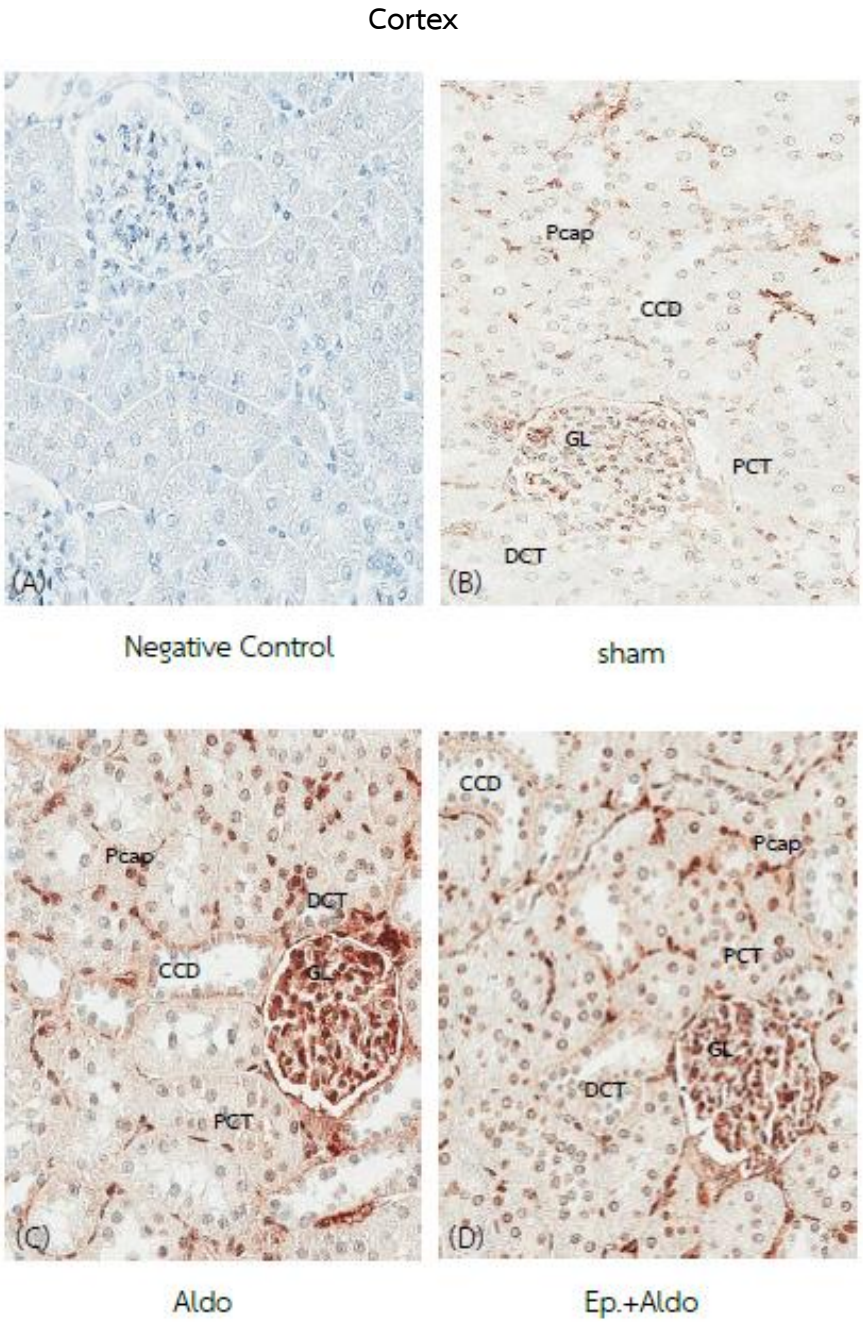

Figure 4. Effects of aldosterone (30 minutes) on renal cav-1 protein localization. Representative immunohistochemical staining micrographs of renal cav-1 protein in the cortex (A-D), from sham (B), Aldo (C), and Ep.+Aldo (D) (n = 5/group). Negative controls: A, Original magnification: A-D, x400.

