ผลของตะกั่วต่อเอนไซม์พาราออกโซเนสในเซลล์เพาะเลี้ยงเฮพจี 2 และเซลล์แมคโครฟาจ
ที่พัฒนามาจาก ทีเอชพี-1

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
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EFFECTS OF LEAD ON PARAOXONASE ENZYMES IN HEPG2 CELLS AND THP-1 DIFFERENTIATED MACROPHAGE CELLS

Miss Wanida Sukketsiri

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biopharmaceutical Sciences

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Thesis Title	EFFECTS OF LEAD ON PARAOXONASE ENZYMES IN HEPG2 CELLS AND THP-1 DIFFERENTIATED MACROPHAGE CELLS
By	Miss Wanida Sukketsiri
Field of Study	Biopharmaceutical Sciences
Thesis Advisor	Associate Professor Pol.Lt.Col. Somsong Lawanprasert, Ph.D.
Thesis Co-advisor	Associate Professor Laddawal Phivthong-ngam, Ph.D. Sureerut Porntadavity, Ph.D.

Accepted by the Faculty of Pharmaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

……. Dean of the Faculty of Pharmaceutical Sciences
(Associate Professor Pinthip Pongpetch, Ph.D.)

THESIS COMMITTEE

…….. Chairperson
(Associate Professor Nongluksna Sriubolmas, Ph.D.)

…….. Thesis Advisor
(Associate Professor Pol.Lt.Col. Somsong Lawanprasert, Ph.D.)

…….. Thesis Co-advisor
(Associate Professor Laddawal Phivthong-ngam, Ph.D.)

…….. Thesis Co-advisor
(Sureerut Porntadavity, Ph.D.)

…….. External Member
(Associate Professor Soontharee Tantrarongroj, M.Sc)

…….. Member
(Associate Professor Piyawan Surinrut, Ph.D.)

…….. Member
(Assistant Professor Rataya Luechapudiporn, Ph.D.)

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วนิดา สุขเกษศิริ: ผลของตะกั่วต่อเอนไซม์พาราออกโซเนสในเซลล์เพาะเลี้ยงเฮพจี 2 และเซลล์แมคโครฟาจที่พัฒนามาจาก ทีเอชพี-1. (EFFECTS OF LEAD ON PARAOXONASE ENZYMES IN HEPG2 CELLS AND THP-1 DIFFERENTIATED MACROPHAGE CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร.พ.ต.ท.หญิงสมทรง ลาวัณย์ประเสริฐ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.ดร. ลัดดาวัลย์ ผิวทองงาม, อ.ดร.สุรีรัตน์ พรธาดาวิทย์, 123 หน้า.

ตะกั่วเป็นโลหะหนักที่พบปนเปื้อนมากในสิ่งแวดล้อม เป็นสาเหตุสำคัญทำให้เกิดปัญหาสุขภาพ พิษจากสารตะกั่วก่อให้เกิดพยาธิสภาพและโรคต่างๆ เช่น โรคหัวใจและหลอดเลือด การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลของตะกั่วต่อเอนไซม์พาราออกโซเนส (PON) ทำการศึกษาผลของตะกั่วต่อ PON1 PON2 และ PON3 ในเซลล์เฮพจี 2 รวมทั้งศึกษาผลของตะกั่วต่อ PON2 โดยใช้เซลล์แมคโครฟาจที่พัฒนามาจาก ทีเอชพี-1 ทำการประเมินการอยู่รอดของเซลล์โดยการบ่มเซลล์ด้วยตะกั่วอะซิเตตที่ความเข้มข้น 0, 0.05, 0.1, 0.5, 1, 10, 100 และ 1000 ไมโครกรัม/มิลลิลิตร เป็นเวลา 24, 48 และ 72 ชั่วโมง ศึกษาผลของตะกั่วต่อสารออกซิเจนที่ว่องไวและปฏิกิริยาออกซิเดชันของไขมัน โดยการบ่มเซลล์ด้วยตะกั่วอะซิเตตที่ความเข้มข้น 0, 0.05, 0.1, 0.5, 1, 10, และ 100 ไมโครกรัม/มิลลิลิตร เป็นเวลา 1 ชั่วโมง ศึกษาผลของตะกั่วต่อสมรรถนะของ PON1 PON2 และ PON3 โดยบ่มเซลล์เฮพจี 2 หรือเซลล์แมคโครฟาจที่พัฒนามาจาก ทีเอชพี-1 ด้วยตะกั่วอะซิเตตที่ความเข้มข้น 0, 0.05, 0.1, 0.5, 1, 10, และ 100 ไมโครกรัม/มิลลิลิตร ศึกษาการแสดงออกของโปรตีนและเมสเซนเจอร์อาร์เอ็นเอ (mRNA) ของ PON โดยวิธี Western blot analysis และ real time RT-PCR ตามลำดับ ผลการศึกษาพบว่า ตะกั่วไม่มีผลลดการอยู่รอดของเซลล์เพาะเลี้ยงทั้งสองชนิด ที่ความเข้มข้นถึง 100 ไมโครกรัม/มิลลิลิตร พบการเพิ่มขึ้นของสารออกซิเจนที่ว่องไวอย่างมีนัยสำคัญแต่ไม่มีผลเปลี่ยนแปลงปฏิกิริยาออกซิเดชันของไขมัน ในเซลล์เพาะเลี้ยงทั้งสองชนิด ตะกั่วไม่มีผลต่อสมรรถนะของ PON1 แต่มีผลลดสมรรถนะของ PON2 อย่างมีนัยสำคัญในลักษณะที่ขึ้นกับความเข้มข้นและระยะเวลาสัมผัสในเซลล์เฮพจี 2 โดยไม่มีผลเปลี่ยนแปลงสมรรถนะของ PON2 ในเซลล์แมคโครฟาจที่พัฒนามาจากทีเอชพี-1 ตะกั่วมีผลลดสมรรถนะของ PON3 เฉพาะที่เวลา 24 ชั่วโมง ในเซลล์เฮพจี 2 การเปลี่ยนแปลงสมรรถนะของ PON2 ไม่สอดคล้องกับผลของตะกั่วต่อการแสดงออกของโปรตีน PON2 และ mRNA แคลเซียมมีผลทำให้ฤทธิ์ยับยั้งของตะกั่วต่อสมรรถนะของ PON2 กลับคืนสู่ปกติได้ เป็นข้อมูลบ่งชี้ว่าตะกั่วลดสมรรถนะของ PON2 โดยการแทนที่แคลเซียมในโครงสร้างของ PON2 การเปลี่ยนแปลงสมรรถนะของ PON3 จากตะกั่วมีความแตกต่างจาก PON2 ที่การลดลงของสมรรถนะของ PON3 มีความสอดคล้องบางส่วนกับการลดลงของโปรตีนและ mRNA ของ PON3

