CHAPTER IV

MATERIALS AND METHODS

4.1 Materials

4.1.1 Chemical materials

Acetic acid
Antifoam B emulsion
Biotinylated antiobody diluent
Biotinylated anti-thrombomodulin
Butylated hydroxytoluene
Buffer RWI and buffer RPE
Chloroform
Chromogen TMB substrate
Deionize-distilled water
DNase and RNase free H2O
10x DNase buffer
DNase
dNTP
Ethanol
70% ethanol
HRP diluent
Hydrogen chloride (HCl)
Iodine
iTaqTM SYBR® Green Supermix
Methanol
N-ethylmaleimide
Potassium iodine
Phosphate-buffered saline (PBS)
RiboZolTM reagent
RNase-free water
RNase out
Sodium citrate
Sodium nitrite
Sodium hydroxide
Tris–HCl (pH 7.4)
Superscript reverse transcriptase
Standard diluent buffer
Stop reagent
Streptavidin-HRP
Thrombomodulin standard
Wash buffer

4.1.2 Instruments

- Applied Biosystems 7500 Fast Real-Time PCR System (Life technology, CA)
- BioTek ELx808 Absorbance Microplate Reader (BIOTEK, VT)

- ANALYZER CLD 88 liquid NO analysis chemiluminescence (Eco Medics, Switzerland)
- EZDO® pH/MV/Temp Meter PL-600 (M.R.C, Israel)
- Labtech® hotplate stirrer (LabTech, Italy)
- Labnet® spectrafuge 24D (Labnet, NJ)
- Labnet® mini plate spinner MPS 1000 (Labnet, NJ)
- Labnet MultigeneTM Mini PCR Thermal Cycler (Labnet, NJ)
- MPW-350R High speed brushless centrifuge (MPW Med. Instruments, Poland)
- Nanodrop® spectrophotometer ND-1000 (Thermo Scientific, DE)
- Nikon® Eclipse TE2000-5 Microscope (Nikon, Japan)
- Shellab CO_2 incubator (SHEL Lab, OR)
- SpextraMax M3 multi-mode Microplate reader (Molecular Devices, Sunnyvale, CA)

4.2 Methods

4.2.1 Subjects

Participants were local residents in Mae Sot District, Tak Province, Thailand in which cadmium contamination was widespread. The subjects who participated in the study were classified into control subjects with urinary cadmium < 0.5 μg/g creatinine, and subjects with urinary cadmium > 5 μg/g creatinine as described previously (13).

4.2.2 Plasma sample collection for the determination of potential NO consumption

Fasting venous blood was collected using 3.8% sodium citrate (9:1) as an anticoagulant. Whole blood was centrifuged at 3500 rpm for 3 min to separate plasma. The plasma samples from each group were thawed on ice. To determine potential NO consumption, samples were injected into a glass vessel containing PBS containing 50 µM DETANONOate (NO donor) purged with helium gas (41).

4.2.3 Preparation of reagents and standard solution for NO consumption measurement

- **DETANONOate stock solution, 40 mM**

DETANONOate stock solution was prepared by dissolving 0.005 g of DETANONOate in 1 M sodium hydroxide (NaOH). An aliquot of stock solution was kept at 4 ^{0}C for 4 weeks.

- **DETANONOate solution, 50 µM**

50 µM DETANONOate solution was prepared by dissolving 62.5 µL of stock DETANONOate solution (40 mM) in 437.5 µL of 0.01M NaOH (41).

- **Phosphate-buffered saline (PBS) solution**

0.1 M PBS stock solution was stored at -20 ^{0}C until used and thawed on ice. 50 mL of 0.01M PBS was prepared by diluting 5 mL of PBS stock solution with water and adjusting pH to 7.4 (41).

- **Tri-iodide solution preparation**

Tri-iodide solution was used for measurement of sodium nitrite ($NaNO_2$) standard solution, and freshly prepared by dissolving 0.4 g potassium iodine and 0.26 g iodine in 8 mL of distilled water and 28 mL of glacial acetic acid. All components were thoroughly mixed by magnetic stirrer for 30 minutes. The tri-iodine solution had a dark brown and ready to use for nitrite measurement.

