การทำงานของอัลโดสเตอโรนแบบนัลจีโนมิกต่อการไดเมอไรเซชั่นของแองจิโอเทนซินรีเซพเตอร์ในไตหนูแรท: บทบาทของมิเนราโลคอร์ติคอยด์รีเซพเตอร์และเอ็นเอดีพีเอชออกซิเดส

นายกิตติศักดิ์ สินพิทักษ์กุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Nongenomic actions of aldosterone on angiotensin receptor dimerization in rat kidney: role of mineralocorticoid receptor and NADPH oxidase

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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กิตติศักดิ์ สินพิทักษ์กุล : การทำงานของอัลโดสเตอโรนแบบนัลจีโนมิกต่อการไดเมอไรเซชั่นของแองจิโอเทนซินรีเซพเตอร์ในไตหนูแรท: บทบาทของมิเนราโลคอร์ติคอยด์รีเซพเตอร์และเอ็นเอดีพีเอชออกซิเดส (Nongenomic actions of aldosterone on angiotensin receptor dimerization in rat kidney: role of mineralocorticoid receptor and NADPH oxidase) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. สมจิตร์ เอี่ยมอ่อง, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ. นพ. กฤษณพงศ์ มโนธรรม, 102 หน้า.

การศึกษาในอดีตในเซลล์เพาะเลี้ยงแสดงให้เห็นว่าอัลโดสเตอโรนเพิ่ม angiotensin II receptor (ATR) dimerization แบบไม่ผ่านจีโนมิกโดยอาศัย transglutaminase type 2 (TG2) และ reactive oxygen species (ROS) อย่างไรก็ตามยังไม่มีการศึกษาดังกล่าวในไตสัตว์ทดลอง วัตถุประสงค์ของการศึกษาในครั้งนี้เพื่อตรวจสอบผลของอัลโดสเตอโรนที่ไม่ผ่านจีโนมิกต่อระดับปริมาณโปรตีน (dimeric และ monomeric forms) ของ angiotensin II type 1 receptor (AT_1R) และ angiotensin II type 2 receptor (AT_2R), TG2 (เอนไซม์ที่ทำให้เกิดการเชื่อมโยงระหว่างโปรตีน) และ p47phox (cytosolic part of NADPH oxidase activation เพื่อสร้าง ROS) และบริเวณที่มีการแสดงออกของโปรตีน TG2 และ p47phox ในไตหนูแรท

ทำการทดลองในหนูแรทพันธุ์วิสต้าเพศผู้ แบ่งออกเป็นกลุ่มที่ได้รับสารละลาย normal saline หรือ อัลโดสเตอโรน (150 µg/kg BW) ฉีดเข้าทางช่องท้องเป็นเวลา 30 นาที หรือ ได้รับ eplerenone [mineralocorticoid receptor (MR) blocker, 15 mg/kg BW] หรือ apocynin (NADPH oxidase inhibitor, 5 mg/kg BW) เป็นเวลา 30 นาที ก่อนฉีดอัลโดสเตอโรน ทำการตรวจวัดระดับโปรตีนของ dimeric และ monomeric forms ของ AT_1R, AT_2R, p47phox ใน plasma membrane และโปรตีนของ TG2 ใน tissue homoginated โดยวิธี Western blot analysis ตรวจวัดบริเวณที่มีการแสดงออกของโปรตีน TG2 และ p47phox โดยวิธี immunohistochemistry ในเนื้อเยื่อไต วิเคราะห์ระดับปริมาณโปรตีน AT_1R/AT_2R heterodimerization โดยเทคนิค co-immunoprecipitation และ Western blot รวมทั้งตรวจวัดบริเวณเนื้อเยื่อไตที่มีการแสดงออกร่วมกันของโปรตีน AT_1R และ AT_2R ด้วยวิธี double labelling immunohistochemistry ผลการทดลองพบว่าภายหลังการฉีดอัลโดสเตอโรน 30 นาที มีปริมาณโปรตีน dimeric forms ของ AT_1R และ AT_2R ($p < 0.001$) เพิ่มขึ้น แต่ monomeric forms ของทั้งสอง receptors ไม่เปลี่ยนแปลง การให้ eplerenone สามารถยับยั้งเฉพาะ dimeric form ของ AT_2R ขณะที่ apocynin ป้องกันการเกิด dimeric formation ของทั้งสอง receptors อัลโดสเตอโรนเพิ่มระดับโปรตีนของ TG2 และ p47phox แต่ผลถูกยับยั้งเมื่อได้รับ eplerenone หรือ apocynin อัลโดสเตอโรนกระตุ้นการแสดงออกของโปรตีน TG2 ส่วนใหญ่ในบริเวณ medulla ขณะที่โปรตีน p47phox มีระดับเพิ่มขึ้นทั้งในบริเวณ cortex และ medulla การให้ eplerenone หรือ apocynin ได้ลดระดับการแสดงออกของทั้ง TG2 และ p47phox พบว่าไม่มีการเปลี่ยนแปลงอย่างมีนัยสำคัญของ plasma membrane AT_1R/AT_2R heterodimerization ในทุกกลุ่มทดลองที่ทำการศึกษา และในทุกกลุ่มเช่นกันที่พบว่าการแสดงออกร่วมกันของ AT_1R และ AT_2R มีมากในบริเวณ glomerulus, distal convoluted tubule และ cortical collecting duct ขณะที่เกิดขึ้นน้อยและกระจายตัวที่บริเวณ proximal convoluted tubule และ peritubular capillary

โดยสรุป ข้อมูลในครั้งนี้เป็นหลักฐานชิ้นแรกที่แสดงว่าอัลโดสเตอโรนเพิ่มระดับการแสดงออกของ TG2 และ p47phox ในไต และกระตุ้น AT_1R และ AT_2R dimerization อัลโดสเตอโรนกระตุ้น AT_1R และ AT_2R dimerization โดยผ่านการทำงานของ NADPH oxidase อย่างไรก็ตามอัลโดสเตอโรนที่ทำให้เพิ่ม AT_1R dimer formation นั้นไม่อาศัย MR แต่ dimeric formation ของ AT_2R นั้นผ่านการทำงานของ MR อัลโดสเตอโรนเพิ่มการแสดงออกของ TG2 และ p47phox ในไตโดยอาศัยการทำงานของทั้ง MR และ NADPH oxidase ส่วนheterodimerization ของ AT_1R/AT_2R มีระดับคงที่ในทุกกลุ่มการทดลอง โดยบริเวณที่มีการแสดงออกร่วมกันของโปรตีน AT_1R และ AT_2R นั้นพบว่ามีเด่นชัดในบริเวณ cortex

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2559 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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KEYWORDS: ANGIOTENSIN II TYPE 1 RECEPTOR / ANGIOTENSIN II TYPE 2 RECEPTOR / MINERALOCORTICOID RECEPTOR / NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE OXIDASE / NONGENOMIC ACTIONS OF ALDOSTERONE / OXIDATIVE STRESS / RAT KIDNEY / RECEPTOR DIMERIZATION / TRANSGLUTAMINASE TYPE 2

KITTISAK SINPHITUKKUL: Nongenomic actions of aldosterone on angiotensin receptor dimerization in rat kidney: role of mineralocorticoid receptor and NADPH oxidase. ADVISOR: ASSOC. PROF. SOMCHIT EIAM-ONG, Ph.D., CO-ADVISOR: KRISSANAPONG MANOTHAM, M.D., 102 pp.

Previous *in vitro* studies demonstrated that aldosterone nongenomically elevates angiotensin II receptor (ATR) dimerization through transglutaminase type 2 (TG2) and reactive oxygen species (ROS). However, there is no *in vivo* study regarding this circumstance in the kidney. The purpose of the present investigation is to examine the nongenomic effects of aldosterone on protein abundances (dimeric and monomeric forms) of angiotensin II type 1 receptor (AT_1R) and angiotensin II type 2 receptor (AT_2R), TG2 (a crosslinking protein enzyme), and p47phox (a cytosolic part of NADPH oxidase activation to generate ROS), and localizations of TG2 and p47phox proteins in rat kidney.

Male Wistar rats were received normal saline solution or aldosterone (150 µg/kg BW) by intraperitoneal injection for 30 minutes or pretreated with eplerenone [mineralocorticoid receptor (MR) blocker, 15 mg/kg BW] or with apocynin (NADPH oxidase inhibitor, 5 mg/kg BW) 30 minutes before aldosterone injection. Plasma membrane protein abundances of renal dimeric and monomeric forms of AT_1R, AT_2R, and p47phox as well as of tissue homogenated TG2 protein were measured by Western blot analysis. Protein localizations of TG2 and p47phox were examined by immunohistochemistry. Protein levels of AT_1R/AT_2R heterodimerization were analyzed by co-immunoprecipitation and Western blot techniques. Finally, the colocalization of AT_1R and AT_2R proteins was determined by double labelling immunohistochemistry. After 30 minutes of aldosterone injection, aldosterone enhanced renal dimeric forms of AT_1R and AT_2R ($p < 0.001$) but monomeric forms of both receptors unaltered. Eplerenone inhibited only the dimeric form of AT_2R while apocynin prevented the dimeric formation of both receptors. Aldosterone increased TG2 and p47phox protein abundances that were blunted by pretreatment with eplerenone or apocynin. Aldosterone stimulated TG2 protein expression mainly in the medulla whereas p47phox protein was increased in both cortex and medulla. Pretreatment with eplerenone or apocynin alleviated the expressions of both TG2 and p47phox. No significant changes in plasma membrane AT_1R/AT_2R heterodimerization were observed in all studied groups. The colocalization of AT_1R and AT_2R proteins was high in glomerulus, distal convoluted tubule, and cortical collecting duct meanwhile the colocalization was weak and diffused in the proximal convoluted tubule and peritubular capillary in all studied groups.

In conclusion, the present data are the first document demonstrating that aldosterone nongenomically increases renal TG2 and p47phox protein expressions and then activates AT_1R and AT_2R dimerizations. Aldosterone-stimulated AT_1R and AT_2R dimerizations are mediated through activation of NADPH oxidase. However, aldosterone-induced AT_1R dimer formation is MR-independent pathway whereas the formation of AT_2R dimer is modulated via MR-dependent manner. Aldosterone-enhanced renal TG2 and p47phox protein expressions are depend on both MR and NADPH oxidase activations. The heterodimerization of AT_1R/AT_2R is maintained in all studied groups. The colocalization of AT_1R and AT_2R proteins is more prominent in the cortex region.

Field of Study: Medical Science
Academic Year: 2016

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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LIST OF ABBREVIATIONS

$^{\circ}C$	Degree Celcius
g	Gram
i.p.	intraperitoneal injection
M	Molar
dL	Decilitre
mL	Millilitre
µg	Microgram
µL	Microlitre
Aldo	Aldosterone
Apo.	Apocynin
AT_1R	Angiotensin II type 1 receptor
AT_2R	Angiotensin II type 2 receptor
CCD	Cortical collecting duct
DCT	Distal convoluted tubule
Ep.	Eplerenone
H_2O_2	Hydrogen peroxide
KCl	Potassium chloride
KH_2PO_4	Potassium di-hydrogen phosphate
MCD	Medullary collecting duct
MR	Mineralocorticoid receptor
NaCl	Sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate

Na_2HPO_4	Di-sodium hydrogen phosphate
NaH_2PO_4 H_2O	Sodium phosphate monobasic monohydrate
NSS	Normal saline solution
PBS	Phosphate buffer solution
PCT	proximal convoluted tubule
Pcap	Peritubular capillary
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TALH	Thick ascending limb of Henle's loop
TBS	Tris buffer solution
TG2	Transglutaminase type 2
tLH	Thin limb of Henle's loop
VR	Vasa recta

CHAPTER I
INTRODUCTION

Background and rational

The steroid hormone aldosterone plays an essential role in controlling blood pressure by regulation of sodium, potassium, and water balance in the kidney by genomic and nongenomic mechanisms [1]. Roles of aldosterone in the distal nephron have been described to a genomic mechanism which the binding of its intracellular receptor, mineralocorticoid receptor (MR), and then the hormone-receptor complex is transferred to the nucleus and binds to hormone-responsive elements in the promoter sites in order to promote the expression of target genes [2]. Besides genomic mechanism of aldosterone, many investigators have recently turned to nongenomic mechanism [3-5]. This mechanism is characterized with a rapid onset (seconds to minutes) [6-8].

In *in vitro* study, aldosterone nongenomically increases the formation of angiotensin II type 1 receptor (AT_1R) dimerization in cultured mouse mesenteric arterioles by transglutaminase 2 (TG2) [9]. TG2 is a catalytic enzyme providing post-translational modification of proteins by crosslinking two proteins with covalent bonds formation [10]. In addition, reactive oxygen species (ROS) elevation induces both dimerization of AT_1R and dimerization of angiotensin II type 2 receptor (AT_2R) in human embryonic kidney cells [11, 12]. In cultured porcine proximal tubular cells, aldosterone nongenomically elevates ROS involving in nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation [13]. This activation is occurred

by p47phox translocation from cytosolic to plasma membrane in order to bind membranous subunit of NADPH oxidase and then generates ROS production [13]. These studies suggest that TG2 and p47phox-induced NADPH oxidase activation by nongenomic action of aldosterone might lead to both AT_1R and AT_2R dimerization. Dimerization of AT_1R or AT_2R has indicated for receptor activation and function [14]. The knowledge of nongenomic action has a great importance for the understanding of aldosterone cross talk with angiotensin II receptors which play significant role in the receptors function. Although there are available *in vitro* data, *in vivo* studies are less established. Furthermore, there are no studies involving in angiotensin II type 2 receptor (AT_2R) dimerization by nongenomic action of aldosterone.

To acquire *in vivo* data, the present study was performed in the rat kidneys following 30-minute of intraperitoneal injection with normal saline solution, or aldosterone; or recieving pretreatment with eplerenone (MR blocker) or with apocynin (NADPH oxidase inhibitor) 30 minutes before aldosterone injection. Plasma membrane protein abundances of renal dimeric and monomeric forms of AT_1R, AT_2R, and p47phox as well as homogenated renal TG2 protein were measured by Western blotting analysis. Localizations of p47phox and TG2 proteins were examined by immunohistochemistry. Protein levels of AT_1R/AT_2R heterodimerization were analyzed by co-immunoprecipitation and Western blot techniques. Finally, the colocalization of AT_1R and AT_2R proteins was determined by double labelling immunohistochemistry.

Research questions

1. After 30-minute aldosterone injection, does it change protein abundances and localizations of dimeric and monomeric forms (AT_1R or AT_2R), of p47phox, and of TG2 in rat kidney as compared with normal saline solution (NSS) treated group?
2. Does pretreatment with eplerenone (MR blocker) or pretreatment with apocynin (NADPH oxidase inhibitor) able to prevent the alterations induced by aldosterone on protein abundances and localizations of dimeric and monomeric forms (AT_1R or AT_2R), of p47phox, and of TG2 in rat kidney as compared with their respective aldosterone-treated groups?

Research objectives

1. To examine changes of protein abundances and localizations of dimeric and monomeric forms (AT_1R or AT_2R), of p47phox, and of TG2 in rat kidney after 30-minute aldosterone injection as compared with NSS treated group.
2. To clarify the role of MR and NADPH oxidase in alterations induced by aldosterone on protein abundances and localizations of dimeric and monomeric forms (AT_1R or AT_2R), of p47phox, and of TG2 in rat kidney as compared with their respective aldosterone-treated groups.

Hypothesis

After 30-minute aldosterone injection, protein abundances and localizations of dimeric forms (AT_1R or AT_2R), of p47phox, and of TG2 in rat kidney will enhance as compared with NSS-treated group. Pretreatments with eplerenone (MR blocker) or apocynin (NADPH oxidase inhibitor) will decrease p47phox and TG2 and then lessen the dimeric forms (AT_1R or AT_2R) induced by aldosterone.

Keywords

nongenomic actions of aldosterone, angiotensin II type 1 receptor, angiotensin II type 2 receptor, receptor dimerization, mineralocorticoid receptor, nicotinamide adenine dinucleotide phosphate oxidase, transglutaminase type 2, oxidative stress, rat kidney

Expected benefit and application

1. This is the first animal study data demonstrates the role of mineralocorticoid receptor and NADPH oxidase in the alterations induced by aldosterone on protein abundances of dimeric and monomeric forms (AT_1R or AT_2R), of p47phox, and of TG2 and colocalization between AT_1R and AT_2R protein expression in rat kidney.
2. This research contributes a fundamental knowledge of rapid effects of aldosterone on angiotensin receptor dimerization *in vivo*.
3. The fundamental knowledge provides further studies in rapid actions of aldosterone on signaling mechanisms beyond the activation of angiotensin receptors, such as $G_{q/11}$ pathway.

CHAPTER II
THEORY AND LITERATURE REVIEW

Aldosterone biosynthesis

Aldosterone is a steroid hormone produced by zona glomerulosa cells of the adrenal cortex [1]. Cholesterol is converted to aldosterone by a series of locus- and orientation-specific enzyme reactions which are located in mitochondria and smooth endoplasmic reticulum (Figure A) [1]. The daily amount of secreted aldosterone is produced only 30-120 ng/hr/kg in human [15] or 250-400 ng/hr/kg in rat [16]. Since there is no specific aldosterone carrier protein in the blood, very little circulating aldosterone is bound. The normal total plasma aldosterone level in adult human is approximately 200-800 pmol/L [17] or 400-2,000 pmol/L in rat [18, 19]. The half-life of aldosterone is short (~15-20 min) [20]. aldosterone is catabolized principally in the liver into the tetrahydroglucuronide derivative, and its metabolites are excreted in the urine [20]. Less than 1% of secreted aldosterone appears in urine in the free form [20]. Another 5% is in form of acid-labile conjugate, and up to 40% is in form of the tetrahydroglucuronide derivative [20].

Aldosterone has a number of important actions on the kidneys. With regard to the regulation of the extracellular volume, aldosterone acts to reduce sodium excretion by stimulating its reabsorption by the thick ascending limb of Henle's loop, distal convoluted tubule, connecting tubule, and collecting duct [21]. Furthermore, aldosterone also induces potassium and hydrogen secretion by the distal nephron, particularly segments of collecting duct [21]. The pivotal regulators of aldosterone

synthesis and secretion are angiotensin II, the concentration of plasma potassium and adrenocorticotropin releasing hormone [1].

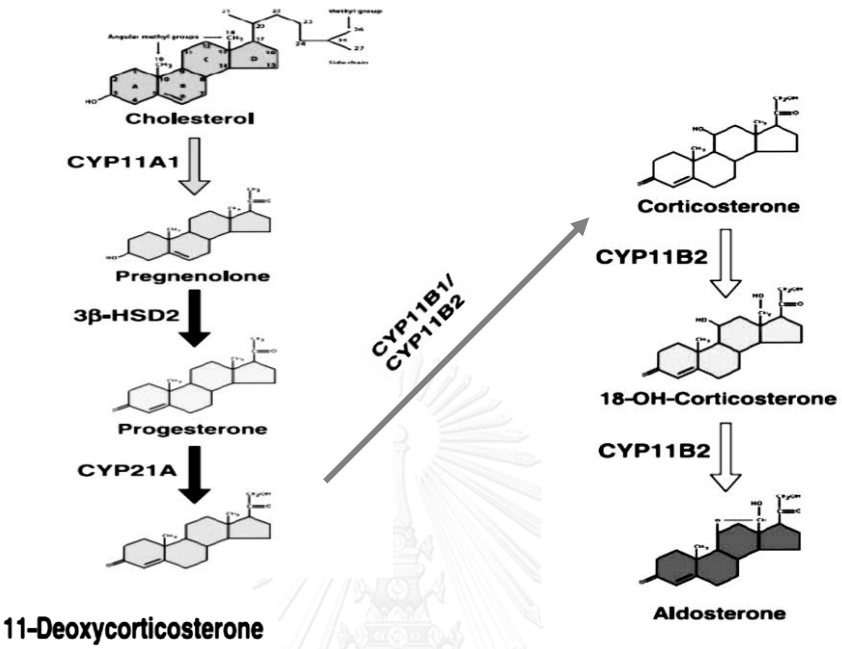

Figure A: Biosynthesis of aldosterone [1]

Mechanism of actions

Steroid hormones modulate many physiological processes. The effects of steroid that are mediated by the modulation of gene expression are known to occur with a time lag of hours or even days [22]. Many studies that have been carried out mainly in the past decade have identified other responses to steroids that are much more rapid and take place in seconds or minutes [4, 22]. These responses follow nongenomic pathways, and they are not rare [22].

Genomic pathway of aldosterone

According to the 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) [23]. 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 [23]. The length time of genomic actions is 1-2 hours before obvious changes in target cell activity, and the whole pathway is sensitive to particular inhibitors, such as actinomycin D or cycloheximide [23].

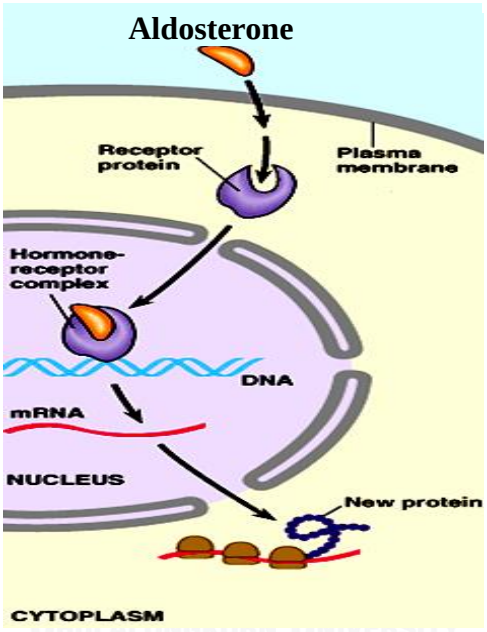

Figure B: Genomic pathway of aldosterone [23]

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 influences gene expression, as does the classical action [22]. The responses take place in seconds to minutes and are independent on transcription and translation. The nongenomic action often involves the generation of intracellular

secondary messengers and various signal transduction cascades such as protein kinase pathways (Figure C) [7, 24, 25]. These actions of aldosterone have been extensively investigated, mostly in cell culture studies [23, 26, 27].

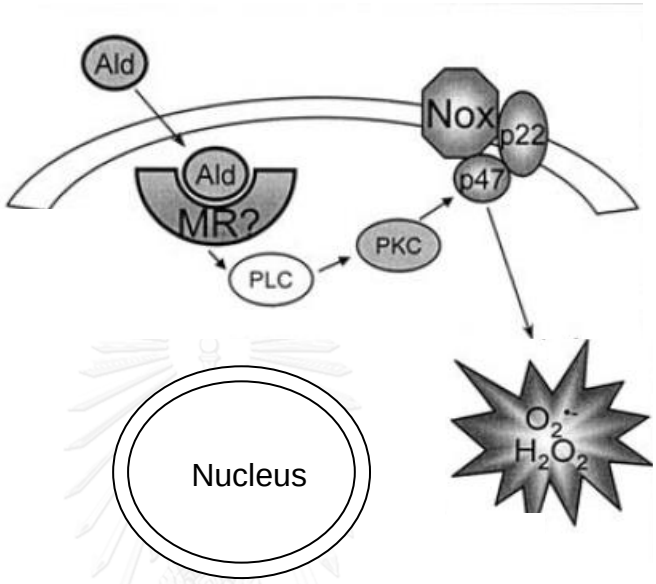

Figure C: Nongenomic pathway of aldosterone [25]

Rapid action of aldosterone on oxidative stress in renal cell culture

The rapid response of changing in intracellular oxidative stress is likely obvious [28]. For superoxide (O_2^-), aldosterone induced double O_2^- production which depends on NADPH oxidase within 30 minutes in cultured mouse macula densa cells [29]. Moreover, aldosterone enhanced this production two folds in cultured porcine proximal tubular (PT) cells and a half fold in Madin-Darby Canine kidney cells [30].

Nongenomic action of aldosterone on angiotensin II receptor

It has been showed that the rapid action of aldosterone is a similar pathway as angiotensin II (Ang II) signaling in the isolated vascular tissue [9]. The results

showed that aldosterone increased angiotensin II type 1 receptor (AT_1R) dimer protein level within 10 minutes. Moreover, an AT_1R blocker could prevent this effect [9]. Nevertheless, the action of aldosterone on the dimer protein level of angiotensin II type 2 receptor (AT_2R) has not been demonstrated.

Forms of angiotensin II receptors

Monomeric form of AT_1R

AT_1R, both in human and rat, contains 359 amino acids and has a molecular mass of 41 kDa [31]. The AT_1R are well conserved between species [31]. Human AT_1R is about 95% identical to that of the rat [32]. Figure D shows a revised secondary-structure model of rat AT_1R [31]. The AT_1R belongs to the seven transmembrane class of G-protein-coupled receptors [33]. Four cysteine residues are located in the extracellular domain, which represent sites of disulphide bridge formation and are critical tertiary structure determinants [34]. The transmembrane domain and the extracellular loop play an important role in Ang II binding [35]. In accordance with the role of Ang II in sodium reabsorption, AT_1R is highly abundant on the brush borders of proximal tubular epithelial cells [36]. Prominent expression has also been found in renal medullary interstitial cells, located between the renal tubules and vasa recta, where Ang II is implied to have a potential role in the regulation of medullary blood flow [37]. In the glomerulus, AT_1R is found in both afferent and efferent arterioles, as well as in the mesangium, endothelium, and podocytes [38].

