ไนตริกออกไซด์ซินเธสที่ไต และการสร้างไนตริกออกไซด์ ในภาวะที่ไตขาดเลือดไปเลี้ยงชั่วคราว : ผลของระบบแองจิโอเทนซิน

นางสาว อยู่เย็น ซื้อจ้าง

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RENAL NITRIC OXIDE SYNTHASE AND NITRIC OXIDE PRODUCTION IN RENAL ISCHEMIC REPERFUSION : EFFECTS OF ANGIOTENSIN SYSTEM

Miss Yuyen Seujange

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By	Miss Yuyen Seujange
Field of Study	Physiology
Thesis Advisor	Assistant Professor Somchit Eiam-Ong, Ph.D.
Thesis Co-Advisor	Associate Professor Pansiri Phansuwan, Ph.D.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master's Degree.

……...Dean of Graduate School
(Professor Suchada Kiranandana, Ph.D.)

Thesis Committee

…….. Chairman
(Associate Professor Prasong Siriviriyakul, M.D.)

…….. Thesis Advisor
(Assistant Professor Somchit Eiam-Ong, Ph.D.)

……..…. Thesis Co-Advisor
(Associate Professor Pansiri Phansuwan, Ph.D.)

…….. Member
(Professor Prasit Futrakul, M.D.)

……. Member
(Associate Professor Chollada Buranakarl, Ph.D., D.V.M.)

อยู่เย็น ซื้อจ้าง: ไนตริกออกไซด์ซินเธสที่ไต และการสร้างไนตริกออกไซด์ ในภาวะที่ไตขาดเลือดไปเลี้ยงชั่วคราว: ผลของระบบแองจิโอเทนซิน (Renal nitric oxide synthase and nitric oxide production in renal ischemic reperfusion: Effects of angiotensin system) อ. ที่ปรึกษา: ผศ. ดร. สมจิตร์ เอี่ยมอ่อง, อ. ที่ปรึกษาร่วม: รศ. ดร. ปานสิริ พันธุ์สุวรรณ 86 หน้า ISBN 974-171-536-6

การวิจัยครั้งนี้เป็นการศึกษาไนตริกออกไซด์ซินเธสที่ไตและการสร้างไนตริกออกไซด์ ในภาวะที่ไตขาดเลือดไปเลี้ยงชั่วคราวข้างเดียว รวมทั้งศึกษาบทบาทของระบบแองจิโอเทนซินต่อภาวะดังกล่าว โดยทำการทดลองในหนูแรทพันธุ์วิสต้าซึ่งแบ่งออกเป็น 2 กลุ่มใหญ่ๆ คือ กลุ่ม sham (S) และกลุ่มที่ชักนำให้เกิดภาวะการขาดเลือดไปเลี้ยงชั่วคราวที่ไตข้างซ้าย โดยการอุดกั้นหลอดเลือดแดงไตเป็นเวลา 30 นาที (IR) ซึ่งกลุ่มนี้จะถูกแบ่งออกเป็นอีก 3 กลุ่มย่อย คือ 1) ได้รับน้ำดื่มเพียงอย่างเดียว (IR) 2) ได้รับน้ำดื่มผสม angiotensin converting enzyme inhibitor (ACEI; Enalapril®; 200 mg/L) และ 3) ได้รับน้ำดื่มผสม angiotensin II receptor type 1 antagonist (ARA; Losartan®; 500 mg/L) สัตว์ทดลองจะได้รับสารดังกล่าว 1 วันก่อนการผ่าตัดทำ S หรือ IR และรับต่อไปอีกเป็นเวลา 1 วันหรือ 7 วันตามกลุ่มการทดลอง เมื่อครบกำหนดการทดลอง ทำการเก็บตัวอย่างปัสสาวะและเลือดเพื่อตรวจวัดระดับไนไตรท์ อิเลคโตรไลต์ BUN ครีเอตินีน (Cr) รวมทั้งเก็บตัวอย่างเนื้อไตเพื่อตรวจวัดเอนโดธีเลียลไนตริกออกไซด์ซินเธส (endothelial nitric oxide synthase) โดยวิธี immunohistochemistry และตรวจวัดระดับการทำลายเนื้อไต ด้วยวิธี Periodic Acid-Shift reaction (PAS) และ Masson's trichrome technique ทำการวัดปริมาณเลือดที่มาเลี้ยงไต (rBF) โดยใช้ laser doppler flowmeter ณ เวลาก่อน ระหว่าง และหลังการอุดกั้นหลอดเลือดแดงไต ผลการทดลองพบว่า เมื่อครบกำหนดการทดลอง 1 วัน ในกลุ่ม IR จะมีระดับเอนโดธีเลียลไนตริกออกไซด์ซินเธสที่เยื่อบุหลอดไต (epithelium) และโกลเมอรูลัสลดลงอย่างมาก เมื่อเทียบกับกลุ่ม S แต่เมื่อได้รับ ACEI หรือ ARA ร่วมด้วย ระดับเอนโดธีเลียลไนตริกออกไซด์ซินเธสจะเพิ่มขึ้นใกล้เคียงกับกลุ่ม S ส่วนกลุ่ม 7 วันหลังทำ IR พบว่าระดับของเอนโดธีเลียลไนตริกออกไซด์ซินเธสมีค่าใกล้เคียงกับกลุ่ม S แต่เมื่อได้รับ ACEI จะมีค่าลดลง นอกจากนี้ยังพบว่าในกลุ่ม IR 1 วัน จะมีระดับซีรั่มไนไตรท์เพิ่มขึ้นอย่างมีนัยสำคัญ ($p < 0.01$) เมื่อเทียบกับกลุ่ม S แต่เมื่อได้รับ ACEI ค่าไนไตรท์จะใกล้เคียงกับกลุ่ม S แต่ถ้าได้รับ ARA จะมีระดับต่ำที่สุด ระดับไนไตรท์จะมีค่าใกล้เคียงกับกลุ่ม S หลังจากทำ IR แล้ว 7 วัน ส่วนระดับการทำลายเนื้อไตในกลุ่ม IR 1 วัน พบว่ามีการขยายกว้าง หลุดลอก และอุดตันของหลอดไตที่บริเวณ cortex ในระดับน้อยถึงปานกลาง ซึ่ง ACEI หรือ ARA สามารถลดระดับการทำลายดังกล่าว อย่างไรก็ตาม กลุ่ม 7 วันนั้นไม่พบการทำลายเนื้อไต เมื่อทำการอุดกั้นหลอดเลือดแดงไตข้างซ้าย ค่า rBF ลดลงร้อยละ 80 ถึง 85 ($p < 0.001$) ซึ่งจะมีค่ากลับมาที่ระดับร้อยละ 50 ณ นาทีที่ 5 และร้อยละ 75 ถึง 80 ณ นาทีที่ 10 หลังการอุดกั้น เมื่อเปรียบเทียบกับระดับก่อนการอุดกั้น โดยค่าดังกล่าวจะกลับสู่ระดับร้อยละ 100 หลังทำ IR 1 วันและ 7 วัน ไม่พบการเปลี่ยนแปลงอย่างมีนัยสำคัญทางสถิติของ rBF ในไตอีกข้าง และการให้ ACEI หรือ ARA ก็ไม่มีผลต่อค่าดังกล่าวของทั้ง 2 ไต ส่วนค่าสัดส่วนการขับทิ้ง (fractional excretion) ของโซเดียม ($FE_{Na}+$) ลดลงอย่างมีนัยสำคัญทางสถิติในกลุ่ม IR 1 วัน มีเพียงกลุ่มที่ได้รับ ARA เท่านั้นที่มีค่าใกล้เคียงกับกลุ่ม S พบว่า FE ของอิเลคโตรไลต์ในทุกกลุ่มมีค่าใกล้เคียงกันเมื่อครบการทดลอง 7 วัน นอกจากนี้ยังพบอีกว่าค่าโซเดียม โปแตสเซียม คลอไรด์ ในซีรั่ม รวมทั้งค่า BUN, Cr และ CCr มีการเปลี่ยนแปลงน้อยซึ่งไม่มีผลทางสถิติในทุกกลุ่มการทดลอง โดยค่าดังกล่าวอยู่ในเกณฑ์ปกติ

จากการศึกษาครั้งนี้ได้แสดงข้อมูลเป็นครั้งแรก ที่เกี่ยวกับภาวะไตขาดเลือดไปเลี้ยงชั่วคราวข้างเดียวว่า ระบบแองจิโอเทนซินมีบทบาทที่สำคัญต่อการควบคุมระดับของไนตริกออกไซด์ซินเธสโปรตีนที่ไต และการสร้างไนตริกออกไซด์ รวมทั้งการขับทิ้งของอิเลคโตรไลต์ในภาวะดังกล่าว

หลักสูตร ..... ลายมือชื่อนิสิต .....

สาขาวิชา ..... ลายมือชื่ออาจารย์ที่ปรึกษา .....

ปีการศึกษา ..... ลายมือชื่ออาจารย์ที่ปรึกษาร่วม .....

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KEY WORD: RENAL ISCHEMIC REPERFUSION/ ANGIOTENSIN CONVERTING ENZYME INHIBITOR/ ANGIOTENSIN II RECEPTOR TYPE 1 ANTAGONIST/ NITRIC OXIDE SYNTHASE
YUYEN SEUJANGE: RENAL NITRIC OXIDE SYNTHASE AND NITRIC OXIDE PRODUCTION IN RENAL ISCHEMIC REPERFUSION: EFFECTS OF ANGIOTENSIN SYSTEM. THESIS ADVISOR: ASSIST. PROF. SOMCHIT EIAM-ONG, PH.D., THESIS CO-ADVISOR: ASSOC. PROF. PANSIRI PHANSUWAN, PH.D. 86 pp. ISBN 974-171-536-6

This study was conducted to investigate renal nitric oxide synthase (NOS) protein expression and nitric oxide (NO) production in unilateral renal ischemic reperfusion (IR) as well as the role of angiotensin system. The male Wistar rats were divided into two main groups; sham operation (S) and IR (30-minute occlusion). In IR groups, the animals were further divided into 3 subgroups treated with: 1) water, 2) angiotensin converting enzyme inhibitor (ACEI; Enalapril®; 200 mg/L), and 3) angiotensin II receptor type 1 antagonist (ARA; Losartan®; 500 mg/L). The treatment was performed one day before the operation (S or IR) and continuously for 1 day or 7 days after the operation. On each experimental due date, 24-hr urine and blood samples were collected. The serum were stored at -80 °C until use for NO production (nitrite), electrolytes, blood urea nitrogen (BUN), creatinine (Cr), and Cr clearance (CCr). The kidneys were removed and fixed for eNOS protein expression and histological study. In addition, the regional renal blood flow (rBF) was measured before, during and after ischemia as well as before sacrificed by a laser doppler flowmeter. By immunohistochemistry, the expression of renal eNOS protein showed more staining in glomerulus as well as in renal tubular epithelial cells in cortex than in medulla. One day after IR caused a marked decrease of eNOS protein expression, especially in cortex. The treatements with ACEI or ARA could ameliorate the loss of renal eNOS protein expression caused by IR in 1-day group. However, this alteration was less observed in 7-day duration after IR, while only the ACEI-treated rats, for 7 days after IR, reduced the expression. One day after IR, serum nitrite concentration was significantly increased ($p < 0.01$). The treatment with ACEI could normalize the heightened nitrite level induced by IR to be that of sham animal. The ARA-treated rats had a greater extent less concentration. However, 7 days after IR, serum nitrite concentration in all groups was comparable and not significant different from 1-day sham animals. The kidney exposed to IR showed mild to moderate dilatation of cortical tubule with few cast formation and mild brush border membrane loss in 1-day post IR group. The treatment with ACEI or ARA could attenuate structural damages. All 7-day groups studied showed normal structure of renal tissue. During left renal artery occlusion, the rBF decreased by 80 to 85% ($p < 0.001$) and restored to be 50% and 75 to 80% of baseline levels at 5^{th} and 10^{th} minute after release, respectively. The values of rBF were completely returned to baseline in either 1-day or 7-day post IR groups. No significant changes of rBF were observed in the right (non-ischemic) kidney. Neither ACEI nor ARA influenced on rBF of both. The fractional excretion (FE) of sodium (FE_{Na^+}) was markedly diminished ($p < 0.001$) in 1 day after IR. Only ARA treatment could restore the value to be near that of the sham animals. However, after 7 days of IR, these FE values in all groups were comparable. In addition, both 1-day and 7-day post IR groups demonstrated similar levels of serum Na^+, K^+, Cl^- as well as BUN, Cr, and CCr. These values are in normal range.

The present data are the first evidence of IR model in that the angiotensin system plays a crucial role in regulation of renal eNOS protein expression, NO production as well as electrolyes excretion.

Department …….. Student's signature ……..
Field of study …….. Advisor's signature ……..
Academic year …… Co-advisor's signature …….……

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LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
ACEI	Angiotensin converting enzyme inhibitor
ANG	Angiotensin
ARA	Angiotensin II receptor type 1 antagonist
AT1	Angiotensin II receptor type 1
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic gaunosine monophosphate
°C	Degree Celsius
DAG	Diacylglyceral
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
eNOS	Endothelial nitric oxide synthase
ET	Endothelin
GFR	Glomerular filtration rate
g	Gram
H_2O_2	Hydrogen peroxide
ICAM	Intracellular adhesion molecule
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP3	Inositol 1,4,5-triphosphate
IR	Renal ischemic reperfusion
Kf	Glomerular capillary ultrafiltration coefficient
L-AMMA	N^G-Monomethyl-L-arginine
L-NAME	N^w nitric-L-arginine methyl ester
L-NNA	N^ω-nitro-L-arginine

LIST OF ABBREVIATIONS (cont.)

LPS	Lipopolysaccharide
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinase
M	Molar
mRNA	Messenger ribonucleic acid
NO	Nitric oxide
NO_3^-	Nitrate
NO_2^-	Nitrite
NOS	Nitric oxide synthase
nNOS	neuronal nitric oxide synthase
$ONOO^\bullet$	Peroxynitrite
$O_2^\bullet$	Superoxide
$OH^\bullet$	Hydroxyradical
PAF	Platelet activating factor
PAS	Periodic Acid-Schiff reaction
PGs	Prostaglandins
PKC	Protein kinase C
PLA2	Phospholipase A2
RBF	Renal blood flow
ROS	Reactive oxygen species
rBF	Regional renal blood flow
rpm	Revolution per minute
TNF	Tumor necrosis factor
TxA2	Thromboxane A2
μl	Microlitre

CHAPTER I

INTRODUCTION

BACKGROUND AND RATIONALE

Renal ischemic reperfusion (IR) is characterized by a decline in renal blood flow (RBF) and oxygen supply (Waz et al., 1998). This often occurs during renal transplantation, surgical revasculization of the renal artery, resuscitation from hypotension states and treatment of suprarenal aortic aneurysms (Rhoden et al., 2001).

IR leads to a series of cellular events including decreased glomerular filtration rate (GFR), glomerular capillary ultrafiltration coefficient (Kf) and causes of renal damage (Honda and Hishida, 1993). With advanced injury, tubular epithelial cells detach from the basement membrane and contribute to intraluminal aggregations of cells and proteins resulting obstruction (Bonventre, 1993). IR might end in renal failure depending on the duration of ischemia (Mashiach et al., 1998).

Many mediators are involved in the pathophysiology of postischemic renal dysfunction including purine metabolites (Waz et al., 1998), reactive oxygen species (ROS) e.g. superoxide ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$) (Brady et al., 2000), and vasoactive substances e.g. endothelin (ET), angiotensin II (ANG II), thromboxane A2 (TxA2), prostaglandins (PGs) and nitric oxide (NO) (Waz et al., 1998). Two molecules that have been widely studied and implicated in IR injury are ANG II and NO (Hennington et al., 1998).

Generation of ANG II acts to constrict vascular smooth muscle cells and also modulates aldosterone secretion, cathecholamine release and secretion of pituitary peptides and hormones (Wong et al., 1990). Within the kidney, ANG II modulates GFR by exerting direct effect on postglomerular vessels tone and on mesangial cell function (Ichikawa and Harris, 1991). ANG II also modulates tubular transport directly (Wong et al., 1990). All respond to ANG II by angiotensin type 1 (AT1) receptor stimulation (Wong et al., 1990). It has been demonstrated that ANG II levels are significantly increased in rats with IR (Kontogiannis and Burns, 1998; Vargas et al., 1994). These result in increased vascular resistance (VR) and mean arterial pressure (MAP), decreased RBF and oxygen consumption causing renal damage (Kontogiannis and Burns, 1998; Vargas et al., 1994). Angiotensin converting enzyme inhibitor (ACEI) and AT1 receptor antagonist (ARA) can ameliorate VR and MAP, increased RBF and oxygen consumption as well as decreased renal damage (Kontogiannis and Burns, 1998; Vargas et al., 1994). In addition, ANG II appears to stimulate proximal tubule (PT) hypertrophy and has been shown to augment epidermal growth factor (EGF) stimulated PT cells mitogenesis (Norman et al., 1987).

Interestingly, ANG II increases $O_2^{\bullet}$ production in rat vascular smooth muscle cells (Griendling et al., 1994), mesangial cells (Jaimes et al., 1998) and human vascular endothelial cells (Berry et al., 2000). Excessive $O_2^{\bullet}$ interacts with many molecules including NO in which also reduces NO bioavailability to generate peroxynitrite ($ONOO^{\bullet}$) (Zbou et al., 2001). $ONOO^{\bullet}$ is a much stronger oxidant and causes endothelial cell impairment (Lane and Gross, 1999). Endothelial cell is an important source of multiple

vasoactive factors, the most widely recognized of which are ET and NO (Lerman and Textor, 2001).