Tri-iodide in the solution was decomposed into free iodide (I^-) and iodine (I_2) forms due to its redox activity. Free iodide reacted with nitrite anion and produced stoichiometric amounts of NO and iodine products.

$$I_3^- \longrightarrow I_2 + I^-$$

$$KI \longrightarrow K + I^-$$

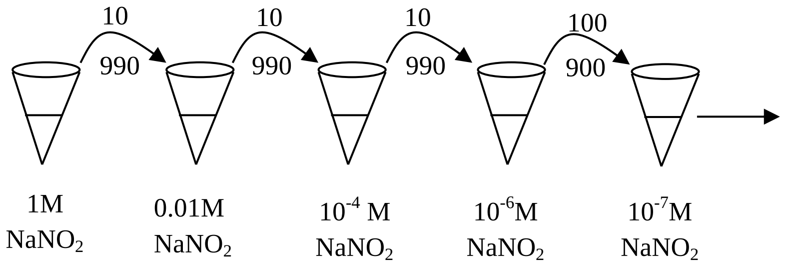
$$2NO_2^- + 2I^- + 4H^+ \longrightarrow 2NO + I_2 + 2H_2O$$

- **Nitrite standard solution preparation, 0.1 µM**

Nitrite standard solution was prepared by weighing $NaNO_2$ in unit of grams and then calculating the volume of distilled water to make 1 M $NaNO_2$ using the following formula.

$$\frac{NaNO_2\ (g)}{69} = \ldots \times 10^{-x}\ \text{mole} \times 10^{6} = \ldots..\ \mu l$$

Next, a serial dilution of standard nitrite solution was conducted as shown in the following diagram.



Injected 10^{-7}M $NaNO_2$ (50, 100, 150, and 200 µL) into an NO chemiluminescence analyzer for a standard measurement

2.2.4 Measurement of potential NO consumption by chemiluminescence detector (CLD)

To measure the potential NO consumption in plasma by a chemiluminescence analyzer, 50 µL of DETANONOate was injected into the purged vessel set at 37°C and fitted with upper cold-water condenser. The purged vessel was connected with the helium gas that carried NO to the NO analyzer to produce a steady-state of NO signal at about 50-70 mV. The signal was generated by release of NO from DETANONOate. Decay of NO signal represents potential NO consumption by plasma (Figure 4.1).

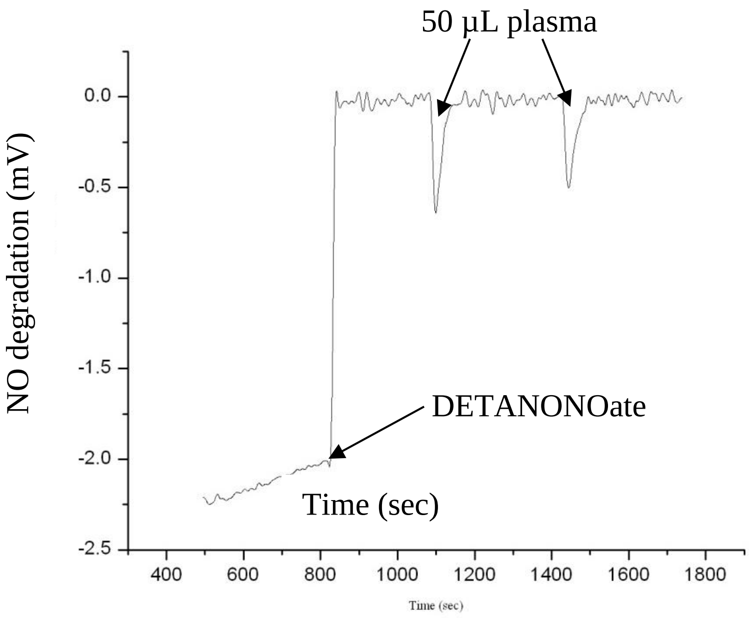
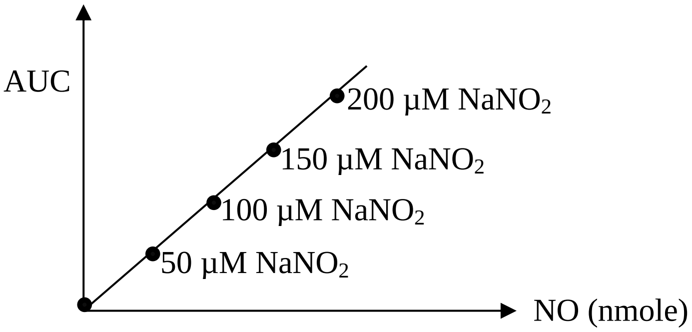


Figure 4.1 Potential NO consumption by plasma

Area under the curve (AUC) of potential NO consumption peak was calculated by Origin 7 (OriginLab Corporation, MA). Then the AUC values were converted into nitrite amount using the standard curve below.



$$NO = \frac{AUC}{Slope}$$

4.2.5 Assessment of the endothelial function in plasma of cadmium-exposed subjects by measurement of soluble thrombomodulin (sTM)

Measurement of sTM was performed by a commercial ELISA kit (American Diagnostica Inc, Greenwich, CT) according to the manufacturer’s instruction (16). The kits contained thrombomodulin microplate (12 x 8 well strips), thrombomodulin standard, primary capture antibody, primary detector antibody, conjugated secondary antibody, and substrate for enzyme-catalyzing colorimetric reaction. The optical density was measured at 450 nm by a SpectraMax M3 multi-mode microplate reader.

