การแสดงออกของตัวกลางสัญญาณ อีจีเอฟอาร์—อีอาร์เค1/2—ซี-เอสอาร์ซี ในวิถีที่ไม่ผ่านจีโนมิกภายหลังได้รับอัลโดสเตอโรนในไตหนูแรท

นายกิตติศักดิ์ สินพิทักษ์กุล

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EXPRESSIONS OF EGFR-ERK1/2-c-Src SIGNALING MEDIATORS IN A NONGENOMIC PATHWAY AFTER ALDOSTERONE ADMINISTRATION IN RAT KIDNEY

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อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. ดร. สมจิตร์ เอี่ยมอ่อง, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : อ. นพ. กฤษณพงศ์ มโนธรรม, 86 หน้า.

อัลโดสเตอโรนเป็น ฮอร์โมนมิเนราโลคอร์ติคอยด์ (mineralocorticoid hormone) ที่สำคัญมีบทบาทในการรักษาสมดุลของโซเดียม โพแทสเซียม และกรด-ด่างในร่างกาย จากหลายการศึกษาในเซลล์เพาะเลี้ยงพบว่าอัลโดสเตอโรนเพิ่มระดับปริมาณโปรตีน phosphorylated epidermal growth factor receptor (pEGFR) และ extracellular signal-regulated kinase 1/2 (pERK1/2) ในวิถีที่ไม่ผ่านจีโนมิก ซึ่งพบว่าหนึ่งในตัวกลางที่เกี่ยวข้องคือ phosphorylated cytosolic tyrosine kinase of Src (pc-Src) อย่างไรก็ตามยังไม่มีการศึกษาในสัตว์ทดลองถึงระดับปริมาณและการแสดงออกของโปรตีนตัวกลางสัญญาณ pEGFR—pERK1/2—pc-Src ในวิถีที่ไม่ผ่านจีโนมิกในไตหนูแรท

การวิจัยครั้งนี้ทำการทดลองในหนูแรทพันธุ์วิสต้าเพศผู้ซึ่งแบ่งออกเป็นกลุ่มที่ได้รับสารละลาย normal saline (NSS) หรืออัลโดสเตอโรน ที่ฉีดเข้าทางช่องท้องเป็นเวลา 30 นาที โดยกลุ่มของอัลโดสเตอโรนจะแบ่งออกเป็นอีก 2 กลุ่มย่อยคือ low dose อัลโดสเตอโรน (LA: 150 µg/kg BW) หรือ high dose อัลโดสเตอโรน (HA: 500 µg/kg BW) ทำการตรวจวัดระดับปริมาณโปรตีน pEGFR pERK1/2 และ pc-Src และตำแหน่งบริเวณที่แสดงออกของโปรตีนดังกล่าวภายในไต โดยวิธี Western blot และ immunohistochemistry ตามลำดับ ผลการทดลองพบว่าภายหลังการฉีดอัลโดสเตอโรน 30 นาที พลาสมาอัลโดสเตอโรนมีระดับเพิ่มขึ้นทั้ง LA และ HA ($p<0.001$) อัลโดสเตอโรนเพิ่มระดับปริมาณโปรตีน pEGFR และ pERK1/2 ($p<0.001$) แต่ไม่เปลี่ยนแปลงระดับโปรตีน pc-Src พบว่า LA เพิ่มระดับการแสดงออกของโปรตีน pEGFR ในบริเวณ inner stripe ของ outer medulla และบริเวณ inner medulla ในขณะที่ HA เพิ่มระดับการแสดงออกในบริเวณ cortex และ medulla ยังพบอีกว่าอัลโดสเตอโรนเพิ่มระดับการแสดงออกของโปรตีน pERK1/2 ในทุกบริเวณของเนื้อไตที่ทำการศึกษา ส่วนการแสดงออกของโปรตีน pc-Src บริเวณ cortex มีค่าเพิ่มขึ้นเมื่อได้รับอัลโดสเตอโรน โดยพบว่ามีการกระจายตัวของโปรตีนที่พบในบริเวณ luminal membrane ของกลุ่ม LA ไปแสดงออกแทนในบริเวณ basolateral membrane ของกลุ่ม HA อย่างไรก็ตามทั้ง LA และ HA เพิ่มระดับการแสดงออกของโปรตีน pc-Src ในบริเวณ cortex แต่ HA ลดระดับการแสดงออกของโปรตีนในบริเวณ inner medulla

ผลการศึกษานี้แสดงเป็นครั้งแรกถึงข้อมูลที่ว่าอัลโดสเตอโรนในวิถีที่ไม่ผ่านจีโนมิกสามารถเพิ่มระดับปริมาณและการแสดงออกของโปรตีน pEGFR และ pERK1/2 ในไตหนูแรท อัลโดสเตอโรนไม่มีอิทธิพลต่อระดับปริมาณโปรตีนทั้งหมดของ pc-Src แต่ผลต่อตำแหน่งบริเวณที่พบการแสดงออกนั้นได้ถูกควบคุมอย่างเด่นชัด

สาขาวิชา สรีรวิทยา ลายมือชื่อนิสิต กิตติศักดิ์ สินพิทักษ์กุล

ปีการศึกษา 2552 ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์หลัก

ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์ร่วม

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KITTISAK SINPHITUKKUL: EXPRESSIONS OF EGFR–ERK1/2–c-Src SIGNALING MEDIATORS IN A NONGENOMIC PATHWAY AFTER ALDOSTERONE ADMINISTRATION IN RAT KIDNEY.
THESIS ADVISOR: ASSOC. PROF. SOMCHIT EIAM-ONG, Ph.D.,
THESIS CO-ADVISOR: KRISSANAPHONG MANOTHAM, M.D.,
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Aldosterone is the major mineralocorticoid hormone which plays an important role in maintenance of body sodium, potassium and acid-base balance. Many *in vitro* studies have demonstrated that aldosterone could enhance protein levels of phosphorylated epidermal growth factor receptor (pEGFR), and extracellular signal-regulated kinases 1/2 (pERK1/2) via nongenomic pathway. One of inter-mediators involved is phosphorylated cytosolic tyrosine kinase of Src (pc-Src). However, there is no *in vivo* study of the protein abundances and expressions of pEGFR–pERK1/2–pc-Src signaling mediators through a nongenomic pathway in rat kidney.

Male Wistar rats were received either normal saline solution (NSS) or aldosterone by intraperitoneal injection for 30 minutes. For aldosterone group, the rats were further divided into two groups: low dose aldosterone (LA: 150 μg/kg BW) or high dose aldosterone (HA: 500 μg/kg BW). Protein abundance and localization of renal pEGFR, pERK1/2, and pc-Src were determined by Western blot and immunohistochemistry, respectively.

After 30 minute of aldosterone injection, plasma aldosterone levels were markedly increased in both LA and HA groups ($p<0.001$). Aldosterone enhanced renal pEGFR and pERK1/2 protein abundances ($p<0.001$) but did not change in pc-Src protein level. LA augmented pEGFR protein expression in the inner stripe of outer medulla and in the inner medulla regions while HA stimulated the expression in both cortex and medulla. Aldosterone elevated pERK1/2 protein expression in all studied areas. The cortical expression of pc-Src protein was increased by aldosterone with the redistribution of staining from the luminal membrane in the LA group to be the basolateral membrane in the HA group. However, both LA and HA activated renal pc-Src protein expression in the cortex but HA decreased the expression in the inner medulla area.

These data first demonstrated that aldosterone, via nongenomic pathway, could elevate pEGFR and pERK1/2 protein abundances and expressions in rat kidney. Aldosterone seems not to influence the total renal pc-Src protein abundance meanwhile the protein localization was obviously modulated.

Field of study: Physiology
Academic year: 2009

Student's signature Kittisak Sinphitukkul
Advisor's signature S. Eiam-Ong
Co-advisor's signature

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LIST OF ABBREVIATIONS

EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
pEGFR	Phosphorylated Epidermal growth factor receptor
ERK1/2	Extracellular signal-regulated kinase 1 and 2
pERK1/2	Phosphorylated extracellular signal-regulated kinase 1 and 2
c-Src	Cytosolic tyrosine kinase
pc-Src	Phosphorylated cytosolic tyrosine kinase
°C	Degree Celcius
g	Gram
ip	intraperitoneal injection
M	Molar
dL	Decilitre
mL	Millilitre
µg	Microgram
µL	Microlitre
Cr	Creatinine
C_{Cr}	Creatinine clearance
H_2O_2	Hydrogen peroxide
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBS	Tris buffer solution
PBS	Phosphate buffer solution
NSS	Normal saline solution
NaCl	Sodium chloride

KCl	Potassium chloride
Na_2HPO_4	Di-sodium hydrogen phosphate
KH_2PO_4	Potassium di-hydrogen phosphate
NaH_2PO_4 H_2O	Sodium phosphate monobasic monohydrate
LA	Low dose aldosterone
HA	High dose aldosterone
OS	Outer stripe of outer medulla
IS	Inner stripe of outer medulla
G	Glomerulus
PCT	Proximal convoluted tubule
PT	Proximal tubule
DT	Distal tubule
T	Thin limb of Henle's loop
TAL	Thick ascending limb of Henle's loop
CD	Collecting duct

CHAPTER I

INTRODUCTION

BACKGROUND AND RATIONALE

Aldosterone is the major physiologic of mineralocorticoid hormone which plays an important role in maintenance of sodium, potassium and acid-base balance (Booth et al., 2002; Good, 2007). Furthermore, it increases extracellular volume in response to volume depletion signaled by the renin-angiotensin system (Vinciguerra et al., 2005). The principal regulators of synthesis and secretion of aldosterone are angiotensin II, the concentration of plasma potassium and adrenocorticotropin releasing hormone (Connell and Davies, 2005).

Renal effects of aldosterone have been ascribed to a genomic mechanism which the binding of its intracellular receptors, then the hormone-receptor complex is transferred to the nucleus where it acts as a transcriptional regulator (Rozansky, 2006). Besides genomic action of aldosterone, its nongenomic action has been extensively investigated (Grossmann and Gekle, 2009). This action is characterized with a rapid onset (seconds to a few minutes) and insensitivity towards inhibitors of transcription and translation (Funder, 2005; Harvey et al., 2008; Thomas et al., 2007). Most studies have been performed in cell culture models.

After aldosterone administration, the rapid alteration in intracellular concentration of various ions is likely obvious (Good, 2007; Thomas et al., 2008). For example, aldosterone rapidly increased cytosolic Na^+ in Madin-Darby canine kidney (MDCK) cells within 2 minutes (Gekle et al., 1998). Furthermore, the effect of aldosterone on intracellular pH has been described recently. In the outer medullary collecting duct microdissected from normal mice, an exposure to aldosterone for 15

minutes increased H^+ extrusion from the acid-load type of an intercalated cell (Winter et al., 2004). Moreover, Watts et al. (2006) have demonstrated that aldosterone decreased HCO_3^- reabsorption in the segment of medullary thick ascending limb within 15 minutes. In addition, Harvey and Higgins (2000) have shown that aldosterone produced a rapid, transient increase in intracellular calcium level in mouse cortical collecting duct cell within 2 minutes.

These nongenomic actions of aldosterone have been extensively identified in capable of transactivation with a multitude of signaling molecules, such as extracellular signal-regulated kinase 1 and 2 (ERK1/2) (Grossmann and Gekle, 2009). ERK1/2 are the member of mitogen-activated protein kinase (MAP kinase) which participate in signal transduction pathway of growth factor hormones such as epidermal growth factor (EGF) (Grossmann and Gekle, 2009). After EGF binding, the receptor of EGF (EGFR) was auto-phosphorylated (pEGFR) and the consequences of phosphorylation in signaling cascade molecules were ensued (Grossmann and Gekle, 2007; Grossmann and Gekle, 2008; Boldyreff and Wehling, 2003a). Then, the phosphorylated forms of ERK1/2 (pERK1/2), a downstream effector, were rapidly induced (Zeng et al., 2009). It has demonstrated that pERK1/2 could activate various protein molecules such as channels or transporters (Pearson et al., 2001; Shi et al., 2002).

Gekle et al. (2002) demonstrated that, in MDCK cells, aldosterone could mimic the actions as EGF performed. These included increases in cytosolic calcium levels, pERK1/2, through EGFR phosphorylation. Furthermore, aldosterone could induce potentiation effect of EGF signaling in Chinese hamster ovary (CHO) cells transfected with human EGFR (Krug et al., 2002).

The upstream-signal that aldosterone transactivated EGFR has been clarified (Boldyreff and Wehling, 2003b). The initiation of the signaling pathway seems to be the interaction of the steroid hormone receptors with signaling molecules, such as cytosolic tyrosine kinase of Src or c-Src (Grossmann and Gekle, 2008). c-Src is a member of membrane-associated non-receptor tyrosine kinases (Roskoski, 2004). Src tyrosine kinases have multiple biological functions, including the role in EGFR phosphorylation (Sandilands and Frame, 2008; Sato et al., 1995). In mouse cortical collecting duct (M-1) cells, aldosterone could rapidly increase both activity and autophosphorylation of Src kinase (Braun et al., 2004). Moreover, in CHO cells, aldosterone could enhance the protein expressions of pEGFR, pERK1/2, and phosphorylated form of c-Src (pc-Src) (Krug et al., 2002). This study also showed that an inhibition of c-Src kinase could prevent the action of aldosterone in enhancing of pEGFR. The authors suggested that aldosterone stimulates the EGFR-ERK1/2 signaling pathway by using c-Src kinase to induce EGFR transactivation.

At present, no *in vivo* study has demonstrated whether aldosterone alters the protein expressions of EGFR–ERK1/2–c-Src signaling mediators in a nongenomic pathway in rat kidney, or not. Therefore, the present study aims to investigate this regards. After 30-min intra-peritoneal injection of aldosterone, the protein abundances of pEGFR, pERK1/2, and pc-Src in rat kidney were measured by Western blot analysis whereas the localization and distribution of these proteins were examined by immunohistochemical method.

RESEARCH QUESTIONS

1. Which areas in rat kidney do pEGFR, pERK1/2, and pc-Src proteins localize or distribute after 30-minute injection of aldosterone or normal saline solution (NSS)?
2. After 30-minute injection, does aldosterone change any localization or distribution of pEGFR, pERK1/2, and pc-Src proteins in rat kidney as compared with NSS treated group?
3. After 30-minute injection, does aldosterone alter protein abundances of pEGFR, pERK1/2, and pc-Src in rat kidney as compared with NSS treated group?
4. After 30-minute injection, does a high dose of aldosterone have more influence on pEGFR, pERK1/2, and pc-Src protein abundances in rat kidney than those of a low dose?

RESEARCH OBJECTIVES

1. To elucidate the areas in rat kidney that pEGFR, pERK1/2, and pc-Src proteins localize or distribute after 30-minute injection of aldosterone or NSS.
2. To clarify whether, after 30-minute injection, aldosterone could alter the protein expressions of pEGFR–pERK1/2–pc-Src signaling mediators in rat kidney, or not.
3. To examine the localization and distribution of pEGFR, pERK1/2, and pc-Src proteins in rat kidney after 30-minute injection of aldosterone as compared with NSS treated group.

4. To quantify the protein abundances of pEGFR, pERK1/2, and pc-Src in rat kidney after 30-minute injection of aldosterone as compared with NSS treated group.
5. To compare the protein abundances of pEGFR, pERK1/2, and pc-Src in rat kidney after 30-minute injection of a low dose to a high dose of aldosterone.

HYPOTHESIS

In nongenomic pathway, aldosterone would enhance the protein expressions of pEGFR, pERK1/2, and pc-Src in rat kidney. The high dose of aldosterone could elevate more protein levels of pEGFR, pERK1/2, and pc-Src as compared with the low dose.

KEY WORDS

aldosterone

nongenomic pathway

phosphorylated epidermal growth factor receptor (pEGFR)

phosphorylated extracellular signal-regulated kinase 1 and 2 (pERK1/2)

phosphorylated cytosolic tyrosine kinase (pc-Src)

protein abundance

immunohistochemistry

rat kidney

EXPECTED BENEFIT AND APPLICATION

The results will provide:

1. The first animal study data demonstrating the protein expressions of pEGFR, pERK1/2, and pc-Src in rat kidney after 30-minute aldosterone administration.
2. Further clarification of nongenomic actions of aldosterone in rat kidney.
3. The fundamental knowledge for further studies in other nongenomic or genomic pathways of aldosterone in regulation of targeted proteins, such as transporters, and channels, etc.

CHAPTER II

THEORY AND LITERATURE REVIEW

ALDOSTERONE

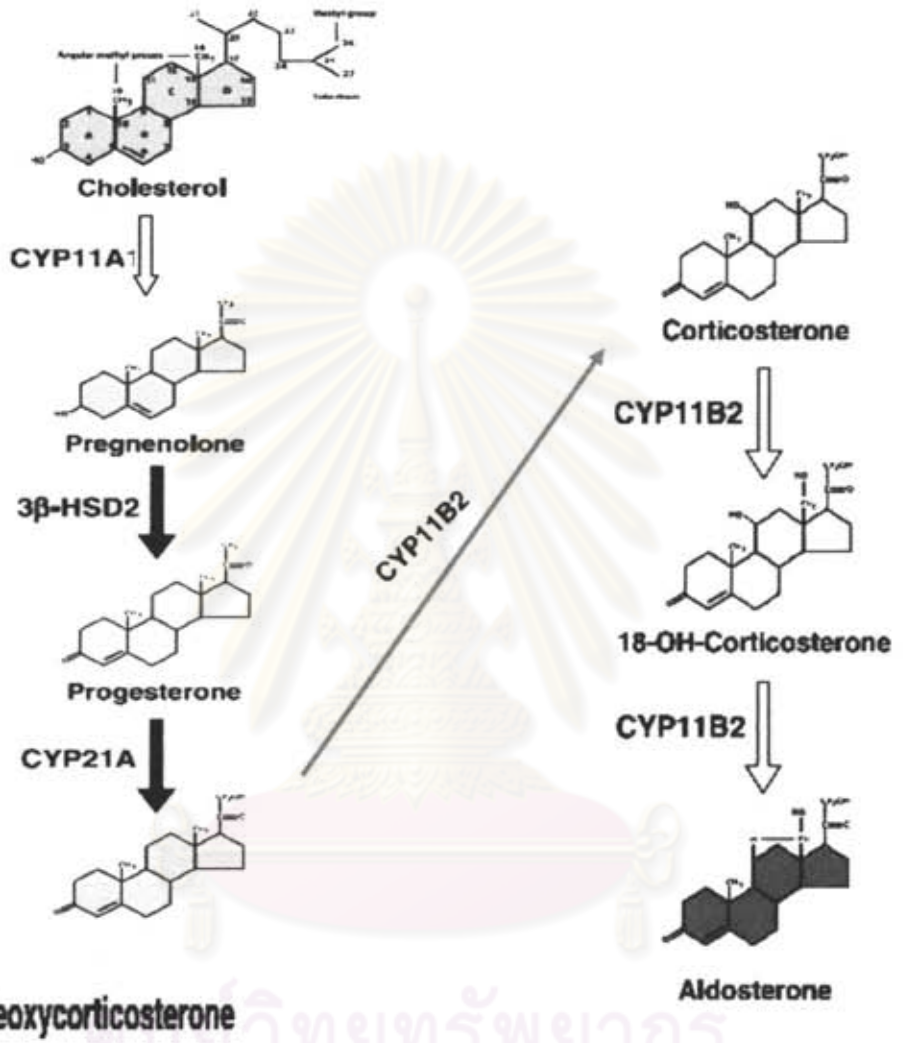
Biosynthesis

Aldosterone is a steroid hormone produced by zona glomerulosa cells of the adrenal cortex (Connell and Davies, 2005). Cholesterol is converted to aldosterone by a series of locus- and orientation-specific enzyme reaction which are located in mitochondria and smooth endoplasmic reticulum (Figure A). The daily amount of secreted aldosterone is produced only 50-200 μg in human or 1.2-2.4 μg in rat (Kaplan, 1988; Moll et al., 1975). 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 138.5-554 pmol/L (Riordan and Malan, 1982) or 400-2,000 pmol/L in rat (Rodriguez-Ayala et al., 2006; Hilfenhaus, 1976).