สาขาวิชา เภสัชศาสตร์ชีวภาพ ..... ลายมือชื่อนิสิต .....

ปีการศึกษา 2554 ..... ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์ร่วม .....

ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5076955333 MAJOR: BIOPHARMACEUTICAL SCIENCES
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WANIDA SUKKETSIRI: EFFECTS OF LEAD ON PARAOXONASE ENZYMES IN HEPG2 CELLS AND THP-1 DIFFERENTIATED MACROPHAGE CELLS. THESIS ADVISOR: ASSOC. PROF. POL.LT.COL. SOMSONG LAWANPRASERT, Ph.D., THESIS COADVISOR: ASSOC. PROF. LADDAWAL PHIVTHONG-NGAM, Ph.D., SUREERUT PORNTADAVITY, Ph.D., 123 pp.

Lead, an ubiquitous contaminant in the environment, is recognized as one of the important health problems. Its toxicities manifests in various pathological symptoms and diseases including cardiovascular diseases. The aim of this study was to explore effects of lead on paraoxonase (PON) enzymes. Effects of lead on PON1, PON2 and PON3 were assessed in HepG2 cells while effect of lead on PON2 was also assessed in THP-1 differentiated macrophage cells. Cell viability was determined by exposing cells to various concentrations (0, 0.05, 0.1, 0.5, 1, 10, 100 and 1000 μg/ml) of lead acetate for 24, 48 and 72 hours. Reactive oxygen species (ROS) generation and lipid peroxidation were assessed by exposing cells to various concentrations (0, 0.05, 0.1, 0.5, 1, 10 and 100 μg/ml) of lead acetate for 1 hour. Effects of lead acetate on PON1, PON2, and PON3 activities were determined using specific substrates after HepG2 cells or THP-1 differentiated macrophage cells were exposed to various concentrations (0, 0.05, 0.1, 0.5, 1, 10 and 100 μg/ml) of lead acetate for 24, 48 and 72 hours. PON protein and mRNA expressions were assessed by Western blot analysis and real time RT-PCR, respectively. The results showed that lead did not significantly decrease cell viability of both cells types at concentrations of lead acetate up to 100 μg/ml. Significant increase of ROS was shown in both cell types. Significant decrease of PON2 activity after lead exposure was observed in a concentration- and time-dependent manner in HepG2 cells but not in THP-1 differentiated macrophage cells. No effect of lead was observed on PON1 activity while decreases of PON3 activity were observed only at 24 hours of lead exposure in HepG2 cells. Modulation of PON2 activity by lead exposure was not associated to the modulation of both PON2 protein and PON2 mRNA expression. Calcium could restore the inhibitory effect of lead on PON2 activity suggesting that lead decreased PON2 activity via replacement of Ca^{2+} in the structure of PON2 enzyme. In contrast to PON2, modulation of PON3 activity by lead exposure was partly associated to the decrease of PON3 protein and mRNA expression.

Field of Study: Biopharmaceutical sciences
Academic Year: 2011

Student's Signature: .....
Advisor's Signature: .....
Co-advisor's Signature: .....
Co-advisor's Signature: .....

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LIST OF ABBREVIATIONS

α	= alpha
ALA	= delta aminolevulinic acid
ALAD	= delta aminolevulinic acid dehydratase
ALAS	= delta-aminolevulinic synthetase
ANOVA	= analysis of variance
AP-1	= activated protein-1
Apo AI	= apolipoprotein AI
ApoE	= apolipoprotein E
ARE	= arylesterase
ATCC	= american type culture collection
ATSDR	= agency for toxic substances and disease registry
β	= beta
bp	= base pair
BSA	= bovine serum albumin
˚C	= degree celcius
Ca^{2+}	= calcium ion
$CaCl_2$	= calcium chloride
CAT	= catalase
cm	= centimeter
Co	= cobalt
Cr^{2+}	= chromium ion
Ct	= threshold cycles
Cu	= copper
Cys	= cysteine
DCF	= 2′,7′-dichlorofluorescin
DCFH	= 2′,7′-dichlorofluorescein
DCFH-DA	= 2′,7′-dichlorofluorescein diacetate
DHC	= dihydrocoumarin
dl	= deciliter

DMSO	= dimethylsulfoxide
EDTA	= ethylenediaminetetraacetic acid
e.g.	= example gratia
EPA	= environmental protection agency
ER	= endoplasmic reticulum
ERK1/2	= extracellular signal-regulated kinase
et al.	= et alii
FBS	= fetal bovine serum
Fe^{2+}	= ferrous ion
γ	= gamma
g	= gram
g	= gravity
GI	= gastrointestinal
GPx	= glutathione peroxidase
HCl	=hydrochloric acid
HDL	= high density lipoprotein
HepG2	= hepatocarcinoma cell line
Hg	= mercury
HMDM	= human monocyte-derived macrophages
HPLC	= high performance liquid chromatography
HRP	= horseradish peroxidase
IARC	= international agency for research on cancer
IQ	= intelligence quotient
KCl	= potassium chloride
kDa	= kilo dalton
kg	= kilogram
K_2HPO_4	= di- potassium hydrogen phosphate
KH_2PO_4	= potassium dihydrogen phosphate
L	= liters
LDL	= low density lipoprotein

