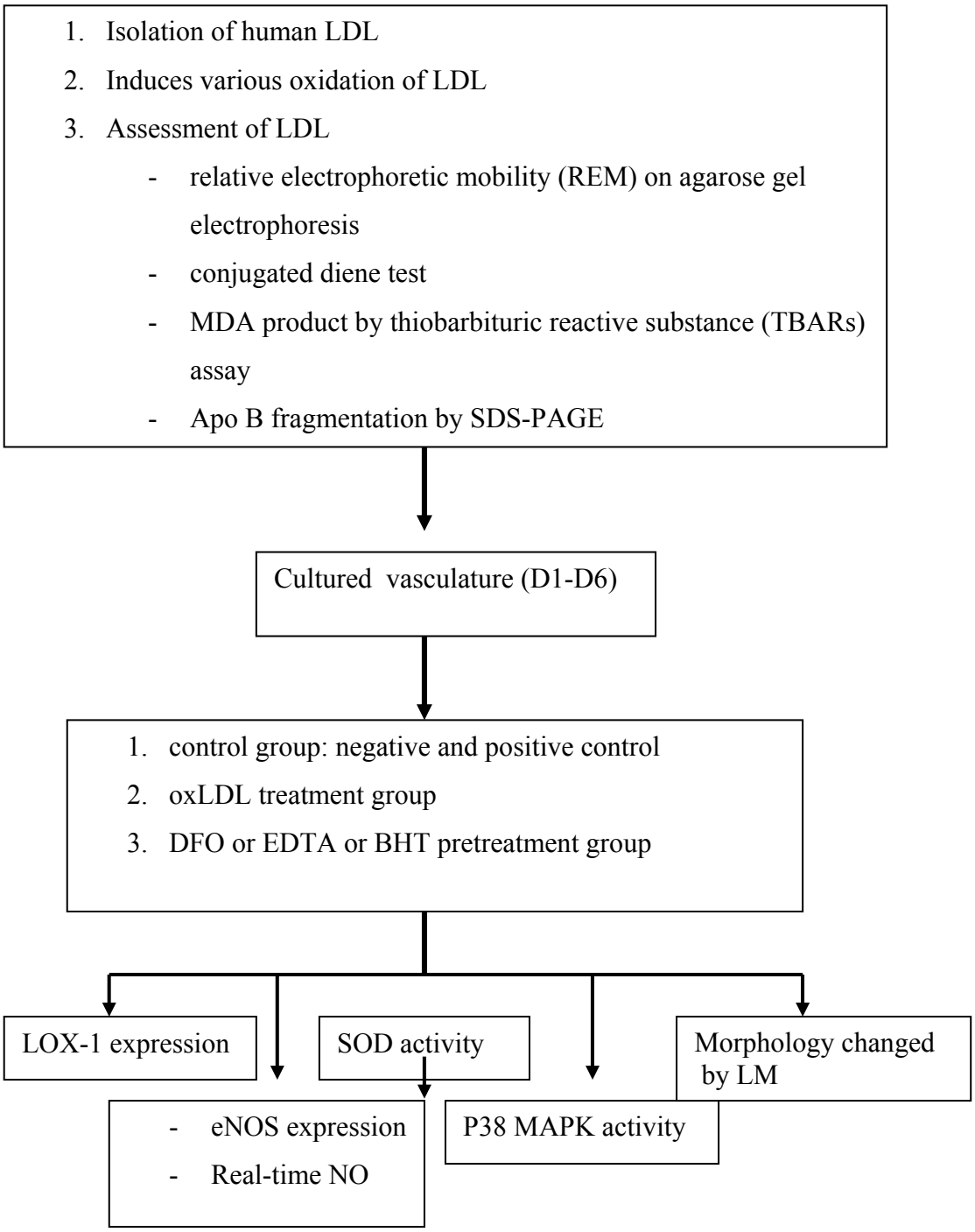
Chapter 3

Methodology

Since we hypothesized that in which species of ROS/RNS involved in the mechanism of oxLDL activation through LOX-1 in cultured vasculature, we have approached the research questions as follows:

1. To induce oxLDL in various degree of oxidation: mildly, moderately and fully oxidation
2. To activate LOX-1 mRNA expression by using oxLDL in various degree of oxidation and doses and designed as treatment groups
3. To perturb the cultured vasculature with iron chelator and peroxyl radical scavenger in pre-incubation for 45 minutes and followed by oxLDL treatment
4. To examine SOD activity of those groups
5. To examine eNOS mRNA expression and real-time NO release
6. To investigate p38 MAPK, redox-sensitive protein, that responds to those treatments
7. To investigate morphological changes by light microscope

We present conceptual framework of our study below.