The half-life of aldosterone is short (20 minute) (Ganong, 2005). Aldosterone is catabolized principally in the liver into the tetrahydroglucuronide derivative, and its metabolites are excreted in the urine. Less than 1% of secreted aldosterone appears in urine in the free form. Another 5% is in form of acid-labile conjugate, and up to 40% is in form of the tetrahydroglucuronide derivative (Ganong, 2005).

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

segments of collecting duct (Hamm, 2004). The pivotal regulators of aldosterone synthesis and secretion are angiotensin II, the concentration of plasma potassium and adrenocorticotropin releasing hormone (Connell and Davies, 2005).



(⇨ Reactions in mitochrondria) (➡ Reactions in endoplasmic reticulum)

Figure A: Biosynthesis of aldosterone (Connell and Davies, 2005)

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 (Lösel and Wehling, 2007). Research that has been carried out mainly in the past decade has identified other responses to steroids that are much more rapid and take place in seconds or minutes. These responses follow nongenomic pathways, and they are not rare (Lösel and Wehling, 2007).

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). This aldosterone-MR complex translocates to nucleus and promotes gene transcription and production of proteins that modulate the expression and activity of channels and other ion transport proteins (Good, 2007). The length time of genomic 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 (Good, 2007).

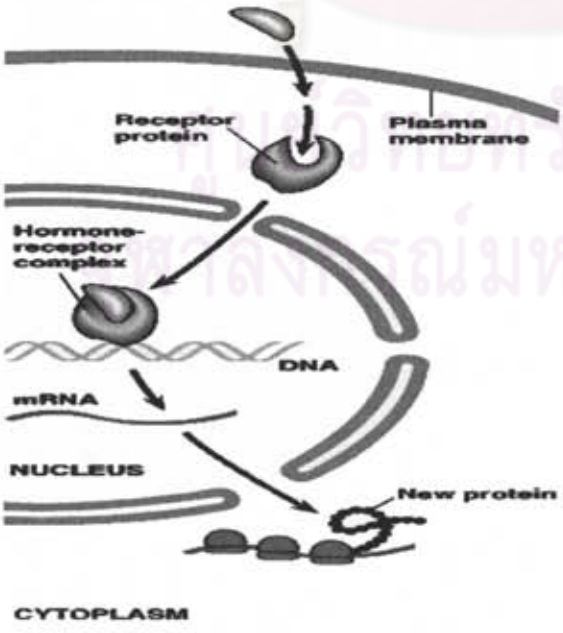


Figure B: Genomic pathway of aldosterone (Good, 2007)

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 (Lösel and Wehling, 2007). The responses take place in seconds to minutes and are independent on transcription and translation (Lösel and Wehling, 2003; Funder, 2005; Lösel and Wehling, 2007). The nongenomic action often involves the generation of intracellular secondary messengers and various signal transduction cascades (Figure C) such as protein kinase pathways. These actions of aldosterone have been extensively investigated, mostly in cell culture studies (Funder, 2005, Good, 2007; Lösel et al., 2003). The mammalian kidney is one of the significant sites of EGF synthesis (Gesualdo, et al. 1996). The actions of EGF have been reported in modulation of glomerular hemodynamics, renal metabolism, and tubular transport (Arar et al., 1999; Grossmann et al., 2004; Harris et al., 1988; Harris et al., 1989).

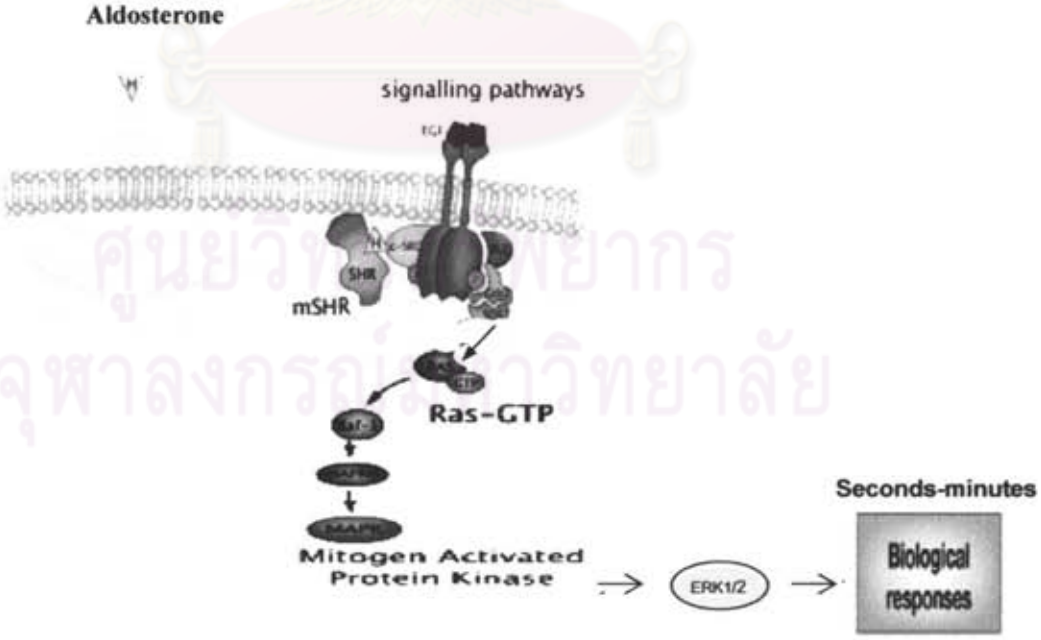


Figure C: Nongenomic pathway of aldosterone (Lösel and Wehling, 2007)

Nongenomic actions of aldosterone in cell culture studies

The rapid response of changing in intracellular concentration of various ions is likely obvious (Good, 2007). Gekle et al. (1998) showed that MDCK cells exhibiting properties of collecting duct intercalated cells responded to aldosterone stimulation. Aldosterone induced a nongenomic increase in cytosolic sodium levels within 2 minutes. This was also accompanied by a significant increase in cell volume with a similar time course compared with the changes in cytosolic sodium concentration (Gekle et al., 1998). Moreover, aldosterone increased H^+ extrusion within 15 minutes in acid loaded collecting duct cells (Winter et al., 2004).

Recently, Watts et al. (2006) showed that aldosterone could decrease HCO_3^- reabsorption within 15 minutes in microdissected segment of medullary thick ascending limb. Furthermore, in M-1 cells, aldosterone also produced a rapid, transient peak increase in cytosolic calcium (Harvey and Higgins, 2000). This alteration was unaffected by pretreatment with spironolactone (mineralocorticoid receptor blocker) or actinomycin D (gene transcription inhibitor) (Booth et al., 2002).

Several studies have indicated that aldostrone induced the rapid nongenomic actions through membrane-associated receptor of epidermal growth factor as epidermal growth factor (EGF) performed (Grossmann and Gekle, 2008; Jorissen et al., 2003).

Epidermal growth factor (EGF)

EGF is a single polypeptide consisting of 53 amino acid residues, of which six are cysteine (Figure D). These cysteines form three intramolecular disulfide bonds that are important for maintaining the biological activity of EGF (Wong and Guillaud, 2004). It is synthesized as a precursor consisting of 1217 amino acid

residues, from which the mature form may be generated by proteolysis. The EGF precursor is a glycosylated transmembrane protein with an apparent molecular weight of 140–150 kDa (Wong and Guillaud, 2004). The mature EGF binds to the EGF receptor (EGFR) on the cell surface and stimulates the intrinsic protein-tyrosine kinase activity of the receptor (Wong and Guillaud, 2004).

Figure D: Structural of epidermal growth factor (EGF) (Wong and Guillaud, 2004)

Epidermal growth factor receptor (EGFR)

The EGFR is one of four transmembrane growth factor receptor proteins that share similarities in structure and function (Singh and Harris, 2005). It is synthesized from a 1210-residue polypeptide precursor (Jorissen et. al, 2003). After cleavage of the N-terminal sequence, the mature 1186-residue protein, a 170-kDa glycoprotein, is inserted into the cell membrane (Jorissen et. al, 2003). It consists of the N-terminal extracellular domain (ECD), a single transmembrane domain, and an intracellular domain consisting of a juxtamembrane sub-domain, intracellular protein tyrosine kinase (PTK) sub-domain, and non-catalytic C-terminal regulatory region (Figure E). The glycosylated ECD is made up of four smaller domains of which two (sub-domain I and III, alternatively called L1 and L2, respectively) are homologous

Leu-rich domains consisting of β-helix folds and the remaining two are Cys-rich (Sub-domain II and IV, or S1 and S2) which are the ligand binding site (Singh and Harris, 2005; Bazley and Gullick, 2005). The short transmembrane domain is typically rich hydrophobic amino acids and is flanked on the intracellular side by a juxtamembrane region that has a regulatory role in receptor downregulation (Singh and Harris, 2005; Bazley and Gullick, 2005). It is predominantly localized to the basolateral cell surface in various epithelial tissues (Gesualdo et al, 1996). The EGFR protein expression was detected mainly along the thick ascending limb of Henle's loop and the distal tubule, in endothelial cells within the glomerulus, as well as in peritubular capillaries and arterioles of the interstitium in normal human kidney (Gesualdo et al, 1996).

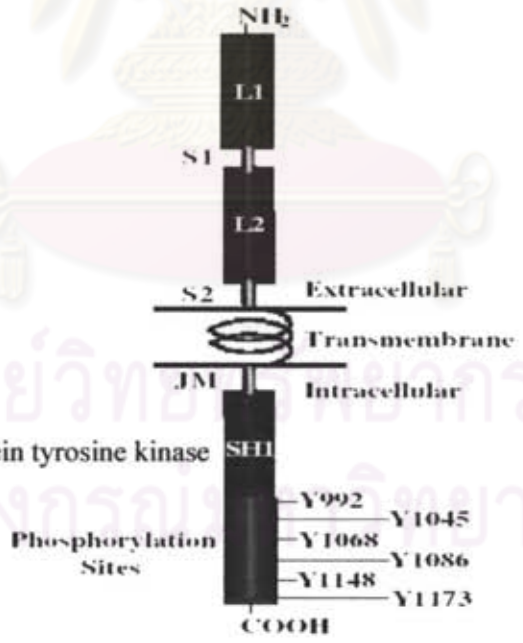


Figure E: Epidermal growth factor receptor (EGFR) (Bazley and Gullick, 2005)

After ligand binding, EGFR will be dimerized, protein kinase of each receptor monomer then phosphorylates a distinct set of tyrosine residues in cytosolic domain of its dimer, partner, a process termed autophosphorylation occurs in two stages (Karp, 2002). First, tyrosine residues in the phosphorylation lip near catalytic site are phosphorylated. This leads to a conformational change that facilitates binding of ATP. The receptor kinase activity then phosphorylates other sites in cytosolic domain. The resulting phosphotyrosines serve as docking sites for other proteins involved in its tyrosine residues-mediated signaling transduction (Figure F). EGFR plays an influential role in cell proliferation, differentiation and ion transport in mammalian cells (Karp, 2002; Singh and Harris, 2005). Finally, the mitogen activated protein kinase (MAPK) cascade is stimulated and then phosphorylated a down stream mediator, such as extracellular signal-regulated kinases 1 and 2 (ERK1/2) (Singh and Harris, 2005).

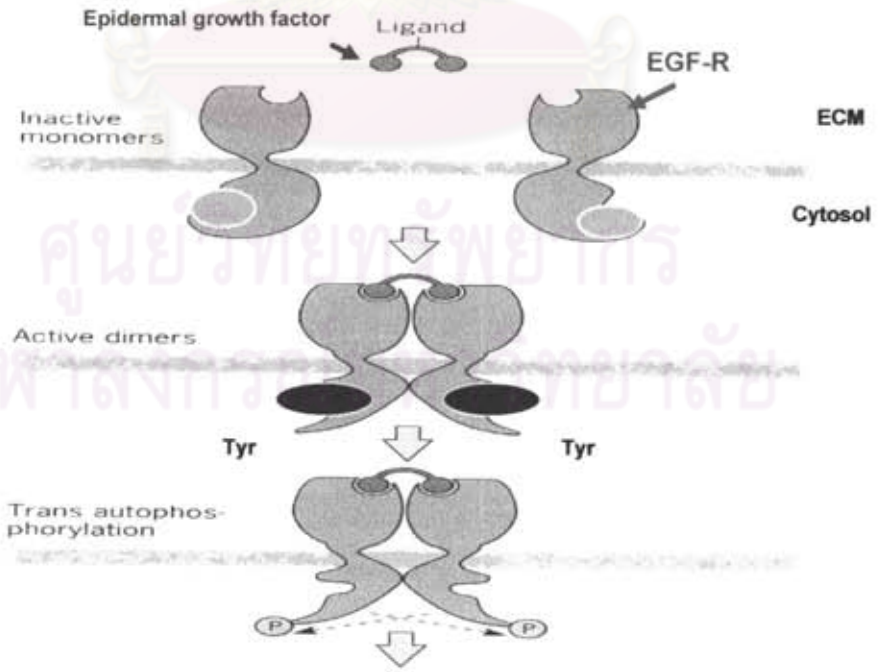


Figure F: Binding of EGF to EGFR (Karp, 2002)

Extracellular signal-regulated kinases 1 and 2 (ERK1/2)

ERK1/2, a member of the mitogen-activated protein kinase (MAPK) family, could be activated by growth factors (Nishimoto and Nishida, 2006). These proteins are 43 and 41 kDa that are nearly 85% identical overall, with much greater identity in the core regions involved in binding substrates (Pearson et al., 2001; Tian et al., 2000). Like other protein kinases, the N-terminal domain is composed largely of β-strands along with two helices, α-helix C and α-helix L16 contributed by a C-terminal domain (Chen et al., 2001). The C-terminal domain consists of primarily α-helices with four short β-strands that contain several residues involved in catalysis (Chen et al., 2001). The surface loop (L12) is referred to as the activation loop of α-helix L16 or phosphorylation lip because it contains the activating site of phosphorylation. There are two phosphoacceptor sites, tyrosine185 and threonine183, which are phosporylated to activate the kinases (Figure G) to be pERK1/2 (Chen et al., 2001).

These protein kinases are widely expressed which are located both in cytoplasm and nucleus. The pERK1/2 consequently could phosphorylate other protein molecules, such as transporters or channels (Nishimoto and Nishida, 2006; Pearson et al., 2001).

Figure G: Structure of extracellular signal-regulated kinase 1 and 2 (ERK1/2) (Chen et al., 2001)

EGF, EGFR, ERK1/2, and aldosterone

Gekle et al. (2002) demonstrated that, in MDCK cells, aldosterone could mimic the actions as EGF performed. These alterations were increases in cytosolic calcium levels, induction of extracellular signal regulated kinase 1/2 phosphorylation (pERK1/2), through phosphorylation of EGF receptor (EGFR). Inhibition of ERK1/2 phosphorylation could reduce the rise in cytosolic calcium (Gekle et al., 2002). Furthermore, Krug et al. (2002) investigated the responses of aldosterone in Chinese hamster ovary (CHO) cells transfected with human EGFR-1. These CHO cells do not express EGFR under control condition (Shi et al., 2000). The data demonstrated that, within 10 minutes, nanomolar concentrations of aldosterone could potentiate the actions of EGF resulting in increases EGFR and ERK1/2 phosphorylation (Krug et al., 2002).

Signaling mediator molecule involved in EGFR/aldosterone interaction

The upstream-signal that aldosterone could transactivate EGFR has been clarified. EGFR is a receptor tyrosine kinases may be activated independently of ligand binding, through the phosphorylation of specific residues in the cytoplasmic domain of the receptor that are distinct from its auto-phosphorylation sites. In such a way EGFR can be phosphorylated at Tyr845 by Src family tyrosine kinases to bring about its activation without binding of EGF (Drube et al., 2006). The Src protein kinases are expressed ubiquitously in vertebrate cells. The transactivating effect on EGFR is mediated by cytosolic tyrosine kinases or c-Src. The c-Src kinase could bind to the cytoplasmic face of the plasma membrane (Figure H).

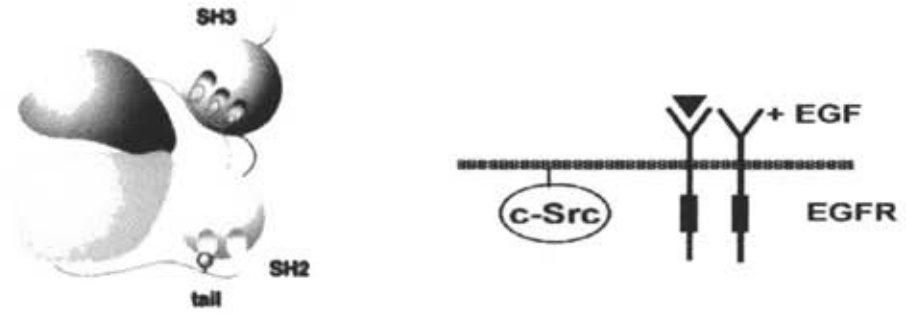


Figure H: Cytosolic tyrosine kinases of Src (c-Src) (Roskoski, 2005)

Cytosolic tyrosine kinase of Src (c-Src)

c-Src is 60 kDa protein composed of six distinct functional regions (Figure I): (a) the Src homology (SH) SH4 domain can attach with the plasma membrane, (b) the unique region, both (c) the SH3 and (d) SH2 domains can bind to other proteins, (e) the protein kinase domain consists of an autophosphorylation site at Tyr416, and (f) the C-terminal regulatory region which consists of a negative regulatory site at Tyr527 (Ingley, 2008; Roskoski, 2004).