NO is a free radical gas that could diverse biomessenger function and participate in the regulation of kidney function by counteracting the vasoconstrictor effects of ANG II (Lerman and Textor, 2001). In addition to its effects on blood flow and tubular reabsorption of sodium (Na^+) (Bachmann and Mundel, 1994), NO inhibits the growth of vascular smooth muscle cells, mesangial cell hypertrophy and hyperplasia, and the synthesis of extracellular matrix (Bachmann and Mundel, 1994). These effects occur in part by decreasing expression of angiotensin converting enzyme (ACE) and AT1 gene (Lerman and Textor, 2001).

NO is synthesized from L-arginine by a family of NOS (Schwobel et al., 2000). NOS isoforms are constitutive NOS (cNOS) and inducible NOS (iNOS) (Marsden et al., 2000). The two cNOS are termed neuronal NOS (nNOS) and endothelial NOS (eNOS). cNOS are transiently activated by agonists inducing elevation of intracellular calcium (Ca^{2+}) (Lane and Gross, 1999). The iNOS isoform can be induced by immunostimulants. These could generate NO production with a continous rate, high output and Ca^{2+} independent (Lane and Gross, 1999).

NO production is rapidly increased in IR rats (Saito and Miyagawa, 2000). In addition, iNOS mRNA and protein are increased and relate with renal injury (Ling et al., 1999; Noiri et al., 1996). The iNOS knockout mice (in macrophage cells of PT) can protect against IR injury (Noiri et al., 1996a). It has been shown that eNOS mRNA and protein are decreased in cell cultured with hypoxia condition (Kakoki et al., 2000; Liao et al., 1995). N^w nitric-L-arginine methyl ester (L-NAME; a non selective NOS

inhibitor) administration significantly increases VR and MAP, decreases RBF and renal function (Kakoki et al., 2000). L-NAME also reduces Na^+ and water excretion (Lahera et al., 1991). These results are similar to ones observed with increased in ANG II (Blantz et al., 1976). An administration of ACEI or ARA could prevent most of the changes in renal and glomerular hemodynamic induced by NOS blockade (De Nicola et al., 1992). In contrast, an acute suprarenal infusion of ANG II increases renal eNOS mRNA expression, while a chronic infusion increases eNOS protein expression (Hennington et al., 1998). These data suggest that ANG II can stimulate eNOS synthesis and this may be one of the mechanisms whereby ANG II enhances NO production (Hennington et al., 1998).

Studies regarding iNOS in IR have yielded inconclusive results, reporting either enhanced or unchanged protein expression (Kakoki et al., 2000; Ling et al., 1999). There were scare data of renal eNOS protein expression in IR state of in vivo study. Administration of L-NAME, a non selective NOS inhibitor, could induce renal abnormalities similar to the ones observed with increased ANG II. In IR, whether renin angiotensin system plays a regulating role on renal NOS protein expression and NO production remains unknown. The present work was conducted to examine the role of renin angiotensin system on renal NOS protein expression and NO production in IR. To accomplish this purpose, ACEI and ARA were utilized in the experimental studies.

CHAPTER II

THEORY AND LITERATURE REVIEW

RENAL ISCHEMIC REPERFUSION

IR is characterized by a decline in RBF and oxygen supply which leads to a series of cellular events including decreased GFR and Kf (Waz et al., 1998). It also causes tubular dysfunction secondarily to obstruction from casts and cellular debris and back-leak of glomerular filtrate through damage tubular epithelia (Waz et al., 1998). Many events are involved in IR including adenosine triphosphate (ATP) depletion, cell swelling, Ca^{2+} influx, enzyme activation, disruption of cytoskeleton, ROS and cytokine activation (Brady et al., 2000).

Adenosine Triphosphate Depletion

A fall in cellular ATP levels is an early event following oxygen deprivation due to ischemia, hypoxia or hypoperfusion (Brady et al., 2000). Oxygen deprivation results in rapid degradation of ATP to adenosine diphosphate (ADP) and adenosine monophosphate (AMP) (Bonventre, 1993). AMP is metabolized further to nucleosides (adenosine and inosine) and to hypoxanthine. These purine metabolites are able to diffuse passively from the cell. As a results, there is a loss of the metabolites that serve as a reservoir for the rapid synthesis of ATP during reperfusion (Brady et al., 2000). A prolong ischemia will also ultimately result in irreversible loss of mitochondria function which further impairs rapid regeneration of ATP following reperfusion. Along with the ability of the cell to survive from

ischemia, is therefore dependent upon the duration of the ischemia period (Brady et al., 2000; Mashiach et al., 1998). ATP depletion impairs many cellular processes including protein synthesis, lipogenesis and membrane transport (Bonventre, 1993).

The major consequences of cellular ATP depletion that lead to cell injury include inhibition of ATP-dependent transport pumps with loss of the ion gradients. Those are normally maintained across cell membranes and polarized epithelia (Bonventre, 1993). The unregulated activation of injurious enzyme systems, such as phospholipase and protease and alteration in the cell cytoskeleton, have been reported (Bonventre, 1993).

Cell Swelling

Cell swelling represents an important response to ischemia (Brady et al., 2000). Cell volume is controlled by the Na^+-K^+ ATPase pump which produces a negative intracellular charge and a low intracellular Na^+ concentration (Brady et al., 2000). During oxygen deprivation and ATP depletion, the Na^+-K^+ ATPase pump becomes inactive. The cells become depolarized and accumulate Na^+ and chloride (Cl^-). As a result, the cells take up water and swell (Mason et al., 1981).

Calcium Influx

An early rise in the concentration of intracellular free Ca^{2+} following ATP depletion has the potential cause of cell injury and death (Burke et al., 1984). During ATP depletion, due to ischemia, Ca^{2+} extrusion from the cell via Ca^{2+}-ATPase is inhibited (Brady et al., 2000). Furthermore, as ATP levels fall, the activity of the Na^+-K^+ ATPase pump at the plasma

membrane decreases and this causes a rise in intracellular Na^+. Therefore, Ca^{2+} is accumulated inside the cell due to the low activity of Na^+-Ca^{2+} exchanger (Bonventre, 1993).

Ca^{2+} that accumulates in cells can be taken up into non-mitochondrial as well as mitochondrial compartments (Cheung et al., 1986). Under normal conditions, the non-mitochondrial strorage sites play a much more important role in the buffering of Ca^{2+} than do the mitochondria (Cheung et al., 1986). If large amounts of Ca^{2+} enter the cells, the mitochondria become more important storage sites, representing a very high capacity system for buffering Ca^{2+} (Lehninger, 1970). Very large amounts of Ca^{2+} can be taken up into mitochondria in the presence of ATP (Lehninger, 1970). Large amounts of mitochondrial uptake of Ca^{2+} could cause mitochondrial swelling, uncoupling of oxidative phosphorylation, and release of free fatty acids, which themselves uncouple oxidative phosphorylation (Bonventre, 1993). Ca^{2+} can be detrimental to cells in a number of additional ways. An increase in intracellular Ca^{2+} stimulates plasma and endoplasmic recticulum Ca^{2+}-ATPase. The increased activity of these Ca^{2+}-ATPase enhances energy consumption and further contributes to ATP depletion (Bonventre, 1993). An increase in cytosolic free Ca^{2+} concentration activates Ca^{2+} dependent protease and phospholipases. These enzyme lead to proteolysis and membrane disruption (Bonventre, 1993). Elevated Ca^{2+} levels also could disrupt the cell cytoskeleton microfilaments (Bonventre, 1993). In addition, increased intracellular Ca^{2+} is able to facilitate the generation of ROS by accelerating the conversion of xanthine dehydrogenase to xanthine oxidase (Figure 1) (Brady et al., 2000).

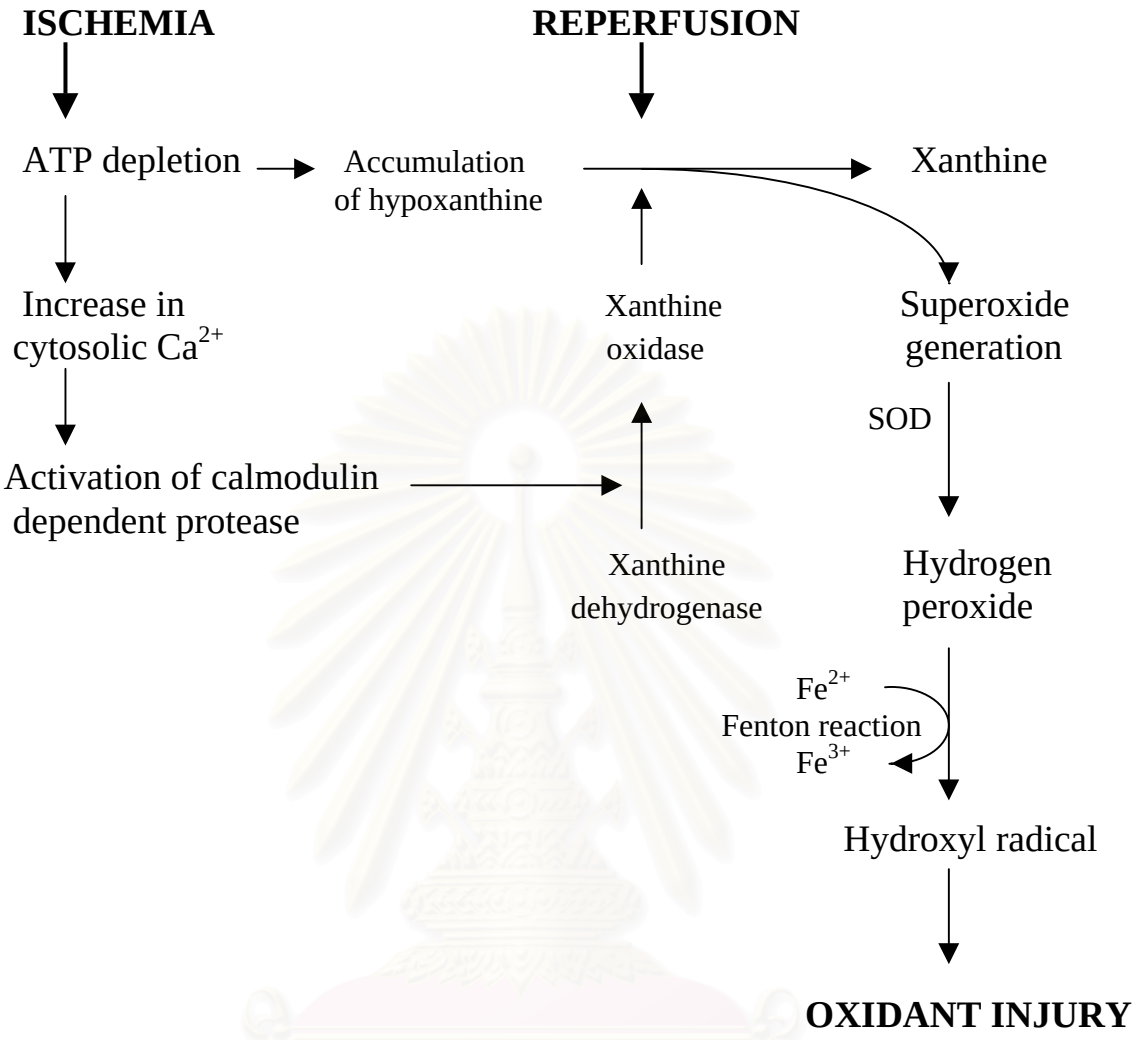


Figure 1 Potential pathway for hydroxyl radical generation following ischemia reperfusion (Brady et al., 2000).

Enzyme Activation

Many forms of enzyme require very high Ca^{2+} concentrations for activation, including phospholipase and protease (Yin and Stossel, 1979). Phospholipase A2 (PLA2), a family of enzymes, hydrolyzes phospholipids to generate free fatty acid and lysophospholipids (Bonventre, 1993). PLA2 likely plays an important role in ischemic cellular injury in many ways (Bonventre, 1993): (1) The PLA2 induces changes in phospholipid

integrity. The toxic action of free fatty acids and lysophosoholipids could alter plasma membrane and mitochondrial membrane permeability properties and bioenergetic capacities. (2) The lipid peroxidation that occurs from ischemia and reperfusion causes an increase in susceptibility of cellular membranes to PLA2 (Bonventre, 1993). (3) As one of the PLA2 products, arachidonic acid is converted to eicosanoids by the prostaglandin endoperoxide synthase and lipoxygenase enzymes, and then ROS are generated. (4) The eicosanoid products of arachidonic acid metabolism are vasoactive and chemotactic for neutrophils, which in turn, may contribute to the tissue injury (Klausner et al., 1989). (5) In addition, when the lipid 1-O-alkyl-2-acyl-phosphorylcholine is the substrate for PLA2, the product becomes the precursor for platelet activating factor (PAF), which can activate platelets and cause capillary occlusion (Bonventre, 1993).

Disruption of the Cytoskeleton

The actin cytoskeleton plays an important role in a number of aspects of epithelial cell function including the maintenance of cellular polarity (Brady et al., 2000). ATP depletion is a marked cause of cytoskeleton defect resulting in the disruption of cell-cell junctional complexes and changes in the expression and disruption of cellular adhesion molecules (Fanning et al., 1999). The junctional complexes include the tight junction, adherens junction, demosomes and gap junction (Sheridan and Bonventre, 2000). The tight junction separates apical and basolateral membrane domain in the polarized cell, and provides a barrier to the paracellular transport of water and solute (Sheridan and Bonventre, 2000). The tight junction is comprised of proteins that provide both a barrier to and channels for paracellular transport (Sheridan and Bonventre, 2000). The adherens

junction is located directly basal to the tight junction and contributes to epithelial cell polarity (Molitoris and Marrs, 1999). The adherens junction contains transmembrane cadherin proteins that link to the actin cytoskeleton and signaling protein via catenins (Molitoris and Marrs, 1999).

Disruption of the tight junction alters both paracellular permeability and cellular polarity. The increase in permeability results in back-leak of glomerular filtrate (Sheridan and Bonventre, 2000). The change in cell polarity has multiple effects resulting from incorrect targeting of membrane proteins (Sheridan and Bonventre, 2000). After 10 minutes of ischemia, Na^+-K^+ ATPase activity usually confined to the basolateral domain, is misdirected to the apical membrane (Figure 2) (Molitoris, 1991). These result in impaired transcellular Na^+ transport and an increase in intraluminal Na^+ delivery to the distal tubule (Molitoris, 1991). The enhanced distal Na^+ delivery may result in afferent arteriole vasoconstriction and a reduction in GFR (Alejandro et al., 1995).

Changes in PT polarity result in misdirected targeting of integrins. Integrins anchor epithelial cells to extracellular matrix protein receptors, which contain the arginine, glycine, aspartic acid sequence (Sheridan and Bonventre, 2000). ATP depletion causes the redistribution of the integrin β-3 subunit from the basolateral to the apical domain. The disruption of both cell-cell adhesion and adhesion to the extracellular matrix allows viable epithelial cells to be shed into the tubular lumen (Molitoris and Marrs, 1999). The apical expression of integrins also promotes intraluminal cell-cell aggregation contributing to obstruction (Zuk and Bonventre, 1998). The large amounts of fibronectin in the lumen

probably potentiates intraluminal cell-cell matrix interaction and cast formation (Zuk and Bonventre, 1998).

Thus, alterations in the cytoskeleton are associated with sublethal injury which manifested by changes in cell polarity and paracellular permeability, as well as desquamation. These functional consequences result in the back-leak of glomerular filtrate and intraluminal obstruction contributing to decreased GFR (Zuk and Bonventre, 1998).

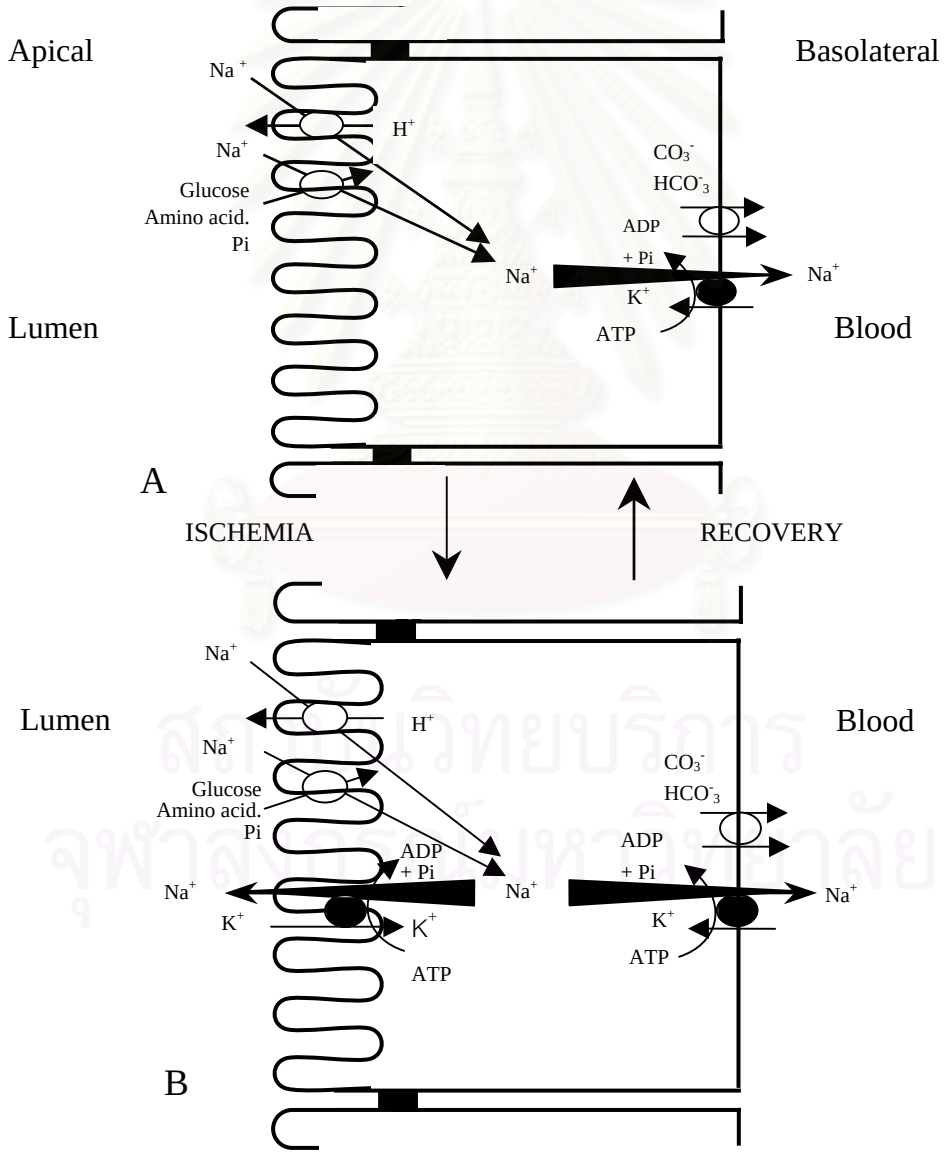


Figure 2 The impairment of transcellular Na^+ transport post-ischemia reperfusion (Molitoris, 1991).