For investigation of those research aims, we design the research protocol as follows:

1. Control group
    - negative control is only vessel ring.
    - positive control is vessel ring with iron chelator or antioxidant pretreatment
2. Experiment batch1: oxLDL treatment
3. Experiment batch 2: pre-incubated with iron chelator (DFO, EDTA) and peroxyl radical scavenger antioxidant (BHT) for 45 minutes of each experiment and then added oxLDL as oxidant after pre-incubation.

DFO: a ferric (Fe^{3+}) chelator in Haber-Weiss reaction and also using 10 μM DFO in the experiment (Steinbrecher, Parthasarathy, Leake, Witztum, & Steinberg, 1984).

EDTA: a ferrous chelator (Fe^{2+}) in Fenton reaction and using 0.3 mM EDTA in the experiment (Steinbrecher, et al., 1984)

Both of iron chelators will limit and reduce catalyst of metal transition oxidation in the culture system. Thus, oxidation will be affected by enzymatic activation in vasculature only.

BHT peroxyl radical scavenger antioxidant and inhibits MDA formation from a polyunsaturated fatty acid. 100 μg/ml BHT was added for preincubation in the experiment (Gavino, Miller, Ikharebha, Milo, & Cornwell, 1981).

3.1. An isolation of native LDL

An isolation of LDL was performed by using floating sequential ultra-centrifugation method (Schumaker & Puppione, 1986). Fresh blood sample was withdrawn from healthy volunteers, who had fasted overnight, and collected into tubes containing 4 mM EDTA as protection of oxidation and 1 mM phenylmethylsulphonyl fluoride (PMSF) as protease inhibitor, respectively. Plasma obtained by centrifugation at 2,500 rpm, 4 °C for 15 min and then adjusted to density of 1.21 g/ml with solid potassium bromide (KBr). High density plasma (d =1.21g/ml) was loaded to the bottom of ultracentifuge tube and then overlaid with gradient salt

solutions density =1.063 g/ml, density =1.019 g/ml, and density =1.005 g/ml, respectively. The gradient density ultracentrifuge tube were centrifuged at 286,000 g, 4°C for 24 hrs. The yellowish LDL fraction was collected at density 1.019-1.063 g/ml and then dialysed agianst phosphate buffer saline (PBS) under nitrogen flow at 4 °C for 24 hrs in order to remove salt containing. The protein concentration of dialysed LDL was determined by using Bradford method and then assessed the purity and susceptible to oxidation by running on 1% agarose gel electrophoresis and assessesment of diene formation before storaged at -80°C. Purified LDL fraction is called native LDL and used in the experiment within 2 weeks. Fresh purified LDL fraction is recommended for the study.

3.2. Oxidative modification of native LDL

We induced purified native LDL to be mildly, moderately and fully oxidation by using Cu_2SO_4 as catalyst. For mildly oxidation, we used 5 μM Cu_2SO_4 added to 1 mg/ml native LDL and let it in 37 °C water bath for 1 hr, 4 hrs for moderately and 18-24 hrs for fully oxidation. Finally, we stop oxidation by adding 0.01 % EDTA to each LDL fraction. Before using in the experiment, we dialyzed oxLDL in order to remove EDTA salt against PBS volume and filtered through a 0.22 μm pore-size membrane (Anthonsen, Stengel, Hourton, Ninio, & Johansen, 2000).

3.3 Characterization of oxidized LDL

We characterized oxidized LDL by four methods as follows:

1. Assessment of protein oxidation
   - quantitate the relative electrophoretic migration by1 % agarose gel electrophoresis
   - Quantitative assessment of apo B fragmentation by SDS-PAGE
2. Assessment of lipid peroxidation
   - Malondialdehyde (MDA) product of lipid peroxidation by TBARs assay