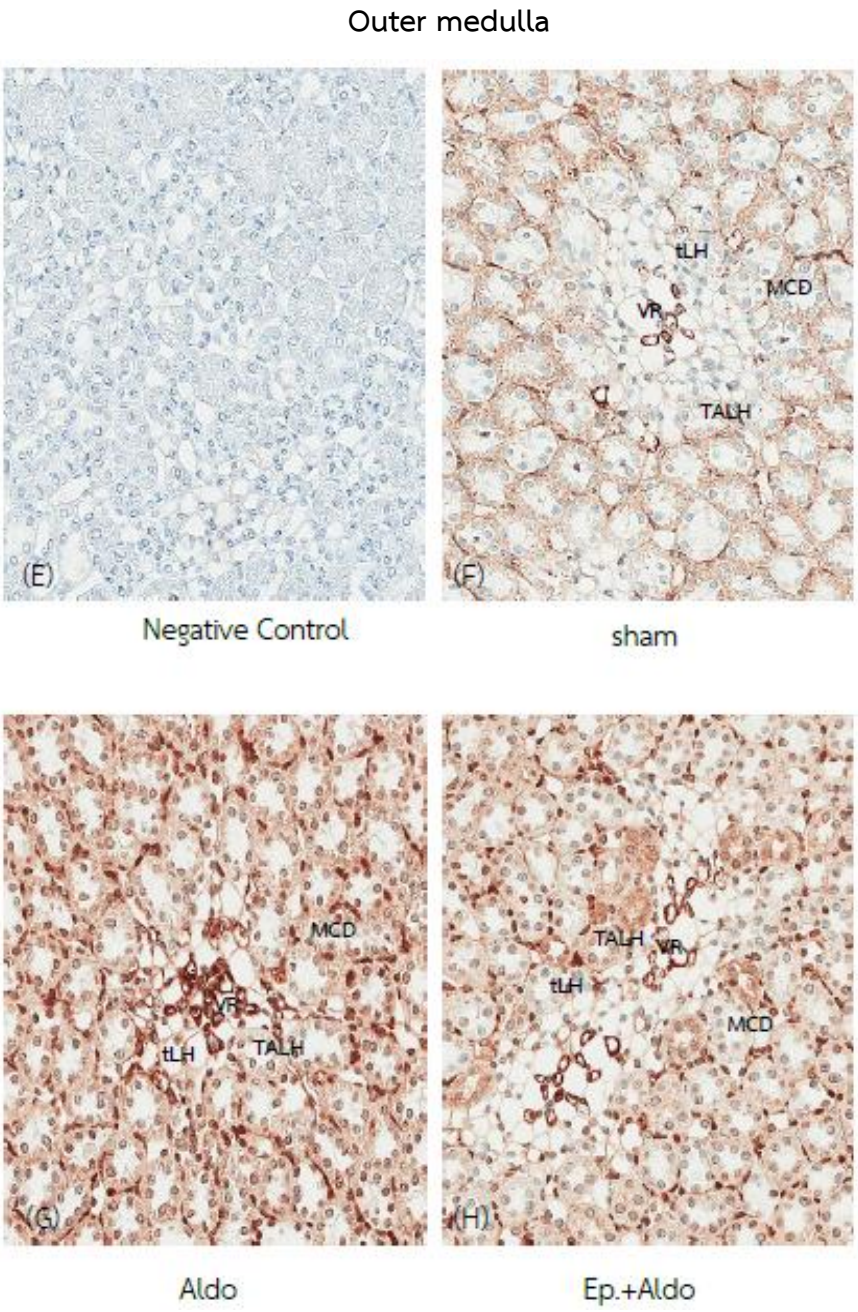

Figure 4. (cont.) Effects of aldosterone (30 minutes) on renal cav-1 protein localization. Representative immunohistochemical staining micrographs of renal cav-1 protein in the outer medulla (E-H), from sham (F), Aldo (G), and Ep.+Aldo (H) (n = 5/group). Negative controls: E, Original magnification: E-H, x200.