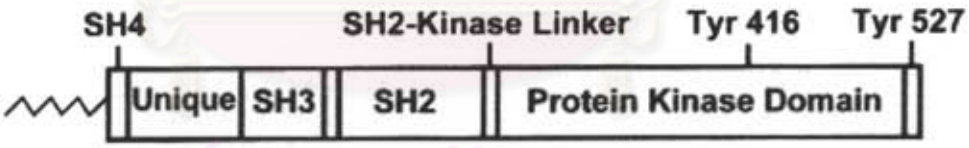


Figure I: Structural domains of c-Src (Roskoski, 2004)

c-Src is a member of the nonreceptor tyrosine kinases (Ingley, 2008; Thomas and Brugge, 1997). It is allosteric enzymes with at least two conformational states that are differentially stabilized by protein-protein interactions and by phosphorylation-dephosphorylation at two different sites (Figure J). When c-Src is in a catalytically inactive or 'closed' conformation, phosphorylated Tyr527 interacts

with the SH2 domain, the SH3 domain is engaged to the SH2-kinase linker region, and Tyr416 in the activation loop of the kinase is unphosphorylated (Roskoski, 2004). Upon activation, Tyr527 is de-phosphorylated, the constraints upon the kinase domain are released, autophosphorylation of Tyr416 occurs, and the SH3 and SH2 domain are then free to recruit other molecules involved in intracellular signaling (Roskoski, 2004). Active c-Src can bind to other proteins through a phosphorylation-independent SH3 domain and a phosphorylation-dependent SH2 domain (Ingley, 2008; Roskoski, 2005).

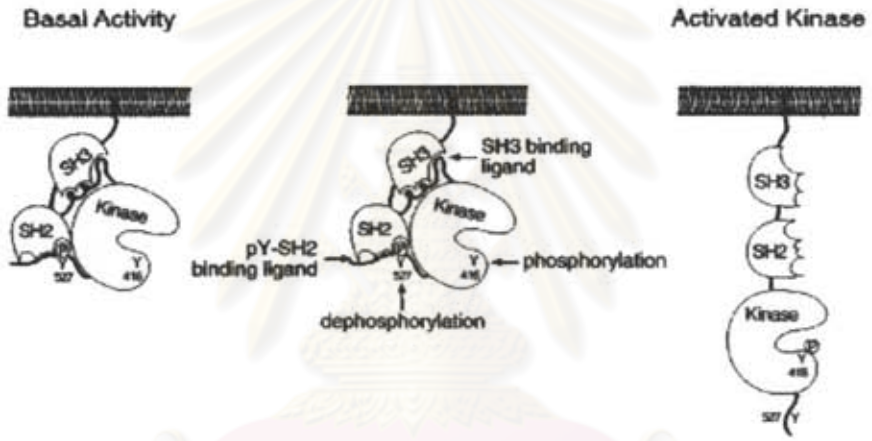


Figure J: Mechanism involved in the activation of c-Src

(Thomas and Brugge, 1997)

In the normal rat kidney, Lin et al. (2004) showed that c-Src protein is highly expressed in principal cells of cortical collecting and medullary collecting tubules, thick ascending limb of Henle's loop, and connecting tubule. The c-Src protein kinases play key roles in cell stimulation of the epidermal growth factor receptor (Parsons and Parsons, 1997).

The interaction of c-Src with EGFR signaling pathway

c-Src appears to communicate with many different receptor protein tyrosine kinases such as EGFR (Sato et al., 1995). Src is also involved in EGFR signaling. Overexpression of Src enhances many different EGF responses protein tyrosine phosphorylation in murine embryo fibroblasts (Wilson and Parsons 1990). The nature of this interaction and its role in the initial activation is unclear. However, the Tyr845 site of EGFR has also been shown to interact with c-Src in vitro study (Sato et al., 1995). The result showed that the EGFR is phosphorylated on tyrosine residue when Src is overexpressed. Furthermore, the proposed position of the Src SH2 binding motif at the juxtamembrane location represents the location of the Src binding sites on EGFR (Figure K).

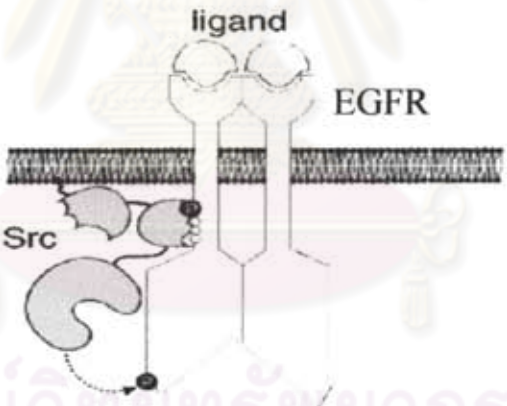


Figure K: The interaction of c-Src with EGFR (Thomas and Brugge, 1997)

c-Src and aldosterone

It was demonstrated that, in M-1 cells, Src kinase activity was rapidly enhanced as early as 2 minutes after administration of aldosterone (Braun et al., 2004). This was accompanied with an increase in autophosphorylation of Src kinase. Moreover, an inhibition of c-Src kinase could prevent the action of aldosterone in

CHO cells (Krug et al., 2002; Grossmann et al., 2005) as well as in M-1 cells (McEneaney et al., 2007).

In cultured rat mesenteric smooth muscle cell, it has shown that c-Src phosphorylation (pc-Src) was rapidly increased as early as 1 and 5 minutes after administration of aldosterone (Callera et al., 2005b; Montezano et al., 2008). In addition, an inhibition of pc-Src could abolish the action of aldosterone (Callera et al., 2005a).

Nongenomic actions of aldosterone in animal studies

Some experiments demonstrated the rapid responses in animals receiving aldosterone injection. For example, an intravenous infusion of aldosterone into rats increased renal sodium excretion within 15 minutes (Rad et al., 2005). Furthermore, Winter et al. (2004) showed that after 30 minutes of intraperitoneal injection of aldosterone at a dose of 150 μg/kg BW, an increase in apical staining of the H^+-ATPase subunit in type A intercalated cells, suggesting the nongenomic action on modulation of transport protein expression. However, in this study, there were no data of plasma aldosterone levels that were enhanced after the injection.

To date, there is no animal study whether, after 30-minute administration, aldosterone changes the protein expressions of pEGFR, pERK1/2, and pc-Src in rat kidney. To accomplish these aims, the nongenomic signaling mediators of aldosterone were elucidated in the present study.

CHAPTER III

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

The study was approved by the Ethics Committee of Research, Chulalongkorn University. Male Wistar rats, weighing 200 to 240 grams, were obtained from the National Center of Scientific Use of Animals (Mahidol University, Salaya, Thailand). The animals were housed with controlled temperature (23-25°C) and 12 hours of controlled light-dark time. The animals were given free access to a laboratory chow and water. At the end of each experiment, the rats were terminated with an overdose of thiopental by intraperitoneal (ip.) injection and their remains were eliminated by burning in an incinerator.

CHEMICALS

Chemical agents

Aldosterone (25 mg), 3, 3′-diaminobenzidine (DAB) solution, protease inhibitor cocktail, Tris base, sodium dodecyl sulfate, and 3-aminopropyltriethyloxy-saline were purchased from Sigma, MO, USA. Thiopental was obtained from Jagsonpal Pharmaceuticals Ltd., Haryana, India. Methanol, acetic acid, absolute ethanol, 95% ethanol, xylene, sodium chloride (NaCl), potassium chloride (KCl), di-sodium hydrogen phosphate (Na_2HPO_4), potassium di-hydrogen phosphate (KH_2PO_4), sodium phosphate monobasic monohydrate (NaH_2PO_4 H_2O), 6% hydrogen peroxide (H_2O_2), mounting medium, Tween-20, sucrose, and 40%

formaldehyde were purchased from Merck, NJ, USA. Paraffin powder embedding medium was purchased from Sigma, USA. Avidin-biotin-peroxidase complex (ABC) -streptavidin horseradish peroxidase complex was purchased from Vector, 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. Modified Lowry protein assay reagent, SuperSignal® West Pico kit, Laemmli sample buffer, ammonium persulfate, 40% acrylamide/Bis, glycine, TEMED, filter paper, nitrocellulose membrane, and CL-XPosure™ Film 5 x 7 inches were purchased from Pierce, IL, USA.

Antibodies

Mouse monoclonal antibody against pEGFR (Catalog No. #2236) rabbit monoclonal antibody against pERK1/2 (Catalog No. #4370) were purchased from Cell signaling, MA, USA. Rabbit monoclonal antibody against pc-Src (Catalog No. AT-7135) was purchased from MBL® International Corporation, MA, USA. Normal horse serum (S-2000), biotinylated goat anti-mouse IgG (H+L) (Catalog No. BA-9200), and biotinylated goat anti-rabbit IgG (H+L) (Catalog No. BA-1000) secondary antibodies were purchased from Vector, USA. ß-actin mouse monoclonal antibody (Catalog No. SC-47778) was purchased from Santa cruz, CA, USA. Blotting grade goat anti-rabbit IgG (H+L) (Human IgG Adsorbed) horseradish peroxidase conjugate (Catalog No. 170-6515) and blotting grade goat anti-mouse IgG (H+L) horseradish peroxidase conjugate (Catalog No. 170-6516) were purchased from Bio-Rad, 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 (Cr) in order to assess kidney function (<1 mg%) (Seujange et al., 2008). The rats were divided into three groups (n = 8 / group) as follows.

Group 1 (Sham): The animals were received normal saline solution (NSS) (0.5 mL/kg BW) by intraperitoneal injection (ip).

Group 2 (Low dose aldosterone, LA): The animals were received aldosterone 150 µg/kg BW; diluted in NSS; ip (Winter et al., 2004) (Sigma, USA).

Group 3 (High dose aldosterone, HA): The animals were received aldosterone 500 µg/kg BW; ip (Loffing et al., 2001).

All groups were received their respective treatment for 30 minutes.

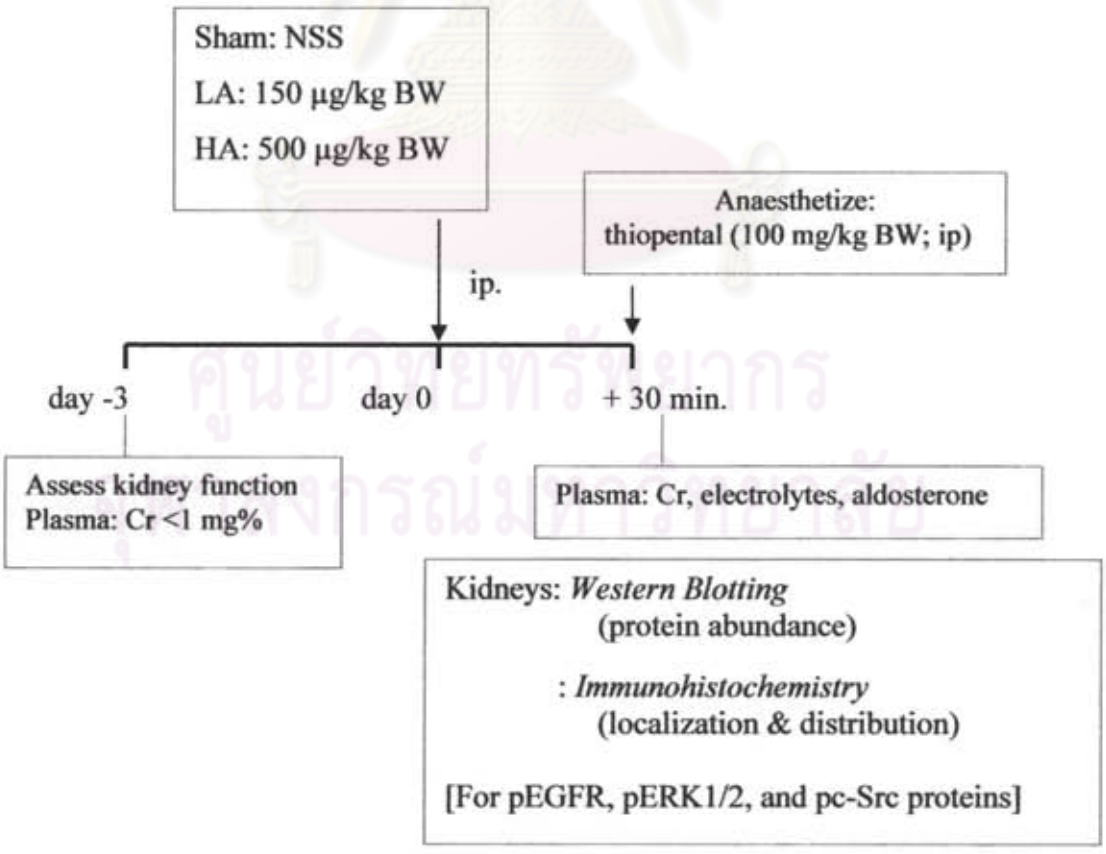
Surgical Operation

One day before the experiment, the animals were placed in metabolic cages for twenty-four hour urine collection. On the experimental date, after 30-min injection of NSS or aldosterone, the rats were anesthetized by thiopental (100 mg/kg BW; ip., Jagsonpal Pharmaceuticals Ltd., India) (Loffing et al., 2001) and the abdomen was opened via a midline incision. Blood sample was collected from the abdominal aorta, and was centrifuged at 1,000 g for 15 minutes at 4°C (H-103N, Kokusan, Tokyo, Japan). Plasma was stored at -80 °C until used for measurement of aldosterone level using a commercial radioimmunoassay kit (Aldo-Riact; CIS Bio International, Gif-Sur-Yvette, France). The radioactivity bound was detected by a gamma scintillation counter (1470 WIZARD Automatic Gamma counters,

PerkinElmer, CT, USA). Blood chemistry was measured for blood urea nitrogen, Cr, electrolytes (ion selection electrode) by indirect method (Model CX3, Beckman, Krefeld, Germany).

The kidneys were removed, fixed in liquid nitrogen and then stored at -80 °C until used for measurement of pEGFR, pERK1/2, and pc-Src protein abundance by Western blot analysis. Additional renal tissue samples were fixed in 10% paraformaldehyde overnight, subjected to tissue processing (dehydration, clearing, and infiltration) by automatic tissue processor (Shandon Citadel 2000, Thermo, PA, USA), and embedded in paraffin for localization of these proteins by immunohistochemistry.

EXPERIMENTAL DESIGN



DETERMINATION OF PROTEIN CONCENTRATION BY LOWRY METHOD

Protein Extraction

Each frozen right kidneys was homogenized 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, USA]. The homogenates were centrifuged at 12,000 g (Biofuge PrimoR, Heracus, Germany) for 20 minutes at 4°C. The supernatant was collected and total protein concentration was measured with modified Lowry assay kit (Pierce, USA) (Seujange et al., 2008).

Protein Assay

Principal: For many years, Lowry's method was the most widely used and cited procedure for protein quantitation (Lowry et al., 1951). The procedure involves the reaction protein with cupric sulfate and tartrate in an alkaline solutions, resoluting in formation of tetradentate copper-protein complexes. When the Folin-Ciocalteu reagent is added, it is effectively reduced in proportion to these chelated copper complexes, producing a water-soluble product whose blue color can be measured at 750 nm. 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) were preferred by dissolving the stock standard BSA solution (2 mg/mL) as indicated in Table A. Pipette double distilled deionized water 180, 450, 250, 250, and

200 µL into tube No.1-5, respectively. Then pipette BSA stock (2 mg/mL) 60 µL into tube No.1 and 50 µL into tube No.2, mixed each tube thoroughly. Then, pipette 250 µL of solution from tube No.2 into tube No.3 and mixed well. Finally, pipette 250 µL of solution from tube No.3 into tube No.4, then mixed each tube thoroughly. There will be sufficient volume of each diluted standard for two replications.

Table A: Preparation of BSA standards

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

Note: DDW = Double distilled deionized water

Preparation of 1X Folin-Ciocalteu reagent

Prepare 1X (1 N) Folin-Ciocalteu reagent by diluting the supplied 2X (2 N) reagent 1:1 with ultrapure water. Because the dilute reagent is unstable, prepare only as much 1X Folin-Ciocalteu reagent as will be used in one day. Each test replicate requires 50 µL of 1X Folin-Ciocalteu reagent in the test tube protocol.

Procedure of Quantitate Total Protein

1. Pipette 0.1 mL of each standard and unknown sample replicate into an approximately labeled test tube.

2. At 15-second intervals, add 0.5 mL Modified Lowry Reagent to each test tube. Mix well and incubate each tube at room temperature for exactly 10 minutes.

3. Exactly at the end of each tube's 10 minutes incubation period, add 50 µL of prepared 1X Folin-Ciocalteu reagent, immediately vortex to mix the contents. Maintain the 15-second interval between tubes established in steps 2.

4. Cover and incubate all tubes at room temperature for 30 minutes.

5. With the spectrophotometer set to 750 nm, zero the instrument by a cuvette filled only with water. Subsequently, measure the absorbance of all the samples.

6. Subtract the average 750 nm absorbance values of the blank standard replicates from the 750 nm absorbance values of all other individual standard and unknown sample replicates.

DETEMINATION OF PROTEIN ABUNDANCE BY USING WESTERN BLOT ANALYSIS

Principal: Immuno-blotting 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 (1970). With this method, it is possible to determine both the purity and the relative molecular weight of an unknown isolated protein or proteins. 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 by boiling at 100 °C, 5 minutes 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, 10% or 12% 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 stacking gel, equal amounts of total protein from each sample were mixed with sample buffer. The amount of total protein used for pEGFR, pERK1/2, and pc-Src was 150 μg.

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 125 volt. Small bubbles should start to be produced and rise off the electrode wire at the bottom of the lower chamber. If no bubbles appear then there is an electrical circuit problem. The gel was run until the dye front reaches the bottom of gel. The power supply was turned off. Then, the gel from the glass plate was removed and placed into the transfer buffer.