Free Radical

A free radical is any species capable of independent existence that posses one or more unpaired electrons and thus is chemically highly reactive (Weight et al., 1996). Foremost, within biological systems are the ROS, especially the superoxide ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) (Weight et al., 1996). The $OH^{\bullet}$ is formed from two precursors, $O_2^{\bullet}$ and H_2O_2. The conversion of H_2O_2 to $OH^{\bullet}$ requires ferrous iron as catalyst (Brady et al., 2000). Both $O_2^{\bullet}$ and H_2O_2 are constantly being produced by normal cells, and their production is increased in many pathophysiologic states (Sheridan and Bonventre, 2000). Cells are normally protected from the injurious effects of $OH^{\bullet}$ formation by a number of scavenging systems. Superoxide dismutase (SOD), a cytosolic and mitochondrial metalloenzyme, catalyses the conversion of $O_2^{\bullet}$ to H_2O_2 (Brady et al., 2000). Catalase, a peroxisomal enzyme, and glutathione peroxidase, a cytosolic and mitochondrial enzyme, catalyze conversion of H_2O_2 to water (Paller et al., 1984). These enzymes and antioxidants represent the major defenses against ROS production within the PT cell. An oxidant injury occurs when these defenses are either deficient or are overwhelmed by excessive production ROS (Paller et al., 1984).

In IR injury, ROS can be formed when oxygen is delivered to tissue during reperfusion (Paller et al., 1984). During ischemia, intracellular Ca^{2+} increases (Burke et al., 1984) and activates calmodulin Ca^{2+} dependent protease that converts xanthine dehydrogenase (the normal form of this enzyme) to xanthine oxidase (Weight et al., 1996). The xanthine oxidase catalyzes hypoxanthine to xanthine during reperfusion. This has been suggested to be an important source of $O_2^{\bullet}$ following ischemia. SOD then

converts $O_2^{\bullet}$ to H_2O_2, which can then be converted to the highly reactive $OH^{\bullet}$ by the iron requiring fenton reaction (Figure 1) (Brady et al., 2000).

ROS are directly toxic to tubular endothelial cells. Mechanisms of ROS induced damage of tubular cells include the peroxidation of lipid membranes, protein denaturation and DNA strand breaks (Brady et al., 2000). Lipid peroxidation by ROS enhances permeability and impairs enzymatic processes as well as ion pumps. ROS induced strand breaks in DNA result in the activation of DNA repair mechanism (Sheridan and Bonventre, 2000).

Cytokines Activation

The inflammatory response associated with ischemia appears to be mediated by the increased renal expression of a number of cytokines including tumor necrosis factor-α (TNF-α) (Donnahoo et al., 1999), interferon-γ, granulocyte-macrophage colony-stimulating factor, interleukin (IL)-1, IL-2 and IL-8. Moreover the expression of endothelial adhesion molecules such as ICAM-1, P-selectin and E-selectin also increase (Brady et al., 2000).

Activated leukocytes could injure endothelial and renal tubular cells directly by oxidants (Lauriat and Linas, 1998). These leukocytes are able to release both $O_2^{\bullet}$ and NO precursors for $OH^{\bullet}$ and peroxynitrite ($ONOO^{\bullet}$) (Bassenge, 1992). The leukocytes also produce myeloperoxidase which catalyzes the formation of hypochlorous acid, another potent oxidant (Weight et al., 1996). However, in addition to causing oxidant injury, the leukocytes probably also contribute indirectly to tubular injury by ischemic

or toxic insult to the kidney. They can promote medullary hypoperfusion in at least two ways (Weight et al., 1996). First, leukocytes mediated to capillary endothelium may exacerbate the congestion of the medullary circulation caused by platelet aggregation and red cell clumping. Second, the activated leukocytes can exacerbate intrarenal vasoconstriction by releasing a number of vasoactive factors including TxA2, leukotriene B4, and PAF (Brady et al., 2000; Weight et al., 1996).

As above described, it has been shown that IR involves a complex inter-related sequence of events. In the majority of renal ischemia primes the tissue for the injury incurred during reperfusion. The injury being principally mediated many mechanisms as noted previously. Besides the loss of renal function, IR causes endothelial cell dysfunction. The endothelial cell is an important source of multiple vasoactive factors, widely recognized of which are ANG II and NO. Both could modulate vascular tone and have been postulated to interact closely in IR condition.

THE RENIN ANGIOTENSIN SYSTEM

The primary function of the renin angiotensin system (RAS) is to maintain adequate capillary perfusion, particularly when body fluid volume is threatened (Gunning et al., 1996). This is accomplished by the dual and interrelated actions of ANG II on vascular resistance and extracellular fluid volume (ECFV) homeostasis (Gunning et al., 1996). ANG II is a potent vasoconstrictor that affects directly on vascular smooth muscle (Gunning et al., 1996).

Renin

Renin is the key rate-limiting enzyme secreted predominantly by the juxtaglomerular cell in the kidney into the blood in response to a number of stimuli including a reduction in blood pressure and a reduction in ECFV (Levens et al., 1992). The active renin cleaves 10 amino acid from its α-2 globulin substrate angiotensinogen to form the decapeptide angiotensin I (ANG I). ANG I is rapidly converted to the biologically organs by the action of the endothelial enzyme, ACE (Gunning et al., 1996).

Angiotensin Converting Enzyme

ACE is a large acidic glycoprotein metalloenzyme composed of a single polypeptide chain. This enzyme is a rather nonspecific exopeptidase that sequentially cleaves dipeptides from the carboxy terminus of polypeptides (Gunning et al., 1996). ACE is distributed throughout the body, most abundantly in kidney, ileum, duodenum, and uterus. The enzyme is thought to be expressed primarily by endothelial, epithelial, and neuroepithelial cells and by some endocrine cells (Gunning et al., 1996). It is membrane bound with the active site directed outward, although in some cells such as sperm, ACE is found on intracellular membrane. Endothelial cell ACE is positioned ideally to act on circulating ANG I to form ANG II. Although it was originally believed that pulmonary endothelium was the principle site of ANG I conversion to ANG II, it is now clear that ANG II formation in peripheral tissue may be equally important. In the kidney, ACE is localized on glomerular endothelial cells and on PT brush border membrane (Gunning et al., 1996). ANG II exerts its biological actions by binding to its specific receptors on target cells (Arima and Ito, 2001).

Angiotensin II Receptor

Two major ANG II receptor subtypes have been defined and designated as type 1 (AT1) and type 2 (AT2) receptors (Matsubara, 1998). Most of the known biological action of ANG II is mediated by AT1 receptor (Horiuchi et al., 1999). Whereas the AT2 receptor is the predominant receptor present in the fetal and early neonate kidney (Aguilera et al., 1994; Kakuchi et al., 1995), disappear shortly after birth and is linked to apoptosis in the other tissues (Shanmugam et al., 1995). The localization and function of AT2 receptor remain unclear (Kontogianinis and Burns, 1998).

Cellular Mechanism of Angiotensin II

AT1 receptors interact with G-protein coupled receptor (Murphy et al., 1991). Stimulation of AT1 receptor activates phospholipase A2, C and D resulting in increased intracellular Ca^{2+}, inositol 1,4,5-triphosphate (IP_3) concentration and diacylglyceral (DAG) which stimulate protein kinase C (PKC) activity (Sasaki et al., 1991). ANG II binds to Gi-protein coupled receptor and then inhibits adenylate cyclase activity and reduces cyclic adenosine monophosphate (cAMP) levels (Inagami, 1999). The synthesis of PGs has been observed as well (Figure 3) (Gunning et al., 1996; Lassegue et al., 1991).

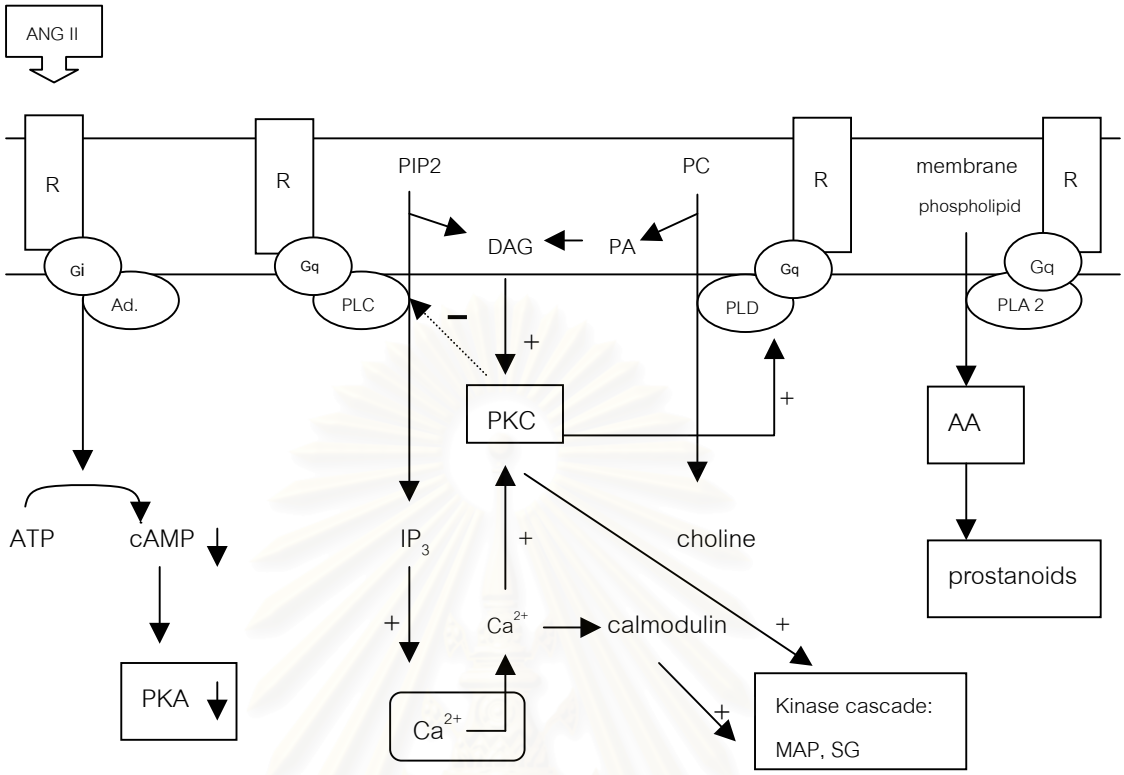


Figure 3 Linkage of angiotensin II receptor type 1 to biochemical processes (Levens et al., 1992). (R = angiotensin II receptor type 1, Ad. = adenylate cyclase, PLC = phospholipase C, PIP2 = phosphoinositoldiphosphate, IP3 = inositol triphosphate, DAG = diacylglyceral, PA = phosphatidic acid, PKC = protein kinase C, PLD = phospholipase D, PC = phosphatidylcholine, PLA2 = phospholipase A2, AA = arachidonic acid, MAP = mitogen activated protein, SG = soluble guanylate cyclase)

The AT1 receptor has been shown to mediate most, if not all, of the known biological responses elicited by ANG II, e.g. vascular smooth muscle contraction, aldosterone secretion, cathecholamine release, secretion of pituitary peptides and hormones, dipsogenic effect and renal sodium reabsorption (Wong et al., 1990).

Intrarenal Action of Angiotensin II

Within the kidney, AT1 receptor distribution is widespread. The AT1 receptors are present in the vasculature on both smooth muscle plasma membranes and endothelial cells including afferent and efferent arterioles (Ardaillou, 1999) as well as in the PT (Gunning et al., 1996).

Action of Angiotensin II on the Glomerular Circulation

The activation of AT1 receptor increases renal vascular resistance and reduces RBF (Arima and Ito, 2001). ANG II preferentially constricts efferent arterioles compared with afferent arteriole. As a result, the GFR is preserved (Yuan et al., 1990). AT1 receptors are present in abundance on mesangial cells, endothelial cells and podocytes (Sharma et al., 1998). In mesangial cells, ANG II causes contraction leading to decreased a capillary surface area and hence Kf (Arima and Ito, 2001). Under pathologic conditions, activation of AT1 receptors stimulates mesangial cell protein synthesis with enhanced production of extracellular matrix proteins and transforming growth factor (TGF)-β (Kagami et al., 1994). In addition, ANG II, through the AT1 receptor, stimulates the mesangial cell to produce several growth factors [such as ET, interleukin (IL)-6 and platelet-derived growth factor (PDGF)] which may contribute to proliferative responses in glomerular diseases (Bakris and Re, 1993; Moriyama et al., 1995). The function of AT1 receptors on podocytes remains unclear. However, Gloy et al. (1998) suggested that stimulation of Ca^{2+} influx and reorganization of the actin cytoskeleton may be involve in the induction of foot-process contractile responses.

Action of Angiotensin II on Renal Tubular Epithelial Transport

Tubular epithelial cells along the entire nephron also express AT1 receptors with a predominant expression in the PT on both apical and basolateral membrane (Li et al., 1994). In the lumen membrane of the PT, the concentration of ANG II is reported to be in the nanomolar range which is approximately 1000 fold greater than that in plasma (Navar et al., 1994). ANG II, through AT1 receptor, affects the PT transport function in vitro with low concentrations ($< 10^{-9}$ mol/L) stimulating and high concentration ($> 10^{-8}$ mol/L) inhibiting transepithelial Na^+ and bicarbonate (HCO_3^-) transport. These occur via the both apical and basolateral Na^+-H^+ exchanger, (Arima and Ito, 2001), HCO_3^- transporter and Na^+-K^+ ATPase activity (Thekkumkara et al., 1998). The biphasic effects of ANG II on PT transport may cause activation of PLA2 and subsequent arachidonic acid release at high concentrations. These lead to an inhibition of Na^+-H^+ exchanger (Li et al., 1994). By contrast, Gi-protein mediated inhibition of adenylate cyclase at low concentrations could decrease production of cAMP which, in tern, enhances the activity of Na^+-H^+ exchanger (Liu and Cogan, 1989; Thekkumkara et al., 1998).

AT1 receptor have also been detected on the macula densa cells and may involve in the ANG II induced enhancement of tubuloglomerular feedback (TGF) (Schnerman and Briggs, 1990). These receptors in the distal tubule have been shown to be linked to the stimulation of undirectional HCO_3^- flux, an effect that appears to be mediated by both apical and basolateral receptor activation (Levine et al., 1994). Finally, AT1 receptor have been identified in both the cortical collecting duct (CCD) and inner medullary collecting duct (IMCD) (Levine et al., 1996).

In the CCD, AT1 receptors may regulate both Na^+ and HCO_3^- transport (Schlatter et al., 1995). In the MCD, the function of AT1 receptors is unclear, though its role in medullary water handling has been suggested (Oliverio et al., 2000). In addition, the expression of AT1 receptors in renal medullary interstitial cell situated between the vasa rectae and tubules has been shown, though the function of these receptors are so far unclear (Arima and Ito, 2001).

Angiotensin II and Reactive Oxygen Species

ANG II increases $O_2^{\bullet}$ production in rat vascular smooth muscle cells (Griending et al., 1994), mesangial cells (Jaimes et al., 1998) and human vascular endothelial cells (Berry et al., 2000), through increased membrane associated NADH/NADPH oxidase activity. Both $O_2^{\bullet}$ and H_2O_2 signaling pathways are associated with the mitogen-activated protein kinase (MAPK) family, but stimulate different signaling pathways (Bass and Berk, 1995). In vascular smooth muscle cells, $O_2^{\bullet}$ activates the p42/44MAPK pathway, while H_2O_2 stimulates the p38 MAPK pathway (Ushio-Fukai et al., 1998). Both p38 MAPK and p42/44 MAPK pathways are required for a full hypertrophic response to ANG II (Bass and Berk, 1995).

Angiotensin II and Renal Ischemic Reperfusion

IR causes an early increase renin release and intrarenal ANG II levels (Vargas et al., 1994). The role of ANG II during IR is extraordinary complex (Lerman and Textor, 2001). Generation of ANG II acts to raise perfusion pressure and to protect glomerular filtration by efferent arteriolar

constriction (Lerman and Textor, 2001). ANG II induces cellular hypertrophy and hyperplasia in several cell types, in addition to stimulating local hormone production and ion transport directly (Lerman and Textor, 2001). AT1 receptors are primary targets of ANG II, expressed on endothelial, epithelial and vascular cells (Lo et al., 1995).

An experimental infusion of ANG II leads to parenchymal renal injury with focal and segmental glomerulosclerosis (Zou et al., 1996). ACEI and ARA in several experimental models diminish renal cell proliferation and suppress the infiltration of mononuclear cells, which trigger the expression of extracellular matrix protein that lead to progressive nephrosclerosis (Geiger et al., 1997). The rat model with IR shows renal damage, increased VR and MAP, decreased RBF and oxygen consumption (Vargas et al., 1994). ACEI (Vargas et al., 1994) and ARA (Kontogiannis and Burns, 1998) could ameliorate renal damage, decrease VR and MAP and also increase RBF and oxygen consumption (Vargas et al., 1994). In addition, ANG II potently induces the expression of renal TGF-β, a cytokine involed in glomerular cell proliferation, monocyte migration and matrix synthesis (Zoja et al., 1998). An administration of ACEI could reduce glomerular cell proliferation and inflammatory cell infiltration (Zoja et al., 1998).