4.2.6 Cell culture

The primary human coronary artery endothelial cells (HCAECs) was purchased from Cell Applications, Inc. (San Diego, CA) and used within 3-9 passages. HCAECs were maintained in the endothelial cell growth medium at 37 ^{0}C, 5% CO_2 humidified incubator. The endothelial cell growth medium was changed every other day until the cells reached 80% confluent. Cells were harvested for using in experiments summarized in Figure 4.2 (42).

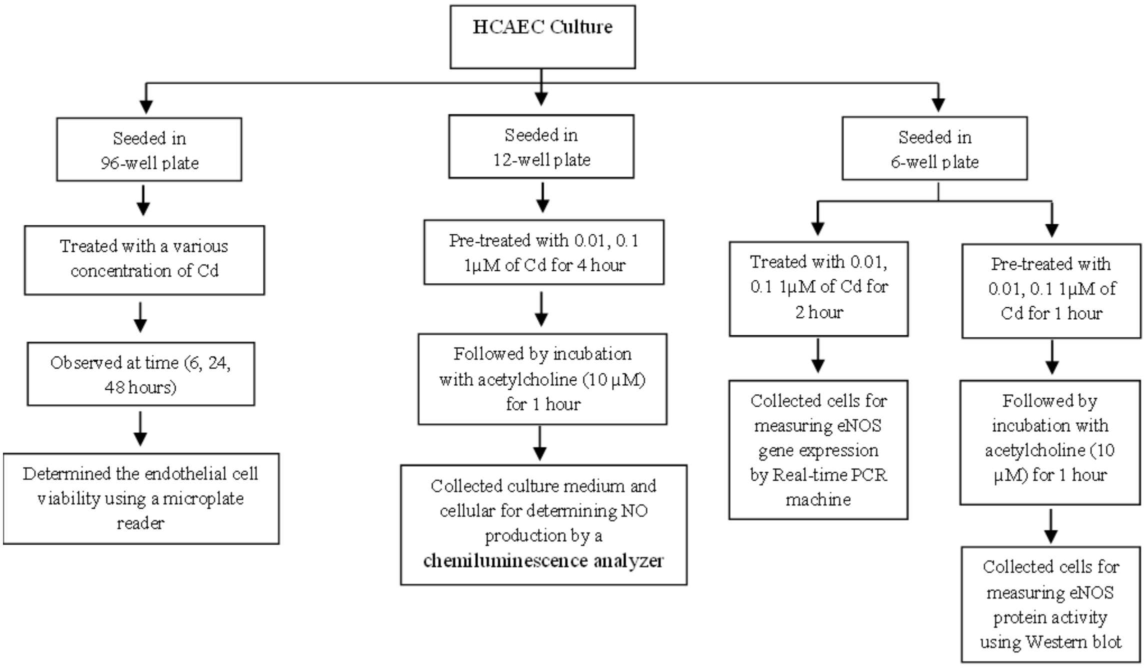


Figure 4.2 Overview of the experimental design aimed to determine the effect of cadmium on NO production in HCAECs

4.2.6.1 Determination of cadmium toxicity by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

HCAECs were seeded into a 96-well plate and treated with various concentrations of cadmium (0.1, 0.3, 1, 3, 10, 30 and 100 μM) for 6 h, 24 h and 48 h. After treatment, the endothelial cell growth medium containing 5 mg/mL 3-(4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was added to each well to obtain the final MTT concentration of 0.5 mg/mL. Cells were then incubated with MTT for 3 h at 37 °C. Afterwards, cells were lysed with 50 µL of dimethyl sulfoxide (DMSO). The optical density (O.D.) was read at 562 and 630 nm using a BioTek ELx808 Absorbance microplate reader (Figure 4.3) (43).

$$\%\ \text{Cell viability} = \frac{\text{(O.D. at 562 nm – O.D. at 630 nm of cadmium treated cells) x 100}}{\text{(O.D.at 562 nm – O.D. at 630 nm of untreated cells)}}$$