Inner medulla

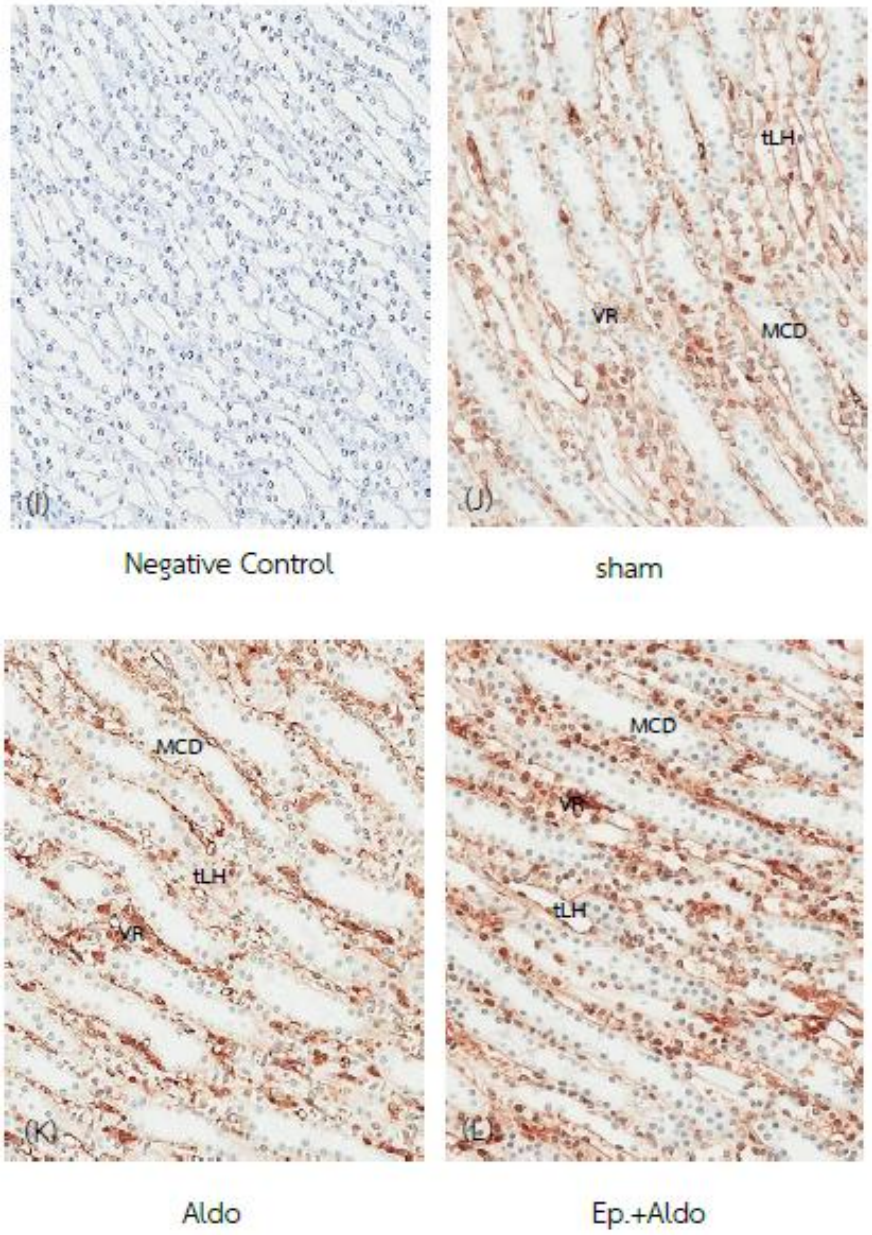

Figure 4. (cont.) Effects of aldosterone (30 minutes) on renal cav-1 protein localization. Representative immunohistochemical staining micrographs of renal cav-1 protein in the inner medulla (I-L), from sham (J), Aldo (K), and Ep.+Aldo (L) (n = 5/group). Negative controls: I, Original magnification: I-L, x200.