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 120 minutes at 100 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 5% non fat dry milk with TBS-T (1:1,000 for pEGFR and for ß-actin), and in 3% bovine serum albumin with TBS-T (1:1,000 for pc-Src and 1:2,000 for pERK1/2). The membrane, in a clean plastic box, was incubated with the respective primary antibody overnight at 4 °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 TBS-T. The goat anti mouse IgG conjugated HRP antibody at 1:5,000 dilution was used for pEGFR and ß-actin. The goat anti rabbit IgG conjugated HRP antibody at 1:5,000 dilution was used for pERK1/2 and pc-Src. 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. In a dark room, the membrane was placed,

protein side up, in a X-ray film cassette. The lights were turned off and a sheet autoradiography film (CL-XPosure™ Film 5 x 7 inches, Pierce, USA) was carefully placed on the top of the membrane, then the cassette was closed and exposed for a certain period, eg. 1 minute (this depends on the amount of target protein on the membrane). The film was developed by X-ray film processor (Optimax® 2010, IGP, Essex, UK) and scanned the intensity by using high resolution scanner (Image class MPC 190, Cannon H12260, Selangor, Malaysia). Each intensity band was quantified by Scion image Release Beta 4.2 (National Institutes of Health, MD, USA). The intensity ratio of each studied protein to ß-actin was calculated.

TO EXAMINE THE LOCALIZATION AND DISTRIBUTION OF STUDIED PROTEINS

Principal: 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 staining is accomplished with antibodies that recognize the target protein. Since antibodies are highly specific, the antibody will bind only to the protein of interest in the tissue section. The antibody-antigen interaction is then visualized 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

Paraffin-embedded kidney sections were cut at 4-μm thickness in a serial section of three for examination of pEGFR, pERK1/2, and pc-Src proteins, respectively. Tissue sections were mounted on 3-aminopropyltriethyloxy-saline-coated slides (Sigma, USA). The slides were deparaffinized in xylene and alcohol, with endogenous peroxidase activity being quenched in 3% hydrogen peroxide for 10 minutes. The non-specific binding of the antibody was blocked by incubating tissue sections with 5% normal horse serum (Vector, USA) in phosphate buffer solution (PBS-T) (PBS + 0.1% Tween) for 30 minutes at room temperature. Then, the section was incubated in monoclonal antibody against pEGFR (1:400) (Cell signaling, USA), or pERK1/2 (1:200) (Cell signaling, USA), or pc-Src (1:400) (MBL® International Corporation, USA) (diluted in 3% normal horse serum) for over one hour at room temperature.

The sections were then rinsed 3 x 10 minutes with PBS-T and incubated with biotinylated goat anti-mouse-rabbit immunoglobulin (Vector, USA) diluted 1:400 in PBS-T for 60 minutes at room temperature. After incubation, tissue sections were rinsed 2 x 10 minutes with PBS-T and then 1 x 10 minutes in PBS. The tissue sections were reacted with ABC-streptavidin horseradish peroxidase complex (Vector, USA) for 60 minutes at room temperature. The sections were then rinsed 2 x 10 minutes in PBS and 10 minutes in 0.05 M Tris-HCl buffer (pH 7.6). The sections were reacted for peroxidative activity in 3, 3′-diaminobenzidine (DAB) solution (Sigma, USA) for 10 minutes. Then, the sections were washed 2 x 5 minutes with distilled water, 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) (Fujigaki et. al, 2007).

CALCULATION FOR ASSESSMENT OF RENAL FUNCTION

$$\textbf{Creatinine clearance } (C_{Cr}) = \frac{U_{Cr} \times V}{P_{Cr}}$$

STATISTICAL ANALYSIS

The results were presented as mean $\pm$ SD. Statistical differences of protein levels among groups were assessed by one way analysis of variance (one-way ANOVA) with post hoc comparison by Tukey's test where appropriate. A probability value (p-value) of less than 0.05 was considered to be statistically significant. The intensity scores of renal protein expressions of pEGFR, pERK1/2, and pc-Src were presented in descriptive statistics by measures of central tendency (Mode). The statistical calculations were performed by means of the statistical package SPSS for Window 16.

CHAPTER IV

RESULTS

Metabolic and renal function parameters in Sham, LA, and HA groups

As illustrated in Table 1, 30-min administration of aldosterone significantly increased plasma aldosterone levels from 1,251.95 $\pm$ 13.83 pmol/L in the sham rats to be 6,521.78 $\pm$ 209.92 pmol/L in the LA group ($p<0.001$) and to be 9,809.62 $\pm$ 251.49 pmol/L in the HA group ($p<0.001$). The HA rats had a 1.5 time higher of plasma aldosterone level when compared with the LA group ($p<0.001$).

No significant changes in plasma sodium, potassium, chloride, creatinine, blood urea nitrogen, creatinine clearance, and urine flow rate were observed among all studied groups.

Effect of aldosterone on renal pEGFR protein abundance in Sham, LA, and HA groups

The pEGFR protein abundance was illustrated at 175 kDa as shown in Figure 1. After 30-min of injection, aldosterone significantly enhanced renal pEGFR protein abundance from 100 % in the sham rat to be 226.24 $\pm$ 9.95% in the LA group, and 356.96 $\pm$ 8.13% in the HA groups ($p<0.001$). Aldosterone enhanced pEGFR protein level dose dependently ($p<0.001$).

Table 1: Metabolic parameter and renal function profiles of Sham, LA, and HA rats

Parameters	Groups		
	Sham	LA	HA
Plasma aldosterone (pmol/L)	1,251.95 ± 13.83	6,521.78 ± 209.92*	9,809.62 ± 251.49*,+
Plasma Na^+ (mmol/L)	141.37 ± 1.50	140.62 ± 1.06	140.75 ± 1.66
Plasma K^+ (mmol/L)	3.58 ± 0.24	3.43 ± 0.32	3.51 ± 0.53
Plasma Cl^- (mmol/L)	112.37 ± 5.09	112.50 ± 2.13	112.12 ± 1.12
Plasma creatinine (mg%)	0.27 ± 0.01	0.23 ± 0.02	0.22 ± 0.01
Blood urea nitrogen (mg%)	20.05 ± 5.29	19.36 ± 2.34	19.32 ± 3.22
Creatinine clearance ($mL.min^{-1}.100g\ BW^{1}$)	0.99 ± 0.08	1.05 ± 0.09	1.10 ± 0.14
Urine flow rate ($x10^{-2}$ mL/min)	1.23 ± 0.12	1.18 ± 0.10	1.22 ± 0.11

Data are expressed as Mean±SD, n = 8/group. *$p<0.001$ *vs.* Sham, +$p<0.001$ *vs.* LA

LA = low dose aldosterone, HA = high dose aldosterone

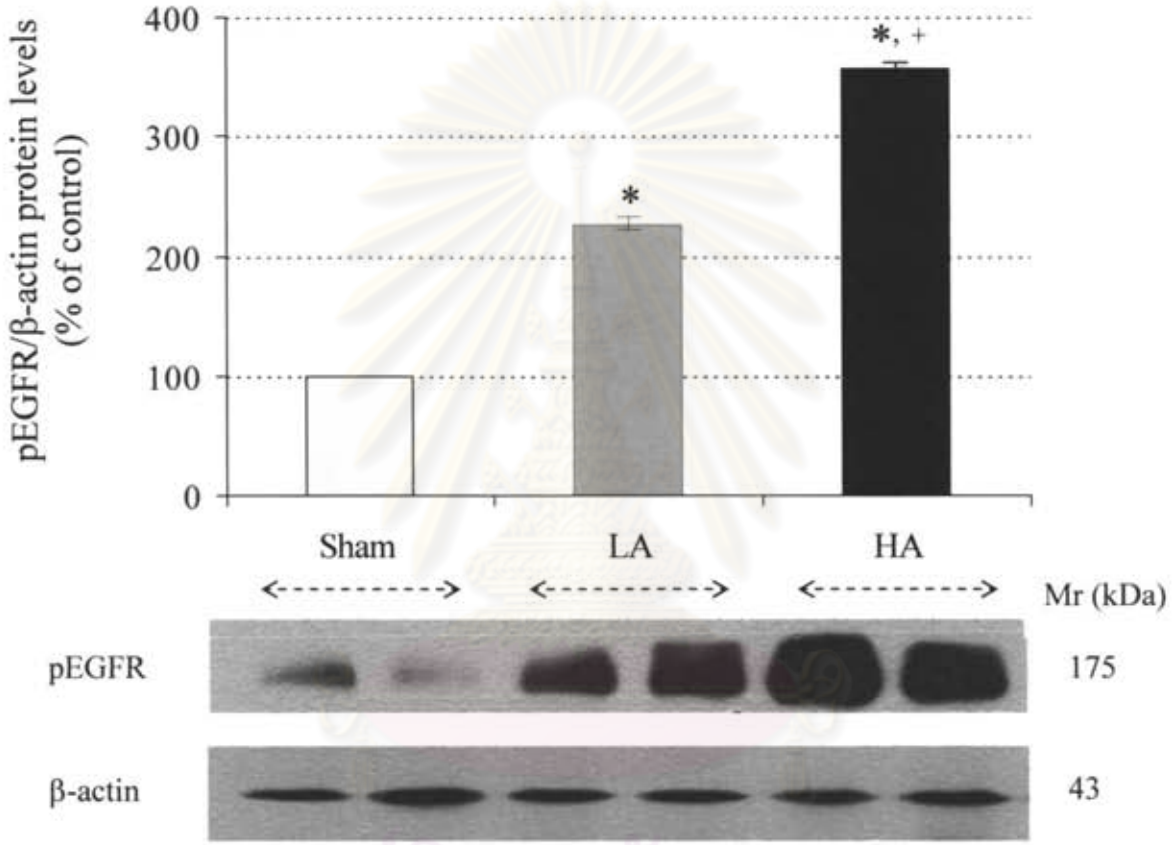


Figure 1: Western blot analysis of renal pEGFR protein abundance in Sham, LA, and HA groups. Protein from renal tissues were separated by SDS-PAGE, transferred to nitrocellulose and subjected to immunoblot analysis. The monoclonal antibody to pEGFR was used. Histogram bars show the densitometric analyses ratios of pEGFR to β–actin intensity, and the representative immunoblot photographs are presented. Data are means ± SD of 8 independent experiments.
*$p<0.001$ *vs.* Sham, $^{+}p<0.001$ *vs.* LA

Effect of aldosterone on renal pEGFR protein localization in Sham, LA, and HA groups

The localization of renal pEGFR protein expression was detected by immunohistochemistry as shown in Figures 2 and 3. In the cortex (Figure 2), the staining was observed mainly in the glomerulus as the score was 1 in the sham group (Figure 2a, Table 2). Low dose aldosterone had no effect on the protein expression (Figure 2b). The intensity score was still 1. However, HA enhanced the intensity score to be 2 (Figure 2c).

In the medulla (Figure 3), the protein expression was localized separately into the outer medulla (Figures 3a-f), and inner medulla (Figures 3g-i). In the outer stripe of outer medulla (Figures 3a-c), there was no staining in the sham and the LA groups (Figures 3a, b) whereas the HA rats showed some trace staining (score 1) in the thick ascending limb of Henle's loop (Figure 3c). In the inner stripe of outer medulla (Figures 3d-f), the staining areas were observed mainly in the vasa recta areas. LA markedly increased the intensity score from 1 in the sham group to be 3 (Figure 3e) whereas the intensity was progressively enhanced to be 4 in the HA animals (Figure 3f). In addition, the trace intensity was noted in some areas of thin and thick limb of Henle's loops caused by aldosterone administration (Figure 3e, f).

In the inner medulla (Figures 3g-i), the staining was noted in the vasa recta areas. The intensity was 2 in the sham animals (Figure 3g). Aldosterone dramatically elevated the intensity to be 4 in both the LA and HA groups with the staining in the vasa recta and some of thin limb of Henle's loops (Figure 3h, i).

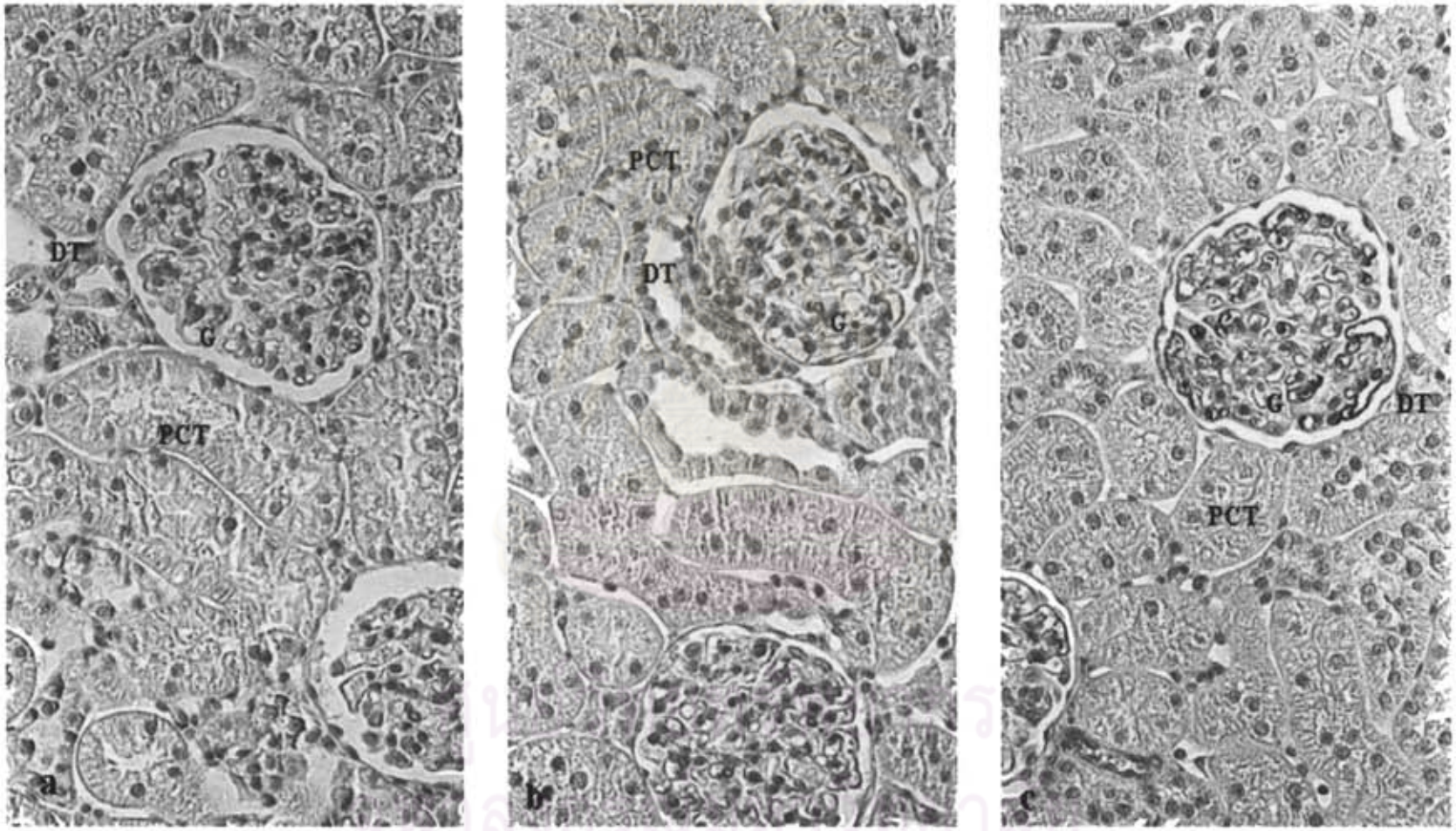


Figure 2: Representative immunohistochemical staining of renal pEGFR protein expression in cortex (original magnification: 200X).
(a): Sham; (b): LA; (c): HA. G: glomerulus, PCT: proximal convoluted tubule, DT: distal tubule

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Table 2: The intensity scores of renal pEGFR, pERK1/2, and pc-Src protein expressions in Sham, LA, and HA rats (Mode, n = 8/group)

Groups	Intensity Score											
	pEGFR				pERK1/2				pc-Src			
	Cortex	Medulla			Cortex	Medulla			Cortex	Medulla		
		Outer medulla		Inner medulla		Outer medulla		Inner medulla		Outer medulla		Inner medulla
		outer stripe (OS)	inner stripe (IS)			outer stripe (OS)	inner stripe (IS)			outer stripe (OS)	inner stripe (IS)	
Sham	1	0	1	2	2	2	2	2	1	1	2	3
LA	1	0	3	4	3	4	4	3	2	1	2	3
HA	2	1	4	4	4	3	3	4	4	1	2	1

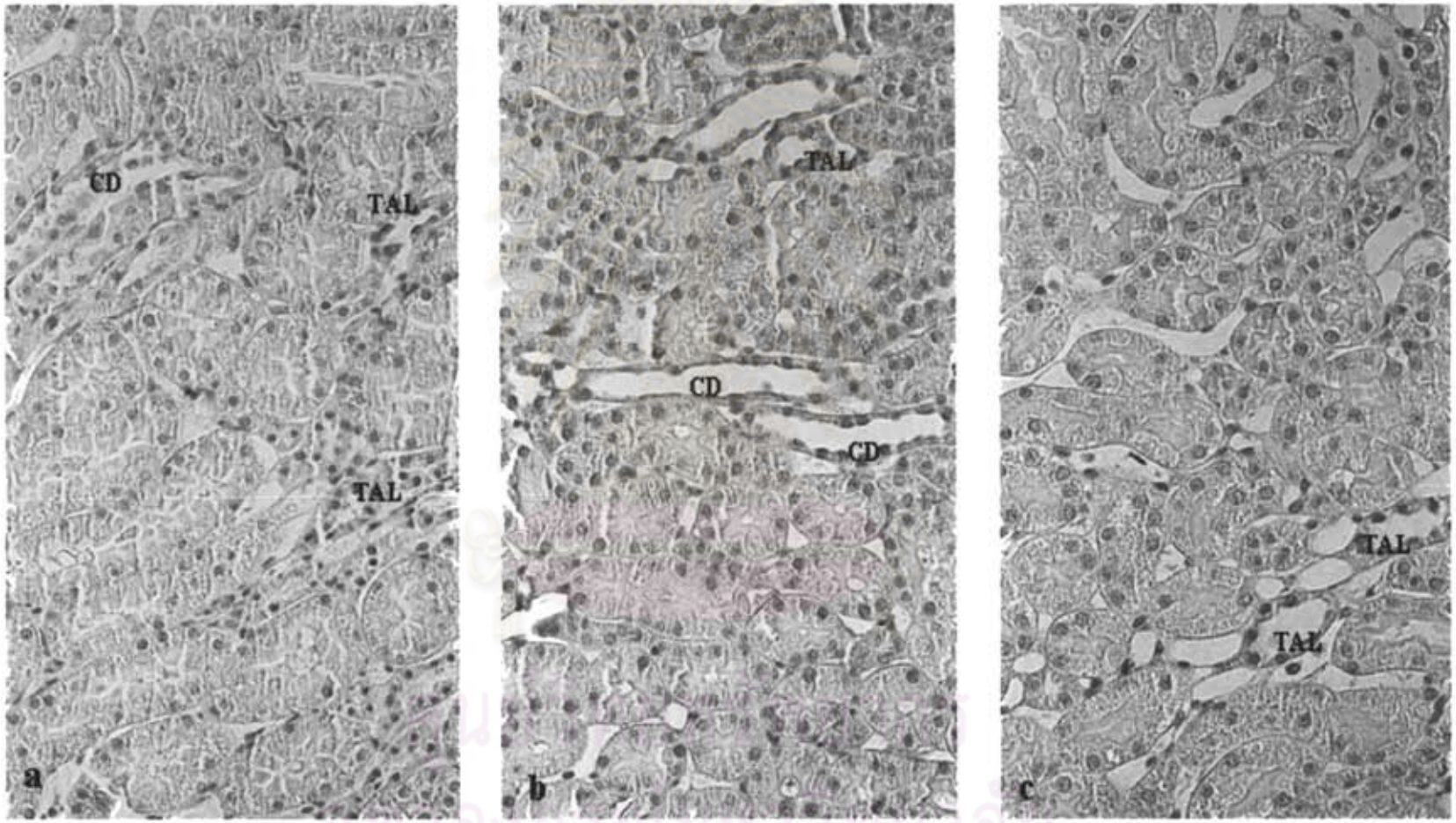

Figure 3: Representative immunohistochemical staining of renal pEGFR protein expression in outer medulla (outer stripe, OS) (original magnification: 200X). (a): Sham; (b): LA; (c): HA. TAL: thick ascending limb, CD: collecting duct