µg	= microgram
µl	= microlitre
µmol	= micromole
µM	= micromolar
M	= molar
MDA	= malondialdehyde
mg	= milligram
mg/kg	= milligram per kilogram body weight
ml	= millitre
mM	= millimolar
MM-LDL	= minimally modified low density lipoprotein
mmole	= millimole
Mn	= manganese
MPM	= mouse peritoneal macrophage
MTT	= 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenltetrazolium bromide
MW	= molecular weight
NaCl	= sodium chloride
NADPH	= nicotinamide adenine dinucleotide phosphate
NaOH	= sodium hydroxide
NHANES II	= national health and nutrition examination survey
NO	= nitric oxide
NPHSII	= northwick park heart study II
OCV	= optimal condition variance
OD	= optical density
OP	= organophosphorus insecticide
Ox-LDL	= oxidized low density lipoprotein
%	= percentage
Pb	= lead
Pb^{2+}	= lead ion
PCR	= polymerase chain reaction

PBS = phosphate buffer saline
PDGFR-β = platelet-derived growth factor receptor-β
pH = potential of hydrogen
PHA = phytoheamagglutinin
PJ = pomegranate juice
PKC = protein kinase C
PI3K = phosphatidylinositol 3-kinase
PMA = phorbol-12-myristate-13-acetate
*p*OHMB = *p*-hydroxymercurybenzoate
PON1 = paraoxonase 1
PON2 = paraoxonase 2
PON3 = paraoxonase 3
PONs = paraoxonases
PPARγ = peroxisome proliferator-activated receptor gamma
Py-5-N = pyrimidine-5′-nucleotidase
RBC = red blood cells
RCV = routine condition variance
ROS = reactive oxygen species
r.p.m. = revolution per minute
SD = standard deviation
SDS = sodium dodecyl sulphate
SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM = standard error of the mean
SH = sulfhydryl
SOD = superoxide dismutase
SREBP-2 = sterol regulatory element-binding protein
TBA = thiobarbituric acid
TBARS = thiobarbituric acid reactive substance
TBST = tris buffered saline-0.05% tween 20

TEP	= 1,1,3,3-tetraethoxypropane
THP-1	= human monocytic cells
Tris	= tris (hydroxymethyl) aminomethane
U	= unit
uPA	= urokinase plasminogen activator
VLDL	= very low density lipoprotein
WHO	= world health organization
Zn^{2+}	= zinc ion

CHAPTER I

INTRODUCTION

Lead is a heavy, bluish-gray metal that occurs naturally in the Earth's crust. It is rarely found naturally as a metal but usually found combined with two or more other elements to form lead compounds. Lead and its salts are widely used in many industries such as lead-based paint, battery, solder, alloy, etc. It has become wildly contaminated in the environment. The amount of lead used in many products has been reduced in recent years to minimize the harmful effect of lead on human health and environment. Because the use of lead as a gasoline additive has been gradually phased out in 1970s and since environmental protection agency (EPA) banned the use of lead containing gasoline for highway transportation in 1996, amount of lead released into the air has further decreased. Although its use in paints was banned in 1978, human exposure to lead continues because unlike other organic chemicals released to the environment, lead does not degrade to other substances [Agency for Toxic Substances and Disease Registry (ATSDR), 2007]. Both occupational and environmental exposures to lead remain a serious problem in many developing and industrialized countries. Lead toxicity manifests in various pathological symptoms of either acute or chronic illness. Its toxicity is closely related to its accumulation in certain tissues and its interference with the bioelements, whose role is critical for several physiological processes. Lead causes many undesired effects, including neurological (Moreira et al., 2001a; Soltaninejad et al., 2003), behavioural (Moreira et al., 2001b; Marco et al., 2005), immunological (Razani-Boroujerdi et al., 1999; Ercal et al., 2000; Bunn et al., 2001a; Bunn et al., 2001b), cardiovascular, renal (Loghman-Adham, 1997; Patra et al., 2001; Vargas et al., 2003), hepatic (Patra et al., 2001), and haematological (Mousa et al., 2002; Siraprasad et al., 2003) dysfunctions. Lead poisoning is defined as a blood lead level equal to or greater than 10 μg/dl. A positive association between lead exposure and cardiovascular disorders including coronary artery disease, stroke, and peripheral arterial disease in general population was reported (Staessen et al., 1995; Lustberg and Silbergeld, 2002; Menke et al., 2006; Schober et al., 2006; Navas-Acien et al., 2007a; Navas-Acien et al., 2007b). *In vivo* and *in vitro* studies suggested that chronic lead exposure caused hypertension, atherosclerosis and cardiovascular disease

by promoting oxidative stress, decreasing nitric oxide availability, impairing nitric oxide signaling, promoting inflammation, disturbing vascular smooth muscle calcium signaling, etc (Vaziri, 2008). Several lines of evidence suggest that cellular damage mediated by oxidants may involved some pathologies associated with lead intoxication. Lead was reported to induce oxidative stress which was attributed to the pathophysiology of lead poisoning (Villeda-Hernandez et al., 2006). Lead-induced oxidative stress was due to the increased the level of reactive oxygen species and depletion of cell antioxidant defense (Lima-Hermes et al., 1991; Monteiro et al., 1995). Furthermore, lead damages a multitude of enzymes and essential cellular structures because lead inhibits action of calcium, affecting calcium-dependent enzymes or the related processes, as well as interacts with protein containing sulfhydryl, amine, phosphate, and carboxyl groups (ATSDR, 2007).