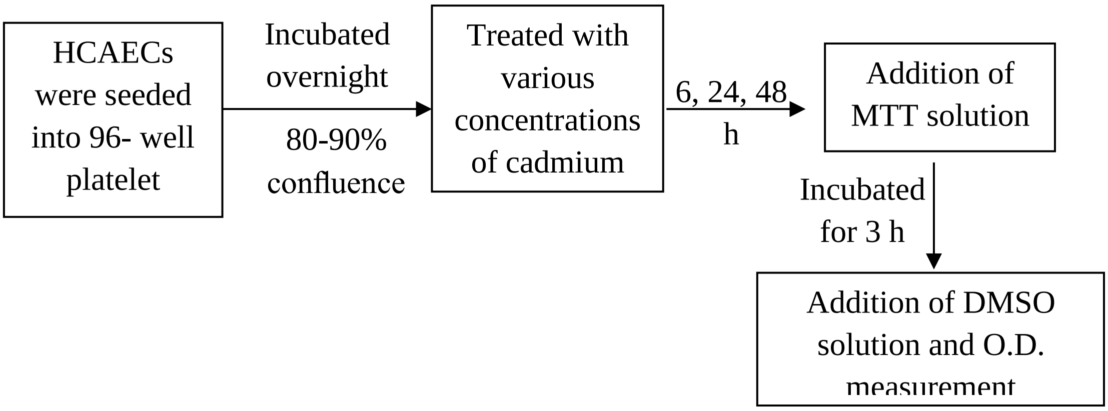


Figure 4.3 The flow chart of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

4.2.6.2 Real-time polymerase chain reaction (Real- time PCR)

To determine the expression of endothelial nitric oxide synthase (eNOS) in human coronary artery endothelial cells, transcripts of endothelial nitric oxide synthase (eNOS) was compared to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In this experiments, HCAECs were seeded into 6-well plates at 3×10^5 cells/cm^2 and treated with cadmium (0.01, 0.1, 1 and 10 µM) for 2 h (43, 44).

RNA extraction

HCAECs were washed twice with cold PBS. Total cellular RNA was extracted using 800 µl Ribozol reagent (Amresco, OH) and 160 µL chloroform. The aqueous and organic phase was separated by 12,000 x g for 15 min at

4 ^{o}C. RNA remained exclusively in the aqueous phase. The upper aqueous phase was carefully removed and transferred to a new tube containing 300 µL of 70% ethanol. After that RNA was purified using the spin column (RNeasy column; 74106 QIAGEN) according to the manufacturer's recommended method. RNA was eluted by 30 µL of RNase free water and RNA concentration was measured using a Nanodrop® spectrophotometer ND-1000.

First-strand complementary DNA (cDNA) synthesis

The first-strand cDNA was synthesized from 1 µg of total RNA extracted from HCAEC culture. The genomic DNA was degraded by incubating each sample with DNase I (Promega) at 37 ^{o}C for 1 h. Afterwards, DNase was inactivated at 70 ^{o}C for 10 min. The random hexamer (Roche) and dNTP were added followed by Superscript III reverse transcriptase (Invitrogen); after that the mixture was incubated at 50 ^{o}C for 1 h as recommended by manufacturer's protocol. Finally, cDNA was diluted 1:1 with sterile water. 4 µL of cDNA was used for each real-time PCR reaction.

Real-time polymerase chain reaction (Real-time PCR)

Quantitative real-time PCR was performed using iTaqTM Universal SYBR® Green Supermix (Bio-Rad) on an Applied Biosystems 7500 Real-Time PCR System. The amplification steps were 95 ^{o}C for 15 sec, 57 ^{o}C for 30 sec and 72 ^{o}C for 30 sec. These steps were repeated for 40 times. The forward and reverse primers of eNOS and GAPDH were as follows: eNOS forward primer 5-ACCCTCACCGCTACAACAT-3 and reverse primer 5-GCCTTCTGC TCATTCTCCA3 (40); GAPDH forward primer 5-AGCCTTCTCCA TGGTGGTGAAGAC-3 and reverse primer 5- CGGAGT CAACGGATTTGGTCG -3 (41). Gene expression was calculated by $2^{\Delta\Delta CT}$ method and normalized with GAPDH mRNA levels. Data were expressed as the relative fold changes (RFC) of mean ± SEM by calculating from the value of eNOS expression divided by GAPDH expression.