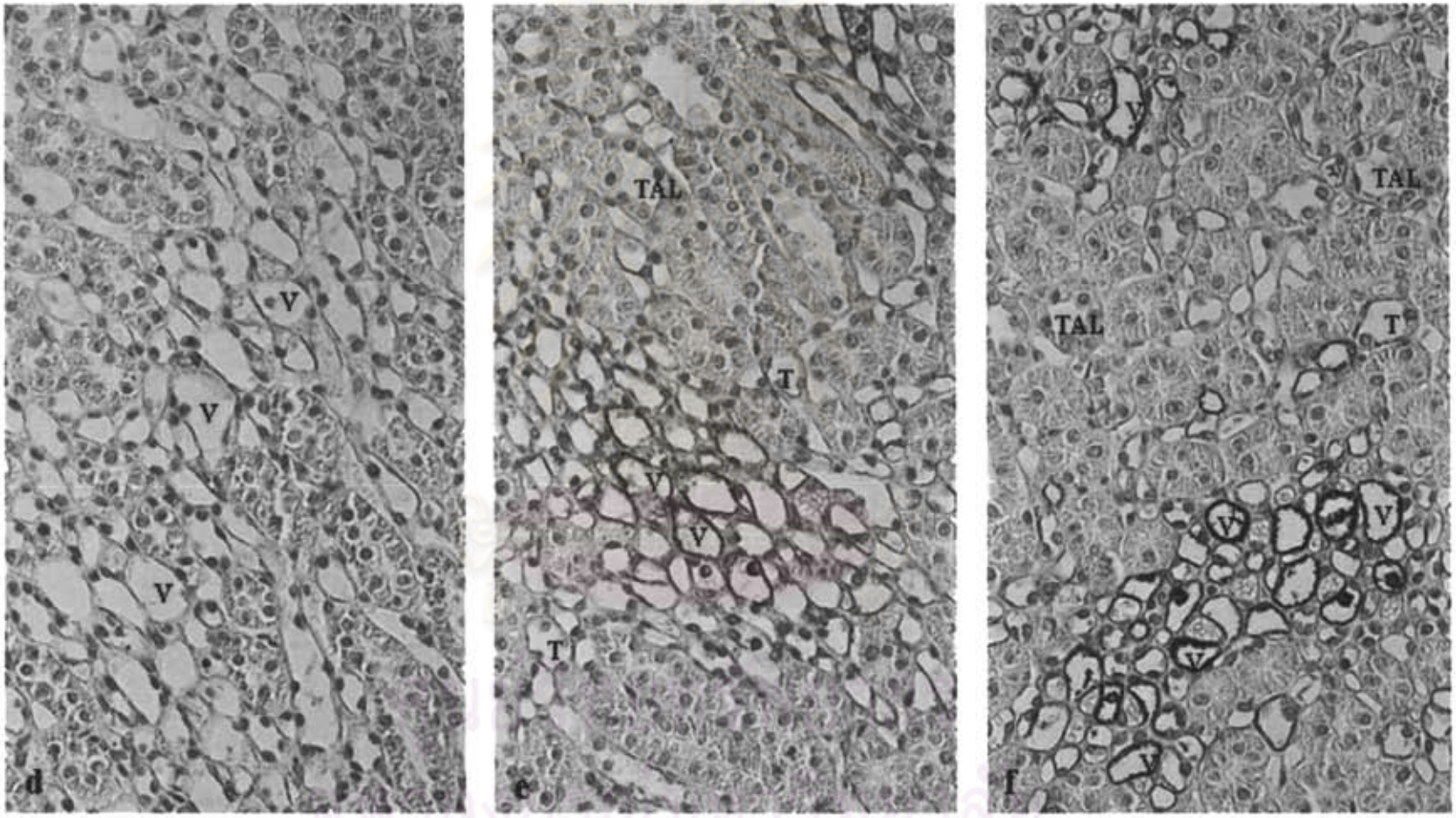

Figure 3 (cont.): Representative immunohistochemical staining of renal pEGFR protein expression in outer medulla (inner stripe, IS) (original magnification: 200X). (d): Sham; (e): LA; (f): HA. T: thin limb, V: vasa recta

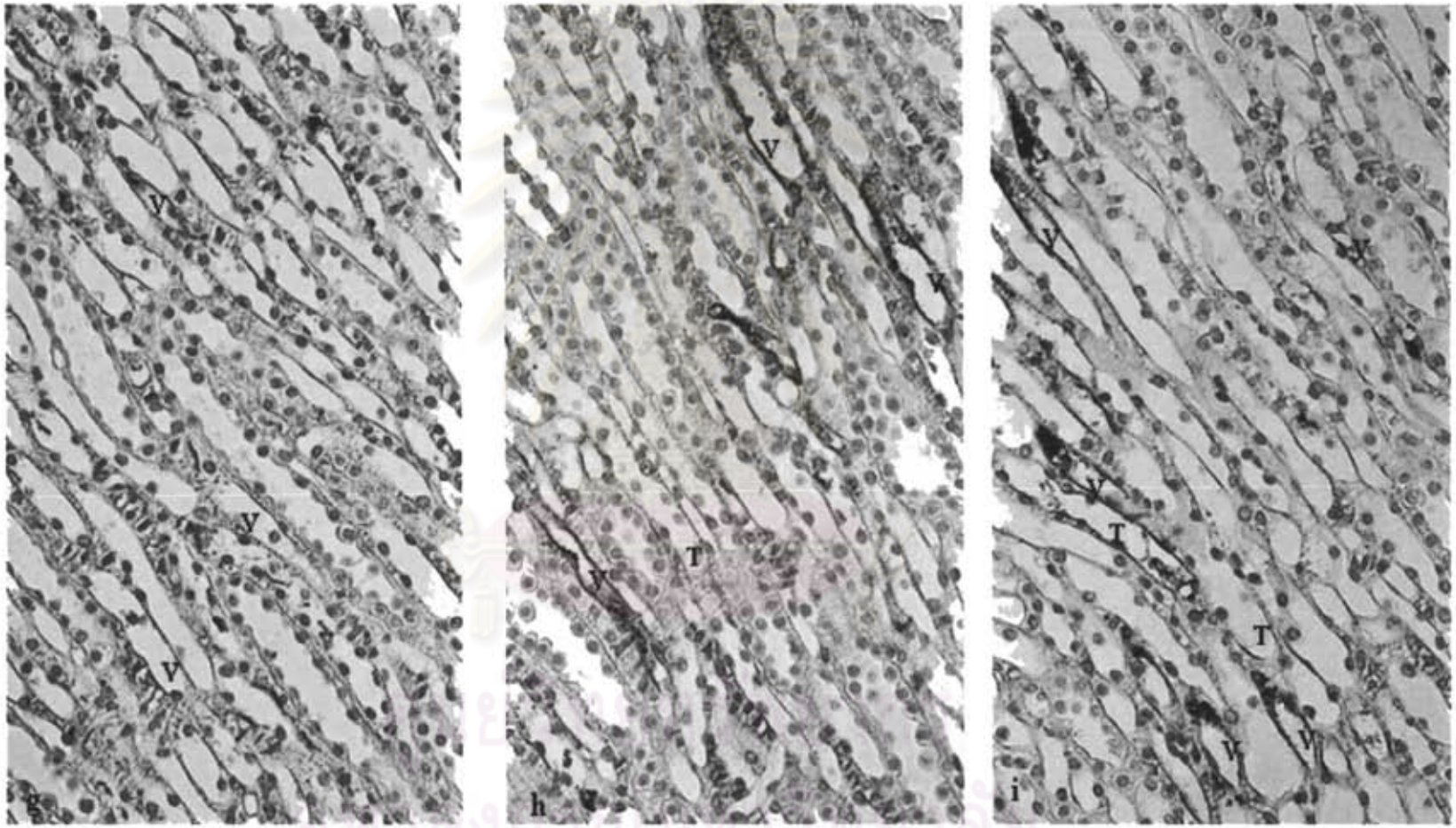

Figure 3 (cont.): Representative immunohistochemical staining of renal pEGFR protein expression in inner medulla (original magnification: 200X). (g): Sham; (h): LA; (i): HA.

Effect of aldosterone on renal pERK1/2 protein abundance in Sham, LA, and HA groups

The pERK1/2 protein abundances were illustrated at 44 and 42 kDa as shown in Figure 4. After 30-min of injection, aldosterone significantly enhanced renal pERK1/2 protein abundance from 100 % in the sham rat to be 303.28 $\pm$ 20.61/ 384.00 $\pm$ 14.87% in the LA group ($p<0.001$) and to be 245.82 $\pm$ 23.52/ 331.72 $\pm$ 8.09% in the HA groups ($p<0.001$). The expression of pERK1/2 protein levels in the LA rats were higher than those observed in the HA group ($p<0.001$).

Effect of aldosterone on renal pERK1/2 protein localization in Sham, LA, and HA groups

The localization of renal pERK1/2 protein expression was detected by immunohistochemistry as shown in Figures 5 and 6. In the cortex (Figure 5), the staining was observed mainly in the glomerulus and some peritubular capillaries as the score was 2 in the sham group; however there was no staining in the proximal convoluted tubules (Figure 5a). Aldosterone increased the intensity scores to be 3 in the LA group (Figure 5b) and to be 4 in the HA rats (Figure 5c). More intense staining in the peritubular capillaries was observed in the HA group (Figure 5c).

In the medulla (Figure 6), the protein expression was localized separately into the outer medulla (Figures 6a-f) and inner medulla (Figures 6g-i). In the outer stripe of outer medulla (Figures 6a-c), the staining in the sham rats was noted in the thick ascending limb and collecting ducts with the score 2, whereas there was still no staining in proximal tubules (Figure 6a). In the LA group, aldosterone markedly enhanced the expression in the collecting duct with the score 4 and a diffused staining in cytosolic area of some proximal tubules (Figure 6b). However, in the HA rats, the

intensity was increased to be only 3 with a trace staining in the proximal tubules (Figure 6c). In the inner stripe of outer medulla (Figures 6d-f), the sham group showed diffused staining in the thick ascending limb of Henle's loops and vascular areas whereas the collecting ducts demonstrated the greater extent with the score 2 (Figure 6d). LA markedly increased the intensity score to be 4 in the collecting ducts with some trace intensity in vasa recta areas and thick ascending limbs (Figure 6e). In the HA animals, the staining was appeared as observed in the sham group with the higher score of 3 (Figure 6f).

In the inner medulla (Figures 6g-i), the staining areas were still noted in the vasa recta, thin limb of Henle's loop and collecting ducts with the score 2 in the sham group (Figure 6g). In the LA rats, aldosterone elevated the intensity to be 3 that was prominent in the thin limb of Henle's loop with a greater extent of nuclear staining (Figure 6h). In the HA animals, the intensity was markedly enhanced in the collecting ducts and vasa recta with the score 4 (Figure 6i).

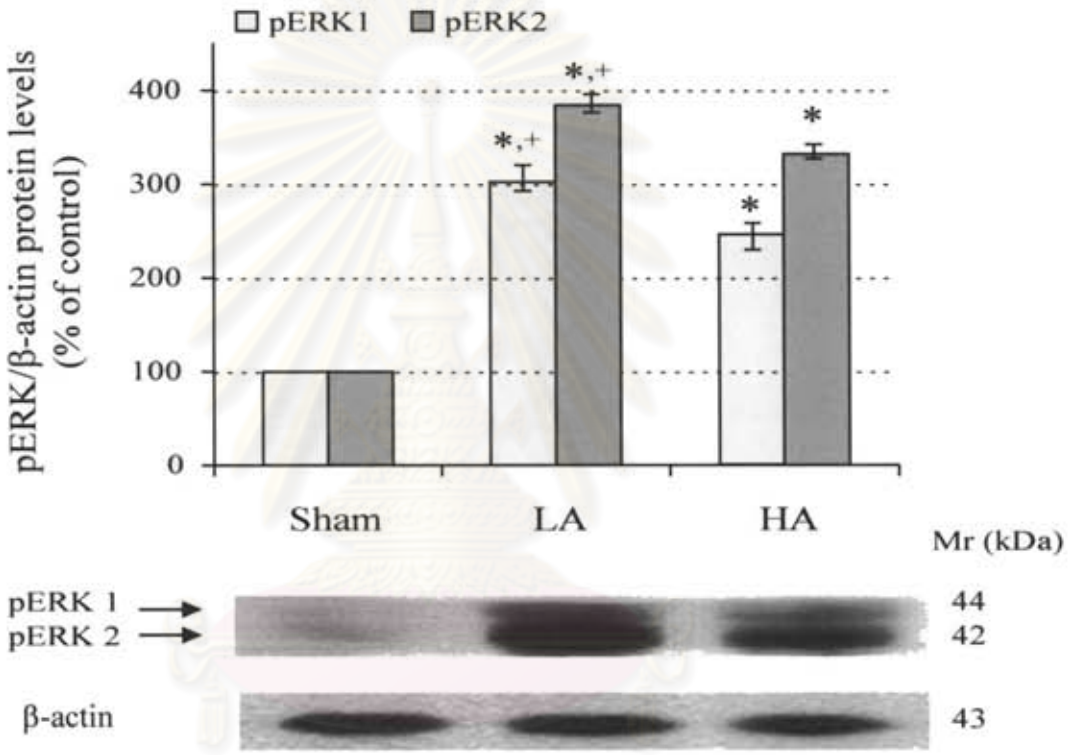

Figure 4: Western blot analysis of renal pERK1/2 protein abundance in Sham, LA, and HA. Protein from renal tissues were separated by SDS-PAGE, transferred to nitrocellulose and subjected to immunoblot analysis. The monoclonal antibody to pERK1/2 was used. Histogram bars show the densitometric analyses ratios of pERK1/2 to β–actin intensity, and the representative immunoblot photographs are presented. Data are means±SD of 8 independent experiments.
*$p<0.001$ *vs.* Sham, $^{+}p<0.001$ *vs.* HA

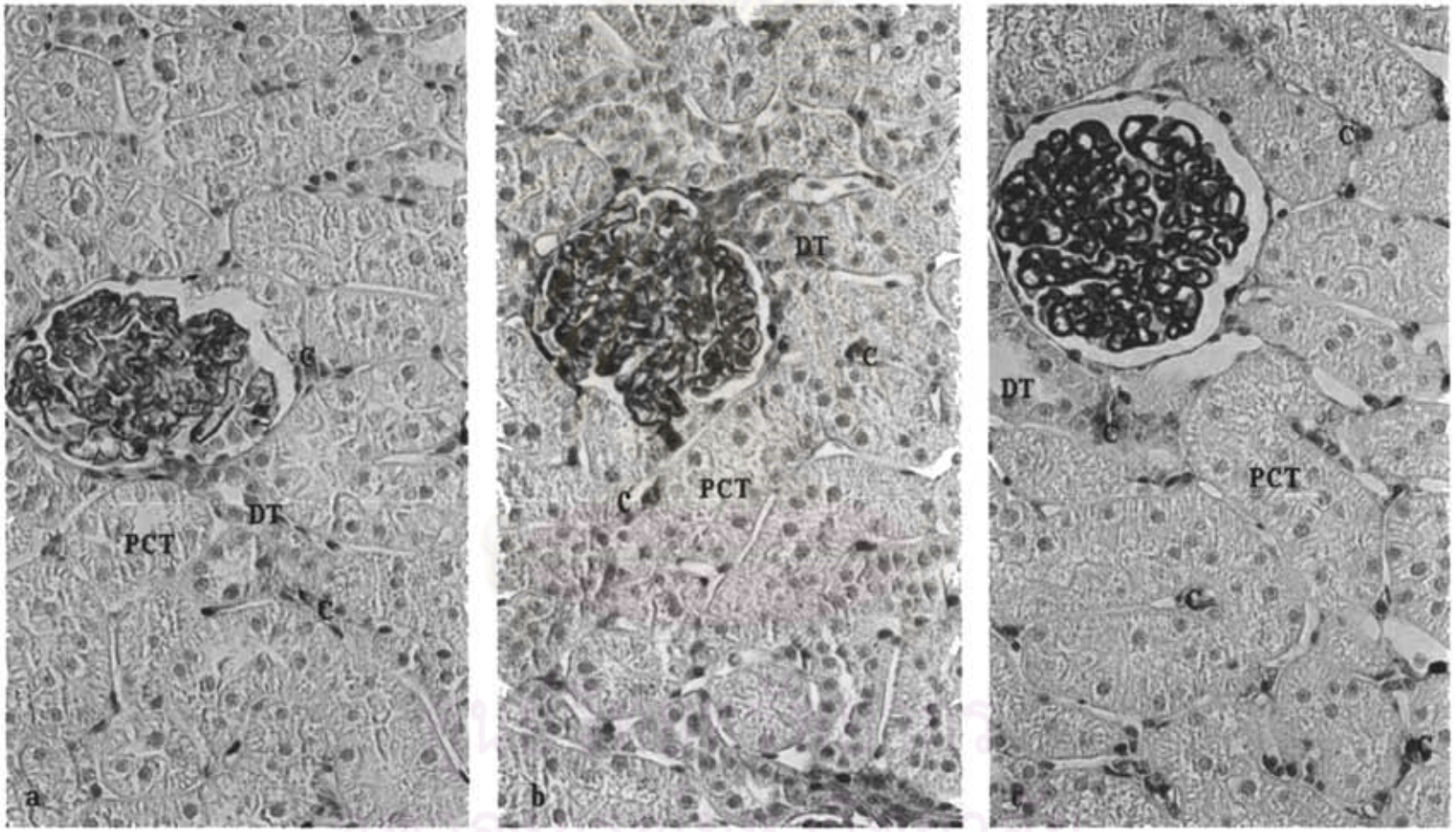

Figure 5: Representative immunohistochemical staining of renal pERK1/2 protein expression in cortex (original magnification: 200X). (a): Sham; (b): LA; (c): HA . C: peritubular capillary