The study in rats with hypercholesterolaemia demonstrated that ANG II is involved in the pathogenesis of atherosclerosis. A Chronic administration of ACEI is able to reduce aortic atherosclerotic lesions (Chobanian et al., 1990). The study in patients with essential hypertension found that ACEI or ARA treatment shows an improvement of small artery structure (Schiffrin et al., 1995).

As noted previously, several observations underscore the dual roles of ANG II for adaptation and maintenance of kidney function and for modulating many steps in the pathologic cascade underlying progressive renal injury. However, IR causes an increases both ROS and ANG II. Besides induced renal dysfunction, ANG II also results in vascular endothelial dysfunction. The vascular endothelium is a source of multiple vasoactive factors, the most widely recognized of which are ET and NO. Especially, NO participates in the regulation of kidney function by counteracting the vasoconstrictor effects of ANG II (Lerman and Textor, 2001).

NITRIC OXIDE AND NITRIC OXIDE SYNTHASE

NO is a lipophilic, highly reactive, free radical gas with diverse biomessenger function (Lane and Gross, 1999). NO is produced by endothelium cells (Marsden et al., 2000). The production is catalyzed by NOS isoforms converting L-arginine and molecular oxygen to L-citrulline and NO (Schwobel et al., 2000).

Nitric Oxide Synthase

Two main NOS isoforms are constitutively expressed in specific cell types in which activity is regulated by changing levels of intracellular Ca^{2+} (cNOS) (Marsden et al., 2000). The two cNOS are termed neuronal NOS (nNOS) and endothelial NOS (eNOS). These are names for the tissue from which they were initially isolated (Lane and Gross, 1999). These NOS are transiently activated by an agonist inducing elevation of intracellular calcium and binding of Ca^{2+}-calmodulin (Lane and Gross, 1999).

This pulsatile activation is ideally suited to a regulatory role by puffs of NO in cells and tissues. Inducible NOS (iNOS), the remaining isoform, can be triggered by immunostimulants in most cell types. This activity is continuous, high output, and Ca^{2+} independent (Lane and Gross, 1999). The iNOS was originally isolated from murine macrophage as an enzyme that contains tightly bound calmodulin, and accordingly, it is fully active at resting low levels of intracellular calcium (Cho et al., 1992). The iNOS could produce a large continuous flux of NO which may be limited only by substrate availability (Lane and Gross, 1999). The transcriptional upregulation of the iNOS gene occurs in response to various inflammatory cytokines [e.g. interleukin (IL)-1. TNF-α or immunostimulants, such as bacterial lipopolysaccharide (LPS) by a mechanism that is synergistic with interferon-γ (Nathan and Xie, 1994)].

Cellular Mechanism of Nitric Oxide

NO is a potent activator of guanylate cyclase, an important hemo-protein target of NO (Lane and Gross., 1999). Binding of NO alters the conformation of the heme moiety and causes activation of the enzyme. The subsequent rise in cGMP levels causes the activation of cGMP-dependent kinases and also augment Ca^{2+}-extrusion through the plasma membrane Ca^{2+}-ATPase (Masden et al., 2000). These result in VSM cells relaxation, suppression of platelet adhesion and aggregation (Azuma et al., 1986). In addition, some evidences reveal an involvement of NO in a remarkable array of key physiological processes including regulation of inflammation (Tiao et al., 1994), neurotransmission (Bredt et al., 1990), learning and memory (Kendrick et al., 1997), penile erection (Morbidelli et al., 1996), cell migration (Noiri et al., 1996b)

and apoptosis (Mannick et al., 1994). Whether the net effects of NO on given cells are either beneficial or deleterious will be determined composition of the surrounding microenvironment (Lane and Gross, 1999).

Intrarenal Action of Nitric Oxide

All three types of NOS are present in the kidney. The expression of nNOS is limited mainly to the macula densa and its regulatory role in signal transduction within the juxtaglomerular apparatus (Mundel et al., 1992). The eNOS is constitutively expressed by the endothelial lining in blood vessels of different caliber. On the other hand, iNOS is constitutived expressed by the PT, and can be induced in the IMCD and mesangial cells (Gologorsky and Noiri., 1999). However, there are clinical and experimental evidences that chronic progressive renal disease and end stage renal disease are associated with NO deficiency (Gologorsky and Noiri., 1999).

Role of Nitric Oxide in the Regulation of Renal Blood Flow and Glomerular Filtration Rate

Evaluation of the role of NO in the regulation of RPF and GFR has heavily relied on the use of various NOS inhibitors with different degrees of specificity with respect to each one of NOS isoforms (Gabbai and Blantz, 1999). These nonspecific NOS inhibitors include N^G-monomethyl-L-arginine (L-AMMA), L-NAME and N^{ω}-nitro-L-arginine (L-NNA) (Gabbai and Blantz, 1999).

Acute administration of the NOS blocker, L-NMMA, to an awake concious rat leads to increases in MAP and decreases in RBF and GFR. Large reductions in RBF relatively to GFR lead to significant increases in filtration fraction (Baylis et al., 1996). In anesthesized rats, a continuous intravenous infusion of NOS blockers increases systemic blood pressure and reduces RPF with variable changes in GFR (Tolins et al., 1990). At the single nephron level, the NOS blockade reduces nephron plasma flow because of increases in afferent and efferent arteriolar resistances (Zatz and De Nueci, 1991). Single nephron GFR is maintained or slightly reduced during L-NMMA infusion because of the opposing effects of an increased glomerular capillary hydrostatic pressure and transcapillary hydrostatic gradient, and the reduction of the Kf and nephron plasma flow (Zatz and De Nueci, 1991).

Experiments performed with renal artery infusion of NOS blockers have been shown that the NOS blockade has a preferential effect on the afferent arteriole and Kf with minimal impact on the efferent arteriole and glomerular capillary hydrostatic pressure (Deng and Baylis, 1993). In mesangial cell culture, the NOS blockade could produce contraction which constitutes a potential mechanism for the reduction of the Kf (Shultz et al., 1990).

Role of Nitric Oxide on Renal Tubular Epithelial Transport

Studies by several groups of investigator have found that NO inhibits the Na^+-H^+ exchanger and Na^+-K^+ ATPase activity in PT (Roczniak and Burna, 1996; Guzman et al., 1995). The NOS blockade is associated with reduced Na^+ and water excretion without affecting autoregulation of total

RBF and GFR (Lahera et al., 1991). NO also modulates tubular reabsorption in other tubular segments including cortical and inner medullary collecting duct (Roczniak et al., 1998). In these segments of the tubule, NO inhibits tubular reabsorption by inhibiting the Na^+-K^+ ATPase activity (Stoos et al., 1994).

Nitric Oxide and Renal Ischemic Reperfusion

The study with real time monitoring of NO in rat with IR demonstrated that NO production is rapidly increased and reaches a plateau (a 13-fold increase on the basal level) approximately 10 - 15 minutes after the clipping of renal artery (Saito and Miyagawa, 2000). But NO is only a slight increase in rats treated with L-NAME (Saito and Miyagawa, 2000). At 30 minutes after ischemia, the clip was removed, NO release returned to almost the basal level immediately (Saito and Miyagawa, 2000). This suggests that the kidney may maintain the necessary blood supply via the circulatory system by dilating arteries as a consequence of released NO during ischemia. A rapid fall in NO current after removal of the clamp reflects the quenching of NO by the $O_2^{\bullet}$ generated upon reoxygeneration rather than the rapid diffusion of NO by recovered blood flow. This agrees with a report studied in rat stomach (Wada et al., 1998).

Many studies demonstrated that iNOS mRNA and iNOS protein were increased in rats with IR (Ling et al., 1999; Noiri et al., 1996a). iNOS produces a large number of NO (Walker et al., 2000). The excessive NO also interacts with $O_2^{\bullet}$ to generate $ONOO^{\bullet}$ (Zbou et al., 2001). The $ONOO^{\bullet}$ is a much stronger oxidant than either the NO or $O_2^{\bullet}$, and appears to be

a key bacteriocidal product of immunstimulant activated macrophages (Walker et al., 2000). Under physiological conditions, $ONOO^{\bullet}$ is sufficiently stable to diffuse some distance before reacting with target molecules such as membrane lipids, protein sulfhydryl groups, DNA and antioxidant (Walker et al., 2000). $ONOO^{\bullet}$, when produced by endothelial cell, is thought to contribute to endothelial injury via modification of the low density lipoprotein within the arterial wall, a process that may contribute to formation of the fatty streak and plaque that are characteristic of the atherosclerotic lesion (Lane and Gross, 1999).

However, a selective iNOS inhibitor can decrease $ONOO^{\bullet}$ production and renal injury (Walker et al., 2000) This suggests that iNOS generated NO mediates damage in IR injury through $ONOO^{\bullet}$ formation (Walker et al., 2000). The iNOS knockout mice (specific in macrophage cell of PT) is also protected against IR injury, whereas iNOS knockout in VSM increases renal injury (Noiri et al., 1996a). These suggest that NO has both cytotoxic and cytoprotective effects on IR injury in many tissues (Noiri et al., 1996a; Saito and Miyagawa, 2000).

The studies in primary culture of human umbilical vein endothelial cells (McQuillan et al., 1994) and bovine aortic endothelial cells (Liao et al., 1995) have shown that eNOS mRNA and protein are decreased in hypoxia condition. In addition, Kakoki et al. (2000) demonstrated that eNOS protein is also decreased in rat with IR, but iNOS protein is not detected. A L-NAME administration significantly increases renal damage and MAP, while RBF and GFR are reduced (Kakoki et al., 2000). These are similar to one observed with increased ANG II (Blantz et al., 1976).

INTERACTION AMONG RENAL ISCHEMIC REPERFUSION, ANGIOTENSIN II AND NITRIC OXIDE

IR causes increases many mediators including ANG II, NO and ROS. The loss of balance between ANG II and NO represents a disturbance of tissue homeostasis and may accelerate tissue damage (Lerman and Textor, 2001). NO participates in the regulation of kidney function by counteracting the vasoconstrictor effects of ANG II (Aki et al., 1997). In addition to its effects on blood flow and tubular reabsorption of Na^+ (Bachmann and Mundel, 1994), NO inhibits the growth of vascular smooth muscle cells, mesangial cell hypertrophy and hyperplasia and the synthesis of extracellular matrix (Bachmann and Mundel, 1994). These effects occur, in part, by decreasing the expression of ACE and AT1 receptors (Raij, 1998).

As mentioned earlier, a NOS blockade induced in the anesthetized rat could change glomerular hemodynamics (increases in glomerular capillary hydrostatic pressure and transcapillary hydrostatic pressure gradient, and reduction in nephron plasma flow and Kf) similarily to the ones observed with increases in ANG II (Blantz et al., 1976). Both ACEI and ARA are able to prevent most of the changes in renal and glomerular hemodynamics induced by NOS blockade (Sigmon and Beierwalts, 1993).

In vitro studies using afferent arteriolar vessel show that NOS blockade is associated with increased response to ANG II, as induced by larger and prolonged reduction of arteriolar lumen (Ito et al., 1993). This response predominates in the afferent arteriole because the change in efferent arteriolar tone induced by ANG II is not modified by NOS blockers

(Gabbai and Blantz, 1999). The studies utilized anesthetized rats undergoing acute (3 hours) or chronic (2 weeks) administration of NOS blockers showed that an acute infusion of the NOS blocker L-NMMA increases systemic blood pressure, but did not modify GFR. Measurements of ANG II reveales a significant reduction in plasma ANG II levels in the absence of changes in kidney tissue ANG II (Garcia et al., 1997). Placement of an aortic snare to prevent increases in renal perfusion pressure during NOS blockade reveales similar changes in plasma and kidney tissue ANG II (Garcia et al., 1997). These imply that the inhibition of renin and plasma ANG II generation is independent of the changes in systemic blood pressure and that NOS blockers directly suppress renin secretion (Gabbai and Blantz, 1999).

The study using isolated perfused rat kidney with perfusion pressure 100 mmHg, have shown that sodium nitroprusside (SNP; NO donor) 30 μmol/L induces 4.5 fold increase of basal renin secretion rate (Kurtz et al., 1998). However, the membrane permeable cGMP analogs 8-bromo-cGMP could inhibit the basal renin secretion and attenuate the stimulation of renin secretion by SNP (Kurtz et al., 1998). If the perfusion pressure is lowered to 40 mmHg in the presence of 30 μmol/L SNP, the renin secretion rate is 12 fold of the basal value. In addition, NO has been to stimulate renin secretion in vivo from conscious (Persson et al., 1993) and anesthesized animal (Johnson and Freeman, 1994). Moreover, NO downregulates the synthesis of ACE in the endothelium and thus can affect ANG II production (Higashi et al., 1995). Furthermore, NO could downregulate AT1 receptor in vascular tissue and adrenal gland and thus mitigates the actions of ANG II (Ichiki et al., 1998).

Besides the effect of NO on production of renin, ACE, and AT1 receptor, ANG II could increase NO production and renal NOS expression. (Hennington et al., 1998). The study performed in an acute ANG II infusion (110 minutes) showed an increase eNOS mRNA by 70% without changing eNOS protein levels, whereas a chronic infusion of ANG II (10 days) increases eNOS protein by 90% without changing eNOS mRNA levels (Hennington et al., 1998). This data suggested that ANG II can increase NO production in the kidney (Hennington et al., 1998). However, ANG II infusion time is only 110 minutes and probably not long enough to allow translation of eNOS protein (Hennington et al., 1998).

There are at least two mechanisms by which ANG II could increase renal NO production. First, ANG II could increase NOS activity by causing an increase in intracellular Ca^{2+} concentration. Secondly, ANG II could increase NO by increasing eNOS synthesis either on a transcriptional level or on translational level. Both would increase NO production (Hennington et al., 1998). In addition, it is possible that ANG II could cause an increase in eNOS synthesis due to an increase in shear stress (Hennington et al., 1998). The shear stress promotes eNOS gene transcription through increase activity of tyrosine kinase (in particular c-Src) (Davis et al., 2001; Ungvari et al., 2001). Schwobel et al. (2000) demonstrated that induced NO production is negatively controlled by AT2, whereas AT1 receptor stimulation enhances NO synthesis in mesangial cells. The overall NO availability depends on the onset of the inflammatory stimuli with respect to ANG II exposure and the available AT receptor.

As above mentioned, the collective data demonstrate that IR causes increases both ANG II and NO. The interaction between ANG II and NO is more complex than just NO opposition of the tonic effect of ANG II on the renal microcirculation (Gabbai and Blantz, 1999). The complex interaction between ANG II and NO is generated by the effect of NO on production of renin and ANG II. However, to date, there is no study of IR related to renal NOS expression and nitric oxide production as well as the role of angiotensin system on this regard. Therefore, the present study aims to investigate this regard.

CHAPTER III

MATERIALS AND METHODS

1. Experimental Animals

The study was performed in male Wistar rats weighing 220 to 250 grams and were obtained from the National Center of Scientific Use of Animals (Mahidol University, Salaya, Nakhonpathom). The animals were housed in a well ventilated room in which the temperature was 23 - 25 °C with an automatic lighting schedule, which provided darkness from 8 p.m. to 6 a.m. The animals were given free access to standard laboratory chow and water as follow the experimental protocol. All rats were used once only and were kept in metabolic cages for 24 hours to collect urine before the experimental and before sacrificed. Urine volume was measured, and the samples were stored at - 80 °C until use.

2. Chemicals

2.1. Chemical Agents

Sodium pentobarbital (Nembutal®) was purchased from Sanofi, France. Enalapril® (20 mg) was obtained from Biolab, Thailand. Losartan® (50 mg) was obtained from M&H, USA. Absolute ethanol, 95% ethanol, Xylene, Dioxane, Di-sodium hydrogen phosphate (Na_2HPO_4), Potassium di-hydrogen phosphate (KH_2PO_4), Trisma® acid, Trisma® base, Triton X-100, 30% hydrogen peroxide and Gelatin were purchased from Merk, Germany. Paraformaldehyde was purchased from Sigma, USA.

Haematoxylin solution (progressive stain) was purchased from C.V. Laboratories, Thailand. ABC-streptavidin-horseradish peroxidase complex was purchased from Vector, USA. Paraffin pour embedding medium was purchased from St. Louis, USA. Nitric oxide (NO_2^- and NO_3^-) assay kits (Catalog No. DE 1500) was purchased from R&D system, USA.

2.2. Antibodies

Mouse monoclonal antibody against NOS II (iNOS, Catalog No. N39020-150) and NOS III (eNOS, Catalog No. N30020-150) were purchased from BD transduction, Japan. Normal swine serum (Code No. X0901, Lot. 110) and Biotinylate swine anti-goat-mouse-rabbit immunoglobulin (Multi-Link, Code No. E0453, Lot. 021) were purchased from Dako, Denmark.