Paraoxonases (PONs) are a family of proteins that play a role in providing relief from both xenobiotics as well as physiological oxidative stress. The paraoxonase (PON) gene family consists of three members: paraoxonase1 (PON1), paraoxonase2 (PON2), and paraoxonase3 (PON3), located adjacent to each other on the long arm of chromosome 7 in humans and chromosome 6 in mice (Primo-Parmo et al., 1996). Of the PONs family, PON1 is the most investigated and best understood member. PON2 is the oldest member in the evolution, followed by PON3 and PON1 (Draganov and La Du, 2004). PON1 and PON3 are exclusively synthesized in the liver and excreted in the blood where they associated with high density lipoprotein (HDL) (Leviev et al., 1997; James and Deakin, 2004). In contrast, PON2 is widely expressed in many tissues, including heart, liver, kidney, lung, placenta, small intestine, spleen, stomach, testis as well as in the cells of the artery wall such as endothelial cells, smooth muscle cells, and macrophage cells (Ng et al., 2001; Ng et al., 2005). It has been suggested that physiological role of PONs is related to the metabolism of lipid mediators produced by the oxidation of polyunsaturated fatty acid (Teilber et al., 2003; Draganov et al., 2005). All PON proteins are implicated in the pathogenesis of several diseases related to oxidative stress and inflammatory condition including atherosclerosis. They inhibit atherogenesis by hydrolyzing lipid hydroperoxides and homocysteine thiolactone, two vascular toxins that enhance risk of such the disease as well as preventing low-density lipoprotein (LDL) oxidative modification (Draganov and La Du, 2004). The common characteristic of PON

proteins is the capability to reverse the development of atherosclerosis via reduction of oxidative stress, promotion of cholesterol efflux from macrophages and normalization of vascular endothelium function (Mackness et al., 2006; Ng et al., 2006a; Shin et al., 2007; Ng et al., 2007).

As mention above, PONs possesses antioxidant properties in an association with antiatherosclerotic effect whereas lead toxicity in part is proposed to be associated to oxidative stress. Whether or not lead toxicity attributed from oxidative stress can be explained by the effect of lead on PONs is questioned. However, lead was recently found to inhibit PON1 activity in serum of workers (Li et al., 2006; Permpongpaiboon et al., 2011) as well as purified PON1 in human serum (Ekinci and Beydemir, 2010). To further elaborate these findings, the aim of study is to investigate effect of lead on paraoxonase (PON1, PON2, and PON3) enzyme activities *in vitro* using HepG2 cells as well as effect of lead on PON2 activity using THP-1 differentiated macrophage cells. The possible mechanisms of lead that modulate paraoxonase enzymes are also investigated.

Hypothesis

Lead inhibits paraoxonase enzymes (PON1, PON2, and PON3) by modulating the enzyme activity or the gene expression of the enzymes.

Objectives

1. To assess effects of lead on paraoxonase (PON1, PON2 and PON3) activity in hepatocarcinoma cell line (HepG2 cells) and effects of lead on PON2 activity in THP-1 differentiated macrophage cells.
2. To investigate cytotoxicity of lead in HepG2 cells and THP-1 differentiated macrophage cells
3. To investigate the possible mechanism of lead to modulate PONs.

Experimental design

To investigate the toxicological effects of lead acetate on paraoxonase enzymes, HepG2 cells and THP-1 differentiated macrophage cells were used. The experiments were performed as followings: Cell viability was measured by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenltetrazolium bromide reduction assay. Lipid peroxide and intracellular

reactive oxygen species production were assessed by spectrofluorometry using thiobarbituric acid reactive substance and 2′,7′-dichlorofluorescein diacetate, respectively. Paraoxonase activities were performed using the selective substrates of PON1, PON2 and PON3. Mechanism of lead to modulate PONs was assessed by quantitatively determining paraoxonase protein and mRNA levels using Western blot analysis and real time RT-PCR, respectively. The process of this study was shown in Figure 1.