- Susceptibility to oxidation by conjugated diene test

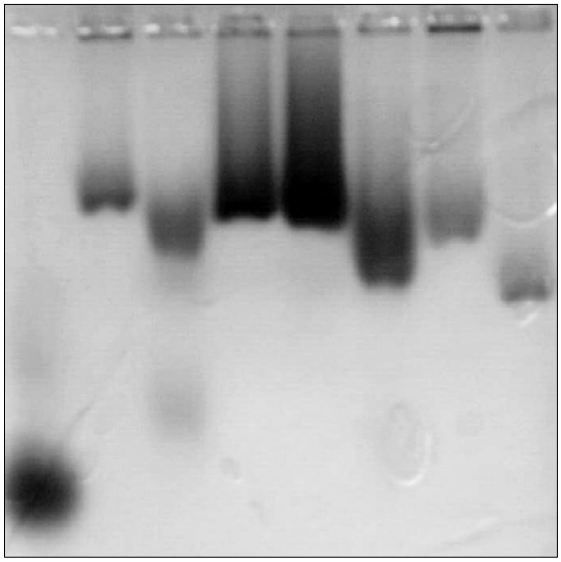
3.3.1 Assessment of protein oxidation

3.3.1.1 Relative Electrophoresis Migration distance (REM) on 1% agarose gel electrophoresis

Oxidized LDL was determined net negative charge resulting from oxidative by electrophoresis (Noble, 1968). Electrophoresis of LDL that suspended in sample buffer containing 10% glycerol, 0.5% bromophenol blue was carried out in 1% agarose gel in 0.05 M barbital buffer, pH 8.6, at 70 voltages. After complete running, the gel was fixed for 2 hr in 25% (v/v) isopropanol, 10% (v/v) glacial acetic acid and then stained with 0.006% (w/v) commassie blue in 10% glacial acetic acid for overnight. The gel was destained with 10% acetic acid until background was seen clearly. The degrees of oxidized LDL were determined by its LDL electrophoresis mobility (REM) compared with BSA as a standard (Figure 3.1).

Figure 3.1

Relative Electrophoresis Migration distance (REM) on agarose gel electrophoresis



3.3.1.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of Apolipoprotein B (apo B) fragment

ApoB-100 is presented in LDL particle and has molecular weight ~ 550 Kda. The structure and property of apoB are fragmented and contain more negatively in oxLDL. Those are determined by SDS-PAGE method (Karpe & Hamsten, 1994) and used 4% acrylamide for stacking gel and 12% acrylamide for separating gel. Sample volume was loaded ~ 1-2.5 μg protein/well and then running in 0.025 M Tris-0.19M glycine at 60-80 volt for 2-3 hrs and fixed with 12% TCA for 2 hr with gently shaking, then staines with 0.015 % commasie blue overnight and destained with 10% acetic acid untiled clearly, respectively.

3.3.2 Assessment of protein oxidation

3.3.2.1 Thiobarbituric reactive substance (TBARs)

The measurment of TBARs as malondialdehyde (MDA) is indicated as an index of lipid peroxidation. Increasing of TBARs values in hypertension, diabetes, hyperlipidemia, atherosclerosis and cardiovascular disease have been reported and related to oxidative stress. Normal TBARs level of isolated LDL were 3.6 $\pm$ 1 nmol/mg LDL protein (Esterbauer, Gebicki, Puhl, & Jurgens, 1992) and high TBARs values (~ 35.7 nmol/mg LDL protein) obtained in the oxLDL (Vieira, Laranjinha, Madeira, & Almeida, 1996). The measurement of TBARs is modified from Yagi (Yagi, 1976) and Wallin (Wallin, Rosengren, Shertzer, & Camejo, 1993). The LDL sample (0.25 mg protein/ml) was mixed in 0.3% NaOH solution with 25% trichloroacetic acid (TCA), 1.3 % thiobarbituric acid (TBA), and boiled at 90 °C for 45 min, cooled on ice for 10 min and centrifuged at 3,000 rpm for 10 min. The supernatant was aspirated and the concentration of TBARs was determined by spectrophotometer at 532 nm. TBARs value was calculated using 1, 1, 3, 3-tetraethoxypropane as a MDA standard curve.

3.3.2.2 Conjugated diene formation

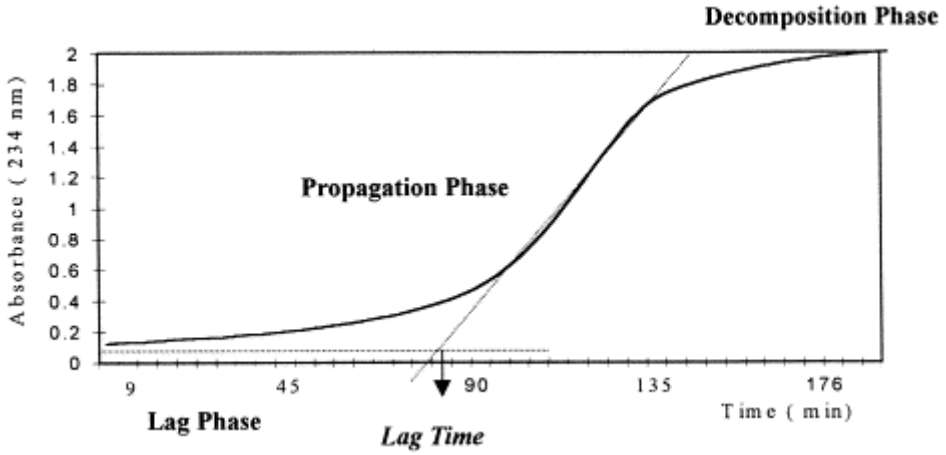
During induction of LDL with Cu_2SO_4, three phase of lipid peroxidation were performed: lag phase, propagation and decomposition. Initial step of LDL oxidation called lag phase which oxidants interact first with the most LDL compound; antioxidants and PUFA. Thus, lag phase time rate is depend on the concentration of the antioxidants (vitamin E) available and followed by the formation of lipid radicals ($LH^{\bullet}$) and minor conjugated dienes (Thomas & Stocker, 2000). LH° can react with O_2 to form peroxyl radicals ($LOO^{\bullet}$) or other oxygenated PUFA derivatives such as alkoxyl radicals, epoxydes, and endoperoxydes. The reaction of $LOO^{\bullet}$ and oxygenated PUFA derivatives are contributed into "propagation phase" of lipid peroxidation, leading to further oxidation of PUFA, cholesterol and other lipids that rapid increase in conjugate dienes. Finally, the oxidation reaction through substrates is depleted and the formation of lipid peroxidation derivatives (aldehydes, alkanes, alcohol, ketone.) is stable that calls "decomposition phase" (Halliwell, 1989).