Cortex

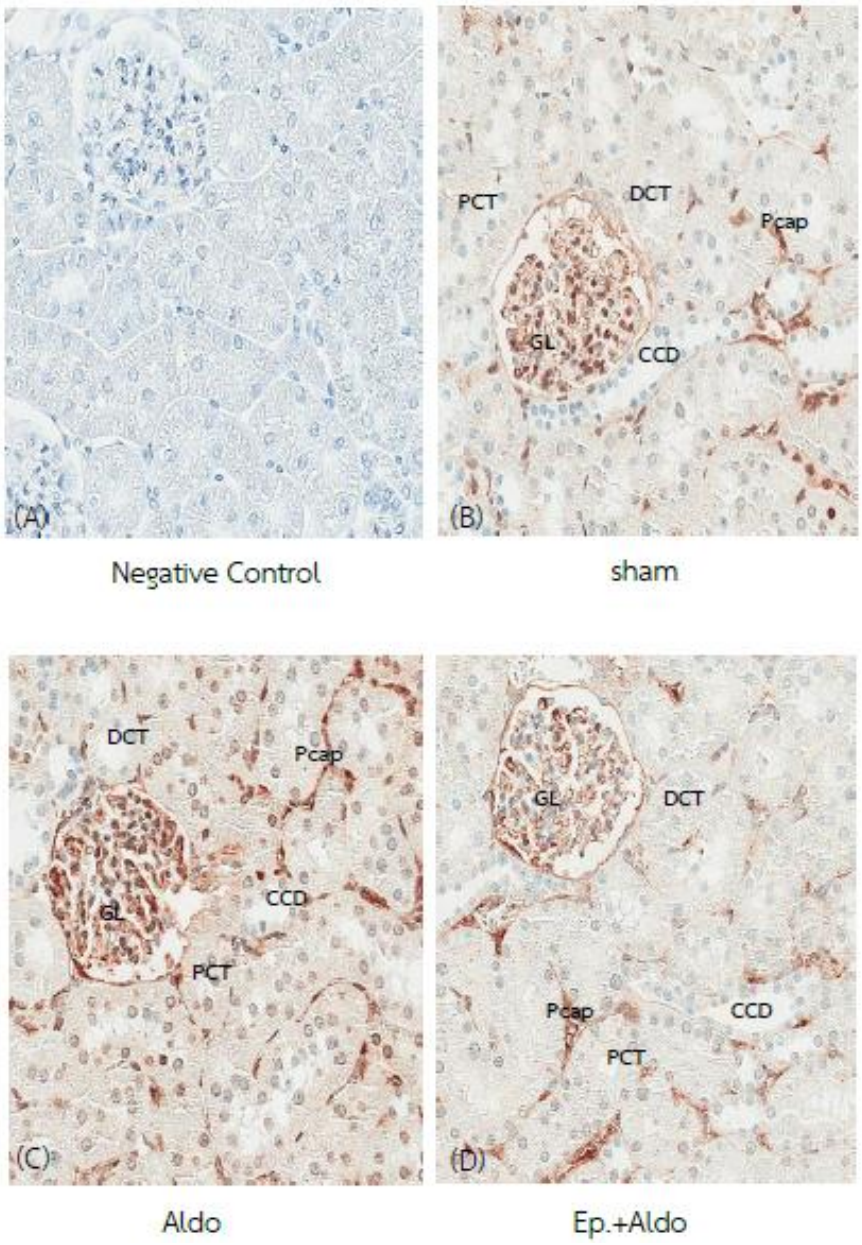

Figure 5. Effects of aldosterone (2 hours) on renal cav-1 protein localization. Representative immunohistochemical staining micrographs of renal cav-1 protein in the cortex (A-D), from sham (B), Aldo (C), and Ep.+Aldo (D) (n = 5/group). Negative controls: A, Original magnification: A-D, x400.

Outer medulla

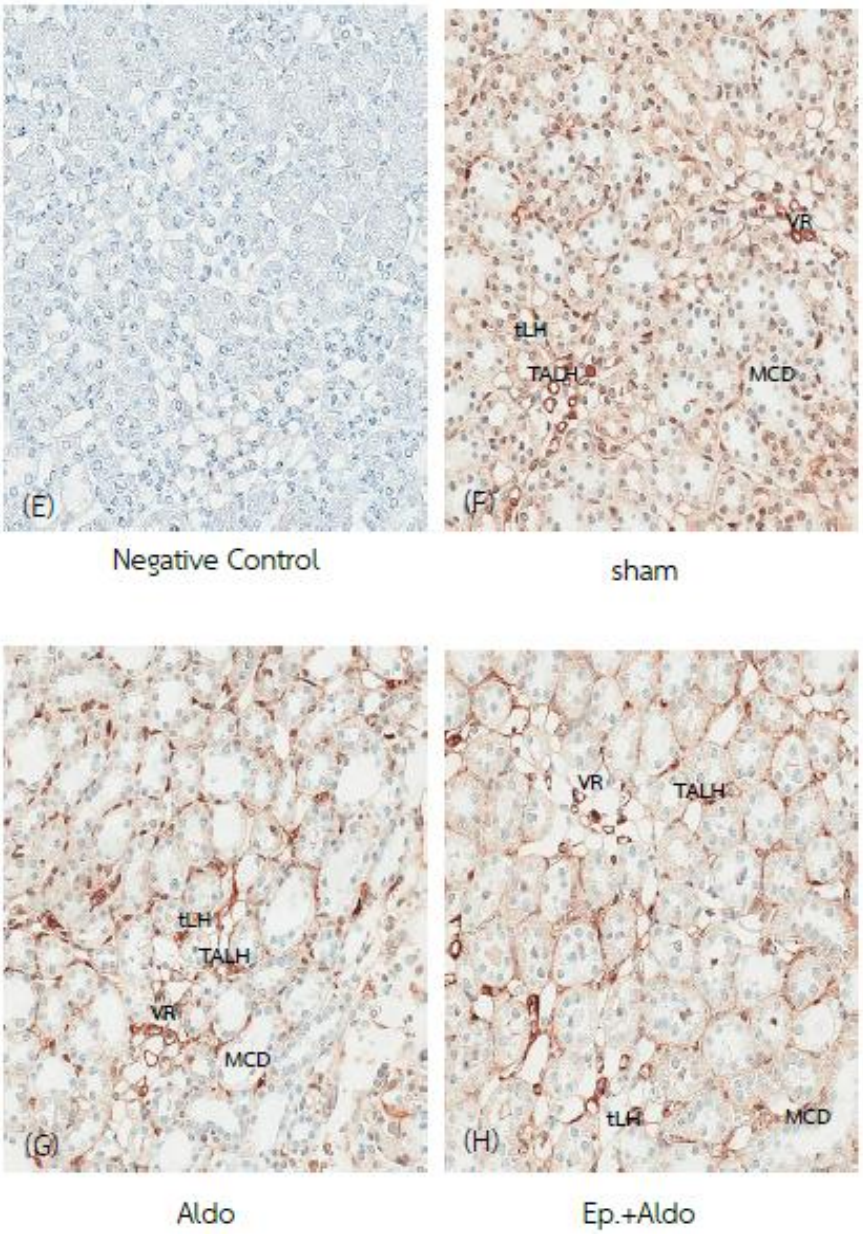

Figure 5. (cont.) Effects of aldosterone (2 hours) on renal cav-1 protein localization. Representative immunohistochemical staining micrographs of renal cav-1 protein in the outer medulla (E-H), from sham (F), Aldo (G), and Ep.+Aldo (H) (n = 5/group). Negative controls: E, Original magnification: E-H, x200.