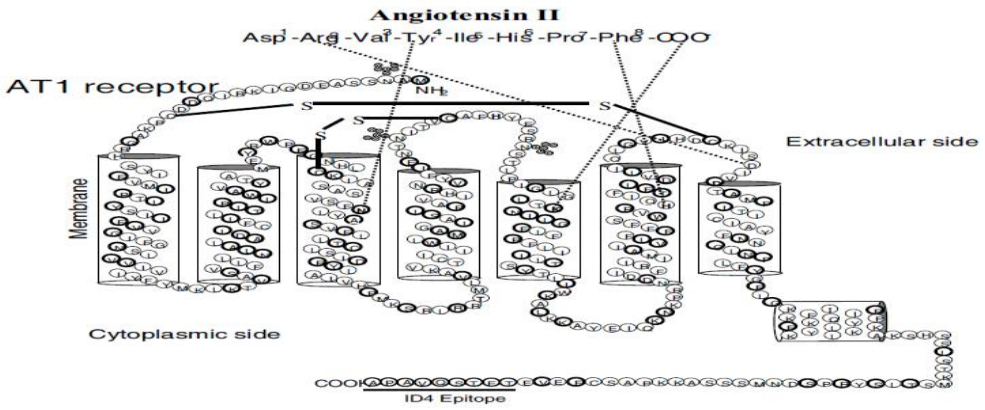

Figure D: Structural model of rat AT_1R [31]

Homodimeric form of AT_1R

Homodimeric form of AT_1R is characterized by an increase in coupling to $G_{q/11}$ generation of inositol phosphate, and intracellular calcium ion $[Ca^{2+}]_i$, as compared with the monomeric form [39]. This indicates that the dimeric AT_1R is hyperactive [39, 40]. Transglutaminase, an enzyme providing a crosslink of two AT_1R proteins, is located to glutamine amino acid at position 315 in the carboxyl terminus of 359- amino acid AT_1R in human embryonic kidney (HEK) cells (Figure E) [40].

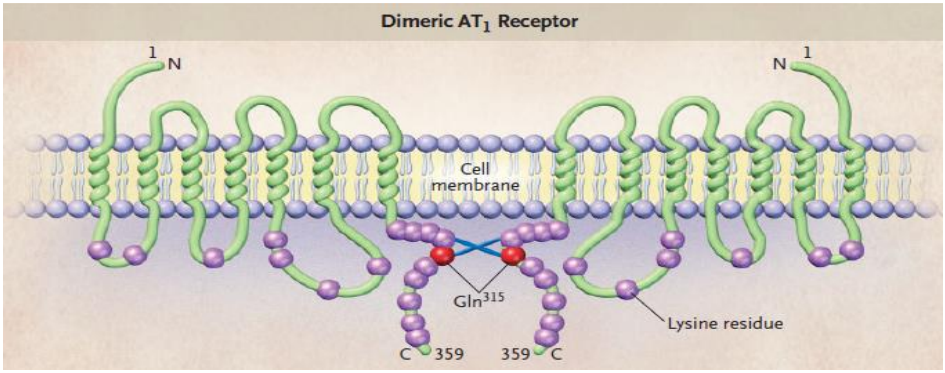

Figure E: AT_1R dimerization [40]

Dimerization of AT_1R can occur by various factors [14]. It has been showed that H_2O_2 induced AT_1R dimerization in AT_1R-transfected HEK cells [11]. In addition, monocytes derived from hypercholesterolemic apolipoprotein E knockout mice also demonstrated increased levels of AT_1R homodimers that covalently cross-linked by transglutaminase enzyme [39]. Recently, vasoconstrictor effect of aldosterone in isolated mouse mesenteric arterioles could be mediated by increasing of intracellular transglutaminase induction and following AT_1R dimerization [9].

Monomeric form of AT_2R

AT_2R is only 34% identical to that of AT_1R, especially in the transmembrane domains [31]. The AT_2R consists of 363 amino acids with molecular weight of about 41 kDa. AT_2R is also well conserved, with 72% identity between rat and human [32]. Like the AT_1R, AT_2R is a seven-transmembrane G protein-coupled receptor [34]. Figure F demonstrates the comparison of structural model between rat AT_2R and AT_1R [41]. The AT_2R protein consists of five potential N-glycosylation sites in the extracellular N-terminal domain and 14 cysteine residues. Ang II binds to the AT_2R with similar affinity as to the AT_1R [42]. Despite substantial research, the actions of the AT_2R are still not well understood and remain somewhat controversial [43]. In general, the actions of AT_2R stimulation oppose those of AT_1R stimulation [43]. For instance, activation of AT_1R causes vasoconstriction and promotes sodium retention, whereas AT_2R stimulation leads to vasodilation [44], and natriuresis [45]. These are consistent with their abundances on the epithelium of the proximal tubule [46]. The vasodilatory effects of AT_2R are mediated by increasing nitric oxide

synthesis and cyclic guanosine 3', 5'-monophosphate (cGMP) synthesis [47]. Inhibition of Na,K-ATPase activity in microdissected rat proximal tubules offers a mechanism for AT_2R-induced natriuresis [48]. In the kidney, besides in the proximal tubules, the expression of AT_2R is also localized in collecting ducts, renal interstitial cells, afferent arterioles, arcuate arteries, interlobular arteries, and outer medullary descending vasa recta [46, 49, 50].

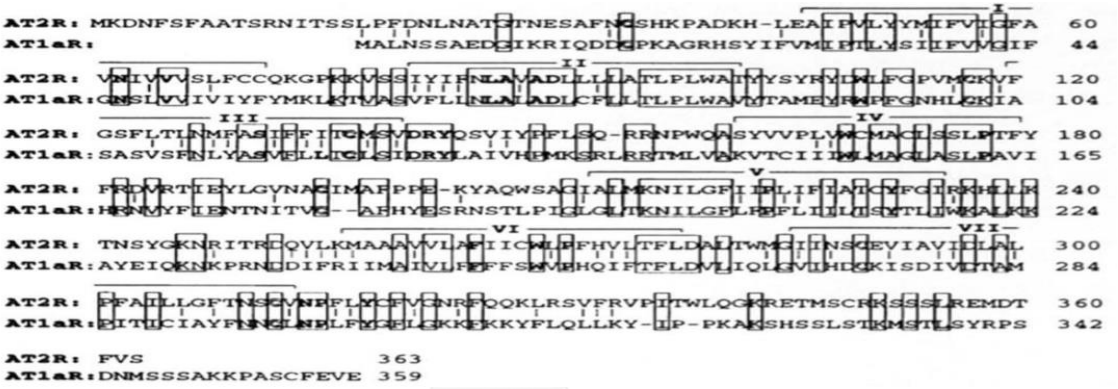

Figure F: The comparison of structural model between rat AT_1R and AT_2R [41]

Homodimeric form of AT_2R

Homodimeric form of AT_2R is characterized by an increase in generation of nitric oxide, and cGMP, as compared with the monomeric form [51]. This suggests that the dimeric AT_2R is hyperactive [51]. Elevation of reactive oxygen species (ROS) mediates oxidative cross-linking of AT_2R monomers to dimers involving tyrosine residues located at putative interreceptor contact sites of the cytoplasmic loop connecting transmembrane helices III/IV in HEK cells [12]. Interestingly, the AT_2R dimerization plays a role in AT_1R signaling inhibition and enhances AT_2R signaling through nitric oxide overproduction [51, 52].

AT_2R homodimerization has been assessed in a few studies [31]. It has shown that Ang II did not alter the dimer formation in cultured rat adrenal medulla (PC12W) cells [53]. By contrast, the dimer formation could be important both for the basal constitutive and can be enhanced after serum-free condition at 24 hours in PC12W cells [54]. In addition, ROS could stimulate AT_2R dimerization in HEK cells expressing NOX-3 [12].

Heterodimeric form of AT_1R/AT_2R

AT_1R and AT_2R could form constitutive heterodimers in PC-12W cells [53]. In normal condition, the heterodimers on the cell surface were lesser than homodimers of AT_1R or AT_2R in cultured human coronary artery smooth muscle cells [55]. Surprisingly, Ang II increased AT_1R/AT_2R heterodimerization by 140% in cultured porcine proximal tubular cells [56]. However, the role of transglutaminase on the heterodimeric formation of AT_1R/AT_2R has not been determined.

Transglutaminase (TG)

Transglutaminases are a family of enzymes that catalyze post-translational modification of proteins by cross-linking protein [57]. Eight TGs have been identified in mammals and humans and they all required Ca^{2+} for catalytic activity [58]. Tissue transglutaminase (tTG or TG2) is the most ubiquitous member of the TG family [59]. This protein is approximately 78 kDa that composed of four distinct domains (Figure G1) [58, 60]. The N-terminal β-sandwich domain contains a high affinity binding site for fibronectin, the catalytic core domain is responsible for Ca^{2+} binding, and the barrel 1 and 2 domains can interact with GTP and phospholipase C, respectively [59]. In physiologic condition, low Ca^{2+} level makes intracellular environment conductive

for TG2 to maintain a catalytically inactive, compact conformation [59]. During activation, the interaction between domain 2 and domains 3 and 4 breaks down after the binding of Ca^{2+}, an essential activator of transamidating activity (Figure G2) [59].

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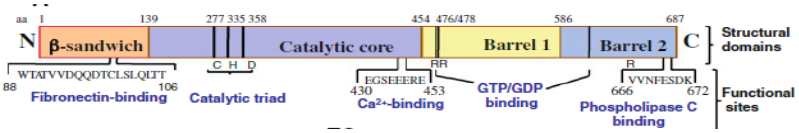
(1)



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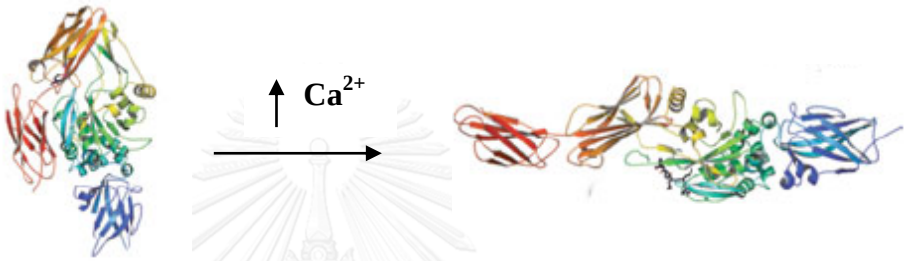


Figure G: (1) Structural and functional of TG2;

(2) Conformation change of TG2 by Ca^{2+} [59]

In the kidney, there was weak and diffuse TG2 immunostaining in glomeruli, interstitial space surrounding the tubules [61-63]. In renal diseases, the intense TG2 staining was observed exclusively within glomeruli and rare staining of tubulointerstium in puromycin aminonucleoside-injected rats, the model of glomerulosclerosis [63]. In addition, TG2 was stained in mesangial cells, cytoplasm of tubular cells (mainly in distal tubuli) and particularly in the interstitial fibrotic area in human IgA nephropathy [64].

One attractive candidate involves in aldosterone-induced AT_1R dimerization [14]. Aldosterone-induced TG2 could stimulate AT_1R dimerization in isolated mouse mesenteric arterioles [9]

Role of transglutaminase on angiotensin II receptor dimerization

After Ca^{2+}-induced TG activation, transamidation can cause a protein cross-linking by forming a Nε-(γ-glutamyl) lysine isopeptide bridge between the deprotonated lysine donor residue of one protein (purple ellipse) and the acceptor glutamine residue of another (blue rectangle) (Figure H) [57]. *In vitro* studies, TG increased the formation of cross-linked AT_1R homodimerization in HEK cells at position 315 of glutamine in the cytoplasmic tail [39]. Although TG2 did not effect on AT_2R dimeric formation, it enhanced AT_2R oligomers after injection of TG2 in mouse hippocampus [65]. However, its effect on AT_1R/AT_2R heterodimerization has not been shown in any studies.

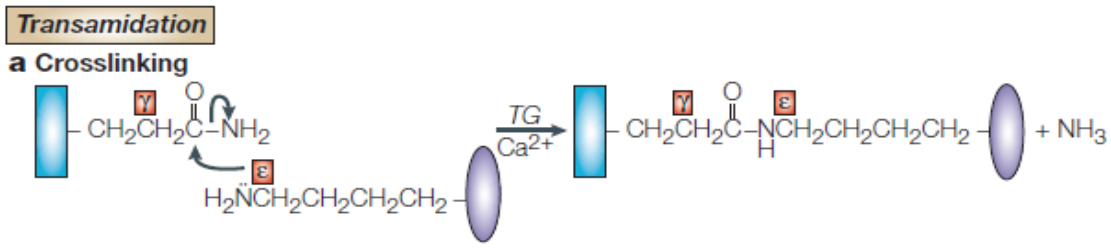


Figure H: Transglutaminases catalyse post-translational modification of proteins [57]

Besides TG2, reactive oxygen species (ROS) is a factor to regulate angiotensin II receptor dimerization [12]. Nongenomic actions of aldosterone participate in increasing of ROS production [14, 28].

Mechanism of aldosterone induces NADPH oxidase activation

Nongenomic actions of aldosterone-induced superoxide (O_2^-) production in cultured mouse macula densa cells and porcine PT cells were inhibited by p47phox inhibition [13, 29]. Moreover, aldosterone also reduced cytosolic p47phox and

activated its insertion into the plasma membrane in porcine PT cells within 30 minutes [13]. These data show that aldosterone can induce NADPH oxidase activation involved in redistribution of p47phox in renal cells [13].

p47phox

p47phox (phox: phagocyte oxidase) is a protein composed of 390 amino acids with approximately molecular mass of 47 kDa which is a component of NADPH oxidase complex [66-68]. The N-terminal amino acid sequence of p47phox has one phox homology domain, the middle amino acid sequence also contains two SH3 domains, and C-terminal sequence has an autoinhibitory region and a proline-rich region (Figure I) [66].

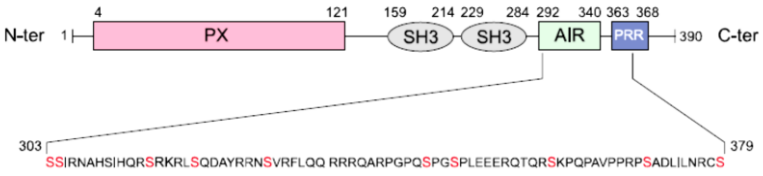


Figure I: Molecular structure of p47phox [66]

In the resting state, the two SH3 domains interact intramolecularly with the C-terminal region of non-phosphorylated protein to keep p47phox in an auto-inhibited state [69, 70]. p47phox is located in the cytosol alone or in a complex containing p40phox and p67phox [71, 72]. When cells are stimulated by protein kinase C activator, eight to nine phosphorylation states of p47phox are observed in cytosol and plasma membrane (Figure J) [73, 74]. The translocation of p47phox to bind membrane associated component of NOX and p22phox causes to activation of NADPH oxidase and then generates O_2^- [73, 75].

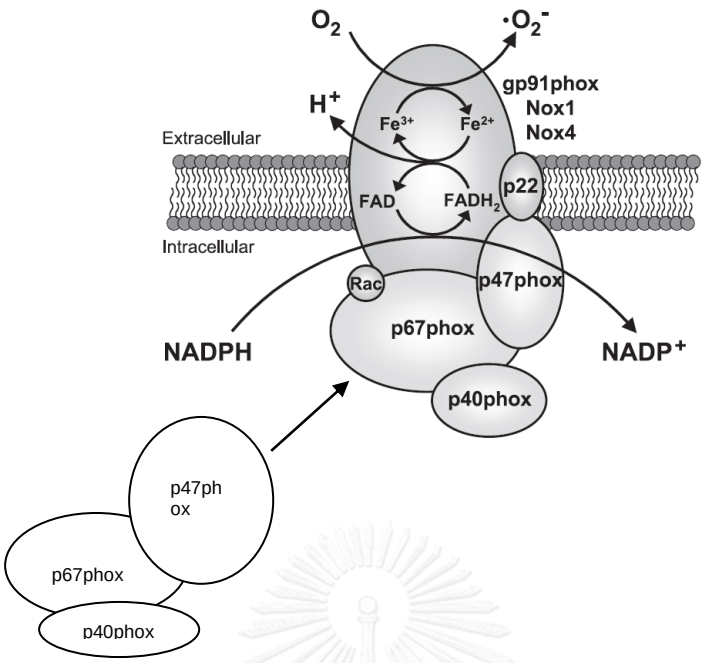


Figure J: Components of NADPH oxidase activation [68]

In the rat kidney, the strongest labeling of p47phox protein is found within the, glomeruli, distal tubules, and collecting ducts of the renal cortex [76]. Lesser staining was detected in the proximal tubules [76]. In renal medulla, the labeling of the p47phox protein appeared to be highest in the vascular bundles, with little labeling within the medullary thick ascending limbs of Henle [76].

DNA-damaging effects of aldosterone are most probably caused by oxidative stress [28]. The induction of DNA strand breaks and chromosomal aberrations could be detected in cultured porcine PT and MDCK cells after 30 minutes aldosterone incubation and continuously rose up to the last timepoint tested (4 hours) [30]. Moreover, spironolactone (MR blocker) partially prevented the action of aldosterone in cultured porcine PT cells [77]. Interestingly, aldosterone-triggered DNA damage was inhibited by pretreatment with apocynin (NADPH oxidase inhibitor) in cultured porcine PT cells [13]. Besides DNA-damaging effects of aldosterone in renal cells, it

has been reported that aldosterone could play a rapid vasoconstrictor effect in isolated rat mesenteric arteries [78]. Furthermore, aldosterone (10^{-12} to 10^{-6} M) produced a dose-dependent vasoconstriction within 5 minutes in isolated mouse mesenteric arterioles [9]. This vasoconstrictor effect did not affect by pretreatment with spironolactone or eplerenone (MR blocker). Surprisingly, cystamine (TG2 inhibitor) significantly suppressed this effect of aldosterone. These data suggest that NADPH oxidase, TG2 and MR are involved in these nongenomic effects of aldosterone [9, 77].

At present, there are no available *in vivo* data regarding the nongenomic actions of aldosterone on protein abundances and localizations of dimeric and monomeric forms (AT_1R and AT_2R), p47phox (NADPH oxidase activation), and TG2 (transglutaminase type 2 which is a major expression in the kidney) in renal tissue. Furthermore, the roles of MR and NADPH oxidase in this regard have not been elucidated. To obtain these results, the nongenomic actions of aldosterone were determined in the present study.

CHAPTER III
MATERIALS AND METHODS

Experimental animals

The study was approved by the Ethics Committee of Research, Faculty of Medicine, Chulalongkorn University. Male Wistar rats weighing 200 to 240 g were obtained from the National Laboratory Animal Center (Mahidol University, Nakorn pathom, Thailand). The animals were housed with controlled temperature (23-25˚C) and 12 hours of controlled light-dark time. The animals were free to access a laboratory chow and distilled water. At the end of each experiment, the rats were terminated with an overdose of thiopental by intraperitoneal (i.p.) injection and their remains were eliminated by burning in an incinerator.

Chemicals

Chemical agents

Aldosterone, eplerenone, dimethyl sulfoxide (DMSO), apocynin, protease inhibitor cocktail, 3, 3'-diaminobenzidine solution, Tris base, glycine, sodium dodecyl sulfate, (3-aminopropyl)triethyloxy-saline, paraffin powder embedding medium, and Bradford protein assay kit were purchased from Sigma, MO, USA. Thiopental was obtained from Jagsonpal Pharmaceuticals, Haryana, India. Methanol, acetic acid, absolute ethanol, 95% ethanol, xylene, sodium chloride, di-sodium hydrogen phosphate, 6% hydrogen peroxide, Tween-20, sucrose, and 40% formaldehyde were purchased from Merck, NJ, USA. Vector Red, Vector SG, and Methyl Green were purchased from Vector Laboratories, CA, USA. Haematoxylin

solution (progressive stain) was purchased from C.V. Laboratory, Bangkok, Thailand. Prestained protein molecular marker was purchased from New England Biolabs, MA, USA. Co-Immunoprecipitation, 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.

Antibodies

Mouse monoclonal antibody against AT_1R (Catalog No. sc-57036, goat polyclonal antibody against AT_2R (Catalog No. sc-7420), mouse monoclonal antibody against p47phox (Catalog No. sc-17845), and β-actin mouse monoclonal antibody (Catalog No. sc-47778) were purchased from Santa Cruz Biotechnology, TX, USA. Mouse monoclonal antibody against transglutaminase type 2 (TG2) (Catalog No. MA5-12915) was purchased from Thermo Scientific, MA, USA. Blotting grade goat anti-mouse IgG (H+L) horseradish peroxidase conjugate (Catalog No. 170-6516) was perchased from Bio-Rad Laboratories, CA, USA. Blotting grade rabbit anti-goat IgG (H+L) horseradish peroxidase conjugate (Catalog No. sc-2768) was perchased from Santa Cruz Biotechnology. ImmPRESS-alkaline phosphatase polymer anti-mouse Ig reagent (Catalog No. MP-5402) and ImmPRESS-peroxidase polymer anti-goat Ig reagent (Catalog No. MP-7405) were purchased from Vector Laboratories, CA, USA.

Experimental procedure

After three days of keeping to familiar with the new housing, the rats were weighed and collected blood samples from tail for measuring creatinine in order to

assess kidney function (<1 mg/dL) [79]. Thirty-two male Wistar rats were divided into four groups (8 rats/group) as follows:

Group 1 (sham): The animals were received normal saline solution (NSS) (0.5 mL/kg BW) by intraperitoneal injection (i.p.) for 30 minutes.

Group 2 (Aldo): The animals were received aldosterone 150 µg/kg BW; diluted in NSS; i.p. for 30 minutes [80].

Group 3 (Ep.+Aldo): The animals were received eplerenone (mineralocorticoid receptor blocker) 15 mg/kg BW; diluted in DMSO; i.p. 30 minutes before aldosterone injection [81].

Group 4 (Apo.+Aldo.): The animals were received apocynin (NADPH oxidase inhibitor) 5 mg/kg BW; diluted in NSS; i.p. 30 minutes before aldosterone injection [82].

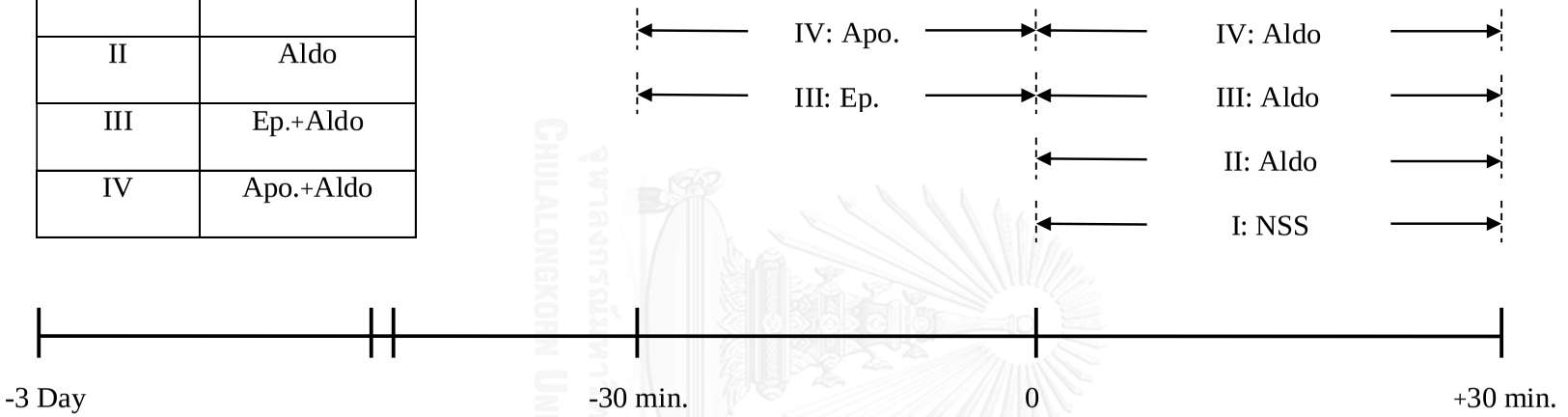
Surgical operation

On the experimental date (between 8.00-11.00 AM), after 30-min injection of NSS or Aldo, or pretreatment with Ep. or Apo. before aldosterone injection, the rats were anesthetized by thiopental (100 mg/kg BW; ip., Jagsonpal Pharmaceuticals) and the abdomen were opened via a midline incision [83]. Blood samples were collected from the abdominal aorta. Blood samples were centrifuged at 1,000 g for 15 minutes at 4°C (H-103N, Kokusan, Tokyo, Japan). Plasma was stored

at -80 °C until use for measurement of blood chemistry. Blood chemistry and electrolytes were measured for sodium, potassium, chloride, bicarbonate, creatinine, and blood urea nitrogen (ion selection electrode) by indirect method (Model CX3, Beckman, Krefeld, Germany). The kidneys were removed, then fixed in liquid nitrogen and then stored at -80 °C until use for measurement of dimeric and monomeric forms of AT_1R or AT_2R, TG2, and p47phox protein abundances by Western blot analysis. In addition, AT_1R/AT_2R heterodimerization was determined by co-immunoprecipitation and then Western blot analysis, respectively. Additional renal tissue samples were fixed in buffered formalin 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 TG2 or p47phox protein by immunohistochemical single staining and colocalization between AT_1R and AT_2R proteins by immunohistochemical double staining.

