CHAPTER III
MATERIALS AND MRTHODS

3.1 Drugs and chemicals

RBP (size ≤ 50 kDa) was obtained from Dr. Supawan Thawornchinsombut, Department of Food and Technology, Faculty of Technology, Khon Kaen University, Khon Kaen, and CUR wan generously provided by The Government Pharmaceutical Organization, Bangkok Thailand. N^{ω}-nitro-L-arginine methyl ester (L-NAME), phenylephrine hydrochloride (PE), angiotensin converting enzyme (ACE) from rabbit lung, angiotensin I (Ang I), angiotensin II (Ang II), hippuryl-L-hitidyl-L-leucine (HHL), *O*-phthalaldehyde (OPA), methoxamine, ethylenediamine tetraacetic acid (EDTA), Glutathione (GSH), thiobabituric acid (TBA), sodium dodecylsulfate (SDS), butylated hydroxyluene (BHT), metaphosphoric acid (MPA), 2,4-dinitrophenylephinenylhydrazine (DNPH), bovine serum albumin fraction V (BSA), *N*-1-nepthylethylenediamine dihydrochloride (NED), sodium nitrate (Na_2NO_2), sodium nitrite (Na_2NO_3), 1,1,3,3-tetraethoxypropane, sulfanilamide, guanidine were purchased from Sigma-Aldrich Pte. Ltd. (Singapore). Di-sodium hydrogen phosphate (Na_2HPO_4), potassium dihydrogen phosephate (KH_2PO_4), di-potassium hydrogen phosphate (K_2HPO_4), acetylcholine chloride (ACh), and sodium nitropusside (SNP) were obtained from Fluka Chemika Co. Ltd. (Buchs, Switzerland). Sodium dihydrogen phosphate (NaH_2PO_4) was obtained from Carlo Erbra (Italy). Paraformaldehyde was obtained from Electron Microscopy Sciences (Hatfield, PA. USA). Nembutal was purchased from Ceva Animal Health Ltd. (Thailand). Heparin sodium was obtained from LEO, (Denmark). Polyethylene glycol (PEG) was obtained from Ajax Finechem Pty Ltd. (Australia). All other chemicals used were of analytical grade quality.

3.2 Instruments

Organ bath, force transducer with Lab chart 7 and PowerLab System (AD. Instruments, Australia), pressure transducer with AcqKnowledge data acquisition

and BIOPAC systems (Santa Barbara Inc., California, U.S.A.), tail cuff IITC model 179 blood pressure analyzer (Victory Boulevard Woodland Hill Inc., Life Science, U.S.A.), electromagnetic flowmeter (Model FM501D, Carolina Medical Electronics, Inc., North Carolina, U.S.A.), UV/Visible spectrophotometer (Ultrospec 6300 *pro*. Bichrom Ltd., UK), luminometer (Turner Biosystems, 2/2n CA, USA), Sunrise absorbance microplate reader (Tecan, Austria GmbH, Austria), centrifuge SIGMA 3K15 (SIGMA Laborzentrifugen Osterode am Harz, Germany).

3.3 Animals and induction of hypertension

3.3.1 Animals

Adult male Sprague-Dawley rats weighing 180- 250 g were obtained from National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom, Thailand. All animals were housed in the HVAC (Heating, Ventilation and Air-Conditioning) System with 12 hours dark/light cycle at the Northeast Laboratory Animal Center, Khon Kaen University, Thailand. All animals were given free access to standard chow diet (Chareon Pokapan Co. Ltd., Thailand) and tap water as libitum. The experimental procedures were reviewed and approved by the Institutional Animal Ethics Committee of Khon Kaen University (AEKKU 17/2553 and AEKKU 01/2551).

3.3.2 Induction of hypertension

The present study comprised 2 models of hypertension-induced in rats.

1) 2K-1C hypertensive rats

2K-1C hypertension was induced in male Sprague-Dawley rats weighing 180-200 g by placing a silver clip (2 mm. i.d.) onto the left renal artery while both kidneys were intact. Sham-operated rats, served as normotensive group, were underwent identical surgical procedures, except that a clip was not applied on the renal artery, A sustained hypertension was found after renal artery clipping for 2 weeks. Animals were weighed before entering the study and also during the time of treatment until sacrificed.

2) L-NAME hypertensive rats

L-NAME hypertension was induced in male Sprague-Dawley rats weighing 220-250 g by administering L-NAME (50 mg/kg/day) in drinking water,

whereas rats in the normal control group received tap water. A sustained hypertension was found after L-NAME administration for 3 weeks. Animals were weighed before entering the study and also during the time of treatment until the end of study. L-NAME hypertensive rats were randomly divided into groups for *in vitro* and *in vivo* studies.