Conjugated dienes develop in LDL through the oxidation of PUFAs with isolated double bonds (= diens), with a UV-absorption maximum at 234 nm. The approximate rate of diene formation during the lag phase is 0.3 nmol/mg protein/min, which means that one molecule of conjugated lipid hydroperoxide is formed on average in each LDL particle every 6 min (Esterbauer, et al., 1992). Native LDL has longer lag phase time than oxLDL.

After dialysis of LDL, 10 uM Cu_2SO_4 was added to LDL solution (100-150 ug protein) in 0.9% normal saline. The kinetics of the LDL oxidation was monitored by the continuous change in absorbance of conjugated dienes at 234 nm at 37 °C by UV-spectrophometer. The absorbance curve is divided into three phases: lag phase, propagation phase, and decomposition phase (Figure 3.2).

Figure 3.2

Kinetics of conjugated diene formation by continuous monitoring of absorbance at 234 nm



(Seghrouchni, Drai, Bannier, Garcia, & Revol, 2001)

3.4 Organ culture preparation

We used organ culture as a powerful model of complete vascular cell function. Human umbilical cord obtianed from normal labor at Thammasat University Hospital and Mahasarakham Hospital . Umbilical cord was resuspended in sterile PBS and then cut into pieces $\simeq$ 3-5 cm under strictly sterile technique. The rings are placed in a 24-well plate containing 1-2 ml of RPMI 1640 culture media with 10% FCS, then added amphotericin B 5 μg/ml and gentamycin 100 μg/ml. Those rings were cultered in incubator with 5 % CO_2 and 95 % O_2 flow. Media were observed and replaced on on day 1^{st} and day 5^{th}. On day 5^{th}, the experiment was performed as described in methodology. For example, in the DFO pretreatment group, we added DFO on D5 for 45 min and then followed by oxLDL for 24 hrs.

3.5 Determination of LOX-1 mRNA expression

LOX -1 mRNA expression was determined by RT-PCR as follows:

3.5.1 Material Preparation

Extensive precautions were taken to ensure that the RNA isolation was done in an RNase-free environment to prevent degradation of the RNA. All instruments used for the dissection, the Sylgard plate, and the Polytron homogenizer were treated with RNase Zap (Ambion) and rinsed with DEPC-treated water. All tubes and tips were RNase-free and disposable, and gloves were worn at all times during tissue processing. The presence and purity of total RNA was detected by spectrophotometry at OD 260/280. The RNA samples were stored at -80 ºC.

The cultured human umbilical artery was pretreated in each experiment group with or without 10 μM DFO, 0.3 mM EDTA or 100 μg/ml BHT for 45 minutes and exposed to various doses and degrees of oxLDL (10-80μg/ml) for 24 hr. The LOX-1 mRNA expression was determined by RT-PCR and quantitated of concentration by densitometer using Quantity one program (GS-800 calibrated densitometer, BIO-RAD).

3.5.2 Isolation of total RNA

Total RNA was extracted from the cultured human umbilical artery with a phenol–guanidine thiocyanate; TRIzol® reagent (Invitrogen). Total RNA was isolated and modified technique as described by Rodrigo (Rodrigo, Martin, Redetzke, & Eyster, 2002). After treatment, the rings were washed in PBS and submerged in 1.5 ml of cold TRIzol reagent (sample volume should not exceed 10% of the volume of TRIzol reagent) and then homogenized and suspended for 15 second. Following this centrifugation step, homogenate was centrifuged at 12,000 × g for 10 min at 4 ºC for remove debris from the sample. Supernatant was aspirated into tube and incubated at 15-30 ºC for 5 min. Chloroform 0.2ml/1 ml of TRIzol reagent was added and vigorous shake for 15 seconds and then incubated at 15-30 ºC for 2-3 min. The sample solution

was centrifuged at 12,000 ×g at 4 ºC for 15 min. After centrifuge, sample solution is divided into three layer; upper layer or aqueous phase, interphase or organic phase, and red phenol-chlorofrom phase. The aqueous layer containing the total RNA was transferred to a phase lock gel-heavy tube for further extraction. The organic phase was transferred to a clean tube, 300 µl of 100% ethanol was added, and this fraction was stored at -20 ºC for further protein extraction.