Inner medulla

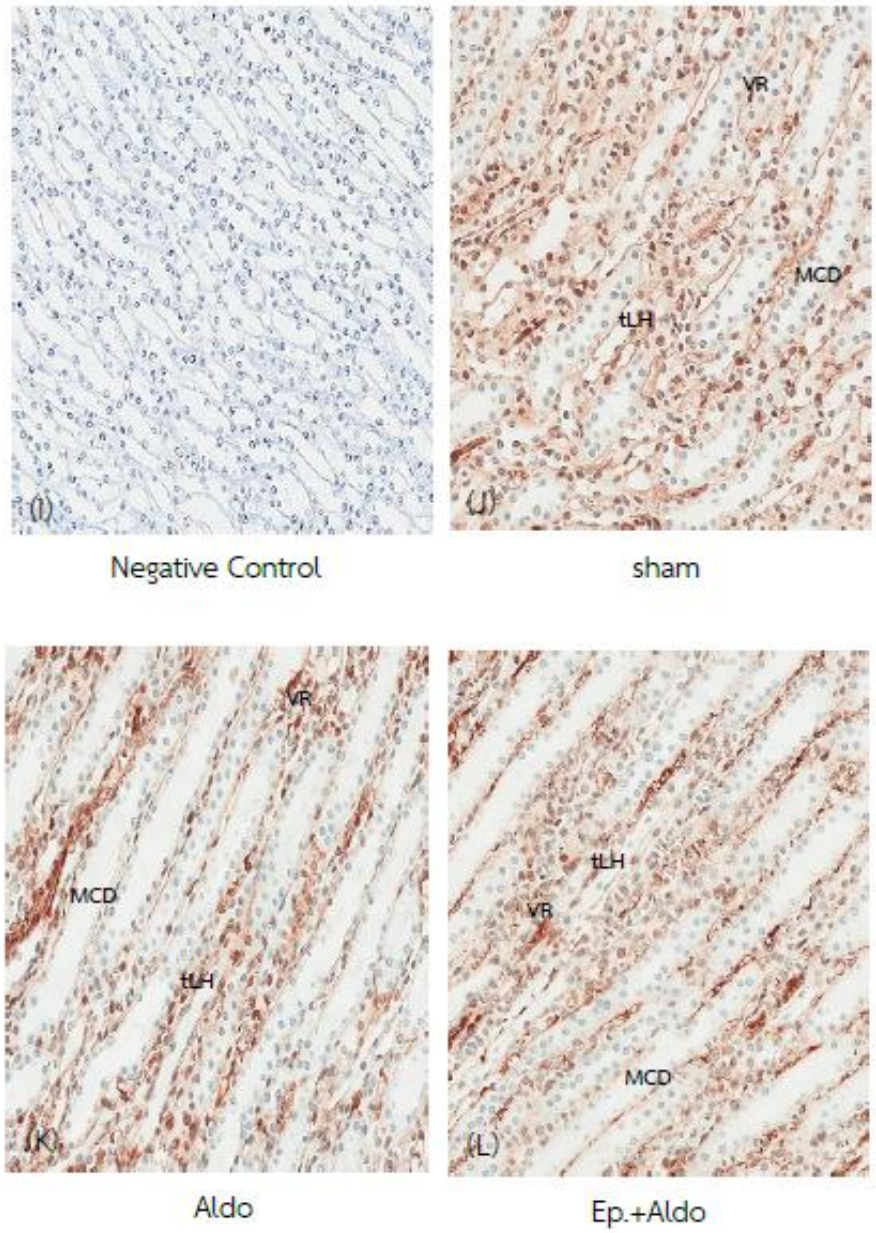

Figure 5. (cont.) Effects of aldosterone (2 hours) on renal cav-1 protein localization. Representative immunohistochemical staining micrographs of renal cav-1 protein in the inner medulla (I-L), from sham (J), Aldo (K), and Ep.+Aldo (L) (n = 5/group). Negative controls: I, Original magnification: I-L, x200.

Protein interaction of striatin/MR, cav-1/MR, or cav-1/striatin

To examine protein interactions of striatin/MR, cav-1/MR, and cav-1/striatin co-immunoprecipitation and Western blotting were applied. As shown in (Figure 6A, 6B, 6D, 6E), either striatin or cav-1 was precipitated, MR proteins were detected from eluted samples both 30 minutes and 2 hours. These results demonstrated that MR interacts with striatin or cav-1. In addition, striatin protein was able to interact with cav-1 protein as well (Figure 6C, 6F).