3.3.3 Experimental groups

All animals were assigned into 16 experimental groups of 16 animals in each group. Rats in normal control groups were served as normotensive animals. Rats in 2K-1C hypertensive groups were operated by placing a silver renal clipping of i.d. 0.2 mm. After recovery for 5 days, rats were administered with PG as vehicle, RBP or CUR for 6 weeks. Rats in L-NAME hypertensive groups were received L-NAME (50 mg/kg/day) via drinking water together with polyethylene glycol (PG) as vehicle, RBP or CUR by intragastrically administration for 3 weeks.

Group 1: Sham-operated control + PG (1.5 ml/kg BW; p.o.) for 6 weeks

Group 2: Sham-operated control + RBP (100 mg/kg BW; p.o.) for 6 weeks

Group 3: Sham-operated control + CUR (100 mg/kg BW; p.o.) for 6 weeks

Group 4: 2K-1C + PG (1.5 ml/ kg BW; p.o.) for 6 weeks

Group 5: 2K-1C + RBP (50 mg/kg BW; p.o.) for 6 weeks

Group 6: 2K-1C + RBP (100 mg/kg BW; p.o.) for 6 weeks

Group 7: 2K-1C + CUR (50 mg/kg BW; p.o.) for 6 weeks

Group 8: 2K-1C + CUR (100 mg/kg BW; p.o.) for 6 weeks

Group 9: Normal control + PG (1.5 ml/kg BW; p.o.) for 3 weeks

Group 10: Normal control + RBP (100 mg/kg BW; p.o.) for 3 weeks

Group 11: Normal control + CUR (100 mg/kg BW; p.o.) for 3 weeks

Group 12: L-NAME + PG (1.5 ml/ kg BW; p.o.) for 3 weeks

Group 13: L-NAME + RBP (50 mg/kg BW; p.o.) for 3 weeks

Group 14: L-NAME + RBP (100 mg/kg BW; p.o.) for 3 weeks

Group 15: L-NAME + CUR (50 mg/kg BW; p.o.) for 3 weeks

Group 16: L-NAME + CUR (100 mg/kg BW; p.o.) for 3 weeks

3.4 Experimental protocols

3.4.1 Indirect blood pressure measurement

A conscious rat was placed in a restrainer and allowed to rest in the warming chamber (33ºC) for 5 min prior to blood pressure measurement. The rat's tail was placed inside the tail cuff sensor, which is attached to the end plate of the restrainer. The photoelectric cuff sensor was connected to the Rat and Mouse Blood Pressure Systems (IITC Life Science, CA, U.S.A.). The mean value of systolic blood pressure (SBP) for each animal was obtained from 3 consecutive recordings.

3.4.2 Hemodynamics measurement

At the end of experiment, rats were anesthetized with an intraperitoneal injection of 60 mg/kg pentobarbital sodium and placed on a heating pad. A tracheotomy was performed to facilitate respiration. The body temperature was kept at 37± 2°C throughout the study with the heating pad. The left femoral artery was cannulated with polyethylene tubing connected to a pressure transducer for continuously monitoring arterial blood pressure by using the AcqKnowledge data acquisition and analysis software (Biopac System Inc., California, U.S.A.).

Hindlimb blood flow (HBF) was continuously measured by placing electromagnetic flow probes around the abdominal aorta were connected to an electromagnetic flow meter (Carolina Medical Electronics, North Carolina, U.S.A.). Hindlimb vascular resistance (HVR) was calculated from the mean arterial pressure (MAP) and HBF.

$$\text{HBF in 100 g tissue unit} = \frac{\text{HBF x 100}}{\text{HLW}} \quad \text{(ml/ 100 g tissue/min)}$$

$$\text{HVR} = \frac{\text{MAP}}{\text{HBF}} \quad \text{(mmHg/min/ 100 g tissue/ ml or Peripheral Resistance Unit: PRU)}$$

Blood samples were withdrawn from abdominal aorta for assays of oxidative stress/antioxidant markers and angiotensin converting enzyme (ACE) level. The aortas and carotid arteries were rapidly excised and used for vascular reactivity testing, analysis of eNOS expression and p47phox NADPH osxidase subunit, morphometric analysis of the arterial wall structure, and assay of $O_2^{\bullet-}$ production.