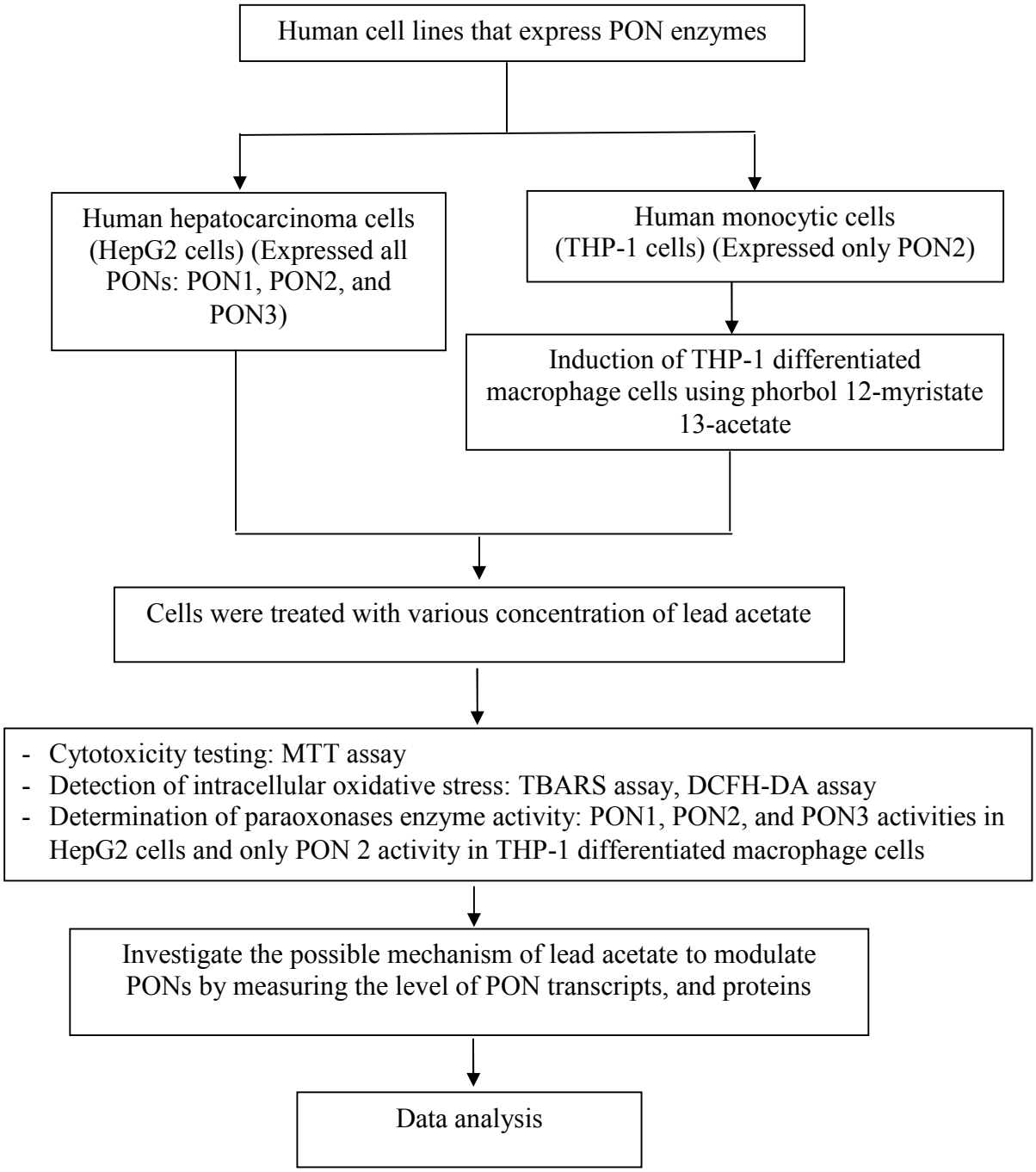

Figure1. Process of the study

Anticipated benefits from the study

The study was provided the information regarding the effect of lead on PONs and the mechanism of lead-induced modulation of PONs. This information will elaborate the explanation for lead toxicities that are related to oxidative stress such as atherosclerosis, cardiovascular diseases as well as other organ toxicities.

Conceptual framework

This study is designed to investigate effect of lead on PONs (PON1, PON2 and PON3) using HepG2 cells because all PONs are expressed in the liver. Effect of lead on PON2 is also investigated using THP-1 differentiated macrophage cells because PON2 is ubiquitously expressed in macrophage cells. Normally, production of oxidative stress is one of the mechanisms of lead-induced organ toxicity and PONs are found to protect cells from oxidative stress. Thus, modulation of PONs by lead (if occur), in term of inhibition, was in part explain the mechanism of lead toxicity such as organ toxicity, atherosclerosis and cardiovascular diseases. Mechanism of lead to modulate PONs was investigated by determining the activity, protein enzyme and mRNA of PONs that are modulated.