The total RNA in the aqueous phase was precipitated by incubation for 10 min at 15-30 ºC with 0.5 ml of 100% isopropanol per 1ml of TRIzol reagent. The isopropanol mixture was centrifuged at 12,000×g for 10 min at 4 ºC to precipitate RNA pellet. Precipitated RNA was seen as gel like or white pellet at bottom of tube. The RNA-containing pellet washed with 70% ethanol by centrifuge at 5,000-7,500×g at 4ºC for 3-5 mins. Alcohol was removed and dried the tube RNA at room temperature for 5-10 mins. Total RNA was dissolved with RNase-free DEPC-treated water (Promega, Madison, WI) and mixed by pipette. The sample RNA was incubated at 50–60 ºC for 10 mins to facilitate dissolution of the RNA pellet. The total RNA samples were treated with RNase-free DNase to remove any contaminating above. The concentration of RNA was quantitated at OD 260/280 by U/V spectrophotometer.

3.5.3 Determination of LOX-1 expression by RT-PCR

Total RNA (1µg) was transferred to microcentrifuge tube. Single-stranded cDNA was synthesized using iScript reverse transcriptase (Bio-rad USA) in combination with an oligo $(dT)_{20}$ primer. Briefly, Template (RNA), Oligo-dT_{15}, and nuclease-free water were mixed and then the mixture was preheated at 65 ºC for 5 min, kept it on ice for 1 min, and spun down at 10 sec, respectively. Then, iScript reverse transcription mix (Bio-Rad USA) were gently mixed. Immediately, the reverse transcription mixture was incubated at 42 ºC for 60-90 min, 85 ºC for 5 mins, respectively. The cDNA product was determined by spectrophotometer at OD 260 and stored at –20 ºC until use.

The cDNA product was amplified with using Hot-start enzyme iTaq DNA polymerase (iQ^{TM} Supermix. Bio-Rad, USA). For polymerase chain reaction (PCR), oligonucleotide of LOX-1 (synthesis from operon) was mixed with 100 nM

per reaction. Synthesis specific for human LOX-1 receptor primer (left primer, 5'-TTACTCTCCATGGTGGTGCC-3'; right primer, 5'-AGCTTCTTCTGCTTGTTGCC-3') was amplified by using thermal cycler at 40 cycles; at 94 °C for 40s, 57 °C for 1 min, and 72 °C for 1 min, respectively. LOX-1 gene product are 193 base pair and then it was load on 1.5% agarose gels and stained using ethidium bromide at dark room for 5 min and then washed in water for 15 min, respectively. Quantitative concentration of LOX-1 expression was determined by densitometer (GS-800 calibrated densitometer, BIO-RAD) and using Quantity one program.

3.6 Determination of eNOS expression

eNOS mRNA expression was determined by RT-PCR as we described in determination of LOX-1 in detail. Total RNA (1μg) was transferred to microcentrifuge tube and single-stranded cDNA was synthesized using iScript reverse transcriptase (Bio-rad USA) in combination with an oligo $(dT)_{20}$ primer. Briefly, Template (RNA), Oligo-dT_{20}, and nuclease-free water were mixed and preheated at 65 ºC for 5 min, kept it on ice for 1 min, and spun down at 10 sec, respectively. Then, iScript reverse transcription mix (Bio-Rad USA) was gently mixed. Immediately, the reverse transcription mixture was incubated at 42 ºC for 60-90 min, 85 ºC for 5 min, respectively. The cDNA product was determined by spectrophotometer at OD 260 and stored at –20 ºC until use.

The cDNA product was amplified with using Hot-start enzyme iTaq DNA polymerase (iQ^{TM} Supermix. Bio-Rad, USA). For polymerase chain reaction (PCR), oligonucleotide eNOS (synthesis from operon) was mixed with 100 nM per reaction. Deternination of eNOS expression using specific synthesis for human eNOS gene; left primer, 5'-CCCTTCAGTGGCTGGTACAT-3'; right primer, 5'-GCTCATTCTCCAGGTGCTTC-3' was amplified by using thermal cycler at 40 cycles; at 94 °C for 40s, 56.5 °C for 1 min, and 72 °C for 1 min, respectively. Expression of eNOS gene product was 250 base pair and then it was load on 1.5% agarose gels and stained by ethidium bromide at dark room for 5 min and then washed in water for 15 min, respectively. Quantitative concentration of eNOS expression was

determined by densitometer (GS-800 calibrated densitometer, BIO-RAD) and using Quantity one program.