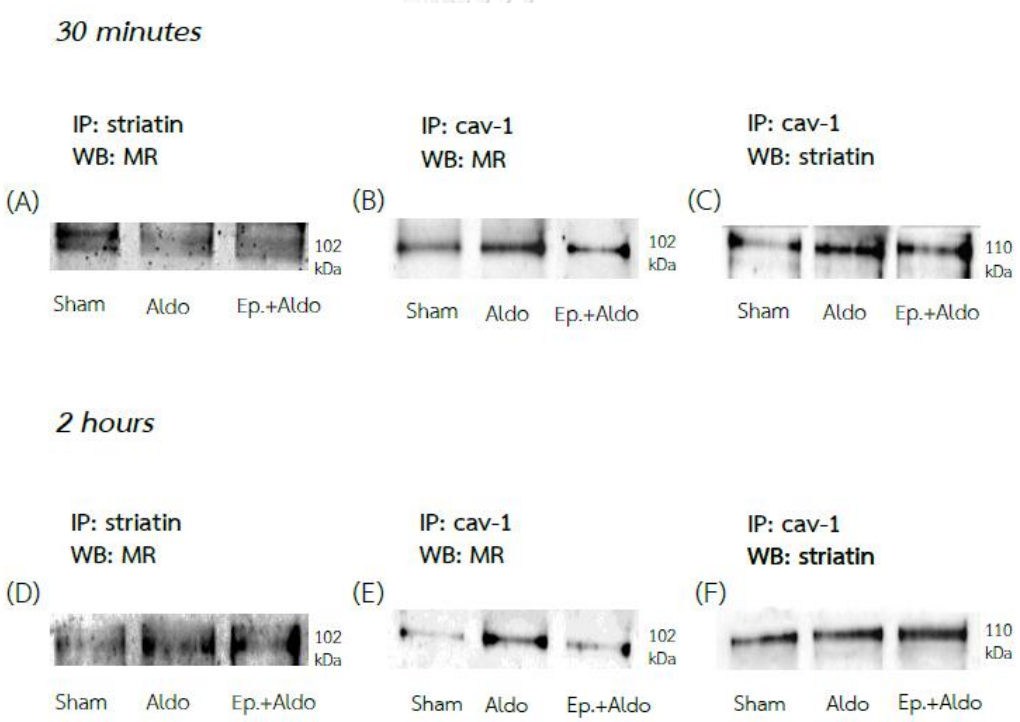

Figure 6. Representative Western blot analysis of protein abundance in sham, Aldo, and Ep.+Aldo groups for 30 minutes (A-C) or 2 hours (D-F). After striatin immunoprecipitated, MR protein was detected by Western blot analysis in 30-minute group (A) and 2-hour group (D). For cav-1 immunoprecipitation, representative Western blots showed MR protein abundances in 30-minute group (B) or 2-hour group (E), and striatin protein levels in 30-minute group (C) or 2-hour group (F).

CHAPTER V
DISCUSSION

The present results provide the first *in vivo* data simultaneously showing renal striatin and cav-1 protein abundances as well as immunoreactivity 30 minutes and 2 hours following aldosterone administration. In 30-minute group, aldosterone significantly enhanced the renal protein abundance of striatin by 50%, and pretreatment with MR antagonist, eplerenone, cannot reverse the effect induced by aldosterone (Figure 1). Aldosterone treatment for 2 hours or pretreatment with eplerenone had no significant effect on striatin levels (Figure 1). Previous *in vitro* and *in vivo* investigation has demonstrated that aldosterone increases striatin protein abundance from 1 hour with a peak at 6 hours and returns to the baseline after 12 hours (Pojoga 2012). Studies in human and mouse endothelial cells revealed that striatin is a critical mediator in rapid actions of aldosterone (Machado-Fiallo 2013, Coutinho 2014).

The precise mechanism by which aldosterone enhances striatin protein abundance has not been established. The explanation may be the linkage of Akt signaling induced by aldosterone to enhance striatin protein levels. An *in vitro* study showed that treatment with another steroid hormone (17β-estradiol, E2) for 5-30 minutes upregulates striatin protein abundance via the Akt pathway (Zheng 2018). It has been reported that aldosterone provides the biphasic effect on Akt phosphorylation either a short (10 minutes) or longer time (24 hours) in cardiomyocytes (Nagoshi 2012). In this regard, aldosterone-induced striatin protein levels in the present study may involve in Akt signaling. In addition, E2-induced striatin expression involved in extracellular signal-regulated kinases 1/2 (ERK1/2) in vascular smooth muscle cells (Zheng 2015). A previously study showed in the rat kidney that aldosterone injection for 30 minutes increases ERK1/2 phosphorylation

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(Sinphitukkul 2011). Therefore, aldosterone rapidly increased striatin protein levels, *per se*, is operated via ERK1/2 activation.