Experimental design

Group	Treatment
I	sham
II	Aldo
III	Ep.+Aldo
IV	Apo.+Aldo



Baseline
Plasma (Creatinine < 1 mg/dL)

- Experimental due date (anesthetized: thiopental 100 mg/kg BW; i.p.)
- Blood sample collection
- Renal tissues
  - :- Protein abundance (Western blotting)
    - dimeric or monomeric forms of ATRs (AT_1R or AT_2R)
    - immunoprecipitation and Western blotting for AT_1R/AT_2R heterodimerization
    - TG2 and p47phox
  - :- Protein localization and distribution (Immunohistochemistry single staining)
    - TG2 and p47phox
  - :- Protein colocalization and distribution (Immunohistochemistry double staining)
    - AT_1R and AT_2R

Determination of protein concentration by Bradford method

Protein extraction

Each frozen kidney was homogenized separately on ice with a homogenizer (T25 Basic, IKA, Selangor, Malaysia) in homogenizing buffer [20 mM Tris-HCl; pH 7.5, 2 mM $MgCl_2$, 0.2 M sucrose, and 5% (v/v) protease inhibitor cocktail (Sigma, MO, USA)] [6, 80, 84]. To collect homoginated protein, the homoginates were centrifuged at 12,000 g for 20 minutes at 4 °C. The supernatant was used as a homoginated protein [79]. To harvest fraction of plasma membrane protein, the homogenates were centrifuged at 4,000 g (Sorvall Legend X1R, Thermo Scientific, Rockford, IL, USA) for 10 minutes at 4 °C. The supernatant was further centrifuged at 17,000 g for 20 minutes at 4 °C. The pellet was dissolved in buffer and used as a plasma membrane protein [85]. Total protein concentration was measured with Bradford protein assay reagent (Pierce, Rockford, IL, USA) following the manufacturer's protocol.

Total protein concentration assay

Principle: For many years, Bradford protein assay was the most widely used and cited procedure for protein quantitation [86]. The procedure is based on the formation of a complex between the dye of Brilliant Blue G and proteins in solution. The protein-dye complex causes a shift in the absorption, producing a water-soluble product whose blue color can be measured at 595 nm by spectrophotometer (SP 3000 Plus, Optima, Tokyo, Japan). The protocol is performed by the sequential step as follow:

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 tube 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 was a 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 of each standard and unknown sample replicate into an approximately labeled test tube.

2. Add 900 µL Bradford reagent to each test tube. Mix well and 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 the samples.

4. Plot standard curve from the absorbance of standard samples and determine protein concentration of unknown samples.

Determination of protein abundance by Western blot analysis

Principle: Immunoblotting is a widely used and powerful technique for the detection and identification of protein using antibodies. The process involves the separation of sample proteins by polyacrylamide gel electrophoresis (PAGE) followed by transferring of the separated protein from the gel onto a thin support membrane. The membrane binds and immobilizes the proteins in the same pattern as in the original gel. The membrane (or "blot") is then exposed to a solution containing antibodies that recognize and bind to the specific protein of interest. The antibodies bound to the membrane are detected by any of a variety of techniques, usually involving treatment with a secondary antibody.

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

Principal: The most widely used method for qualitative analysis of a protein mixture is SDS-PAGE by using the buffer system of Laemmli [87]. In the process, proteins migrate in response to an electrical field through pores in the gel

matrix and separate based on molecular size after sample proteins are solubilized in the presence of anionic detergent and 2-Mercaptoethanol (2-ME). The 2-ME is a disulfide reducing agent, and serves to reduce disulfides holding together the tertiary structure of the protein. The anionic SDS detergent binds strongly to the protein thus disrupting its secondary, tertiary and quaternary structure, resulting in a linear polypeptides chain coated with negatively charged SDS molecules. The binding efficiency of the SDS is generally one SDS molecule for every two amino acid residues. Under this condition, the polypeptide chains are unfold and assumed a rod-like structure and have negative charge, 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. Separation is determined by size and therefore when compared to standards of known molecular weight, 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:

Assembly of apparatus

The reagents used in preparing the gel should be removed from the refrigerator and allowed to warm and degas for one hour period to the preparation of the gel. Meanwhile, the sandwich plates were set up for casting the gel. A sandwich consists of two rectangular glass plates: the outer is 10.1 cm (width) x 8.3 cm (height) and the inner is 10.1 cm (width) x 7.3 cm (height) separated by spacer of 0.75 mm thickness (Mini-Protein® 3 cell, Bio-Rad, USA). In order to prepare flawless gel, one containing has no air bubble or debris, the glass plates must be perfectly cleaned and dry with absolute ethanol. The casting clamps were used to mount the outer and inner glass plates facing together.

Preparation of slab gel

For separating gel, 8% 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. The gel was allowed to polymerize at room temperature for 30 minutes. 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. It should be made sure that no air bubbles formed around the teeth of comb, as they will impede the migration and separation of the proteins. The gel was allowed to polymerize for one hour at room temperature.

Preparation of sample

During polymerizing the 4% stacking gel, equal amounts of total protein from each sample were mixed with sample buffer. The amount of total protein was used 250 µg for AT_1R or AT_2R or p47phox and 150 µg for TG2.

Elecrophoresis

After polymerization was complete, the comb was gently removed. The wells were filled with running buffer (Appendix). The gel was mounted in the electrophoresis apparatus and the upper buffer chamber was filled with running buffer. Remove any air bubbles trapped at the bottom of the wells. This will disrupt the electrical circuit and an uneven electrophoresis. Each protein sample was loaded into the bottom of each well. 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 volts. 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.

Protein transfer

A nitrocellulose membrane (Trans-Blot®, Bio-Rad, USA) and two sheets of absorbent filter paper were cut into the same size of the gel. The membrane, filter papers, and support pads were soaked in the transfer buffer for 10 minutes. The transfer cassette was assembled by lying the black side down, then plate a support pad, filter paper, gel, membrane, filter paper, and support pad. Lock 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 120 volt. After transfer, the membrane was removed from the cassette and immersed into the blocking solution.

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 a platform rocker (Stuart Orbital Shaker SO3, Manchester, UK). This process will reduce the background of non-specific binding site with irrelevant protein. After blocking, the membrane was washed for 5 minutes three times by TBS-T washing buffer.

Detection of bound antibody

The dilution of primary antibody was prepared in TBS-T (1:200 for AT_1R, sc-57036, Santa Cruz Biotechnology), (1:300 for AT_2R, sc-7420, Santa Cruz Biotechnology), (1:250 for p47phox, sc-17845, Santa Cruz Biotechnology), (1:2000 for β-actin, sc-47778, Santa Cruz Biotechnology) and in 3% non fat dry milk with TBS-T (1:1,000 for TG2, MA5-12915, Thermo Scientific). The membrane, in a clean plastic box, was incubated with the respective primary antibody overnight at 4 $^{\circ}$C on a platform rocker. 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 with TBS-T. The goat anti-mouse IgG conjugated HRP antibody at 1:5,000 dilution was used for AT_1R, p47phox, TG2, and β-actin. The rabbit anti-goat IgG conjugated HRP antibody at 1:5,000 dilution was used for AT_2R. The membrane was incubated in the secondary antibody for 1 hour at room temperature on a platform rocker. After that, the blotting membrane was washed with TBS-T for 10 minutes three times on a platform rocker. Finally, it was washed with TBS for 5 minutes two times on a platform rocker.

Protein detection and image analysis

The blot membrane was placed on a cleaned glass plate. The detection reagent (SuperSignal® West Pico kit, Pierce, USA) was prepared by mixing equal parts of the stable peroxide solution and the luminal/enhancer solution, 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. It is necessary to work quickly once the membrane has been exposed to the detection solution. The immunoreactive proteins were documented by using a Molecular imager ChemiDoc XRS system (Bio-Rad Laboratories). 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 Laboratories). The intensity ratio of each studied protein to β-actin, respectively was calculated.

Isolation of native protein complexs by co-immunoprecipitation

Principle: Co-immunoprecipitation (co-IP) is a common approach to study two proteins interaction that use an antibody to immunoprecipitate the antigen (bait protein) and co-immunoprecipitate any interacting protein (prey protein). Isolation of native protein complexes from a tissue lysate or other complex 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 instructions

A. Antibody Immobilization using AT_1R 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 cut pipette tip, add 50 µL of the resin slurry into a Pierce spin column. Place column into a microcentrifuge tube and centrifuge at 1,000 × g for 1 minute. Discard the flow-through.
4. Wash resin twice by adding 200 µL of 1X Coupling buffer, centrifuge at 1,000 × 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-AT1R antibody (sc-57036, 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 90 µL of ultrapure water, 10 µL of 20X Coupling buffer, and 100 µL of antibody at 1 µg/ 10 µL directly to the resin in the spin column.

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 120 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 1,000 × 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 1,000 × 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 1,000 × 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 1,000 × 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 1,000 × g for 1 minute after each wash.

16. Wash the resin six times with 150 µL of Wash solution, centrifuging at 1,000 × g for 1 minute after each wash.

B. Co-IP

• Perform all co-IP steps at 4°C.

1. Mix the bait and prey proteins from a tissue lysate (plasma membrane protein).

2. Dilute 300 µg of plasma membrane protein samples in 200 µL IP Lysis/Wash buffer, load total sample volume into the spin column, centrifuge at 1,000 × g for 1 minute, and discard the flow-through.

3. Wash the resin twice by adding 200 µL of IP Lysis/Wash buffer to the spin column containing the antibody-coupled resin, centrifuge at 1,000 × g for 1 minute and discard the flow-through.

4. Gently tap the bottom of the spin column on a paper towel to remove excess liquid and insert the bottom plug.

5. Add the diluted plasma membrane protein to the appropriate resin. Attach cap and incubate with gentle mixing for overnight at 4 ºC.

6. Remove the bottom plug, loosen the screw cap and place the column in a collection tube. Centrifuge the spin columns at 1,000 × g for 1 minute. Save the flow-through for future analysis.

7. Remove the screw cap, place the column into a new tube, add 200 µL of IP Lysis/Wash buffer and centrifuge.

8. Wash the sample two more times with 200 µL IP Lysis/Wash buffer and centrifuge at 1,000 × g for 1 minute after each wash.

C. Elution of Co-IP

1. Place the spin column into a new collection tube. Add 10 µL of Elution Buffer and centrifuge at 1,000 × g for 1 minute.

2. Keep the column in the tube and add 50 µL of Elution Buffer. Incubate for 5 minutes at room temperature. The column does not need to be mixed.

3. Centrifuge the tube and collect the flow-through. Analyze the flow-through for protein. Perform additional elutions (i.e., Steps C1-C3) as needed. Aproximatly 50 μL of eluted protein sample contains all precipitated protein which can interact with AT_1R or monomeric AT_1R form. Analyze each flow-through fraction separately to ensure that the antigen has completely eluted. 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.

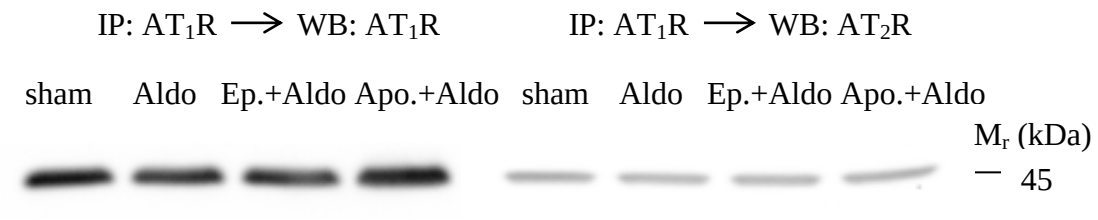
Procedure of Western blotting for AT_1R/AT_2R heterodimers detection

1. In order to examine AT_1R/AT_2R heterodimers in eluted protein samples, 15 μg of each plasma membrane protein from eluted samples was mixed with Laemmli sample buffer and loaded on 8% SDS-PAGE in each lane.
   - Lane 1-4 were loaded with protein samples (sham, Aldo, Ep.+Aldo, Apo.+Aldo)
   - Lane 5 was omitted.
   - Lane 6-10 were loaded with the same pattern samples as in lane 1-4.
2. Protein samples in SDS-PAGE were transferred into a nitrocellulose membrane. The membrane was cut to separate into 2 pieces: lane 1-4 and lane 6-10.

3. To examine AT_1R protein interacted with AT_2R protein, the lane 1-4 membrane was probed with monoclonal anti-AT_1R antibody (1:200, sc-57036, Santa Cruz Biotechnology) at 4 °C overnight. [56, 88]. The lane 6-10 membrane was probed with polyclonal anti-AT_2R antibody (1:200, sc-7420, Santa Cruz Biotechnology) at 4 °C overnight [56, 88].
4. The membranes were washed three times for 10 minutes each with 15 mL of TBS-T.
5. The membranes were incubated with respective horseradish peroxidase-linked secondary antibody (1:5000) 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).
9. The mean AT_2R/AT_1R immunoreactive signal ratio in arbitrary unit (a.u.) represents AT_1R/AT_2R heterodimerization [56, 89].

Calculation for the mean AT_2R /AT_1R immunoreactive signal ratio in arbitrary unit (a.u.)

For example



Step.1 Measure band intensity

a_1 b_1 c_1 d_1 a_2 b_2 c_2 d_2

Step.2 Calculate the ratio of AT_2R to AT_1R intensity

Ratio of AT_2R to AT_1R intensity in sham = a_2/a_1

Ratio of AT_2R to AT_1R intensity in Aldo = b_2/b_1

Ratio of AT_2R to AT_1R intensity in Ep.+Aldo = c_2/c_1

Ratio of AT_2R to AT_1R intensity in Apo.+Aldo = d_2/d_1

Step. 3 Calculate the AT_2R/AT_1R immunoreactive signal ratio in a.u.

$$AT_2R/AT_1R \text{ immunoreactive signal ratio in a.u.} = \frac{\text{Ratio of } AT_2R \text{ to } AT_1R}{\text{Ratio of } AT_2R \text{ to } AT_1R \text{ in sham}}$$

AT_2R/AT_1R immunoreactive signal ratio in a.u. of sham $= \dfrac{a_2/a_1}{a_2/a_1} = 1$

AT_2R/AT_1R immunoreactive signal ratio in a.u. of Aldo $= \dfrac{b_2/b_1}{a_2/a_1}$

AT_2R/AT_1R immunoreactive signal ratio in a.u. of Ep.+Aldo $= \dfrac{c_2/c_1}{a_2/a_1}$

AT_2R/AT_1R immunoreactive signal ratio in a.u. of Apo.+Aldo $= \dfrac{d_2/d_1}{a_2/a_1}$

Step. 4 Calculate the mean AT_2R/AT_1R immunoreactive signal ratio in a.u.

Mean AT_2R/AT_1R immunoreactive signal ratio in a.u. $= \dfrac{\sum X}{n}$

$\sum X$ = summation of the AT_2R/AT_1R immunoreactive signal ratio in a.u.

n = sample size

$$= \frac{\left\{ \dfrac{(b_2/b_1)_i}{(a_2/a_1)_i} + \dfrac{(b_2/b_1)_{ii}}{(a_2/a_1)_{ii}} + \dfrac{(b_2/b_1)_{iii}}{(a_2/a_1)_{iii}} + \dfrac{(b_2/b_1)_{iv}}{(a_2/a_1)_{iv}} + \dfrac{(b_2/b_1)_v}{(a_2/a_1)_v} + \dfrac{(b_2/b_1)_{vi}}{(a_2/a_1)_{vi}} + \dfrac{(b_2/b_1)_{vii}}{(a_2/a_1)_{vii}} + \dfrac{(b_2/b_1)_{viii}}{(a_2/a_1)_{viii}} \right\}}{n}$$

i to viii = membrane number

For example

Calculation the mean AT_2R/AT_1R immunoreactive signal ratio in a.u. of Aldo from membranes i-viii

$$= \left\{ \begin{matrix} (322{,}435.17/\ 1{,}171{,}486.49) \\ (351{,}834.20/\ 1{,}301{,}637.53) \end{matrix} \right. + \begin{matrix} (103{,}283.32/\ 331{,}345.32) \\ (102{,}032.54/\ 330{,}013.32) \end{matrix} + \left. \begin{matrix} (103{,}486.55/\ 338{,}354.41) \\ (102{,}674.32/\ 330{,}332.45) \end{matrix} \right\} +$$

$$\begin{matrix} (73{,}382.81/\ 157{,}453.22) \\ (63{,}245.32/\ 159{,}352.37) \end{matrix} + \begin{matrix} (107{,}840.88/\ 243{,}214.95) \\ (115{,}716.23/\ 270{,}082.50) \end{matrix} + \begin{matrix} (328{,}721.17/\ 960{,}254.80) \\ (335{,}009.20/\ 991{,}172.20) \end{matrix} +$$

$$\left. \begin{matrix} (70{,}438.46/\ 154{,}532.54) \\ (64{,}538.26/\ 154{,}653.74) \end{matrix} + \begin{matrix} (105{,}367.16/\ 254{,}230.45) \\ (105{,}374.89/\ 253{,}947.50) \end{matrix} \right\} \div 8$$

$$= (1.018 + 1.008 + 0.984 + 1.174 + 1.034 + 1.012 + 1.092 + 0.998) \div 8$$

$$= 1.04$$

The mean AT_2R/AT_1R immunoreactive signal ratio in a.u. of Aldo = 1.04

To examine the distribution and localization of studied proteins

Principle: Immunohistochemistry (IHC) is a method for demonstrating the presence and location of proteins in tissue section. It enables the observation of processes in the context of intact tissue. Immunohistochemical single staining is accomplished with antibodies that recognize the target protein. The antibodies will bind only to the protein of interest in the tissue section. Besides single staining, immunohistochemical double staining is a valuable technique that permits precise co-

localization of two specific proteins (antigens) within a single cell or in different cell types within the same tissue. 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 single staining for TG2 or p47phox

Paraffin-embedded kidney sections were cut at 4-μm thickness in a serial two sections for examination of TG2 or p47phox protein. Tissue sections were mounted on 3-aminopropyltriethyloxy-saline-coated slides (Sigma, USA). Antigen retrieval was performed by antigen unmasking solution (Vector Laboratories, USA) in the PreTreatment Module (PT Link; Thermo Fisher Scientific/Labvision, USA) for 20 minutes at 95 ^{0}C and cool-down to 60 ^{0}C and then incubate in phosphate buffer solution and 0.1% Tween (PBS-T) for 20 minutes at room temperature. After wash the slides 2 x 5 minutes with PBS-T, endogenous peroxidase activity was quenched in 3% hydrogen peroxide for 5 minutes. The slides were washed with running tap water for 5 min and 2 x 5 minutes with PBS-T, respectively. The non-specific binding of the antibody was blocked by incubating slides with 2.5% normal horse serum (Vector Laboratories) for 20 minutes at room temperature. Next, the slides were incubated in 150 μL primary antibody against TG2 (1:20,000, MA5-12915, Thermo Scientific) or p47phox (1:150, sc-17845, Santa Cruz Biotechnology) in 2.5% normal horse serum for overnight at room temperature. The slides were then rinsed 3 x 5 minutes with PBS-T and incubated with 150 μL Polymer horse anti-mouse secondary antibody (Vector Laboratories) (ready to use) for 30 minutes at room temperature. After

incubation, the slides were rinsed 3 x 5 minutes with PBS-T and then 1 x 5 minutes in PBS. The sections were reacted for peroxidative activity in 3, 3′-diaminobenzidine (DAB) solution (Sigma, USA) for 5 minutes. Then, the sections were washed with running tap water for 5 min and 2 x 5 minutes with distilled water, respectively. Next, the sections were counterstained with haematoxylin (CV Laboratories, Thailand) and coverslipped with permount.

Areas of staining were identified 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) [90].

Immunohistochemical double staining for AT receptors (AT_1R and AT_2R)

1. Paraffin-embedded kidney sections were cut at 4-µm thickness in a serial cutting of three tissue sections for examination AT_1R protein (single staining, red color), AT_2R protein (single staining, blue-grey color), AT_1R and AT_2R proteins (double staining, mixed color).
2. Tissue sections were mounted on 3-aminopropyltriethyloxy-saline-coated slides (Sigma) and then labeled slide title (AT_1R, AT_2R, AT_1R and AT_2R).
3. Antigen retrieval was performed by antigen unmasking solution (Dako, Glostrup, Denmark) in the PreTreatment Module (PT Link; Thermo Scientific) for 20 minutes at 95 °C and cool-down to 60 °C. And then the slides were incubated in PBS-T for 20 minutes at room temperature.
4. The slides were washed 2 x 5 minutes PBS-T.

5. Next, Ultra V Block, an endogenous horse radish peroxidase/alkaline phosphatase blocking solution (Thermo Scientific) was applied for 10 minutes at room temperature and then washed 1 x 5 minutes PBS-T.

6. Tissue sections were incubated in 2.5% normal horse serum (Vector Laboratories) for 20 minutes at room temperature and then rinse out the serum.

7. Tissue sections were incubated in primary antibodies in 2.5% normal horse serum for overnight at room temperature.

   - Labeled AT_1R slides were incubated with mouse monoclonal antibody against AT_1R (1:20, sc-57036, Santa Cruz Biotechnology).

   - Labeled AT_2R slides were incubated with goat polyclonal antibody against AT_2R (1:20, sc-7420, Santa Cruz Biotechnology)

   - Labeled AT_1R and AT_2R slides were firstly incubated with goat polyclonal antibody against AT_2R (1:20, sc-7420, Santa Cruz Biotechnology)

8. Tissue sections were washed 3 x 5 minutes PBS-T. Tissue sections were added secondary antibodies (ready-to-use, Vector Laboratories) for 30 minutes at room temperature.

   - Labeled AT_1R slides were incubated with ImmPRESS-alkaline phosphatase polymer anti-mouse Ig reagent.

   - Labeled AT_2R slides were incubated with ImmPRESS-peroxidase polymer anti-goat Ig reagent.

- Labeled AT_1R and AT_2R slides were firstly incubated with ImmPRESS-peroxidase polymer anti-goat Ig reagent.

9. Tissue sections were washed 3 x 5 minutes PBS-T. Tissue sections were applied a proper substrate (Vector Laboratories) at room temperature.
   - Labeled AT_1R slides were incubated with Vector Red substrate for 30 minutes.
   - Labeled AT_2R slides were incubated with Vector SG substrate for 2 minutes.
   - Labeled AT_1R and AT_2R slides were firstly incubated with Vector SG substrate for 2 minutes.
10. The slides were rinsed 5 minutes in running tap water.
11. The slides were counterstained with Methyl Green (Vector Laboratories) for 4 minutes at 60 °C, washed with acetone containing 0.05% acetic acid and then coverslipped with permount. Exception of labeled AT_1R and AT_2R slides were not counterstained but they were repeated in steps 6-13 and treated them as labeled AT_1R slides.

A tissue section was randomly selected five different fields in each renal cortex, outer medulla, and inner medulla. Total tissue sections are 32 sections from sham, Aldo, Ep.+Aldo, and Apo.+Aldo groups (n = 8 rats/group). The percentage of cell area positive both AT_1R and AT_2R and cell area positive for AT_2R were measured from the same field of photograph and analyzed using a computer image analyzer (Image-Pro Plus version 6.0, Media Cybernetics, MD, USA) [91].