4.2.6.3 Western blot analysis

To determine effects of cadmium on the activation and protein expression of eNOS, Western blot analysis of cell lysates using specific antibodies against endothelial nitric oxide synthase (eNOS) and phospho-specific eNOS (pS1177) was performed. HCAECs were seeded at 3 × 10^5 cells/ cm^2 into 6-well plates and pre-treated with 0.01, 0.1, 1, and 10 µM $CdCl_2$ for 4 h, followed by incubating with 10 µM acetylcholine for 1 h (45). HCAECs were washed twice with ice-cold phosphate-buffered saline (PBS) and cells were lysed with 0.5 ml of lysis buffer (50 mM Tris-base, 150 mM NaCl, 0.5% NP-40, pH 7.4) at 4 ^{0}C for 30 min. Cell lysate was collected on ice using a cell scraper and centrifuged at 12,000 g at 4 ^{0}C for 20 min to remove pellets. Protein mixed with loading dye and boiled at 95 ^{0}C for 5 min to denature protein. After that, 30 µg of protein was separated by 8% SDS–PAGE gels at 80 V for 2.5 h Proteins on gels were electrotransferred to PVDF membrane at 0.22 A for 2 h. All blots were blocked with 5% non-fat dried milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) for 1.5 h and then membranes were washed 5 times with TBS-T for 10 min each time. After that, membrane were probed with mouse anti-phospho-eNOS (pS1177) (1:1,000 dilution, BD Transduction), mouse anti-total eNOS (1:1000 dilution, BD Transduction) and mouse anti-β-actin antibody (loading control) (1:2000 dilution, BD Transduction) at 4 ^{0}C for overnight (47) followed by incubation with mouse anti-secondary antibody conjugated with horseradish peroxidase (HRP) (1:4000 dilution, Jackson ImmunoResearch, PA) for 1 h. Proteins on blots were visualized using chemiluminescent ECL assay kit (Bio-rad, CA), and the immunoreactive protein was detected on Hyperfilm ECL (Santa Cruz, Texas). The relative intensity of protein bands were quantified by Image J analysis program (NIH website by Scion Corporation, Frederick, MD) (45). Phospho-eNOS expression was normalized to total eNOS expression while total eNOS expression was normalized to β-actin. Relative protein expression was reported as mean ± SEM.

4.2.6.4 Total protein measurement by a Bradford assay

The concentration of total protein in cell lysate was determined using a dye reagent concentrate protein assay**.** After protein collection, protein was diluted 1:1 with distilled water (3A) 1:1 and 8 µL of diluted protein was added into 96-well plates. Dye reagent was diluted 1:4 with distilled water and 160 µL was added into each well. The reaction of protein and dye reagent was occurred and changed to blue color. The O.D. was shifted from 465 nm to 595 nm when protein binding occurred. Protein concentration was determined at $O.D._{595nm}$ (45).

4.2.6.5 Measurement of nitrite anion (NO_2^-) in cell supernatant and cellular levels

Nitrite, an oxidized stable derivative of NO, was measured in cell supernatant and cells. HCAECs (10^5 cells/cm^3, 800 µL) were seeded in 12-well plates, and allowed to be 80% confluences. Once confluenced, the endothelial cells was pre-treated with 0.01, 0.1, 1, and 10 µM $CdCl_2$ for 4 h followed by incubating with 10 µM acetylcholine for further 1 h (46). Afterward, lysis buffer (50 mM Tris-base, 150 mM NaCl, 0.5% NP-40, pH 7.4) was added into wells and cells were scrapped using cell scrapers. The supernatant and cell lysate were collected for nitrite measurement using a chemiluminescence NO detector (47).

Nitrite measurement by a chemiluminescence detector

30 µL of culture media and cell lysate were injected into a purge vessel containing tri-iodide solution. Sodium nitrite ($NaNO_2$) was used to generate a standard calibration curve, primarily described in 4.1. Tri-iodide reduces nitrite to NO rapidly. NO reacts with ozone (O_3) inside the chemiluminescence, forming excited NO_2 ($*NO_2$) as shown in the following equations (47).

$$NO + O_3 \longrightarrow *NO_2 + O_2^-$$

$$NO_2 \longrightarrow NO_2^- + hv$$

The reaction of NO with O_3 produces excited nitrogen dioxide (NO_2) which emits luminescence. The emitted chemiluminescence is detected and quantified by a photomultiplier tube of chemiluminescence detector. Nitrite is reduced to NO in tri-iodide solution.

Area under curve was calculated using Origin 7, and converted into nitrite amount using the standard curve.