3.4.3 Biochemical and molecular assessments

1) Assay of superoxide production ($O_2^{\bullet-}$) in carotid arteries

Principle

Lucigenin was widely used as a chemiluminescence probe for detecting $O_2^{\cdot-}$ production in biological system. Before photon emission, lucigenin (Luc^{2+}) must first be reduced by one electron to produce a cationic radical ($Luc^{\cdot+}$). Its reaction with $O_2^{\cdot-}$ produces an unstable dioxetane, which decomposes into two molecules of N-methylacridone, one of which is in an electronically excited state and returns to ground state upon release of a photon

Procedure

Production of $O_2^{\cdot-}$ in the carotid artery was determined by luciginin-enhanced chemiluminescence as previously described with some modifications (Nakmareong *et al.*, 2011). In brief, the carotid artery was rapidly excised, cleaned, and cut into segment 4-5 mm in length). The vessel segment was incubated in 900 μL oxygenated Krebs- Ringer bicarbonate solution at 37°C for 30 min. The chemiluminescence signal was measured after the addition of luciginin (60 μM), and counted in a luminometer (Turner Biosystems, 23 CA, USA). The photon counts were integrated every 10 s for 5 min and averaged. The vessels were dried for 24 h at 45°C and weighed. $O_2^{\cdot-}$ production in carotid artery tissue was expressed as relative light unit count/mg dry wt/ min.

2) Assay of malondialdehyde and protein carbonyls

Plasma malondialdehyde (MDA) was determined by measuring thiobarbituric acid reactive substances and oxidizing protein damage was assessed by measuring the formation of carbonyl groups, by reaction with 2,4-dinitrophenylhydrazine, again following a previously described method (Nakmareong *et al.*, 2012).

3) Assay of nitrate/nitrite in plasma

Principle

Accumulation of nitrate/nitrite, the end producs of NO metabolism used as indices of NOS activity, by using Griess reagents, and the resulting reaction product, azo compound, can be measured spectrophotometrically at 450 nm.

Procedure

Plasma samples were deprotinization by ultrafiltration using centrifugal concentrators (NANOSEP^{TM}, Pal Filtration, U.S.A.) The supernatant was mix with 1.2 μM NADPH, 4 mM G-6-P, 1.28 units/ml G-6-PD and 0.8 unit nitrate reductase, and then incubated at 30 °C for 30 min. After that, themixure was reacted with a Griess solution (4% sulfanilamide in 0.3% naphthylenediamine dichloride, NED) for 15 min. The absorbance of sample was measured on an enzyme-linked immunosorbbent assay (ELISA) plate reader with a filter wavelength of 540 nm (tecan GmbH., Grodig, Australia). A standard curve was established with a set of serial dilution of $NaNO_2$.

4) Assays of Angiotensin converting enzyme activity in plasma

Principle

ACE activity can be measured using the substrate, a synthetic peptide, hippuryl-L-histidyl-L-leucine (HHL). L-histidyl-L-leucine (HL) is released by the action of ACE, which can be labeled with *o*-phthaldialdehyde (OPA) to quantify the level of activity from the following reaction:

$$\text{Hippuryl-histidyl-leucine} \xrightarrow{\text{ACE}} \text{histidyl-leucine + hippurate}$$

ACE- activity assay procedures

The level of ACE activity in plasma was determined using the *o*-phthalaldehyde (OPA)-chromogenic reaction for histidyl-leucine following a previously described method (Chang *et al.*, 2001) with slight modifications. In brief, 25 μL sample of plasma and 50 μL of 15 mM Hip-His-Leu solution were mixed in 100 μL buffer (20 mM sodium borate and 300 mM NaCl, pH 8.3), and incubated at 37 °C for 30 min. Background absorbance was determined from a plasma sample diluted in 150 uL buffer. The color reaction was formed by adding OPA reagent (1 mM OPA and 1 mM 2-meraptoethanol in buffer containing 0.1M sodium borate and 0.2 M NaOH, pH 12). After incubation at room temperature for 20 min, the absorbance was measured at 390 nm with a spectrophotometer (Ultrospec 6300 *pro*. Bichrom Ltd., U.K.). Results were calibrated according to a standard curve of ACE solution (15- 120 mU/mL).