3. Experimental Procedure

Animals were divided into 2 main groups as follow:

1. Sham group (n = 8)
2. IR groups (n = 48)

3.1. IR and Sham Operation

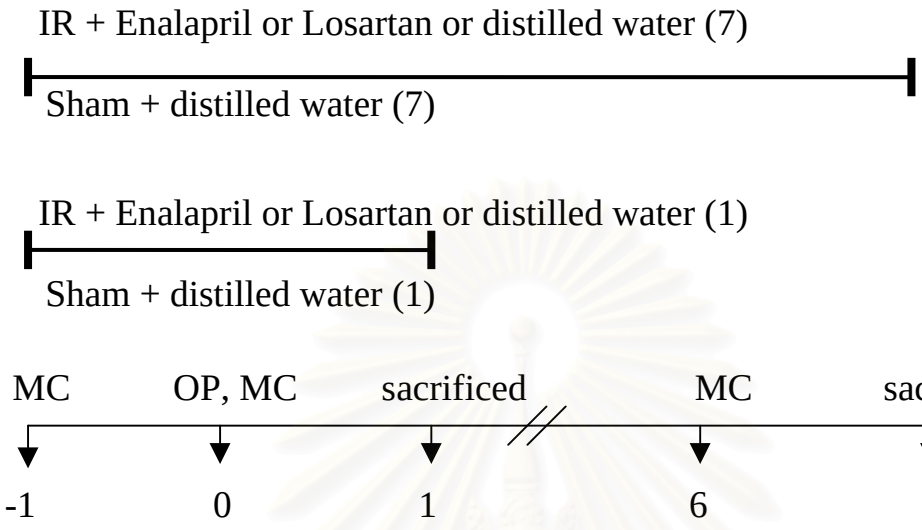
After three days to familiar with the new housing, the animals were weighed and collected blood sample from the tail for measuring BUN in order to assess kidney function (less than 30 mg %). After 24-hour urine collection, the rats were anesthetized by intraperitoneal (i.p.) injection of sodium pentobarbital (60 mg/kg body weight). Using aseptic technique, a midabdominal incision was made to expose the kidneys. The rBF was

measured in both sides. Then, an unilateral left renal pedicle cross clamp was performed for 30 minutes with microvascular clamps for IR groups. The rBF was monitored in both kidneys every five minutes, during the ischemia phase (30 minutes) and the reperfusion phase (20 minutes). The incision was closed. The sham group comprised of the rats that were operated and only wiped left renal pedicle. The rBF was also monitored as performed in IR group. All rats were allowed to wake up and return to a clean cage with free access to food but with three different kinds of drinking solution as follow:

1. Water (only distilled water)
2. Water + ACEI (Enalapril® 200 mg/L)
3. Water + ARA (Losartan® 500 mg/L)

The volume of drinking water was approximately 30 ml/rat/day. ACEI or ARA were provided one day before the operation and continuously for 1 day or 7 days after IR or S (n = 8 rats/group). Twenty-four hour urine was collected before the due date. On each experimental due date, the animals of respective groups were re-operated under anesthesia and rBF of both kidneys were measured. Blood samples were collected from the aorta through an abdominal incision and centrifuged at 3500 rpm. Serum were stored at -80 °C until use for BUN, Cr, and electrolyte measurements, as well as NO metabolite assay (NO_2^- and NO_3^-). The kidneys were removed and fixed in 4% paraformaldehyde overnight then transferred into 0.1 M sodium phosphate buffer (PBS) and stored at 4 °C. Tissues were embedded in paraffin, cut into 4-5 μm thick sections for eNOS, iNOS protein expression and histological evaluation.

3.2. Experimental Design



(MC = metabolic cage and OP = operation)

3.3. Measurement of Regional Renal Blood Flow

The rBF in the rat was measured with a Laser Doppler flowmeter (Advance ALF 21, Advance Co., Japan). Under sodium pentobarbital anesthesia, the fiber optic needle probe of Laser Doppler flowmeter was fixed 1-2 millimeter above the cortical surface of the kidney. The blood flow was measured before, during and after ischemia from both kidneys every 5 minutes and recorded by the polygraph (Nihon RM 6000, Japan). The percentage of changes in rBF was calculated.

3.4. Immunohistochemical Study

Paraffin-embedded kidney sections were cut at 4 μm. 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 swine serum in PBS-A (PBS + 1% BSA + 0.3% Triton X-100) for 30 minutes at room temperature. Then, the section was incubated in primary antibody for eNOS or iNOS in concentrations of 1:100 and 1:200, respectively (diluted in PBS-A) over one hour at room temperature.

After incubation, the sections were rinsed 3 x 10 minutes with PBS-B (PBS + 0.25% BSA + Triton X-100) and were then incubated with biotinylated swine anti-goat-mouse-rabbit immunoglobulin (Multi-Link) diluted 1:50 in PBS-B for 60 minutes at room temperature. After incubation, tissue sections were rinsed 2 x 10 minutes with PBS-B and then 1 x 10 minutes in PBS. The tissue sections were related with ABC-streptavidin horseradish peroxidase complex (diluted in PBS) 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 a solution containing 0.025% 3, 3'-diaminobenzidine (DAB) and 0.01% H_2O_2 in 0.05 M Tris-HCl buffer (pH 7.6) for 30 minutes. Then, sections were washed 2 x 5 minutes with distilled water. Finally, they were couterstained with haematoxylin and dehydrated. The slides were mounted and coverslipped with permount.

Areas of staining were identified, and the intensity of staining was scored from 0 to 3 (0 = no staining, 1 = weak positive, 2 = moderate staining and 3 = strongly positive staining). All slides were viewed and scored by three blinded observers (Hegarty et al., 2001).

3.5. Nitric Oxide (NO_2^- and NO_3^-) Assay

Since most of NO is oxidized to nitrate (NO_3^-) and nitrite (NO_2^-), the concentration of these anions have been used as a quantitative measure of NO production (R&D system, USA). The assay involves the conversion of NO_3^- to NO_2^- by nitrate reductase. Prepare all reagents, working standard as directed in the handout. Serum samples were diluted 2-fold into Reaction Buffer (1x). The reaction is followed by a calorimetric detection of NO_2^- as on azo dye product of the Griess reaction. The Griess reaction is based on the two step diazotization reaction in which acidified NO_2^- produces a nitrosating agent which reacts with sulfanilic acid to produce the diazonium ion. This ion is then coupled to N-(1-naphthyl) ethylenediamine to form the chromophoric azo-derivative which absorbs light at 540 nm (all NO_2^- and NO_3^- assays were run in duplicate).

Calculation of results:

Create a standard curve by reducing the data using computer software (Anthos 2010 software version 1.7 with printer, Anthos labec instruments, Austria) capable of generating a linear curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance of each standard on the Y-axis against the endogenous nitrite or total nitrite concentration on the X-axis. Calculate the concentration of nitrite corresponding to the mean absorbance from the nitrite standard curve. To determine the concentration of nitrate in the sample as follow:

1. Measure the endogenous nitrite concentration (X μmol/L) using the nitrite assay.
2. Measure the total nitrite concentration (Y μmol/L) after the conversion of nitrate to nitrite using the nitrate reduction assay procedure.
3. Determine the nitrate concentration in the sample by subtracting the endogenous nitrite concentration from the total nitrite concentration.

$$\text{Nitrate concentration} = Y - X \ \mu\text{mol/L}$$

3.6. Morphologic Evaluation of Kidney

Renal tissue injury was assessed in tissue sections stained using the Periodic Acid-Schiff (PAS) reaction and Masson's trichrome technique. Sections were scored in a blinded semiquantitative manner. The numerical scores indicate the following: 0 = normal structure, 1 = areas of tubular epithelial cell swelling, vacuolar degeneration, necrosis, and desquamation involving less than 25% of cortical tubules, 2 = similar changes involving greater than 25% but less than 50% of cortical tubules, 3 = similar changes involving greater than 50% but less than 75% of cortical tubules, 4 = similar changes involving greater than 75% of cortical tubules, and 5 = complete cortical necrosis (Walker et al., 2000).

3.7. Calculation for Assessment of Renal Function

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

Urinary electrolyte excretion $= U_e \times V$

Fractional electrolyte excretion (FE_e) $= \frac{U_e V/P_e \times 100}{CCr}$

4. Statistical Analysis

All data were expressed as mean $\pm$ S.E. The results of rBF were presented in percent change of baseline and compare serial change by using ANOVA for repeated measurement. The results of blood and urine parameters were compared by using ANOVA. Probability values of less than 0.05 were considered to be statistically significant. The intensity scores of renal eNOS protein expression and renal pathological scores were present in descriptive statistics by measures of central tendency (Mode).

CHAPTER IV

RESULTS

1. Renal NOS Protein Expression

The expression of eNOS protein in renal tissues detected by immunohistochemical method is shown in Figures 4 - 6. In sham group (Figure 4A), the expression was occuring in both glomeruli and renal tubular epithelium. The more staining of eNOS protein was presented in cortex than that in the medulla. One day after 30-minute of IR caused a marked decrease of eNOS protein expression, especially in cortex (Figure 4B). The intensity score diminished from 3 to be 1 (Table 1). However, this alteration was less observed in seven-day duration after IR in that the score reduced to be 2 (Table 1 and Figure 4C).

The treatments with ACEI or ARA could ameliorate the loss of renal eNOS protein expression caused by IR in 1-day group (Figures 5C and 5D). The intensity score could restore to be 2 (Table 1). Interestingly, only the ACEI treated rats, for 7 days after IR, reduced the expression to be the score of 1 (Table 1 and Figure 6C).

In renal medulla area, the eNOS protein expression showed the comparable level in all groups studied (Table 1). Of interest, the right kidney (non-ischemia), demonstrated the eNOS protein expression in the same pattern as the IR kidney did (Table 1).

Regard to iNOS, the 30-minute IR condition in the present study did not influence the protein expression (data not shown).

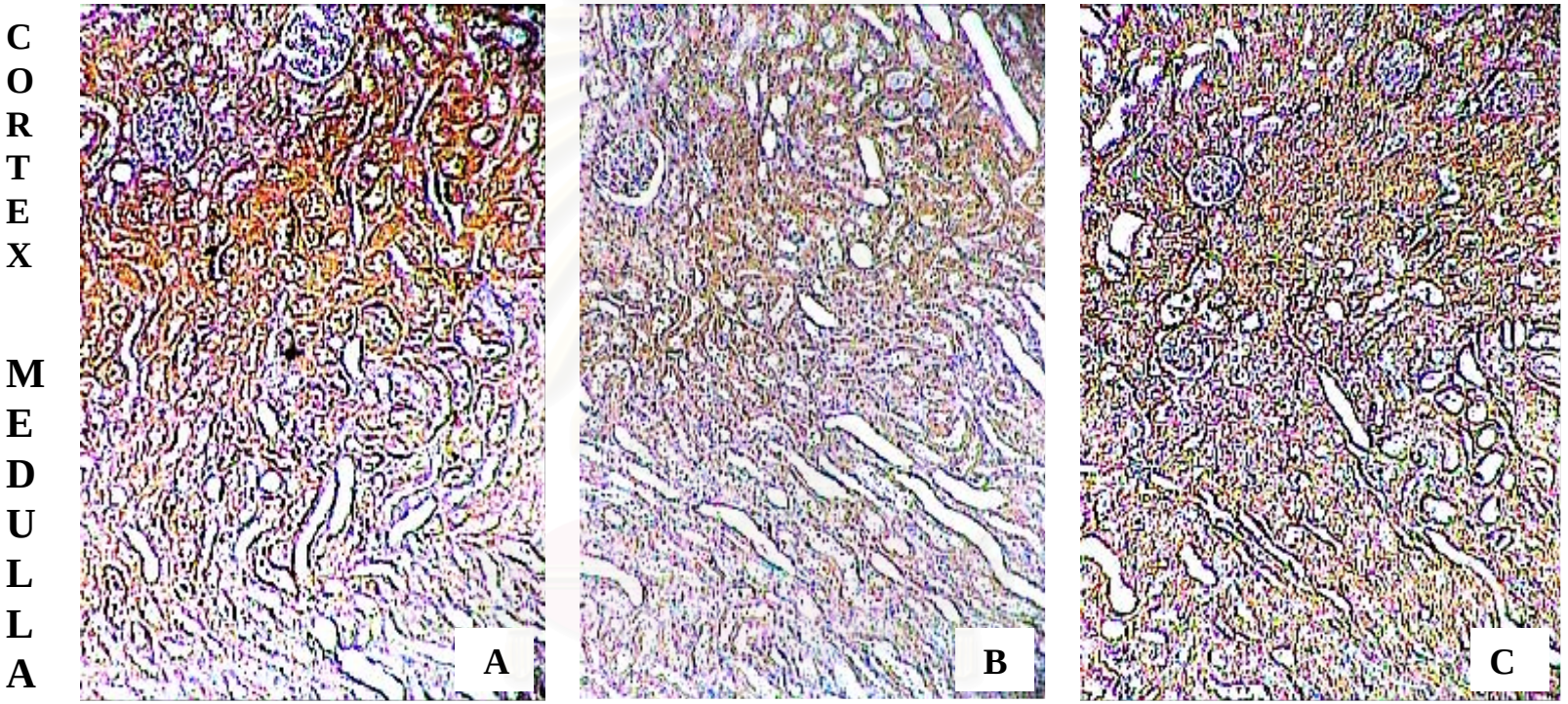


Figure 4 Immunohistochemical staining of renal eNOS protein expression. A: Sham, B: 1 day after IR, and C: 7 days after IR. Intensity scores are presented in parenthesis (C = cortex, M = medulla). Original magnification: 40X.

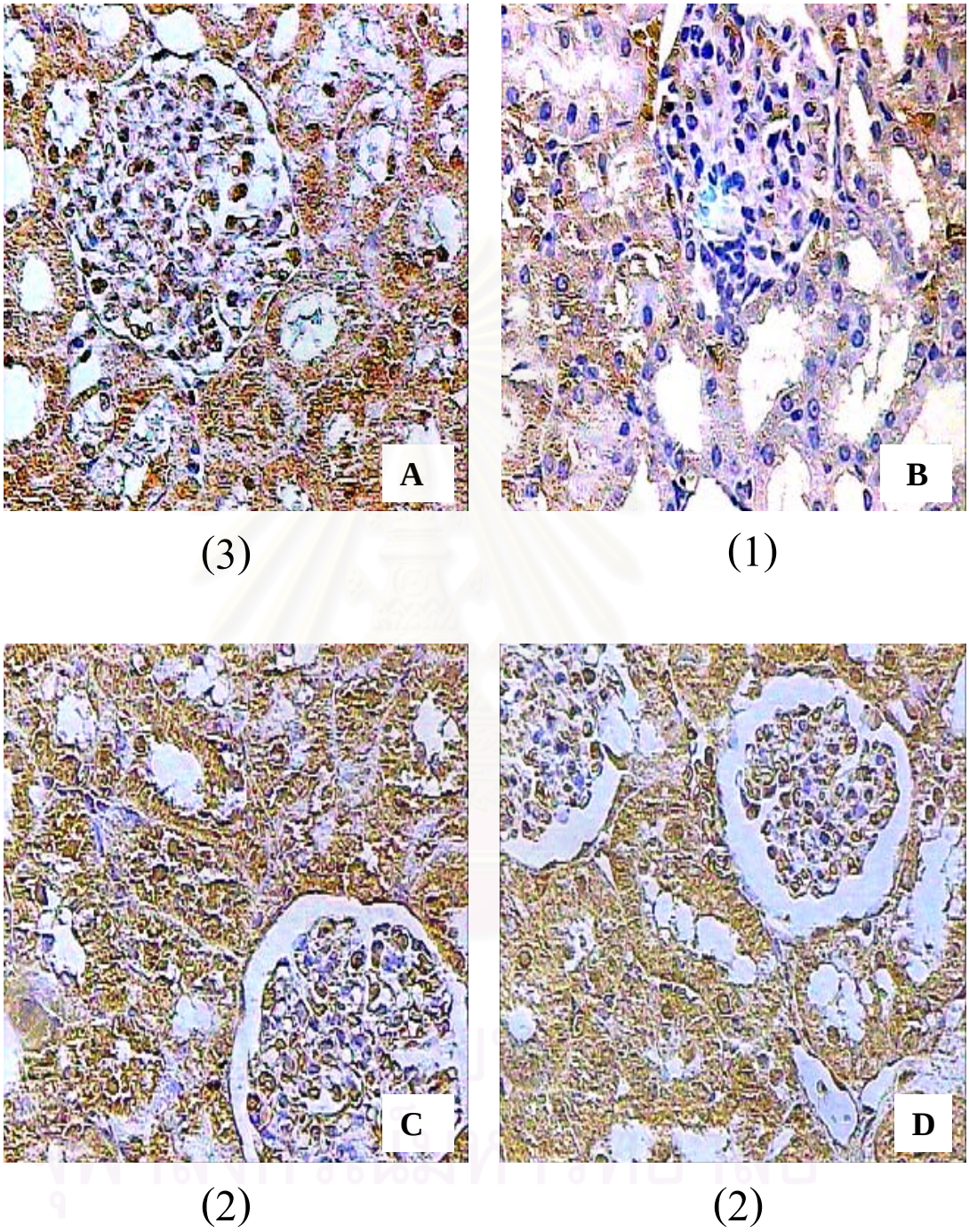


Figure 5 Immunohistochemical staining of renal eNOS protein expression in cortex from 1-day IR groups. A: Sham, B: IR, C: IR+ACEI, and D: IR+ARA. Intensity scores are presented in parenthesis. Original magnification: 200X.