Calculation for the mean percentage of cell area positive for AT_2R and the mean percentage of cell area positive both AT_2R and AT_1R in the cortex

For example

Step. 1 Measure the cell area

1.1 Measure the cell area positive for AT_2R in the cortex = a

1.2 Measure the cell area positive both AT_2R and AT_1R in the cortex = ab

1.3 Measure the total area of studied field = T

Step.2 Calculate the percentage of cell area positive

$$\text{Percentage of cell area positive} = \frac{\text{cell area positive}}{\text{total area of studied field}} \times 100$$

2.1 Percentage of cell area positive for AT_2R in the cortex = $\frac{a}{T} \times 100$

2.2 Percentage of cell area positive both AT_2R and AT_1R in the cortex = $\frac{ab}{T} \times 100$

Step. 3 Calculate the mean percentage of cell area positive

$$\text{Mean percentage of cell area positive} = \frac{\sum X}{n}$$

$\sum X$ = summation of the percentage of cell area positive

n = number of studied fields

3.1 Mean percentage of cell area positive for AT_2R in the cortex ($\overline{a_1}$) $= \dfrac{[(a_1/T_1)_i + (a_2/T_2)_{ii} + (a_3/T_3)_{iii} + (a_4/T_4)_{iv} + (a_5/T_5)_v]}{n}$

3.2 Mean percentage of cell area positive both AT_2R and AT_1R in the cortex ($\overline{ab_1}$) $= \dfrac{[(ab_1/T_1)_i + (ab_2/T_2)_{ii} + (ab_3/T_3)_{iii} + (ab_4/T_4)_{iv} + (ab_5/T_5)_v]}{n}$

$\overline{a_1}$ = mean percentage of cell area positive for AT_2R in the cortex from the rat number 1 of each group (sham, Aldo, Ep.+Aldo, Apo.+Aldo)

$\overline{ab_1}$ = mean percentage of cell area positive both AT_2R and AT_1R in the cortex from the rat number 1 of each group (sham, Aldo, Ep.+Aldo, Apo.+Aldo)

i to v = number of studied fields in the cortex

For example

Calculation the mean percentage of cell area positive for AT_2R and the mean percentage of cell area positive both AT_2R and AT_1R in the cortex of sham group

There are 8 rats in each group. Therefore, the average values of sham group are calculated as follows.

Mean percentage of cell area positive for AT_2R in the cortex of sham group

$$= \frac{(\overline{a_1} + \overline{a_2} + \overline{a_3} + \overline{a_4} + \overline{a_5} + \overline{a_6} + \overline{a_7} + \overline{a_8})}{(n = 8)}$$

$$= \frac{(0.37 + 0.84 + 0.42 + 0.39 + 0.80 + 0.37 + 0.67 + 0.38)}{8}$$

$$= 0.53$$

Mean percentage of cell area positive both AT_2R and AT_1R in the cortex of sham group

$$= \frac{(\overline{ab_1} + \overline{ab_2} + \overline{ab_3} + \overline{ab_4} + \overline{ab_5} + \overline{ab_6} + \overline{ab_7} + \overline{ab_8})}{(n = 8)}$$

$$= \frac{(0.91 + 1.29 + 1.13 + 2.16 + 1.33 + 1.38 + 1.31 + 2.14)}{8}$$

$$= 1.46$$

Statistical analysis

The results were presented as mean ± SD (dimeric or monomeric form of ATRs, AT_1R/AT_2R heterodimerization, TG2, p47phox, and the percentage of cell area positive for both AT_1R and AT_2R or cell area positive for only AT_2R). Statistical differences of protein abundances among groups were assessed by one way analysis of variance (one-way ANOVA) with post hoc comparison by Tukey's test where appropriate while the percentages of cell area positive for both AT_1R and AT_2R and cell area positive for only AT_2R within each group were evaluated by Paired t-test. A probability value (p-value) of less than 0.05 was considered to be statistically significant. The intensity scores of renal protein expressions of p47phox and TG2 were presented in descriptive statistics by measures of central tendency (median). The statistical calculations were performed by IBM SPSS Statistics program version 22.0 (SPSS Inc., Chicago, IL, USA).

CHAPTER IV
RESULTS

Metabolic parameters

All studied groups had no significant changes in plasma sodium, potassium, chloride, bicarbonate, creatinine, and blood urea nitrogen as shown in Table 1.

Effect of aldosterone on plasma membrane dimeric forms of AT_1R and AT_2R

By Western blotting analysis, representative bands of plasma membrane protein abundances of dimeric form of AT_1R (100 kDa) and monomeric form of AT_1R (50 kDa) were shown in Figure 1. Aldosterone enhanced the protein abundance of dimeric form of AT_1R from sham 100% to be 278.2 ± 22.5% ($p < 0.001$). Pretreatment with eplerenone (MR blocker) did not alleviate the dimeric form of AT_1R (276.2 ± 21.1%, $p < 0.001$) whereas pretreatment with apocynin (NADPH oxidase inhibitor) prevented the formation of dimerized AT_1R (102.3 ± 3.5%, $p = 0.83$). No significant changes in monomeric form of AT_1R were observed in all studied groups (sham = 100%; Aldo = 98.6 ± 7.2%; Ep.+Aldo = 99.5 ± 5.3%; Apo.+Aldo = 99.8 ± 3.2%) (Figure 1).

For AT_2R, representative bands of plasma membrane protein abundances of dimeric form (80 kDa) and monomeric form (41 kDa) were shown in Figure 2. Aldosterone stimulated the protein abundance of dimeric form of AT_2R from sham 100% to be 182.3 ± 15.3% ($p < 0.001$). Pretreatment with eplerenone or apocynin alleviated the dimerized AT_2R proteins (108.1 ± 10.3%, $p = 0.32$ and 102.71 ± 4.3, $p = 0.52$), respectively. No significant changes in monomeric form of AT_2R were

noted in all studied groups (sham = 100%; Aldo = 95.2 ± 6.1%; Ep.+Aldo = 94.3 ± 3.8%; Apo.+Aldo = 100.5 ± 4.8%) (Figure 2).

Table 1: Metabolic parameters in sham, Aldo, Ep.+Aldo, and Apo.+Aldo groups

Parameters	Groups				
	sham	Aldo	Ep.+Aldo	Apo.+Aldo	p-value
Plasma Na^+ (mmol/L)	143.38 ± 1.56	140.33 ± 5.31	145.25 ± 2.69	144.15 ± 2.52	0.09
Plasma K^+ (mmol/L)	3.42 ± 0.27	3.55 ± 0.24	3.40 ± 0.34	3.67 ± 0.55	0.55
Plasma Cl^- (mmol/L)	102.88 ± 1.39	102.22 ± 3.61	104.68 ± 1.66	104.17 ± 1.31	0.22
Plasma HCO_3^- (mmol/L)	24.62 ± 1.24	23.63 ± 1.78	27.00 ± 2.09	24.53 ± 3.73	0.12
Plasma creatinine (mg/dL)	0.24 ± 0.02	0.24 ± 0.02	0.24 ± 0.03	0.28 ± 0.05	0.14
Blood urea nitrogen (mg/dL)	18.25 ± 1.70	20.60 ± 3.35	17.75 ± 3.64	20.15 ± 4.79	0.44

Data are expressed as mean ± SD, n = 8 rats/group.
Aldo = aldosterone group, Ep.+Aldo = pretreatment with eplerenone (MR blocker) before aldosterone injection,
Apo.+Aldo = pretreatment with apocynin (NADPH oxidase inhibitor) before aldosterone injection

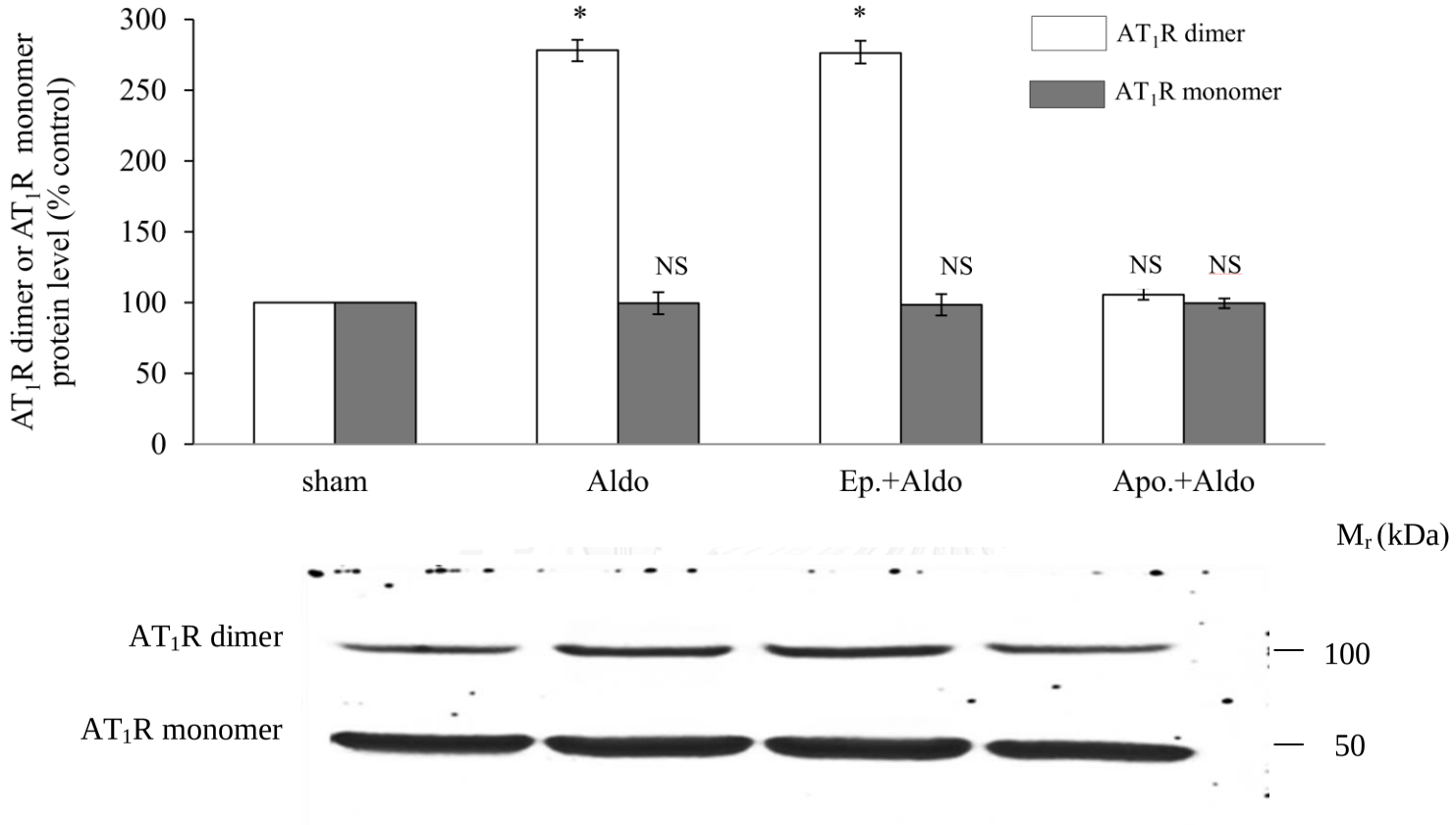


Figure 1: Renal dimeric and monomeric forms of AT_1R proteins abundance in sham, Aldo, Ep.+Aldo, and Apo.+Aldo groups by Western blot analysis. Plasma membrane proteins from renal tissues were separated by SDS-PAGE, transferred to nitrocellulose membrane and subjected to immunoblot analysis. The monoclonal antibody to AT_1R was used. Histogram bars show the densitometric analyses of AT_1R dimer or AT_1R monomer, and the representative immunoblot photographs are presented. Data are means $\pm$ SD of 8 independent experiments. *$p < 0.001$ *vs.* sham

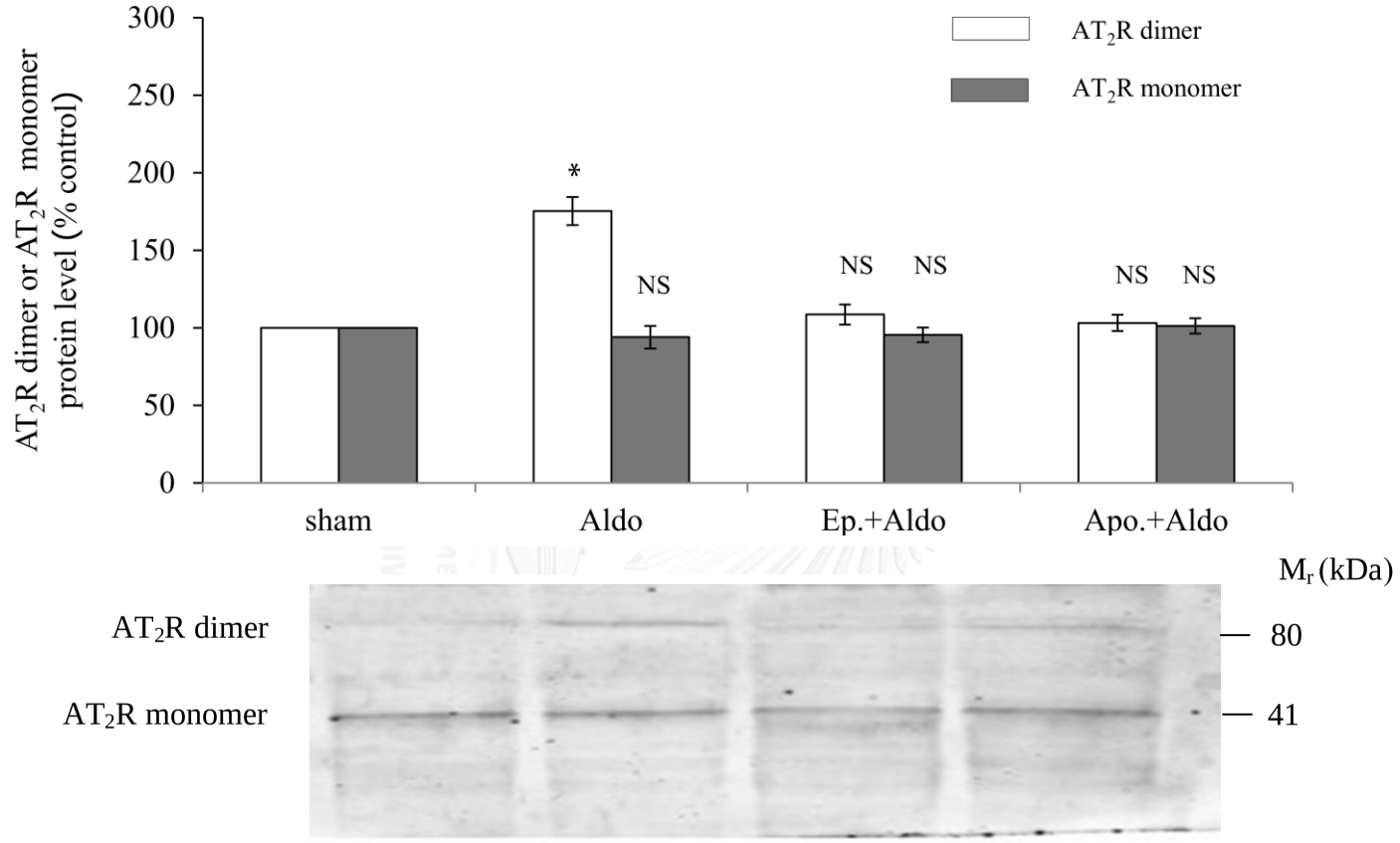


Figure 2: Renal dimeric and monomeric forms of AT_2R proteins abundance in sham, Aldo, Ep.+Aldo, and Apo.+Aldo groups by Western blot analysis. Plasma membrane proteins from renal tissues were separated by SDS-PAGE, transferred to nitrocellulose membrane and subjected to immunoblot analysis. The polyclonal antibody to AT_2R was used. Histogram bars show the densitometric analyses of AT_2R dimer or AT_2R monomer, and the representative immunoblot photographs are presented. Data are means $\pm$ SD of 8 independent experiments. *$p < 0.001$ *vs.* sham

Effect of aldosterone on plasma membrane AT_1R/AT_2R heterodimers

To examine protein abundance of heterodimeric forms between AT_1R and AT_2R. By co-IP technique, AT_1R was first immunoprecipitated with anti-AT_1R antibody and then the nitrocellulose membrane was probed with anti-AT_2R antibody by Western Boltting. The density of 45-kDa band is AT_2R protein, (the upper band, Figure 3). Mean intensity values of AT_2R in each group were presented (sham = 155,053.1 ± 117,897.4, Aldo = 151,869.4 ± 108,208.0, Ep.+Aldo = 153,083.7 ± 117,535.4, Apo.+Aldo = 160,385.3 ± 130,986.2). Next, the identical loading protein from immunoprecipitated with anti-AT_1R antibody was determined in each experiment (the lower band, Figure 3). Mean intensity values of AT_1R in each group were demonstrated (sham = 473,899.0 ± 428,421.4, Aldo = 451,359.0 ± 389,395.5, Ep.+Aldo = 458,463.8 ± 421,095.3, Apo.+Aldo = 469,785.0 ± 458,709.3). Mean ratio of AT_2R to AT_1R intensity values in all groups were demonstrated (sham = 0.36 ± 0.06, Aldo = 0.37 ± 0.08, Ep.+Aldo = 0.37 ± 0.08, Apo.+Aldo = 0.38 ± 0.08). Finally, mean AT_2R/AT_1R immunoreactive signal ratios in arbitrary unit (a.u.) in all groups were shown in Figure 3 (sham = 1, Aldo = 1.04 ± 0.06, Ep.+Aldo = 1.04 ± 0.06, Apo.+Aldo = 1.05 ± 0.05). The results demonstrated that aldosterone did not alter the formation of plasma membrane AT_1R/AT_2R heterodimerization (Aldo = 1.04 ± 0.06 a.u., p = 0.342) as compared with the sham group (sham = 1 a.u.) (Figure 3). Moreover, pretreatment with eplerenone or apocynin for 30 minutes before aldosterone administration had no effect on AT_1R/AT_2R heterodimerization (Ep.+Aldo = 1.04 ± 0.06 a.u., p = 0.229; Apo.+Aldo = 1.05 ± 0.05 a.u., p = 0.072, respectively).

Effect of aldosterone on renal transglutaminase 2 (TG2)

As shown in Figure 4, aldosterone increased homoginated protein abundance of TG2 (165.6 ± 8.2%, $p < 0.001$) as compared with the sham group (100%) while pretreatment with eplerenone or apocynin abolished the effect of aldosterone (97.3 ± 5.1%, $p = 0.48$; 110.3 ± 10.2%, $p = 0.40$, respectively).

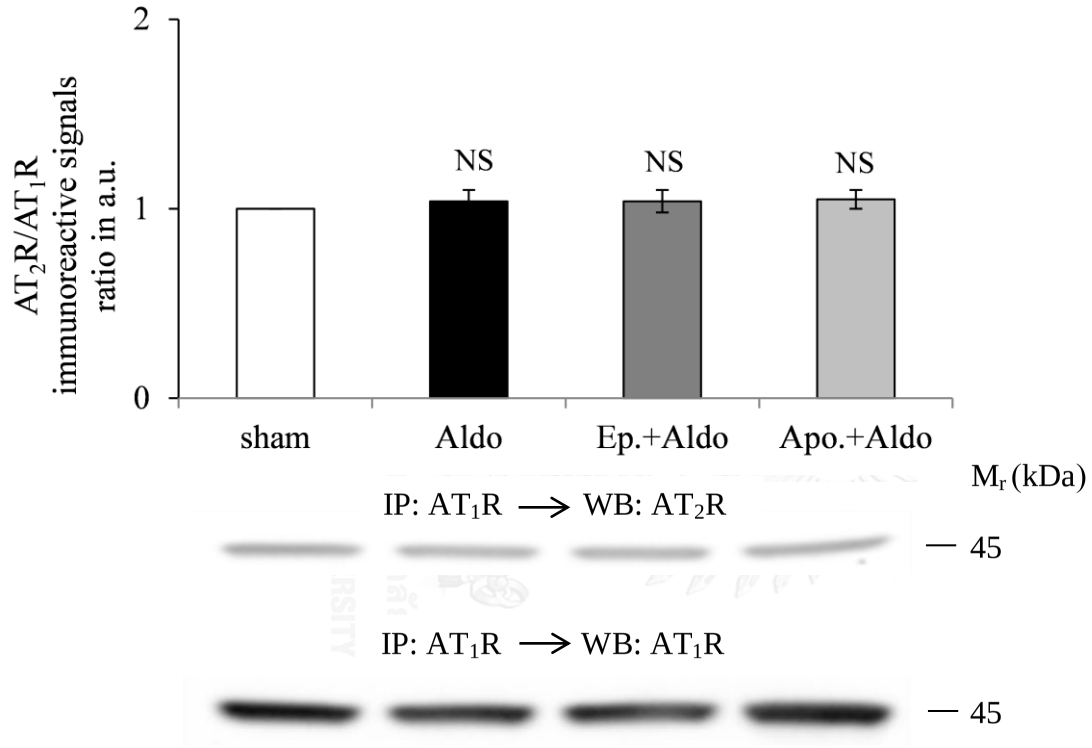


Figure 3: Renal AT_1R/AT_2R heterodimerization protein abundance in sham, Aldo, Ep.+Aldo, and Apo.+Aldo groups by Western blot analysis. Plasma membrane fractions from renal tissues in each group were immunoprecipitated with monoclonal anti-AT_1R antibody and then examined by Western blotting with polyclonal anti-AT_2R antibody (IP: $AT_1R \longrightarrow$ WB: AT_2R). The identical protein loading of plasma membrane fractions were probed with monoclonal anti-AT_1R antibody (IP: $AT_1R \longrightarrow$ WB: AT_1R). Histogram bars show densitometric analysis ratios between AT_2R and AT_1R. Data are means ± SD of 8 independent experiments. (IP = immunoprecipitation, WB = Western blotting).

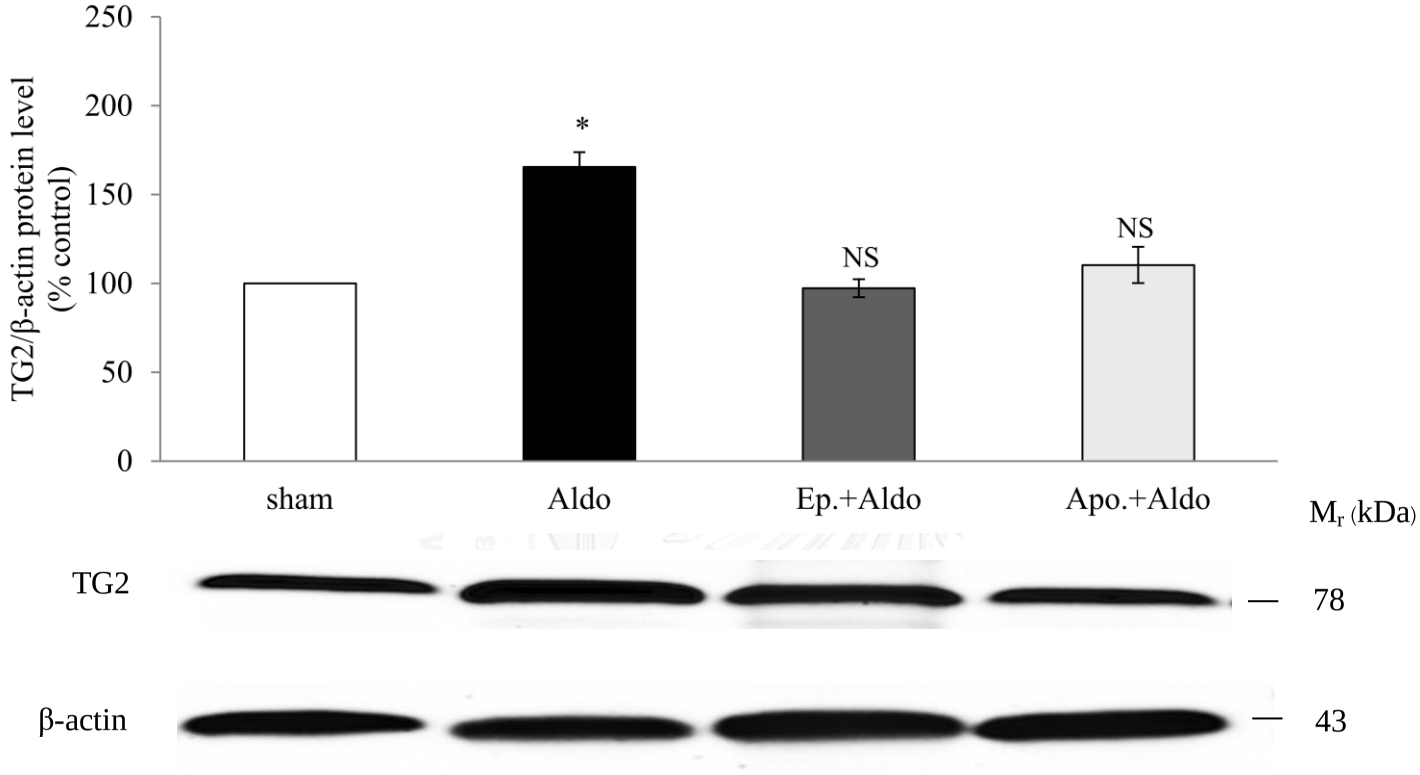


Figure 4: Renal TG2 protein abundance in sham, Aldo, Ep.+Aldo, and Apo.+Aldo groups by Western blot analysis. Homoginated proteins from renal tissues were separated by SDS-PAGE, transferred to nitrocellulose membrane and subjected to immunoblot analysis. The monoclonal antibody to TG2 was used. Histogram bars show the densitometric analyses ratios of TG2 to β-actin intensity, and the representative immunoblot photographs are presented. Data are means ± SD of 8 independent experiments. *$p < 0.001$ *vs.* sham

Effect of aldosterone on renal TG2 protein localization

In the cortex, TG2 protein expression of sham group expressed in grade 1 (trace) in the glomerulus and markedly expressed in grade 4 (strong) in the peritubular capillary (Pcap) (Figure 5a and Table 2). The staining intensity was diffuse in grade 3 (moderate) in the proximal convoluted tubule (PCT). There are no staining intensity in distal convoluted tubule (DCT) and cortical collecting duct (CCD). Aldosterone did not change the staining intensity in these areas (Figure 5b). Of note, Aldosterone increased TG2 protein localization at the luminal membrane of PCTs as compared with the sham group (Figure 5a and b). Pretreatment with eplerenone or apocynin normalized the localization pattern of TG2 protein in PCT to be the same as in the sham group (Figure 5c and d).