3.7 Determination of nitric oxide (NO) concentration

Endothelial dysfunction elicited by oxLDL has been implicated in pathogenesis of atherosclersis (Ross, 1999). Thus, the measurement of NO release is the assessment of endothelial cell function. Electrochemical NO measurement is direct real-time of NO production which based on the direct oxidation of NO to nitrate (Shibuki, 1990).

Method

Quantitation of NO concentration was determined by amino-700 probe (inNO nitric oxide measuring system, model inNO-T S/N 3782-G, Tampa, FL, USA). This electrochemistry technique is based on nitrite level because NO is electrochemical reactive species that oxidized on the surface of carbon fiber microelectrode. The reaction of NO that oxidized by carbon fiber microelectrode is showed.

$$\underset{\text{Nitric oxide}}{NO} \xrightarrow{-e^-} \underset{\text{nitrosonium cation}}{NO^+} \xrightarrow{+OH^-} HONO \xrightarrow{-H^+} \underset{\text{nitrite}}{NO_2^-}$$

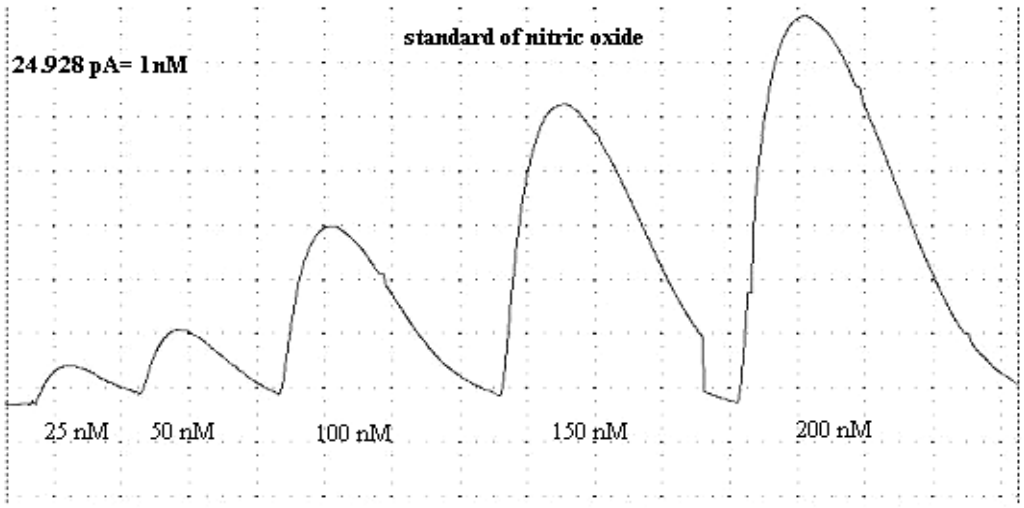
We measured the electrical current (pA) which is linear proportion to NO concentration. The sensitivity of measurement was approximately 20 nM. Usually, microelectrode probe was calibrated by calibration solution containing 2ml of 1M H_2SO_4, 20 mg KI, and 18 ml of ddH_2O and nitrite standard. Microelectrode probe was dipped into calibration solution with gently mixed and set background of sensor to zero (pA was showed zero or nearly). Nitrite standard was added into calibration solution at 25 nM, 50 nM, 100 nM, 150 nM, and 200 nM, respectively. The reaction of calibration showed the proportion of nitrite and NO was 1:1. Standard curve of nitrite standard was showed in figure 3.3.

$$2NO^-_2 + 2I^- + 4H^+ \longrightarrow 2NO + I_2 + 2H_2O$$

Equation of the reaction of calibration

Figure 3.3

Measurement of standard curve of NO at 25, 50, 100, 150, and 200 nM and conversion of current in pA to concentration (nM)



3.8 Determination of SOD activity

Superoxide dismutase (SOD) is one of antioxidant enzymes that scavenge superoxide anion. We determined SOD activity by using xanthine oxidase method (Figure 3.4).

Method

Cultured human umbilical artery was homogenized in ddH_2O with homoginizer. Homogenate was centrifuged at 12000×g for 15 mins. Aqueous of homogenated was aspirated. SOD activity was measured by using SOD determination kits (Fluka, Switzerland). SOD assay was determined by utilizing a highly water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) producing a water-soluble formazan dye upon reduction with $O_2^{\circ-}$. The rate of reduction with O_2 was linearly related to the

xanthine oxidase (XO) activity, and was inhibited by SOD. Standard curve was constructed and used for SOD determination in unit concentration (U/ml) (Figure 3.5).