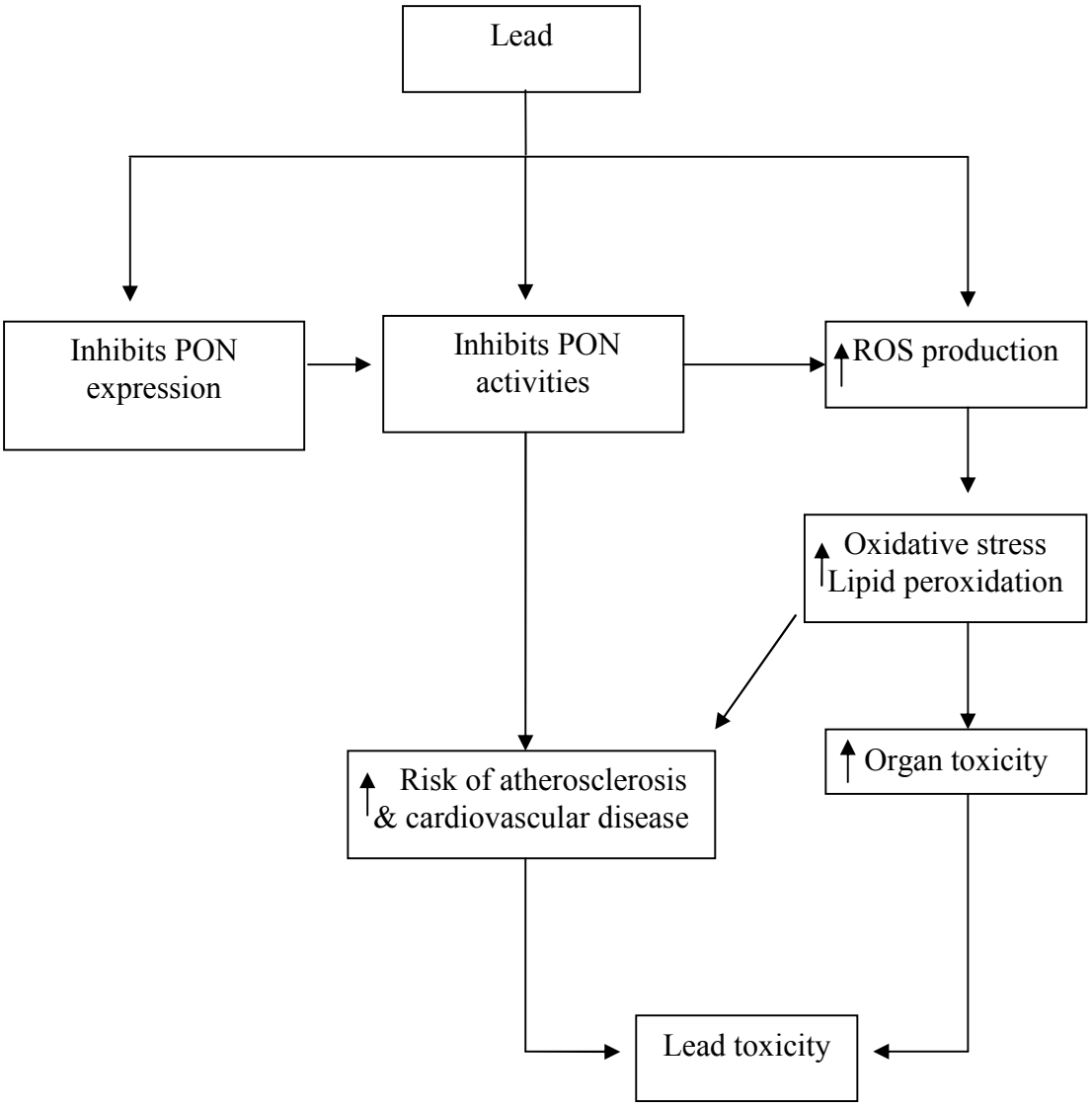

Figure2. Conceptual framework of the study

CHAPTER II

LITERATURE REVIEWS

1. Lead

1.1 Backgrounds

1.1.1 Chemical and physical information

Lead is a naturally occurring element and is a member of Group 14 (IVA) of the periodic table. Natural lead is a mixture of four stable isotopes, ^{208}Pb (51-53%), ^{206}Pb (23.5-27%), ^{207}Pb (20.5-23%), and ^{204}Pb (1.35-1.5%). Lead isotopes are the stable decay product of three naturally radioactive elements: ^{206}Pb from uranium, ^{207}Pb from actinium, and ^{208}Pb from thorium. It has an atomic number of 82. The atomic weight is 207.19 with the density of 11.34, melting point of 327.5 ^{o}C and boiling point of 1725 ^{o}C. Lead exists in three oxidation states: 0, +2 and +4, but it is usually in the state of +2 in inorganic compounds. It is soluble in nitric acid, but relatively insoluble in sulfuric acid and hydrochloric acid. Lead sulfide, phosphate, carbonate, and oxides are insoluble or practically insoluble in water; lead chloride is slightly soluble; and lead acetate, subacetate, and nitrate are soluble in water [International Agency for Research on Cancer (IARC), 1980; Goyer, 1988; Budavari et al., 1989]. Lead has an ability to form alloy with other metals which have defined utilities in many industries (Reilly, 1991).

1.1.2 The uses of lead

Lead is widely used in many industries and everyday lives. Lead may be used in the form of metal, either pure or alloyed with other metals, or as chemical compounds. The commercial importance of lead is based on its ease of casting, high density, low melting point, low strength, ease of fabrication, acid resistance, electrochemical reaction with sulfuric acid, and chemical stability in air, water, and soil (Sutherland and Milner, 1990; King and Ramachandran, 1995; Shea, 1996). Lead is used in the manufacture of batteries; alloys, brass and bronze and some solders; sheets and pipe for nuclear and x-ray shielding, cable covering, noise control materials; chemical resistant linings; ammunition; and pigments. Lead compounds are also used in glass making, ceramic glazes, plastic stabilizers, caulk, and paints.

Certain dispersive uses of lead that led to widespread exposure, such as tetraethyl lead in gasoline, water pipe, solder in food cans, lead shot and sinkers, paints, have been or are being phased out due to environmental and health concerns. New environmentally safe uses for lead include radiation protection in computer, television, diagnostic magnetic imaging, and other nuclear medical technology; circuit boards in computers and other electronic equipment; piezoelectric ceramics; superconductor technology; and high purity lead oxides used in optical technology (ATSDR, 2007). Also, the use of lead based anti-knock agent was discontinued in many countries due to the impact on the environmental and health (Boontherawara, 1994).

1.2 Human exposure to lead

Lead occurs naturally in the environment. However, most of the high levels of lead found throughout the environment come from human activities. Environmental levels of lead have increased more than 1,000-fold over the past three centuries as a result of human activity. Lead can enter the environment through releases from mining lead and other metals, and from factories that make or use lead, lead alloys, or lead compounds. Lead is a natural element that persists in water and soil. Lead particles in the atmosphere have a residence time of about 10 days. Lead is commonly found in soil especially near roadways, older houses, old orchards, mining areas, industrial sites, near power plants, incinerators, landfills, and hazardous waste sites. People living near hazardous waste sites may be exposed to lead and chemicals that contain lead by breathing air, drinking water, eating foods, or swallowing dust or dirt that contain lead.

General population exposure: In the general population, food and beverages are the major sources of lead exposure. Lead aerosol is main source of lead contamination in food (Flegal et al., 1990). Contamination of lead in food is also from cooking with lead-contaminated water and storage in lead-contaminated containers. Atmospheric lead aerosols may be the major source of lead contamination in drinking water. Lead toxicity may result from storage of alcoholic and the other beverage in unsuitable metal or glaze earthenware containers (Reilly, 1991). However inhalation can also be a route to exposure of lead (Goyer, 1991). Lead in the atmosphere comes from a huge variety of natural sources such as dusts, volcanic output and human activities such as mining, industrial uses and petrol combustion (Reilly, 1991). It has

already been noted that the concentration of airborne lead depended on proximity to traffic, whether indoor or outdoor and time of the day. The resident time of lead in the atmosphere vary with a number of factors including particle size (Harrison and Laxen, 1981). Lead contamination in children contrast with in adult, generally, the main source of lead in their diet is often from the ingestion of nonfood item such as soil, leaded paint chips, and toy through normal oral exploratory behavior and normal hand-to-mouth activity [World Health Organization (WHO), 2000; ATSDR, 2007].

Occupational lead exposure: People who are exposed at work are usually exposed by breathing in air that contains lead particles. Lead and lead compounds play a significant role in modern industry, with lead being the most widely used nonferrous metal. There are several occupations and industries that associate with lead exposure such as battery manufacturing, chemical industry, construction workers, demolition workers, foundry workers, gas-station attendants, gasoline additives, jewelers, lead miners, lead smelters, pigment manufacturing, pipe fitters, plastics industry, pottery workers, printers, rubber industry, soldering of lead, stained-glass makers, and welders (WHO, 2000; ATSDR, 2007). Workers are exposed to lead by breathing in lead dust or fumes from work activities, or particles of lead may be swallowed from eating, drinking or smoking in lead-contaminated areas. Between 0.5 and 1.5 million workers are exposed to lead in the workplace.