In the outer medulla, aldosterone increased the staining intensity in the thick ascending of Henle's loop (TALH), the medullary collecting duct (MCD), vasa recta (VR), and thin limb of Henle's loop (tLH) (Figure 5f). Pretreatment with eplerenone or apocynin inhibited the effect of aldosterone on the staining intensity in all areas, except the staining intensity at MCD was sustainable (Figure 5g and h). In the inner medulla, aldosterone also elevated the staining intensity in MCD, VR, and tLH (Figure 5j). Pretreatment with eplerenone or apocynin abolished the effect of aldosterone on the staining intensity to be similar to the sham group (Figure 5k and l; Table 2).

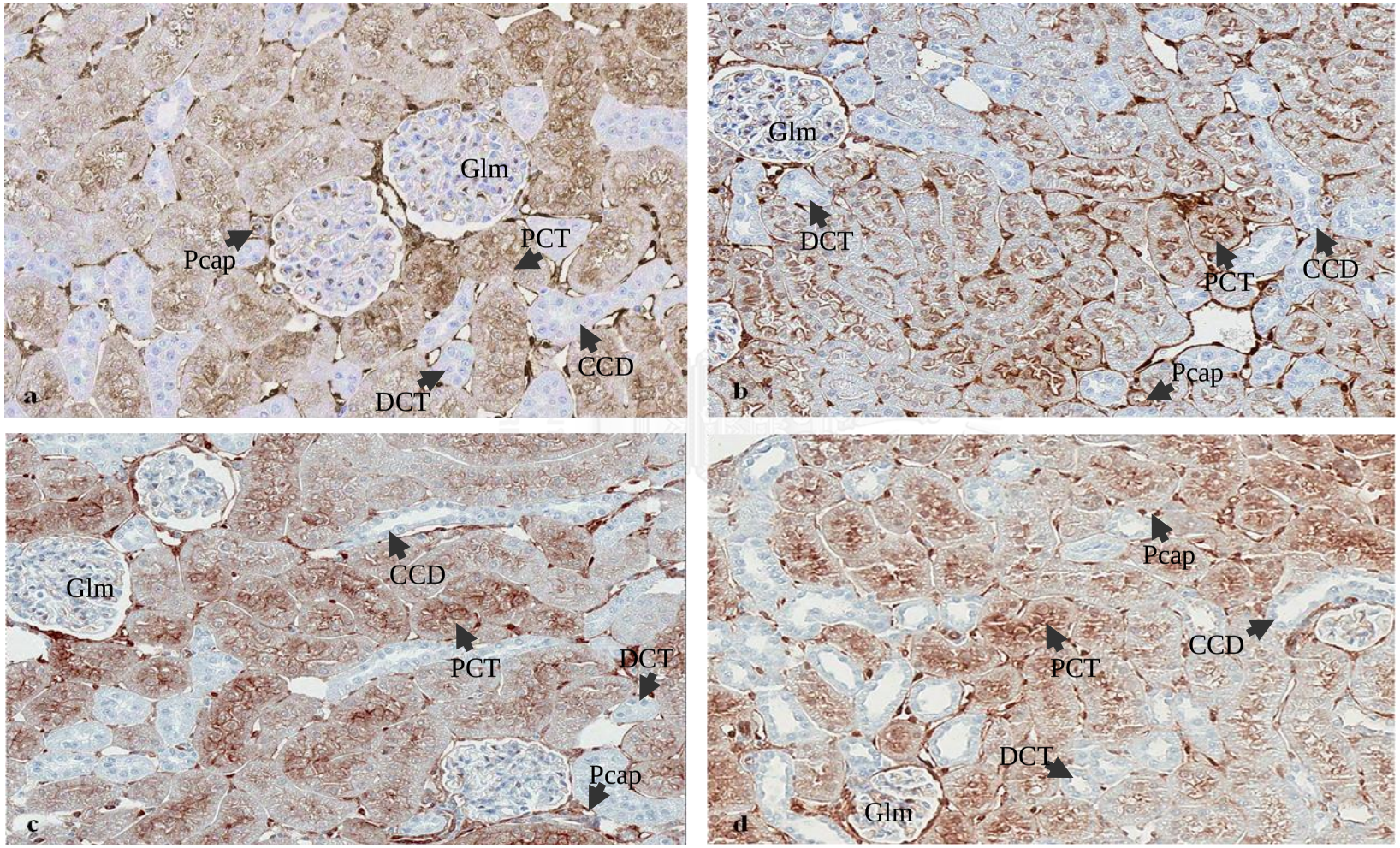


Figure 5: Representative immunohistochemical staining of renal TG2 protein expression in the cortex(original magnification x400). (a): sham; (b): Aldo; (c): Ep.+Aldo; (d): Apo.+Aldo. Glm = glomerulus; PCT = proximal convoluted tubule; DCT = distal convoluted tubule; CCD = cortical collecting duct; Pcap = peritubular capillary.

Table 2 Median staining intensity (score) of renal TG2 and p47phox protein expressions

	Median staining intensity (score)							
	TG2				p47phox			
	sham	Aldo	Ep.+Aldo	Apo.+Aldo	sham	Aldo	Ep.+Aldo	Apo.+Aldo
Cortex								
Glomerulus	1	1	1	1	2	4	2	1
PCT	3	3	3	3	2	4	3	2
DCT	0	0	0	0	2	4	3	2
CCD	0	0	0	0	1	3	1	1
Pcap	4	4	4	4	2	4	3	2
Outer medulla								
TALH	0	1	0	0	1	2	1	1
MCD	0	1	1	1	1	1	1	1
VR	3	4	3	1	4	4	4	4
tLH	2	3	2	1	1	3	2	1
Inner medulla								
MCD	3	4	2	3	1	4	1	3
VR	2	3	2	2	3	4	3	3
tLH	1	3	1	1	2	4	2	3

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.

PCT = Proximal convoluted tubule; DCT = distal convoluted tubule; CCD = cortical collecting duct; Pcap = peritubular capillary; TALH = thick ascending limb of Henle'sloop, MCD = medullary collecting duct; VR = vasa recta, tLH = thin limb of Henle's loop.

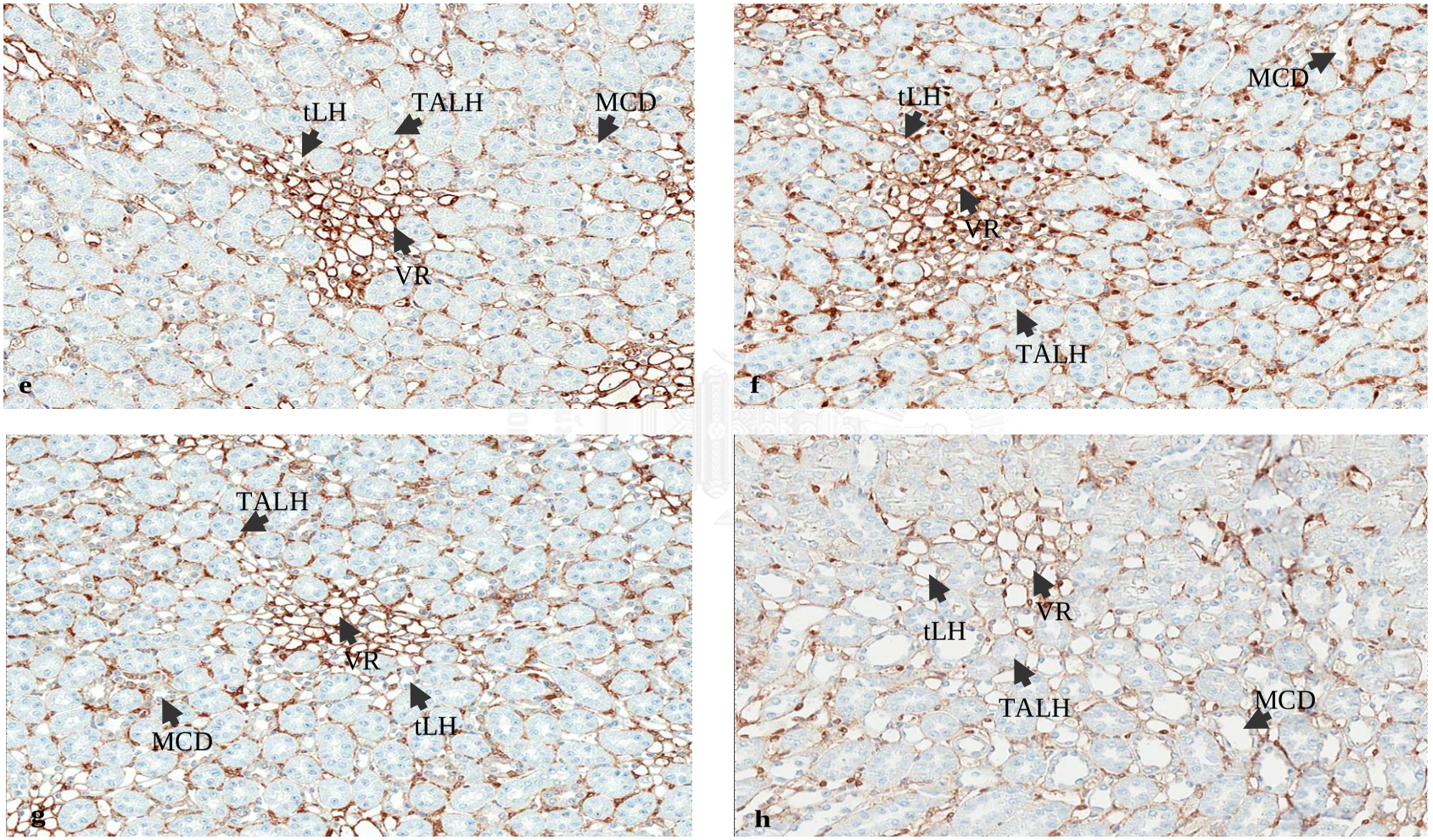


Figure 5 (cont.): Representative immunohistochemical staining of renal TG2 protein expression in the outer medulla (original magnification x200). (e): sham; (f): Aldo; (g): Ep.+Aldo; (h): Apo.+Aldo. TALH = thick ascending limb of Henle's loop; MCD = medullary collecting duct; VR = vasa recta; tLH = thin limb of Henle's loop.

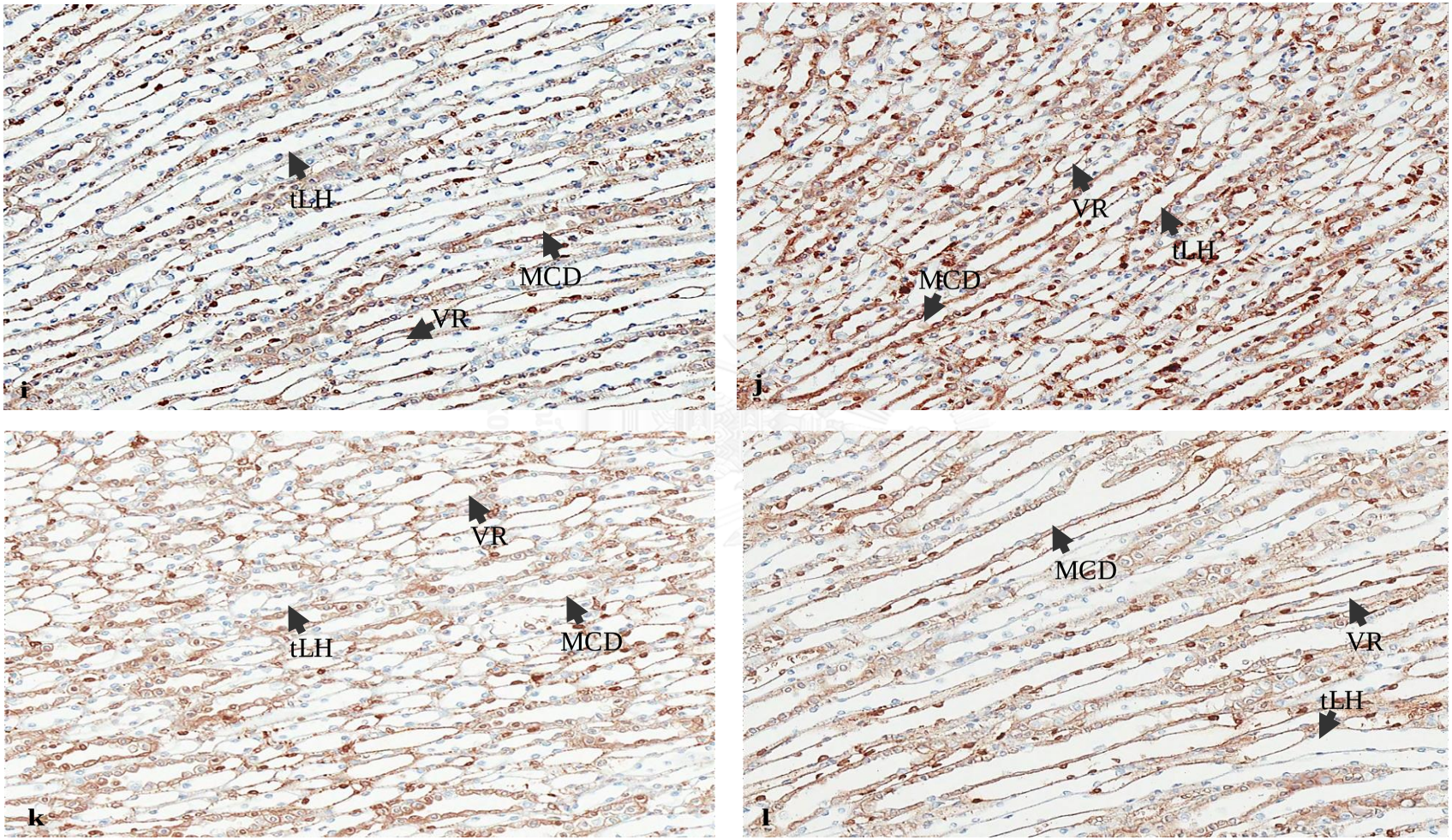


Figure 5 (cont.): Representative immunohistochemical staining of renal TG2 protein expression in the inner medulla (original magnification x200). (i): sham; (j): Aldo; (k): Ep.+Aldo; (l): Apo.+Aldo. MCD = medullary collecting duct; VR = vasa recta; tLH = thin limb of Henle's loop.

Effect of aldosterone on renal plasma membrane protein abundance of p47phox

As shown in Figure 6, aldosterone enhanced plasma membrane protein abundance of p47phox (218.2 ± 11.7%, $p < 0.001$) as compared with the sham group (100%) while pretreatment with eplerenone or apocynin inhibited the effect of aldosterone (101.6 ± 5.2%, $p = 0.90$; 104.5 ± 4.7%, $p = 0.40$, respectively).

Effect of aldosterone on renal p47phox protein localization

In the cortex, p47phox protein in the sham group expressed at the basolateral membrane of renal tubular cells and renal vasculature (Figure 7). Aldosterone increased the staining intensity of p47phox to be grade 4 (strong) in the glomerulus, PCT, DCT, and Pcap; as well as to be grade 3 (moderate) in the CCD (Figure 7b). Pretreatment with eperenone or apocynin prevented the effect of aldosterone on p47phox stimulation to be near the sham group (Figure 7c and d).

In the outer medulla, p47phox in the aldosterone group was higher expression in the TALH and tLH (Figure 7f). Pretreatment with eplerenone or apocynin inhibited aldosterone-induced the expression in these areas while the staining intensity was unaltered in the MCD and VR in all groups (Figure 7e-h). In the inner medulla, aldosterone increased the staining intensity of p47phox to be grade 4 (strong) in all studied areas (Figure 7j). Pretreatment with eplerenone lessened the staining intensity to be the same as in the sham group whereas pretreatment with apocynin declined the staining intensity to be grade 3 (moderate) in all studied areas (Figure 7k and l; Table 2).

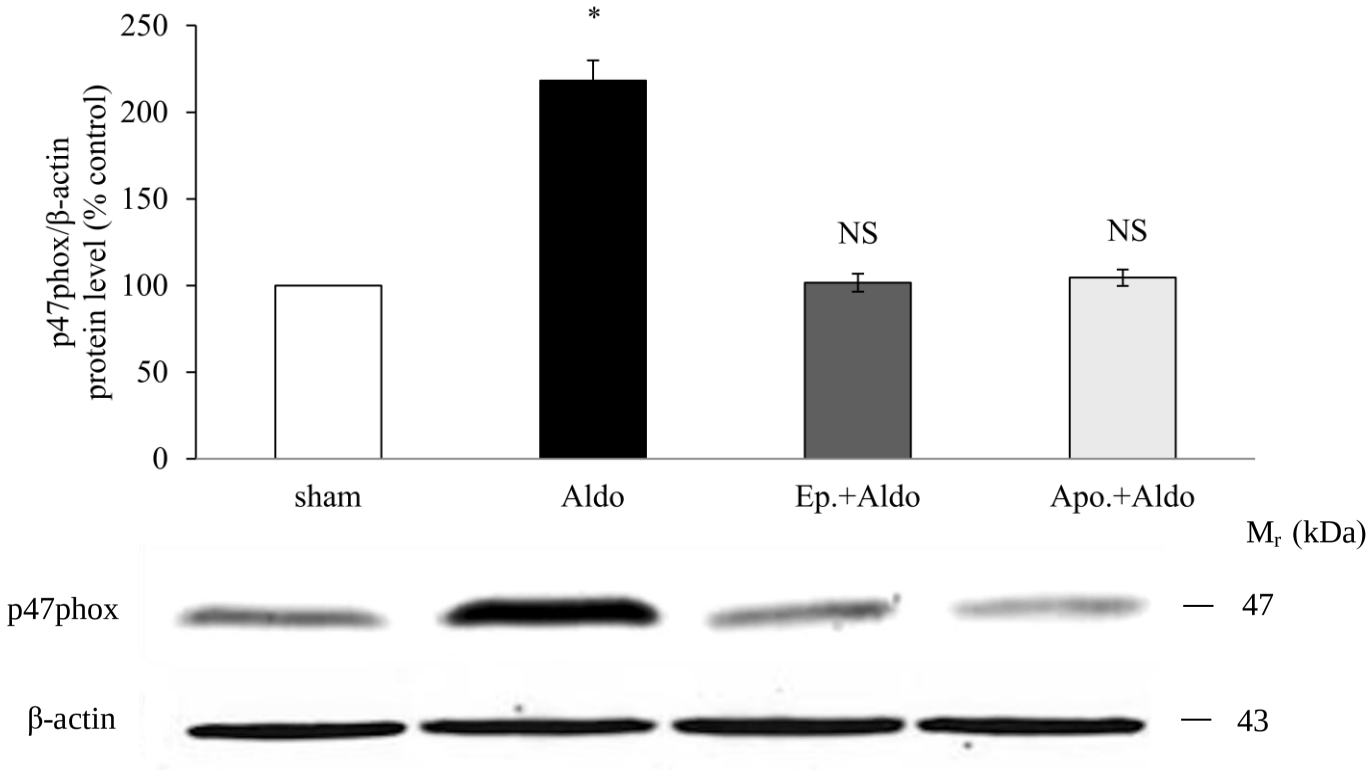


Figure 6: Renal p47phox protein abundance in sham, Aldo, Ep.+Aldo, and Apo.+Aldo groups by Western blot analysis. Plasma membrane proteins from renal tissues were separated by SDS-PAGE, transferred to nitrocellulose membrane and subjected to immunoblot analysis. The monoclonal antibody to p47phox was used. Histogram bars show the densitometric analyses ratios of p47phox to β-actin intensity, and the representative immunoblot photographs are presented. Data are means ± SD of 8 independent experiments. *$p < 0.001$ *vs.* sham

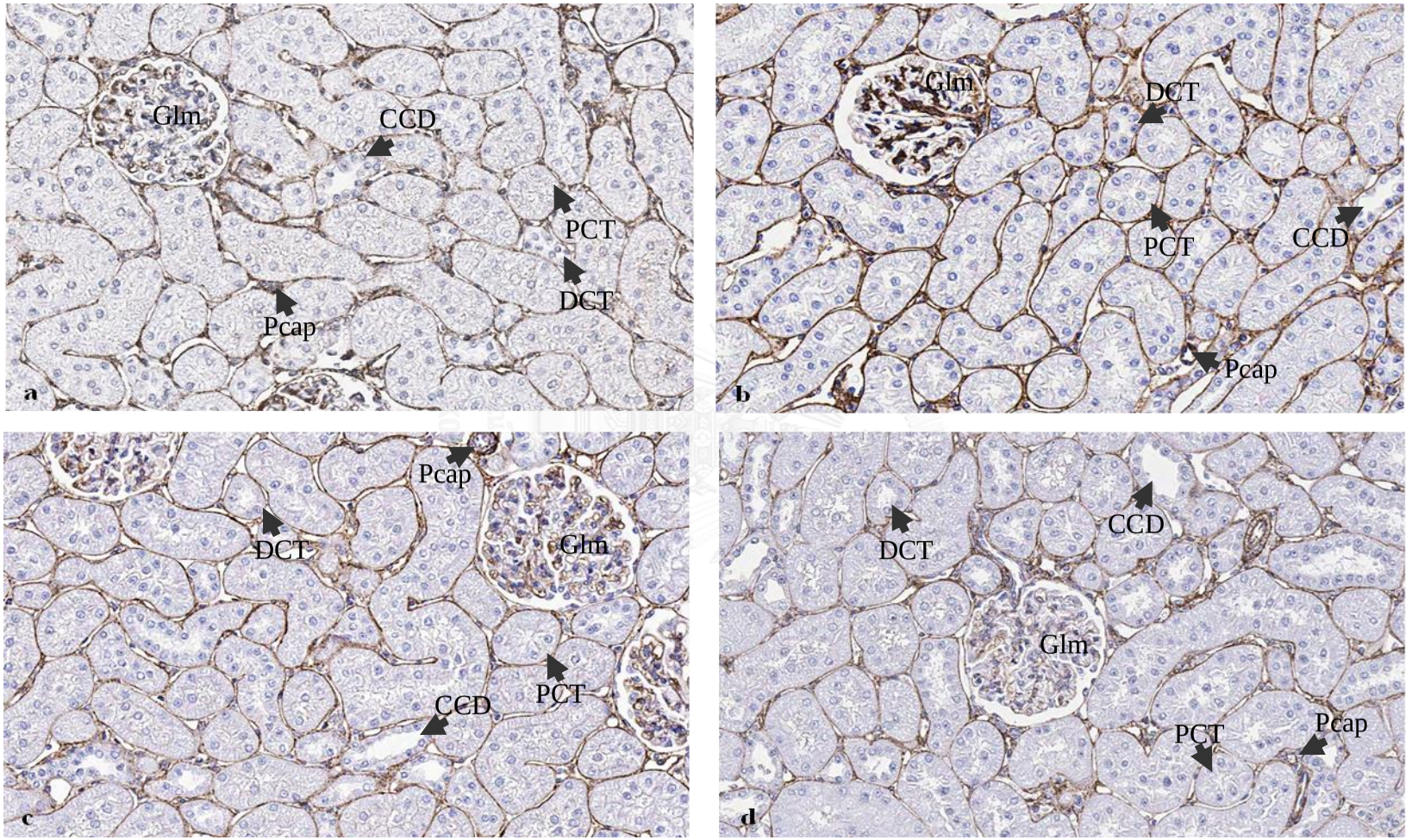


Figure 7: Representative immunohistochemical staining of renal p47phox protein expression in the cortex (original magnificationx400). (a): sham; (b): Aldo; (c): Ep.+Aldo; (d): Apo.+Aldo. Glm = glomerulus; PCT = proximal convoluted tubule; DCT = distal convoluted tubule; CCD = cortical collecting duct; Pcap = peritubular capillary