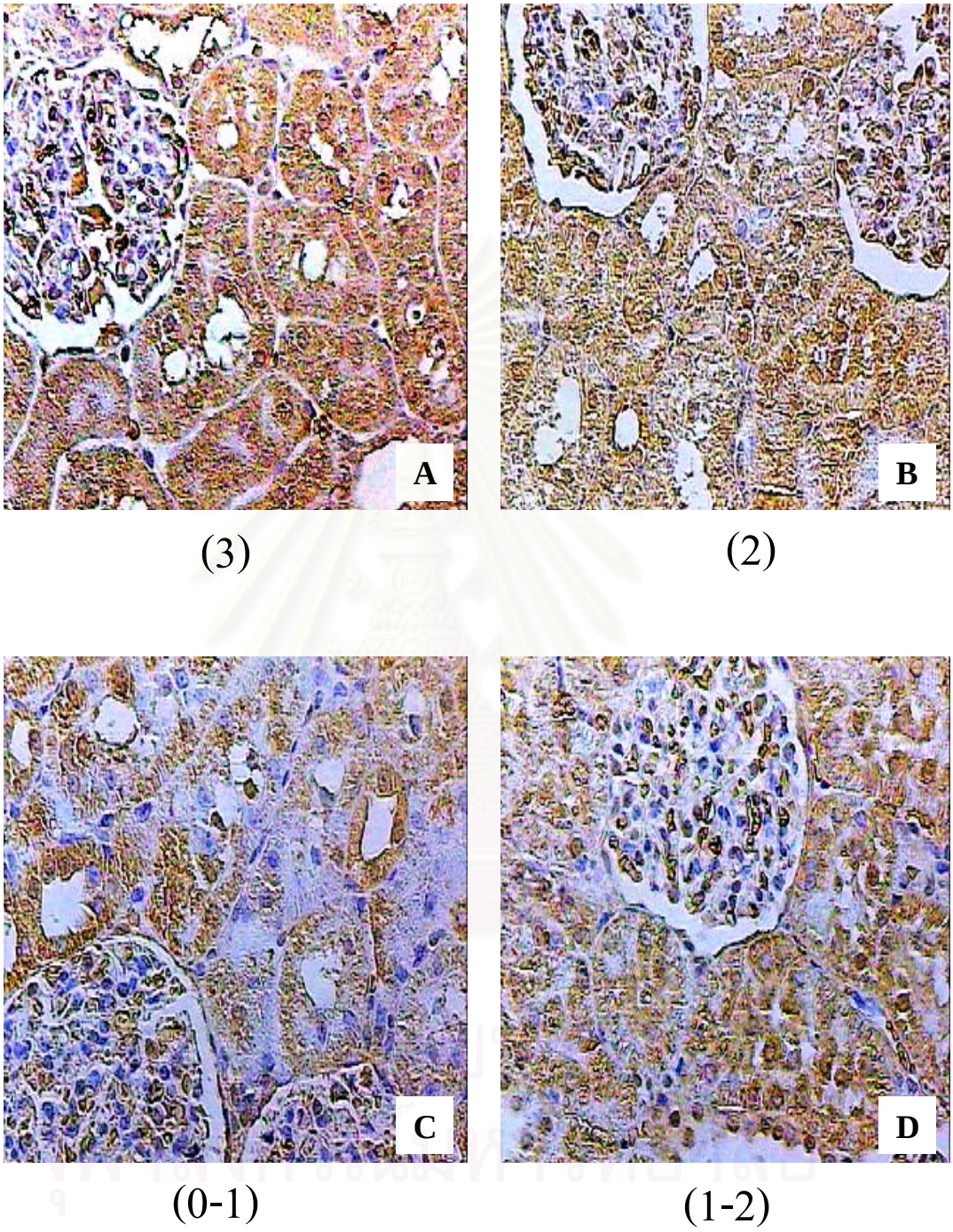


Figure 6 Immunohistochemical staining of renal eNOS protein expression in cortex from 7-day IR groups. A: Sham, B: IR, C: IR+ACEI, and D: IR+ARA. Intensity scores are presented in parenthesis. Original magnification: 200X.

Table 1 The intensity scores of renal eNOS protein expression in cortex and medulla from left (ischemic) and right (non-ischemic) kidney of rats in Sham, IR, IR+ACEI, and IR+ARA after 1 day or 7 days post IR.

Groups		**Duration period after IR**			
		1 day		7 days	
		Cortex	Medulla	Cortex	Medulla
Sham	Lt.	3	2	3	2 - 3
	Rt.	3	2	3	2
IR	Lt.	1	1 - 2	2	2
	Rt.	1	2	2	2
IR + ACEI	Lt.	2	2 - 3	0 - 1	1 - 2
	Rt.	2	2	1	1- 2
IR + ARA	Lt.	2	1 - 2	1 - 2	1 - 2
	Rt.	1 - 2	2	2	1 - 2

Renal NOS protein expression was assessed by immunohistochemistry. The intensity of NOS staining was scored from 0 to 3 (0 = no staining, 1 = weak positive, 2 = moderate staining, and 3 = strongly positive staining) (n = 4 - 5/group). All slides were viewed and scored by three blinded observers. (IR = renal ischemic reperfusion, ACEI = angiotensin converting enzyme inhibitor, ARA = angiotensin II receptor type 1 antagonist, Lt. = left kidney, Rt. = right kidney).

2. Nitric Oxide Production

The stable metabolite of NO, nitrite, was ultilized to assess NO production. As shown in Figure 7 (left panels), one day after IR, the serum nitrite concentration significantly rose from 18.26 $\pm$ 2.72 μmol/L in sham group to be 34.48 $\pm$ 2.72 μmol/L ($p < 0.01$). The treatment with ACEI could normalize the heightened nitrite level induced by IR to be that of sham animals. Moreover, the ARA-treated rats had a greater extent less concentration ($p < 0.001$) as compared with IR group. However, after 7 days post IR, serum nitrite concentration in all groups was comparable and not significant difference from the 1-day sham animals (Figure 7, right panels).

3. Histopathological Study

The pathological study in renal tissue was defined by histological examination and shown in Figures 8 and 9. One day after 30-minute of IR, the kidney showed mild to moderate dilatation (scores 2 - 3; Table 2) of cortical tubule with few cast formation and mild brush border membrane loss (Figure 8B). However, there was no cell infiltration or fibrosis. Slight changes were noted in medulla area. The treatments with ACEI or ARA could attenuate those structural damages (Figures 9C and 9D, respectively).

In the longer period, 7 days post IR, all groups studied demonstrated normal structure of renal tissue. As predicted, the right kidney (non-ischemic) of rats undergone IR had normal histological study both 1 day and 7 days after IR (Figure 8C).

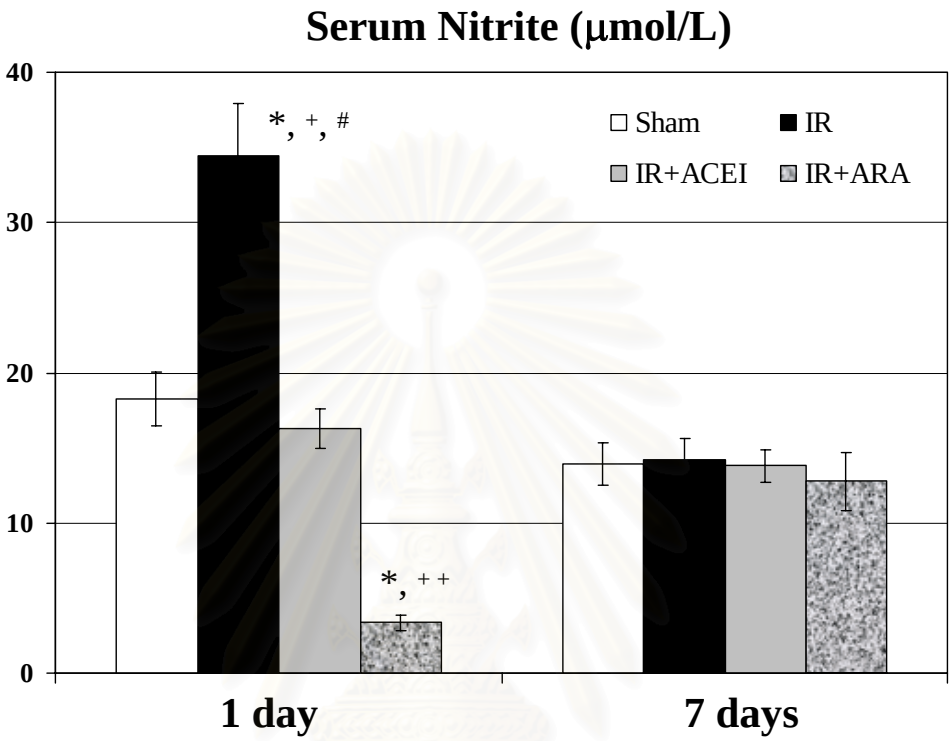


Figure 7 Serum level of nitrite concentration in Sham, IR, IR+ACEI and IR+ARA groups from 1 day and 7 days after IR (n = 6 ± 1/group). * $p < 0.01$ vs Sham; $^{+}$ $p < 0.001$ vs IR+ACEI; $^{\#}$ $p < 0.001$ vs IR+ARA; $^{++}$ $p < 0.05$ vs IR+ACEI.

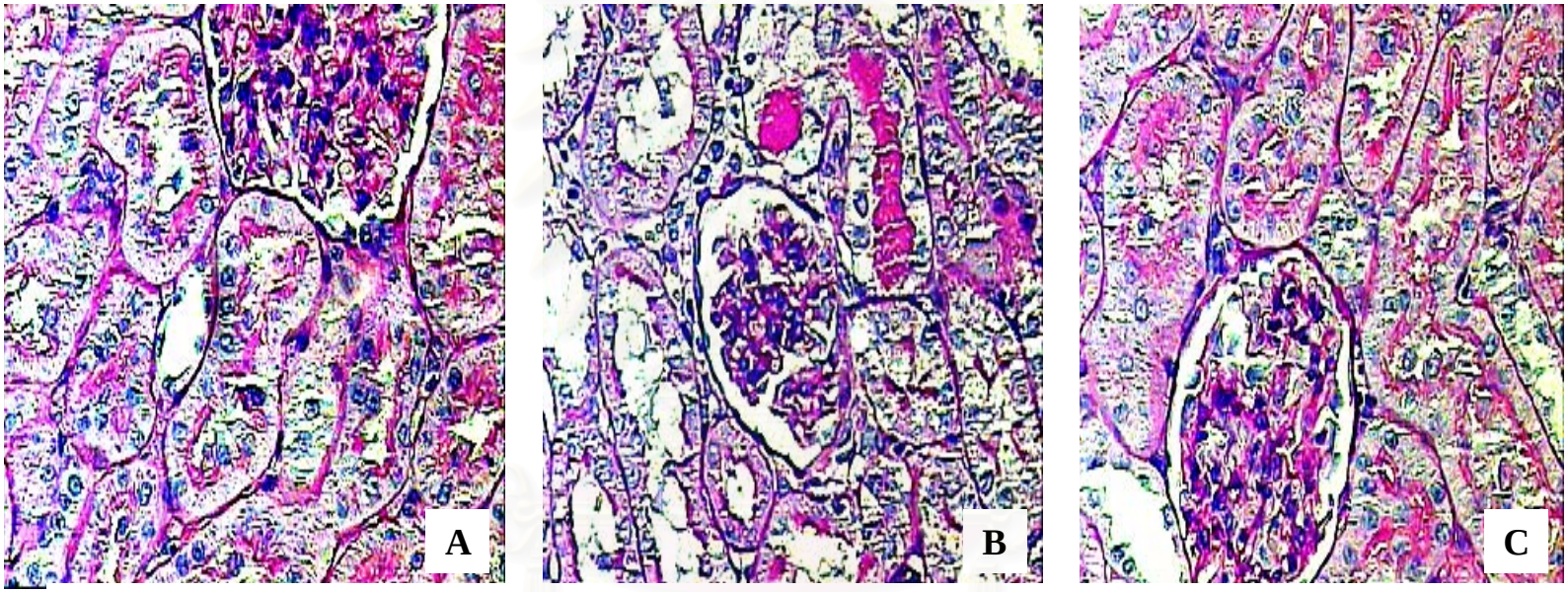


Figure 8 Representative PAS-stained tissue sections of renal cortex. A: Sham, B: 1 day after IR: left (ischemic) kidney, and C: 1 day after IR: right (non-ischemic) kidney. Pathological scores are presented in parenthesis. Original magnification: 200X.

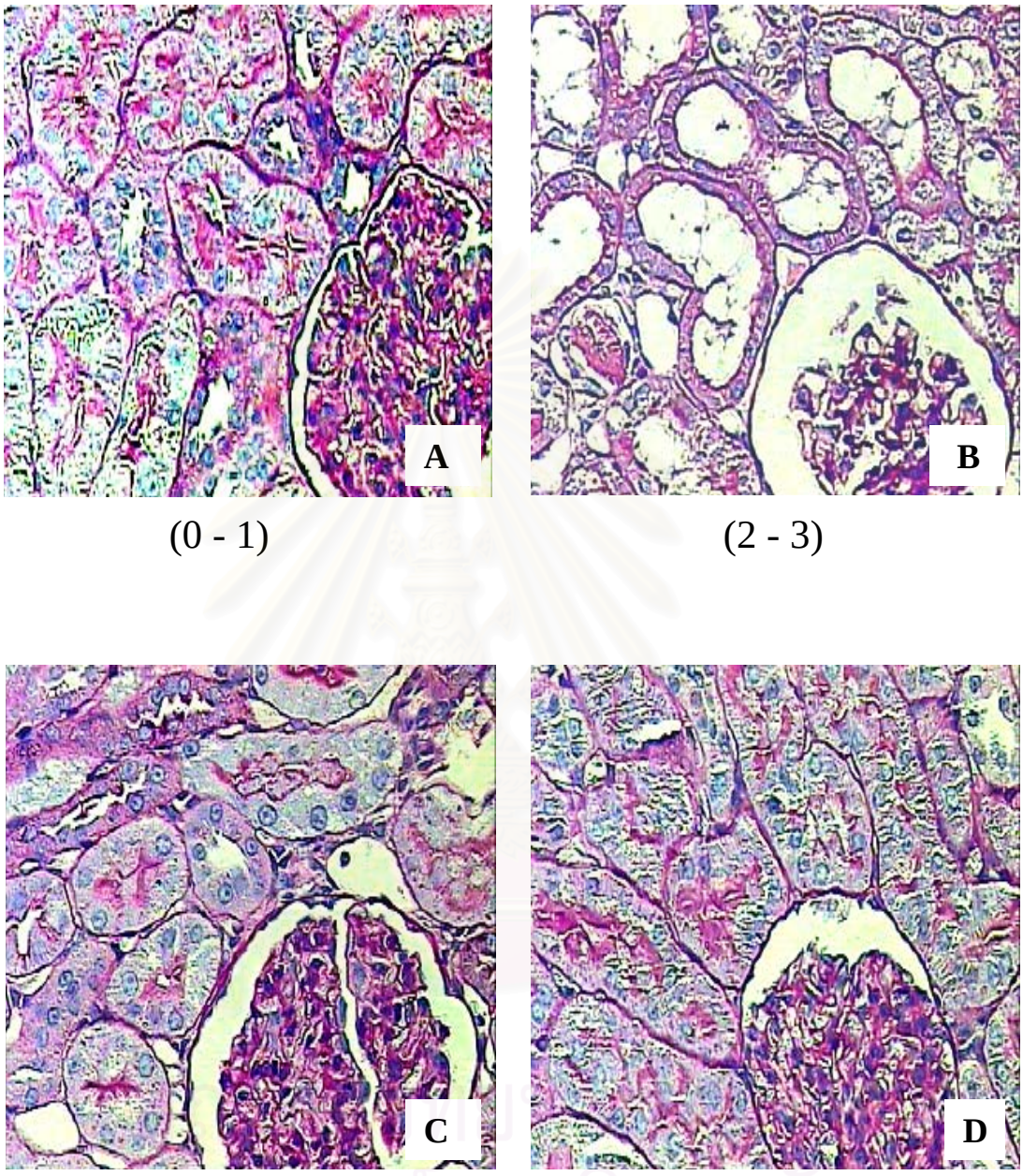


Figure 9 Representative PAS-stained tissue sections in renal cortex of left (ischemic) kidney from 1 day after IR groups. A: Sham, B: IR, C: IR+ACEI, and D: IR+ARA. Pathological scores are presented in parenthesis. Original magnification: 200X.

Table 2 The pathological scores in renal cortex and medulla from left (ischemic) and right (non-ischemic) kidney of rats in Sham, IR, IR+ACEI, and IR+ARA after 1 day or 7 days post IR.

Groups		**Duration period after IR**			
		1 day		7 days	
		Cortex	Medulla	Cortex	Medulla
Sham	Lt.	0 - 1	0 - 1	0 - 1	0 - 1
	Rt.	0 - 1	0 - 1	0 - 1	0 - 1
IR	Lt.	2 - 3	1 - 2	1	0 - 1
	Rt.	0 - 1	0 - 1	0 - 1	0 - 1
IR + ACEI	Lt.	1	1	0 - 1	0 - 1
	Rt.	0 - 1	0 - 1	0 - 1	0 - 1
IR + ARA	Lt.	0 - 1	1	0 - 1	0 - 1
	Rt.	0 - 1	0 - 1	0 - 1	0 - 1

Renal tissue injury was assessed in PAS and Masson's trichrome technique stained tissue sections. Sections were scored in a blinded, semiquantitative manner. (n = 4 – 5/group) The numerical scores indicate the following: 0 = normal structure; 1 = areas of tubular epithelial cell swelling, vacuolar degeneration, necrosis, and desquamation involving less than 25% of cortical tubules; 2 = similar changes involving greater than 25% but less than 50% of cortical tubules; 3 = similar changes involving greater than 50% but less than 75% of cortical tubules; 4 = similar changes involving greater than 75% of cortical tubules, and 5 = complete cortical necrosis. (IR = renal ischemic reperfusion, ACEI = angiotensin converting enzyme inhibitor, ARA = angiotensin II receptor type 1 antagonist, Lt. = left kidney, Rt. = right kidney).

4. Regional Renal Blood Flow

The percent of changes in regional renal blood flow (rBF) was measured before, during and after ischemia from both kidneys. The results are presented in Figures 10 [from left (ischemic) kidney] and 11 [from right (non-ischemic) kidney]. Clamping the left renal pedicle of rats decreased rBF approximately 80 to 85% of the basal level measured before (Figure 10, $p < 0.001$). This level was maintained throughout the 30-minute occlusion. After removal of the clamps, the rBF rapidly returned to the basal value within 1 minute. However, the flow progressively reduced to be around 50% at the 5^{th} minute ($p < 0.001$) and then restored to be 75 to 80% of basal level at the 10^{th} minute and maintained (Figure 10). Interestingly, the rBF of rats from either 1-day or 7-day group, completely returned to basal level. Moreover, the three groups of rats subjected to IR (treated with ACEI or ARA) demonstrated the same pattern of changes in rBF.

Regard to the right kidney (non-ischemic), the rBF slightly reduced during IR; however, it value was not significant (Figure 11, $p = 0.1$). Although the rBF tended to diminish within 1 minute after release the clamps ($p = 1$), the value then progressively returned to the baseline level within 10 minute and maintained. After 1 day or 7 days of IR, the rBF in the right kidney still showed a comparable degree as that in the sham group. Again, the treatments with ACEI and ARA had no influence on this alteration (Figure 11).