1.3 Toxicokinetics

Absorption via inhalation is a major route of absorption for occupational exposure. Absorption of lead from the respiratory tract is dependent on the size of the particles inhaled and the fraction deposited in the lungs. More than 90% of the lead contained in particles deposited in the lungs is absorbed into the blood. In a general population, the main route of absorption is gastrointestinal (GI) tract (WHO, 1995). The extent and rate of GI absorption of ingested inorganic lead are influenced by physiological states of the exposed individual including age, fasting, nutritional calcium, iron status, pregnancy and physicochemical characteristics of the medium ingested such as particle size, mineralogy, solubility, and lead types. GI absorption of water-soluble lead appears to be higher in children than in adults. GI absorption in children appears to be higher 50% for lead in food and beverages. In adult humans, absorption of lead was reported to be 10-15% of ingested lead. Lead absorption in

children is affected by nutritional status. Children who are iron deficient have higher blood lead concentrations, which would suggest that iron deficiency increase the absorption rate of lead. Dietary deficiencies of copper, zinc, calcium, iron and protein, and increase in dietary fat cause an increase in absorption of lead.

Three major compartments for the distribution of lead are blood, soft tissue, and bone. In circulating blood, almost all (99%) blood lead is associated with erythrocytes, where much of it is bound to hemoglobin. Biological half-life of blood lead is 25-28 days when blood lead is in equilibrium with other compartments. Soft tissues that take up lead are liver and kidneys, with smaller amounts taken up by brain and muscle. The largest fraction of lead retained in the body is found in bone. Total body lead in bone is about 95% in adults as compared to only 73% in children. However, some lead can leave from bones and reenter to blood and organs under certain circumstances (e.g., during pregnancy and periods of breast feeding, after a bone is broken, and during advancing age). Although bone lead is a large, relatively inert fraction with a half-life of greater than 20 years, there is a "labile" fraction that is in equilibrium with soft tissue lead.

Body does not change lead into any other form as well as inorganic lead ion is not metabolized in the body. Approximately 75% of inorganic lead absorbed into the body is excreted in urine and less than 25% is excreted in feces. Lead is also excreted in breast milk and therefore, available for intake by infants (Gulson et al., 1995).

1.4 Toxic effects and mechanism of toxicity

The systemic uptake resulting from exposure to lead in the various media (air, water, diet, soil) contributes to the total body lead burden, which in turn is linked to the adverse effects. Concentration of lead in blood is used as a measure of exposure. Therefore, effects of lead cannot be described in terms of route specificity. Lead can induce a wide range of adverse effects in humans depending on the dose and duration of exposure. Toxic effects from exposure to lead involve several organs, systems and biochemical activities. Children are most sensitive to effects in the central nervous system, while adults peripheral neuropathy, chronic nephrophathy, and hypertension (ATSDR, 2007). Due to the multi-modes of action of lead in biological systems, lead could potentially affect many system or organs in the body (Figure 3).