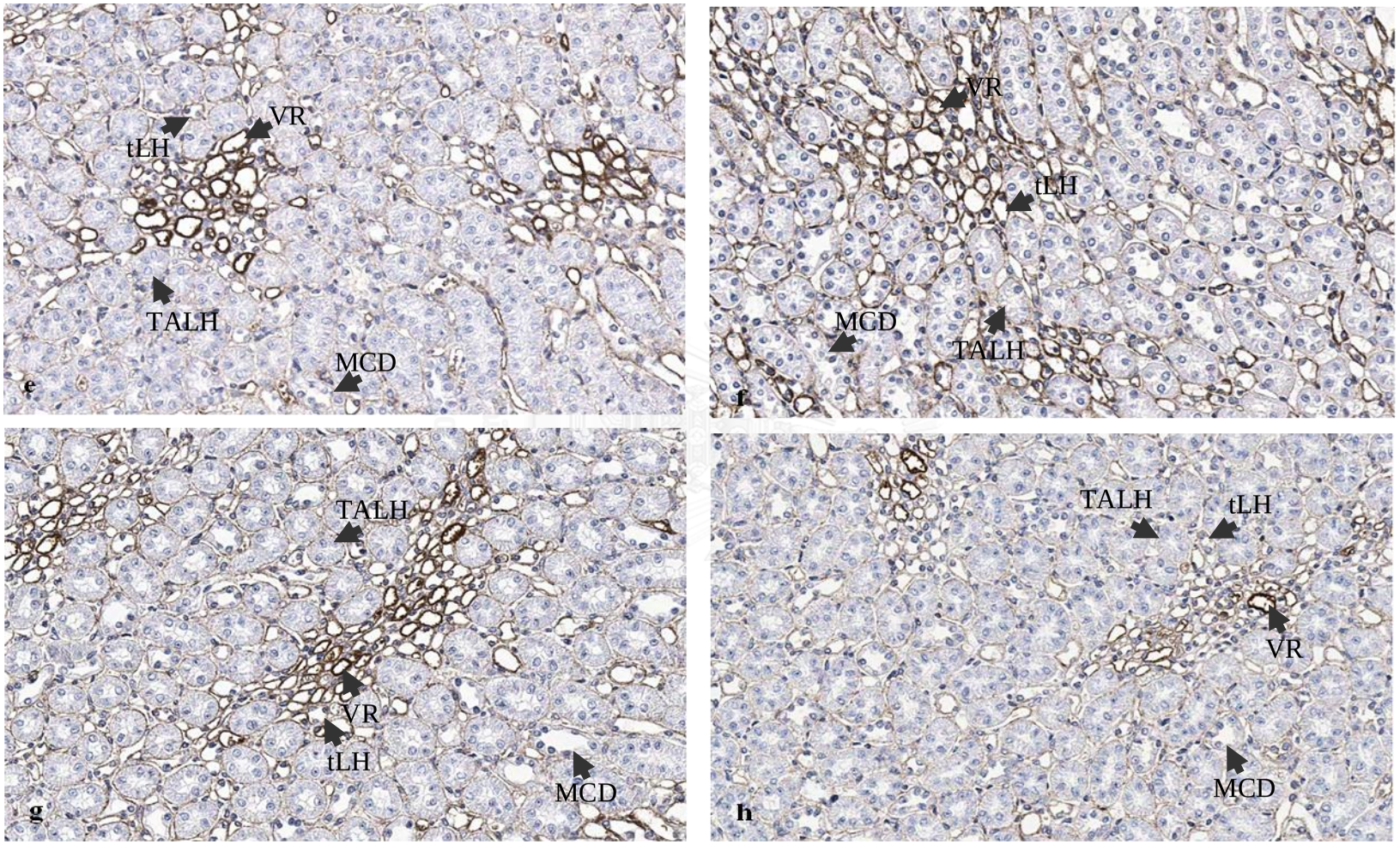


Figure 7 (cont.): Representative immunohistochemical staining of renal p47phox protein expression in the outer medulla (original magnification x200). (e): sham; (f): Aldo; (g): Ep.+Aldo; (h): Apo.+Aldo. TALH = thick ascending limb of Henle's loop; MCD = medullary collecting duct; VR = vasa recta; tLH = thin limb of Henle's loop.

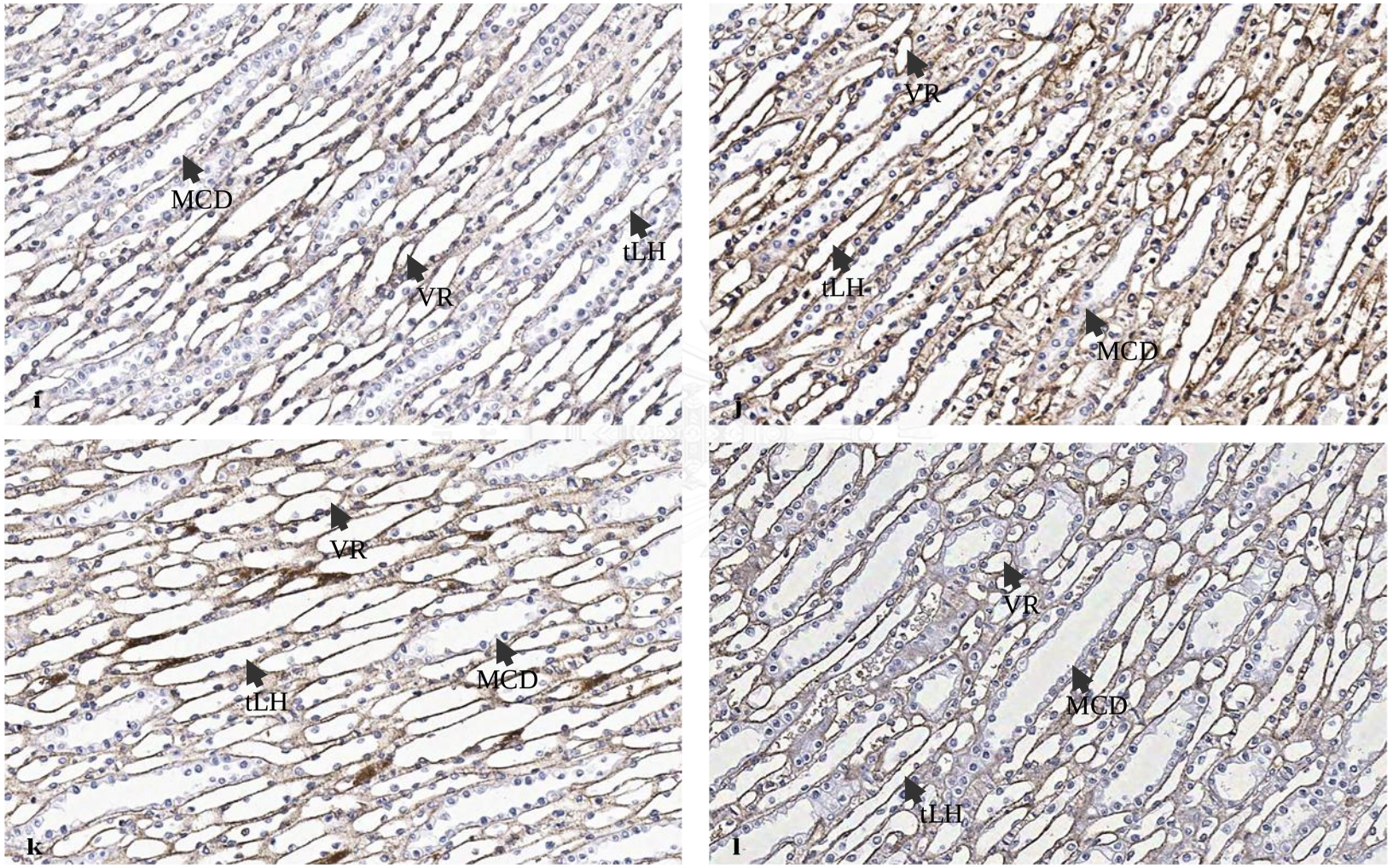


Figure 7 (cont.): Representative immunohistochemical staining of renal p47phox protein expression in the inner medulla (original magnification x200). (i): sham; (j): Aldo; (k): Ep.+Aldo; (l): ApoMCD = medullary collecting duct; VR = vasa recta; tLH = thin limb of Henle's loop.

Protein expression of AT_1R and AT_2R colocalization

In the cortex, all groups had more significant AT_2R expression in the cell areas that expressed AT_1R (Table 3). The colocalization of AT_1R and AT_2R expressions was high intense in the glomeruli, DCT and CCD, especially the basolateral membrane. The diffused staining was observed in PCT and Pcap (Figure 8).

In the medulla, outer and inner regions, there were no significant differences in AT_2R expression in areas presenting negative or positive staining of AT_1R protein (Table 3). The colocalization of AT_1R and AT_2R in the TALH and MCD was faint while it was rare in the tLH and VR (Figure 9 and 10).

Table 3 Protein expression of AT_1R and AT_2R colocalization in sham, Aldo, Ep.+Aldo, and Apo.+Aldo groups

	sham (n = 8)			Aldo (n = 8)		
	AT_1R			AT_1R		
	Negative	Positive	p-value	Negative	Positive	p-value
Expression of AT_2R (%)						
Cortex	0.53 ± 0.20	1.46 ± 0.45	0.002	0.54 ± 0.19	1.56 ± 0.41	0.001
Outer medulla	0.49 ± 0.20	0.79 ± 0.37	0.071	0.50 ± 0.15	0.63 ± 0.27	0.138
Inner medulla	0.43 ± 0.09	0.48 ± 0.08	0.220	0.49 ± 0.06	0.57 ± 0.12	0.161

	Ep.+Aldo (n = 8)			Apo.+Aldo (n = 8)		
	AT_1R			AT_1R		
	Negative	Positive	p-value	Negative	Positive	p-value
Expression of AT_2R (%)						
Cortex	0.54 ± 0.20	1.45 ± 0.29	0.001	0.54 ± 0.18	1.64 ± 0.54	0.002
Outer medulla	0.49 ± 0.19	0.78 ± 0.32	0.071	0.50 ± 0.15	0.63 ± 0.27	0.120
Inner medulla	0.42 ± 0.05	0.48 ± 0.07	0.088	0.49 ± 0.06	0.56 ± 0.12	0.171

Values are expressed as mean ± SD.

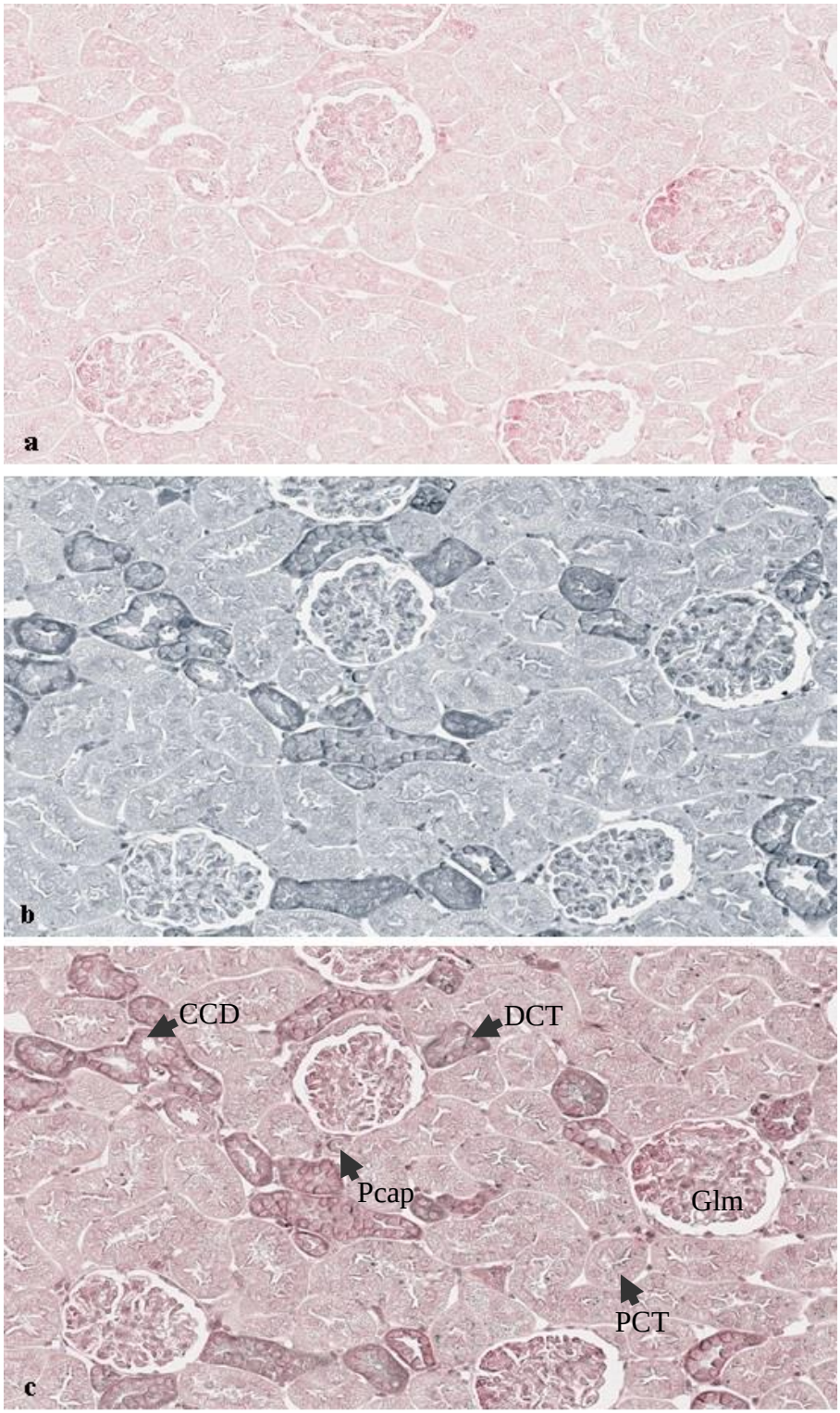


Figure 8: Representative immunohistochemical double staining of renal ATRs protein expression in cortex [AT_1R (a; red color), AT_2R (b; blue-grey color), and AT_1R and AT_2R (c; purple color)] (original magnification x400) (a-c): sham.
Glm = glomerulus; PCT = proximal convoluted tubule;
DCT = distal convoluted tubule; CCD = cortical collecting duct;
Pcap = peritubular capillary.

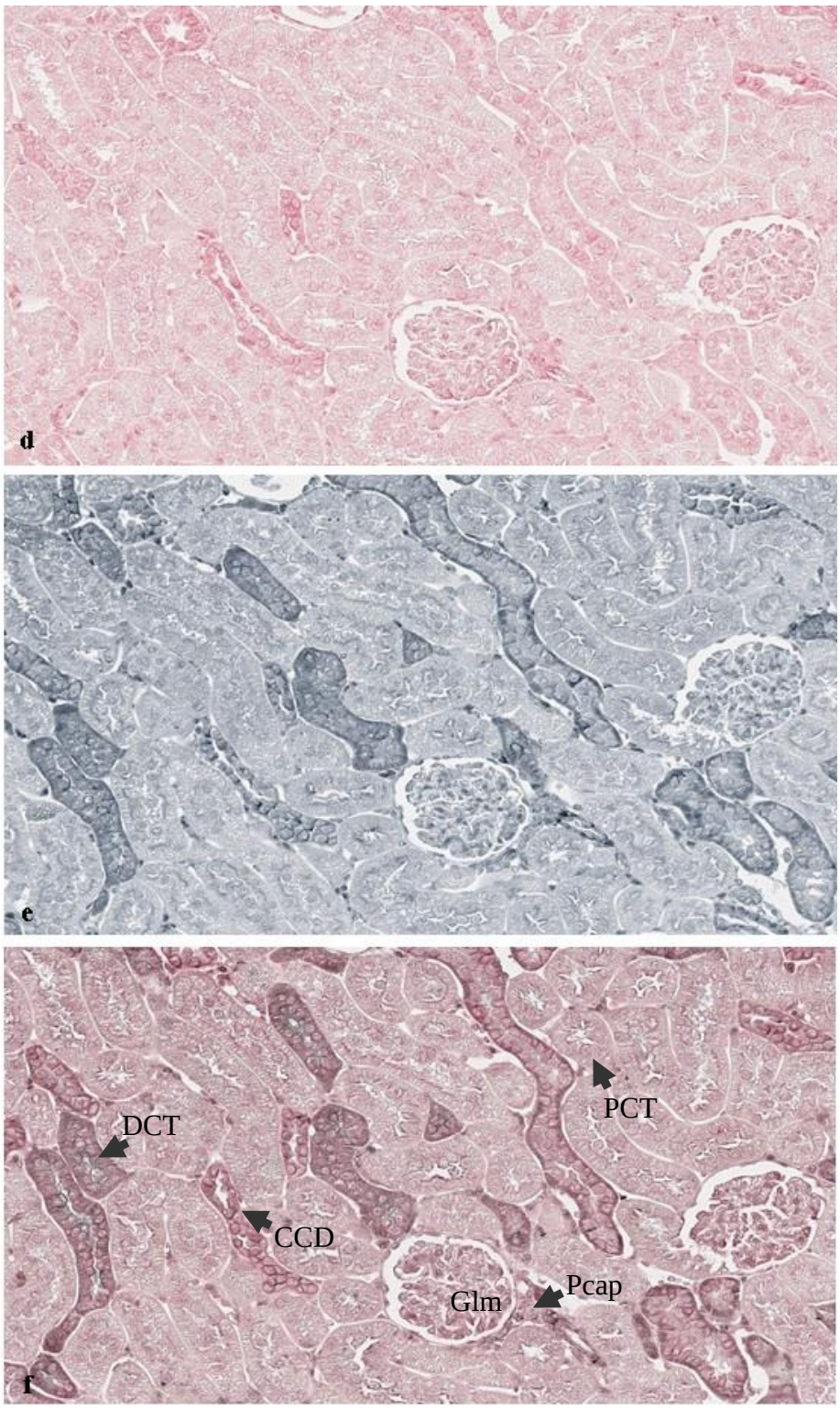


Figure 8 (cont.): Representative immunohistochemical double staining of renal ATRs protein expression in cortex [AT_1R (d; red color), AT_2R (e; blue-grey color), and AT_1R and AT_2R (f; purple color)] (original magnification x400) (d-f): Aldo.
Glm = glomerulus; PCT = proximal convoluted tubule;
DCT = distal convoluted tubule; CCD = cortical collecting duct;
Pcap = peritubular capillary.

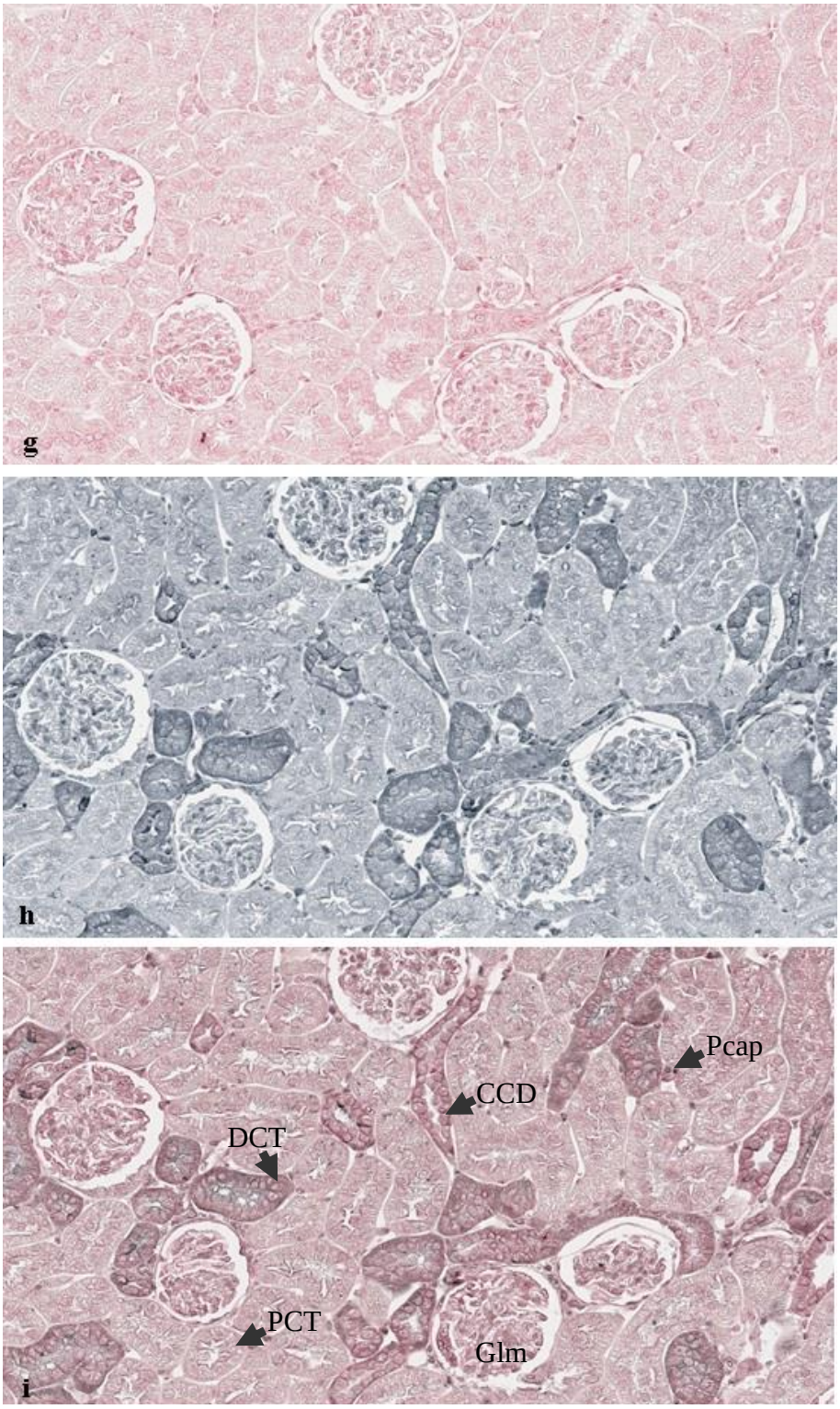


Figure 8 (cont.): Representative immunohistochemical double staining of renal ATRs protein expression in cortex [AT_1R (g; red color), AT_2R (h; blue-grey color), and AT_1R and AT_2R (i; purple color)] (original magnification x400) (g-i): Ep.+Aldo.
Glm = glomerulus; PCT = proximal convoluted tubule; DCT = distal convoluted tubule; CCD = cortical collecting duct; Pcap = peritubular capillary.

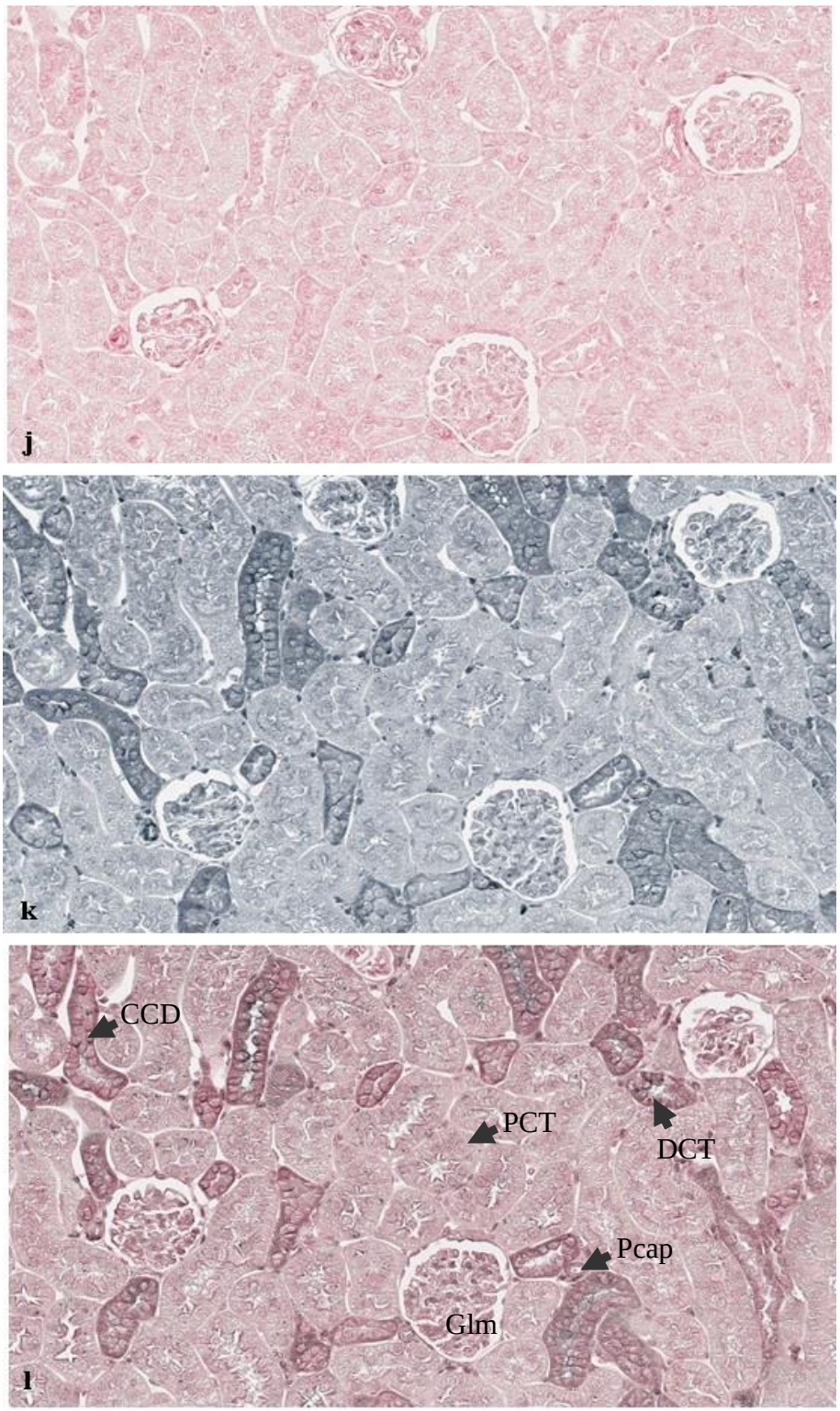


Figure 8 (cont.): Representative immunohistochemical double staining of renal ATRs protein expression in cortex [AT_1R (j; red color), AT_2R (k; blue-grey color), and AT_1R and AT_2R (l; purple color)] (original magnification x400) (j-l): Apo.+Aldo.
Glm = glomerulus; PCT = proximal convoluted tubule;
DCT = distal convoluted tubule; CCD = cortical collecting duct;
Pcap = peritubular capillary.

