CHAPTER III

MATERIALS AND METHODS

Experimental animals

Adult male Wistar rats of body weight between 250-300 g were obtained from National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom. The animals were acclimatized in animal care facility at the Faculty of Pharmaceutical Sciences, Chulalongkorn University for 1-2 week prior to use. Municipal tap water and rat feed 082 (National Laboratory Animal Center, Salaya, Nakornpathom) were provided *ad libitum*.

The use of laboratory animals in this research was approved by the ethic committee of Chulalongkorn University in Thailand.

Chemicals

All the chemicals such as reduced Glutathione, Acetylcholine HCl (Ach), phenylephrine (PE), serotonin (5-HT), phorbol-12-myristate-13-acetate (PMA), caffeine, tetraethylammoniun chloride (TEA), methylene blue, N-nitro-L-arginine (L-NAME), bis(o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid (BAPTA-AM), ethyleneglycol-bis-(β-aminoethylether)-N,N'-tetraacetic acid (EGTA) and all drugs such as glibenclamide and Ibuprofen were purchased from Sigma Chemicals Co. (Germany). Other chemicals such as NaCl, KCl, $CaCl_2$, $MgCl_2$, KH_2PO_4, $NaHCO_3$, and glucose were purchased from APS chemical, Australia which used to prepare Krebs-Henseleit solution. The Ca^{2+} -free solution was prepared by removing $CaCl_2$ from the normal KHB solution and adding ethyleneglycol-bis-(β-aminoethylether)-N,N'-tetraacetic acid (EGTA, Sigma) at a final concentration of 0.6 mM.

Preparation of aortic rings

The animals were sacrificed by cervical dislocation and the thoracic aorta was removed and placed in Petri-dish containing Krebs-Henseleit solution (KHS) of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.52, $MgSO_4$ 1.64, KH_2PO_4 1.18, $NaHCO_3$ 7 and glucose 5.5. Segments of approximately 0.3 cm long was obtained and suspended in double walled organ baths (Harvard type Organ bath) which contained 15 ml of KHS solution at 37°C and bubbled with a mixture of 95%O_2 and 5%CO_2. The system was connected to an isotonic force transducer (MLT 050/A, AD Instruments Australia) under a resting tension of 1.0 g. The tension was recorded with the computer equipped Software Chart 5.0 of Powerlab 4/SP data acquisition system (AD Instruments Australia). During an initial stabilization period of approximately 60 min, the bathing solution was replaced every 15 min. Then the aortic rings were contracted with phenylephrine (PE; 1 μM) in order to obtain basal contractile responses. In the aortic preparations without endothelium cells, the endothelium were removed by gentle rubbing the lumen with cotton swab. The absence of the endothelium was confirmed by the relaxant response of less than 10% when they were challenged by acetylcholine (Ach; 10 μM).

Experimental procedures

1. Relaxant effects of GSH on aortic preparations

The aortic preprarations were placed in normal KHS until the tension was stable. Each contractant including PE (1 μM) or KCl (60 mM) or Bay K8644 (1 μM) was added in to the preparations to induce aortic contraction which was referred as maximum contraction. When the contraction reached plateau, GSH at the concentrations ranging from 2 to 8 mM were added cumulatively to produce

relaxation. The relaxations were calculated as a percentage in relative to the contraction provoked by specific contractants. The investigation of GSH induced vasorelaxation was performed in either endothelium-intact and endothelium-denude aortic preparations specific in experiment of PE (1 μM) induce aortic contraction but in experiment of KCl (60 mM) or Bay K8644 (1 μM) was induce aortic contraction were performed only in endothelium-denude aortic preparations. In addition to GSH, NAC was also tested for its effects in some experiments.