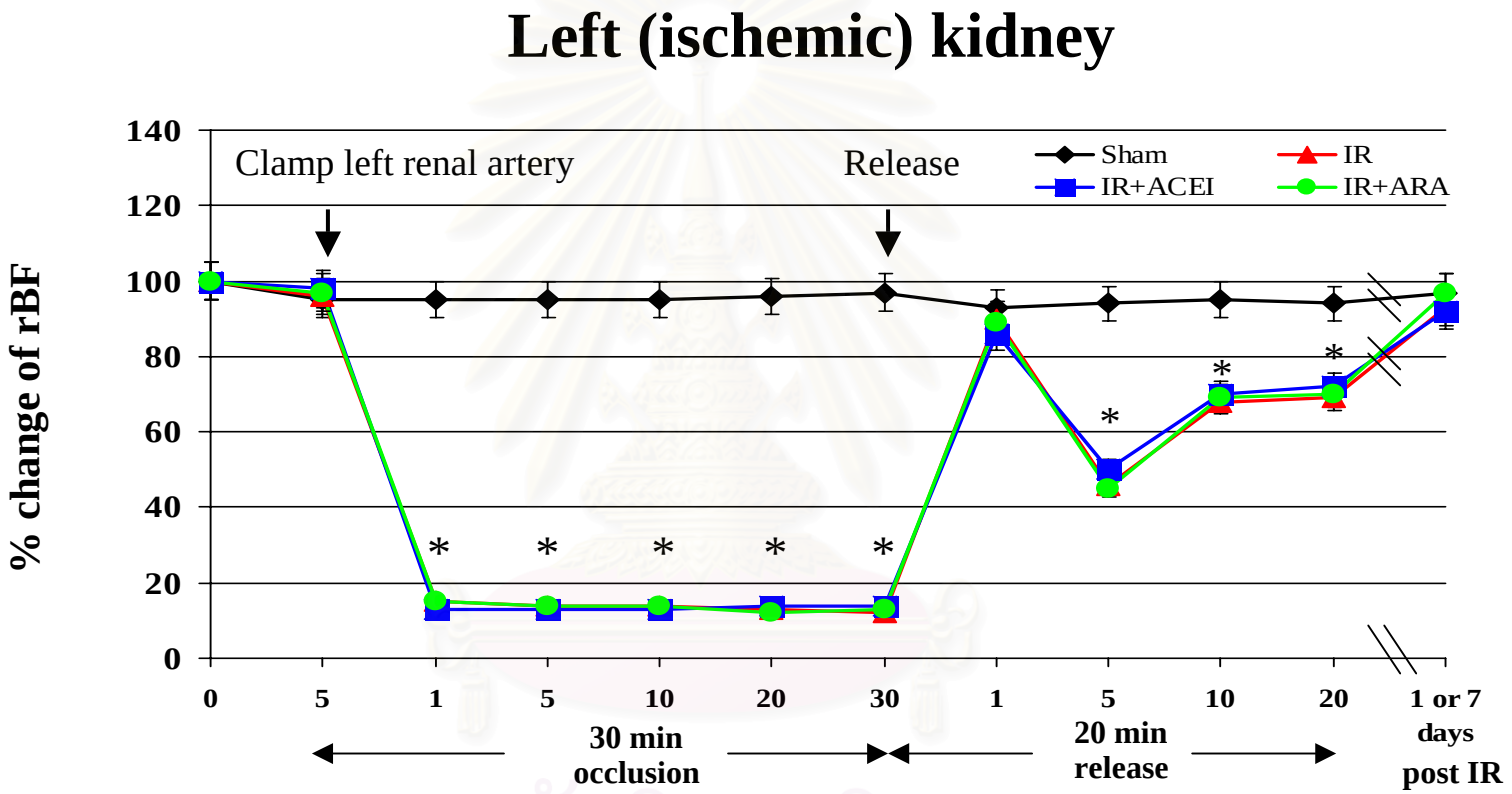


Figure 10 The percent changes of rBF in left (ischemic) kidney from Sham, IR, IR+ACEI, and IR+ARA rats (n = 8 $\pm$ 1/group, * $p < 0.001$ compared with baseline).

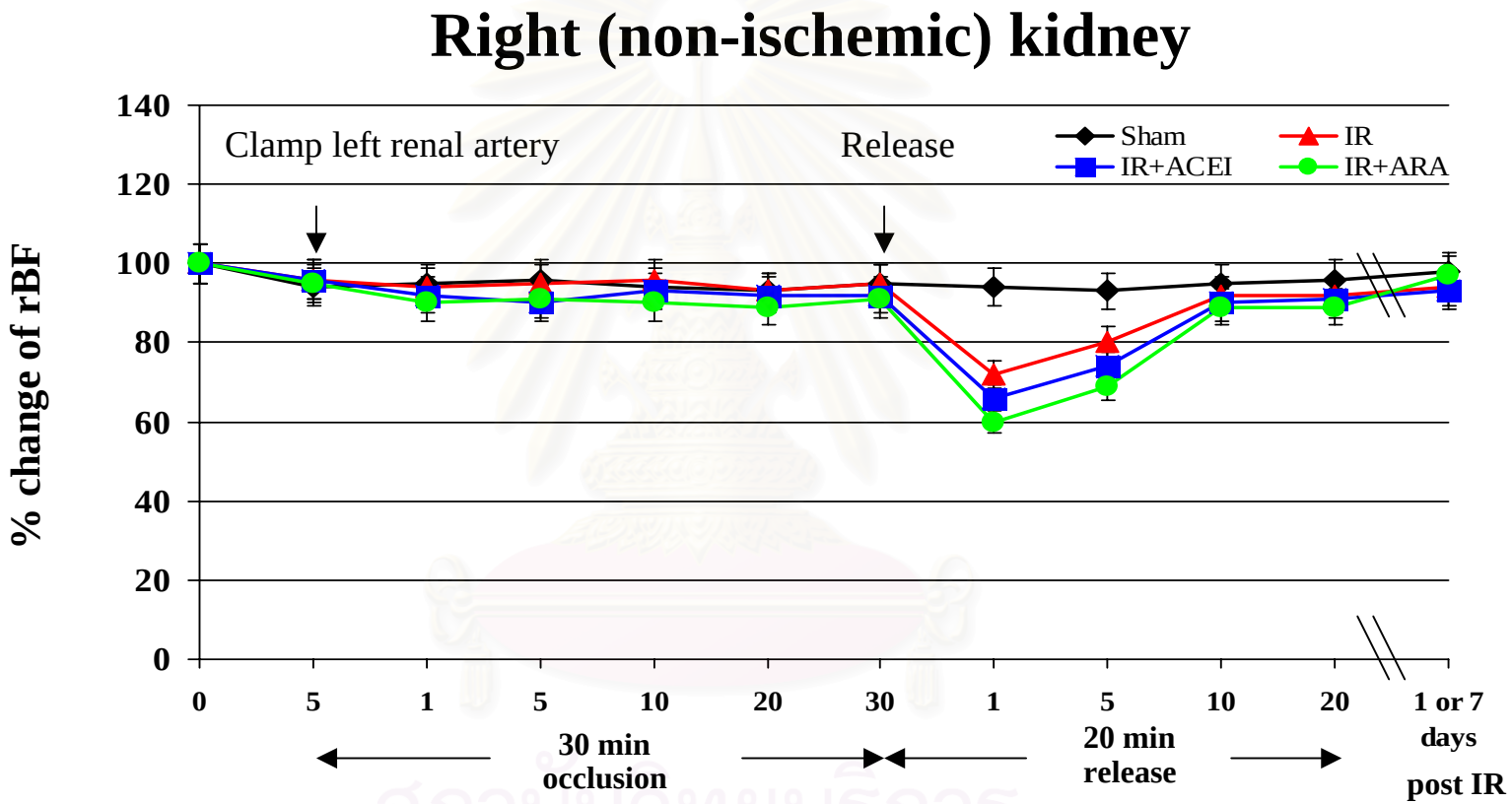


Figure 11 The percent changes of rBF in right (non-ischemic) kidney from Sham, IR, IR+ACEI, and IR+ARA rats (n = 8 $\pm$ 1/group).

5. Fractional Excretion of Electrolytes

One day after IR, the fractional excretion of Na^+ (FE_{Na^+}) markedly diminished approximately 10 times ($p < 0.001$, Figure 12A: left panels). Interestingly, only the treatment with ARA could restore the value to be near that of the sham animals. The FE_{K^+} tended to decrease but not significantly whereas the FE_{Cl^-} did not change during IR (Figure 12B and 12C: left panels, respectively). The ARA-treated animals demonstrated comparable values of FE_{K^+} and FE_{Cl^-} as did in the sham group. Although the ACEI treatment slightly reduced these two FE levels, the differences were not significant. By contrast, in 7-day IR groups, both treatments could restore all FE values back to those of sham levels (Figure 12: right panels).

6. Metabolic Parameters in Serum and Renal Function

Serum concentrations of Na^+, K^+, Cl^-, Cr and BUN in all groups studied showed comparable levels (Table 3). As predicted, creatinine clearance and urine flow rate (Table 4) were not significantly altered by IR or treatment with either ACEI or ARA (Table 4). All these parameters from 1-day study demonstrated the same values as observed from 7-day experiment.

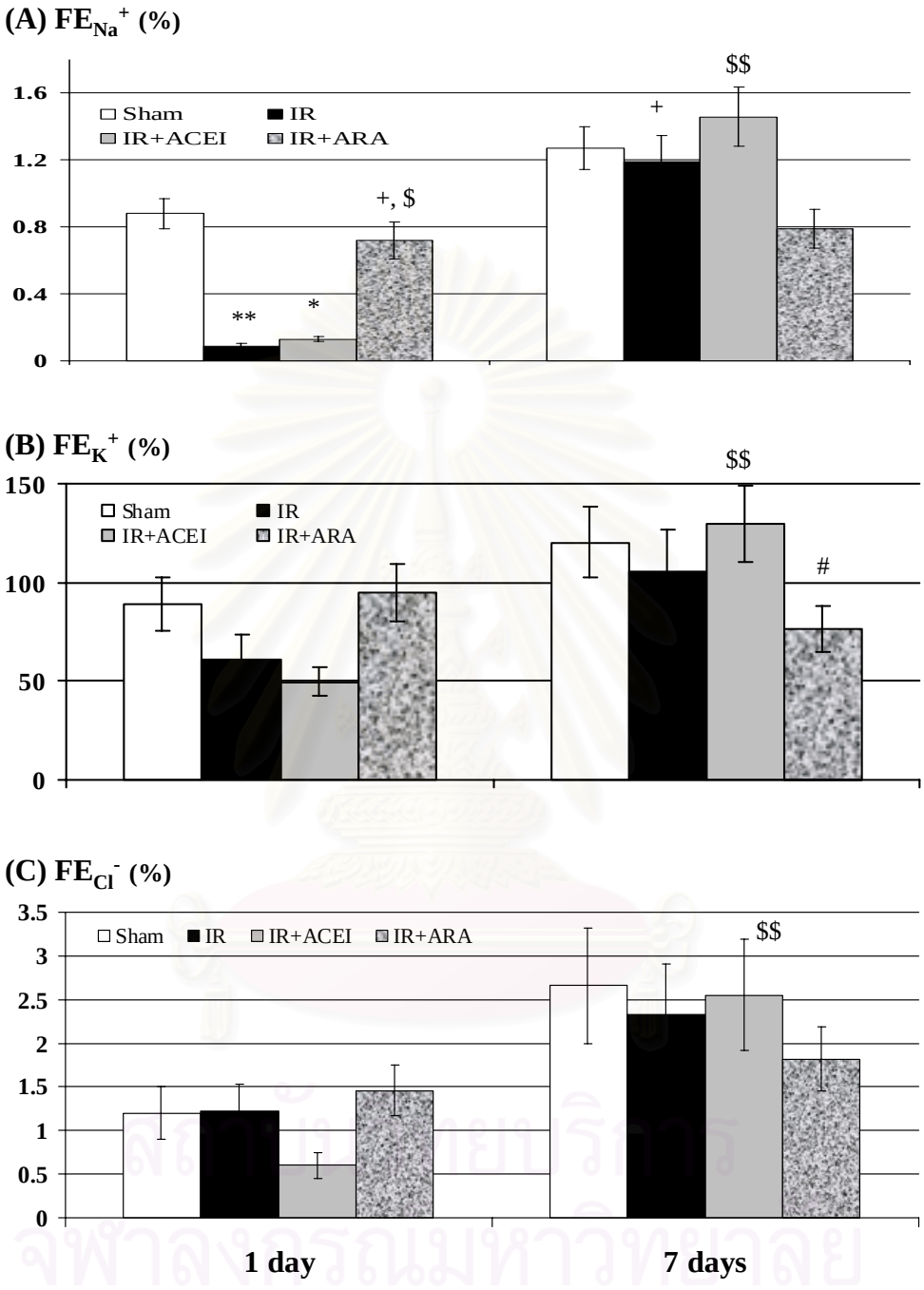


Figure 12 Fractional excretion of Na^+, K^+, and Cl^- in Sham, IR, IR+ACEI, and IR+ARA groups from 1 day and 7 days after IR (n = 8±1/ group, * $p < 0.01$, ** $p < 0.001$ vs Sham; $^{+}$ $p < 0.0$ vs 1-day IR; $^{\$}$ $p < 0.01$, $^{\$\$}$ $p < 0.001$ vs 1-day ACEI; $^{\#}$ $p < 0.05$ vs 7-day ACEI;).

Table 3 Serum levels of Na^+, K^+, Cl^-, Cr and BUN in Sham, IR, IR+ACEI, and IR+ARA rats on day 1 or day 7 after IR (Mean ± S. E.; n = 8 ± 1/group).

Groups	Na^+ (mmol/L)		K^+ (mmol/L)		Cl^- (mmol/L)		Creatinine (mg%)		BUN (mg%)	
	Duration period after IR		Duration period after IR		Duration period after IR		Duration period after IR		Duration period after IR	
	1 day	7 days	1 day	7 days	1 day	7 days	1 day	7 days	1 day	7 days
Sham	140 ± 1.47	143 ± 1.47	3.93 ± 0.23	4.13 ± 0.23	101 ± 1.63	103 ± 1.63	0.57 ± 0.05	0.60 ± 0.05	25.77 ± 2.68	26.72 ± 2.76
IR	135 ± 1.04	142 ± 1.04	4.08 ± 0.16	3.95 ± 0.16	100 ± 1.16	102 ± 1.16	0.51 ± 0.04	0.52 ± 0.04	23.37 ± 1.89	26.70 ± 1.91
IR + ACEI	134 ± 1.04	138 ± 1.04	4.35 ± 0.16	4.41 ± 0.16	97 ± 1.16	103 ± 1.16	0.45 + 0.04	0.62 ± 0.04	25.30 ± 1.89	34.01 ± 1.91
IR + ARA	139 ± 1.04	142 ± 1.04	4.01 ± 0.16	4.25 ± 0.16	102 ± 1.16	105 ± 1.16	0.64 ± 0.04	0.56 ± 0.04	27.44 ± 1.89	23.80 ± 2.00

(IR = renal ischemic reperfusion, ACEI = angiotensin converting enzyme inhibitor, ARA = angiotensin II receptor type 1 antagonist)

Table 4 Creatinine clearance and urine flow rate in Sham, IR, IR+ACEI, and IR+ARA rats on day 1 or day 7 after IR (Mean ± S. E.; n = 8 ±1/ group)

Groups	**Creatinine clearance** (ml/min/ 100 g BW)		**Urine flow rate** (ml/min)	
	Duration period after IR		Duration period after IR	
	1 day	7 days	1 day	7 days
Sham	0.70 ± 0.11	0.57 ± 0.11	0.021 ± 0.004	0.026 ± 0.004
IR	0.58 ± 0.08	0.74 ± 0.08	0.021 ± 0.003	0.03 ± 0.003
IR + ACEI	0.80 ± 0.08	0.53 ± 0.08	0.022 ± 0.003	0.026 ± 0.003
IR + ARA	0.47 ± 0.08	0.69 ± 0.08	0.022 ± 0.003	0.019 ± 0.003

(IR = renal ischemic reperfusion, ACEI = angiotensin converting enzyme inhibitor, ARA = angiotensin II receptor type 1 antagonist, BW = body weight)

CHAPTER V

DISCUSSION AND CONCLUSION

It has long been known that eNOS is constitutively expressed by endothelial lining in blood vessels (Lane and Gross, 1999; Marsden et al., 1996). Of interest, although the expression of eNOS protein in renal tubule segments has not yet well been reported, the eNOS-like immunoreactivity has been observed in the porcine kidney epithelial cell line (LLC-PK1) (Tracey et al., 1994). The present study is the first in vivo evidence demonstrated that eNOS protein is well expressed constitutively in cytoplasmic area of renal tubular epithelial cells. One day after IR, the expression diminished markedly, especially in the cortical region (Figure 4). The explanation for this alteration may involve in various mechanisms as follow:

The eNOS protein expression is regulated by hypoxia (Liao et al., 1995; McQuillan et al., 1994). Several in vitro studies have shown that hypoxia decreases eNOS expression in human umbilical vein endothelial cells (McQuillan et al., 1994), in human pulmonary artery endothelial cells (Ziesche et al., 1996), and in bovine pulmonary artery endothelial cells (Liao et al., 1995). The mechanisms by which hypoxia inhibited eNOS expression involve in both transcriptional and posttranscriptional processes (McQuillan et al., 1994). In addition, the in vivo study of IR (Kakoki et al., 2000) demonstrated that the dimeric (active) form of eNOS, from renal tissue homogenate, decreased in the ischemic kidney due to depletion of tetrahydrobiopterin (BH_4). BH_4 is a cofactor of all isoforms of NOS to form

dimerization (Barbara et al., 1997; Berka and Tsai, 2000; Crespo et al., 1996; Venema et al., 1997). Furthermore, BH_4 has a direct antioxidant effect and reduces reactive oxygen species occurred during reperfusion (Wever et al., 1997). It has been demonstrated that, upon renal artery occlusion, NO release rapidly increased in renal cortex (within 5 minutes), and it reached a plateau (a 13-fold increase on the basal level) approximately 10 to 15 minutes later (Saito and Miyagawa, 2000). It could be postulate that the available BH_4 becomes lesser at the same rate increment of NO production. This indicates that BH_4 depletion occurs not only in reperfusion period but also in ischemic status. An administration of BH_4 to rats before IR could restore NOS activity and eNOS protein expression (Kakoki et al., 2000).