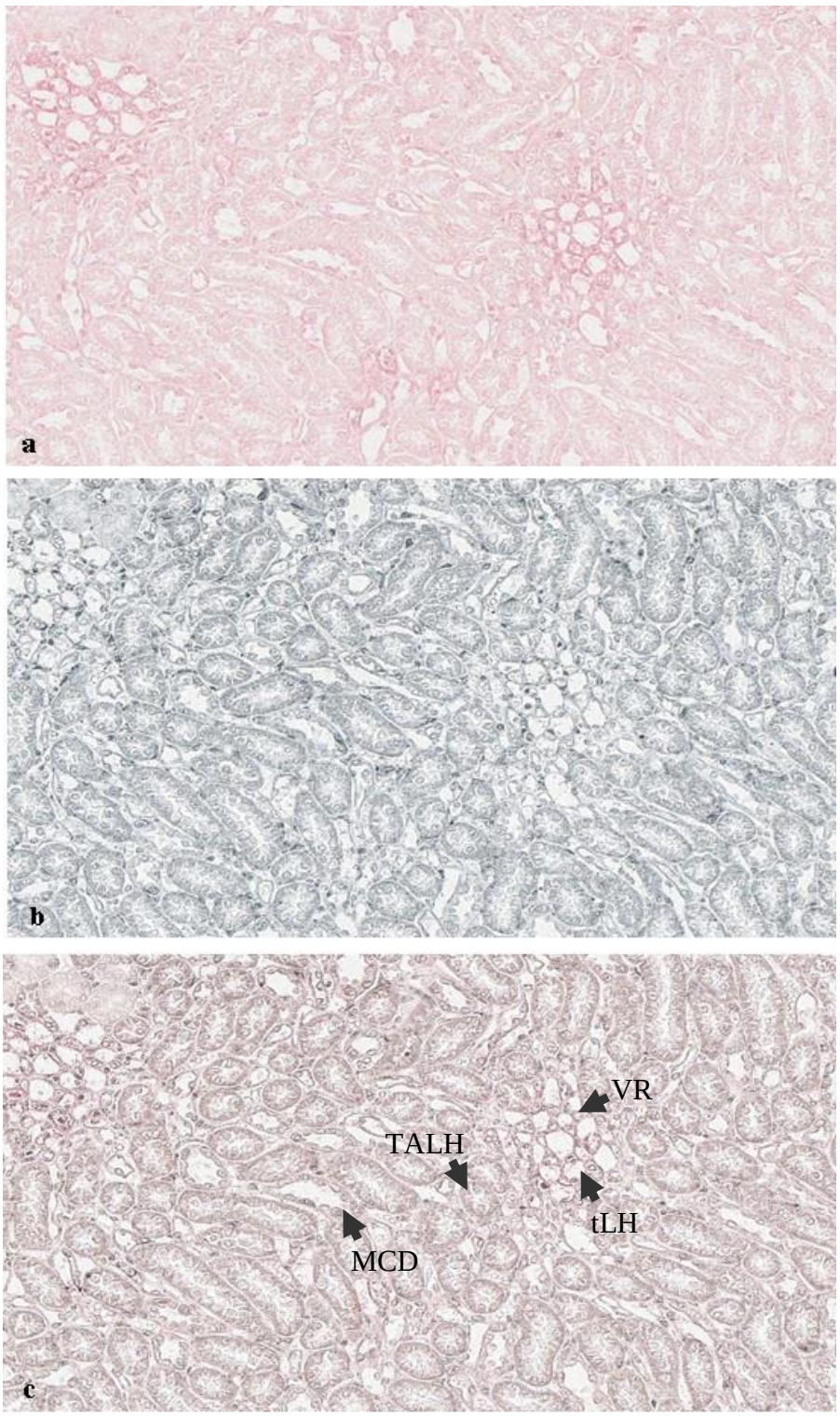


Figure 9: Representative immunohistochemical double staining of renal ATRs protein expression in outer medulla [AT_1R (a; red color), AT_2R (b; blue-grey color), and AT_1R and AT_2R (c; purple color)] (original magnification x200) (a-c): sham.
TALH = thick ascending limb of Henle's loop; MCD = medullary collecting duct; VR = vasa recta; tLH = thin limb of Henle's loop.

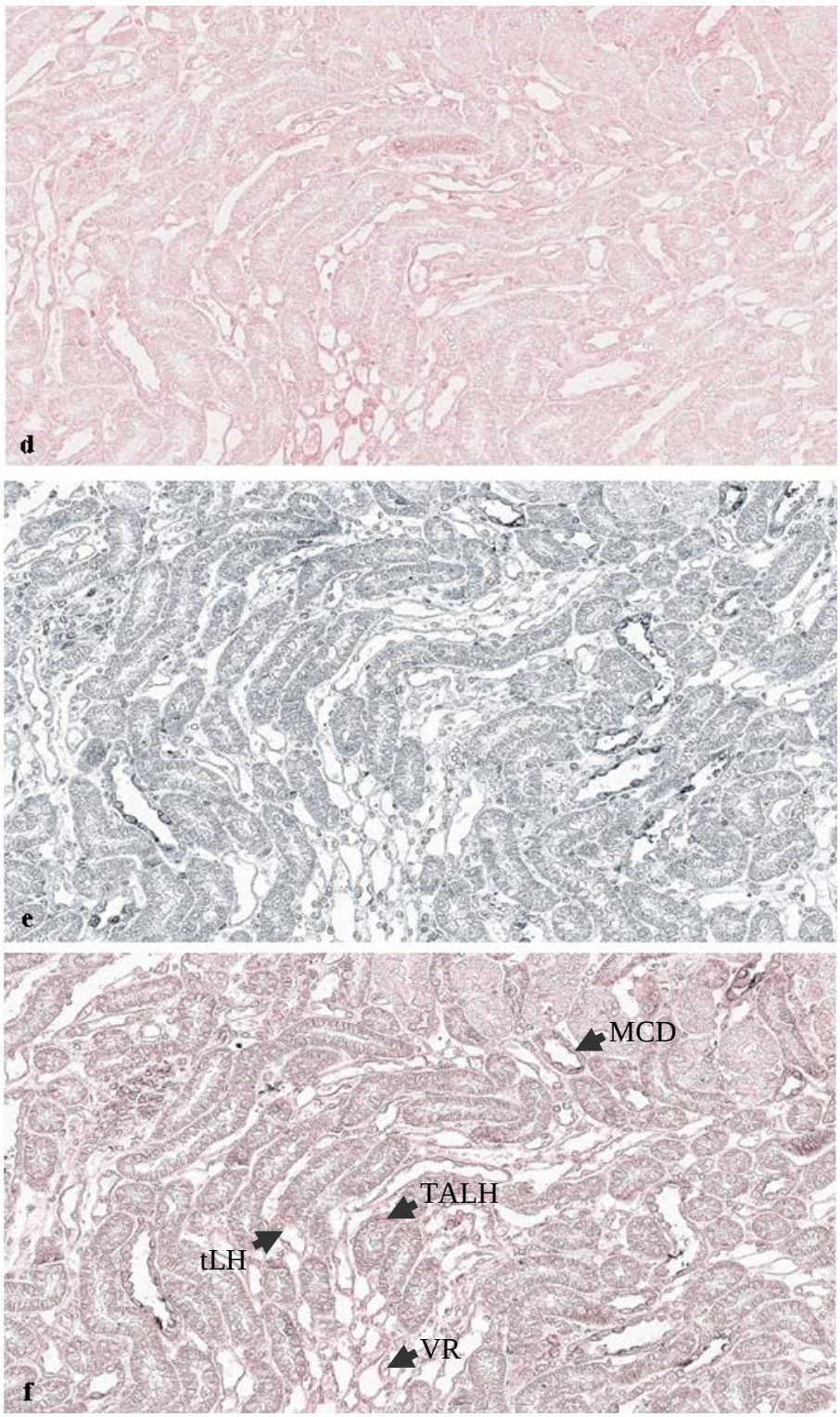


Figure 9 (cont.): Representative immunohistochemical double staining of renal ATRs protein expression in outer medulla [AT_1R (d; red color), AT_2R (e; blue-grey color), and AT_1R and AT_2R (f; purple color)] (original magnification x200) (d-f): Aldo.
TALH = thick ascending limb of Henle's loop; MCD = medullary collecting duct; VR = vasa recta; tLH = thin limb of Henle's loop.

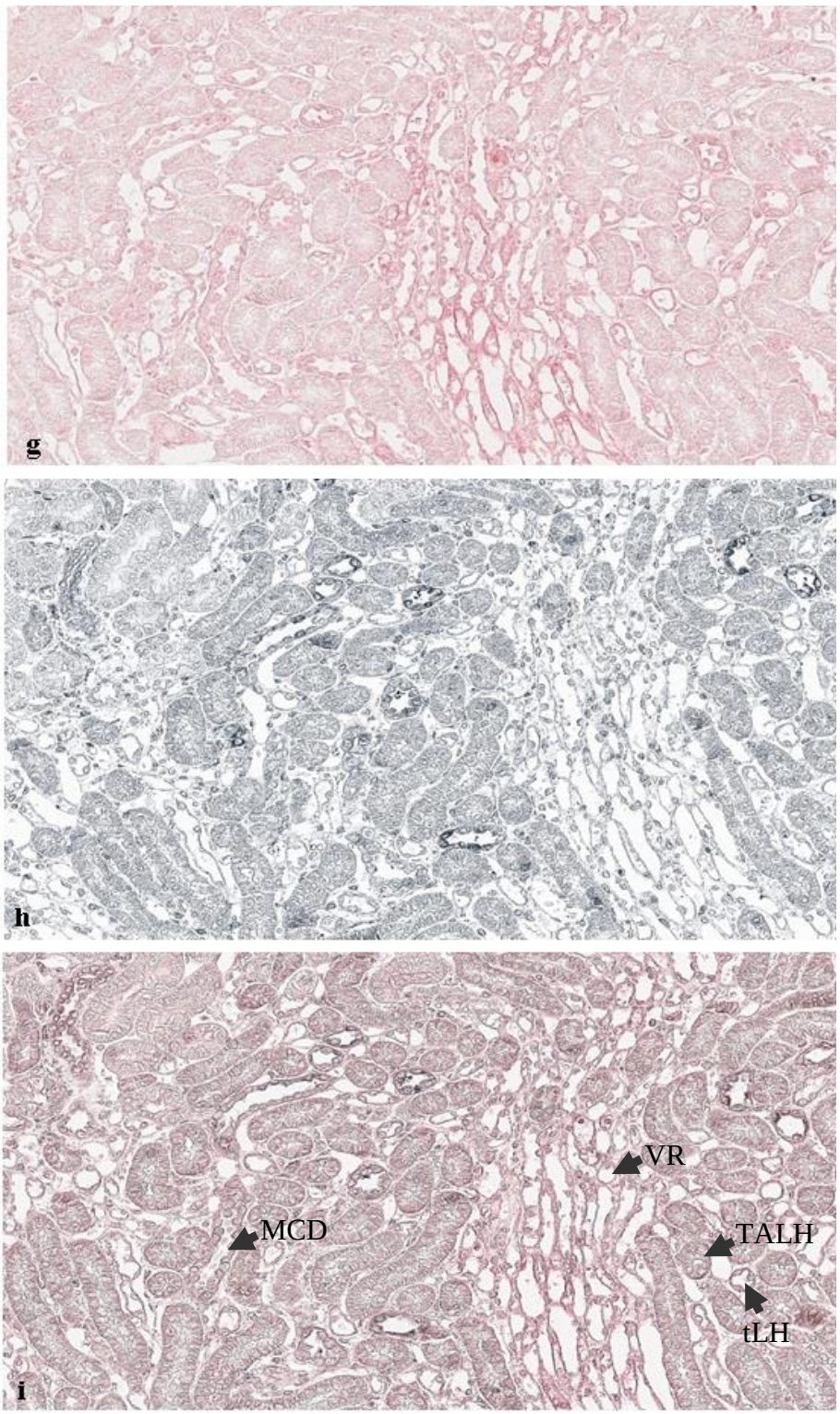


Figure 9 (cont.): Representative immunohistochemical double staining of renal ATRs protein expression in outer medulla [AT_1R (g; red color), AT_2R (h; blue-grey color), and AT_1R and AT_2R (i; purple color)] (original magnification x200) (g-i): Ep.+Aldo.
TALH = thick ascending limb of Henle's loop; MCD = medullary collecting duct; VR = vasa recta; tLH = thin limb of Henle's loop.

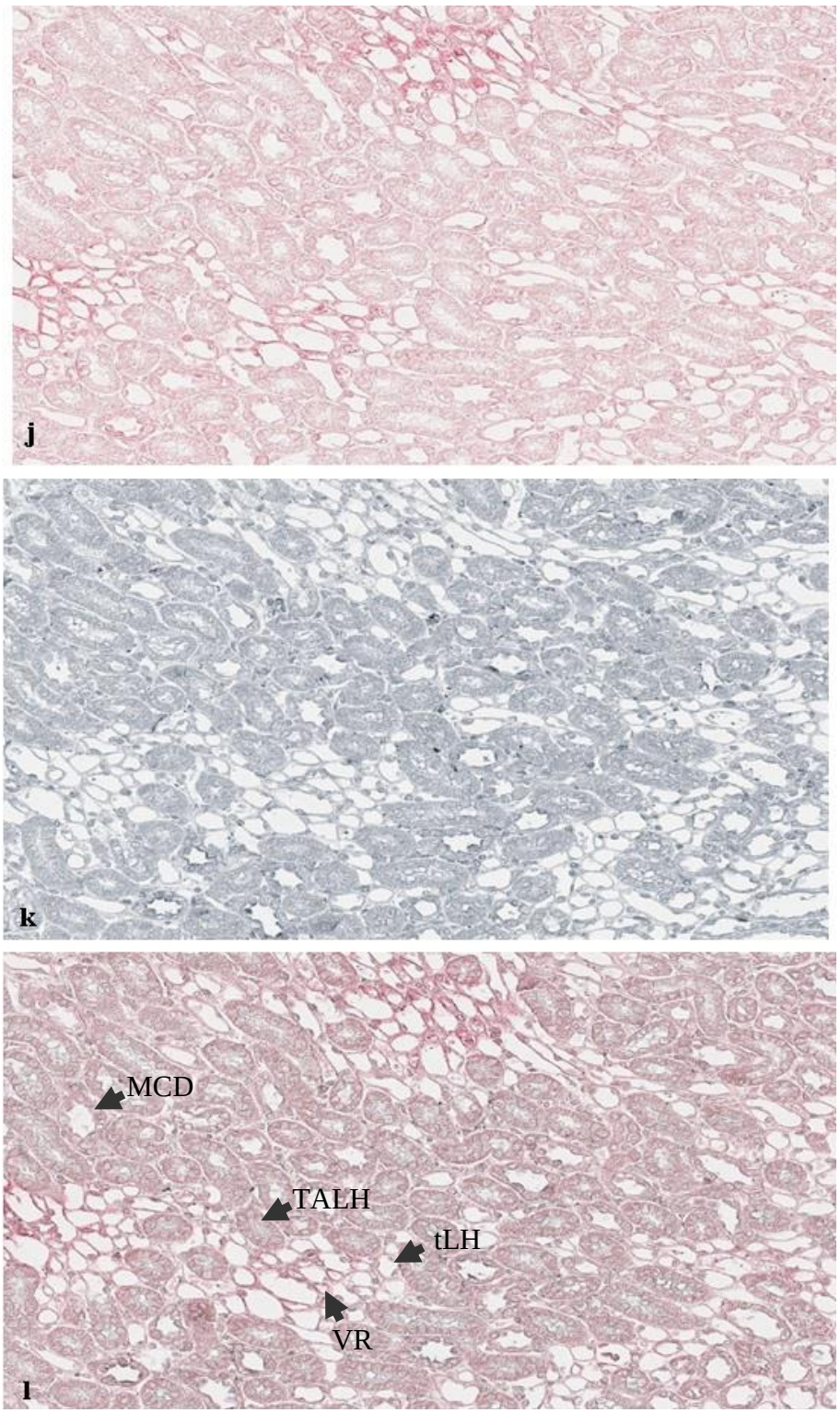


Figure 9 (cont.): Representative immunohistochemical double staining of renal ATRs protein expression in outer medulla [AT_1R (j; red color), AT_2R (k; blue-grey color), and AT_1R and AT_2R (l; purple color)] (original magnification x200) (j-l): Apo.+Aldo.
TALH = thick ascending limb of Henle's loop; MCD = medullary collecting duct; VR = vasa recta; tLH = thin limb of Henle's loop.

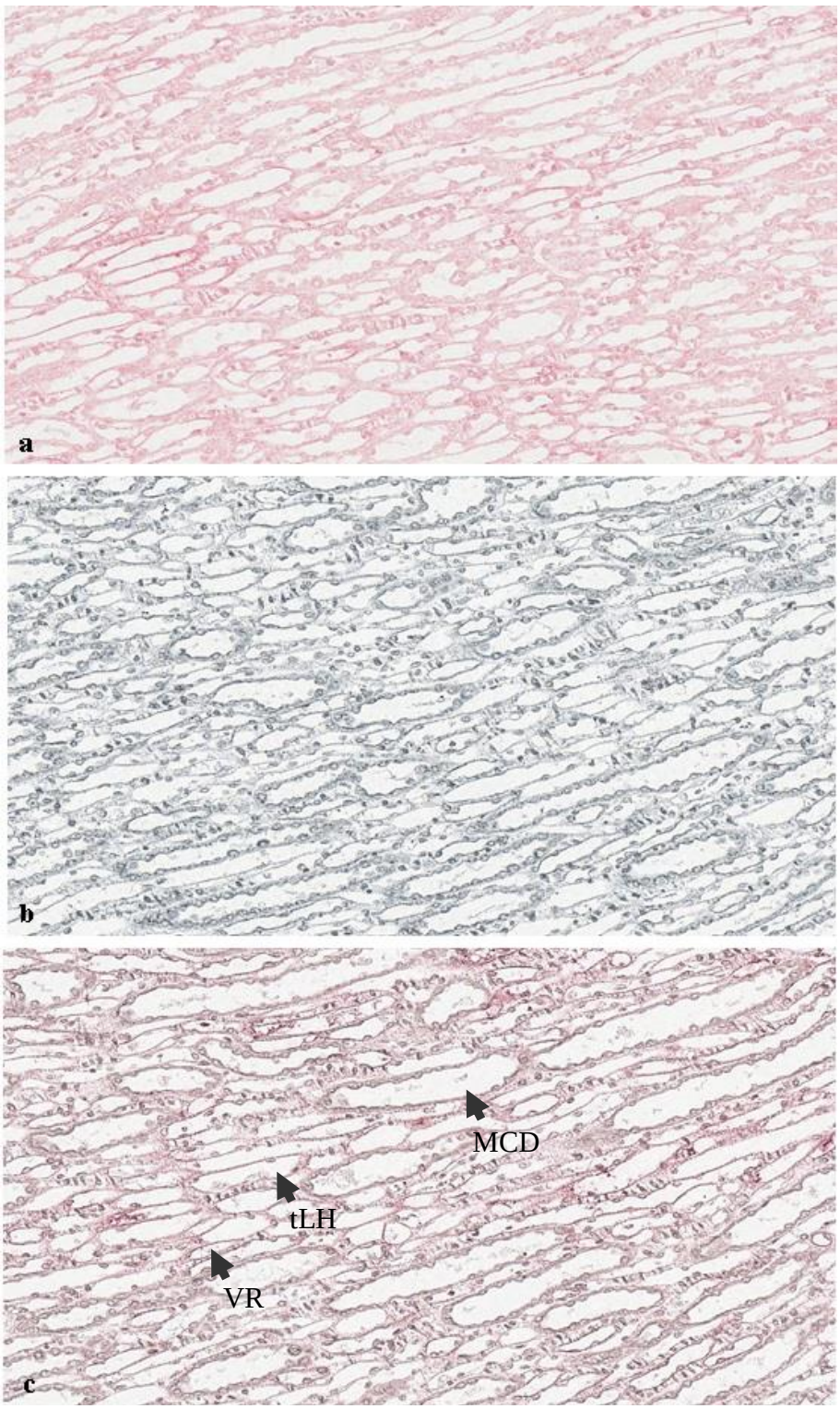


Figure 10: Representative immunohistochemical double staining of renal ATRs protein expression in inner medulla [AT_1R (a; red color), AT_2R (b; blue-grey color), and AT_1R and AT_2R (c; purple color)] (original magnification x200) (a-c): sham.
MCD = medullary collecting duct; VR = vasa recta;
tLH = thin limb of Henle's loop.

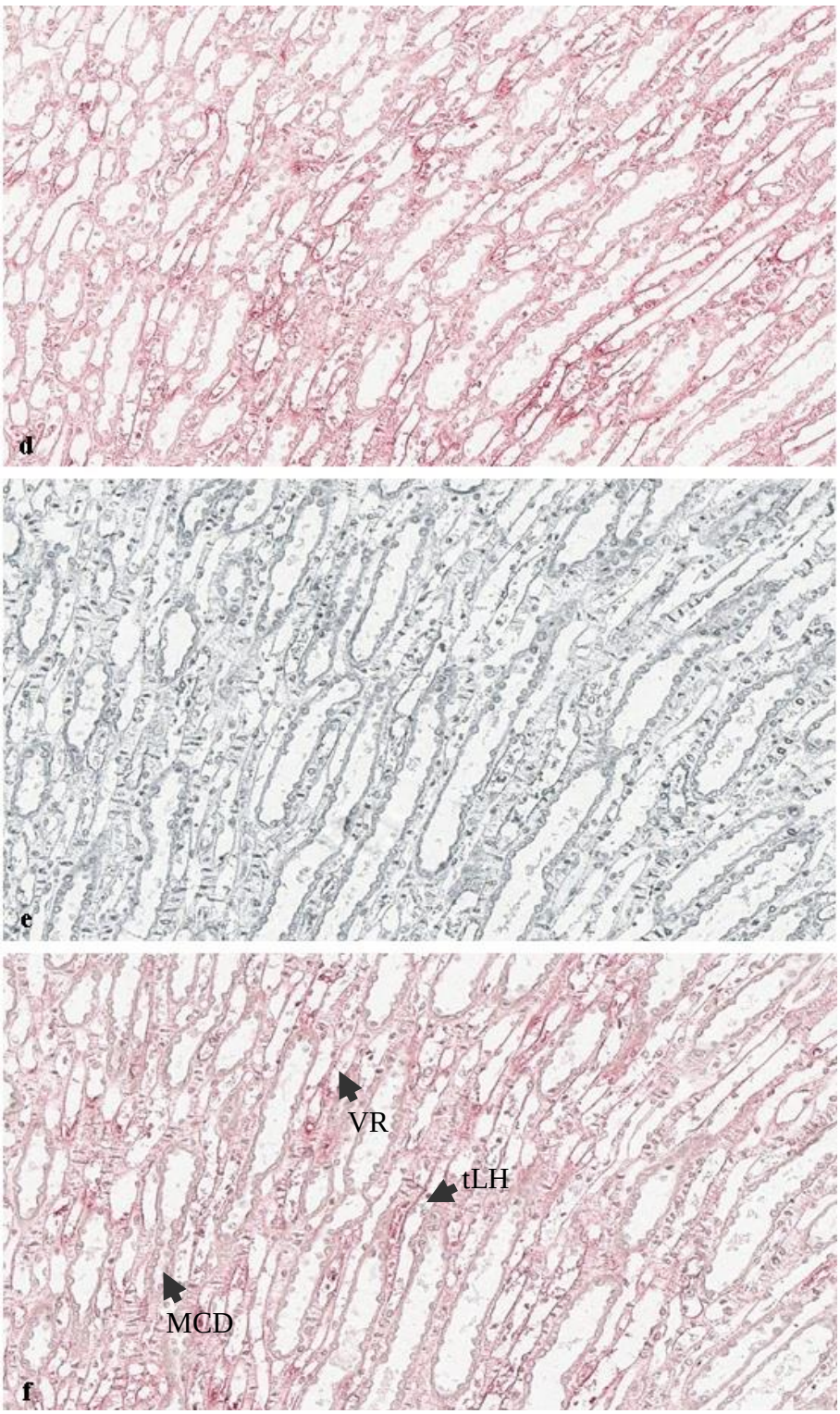


Figure 10 (cont.): Representative immunohistochemical double staining of renal ATRs protein expression in inner medulla [AT_1R (d; red color), AT_2R (e; blue-grey color), and AT_1R and AT_2R (f; purple color)] (original magnification x200) (d-f): Aldo.
MCD = medullary collecting duct; VR = vasa recta;
tLH = thin limb of Henle's loop.