Figure 3.4

The reaction of determined xanthine oxidase given SOD

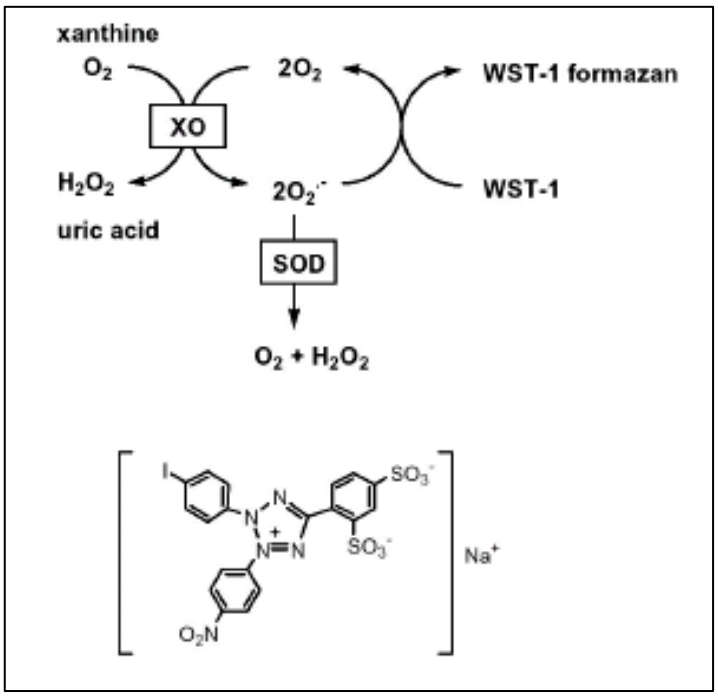


Figure 3.5

Standard curve of superoxide dismutase (SOD) concentration

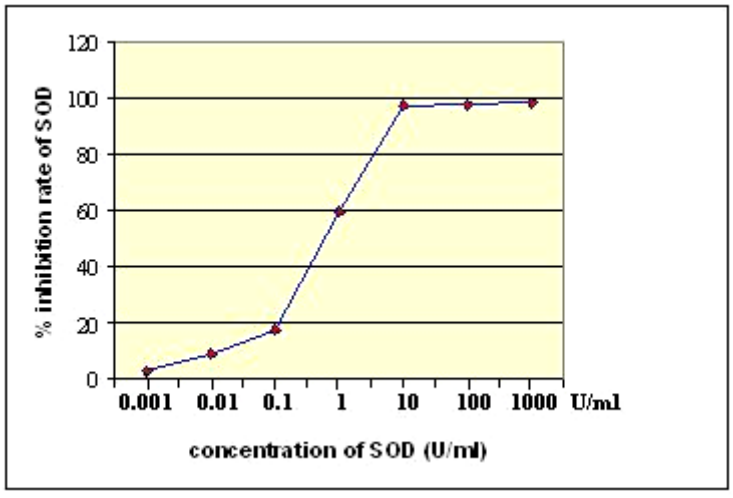


Table 3.1

Protocol of SOD working sheet

	Sample	Blank 1	Blank2	Blank3
Sample solution	20µl		20µl	
ddH_2O		20µl		20µl
WST working solution	200µl	200µl	200µl	200µl
Enzyme working solution	20µl	20µl		
Dilution buffer			20µl	20µl

All sample and standard tube were mixed and incubated at 37 °C for 20 min and presented in table 3.1. SOD activity was determined at λ 450 nm. Calculated the SOD activity in % inhibition rate as the equation and reported in U/ml/mg tissue.

SOD activity (%inhibition rate)

= {[(**A**blank1-**A**blalank3)-**A**sample-**A**blank2]/(**A**blank1-**A**blank3)}× 100

*** A = value at absorbance 450 nm**

SOD activity was calculated from standard curve and presented in Unit/ml/mg tissue.

3.9 Immunohistochemistry of p38MAPK

We examined p38MAPK as redox-sensitive protein through ROS/RNS pathway activated by oxLDL via LOX-1 receptor by immunohistochemistry technique as described by Mayer (Mayer, Leone, Hainfeld, & Bendayan, 2000).

Method

Cultured human umbilical artery was washed ×3 times with PBS. The tissues was fixed with 4% formaldehyde for 10 min at room temperature and 10 min at 4 °C, washed × 3 times with PBS and immersed the tissue in PBS, respectively. Frozen section medium was poured cover the frozen chuck. Place the tissue and cover on the frozen section by frozen medium. Allow the tissue and frozen section medium was freezed (10 min at −22 °C). Tissue in frozen medium was cut off to 4 μM thickness and tissue section was placed on a poly-L-lysine (HistoGrip™) coated slide. The glass slide with the frozen section was stored at –80 °C until used. Using a Pap pen, outline the tissue sections on the glass slide in order to prevent the reagent from spreading over the entire surface of the slide.