5) Western blot analysis in the aortas

Western blotting was performed on aortic homogenates in order to detect the protein expressions of eNOS and the $p47^{phox}$ NADPH oxidase subunit following previously described methods (Nakmareong *et al.*, 2011; Sanchez *et al.*, 2006). In brief, the rat thoracic aortas were homogenized in cell lysis buffer (Cell Signaling Technology, Inc., MA, U.S.A.) and centrifuged at 4 °C and 12000 rpm for 30 min. The supernatant was collected and the protein content was analyzed by the Bradford dye-binding method (Bradford, 1976). A total of 30 μg of protein per sample was separated on 10% sodium dodecyl sulfate polyacrylamide gel by an electrophoresis system. The proteins were transferred electrophoretically onto a polyvinylidene difluoride membrane, blocked with 5% skimmed milk in Tris buffer saline with 0.1% Tween-20 and incubated overnight with primary antibody of either mouse monoclonal anti-eNOS (1:2000 dilution; BD Biosciences, CA, U.S.A.) or mouse monoclonal anti-$p47^{phox}$ (1:1500 dilution; Santa Cruz Biotechnology, Indian Gulch, CA, U.S.A.). The membranes were repeatedly washed with TBS and incubated for 2 h at room temperature with the secondary antibody horseradish peroxidase goat anti-mouse IgG (1:2000 dilution; Santa Cruz Biotechnology). The blots were incubated in the enhanced chemiluminescent substrate solution (Thermo Fisher Scientific Inc., IL, U.S.A.). The intensity of specific eNOS or $p47^{phox}$ NADPH oxidase and β-actin bands were imaged and captured using a digital imaging system for quantitative imaging of gels and blots (Imagequant 400, GE Healthcare Pittsburgh, PA, U.S.A.). The intensity of the bands was normalized to β-actin expression from the same sample. The intensities were expressed as percentages of those from the aorta of normal controls.

3.4.4 Vascular reactivity testing

1) The isolated thoracic aortic rings

Preparation

After obtained stable baseline blood pressure measurements, rats were sacrificed by overdoses of anesthetic drug, the chest was opened and the descending thoracic aortas were dissected from surrounding connective tissues and cut into 3- 4 mm. in length. Each ring was transferred into the organ bath with physiological salt solution (PSS) at 37°C containing (mM): NaCl 118.2, KCl 4.7,

KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.18, Glucose 11.0, $NaHCO_3$ 25 and $CaCl_2.2H_2O$ 2.5 (pH 7.4); bubbled with 95% O_2 and 5% CO_2. Ring was sustained on stainless-steel hooked, and connected to isometric force transducer and Powerlab System (AD. Instruments, Australia) recording system. The aortic rings were equilibrated for 60 min at resting tension 1.0 g. before starting experimental protocols. To test maximal contraction each ring was exposed to Kreb's solution containing KCl 125 mM for 15 min. The endothelium intact was tested by acetylcholine (ACh) 1.0 μM to induce more than 80% relaxation pre-contracted with PE to 50-60% of maximal contraction of KCl 125 mM.

All of the following experiments were conducted on the aortic rings of normotensive and hypertensive rats. In all experiments, each ring was used only once and data of each set of experiments are collected from six to eight aortic rings.

To examine endothelium-dependent and endothelium-independent relaxation, aortic rings were pre-contracted with phenylephrine (PE) to about 60 % of the maximal contraction of KCl (125 mM), were used to construct cumulative concentration-response curve to ACh ranging from 0.001 to 30 μM and SNP ranging from 0.001 to 30 μM. A percentage of vasorelaxation was calculated from the beginning raised tone.

2) The mesenteric vascular beds

Preparation

The main branch of the superior mesenteric artery was identified, cleaned of connective tissues and cannulated with a blunted hypodermic needle. The mesenteric bed preparation was placed on a stainless steel grid (7x5 cm.) in a warm humid chamber (37°C) and perfused at a constant flow rate of 5 mL/min, using a periplastic pump (07534-04, Cole-Palmer Instrument, Illinois, USA). Kreb's solution was composed of the following (mM): NaCl 118.2, KCl 4.7, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.18, Glucose 11.0, $NaHCO_3$ 25 and $CaCl_2.2H_2O$ 1.25 (pH 7.4). The solution was maintained at 37°C and continually gassed with 95% O_2 and 5% CO_2. Mean perfusion pressure was monitored using a pressure transducer and the data recorded using BIOPAC system (California, U.S.A.).

After 30 min equilibration period, methoxamine was added to raise tone in each preparation (50-70 mmHg above baseline) before endothelial function testing by using 1μM ACh. Under methoxamine raised tone condition, the endothelium-dependent and endothelium-independent relaxations were performed. ACh ranging from 0.001 to 30 μM and SNP ranging from 0.001 to 30 μM are directly inject into the perfusate proximal to the arterial cannula with an infusion pump. A volume of 100 μL for each concentration was injected for 10 s.