In order to investigate the mechanisms in modulating the vascular tension of GSH, several known inhibitors of vasorelaxation were applied in some experiments. After the resting tension was stable, each of the vasorelaxant inhibitors including ibuprofen (10 μM), propranolol (10 μM), atropine (10 μM), L-NAME (10 μM), methylene blue (10 μM) and glibenclamide (10 μM) were preincubation for 30 minutes prior to start the experiment of GSH-induced vasorelaxation, as described above. This investigation was performed in either endothelium-intact and endothelium-denude aortic preparations

1.1 Influence of calcium on vasorelaxation

The roles of extracellular Ca^{2+} on vasorelaxation and the effects of GSH were determined using the methods described by Yang *et al.*, 1999. In this study, the intact aortic rings were incubated with 10 μM BAPTA-AM for 15 min after pre-contraction with PE (1 μM) at steady state. Then, GSH was added cumulatively to induce the relaxant responses. As known, BAPTA-AM was a member permeable Ca^{2+} chelater which was able to chelate intracellular Ca^{2+} of the endothelium and smooth muscle cells. In another experiment, Ca^{2+}-free medium containing EGTA (0.2 M), which is intracellular Ca^{2+} chelating agent, was incubated with this at least 90 min before starting the experiments. The relaxant responses of GSH cumulative dose were test at steady state of the pre-contraction with PE (1 μM) in the free medium containing EGTA (0.2 M).

1.2 Potentiation effects of GSH on the Ach- or SNP-induced relaxation

The potentiation of GSH on Ach- or SNP- induced relaxation was tested in endothelium-intact aortic preparations. After the resting tension was stable, KCl (60 mM) was added to provoke the contraction. When the contraction reached a plateau state, Ach (0.01-100 μM) or SNP (0.001-10 μM) were added cumulatively to induced relaxation. The potentiation effects of GSH were tested by preincubating the tissues with GSH 5 min prior to addition of KCl (60mM). The relaxation were calculated as a percentage in relative to the contraction provoked by KCl (60mM) in the absence of GSH.

2. Effect of GSH on aortic contraction

2.1 Contraction provoked by cumulative addition of PE

After the resting tension was stable, PE at the concentrations ranging from 0.001-10 μM was added cumulatively to provoke the contraction. The maximum contraction produced in this experiment was referred as a 100% contraction, and used as a reference for further calculation of contractile responses. In order to test the effect of GSH on PE-induced contraction, GSH at desired concentration was preincubated for 5 minutes prior to start adding PE cumulatively. The residual contraction was expressed as % response which was calculated as a percentage of the maximum contraction induced by PE in the absence of GSH.

The experiment were performed in either endothelium-intact and endothelium-denude aortic rings. In addition to GSH, NAC was also tested for its effects in some experiments.

2.2. Contraction provoked by various contractants

In these experiments, the inhibitory effects of GSH were tested against endothelium-independent contraction provoked by several known contractants. Those

contractants included phenylephrine (PE; 1 μM), serotonin (5-HT; 1 μM), histamine (His; 1 μM), phorbol-12-myristate-13-acetate (PMA; 1 μM), tetraethylammoniun chloride (TEA; 1 mM) and KCl (60 mM). After the resting tension was stable, each contractant was added to induce contraction, which was referred a 100% contraction in this experiment. After a washout period, the tissues were incubated in fresh KHS and gassed until tension stable. Then, GSH was incubated for 5 minute prior to addition of each contractant. The residual contraction was expressed as % response which was calculated as a percentage of the maximum contraction induced by each contractant in the absence of GSH. In addition to GSH, NAC was also tested for its effects in some experiments.

2.3. Influences of GSH and other thiol-containing compounds on endothelium-independent contraction

In this experiment, the effects of GSH and other thiol-containing compounds were tested against PE-induced contraction of endothelium-denude aorta. After the resting tension was stable, single treatment of PE at the desired concentration was added to induce contraction which was defined a 100% contraction in this experiment. After a washout period, the tissues were reincubated in fresh and gassed KHS until tension was stable. Then, GSH and other thiol-containing compounds including N-acetylcysteine (NAC), homocysteine or captopril, at the equimolar concentration were preincubated for 5 minute prior to addition of PE. The residual contraction was expressed as % response which was calculated as a percentage of the maximal contraction induced by PE. In addition, L-valine was also applied in place of GSH in some experiments.