A previous study showed that aldosterone treatment increases striatin protein abundances with a peak at 2 hours in mouse heart (Pojoga 2012). In the present study, aldosterone administration for 2 hours did not significantly alter striatin protein in rat kidney. This may be due to tissue specific different responses. However, the precise mechanisms need to be clarified. Another suggested explanation in this regard may be related to the level of aldosterone concentration. As a single dose of aldosterone was injected the remaining level of circulating aldosterone progressively declined. This caused less effectiveness of aldosterone after 2 hours. It has been indicated in mouse that plasma aldosterone concentration was remained at highest levels for 30 minutes after injection and then returned to the initial value after 60 minutes (Fakitsas 2007). Moreover, in liver tissues, after aldosterone injection, radioactivity of labeled aldosterone was spread rapidly within 10-30 minutes and returned to baseline at 120 minutes (Tait 1961). In addition, a previous *in vitro* study in human endothelial cell line showed that aldosterone-induced striatin protein levels were reversed by MR blocker, spinorolactone (Pojoga 2012). By contrast, the present study in rat kidney revealed that striatin levels are still high even though pretreatment with MR antagonist, eplerenone (Figure 1). Further *in vivo* investigations are required to explain this discrepancy. Thus, in the kidney, aldosterone enhanced striatin protein levels is MR-independent pathway.

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For cav-1, the present data in 30-minute group showed that aldosterone rapidly increases cav-1 protein abundance to be double in the rat kidney (Figure 1). It has been demonstrated *in vitro* that aldosterone incubation for 48 hours induces cav-1 protein abundance (Igarashi 2013). In liver sinusoidal endothelial cells, aldosterone treatment in time-dependent from day 1 till day 3 increased the membrane protein level of cav-1 (Luo 2017). The mechanism by which aldosterone

stimulates caveolin protein may relate to Src activation. In M-1 cell, aldosterone rapidly induced Src protein levels in a dose-dependent manner (Braun 2004). Furthermore, the protein abundance of Src in vascular smooth muscle cells was time dependently increased by aldosterone (from 15 to 45 minutes) (Callera 2005, Callera 2005). It has been noted that Src tyrosine kinases induce caveolin phosphorylation (Li 1996). Aldosterone-induced cav-1 levels in 30-minute group may link to Src activation. In addition, incubation of PKC**α** increased cav-1 protein abundance in human lung fibroblasts (Tourkina 2005). In the rat kidney, a previous study documented that aldosterone rapidly enhances PKC**α** protein abundance (Eiam-Ong 2014). Therefore, aldosterone injection for 30 minutes induced cav-1 protein levels in the present investigation through PKC**α** activation. Of note, a previous *in vitro* examination reported that aldosterone-induced cav-1 protein is completely inhibited by spironolactone (Igarashi 2013). In the present study, eplerenone was able to partially ameliorate cav-1 protein levels induced by aldosterone (Figure 1). This suggests that, in the kidney, aldosterone regulates cav-1 protein levels, in part, through MR pathway. For 2-hour group, protein abundances of cav-1 were comparable between sham and aldosterone groups. The less aldosterone concentration alter 2 hours of aldosterone injection also had no effectiveness on cav-1 protein abundance. In addition, this study investigated the effect of aldosterone on cav-1 protein abundance in plasma membrane. It may be speculated that the internalization of cav-1 protein from plasma membrane possibly occurred at 2 hours. Parton et al. (2013) reported the pathway of cav-1 synthesis from the rough endoplasmic reticulum (rER) and its trafficking through the Golgi complex to the cell surface. After trafficking to the plasma membrane, caveolae has been shifted through endocytosis which could be targeted to early endosomes and could be recycled back to the plasma membrane. This recycling from the endosome maintains caveolar density which their relative rates must be balanced (Parton and del Pozo 2013). For

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another pathway, cav-1 proteins also localize at the plasma membrane as lipid droplets and then translocated to the ER (Parton and del Pozo 2013). Interestingly, caveolae internalization was regulated by PKCα-mediated filamin A phosphorylation and the actin cytoskeleton (Muriel 2011). It has demostrated that an increase of PKCα phosphorylation induces caveolae internalization from plasma membrane to vesicles (Smart 1995). In mesangial cells, PKC activation by phorbol 12-myristate 13-acetate declines cav-1 protein levels at 2 hours (Tamai 2001). However, the duration of cav-1 trafficking is remain unclear. Therefore, it was possible that, after 2-hour administration, aldosterone concentration levels were not sufficient and had no effectiveness to enhance cav-1 protein abundance in rat kidney. Pretreatment with eplerenone reduced cav-1 protein levels. The precise mechanism in this regard is still unknown. One possible explanation is that MR blocked cav-1 protein synthesis via genomic pathway.

For immunolocalization of striatin, no previous data are available in the rat kidney. In MDCK cells, striatin protein expressed in the cytosolic compartment and plasma membrane (Breitman 2008). In canine cardiac myocytes, striatin localized within the intercalated discs (Meurs 2010). The present study is the first document demonstrating striatin distribution in kidney tissues. For 30 minutes, in sham rats, prominent immunoreactivity was noted in the medulla region, especially in the MCD and VR (Figure 2 and Table 1). Aldosterone rapidly increased the immunostaining in most studied areas. Pretreatment with eplerenone attenuated the intensity scores in the cortex region but the immunoreactivity in the tLH and MCD was progressively enhanced. After 2 hours of injection, aldosterone also stimulated immunoreactivity of striatin in medulla region. Aldosterone markedly enhanced immunostaining in VR, tLH, and MCD. Of interest, immunoreactivity was decreased by eplerenone (Figure 3 and Table 1). The mechanism to explain this phenomenon remains to be clarified.