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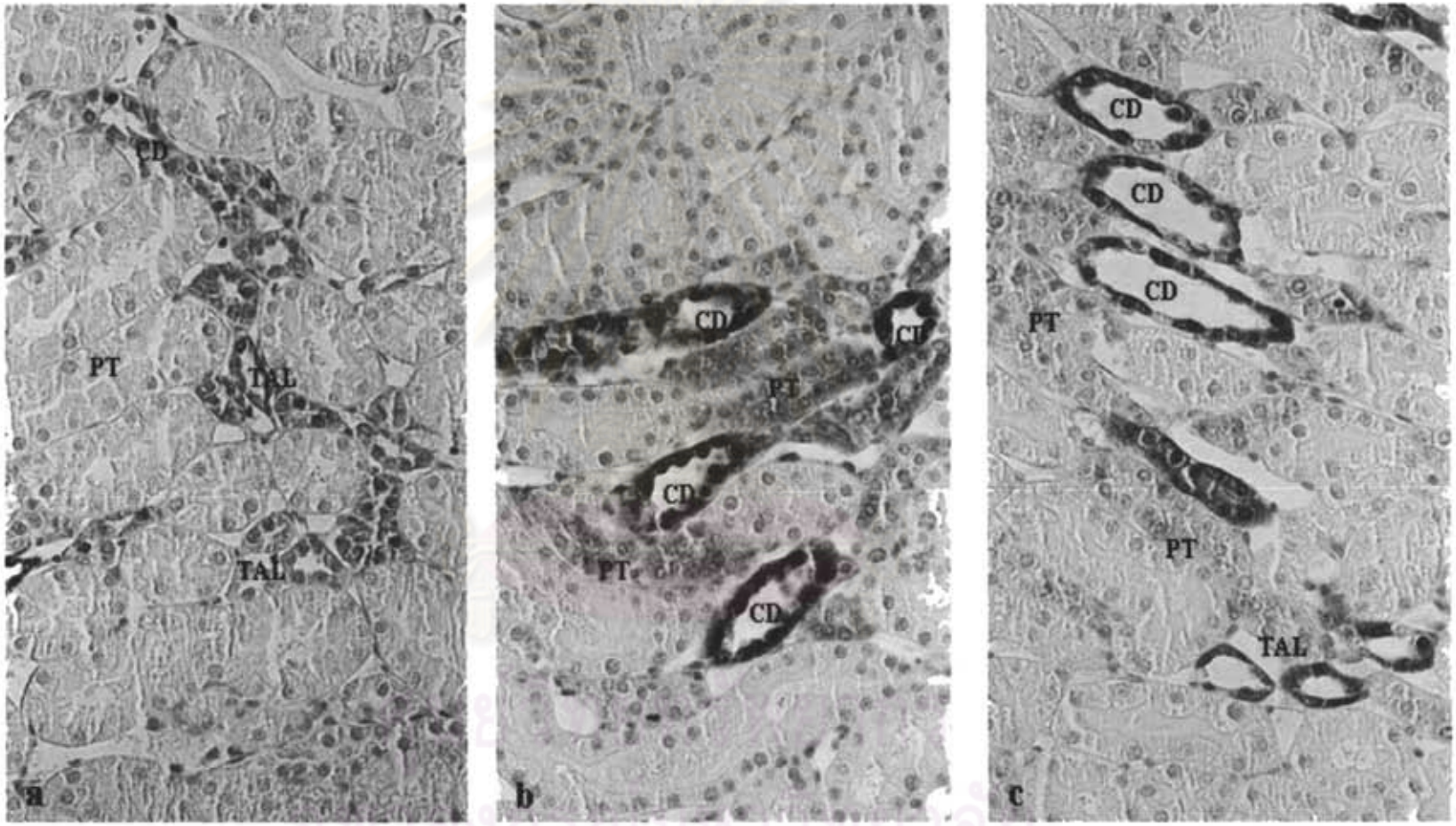

Figure 6: Representative immunohistochemical staining of renal pERK1/2 protein expression in outer medulla (outer stripe, OS) (original magnification: 200X). (a): Sham; (b): LA; (c): HA. PT: proximal tubule

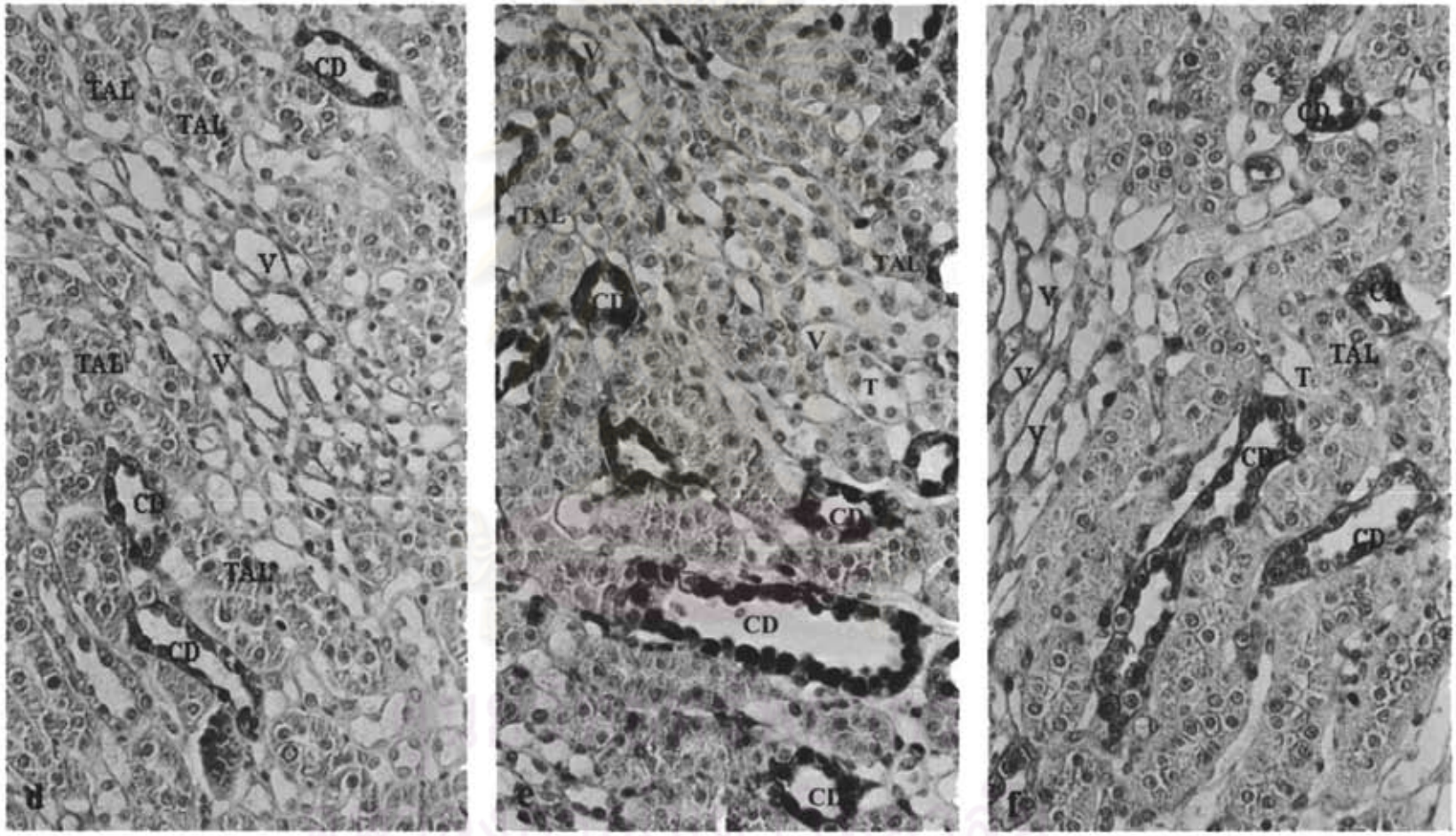

Figure 6 (cont.): Representative immunohistochemical staining of renal pERK1/2 protein expression in outer medulla (inner stripe, IS) (original magnification: 200X). (d): Sham; (e): LA; (f): HA.

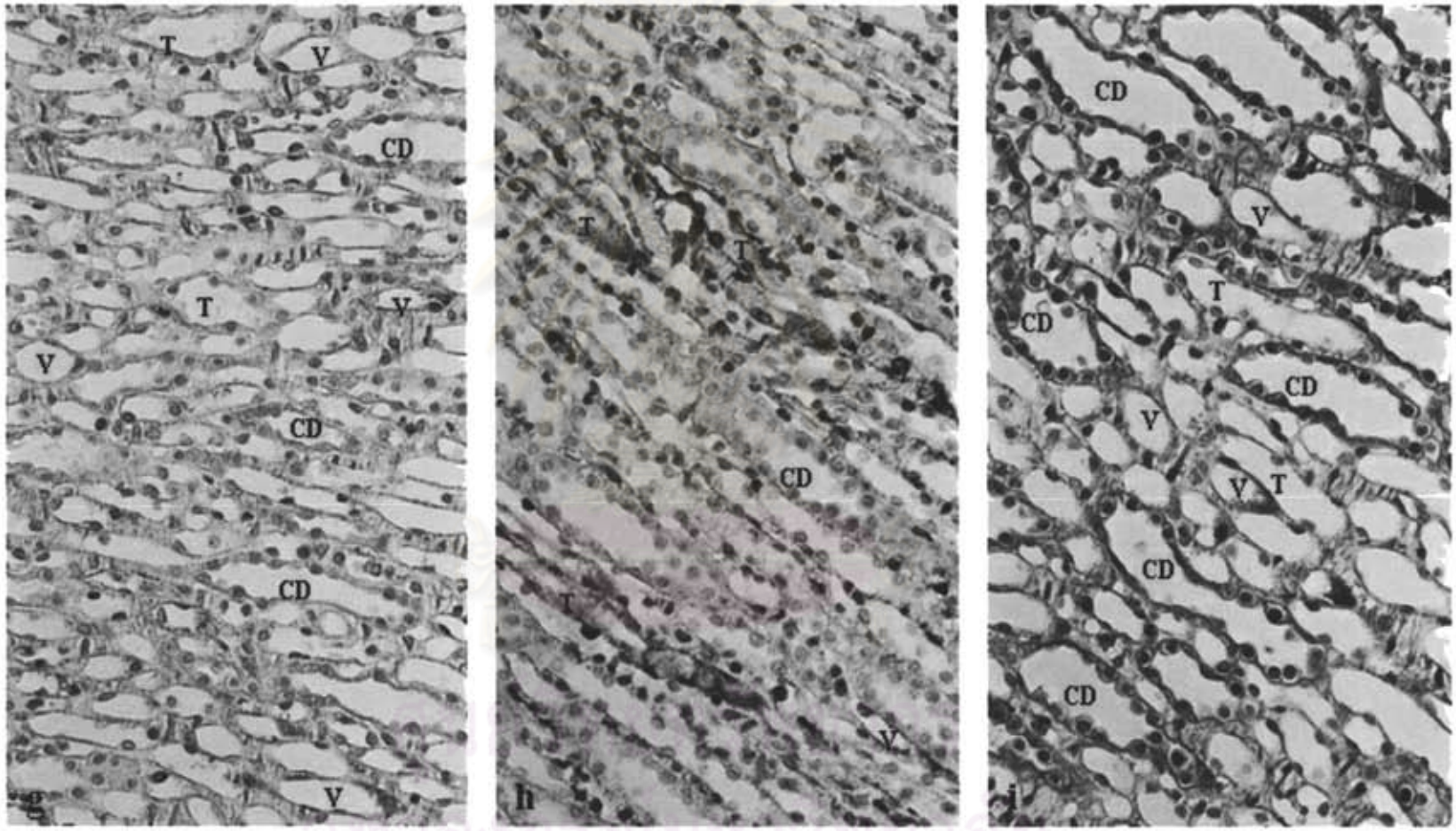

Figure 6 (cont.): Representative immunohistochemical staining of renal pERK1/2 protein expression in inner medulla (original magnification: 200X). (g): Sham; (h): LA; (i): HA.

Effect of aldosterone on renal pc-Src protein abundance in Sham, LA, and HA groups

The pc-Src protein abundance was illustrated at 60 kDa as shown in Figure 7. After 30-min of injection, aldosterone had no effect on renal pc-Src protein abundance in all groups. The expressions were 100 % in the sham rat, 105.01 $\pm$ 8.91% in the LA group ($p = 0.39$, NS), and 106.02 $\pm$ 9.39% in the HA group ($p = 0.26$, NS).

Effect of aldosterone on renal pc-Src protein localization in Sham, LA, and HA groups

The localization of renal pc-Src protein expression was detected by immunohistochemistry as shown in Figures 8 and 9. In the cortex (Figure 8), the trace staining was observed in the glomerulus, luminal membrane of distal tubules, and collecting ducts as the score was 1 in the sham group; however there is no staining in proximal convoluted tubules (Figure 8a). Aldosterone increased the intensity score to be 2 in the LA group with more obvious staining in the luminal membrane of proximal convoluted tubules (Figure 8b). In the HA rats, the intensity was dramatically increased in the glomerulus as the score was 4 (Figure 8c). Of interest, in the HA animals, the protein expression in the proximal convoluted tubules was redistributed from the luminal membrane to the basolateral membrane (Figure 8c).

In the medulla (Figure 9), the protein expression was localized separately into the outer medulla (Figures 9a-f), and inner medulla (Figure 9g-i). In the outer stripe of outer medulla (Figures 9a-c), the staining in the sham rats was noted in luminal membrane of thick ascending limb of Henle's loop and collecting duct with the score 1 (Figure 9a). Aldosterone did not change the intensity scores in both LA and HA groups (Figures 9b, c). Indeed, aldosterone induced some trace expression in the

peritubular capillaries (Figures 9b, c). In the inner stripe of outer medulla (Figures 9d-f), the sham and LA groups showed the most staining in the vasa recta areas with the score 2 (Figures 9d, e) and some trace staining in the thick and thin limb of Henle's loops. In the HA animals, the staining was appeared as observed in the sham and LA groups with more diffused staining in tubular areas including collecting duct (Figure 9f).

In the inner medulla (Figures 9g-i), the staining was noted more in the vasa recta but less in the tubular areas (thin limb of Henle's loop and collecting duct) with the score 3 (Figure 9g). LA did not alter the intensity score; however more nuclear staining was noted in the tubular regions (Figure 9h). Of interest, in the HA animals, the intensity was fall to be 1 (Figure 9i).

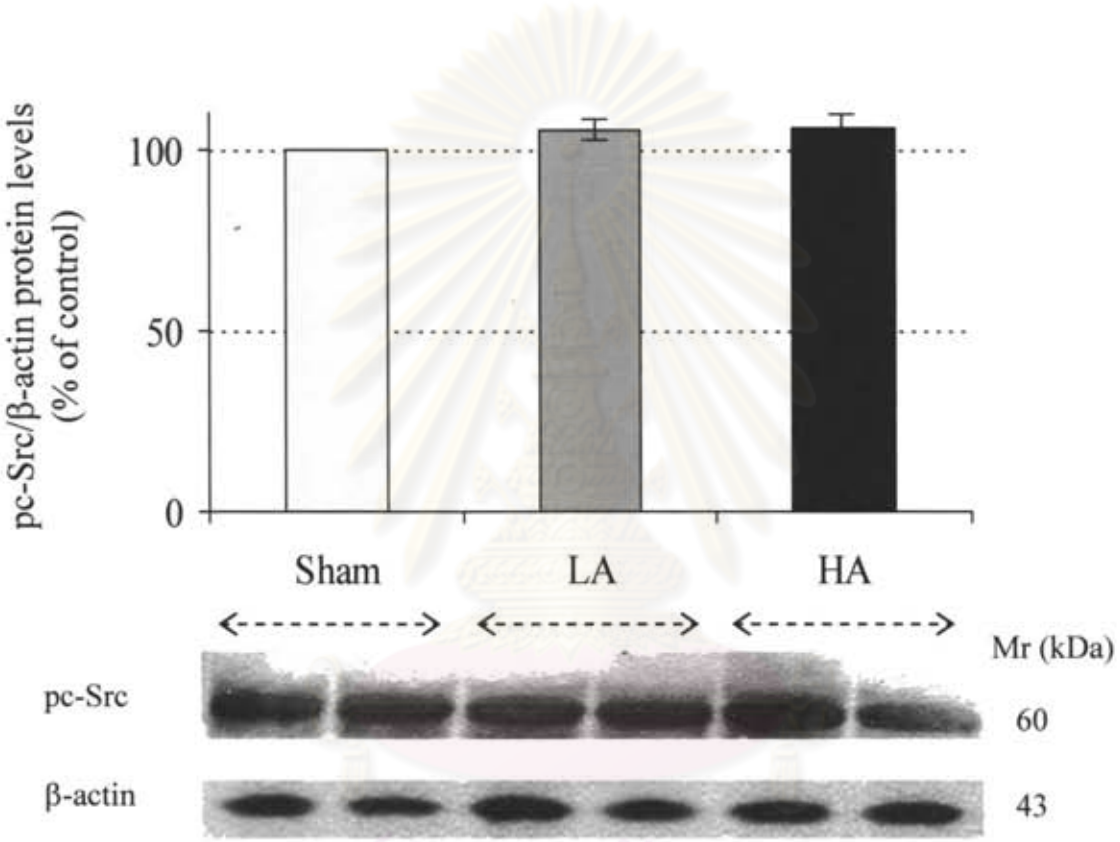

Figure 7: Western blot analysis of renal pc-Src protein abundance in Sham, LA, and HA. Protein from renal tissues were separated by SDS-PAGE, transferred to nitrocellulose and subjected to immunoblot analysis. The polyclonal antibody to pc-Src was used. Histogram bars show the densitometric analyses ratios of pc-Src to β–actin intensity, and the representative immunoblot photographs are presented. Data are means±SD of 8 independent experiments.