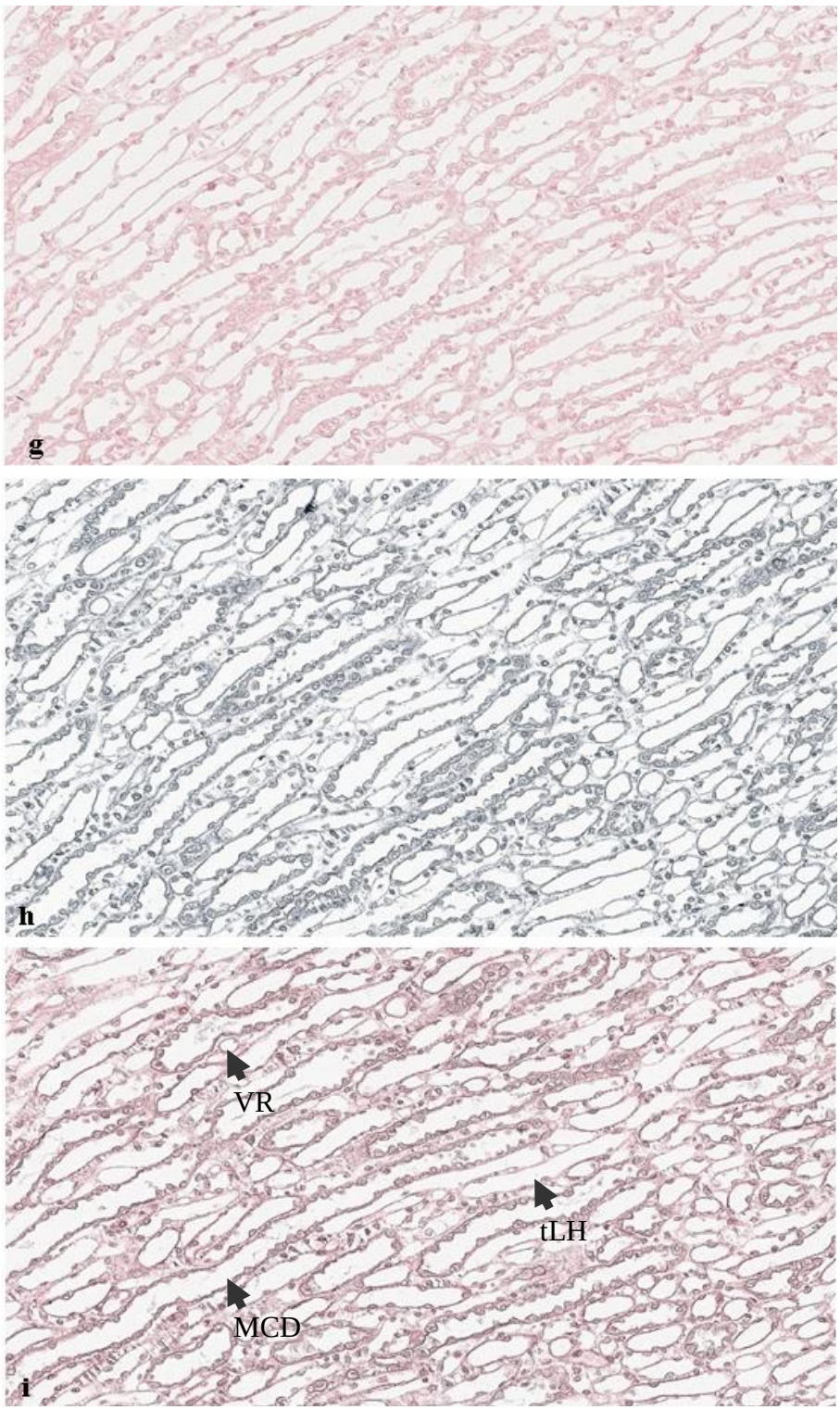


Figure 10 (cont.): Representative immunohistochemical double staining of renal ATRs protein expression in inner medulla [AT_1R (g; red color), AT_2R (h; blue-grey color), and AT_1R and AT_2R (i; purple color)] (original magnification x200) (g-i): Ep.+Aldo.
MCD = medullary collecting duct; VR = vasa recta;
tLH = thin limb of Henle's loop.

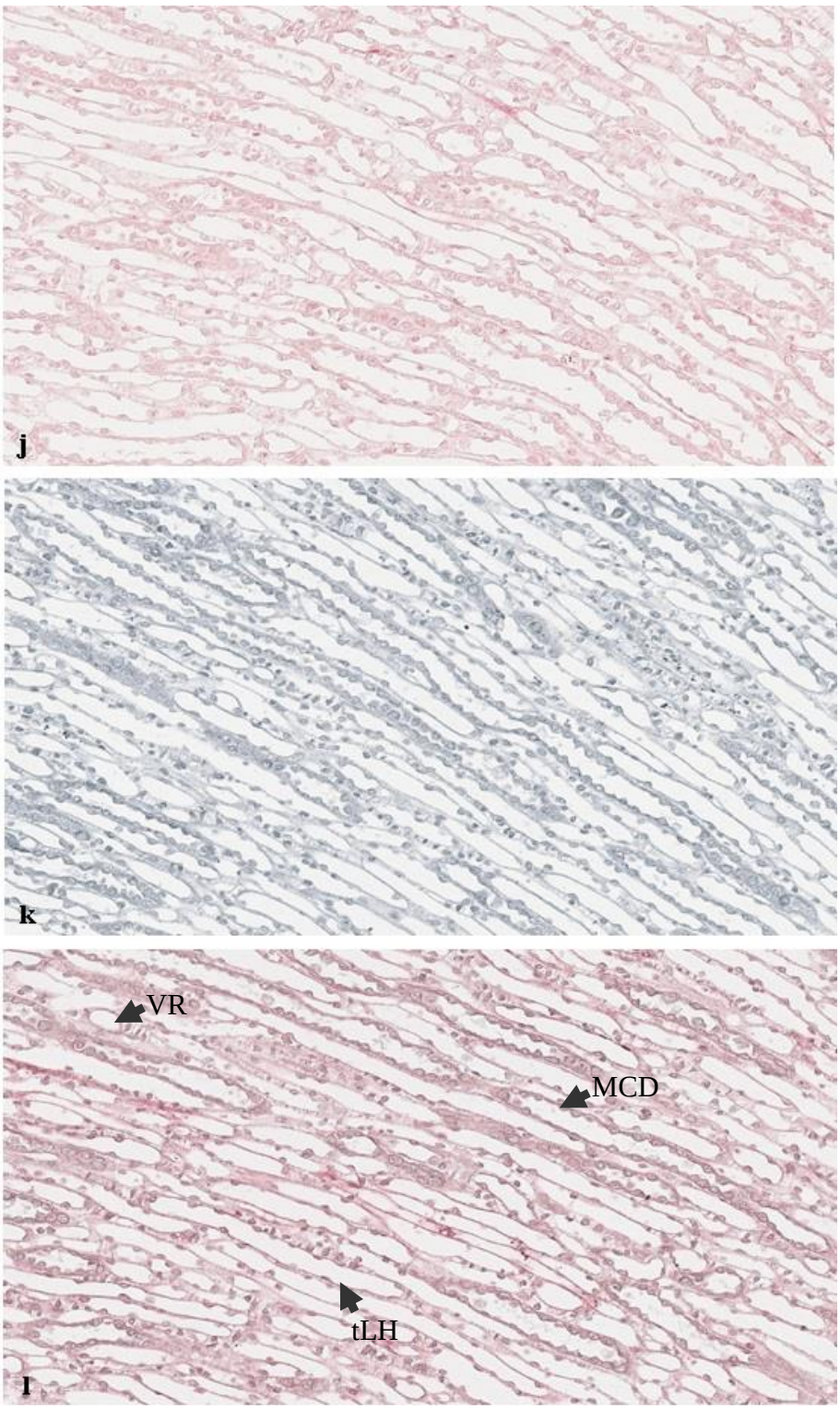


Figure 10 (cont.): Representative immunohistochemical double staining of renal ATRs protein expression in inner medulla [AT_1R (j; red color), AT_2R (k; blue-grey color), and AT_1R and AT_2R (l; purple color)] (original magnification x200) (j-l): Apo.+Aldo.
MCD = medullary collecting duct; VR = vasa recta;
tLH = thin limb of Henle's loop.

CHAPTER V
DISCUSSION AND CONCLUSION

In this study, the results demonstrate *in vivo* for the first time that aldosterone nongenomically enhances the renal protein abundances of AT_1R and AT_2R dimerizations (Figure 1 and 2). The present study shows that aldosterone elevates dimeric forms of AT_1R and AT_2R but did not change monomeric forms of both receptors. A former study has demonstrated that aldosterone enhances the dimeric form of AT_1R but unaltered the monomeric form of AT_1R [9]. There are no data of AT_2R dimerization by aldosterone. A proposed mechanism of cellular receptor dimerization has been established [92]. ATRs are the seven membrane classes of G-protein-coupled receptors (GPCRs) which form as dimers in the endoplasmic reticulum (ER) and then translocate into the plasma membrane [92]. Thus, aldosterone might elevate both AT_1R and AT_2R dimerizations within the plasma membrane of ER before the dimers are transported into cell membrane, resulting in the unchanged monomers.

This finding is the first that aldosterone-induced AT_1R dimer formation is MR-independent pathway whereas the formation of AT_2R dimer is modulated via MR-dependent manner (Figure 1 and 2). Interestingly, the elevations of AT_1R and AT_2R dimerizations are inhibited by apocynin (Figure 1 and 2). The results indicate that the role of NADPH oxidase is an influence on dimerization of both AT_1R and AT_2R.

Previous *in vitro* studies have demonstrated that intracellular relationship among p47phox, NADPH oxidase, ROS, and TG2 responses on AT_1R dimerization

by nongenomic action of aldosterone [9, 11, 13]. To support this concept, the present study reports that aldosterone rapidly elevates renal TG2 and p47phox. These effects are prevented by apocynin (Figure 4 and 6). Therefore, an MR-independent pathway, aldosterone might stimulate ROS via NADPH oxidase and then activate both TG2 and AT_1R dimerization in rat kidney.

For the mechanism of AT_2R dimerization, this study shows that the nongenomic action of aldosterone elevates renal TG2 protein abundance via MR dependance (Figure 4). A former study has demonstrated that TG2 contributes a crosslinking formation of AT_2R into oligomers in human embryonic kidney cells [12]. Nevertheless, TG2-induced AT_2R dimerization has not been demonstrated in any study. It is a plausible that aldosterone elevates TG2 via MR and then AT_2R dimerization. The translocation of p47phox into plasma membrane is a representative of NADPH oxidase activation (ROS production) [93]. The present study illustrates that aldosterone-induced AT_2R dimerization is dependent on both MR and NADPH oxidase activations (Figure 2). A previous study has demonstrated that aldosterone rapidly decreases cytosolic p47phox but the plasma membrane p47phox on AT_2R dimerization has not been examined [13]. This study showed that aldosterone-induced AT_2R dimerization is inhibited by apocynin (Figure 2). The results indicate that aldosterone-induced p47phox subunit of NADPH oxidase via MR has a potential role on AT_2R dimerization.

According to AT_1R and AT_2R are G-protein-coupled-receptors (GPCRs), they can form dimerization as homodimers (AT_1R-AT_1R or AT_2R-AT_2R) or undergo complex formation with each other (AT_1R-AT_2R) or interact with other GPCRs to form heterodimers [14]. Many studies have shown that ATRs dimerization also

involves in receptor functions in renal cells [56, 88, 89, 94]. Under physiologic condition, in dimerization of ATRs, it has been demonstrated that heterodimerization of AT_1R/AT_2R stimulates Na^+-ATPase activity at basolateral membrane of isolated rat proximal tubules and then induces sodium reabsorption [88]. Moreover, the heterodimer formation also enhances Ca^{2+}-ATPase activity of the sarco(endo)plasmic reticulum in cultured porcine proximal tubular cells and then controls calcium homeostasis [56]. Furthermore, the heterodimer formation inhibits Ca^{2+}-ATPase activity at basolateral membrane of sheep proximal tubule [94]. Under pathologic condition, a previous study has indicated that the heterodimer formation is elevated in renal cortex from preeclampsia rat model [89]. Although AT_1R/AT_2R heterodimer is a part of ATR dimerization, the ATR homodimers- or other pattern of ATR heterodimers-induced by aldosterone is unestablished. The present study hypothesized that aldosterone-induced AT_1R or AT_2R dimerization may occur through AT_1R and AT_2R heterodimer. By co-immunoprecipitation (co-IP) and Western blotting (WB) techniques, the present results first demonstrate that nongenomic action of aldosterone has no effect on AT_1R/AT_2R heterodimer formation in rat kidney. There are no precise mechanisms to explain these phenomena. Indeed, the AT_2R that forms a heterodimer with AT_1R could stabilize a conformation change on AT_1R and prevents G protein dissociation [53]. Furthermore, it has demonstrated that AT_1R-interacted with D_3 dopamine receptor is decreased whereas AT_1R-interacted with endothelin receptor type B is elevated after angiotensin II treatment in cultured rat renal proximal tubular cells [95, 96]. In addition, AT_2R has been proposed that it could heterodimerize with these receptors (AT_2R-D_3 dopamine receptor, AT_2R-endothelin receptor type B) [51]. For AT_1R homodimerization, a previous study has shown that

the homodimerized AT_1R (AT_1R-AT_1R) expresses in cultured human embryonic kidney cells after angiotensin II stimulation [39]. Moreover, constitutively active homodimerized AT_2R (AT_2R-AT_2R) is localized in plasma membrane after 24 hours under serum-free condition in transfected Chinese Hamster ovary cells with AT_2R [54]. The technique of co-IP and WB performed in the present study is utilized for protein-protein heterodimer detection. It has demonstrated that the biophysical technique with bioluminescence resonance energy transfer could detect protein-protein homodimerization [97]. Therefore, more studies should be performed to reveal the ATR homodimer formation.

Localization of TG2 protein in rat kidney was shown in Figure 5. TG2 protein in the sham group expressed weak and diffuse immunostaining in glomeruli and tubules. These results are in agreement with many previous studies in rat kidney [61, 63]. In the present study, aldosterone markedly enhanced the TG2 expression in the renal medulla (Figure 5). Although the expressions in renal cortex were unaltered, nongenomic action of aldosterone translocated the TG2 protein from the cytosol into luminal membrane (Figure 5b). It has been shown that translocation of TG2 from the cytosol into the plasma membrane involves in phosphoinositide-dependent recruitment of cytoplasmic TG2 protein to the perinuclear recycling compartment, and then delivery TG2 into the plasma membrane in mouse embryonic fibroblast cells [98]. Moreover, Pcap and VR highly expressed TG2 protein after aldosterone treatment (Table 1). Pretreatment with eplerenone or apocynin could alleviate the immunoreactivity (Figure 5). The results suggest that aldosterone-enhanced TG2 protein expression via MR and NADPH oxidase pathway and TG2 might play a

significant role in transamidation and protein-protein crosslinking along the renal tubule and renal vasculature.

Localization of p47phox protein in rat kidney was shown in Figure 7. p47phox protein of sham group expressed at the glomeruli and renal vasculatures in renal cortex and medulla. The basal expression of p47phox protein in the glomeruli and renal vasculature is similar to former studies in rat kidney [76, 99, 100]. Moreover, the results showed that p47phox markedly expressed at the basolateral membrane of renal tubules (PCT, DCT, TALH, CCD, and MCD) (Figure 7). These results are different from those previously studies [76, 99, 100]. Since the high specific monoclonal of p47phox antibody was used in the present study. Aldosterone increased basolateral membrane of p47phox protein expression in both cortex and medulla and this elevation was alleviated by eplerenone or apocynin (Figure 7). Therefore, aldosterone *per se*, nongenomically stimulates NADPH oxidase in rat kidney.

From double immunohistochemistry, the colocalization of renal protein expressions of AT_1R and AT_2R mainly presented in the cortex area in all studied groups (Table 3). The colocalization of AT_1R and AT_2R proteins was presented in the glomerulus and basolateral membrane of PCT, DCT, and CCD in all studied groups (Figure 8). It has been demonstrated that the colocalization of AT_1R and AT_2R proteins presents in cultured rat renal proximal tubular epithelial cells [101]. The present results suggest that there is a constitutive colocalization in the glomerulus, PCT, DCT, and CCD in rat kidney.

Both AT_1R and AT_2R locate in the same areas of nephron segments may suggest some significances. For example, in the process of podocyte cells, proteinuria

induced destruction of nephrin, a barrier molecule, was suppressed by administration of AT_1R antagonist, whereas AT_2R agonist could stimulate nephrin expression [102]. In this regard, angiotensin II type 2 receptors play opposite roles to modulate nephrin expression. It has been reported that AT_1R exerts some ion transports in DCT and CCD [103, 104]. AT_1R stimulates thiazide-sensitive NaCl cotransport (NCC) activity in microdissected mouse DCTs [103]. In addition, AT_1R enhances epithelial Na^+ channel (ENaC) activity in microdissected rat CCDs [104]. Nevertheless, the functions of AT_2R on NCC in DCT or on ENaC in CCD have not been established. The further *in vivo* study is required to examine this notion.

In conclusion, the present data are the first document demonstrating that aldosterone nongenomically increases renal TG2 and p47phox protein expressions and then activates AT_1R and AT_2R dimerizations. Aldosterone-stimulated AT_1R and AT_2R dimerizations are mediated through activation of NADPH oxidase. However, aldosterone-induced AT_1R dimer formation is MR-independent pathway whereas the formation of AT_2R dimer is modulated via MR-dependent manner. Aldosterone-enhanced renal TG2 and p47phox protein expressions are depend on both MR and NADPH oxidase activations. The heterodimerization of AT_1R/AT_2R is maintained in all studied groups. The colocalization of AT_1R and AT_2R proteins is more prominent in the cortex region.

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APPENDIX

PREPARATION OF BUFFERS AND REAGENTS

Immunohistochemistry

10% Buffered Neutral formalin (pH 7.0) 1 litre

40% Formaldehyde	100.00	mL
dH_2O	900.00	mL
Sodium phosphate monobasic monohydrate	4.00	g
Di-sodium hydrogen phosphate	6.50	g

10x PBS washing buffer (pH 7.4) 1 litre

Sodium chloride	80.00	g
Potassium chloride	2.00	g
Di-sodium hydrogen phosphate	14.40	g
Potassium di-hydrogen phosphate	2.40	g

Adjust the pH to 7.4 with conc. HCl.
Adjust the volume to 1 litre with dH_2O

1x PBS washing buffer 1 litre

10x PBS	100.00	mL
dH_2O	900.00	mL

Mix and store at room temperature

1x PBS/0.1% Tween-20 washing buffer 1 litre

1x PBS	999.00	mL
Tween-20	1.00	mL

Mix and store at room temperature

Blocking solution (3% horse serum in 1x PBS/0.1% Tween-20) 10 mL

5% horse serum	6.00	mL

Adjust the volume to 10 mL with PBS/0.1% Tween-20

3% Hydrogen peroxide 10 mL

6% Hydrogen peroxide	5.00	mL
dH_2O	5.00	mL

Vector SG substrate working solution

PBS (10 mM sodium phosphate, 0.9% NSS, pH 7.5)	1	mL
Vector SG Chromogen	15	µL
H_2O_2 solution	24	µL

Vector Red substrate working solution

TBS (100 mM Tris-HCl, 0.9% NSS, pH 8.2)	1	mL
Vector Red Chromogen 1	16	µL
Vector Red Chromogen 2	16	µL
Vector Red Chromogen 3	16	µL

Stock DAB in Tris

Dissolve DAB 60 mg/Tris 12 mL
Filter
Pipette 1 mL into polypropylene tube (about 10 tubes then freeze in the refrigerator)

Western blot

1.5 M Tris base (pH 8.8) 100 mL

Tris base	18.21	g
dH_2O	80.00	mL

Adjust the pH to 8.8 with conc. HCl
Adjust the volume to 100 mL with dH_2O

20% SDS 25 mL

SDS	5.00	g

Adjust the volume to 100 mL with dH_2O

10% Ammonium persulfate 10 mL

Ammonium persulfate	1.0	g

Adjust the volume to 10 mL with dH_2O

1x Triton lysis buffer 100 mL

25 mM Tris-HCl (pH 8.0)	0.40	g
150 mM NaCl	0.88	g
0.5% Triton X-100	0.50	mL
5 mM EDTA	0.186	g
dH_2O	100.00	mL

10x Laemmli running buffer (pH 8.3) 1 litre

Tris base	30.00	g
glycine	144.00	g
SDS	10.00	g
dH_2O	900.00	mL

Adjust the pH to 8.3 with conc. HCl and conc. NaOH
Adjust the volume to 1 litre with dH_2O

1x Transfer buffer 1 litre

Tris base	3.04	g
glycine	14.40	g
dH_2O	700.00	mL
100% Methanol	200.00	mL

Adjust the volume to 1 litre with dH_2O

10x TBS washing buffer (pH 7.6) 1 litre

Tris base	24.20	g
NaCl	80.00	g

Adjust the pH to 7.6 with conc. HCl
Adjust the volume to 1 litre with dH_2O

1x TBS washing buffer/0.1% Tween-20 1 litre

10x TBS washing buffer	100.00	mL
dH_2O	800.00	mL
Tween-20	1.00	mL

Adjust the pH to 7.6 with conc. HCl and conc. NaOH
Adjust the volume to 1 litre with dH_2O

1x TBS/0.1% Tween-20 washing buffer 1 litre

1x TBS	999.00	mL
Tween-20	1.00	mL

Mix and store at room temperature

The solutions of Tris/glycine SDS-polyacrylamide gel electrophoresis for Western blot

Reagents	8% Separating gel (mL)	4% Stacking gel (mL)
dH_2O	5.16	3.08
40% acrylamide mix	2.00	0.50
1.5 M Tris (pH 8.8)	2.50	1.25
100% Glycerol	0.24	0.12
20% SDS	0.050	0.025
10% APS	0.0375	0.01875
TEMED	0.0125	0.00625
Total volume	10.00	5.00

VITA

My name is Mr. Kittisak Sinphitukkul. I was born on August 18, 1983, in Chantaburi, Thailand. I graduated my Bechelor degree of Science (Physical Therapy) from Naresuan University in 2006. For my Master degree, I received the Master of Science in Physiology from Chulalongkorn University in 2009. And then I have entered in the Doctor of Philosophy (Physiology) at Chulalongkorn University since 2012, and finished my education in 2016. I was a research assistant during 2009-2012 at the Renal Physiology Laboratory Unit, Faculty of Medicine, Chulalongkorn University. My research publications are as follow: 1) Sinphitukkul K, Eiam-Ong S, Manotham K, Eiam-Ong S. Am J Nephrol. 2011; 33:111-120. 2) Eiam-Ong S, Sinphitukkul K, Manotham K, Eiam-Ong S. Biomed Res Int. 2013; 2013: doi:10.1155/2013/346480. 3) Eiam-Ong S, Sinphitukkul K, Manotham K, Eiam-Ong S. J Steroids Horm Sci. 2014; 5: doi:10.4172/2157-7536.1000125.