3.4.5 Morphometric analysis and composition of vascular wall

These measurements were carried out on a separate set of animals (six per group). After sacrifice by an overdose of anesthetic drug, thoracic aortas and superior mesenteric arteries were cleaned of loosely adhering fibrous tissue and fixed with 4% phosphate-buffered formaldehyde. The vessels were cut to 5 mm in length, and embedded vertically in paraffin blocks using standard histological procedures. Five-μm-thick sections were cut and stained with hematoxylin and eosin (H&E), Picrosirius Red, and Miller's elastic stain to determine the number of VSMCs and the area fraction of collagen and elastin in the aortic and mesenteric media layer, these being taken as measures of their concentration in the specimens. Medial cross-sectional area (CSA) was calculated by subtracting the lumen internal area (A_i) from the external area (A_e), which was measured in tissue sections (×40). The external radius (R_e) and the internal radius (R_i) were calculated as the square root of A_e/π and A_i/π, respectively. Medial thickness (M) was calculated as R_e minus R_i. Finally, media to lumen ratio (M/L) was calculated as the wall thickness divided by radius of the lumen (Castro *et al.*, 2009).

The stained sections were examined with light microscopy (Nikon ECLIPSE Ni-u, Nikon Instruments Inc., NY, USA) and the images were captured at ×200 with a digital microscope camera (Nikon DS-Ri1 Camera). Twelve images from three non-consecutive sections per animal were captured and used to count the number of VSMCs and measure the lumen and medial areas and the areas within the media of stained collagen and elastin by means of image analysis software (Image-Pro Plus, Media Cybernetics, MD, USA). The number of VSMCs was obtained by counting their nuclei of in the sections stained with H&E. The area fraction of collagen or elastin in the aortic medial layer was assessed by automatically counting

thresholded pixels stained with Picrosirius Red or Miller's elastic staining and dividing by the total number of medial pixels. All measurements were made by one observer and preliminary observations of intra-observer repeatability showed a coefficient of variation of less than 5% for all estimations of thresholded area.

3.4.6 Immunohistochemistry

To determine smooth muscle actin (SMA), MMP-2 and MMP-9 levels in the thoracic aortas and mesenteric arteries, the de-waxed aortic and mesenteric sections were stained with antibodies specific to each: (ab5694; Abcam, for SMA, ab37150; Abcam, for MMP-2 and AB19016; Millipore, for MMP-9) and the R.T.U. Vectastain ABC kit **(**Vector Laboratories, Inc., CA, U.S.A.) as recommended by the manufacture.

The methods for assessment, MMP-2, sections were sequentially treated with the following: antigen retrieval using microwave full power in Tris-EDTA-citrate unmasking solution, pH 8.1 for 35 minutes. Whereas, MMP-9 were treated with antigen retrieval using microwave full power in Vector unmasking solution, pH 6 for 35 minutes. MMP-2 and MMP-9 sections were covered with 3% endogenous peroxidise for 15 min: normal blocking serum for 20 minutes (Normal Horse Serum from the R.T.U. kit): incubate slices with the primary antibody solution dilution using antibody diluents, whereas negative control sections apply antibody diluents alone for 3 hrs at room temperature (MMP-2 1:300, MMP-9 1:150 and SMA 1:150). The Universal Biotinylated Secondary Antibody (Biotinylated Anti Rabbit and Mouse from the R.T.U. kit) were used to cover each section for 30 minutes, and applied ABC reagent (the R.T.U. kit) for 30 minutes. Finally, the slide was covered with the DAB solution for 5 minutes. The binding was shown as a dark brown color. The images were captured using light microscopy.

The percentage of immuno-stained SMA, MMP-2 or MMP-9 in the aortic wall was quantified in a similar way as collagen and elastin, by counting the thresholded pixels stained for SMA, MMP-2 or MMP-9 using the Image-Pro Plus Program.

Immunohistochemical stained of MMP-2 and MMP-9 were semi-quantified intensity score by arbitrary scale of weakness staining as 1, moderate staining as 2 and strong staining of brown color as 3. MMP-2 and MMP-9 were

calculated from percentage of area fraction multiply by intensity score and expressed as arbitrary unit.

3.5 Statistical analysis

Results were expressed as mean ± SEM of measurements. The differences among various groups were compared by using one-way analysis of variance (ANOVA) followed by a post-hoc Turkey test. Other comparisons were made using a student's paired t-test. A value of $P < 0.05$ was considered statistically significant.

3.6 Overview of the research protocol