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Cav-1 immunoreactivity in the present study showed the similar baseline distribution as previous investigations (Breton 1998, Fujigaki 2007). In Figure 4, cav-1 immunostaining in 30 minutes induced by aldosterone was obvious in vasculatures: the GL, peritubular capillaries, and vasa recta (Table 2). The staining intensity scores in the GL, TALH, MCD, and tLH were slightly minimized by eplerenone. Aldosterone injection for 2 hours increased cav-1 protein immunoreactivity in all studied areas which declined by eplerenone (Figure 5 and Table 2). The protein distribution of striatin and cav-1 induced by aldosterone along the nephron segments is diversely operated. This implies some significant roles of both proteins in the kidney.

For protein interaction, the present data showed that MR interact with striatin and cav-1 (Figure 6). It was already known that striatin function as a scaffolding protein that interacts with mediators of vesicular trafficking (Hwang and Pallas 2014). It has been studied that striatin mediates the nongenomic effect of estrogen receptor-**α** (ER**α**). Striatin serves as a scaffold directly bind the ER**α** that localization of ER**α** to the plasma membrane and bridges ER**α** with the GPCR ($G_{\alpha i}$) for the formation of an ER**α**-$G_{\alpha i}$ complex (Lu 2004). It has been evidenced that the N-terminus of striatin bound to the N-terminus of ER**α** while the C-terminal WD repeat domain bound the $G_{\alpha i}$ complex (Lu 2004). Therefore, Pojoga et al. (2012) hypothesized that striatin may also interact with other steroid hormone receptors, such as MR. A previous study both *in vitro* and *in vivo* demonstrated that striatin is present and co-precipitates with the MR in mouse aortic endothelial cells, EA.hy926 cells, and mouse heart tissues (Pojoga 2012). Another interesting of the scaffold protein, cav-1 is a scaffold protein that was interacted with signaling proteins via N-termini termed the caveolin scaffolding domain (Li 1996). It has been reported that cav-1 was associated with MR (Pojoga 2010). MR is in the cell surface which connects with caveolae via cav-1 protein (Coutinho 2014). In addition, Pojoga et al (2010) demonstrated that cav-1 interacts with MR in the wild type group. The cav-1 knockout mice lost this property.

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The N-terminal domain of the MR has been found interact with cav-1 (Pojoga 2010). As previously described, striatin contains several protein-protein interaction domains, including caveolin-binding domain (Hwang and Pallas 2014). The present results demonstrated that striatin also interacts with cav-1 protein. It has been shown that all isoforms of striatin were interacted with cav-1 (Gaillard 2001). Moreover, cav-1 was directly interacted with striatin in mouse aortic endothelial cells, EA.hy926 cells, and mouse heart tissues (Pojoga 2012). Collectively, the present study shows that either striatin or cav-1 forms a complex with MR, and striatin also interacts with cav-1 protein in rat kidney in both 30-minute and 2-hour groups of sham, aldosterone, and eplerenone pretreatment groups (Figure 6).

Of note, striatin and cav-1 proteins are associated in modulating various cellular functions through multifunctional signals (Baudrand 2014, Hwang and Pallas 2014). The critical role of striatin on salt sensitive blood pressure and vascular responses have been clearly documented (Garza 2015, Garza 2015, Gupta 2017). Unfortunately, studies of striatin related to kidney function have not been established. For cav-1, it exhibits a wide range modulation of health and disease (Cohen 2004). An *in vivo* study suggested that cav-1 regulates aldosterone-mediated pathways of glucose and lipid homeostasis (Baudrand 2016). Aldosterone-induced oxidation promoting cell defenestration is cav-1-related autophagy (Luo 2017). In the kidney, cav-1 promotes renal water and salt reabsorption via modulation of sodium-chloride cotransporter function and regulation of vascular endothelial nitric oxide (Willière 2018). More *in vivo* examinations are needed to elucidate the molecular mechanisms of striatin and cav-1 in modulating aldosterone's pathways and consequently regulating kidney functions.

In conclusion, this is the first *in vivo* study demonstrating that aldosterone differently modulates striatin and cav-1 protein levels in rat kidney. In a rapid action, aldosterone increases striatin via MR-independent manner, whereas it partially

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induces cav-1 through MR-dependent pathway. For a longer effect, aldosterone had no significant alterations on striatin and cav-1 protein levels. Blockage of MR could disturb aldosterone actions on cav-1 protein. Furthermore, this study suggested that striatin and cav-1 are scaffolding proteins that interact with MR in rat kidney.

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