2.4 Contraction in Ca^{2+}-free environment

The methods were performed as described by Ko *et al.*, 2000, in order to determine the effects of GSH and other thiol-containing compounds on endothelium-independent contraction in Ca^{2+}-free environment. Initially, the aortic ring was placed in normal KHS until the tension was stable. In this condition, PE (1 μM) was added to induce contraction. Then the bathing solution was replaced with Ca^{2+}-free KHS. PE was added to provoke the contraction, and developed tension was definded as a 100% contraction. In order to investigate the effects of GSH and other thiol-containing compounds, the tested compounds were incubated in this Ca^{2+}-free condition for 10 minutes, followed by addition of PE to induce contraction. The tensions were recorded and calculated for the % response or residual contraction in relative to those of the PE-induced contraction in the absence of tested compounds.

Another experiment in Ca^{2+}-free condition was performed, using caffeine (10 mM) to induce contraction. In addition to GSH, NAC was also tested for its effects in this experiments.

2.5 Contraction in high K^+-Ca^{2+}-free depolarizing solution.

The methods were performed as described by Hof and Vuorela, 1983, in order to determine the effects of GSH and NAC on the Ca^{2+} influx through voltage operated Ca^{2+} channel (VOC). In this study, after the tensions of aortic rings were stable in normal KHS, the bathing solution was changed to high K^+-Ca^{2+}-free depolarizing solution. Then, the contraction was provoked by addition of $CaCl_2$ cumulatively at the concentrations ranging from 10μM to 10mM. In order to investigate the effects of GSH, the experiment procedure was repeated with the preincubation of GSH for 5 minutes prior to addition of $CaCl_2$. The contractile responses were expressed as the percentage of maximum developed tension in the absence of GSH.

Another experiment was set up to investigate the effect of GSH on the contraction induced by different types of stimuli in Ca^{2+}-free environment, and followed by addition of $CaCl_2$ (Iesaki and Wolin, 2000). Initially, the contraction was provoked with 30 mM KCl in KHS. Then, the bathing solution was changed to Ca^{2+}-free solution containing 0.1 mM EGTA. The contraction was induced by addition of each stimuli including 5-HT (1 μM), KCl (30 mM) or BayK8644 (1 μM) in the absence or presence of the tested compounds (GSH or homocysteine at the concentration of 5 mM). Subsequently, $CaCl_2$ at the concentration of 1mM was added to the bathing system. The experimental design revealed the influence of GSH on the contractile responses originating from the initial release of intracellular Ca^{2+} and subsequent influx of extracellular Ca^{2+}.

2.6 Spontaneous contraction of Ca^{2+}-depleted tissues during Ca^{2+} replenishment

Under specific condition, intracellular Ca^{2+} could be depleted, followed by Spontaneous Ca^{2+} replenishment, resulting in spontaneous contraction. Initially, the aortic strip was depleted of intracellular Ca^{2+} by repetitive treatment of PE in Ca^{2+}-free solution, as previously described by Noguera *et al.*, 1998. Upon changing the bathing solution from Ca^{2+}-free solution to Ca^{2+}-containing solution in this condition, a spontaneous contraction or an increase in the resting tone (IRT) of the aorta was observed. The IRT was expressed as % residual contraction in relative to the IRT response in the absence of tested compounds.

3. Antioxidant activity by DPPH assay.

Radical scavenging activity of GSH, NAC, homocysteine and captopril were measured by the modified DPPH method (Mazor *et al.*, 2006; Brand-Williams *et al.*,, 1995). DPPH is stable radical, dark violet compound which can be dissolved with ethanol. Upon reaction with a reducing agent, the color of DPPH is bleached, and can be quantified by spectrophotometry at 510 nm (Anthos Labtec HT2 version 1.21E). Various

concentrations of each thiol-containing compounds (0.5, 5, 50 and 500 mM) were incubated in the reaction mixture containing DPPH (50 mM) for 20 minutes at room temperature in the dark condition, prior to spectrophotometic measurement at 510 nm.

Statistical Data Analysis

Results are expressed as the mean ± standard error of mean (SEM) of the percentage change in aortic ring tension. The median of effective concentration (EC_{50}) are calculated from dose-response curves by linear regression. Data were compared by Student's *t-test* or one-way and two-way analysis of variance (ANOVA) where appropriate. *P* values which were less than 0.05 are considered statistically significance. *n* represents the number of animals in each group.