Tissue was stained as following: placed the frozen section slide in humidify staining chamber, then, PBS was added to cover the slide and incubated 5 min at room temperature. PBS was removed carefully and treated the slides with 0.3% H_2O_2/PBS (Peroxo-Block™) for 45 sec for eliminated endogenous peroxidase activity, and washed immediately. The slide was added 2 drops (100 μL) of serum blocking solution (1% BSA in 0.01M PBS) for blocking non-specific binding and then incubated for 15 min at room temperature in a humidified chamber. Tissue section was blotted off solution and let it dried without any perturbation. It was added by diluted primary antibody for p38 MAPK [(rabbit anti-phospho-p38MAPK (thr180/tyr182)] (Zymed®Laboratories, invitrogen immunodetection, USA) (1:100) in 0.01M PBS and incubated for 1 hr at room temperature. Then the tissue slide was rinsed by PBS-T × 3 times with 2 min of each period and followed by 0.01 M PBS. For detected by secondary antibody, the tissue section was dropped by diluted secondary antibody Goat F $(ab')_2$ anti-rabbit Ig's HRP (1:300) in 0.01M PBS (Invitrogen, USA), incubated for 15 min at room temperature. After that, those tissue slide was rinsed by PBS-T × 3 times with 2 min of each period.

To detect bound antibodies, DAB solution (DAB substrate kit, Zymed® laboratory, Invitrogen, USA) was applied for 2 drops (100ul) that enough for covered slide incubated for 30 min at room temperature and then washed 2-3 times in 0.01M PBS with of 10 min of each period. The tissue was count-stained with hematoxylin for 30 seconds and washed with ddH_2O until clear. The tissue slide was

dried, mounted with mounting media (Histomount ^{TM}, Invitrogen, USA), covered by coverslip, and took photograph under light microscope. The p38 MAPK activity was quantitated the density by using Image proplus software analysis Version 6.3 (MediaCybernetic, USA).

3.10 Morphological changes by light microscope

Method

Cultured human umbilical artery was sectioned by paraffin method. The tissues were sliced very thin and then processing which involved the following steps; fixation, dehydration, embedment and subsequent sectioning with a microtome**.** Routine fixation involved the chemical cross-linking of proteins (to prevent enzyme action and digestion) and the removal of water. The cultured human umbilical artery was fixed in diluted 4% formaldehyde in PBS for preserve the general organelle structure of the cell. The fixation of cultured artery was performed at room temperature for 15 min and followed at 4 °C for 15 min, respectively. Consequently, any remaining fixative was washed out with PBS at room temperature. Removal of PBS let the tissue to be dehydrated.

The dehydration process was accomplished by passing the tissue through a series of increasing alcohol concentrations. The blocks of tissue was transferred sequentially to 50% alcohol and followed by different concentration of alcohol for 30 min of each period; twice in 70%, twice in 95%, and 3 × times in 100% alcohol, respectively. Notably, alcohol was hydroscopic and absorbed water vapor from the air. After dehydration, the tissues went to embed in paraffin. Before paraffin embedding, the tissues was sequentially suspended twice in 100% of xylene about 20 min of each period. Then, the tissue was embedded with pure paraffin; xylene: paraffin (2:1), xylene: paraffin (1:1), and 2 × paraffin for about 20-30 min of each period and placed in an oven at 56-58 °C, respectively (the melting temperature of paraffin). Paraffin was infiltrated into tissue, then the tissue was placed in embedded mold and melted paraffin was poured into the mold to form a block. The blocks were allowed to cool. The tissue paraffin was sectioned about 4-5 μm thickness by microtome and placed on slide. Hematoxylin and eosin was used in the staining

processing.. First, deparaffinization of paraffin sections was done in an oven at 56-58 °C and rehydrated as follows: 3 × xylene, 2 × 100% alcohol, 2 × 95% alcohol, once in 80% alcohol, and once in water about 20 min of each period. Blotted excess water from slide holder before going to hematoxylin was performed. The section was stained with hematoxylin for 3 min and sequentially rinsed in tap water for 5 min. Then, sections were fast de-stained in blue solution and rinsed in tap water for 5 min and then stained with once for 30 second in eosin. Next, dehydration of sections was transferred sequentially to three times in 95% alcohol for 5 min of each period, three times in 100% alcohol for 5 min of each period and blotted any excess alcohol. Finally, section was transferred to three times in xylene for 15 min. The section was covered with permount solution, followed with coverslip on to slide and dried. Morphology changes were observed under light microscope.

3.11 Statistic analysis

All data are expressed as mean $\pm$ SEM. Data were analyzed by ANOVA: Dunett's test. Significant difference was taken at P value < 0.05.