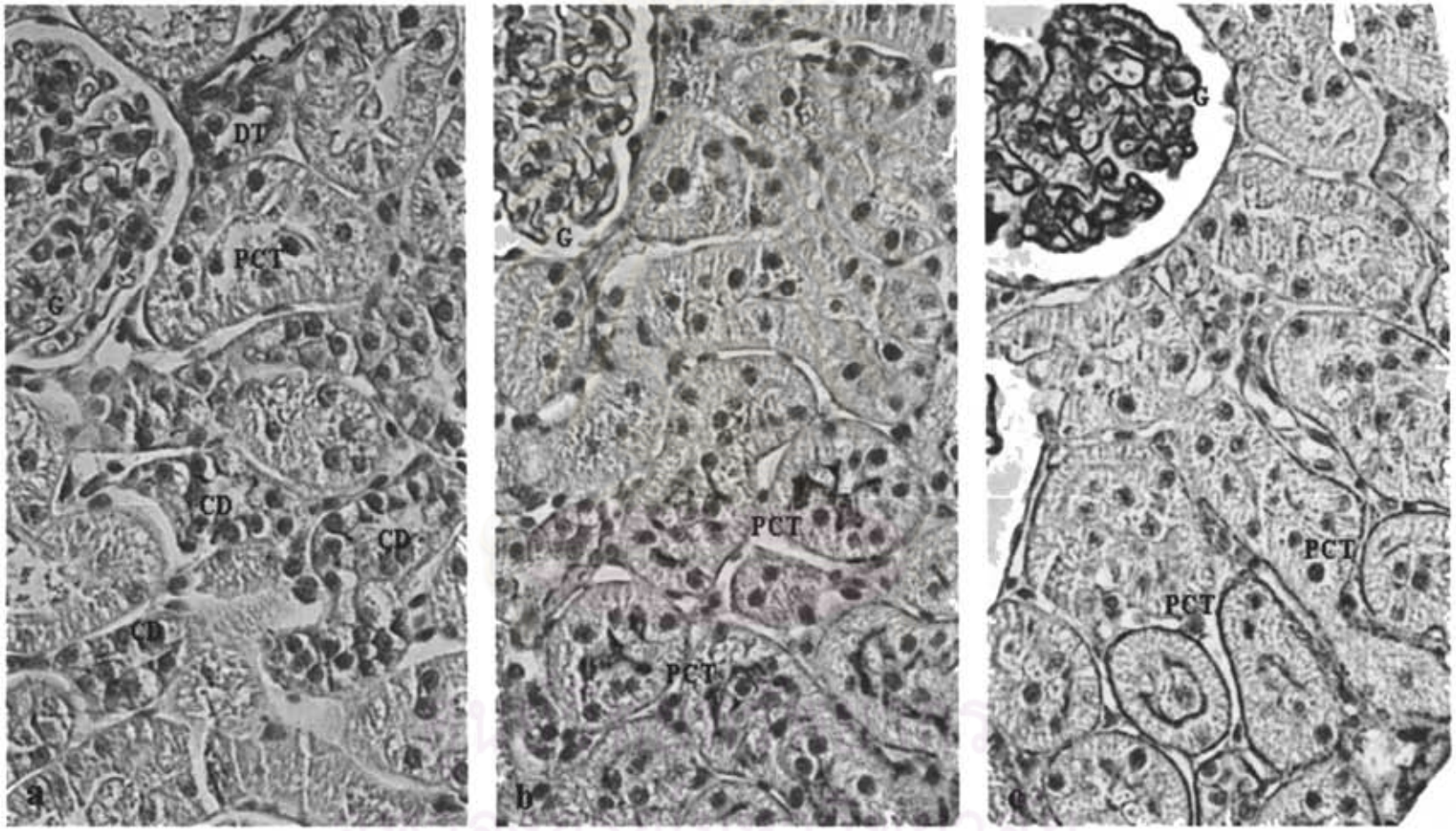

Figure 8: Representative immunohistochemical staining of renal pc-Src protein expression in cortex (original magnification: 400X). (a): Sham; (b): LA; (c): HA.

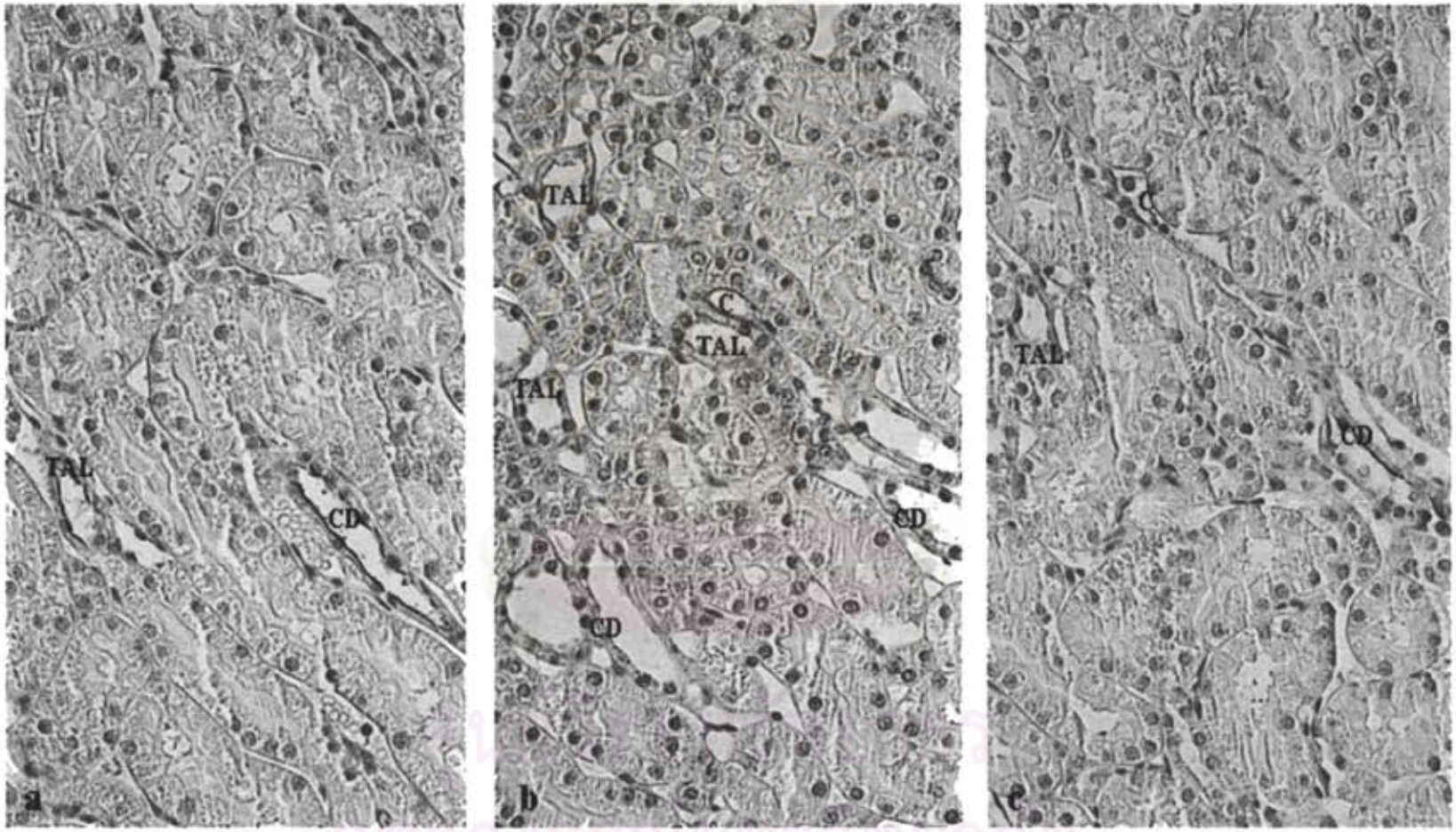

Figure 9: Representative immunohistochemical staining of renal pc-Src protein expression in outer medulla (outer stripe, OS) (original magnification: 200X). (a): Sham; (b): LA; (c): HA.

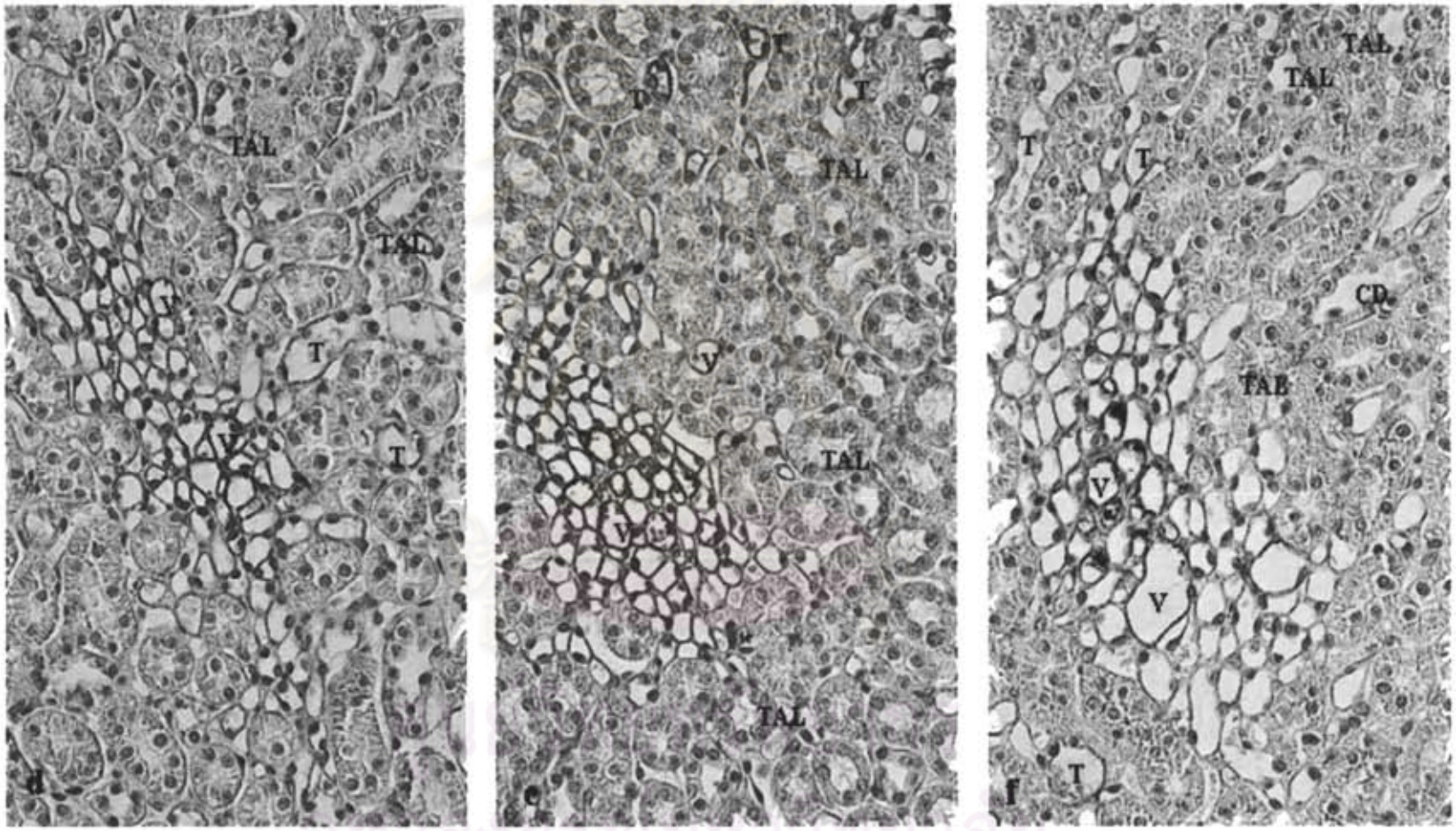

Figure 9 (cont.): Representative immunohistochemical staining of renal pc-Src protein expression in outer medulla (inner stripe, IS) (original magnification :200X). (d): Sham; (e): LA; (f): HA.

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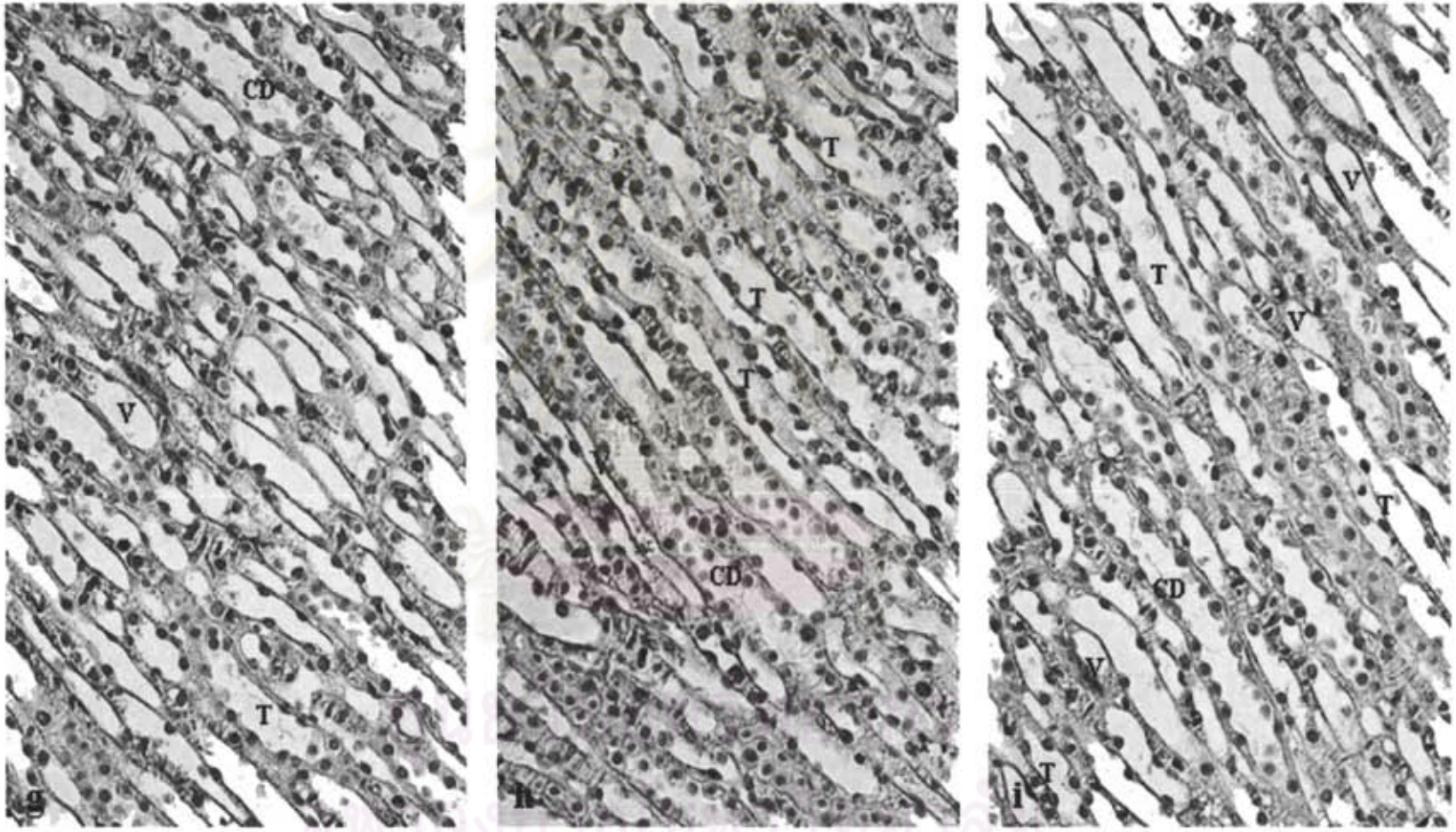

Figure 9 (cont.): Representative immunohistochemical staining of renal pc-Src protein expression in inner medulla (original magnification: 200X). (g): Sham; (h): LA; (i): HA.

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CHAPTER V

DISCUSSION AND CONCLUSION

Besides its classical actions mediated via the traditional genomic signaling pathway, new interests in the nongenomic actions of aldosterone have arouse (Grossmann and Gekle, 2009). It has been first proposed that aldosterone is capable of interacting in a non-genomic fashion with the mitogen-activated protein kinase (MAPK) signaling pathway (Manegold et al., 1999). MAPK, a family of ubiquitous Ser/Thr kinases, plays an important role in various signal transduction cascades including those activation via epidermal growth factor receptor (EGFR) in the kidney (Grossmann and Gekle, 2008; Shaul and Seger, 2007; Tian et al., 2000; Yoon and Seger, 2006). One of downstream signal molecules in this pathway is the extracellular signal-regulated kinase 1 and 2, ERK1/2 (Ramos, 2008).

The results in the present study have demonstrated that a nongenomic pathway of aldosterone enhanced pEGFR and pERK1/2 protein abundances and expressions in rat kidney. This is the first *in vivo* investigation showing that aldosterone could transactivate EGFR phosphorylation and then increase the downstream signal, pERK1/2, activation. These data are in agreement with those performed previously in cell-cultured examinations.

It has been indicated that aldosterone rapidly enhanced ERK1/2 phosphorylation in kidney cell culture, such as human renal proximal tubular epithelial cells (Xu et al., 2008), MDCK cells (Gekle et al., 2001; Gekle et al., 2002), M-1 cells (Markos et al., 2005; McEneaney et al., 2010; Rossol-Haseroth et al., 2004), mesangial cells (Han et al., 2009; Huang et al., 2009; Nishiyama et al., 2005), and renal fibroblast (Nagai et al., 2005). This aldosterone induced pERK1/2 activation

was mediated by EGFR transactivation (Gekle et al., 2002; Grossmann et al., 2005; Krug et al., 2002; McEneaney et al., 2007). Evidences from pharmacological experiments have shown that various inhibitors of the EGFR tyrosine kinase, tyrphostin AG1478 or c56, were able to prevent rapid aldosterone-induced ERK1/2 phosphorylation (Gekle et al., 2002; Grossmann et al., 2005; Krug et al., 2002; McEneaney et al., 2007). Moreover, an increase in Tyr845 or Tyr1086 phosphorylation of the EGFR was detected after a short incubation with aldosterone (Grossmann et al., 2005; McEneaney et al., 2007). This rapid activation of the ERK1/2 cascade which was preceded by MEK phosphorylation is in line with previous studies. Inhibition of MEK activity by PD98059 or U0126 could abolish ERK1/2 phosphorylation (Gekle et al., 2001; Grossmann et al., 2005; Krug et al., 2002; Markos et al., 2005; Nagai et al., 2005; Rossol-Haseroth et al., 2004). This confirms that aldosterone induced activation of the ERK1/2 cascade involves the EGFR transactivation.