Hypoxia also has an additionally indirect effect on eNOS expression. In primary cultures of renal proximal tubular epithelial cells prepared from human kidneys, hypoxia for 60 minutes increased osteopontin (OPN) expression (Hwang et al., 1994a). The stimulation of OPN synthesis involves in PKC and p38 MAPK pathways (Sodhi et al., 2001). OPN is a secreted, Arg-Gly-Asp-containing phosphoprotein expressed at high level in the kidney (Verhulst et al., 2002; Xie et al., 2001). OPN is mainly present in the loop of Henle and distal nephrons in normal kidneys in animals and humans (Xie et al., 2001). After renal damage, OPN expression may be significantly up-regulated in all tubule segments and glomeruli (Xie et al., 2001). This upregulation of OPN is mediated by angiotensin II (Hwang et al., 1994b). It has been noted that OPN inhibits iNOS expression in primary mouse kidney proximal tubular epithelial cells (Yu et al., 2000) as well as decreases eNOS abundance in human kidney proximal tubular epithelial cells (Hwang et al., 1994a). Therefore, the

induction of OPN during IR may be another reason for reduction of eNOS expression in the present study.

The present findings show that, in 1 day after IR, rats treated with ACEI or ARA could restore the eNOS expression. It has been demonstrated that IR causes an increase of ANG II level both in renal tissues and blood (Kontogiannis and Burns, 1998; Vargas et al., 1994). ANG II increases O_2^- production (Berry et al., 2000; Griendling et al., 1994; Jaimes et al., 1998), which in turn binds to BH_4 and then decreases eNOS active form. Therefore, blockade of angiotensin system could reduce O_2^- generation and subsequently maintain BH_4 concentration being available for dimerization of eNOS protein. Moreover, ACEI itself is an antioxidant which could scavenge many oxidative stresses mediated via ROS induced during IR (Levens et al., 1992). However, the long-term (7 days) treatment of ACEI in the present study slightly suppressed eNOS expression. This may result from a greater extent in reduction of shear stress (Ito et al., 1991) in that ACEI could diminish not only ANG II level (vasoconstrictor) but also raise bradykinin concentration (vasodilator) (Levens et al., 1992). Thus, the need of product from eNOS is modest, and then is subsequently down-regulated. All together, these results indicate that renin angiotensin system plays an essential role in renal eNOS protein expression.

Beside the effect of IR on eNOS expression, many investigators reported that IR causes increased iNOS mRNA and protein expression (Ling et al., 1999; Noiri et al., 1996). iNOS could produces a large continuous flux of NO (Walker et al., 2000). The excessive NO interacts with O_2^- to generate $ONOO^-$ (Zbou et al., 2001). $ONOO^-$ is a potent and

versatile oxidant which can react with lipids, proteins and DNA (Pryor and Squadrito, 1995). These reactions have been used for explanation many cytotoxic actions of NO and O_2^- induced by IR (Walker et al., 2000). The transcriptional upregulation of iNOS gene occurs in response to various inflammatory cytokines. However, at present study, renal iNOS protein expression was not detected in any groups of rats (data not shown). These are accompanies with some authors who indicated that hypoxia fails to induce iNOS expression both in vitro (Archer et al., 1995) and in vivo (Gess et al., 1997; Kakoki et al., 2000). It is possible that IR induced iNOS expression depending on the duration of ischemia. Most previous studies were performed with 45 to 60-minute occlusion in uninephrectomized rats, but the present study was performed only 30 minutes in intacted kidney animals. In addition, although the ischemic kidney showed a mild to moderate of cortical tubule damage, there was no cell infiltration or fibrosis. Therefore, no sources of iNOS could be detected.

Regards to NO production, as mentioned earlier, NO releases from renal tissue immediately upon ischemia; however, the level returns to the baseline after reperfusion (Saito and Miyagawa, 2000). On the other hand, blood NO concentration in IR remains controversial. Some studies have demonstrated a lower level of NO after IR (Waz et al., 1998) but the other revealed an increase in serum NO (Ozer et al., 2001). The present study also shows that, 1 day post IR, serum NO significantly rose almost double. This may be the result from the secondary consequences of ANG II-triggered NO release (Henington et al., 1998; Saito et al., 1996). The inhibition of rennin angiotensin system by ACEI or ARA could attenuate the heightened level caused by IR. Again, this confirms that the angiotensin system performs a linkage relationship to the nitric oxide system.

The present results shows that FE_{Na^+} decreased markedly in 1 day after IR. This may be the influence of ANG II and NO on Na^+ transport. ANG II could potentiate Na^+ reabsorption via activation Na^+-K^+ ATPase activity (Gunning et al., 1996), whereas NO stimulates Na^+ transport via Na^+-HCO_3^- cotransporter (Wang, 1997), and via Na^+ channel (Ortiz and Garvin, 2002).

Of interest, the alterations occurred in 1 day after IR, both structural and functional, returned to the comparable levels with those of the sham within 7 days. This may be a beneficial supporting report in that the 30-minute ischemia is a safe period applying in renal transplantation. Several studies have demonstrated that a prolonged ischemic time is the major cause of renal dysfunction and graft rejection (Dragun et al., 2001; Kouwenhoven et al., 1999; Lee et al., 2000; Sammut et al., 2000).

In conclusion, the present data are the first evidence of IR model in that the rennin angiotensin system plays a crucial role in regulation of renal eNOS protein expression, NO production as well as electrolyte excretion.

The proposed mechanisms that IR reduces renal eNOS protein expression may involve in various mechanisms show in Figure 13 as follow:

As mentioned earlier, NO production is immediately released from renal tissue upon ischemia, corresponding with the reduction of renal eNOS protein (eNOS was used up for NO production). The reduction of renal eNOS protein may associate with a decrease of BH_4, a cofactor of all isoforms of NOS to form dimerization (NOS active form). In addition, IR causes an increase both $O_2^{\bullet}$ and ANG II. ANG II also increases both $O_2^{\bullet}$ and OPN. $O_2^{\bullet}$ is a oxidative stress which binds to BH_4 and then decreases eNOS active form. ANG II stimulates OPN synthesis via PKC and p38 MAPK pathways. OPN suppresses renal eNOS protein expression through inhibition of NOS mRNA synthesis. All of these mechanisms could involve either directly or indirectly in a reduction of renal eNOS protein expression caused by ischemia. The present study shows that blockadge of rennin angiotensin system by ACEI or ARA could restore the renal eNOS protein expression via suppressing of ANG II level, and then decreasing $O_2^{\bullet}$ and OPN production.

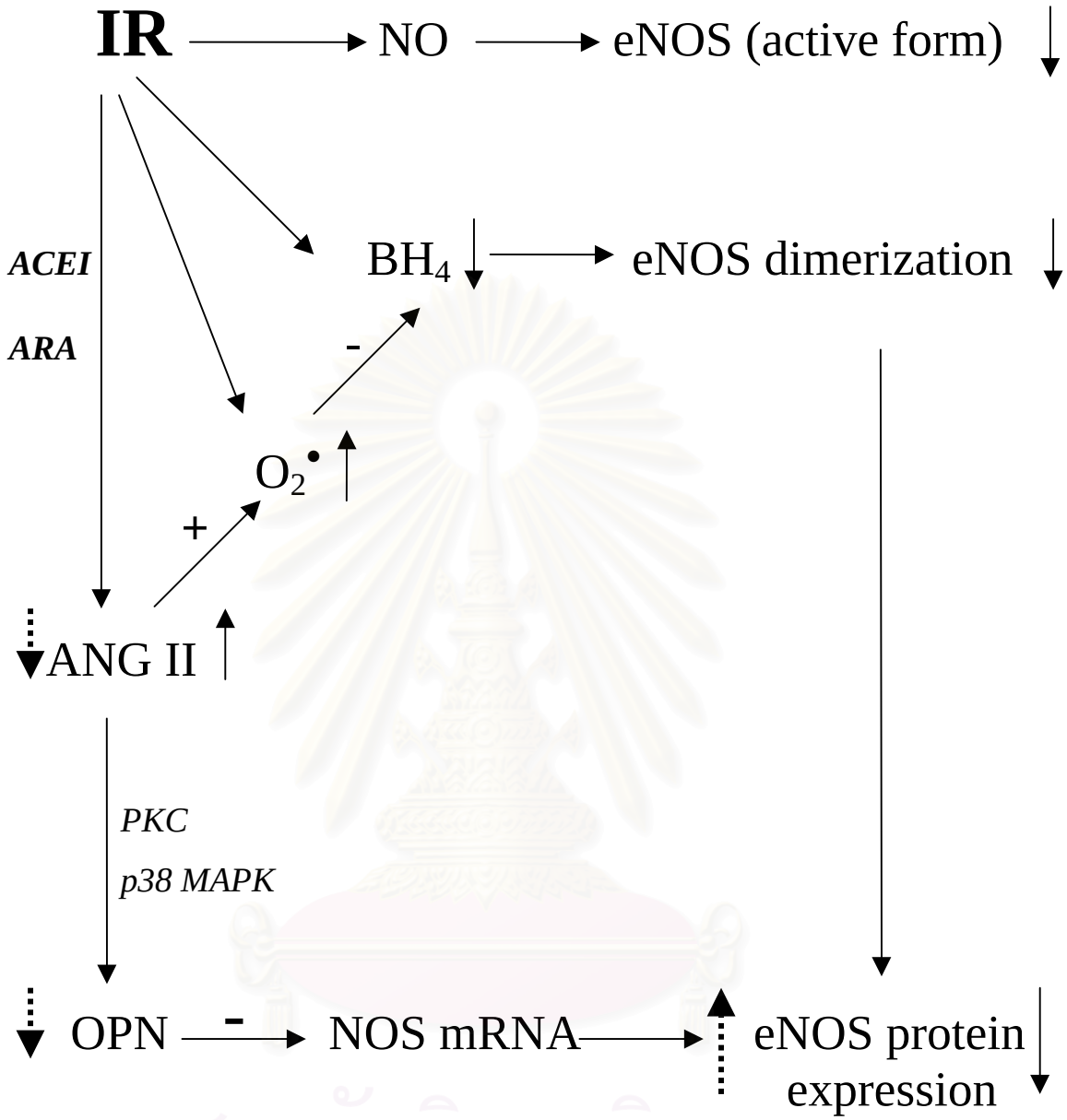


Figure 13 The proposed mechanisms that IR reduces eNOS protein expression in renal epithelial cells.

Note, ····· Refers to IR treated with ACEI or ARA.

(IR = renal ishemic reperfusion, NO = nitric oxide, eNOS = endothelial nitric oxide synthase, ACEI = angiotensin converting enzyme inhibitor, ARA = angiotensin II receptor type 1 antagonist, BH_4 = tetrahydrobiopterin, $O_2^{\bullet}$ = superoxide, ANG II = angiotensin II, OPN = osteopontin, PKC = protein kinase C, MAPK = mitogen-activated protein kinase)

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APPENDIX

APPENDIX

Buffer and Reagent for Immunohistochemistry

1. Buffer solution preparation

0.2 M Na-KPB pH 7.4

Na_2HPO_4 = 0.162 M = 22.98 gm / H_2O

KH_2PO_4 = 0.038 M = 5.16 gm / 1000 ml

0.1 M Na-KPB pH 7.4

0.2 M Na-KPB = 1000 ml

H_2O = 1000 ml

0.1 M Na-KPB (saline) pH 7.4

0.1 M Na-KPB = 100 ml

NaCl = 0.8 gm

0.05 M Tris-HCl pH 7.6

Dissolve Tris 6.1 gm in H_2O = 800 ml

Add 5 N HCl until pH 7.6

Add H_2O until = 1000 ml

4% Paraformaldehyde in 0.1 M Na-KPB pH 7.4 : 100 ml

Paraformaldehyde = 4 gm

Add H_2O = 50 ml (in hood)

Heat at 60°C and stir

Add 1N NaOH until clear in color

Add 0.2 M Na-KPB = 50 ml

PBS-A (0.3 % Triton X-100, 1% BSA)

PBS	=	100 ml
BSA	=	1 gm
Triton X-100	=	0.3 ml

PBS-B (0.1 % Triton X-100, 0.25% BSA)

PBS	=	100 ml
BSA	=	0.25 gm
Triton X-100	=	0.1 ml

2. Stock DAB in Tris

Dissolve DAB 60 mg / Tris 12 ml

Filter

Pipette 1 ml into polypropylene tube (about 10 tubes then freeze in refrigerator).

3. Working DAB (0.05% DAB in Tris) 0.01% H_2O_2

- Stock DAB 1 tube + Tris 9 ml
- Add 3.3 μl of 30% H_2O_2

Sample and Reagent Preparation for Nitrate and Nitrite Assay

Sample preparation

All sample require at least a 2-fold dilution into reaction buffer (1x). After dilution, samples must be ultrafiltered through a 10,000 molecular weight cutoff filter to eliminate proteins.

Reagent preparation

1. *Reaction buffer concentration (1x)*

   Dilute 30 ml of reaction buffer concentration (10x) into distilled water to prepare 300 ml of reaction buffer (1x).

2. *NADH reagent*

   Reconstitute, the NADH with 1 ml distilled water. Allow the NADH to sit for 3 minutes with gentle agitation prior to use. (Keep tightly capped on ice for the duration of the assay).

   Dilution, immediately before use, dilute 900 μl of NADH with 1.8 ml of distilled water (Keep on ice for the duration of the assay).

3. *Nitrate reductase*

   Reconstitute, the nitrate reductase with 1 ml nitrate reductase storage buffer. Vortex vigorously and allow to sit for 15 minutes at room temperature. Vortex again and allow to sit for an additional 15 minutes at room temperature. Vortex again. (Keep on ice for the duration of the assay).

Dilution, immediately before use, dilute the nitrate reductase using the following equation. Determine the number of wells to be used (all samples and standards should be assayed in duplicate).

a. Nitrate reductase (µl) = (# wells + 2) x 10 µl.

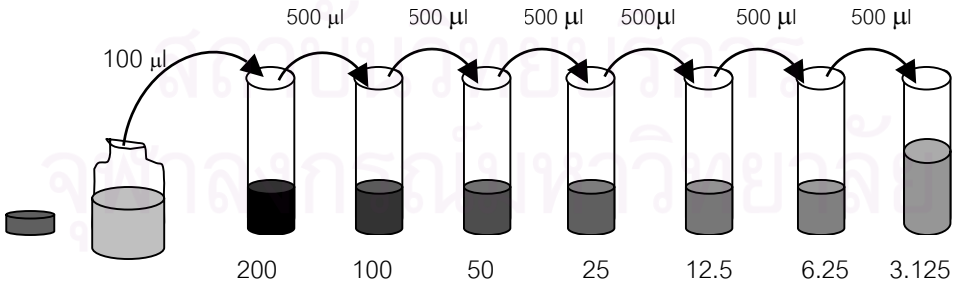
b. Reaction buffer (µl) = volume from step a x 1.5.

c. Add volumes from steps a and b to a tube, vortex.

d. Place on ice and use within 15 minutes of dilution.

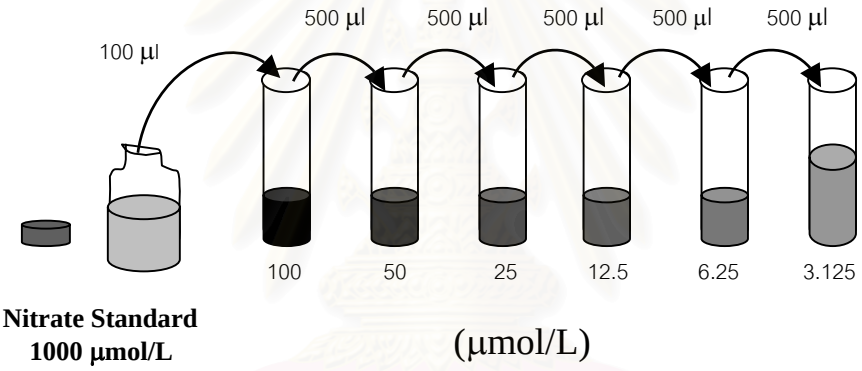
4. Nitrite standard

Pipette 900 µl of reaction buffer (1x) into the 200 µmol/L tube. Pipette 500 µl of reaction buffer (1x) into the remaining tubes. Use the 2,000 µmol/L standard stock to produce a dilution series (below). Mix each tube thoroughtly and change pipette tips between each transfer. The 200 µmol/L standard serves as the high standard and the reaction buffer (1x) serves as the zero standard (0 µmol/L).



5. Nitrate standard

Pipette 900 µl of reaction buffer (1x) into the 100 µmol/L tube. Pipette 500 µl of reaction buffer (1x) into the remaining tubes. Use the 1,000 µmol/L standard stock to produce a dilution series (below). Mix each tube thoroughtly and change pipette tips between each transfer. The 100 µmol/L standard serves as the high standard and the reaction buffer (1x) serves as the zero standard (0 µmol/L).



BIOGRAPHY

Miss. Yuyen Seujange was born on August 24, 1974 in Trang province, Thailand. She received the Bachelor degree of Nursing Science in 1997 from Mahidol University, Bangkok, Thailand. She has entrolled at Chulalongkorn University in graduate program for the Degree of Master of Science in Physiology and graduated in 2003.