Interestingly, the present data show that aldosterone could increase pEGFR protein abundance in dose-dependent manner (Fig. 1). This is similar to the previous study (Huang et al., 2009). However, the downstream signal, pERK1/2, protein level in HA groups was enhanced less than that in LA animals (Fig. 4). One of several explanations on this phenomenon is the negative feedback loop of MEK/ERK cascade (Ramos, 2008). MEK is the target and can be inhibited by ERK phosphorylation of MEK1/2 at Thr292 (Eblen et al., 2004) and Thr212 (Sundberg-Smith et al., 2005), and then reduced activation of ERK. Therefore, the negative feedback pathway to prevent the over phosphorylation of ERK1/2 in the present study is likely to be appeared.

From Western blot analysis, the present data demonstrate the total protein abundances of pEGFR and pERK1/2, which were increased by the nongenomic action

of aldosterone, meanwhile whether these enhanced proteins are localized or distributed in renal tissue homogenously. Moreover, these protein expressions could be altered by aldosterone, or not. The present results, from immunohistochemistry, first clearly illustrate that both phosphorylated proteins were not expressed in the similar pattern or the same intensity degree either sham or aldosterone groups (Table 2; Fig. 3 and 5). For pEGFR, the sham rats showed not much expression (score 0-2) in both cortex and medulla. The staining was observed in the glomeruli, loop of Henle, vasa recta, but not in proximal tubule. Aldosterone increased the expression dose dependently with the greater extent in medulla region (Table 2; Fig. 3). However, as compared to pEGFR, the pERK1/2 protein expression was presented in a higher intensity (score 2) with the more involving areas, such as peritubular capillary, collecting duct (Table 2; Fig. 5). Aldosterone markedly enhanced the expression in glomeruli, collecting duct, loop of Henle, as well as induced the expression in proximal tubule (Fig. 5). Of interest, the HA rats had less pERK1/2 protein expression (score 3) in the outer medulla than those observed in the LA group (score 4). This could be the evidence supporting again that the negative feedback loop of MEK/ERK cascade is exist in the present, *in vivo*, study.

Logically, after transactivating on EGFR-ERK1/2 phosphorylation by nongenomic effect of aldosterone, the expression of both pEGFR and pERK1/2 proteins should be increased in a proportional pattern. However, the present data demonstrate that the expression of pERK1/2 was greater than those of pEGFR, especially in the cortex and outer stripe of outer medulla. This may explain by the ERK pathway regulation (Ramos, 2008). There are multiple signals which can activate the core Raf/MEK/ERK signaling module. For example, the phosphorylation of Raf at various sites by kinases, such as protein kinase C (PKC), was demonstrated

(Wellbrock et al., 2004; Wetzker and Böhmer, 2003). Once activated, Raf phosphorylates and activates MEK, then subsequently enhances ERK1/2. It has been shown that aldosterone could rapidly activate PKC in several cell lines and then elevate ERK1/2 phosphorylation (Christ et al., 1995; Harvey and Higgins, 2000; Mihailidou et al., 2004). Further study in rat kidney related to PKC/Raf/ERK interaction is required to elucidate this mechanism pathway.

It has long been known that aldosterone classically binds to the cytoplasmic mineralocorticoid receptor (MR) which then acts as a transcription factor for genomic pathway (Odermatt and Atanasov, 2009). However, one of the most controversial questions about the nongenomic aldosterone actions on EGFR phosphorylation is the requirement of MR for the rapid effects.

Several reports have indicated that nongenomic effect of aldosterone on activating EGFR-ERK1/2 phosphorylation is MR dependent. This evidence has been clarified by various investigations. Aldosterone-induced ERK1/2 phosphorylation was abolished by pretreatment with MR antagonist, such as spironolactone, RU38486, eplerenone, RU28318 or canrenoate (Grossmann et al., 2005; Han et al., 2009; McEneaney et al., 2007; McEneaney et al., 2010; Nagai et al., 2005).

It has remained elusive how the aldosterone/MR signal is transmitted to the ERK1/2 cascade. One of candidate mediators, the cytosolic tyrosine kinase (c-Src), has been shown to involve EGFR transactivation. Several investigators identified c-Src as the link between aldosterone-bound MR and ERK1/2 often with EGFR as intermediate (Braun et al., 2004; Callera et al., 2005a; Callera et al., 2005b; Grossmann et al., 2005; Krug et al., 2002; McEneaney et al., 2007). Aldosterone rapidly enhanced pc-Src protein abundance and activated EGFR-ERK1/2 signaling cascade (Braun et al., 2004; Callera et al., 2005a; Grossmann et al., 2005; Krug et al.,

2002; McEneaney et al., 2007). The increase in EGFR-ERK1/2 phosphorylation mediated by aldosterone/c-Src induction was inhibited by the c-Src inhibitor, PP2 (Callera et al., 2005a; Grossmann et al., 2005; Krug et al., 2002; McEneaney et al., 2007).

Surprisingly, the present, *in vivo*, results show that aldosterone, *per se*, did not lead to an increase in total pc-Src protein abundance but could modulate this protein expression in rat kidney (Fig. 7). Obviously, in the cortex area, no protein expression of pc-Src was noted in the proximal convoluted tubule in control kidney (Fig. 8a). Aldosterone could induce this expression with the prominent staining at the luminal membrane in the LA group (Fig. 8b). The higher aldosterone level, in HA rats, could redistribute this expression to be the basolateral membrane (Fig. 8c). The precise mechanism to explain this first report is still unclear. However, these exclusive data could be the strong supporting evidence that aldosterone/c-Src interaction in the nongenomic pathway plays more important roles on the proximal tubular functions. This notion is now firmly established. For example, Pergher et al. (2009) recently have demonstrated that aldosterone caused a significant increase in HCO_3^- reabsorption in luminally perfused proximal tubules. Furthermore, aldosterone could stimulate Na^+/H^+ exchanger isoform 1 activity in an isolated superfused proximal S_3 segment of rat (Leite-Dellova et al., 2008), and elevate Ca^{2+} influx into the proximal tubular epithelial cell culture isolated from human renal cells (Köppel et al., 2003).

In addition, via nongenomic action, aldosterone could inhibit Na^+/H^+ exchanger isoform 3 activity and decrease HCO_3^- reabsorption in renal medullary thick ascending limb (Good et al., 2002; Good et al., 2006; Watts et al., 2006), but enhance Ca^{2+} reabsorption in distal tubule through L- and T- type Ca^{2+} channels (Leclerc et al., 2004). Nongenomic actions of aldosterone in the renal tubule as well

as aldosterone-induced cation transport in the distal nephron have been reviewed (Good, 2007; Thomas et al., 2008).

Indeed, it has been shown that c-Src tyrosine kinase could regulate renal outer medullary K^+ channel location in the cortical collecting duct (Lin et al., 2004), activate the K^+/Cl^- cotransporter in the hippocampal neurons (Kelsch et al., 2001), modulate K^+ channels in Chinese hamster ovary cells (Gamper et al., 2003), but inhibit the epithelial Na^+ channel in the fibroblast cells (Gilmore et al., 2001). Thus, the further *in vivo* study is required to illustrate the aldosterone/c-Src interaction on cation/anion transports in various renal tubule segments.

In contrast, aldosterone could operate its nongenomic actions through MR-independent. Aldosterone induced EGFR-ERK1/2 phosphorylation was unaffected by the MR antagonists, spironolactone or RU26752 (Markos et al., 2005; Rossol-Haseroth et al., 2004). The alternative mechanisms of EGFR transactivation by aldosterone are currently the major topics of signal transduction research and recently several interesting findings. These include the reactive oxygen species (ROS) and angiotensin II (ANG II).

Oxidative stress is known to activate MAPKs (Cobb, 1999). Dong et al. (2004) demonstrated that H_2O_2 induced ERK1/2 phosphorylation was dependent on EGFR activation in pig renal proximal tubular epithelial cells. Recently, Huang et al. (2009) clearly illustrated that aldosterone increased ROS generation and enhanced EGFR phosphorylation in human mesangial cells. The mechanism for elevation of ROS in this regard is explained via the rapid activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Aldosterone could stimulate ROS production through activation of NADPH oxidase in human mesangial cells (Huang et al., 2009), ventricular cardiomyocytes (Rude et al., 2005), and vascular smooth

muscle cells (Callera et al., 2005a). Furthermore, inhibition of NADPH oxidase by apocynin, an NADPH oxidase inhibitor, abolished aldosterone-induced ERK1/2 phosphorylation (Rude et al., 2005).

For ANG II, it seems increasingly evident demonstrated the existence of cross-talk between ANG II and aldosterone which could potentially modulate ANG II signal transduction (Lemarié et al., 2008). These interactions between ANG II and aldosterone stimulate other specific signaling pathways in which are distinct from those usually induced on their own (Lemarié et al., 2008). It has been shown that ANG II dose-dependently increased EGFR phosphorylation through ANG II subtype 1 receptor (AT_1R) in renal proximal tubular cells (Chen et al., 2006), and enhanced pERK1/2 in preglomerular smooth muscle cells (Andresen et al., 2003). Moreover, aldosterone could augment AT_1R-dependent activation of ERK1/2 in mesenteric vascular smooth muscle cells (Lemarié et al., 2009). Indeed, AT_1R has traditionally been thought to act as a monomer (Aplin et al., 2009). Recent evidence has revealed that aldosterone rapidly increased the formation of AT_1R dimer in mesenteric arterioles and caused vasoconstriction (Yamada et al., 2008). Taken together, these indicate that aldosterone could induce EGFR-ERK1/2 transactivation via AT_1R/NADPH/ROS linkage. Further *in vivo* investigations in rat kidney are required to elucidate this interaction.

In conclusion, the present *in vivo* data demonstrate that aldosterone, via nongenomic pathway, could elevate pEGFR and pERK1/2 protein abundances and expressions in rat kidney. Aldosterone seems not to influence the total renal pc-Src protein abundance meanwhile the protein localization was obviously modulated.

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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

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	12% Separating gel (mL)	10% Separating gel (mL)	4% Stacking gel (mL)
H_2O	4.17	4.67	3.08
40% acrylamide mix	3.00	2.50	0.50
1.5 M Tris (pH 8.8)	2.50	2.50	1.25
100% Glycerol	0.24	0.24	0.12
20% SDS	0.05	0.05	0.025
10% APS	0.03	0.03	0.01875
TEMED	0.01	0.01	0.00625
Total volume	10.00	10.00	5.00

Calculation for assessment plasma aldosterone (pmol/L)

1 (pg/mL) = 2.77 (pmol/L)

Table B: The intensity scores of renal pEGFR protein expression in cortex, outer medulla, and inner medulla from each rat of sham, LA, and HA groups. Sections were scored in a blinded, semi-quantitative manner by three pathologists.

Groups		Cortex					Outer medulla										Inner medulla				
							outer stripe					inner stripe									
		Pathologist					Pathologist					Pathologist					Pathologist				
		(1)	(2)	(3)	Mode		(1)	(2)	(3)	Mode		(1)	(2)	(3)	Mode		(1)	(2)	(3)	Mode	
Sham	1	0	1	1	1		0	0	1	0		1	1	0	1		2	1	2	2	
	2	1	0	0	0		0	0	0	0		1	2	1	1		1	1	1	1	
	3	1	1	1	1		1	1	0	1		0	1	1	1		2	3	3	3	
	4	1	0	1	1	1	1	0	0	0	0	0	1	0	0	1	1	2	2	2	2
	5	1	1	1	1		0	0	0	0		1	1	0	1		2	2	2	2	
	6	1	0	0	0		1	0	1	1		0	0	0	0		2	1	1	1	
	7	0	1	1	1		0	0	1	0		0	0	1	0		2	1	2	2	
	8	1	1	0	1		0	1	1	1		1	0	1	1		3	2	3	3	
LA	1	1	1	0	1		0	1	1	1		2	3	2	2		3	3	4	3	
	2	2	1	2	2		1	0	0	0		3	3	2	3		4	4	3	4	
	3	1	0	1	1		0	0	1	0		2	3	2	2		4	4	4	4	
	4	0	1	1	1	1	0	1	0	0	0	3	3	3	3	3	4	3	3	3	4
	5	0	0	1	0		0	0	1	0		4	4	3	4		3	4	3	3	
	6	1	1	1	1		0	0	0	0		2	3	2	2		4	4	3	4	
	7	0	1	1	1		1	0	0	0		2	3	3	3		4	3	4	4	
	8	1	0	0	0		1	0	1	1		3	3	4	3		4	4	4	4	
HA	1	1	2	2	2		1	2	1	1		3	4	4	4		4	3	4	4	
	2	1	2	1	1		0	1	1	1		4	3	4	4		4	3	3	3	
	3	2	1	2	2		0	0	0	0		3	3	3	3		4	4	4	4	
	4	1	0	1	1	2	0	1	1	1	1	4	3	4	4	4	4	3	4	4	4
	5	1	1	1	1		1	1	0	1		4	4	4	4		3	4	3	3	
	6	1	2	2	2		0	1	0	0		4	3	4	4		4	4	4	4	
	7	2	2	1	2		1	1	0	1		3	4	3	3		4	4	3	4	
	8	2	2	2	2		1	0	1	1		3	4	4	4		3	4	4	4	

Table C: The intensity scores of renal pERK1/2 protein expression in cortex, outer medulla, and inner medulla from each rat of sham, LA, and HA groups. Sections were scored in a blinded, semi-quantitative manner by three pathologists.

Groups		Cortex					Outer medulla										Inner medulla				
							outer stripe					inner stripe									
		Pathologist					Pathologist					Pathologist					Pathologist				
		(1)	(2)	(3)	Mode		(1)	(2)	(3)	Mode		(1)	(2)	(3)	Mode		(1)	(2)	(3)	Mode	
Sham	1	2	1	2	2	2	3	3	2	3	2	2	3	2	2	2	2	1	2	2	2
	2	2	1	1	1		3	2	2	2		2	2	2	2		1	2	2	2	
	3	2	2	2	2		2	2	2	2		2	2	3	2		1	2	2	2	
	4	2	1	2	2		3	2	2	2		2	3	2	2		1	2	1	1	
	5	1	1	2	1		3	3	2	3		3	3	2	3		1	2	2	2	
	6	2	2	2	2		2	2	3	2		3	3	3	3		2	1	1	1	
	7	2	1	2	2		2	2	2	2		2	3	2	2		1	2	2	2	
	8	2	1	1	1		2	3	2	2		3	2	2	2		2	1	2	2	
LA	1	3	2	3	3	3	4	4	3	4	4	4	3	4	4	4	3	2	3	3	3
	2	2	3	2	2		4	3	3	3		4	4	4	4		3	3	3	3	
	3	3	3	2	3		4	4	4	4		3	4	3	3		3	2	3	3	
	4	2	3	3	3		3	4	4	4		3	4	4	4		3	2	2	2	
	5	3	3	3	3		3	4	3	3		4	3	4	4		3	3	3	3	
	6	3	2	2	2		4	3	4	4		3	4	4	4		2	3	2	2	
	7	3	2	3	3		4	4	4	4		4	4	4	4		3	2	3	3	
	8	2	3	3	3		4	3	4	4		3	4	3	3		2	3	3	3	
HA	1	4	4	3	4	4	3	3	4	3	3	3	4	3	3	3	4	3	4	4	4
	2	4	4	4	4		3	3	4	3		3	4	4	4		4	4	4	4	
	3	3	4	3	3		4	4	3	4		3	4	3	3		4	4	3	4	
	4	3	3	4	3		3	3	4	3		3	4	4	4		4	3	4	4	
	5	4	4	4	4		4	4	3	4		4	4	3	4		3	3	4	3	
	6	4	3	4	4		3	3	3	3		4	3	3	3		3	4	4	4	
	7	4	4	3	4		4	4	3	4		3	4	3	3		4	3	4	4	
	8	3	4	3	3		3	4	3	3		3	3	3	3		3	4	4	4	

Table D: The intensity scores of renal pc-Src protein expression in cortex, outer medulla, and inner medulla from each rat of sham, LA, and HA groups. Sections were scored in a blinded, semi-quantitative manner by three pathologists.

Groups		Cortex					Outer medulla									Inner medulla				
							outer stripe					inner stripe								
		Pathologist					Pathologist					Pathologist					Pathologist			
		(1)	(2)	(3)	Mode		(1)	(2)	(3)	Mode		(1)	(2)	(3)	Mode		(1)	(2)	(3)	Mode	
Sham	1	1	1	2	1	1	1	2	1	1	1	2	3	2	2	2	2	2	2	2	3
	2	1	2	1	1		2	2	1	2		1	2	2	2		2	3	2	2	
	3	2	2	1	2		2	1	1	1		2	3	2	2		2	3	3	3	
	4	2	1	1	1		1	2	2	2		3	2	3	3		3	2	3	3	
	5	1	2	1	1		1	1	1	1		2	2	1	2		2	3	3	3	
	6	1	1	1	1		2	1	1	1		1	2	2	2		3	3	2	3	
	7	1	2	2	2		1	2	2	2		2	2	2	2		2	3	3	3	
	8	1	1	2	1		1	2	1	1		2	3	3	3		2	3	2	2	
LA	1	2	1	1	1	2	1	2	1	1	1	2	2	3	2	2	4	3	4	4	3
	2	2	1	2	2		1	1	1	1		3	2	3	3		4	3	3	3	
	3	2	2	1	2		1	2	2	2		2	3	2	2		3	3	3	3	
	4	2	1	1	1		1	1	2	1		3	2	2	2		3	4	3	3	
	5	1	2	2	2		2	1	1	1		3	2	2	2		3	3	4	3	
	6	1	2	2	2		1	1	2	1		2	2	2	2		4	3	4	4	
	7	2	1	2	2		1	1	1	1		2	3	2	2		4	3	3	3	
	8	2	1	1	1		2	1	1	1		2	2	3	2		4	3	3	3	
HA	1	4	4	3	4	4	1	2	1	1	1	2	3	3	3	2	1	2	1	1	1
	2	4	3	4	4		1	2	1	1		2	3	2	2		2	2	1	2	
	3	3	3	4	3		2	1	1	1		2	2	2	2		1	1	0	1	
	4	4	3	4	4		1	1	1	1		3	2	2	2		1	1	1	1	
	5	3	4	4	4		2	1	1	1		2	2	2	2		1	2	1	1	
	6	3	3	4	3		2	1	2	2		2	3	2	2		2	1	2	2	
	7	4	4	4	4		1	2	1	1		2	3	3	3		1	1	2	1	
	8	3	3	3	3		2	2	1	2		3	2	2	2		2	1	1	1	

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