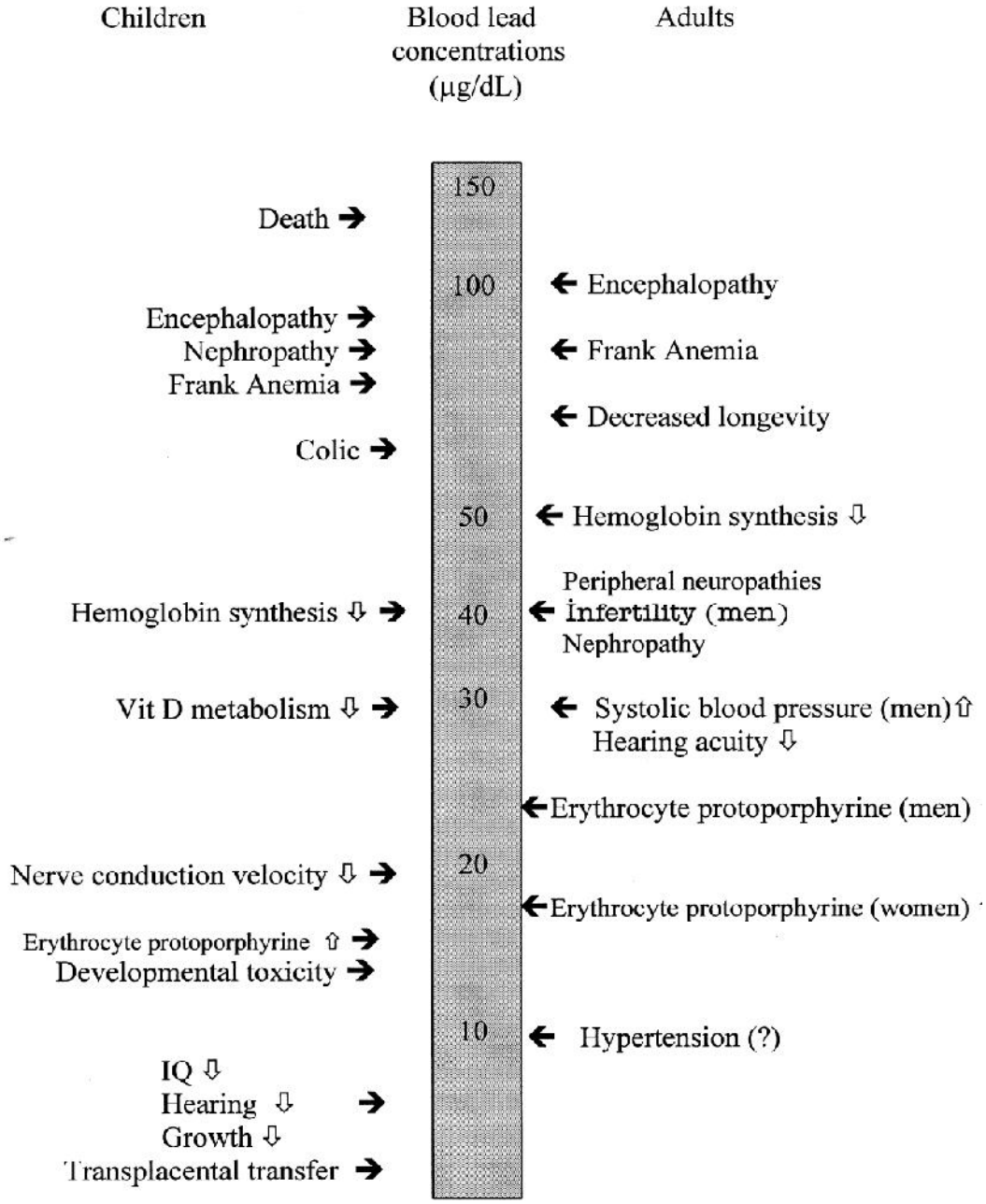

Figure 3. Effects of inorganic lead in an association to blood lead concentration in children and adults. (Gurer and Ercal, 2000)

1.4.1 Neurotoxic effects

Lead can impair cognitive function in children and adults, but children are more vulnerable than adults. The increased vulnerability is due in part to the

relative importance of exposure pathways and differences in toxicokinetics. Clinically, overt lead encephalopathy may occur in children with high exposure to lead, probably at blood lead level of 70 μg/dl or higher. Symptoms of lead encephalopathy begin with lethargy, vomiting, irritability, loss of appetite, and dizziness, progressing to obvious ataxia, and a reduced level of consciousness, which may progress to coma and death (Goyer, 1990; Bellinger, 2004; Laraque and Trasande, 2005; ATSDR, 2007). The most sensitive indicators of adverse neurological outcomes are psychomotor tests or mental development indices, and broad measures of intelligence quotient (IQ). Most studies report a 2- to 4-point IQ deficit for each μg/dl increase in blood lead level within the range of 5-35 μg/dl. Recent studies found that deficits in cognitive and academic skills could occur with blood lead level < 5.0 μg/dl (Lamphear et al., 2000). A cohort study of children from pregnancy through 10 years of age found that lead exposure around 28 weeks of gestation is a critical period for later child intellectual development, and lead's effect on IQ occurs with a few of blood lead level (Schnaas et al., 2006). Adults with occupational exposure may demonstrate abnormalities in a number of measures in neurobehavior with cumulative exposures resulting from blood lead level > 40 μg/dl (Lindgren et al., 1996). Peripheral neuropathy is a classic manifestation of lead toxicity in adults. Peripheral neuropathy is characterized by segmental demyelination and possibly axonal degeneration. Motor nerve dysfunction, assessed clinically by electrophysiological measurement of nerve conduction velocities, occurred with blood lead level as low as 40 μg/dl (Goyer, 1990). Nerve conduction velocity has provided a more sensitive indicator of peripheral nerve function. In adult, prolonged and severe lead poisoning with blood lead levels above 80-100 μg/dl causes distal motor neuropathies. The clinical sign of the disease is characterized by extensor weakness, wrist drop and foot drop without sensory impairment (WHO, 1995; Bloom and Brandt, 2000). For the central nervous system, lead can cause an acute encephalopathy, which is a pathologic change and cerebral edema. In adult, acute encephalopathy is associated with blood lead levels greater than 120 μg/dl. Clinical manifestation of acute lead encephalopathy in adults is often characterized by a predromal syndrome, including vomiting, irritability, loss of appetite, dizziness, decrease alertness and loss of memory, orientation and perception. In addition, it may develop rapidly to massive cerebral edema, convulsion, coma and death (Castellino et

al., 1995; WHO, 1995; Bloom and Brandt, 2000). Lead can affect the brain by multiple mechanisms (Goyer, 1996; ATSDR, 2007). It may act as a surrogate for calcium and/or disrupt calcium homeostasis. The stimulation of protein kinase C (PKC) may result in alteration of blood brain barrier and inhibition of cholinergic modulation of glutamate-related synaptic transmissions. Lead affects virtually every neurotransmitter system in the brain, including glutamatergic, dopaminergic, and cholinergic systems. All these systems play a critical role in synaptic plasticity and cellular mechanisms for cognitive function, learning, and memory.

1.4.2 Hematologic effects

Blood lead is distributed between plasma and erythrocyte. Lead has a high affinity binding to erythrocytes up to blood lead levels approximately 50 μg/dl. Lead is bound to hemoglobin in blood and has greater affinity for fetal than adult hemoglobin. Free form of lead in blood is important in relation to biological activity. Lead has long been known to alter the hematological system. The anemia induced by lead is microcytic and hypochromic and results primarily from both inhibition of heme synthesis and shortening of the erythrocyte lifespan (Goyer, 1991; Goyer, 2007). The adverse hematological effects of lead are mainly the result of its perturbation of the heme biosynthesis pathway. Lead interferes with heme synthesis by altering the activities of δ-aminolevulinic acid dehydrogenase (ALAD) and ferrochelatase. As a consequence of these changes, heme biosynthesis is decreased and the activity of the rate-limiting enzyme of the pathway, δ-aminolevulinic synthetase (ALAS), which is feedback inhibited by heme, is subsequently increased. The end results of these changes in enzyme activities are increased urinary porphyrins, coproporphyrin, and δ-aminolevulinic acid (ALA); increased blood and plasma ALA; and increased erythrocyte protoporphyrin (Figure 4). Four possible ways for lead-inhibited ALAD. Firstly, it is a direct inhibitory effect by the binding of lead ion (Pb^{2+}) to the sulfhydryl (–SH) group in the active site (Masci et al., 1995; Ahamed and Siddiqui, 2007). In addition, lead can also inhibit ALAD enzyme by displacing a zinc ion at the metal binding site, not the active site. The inhibition causes a change in the enzyme's quaternary structure leads to denaturation of protein (Warren et al., 1998).

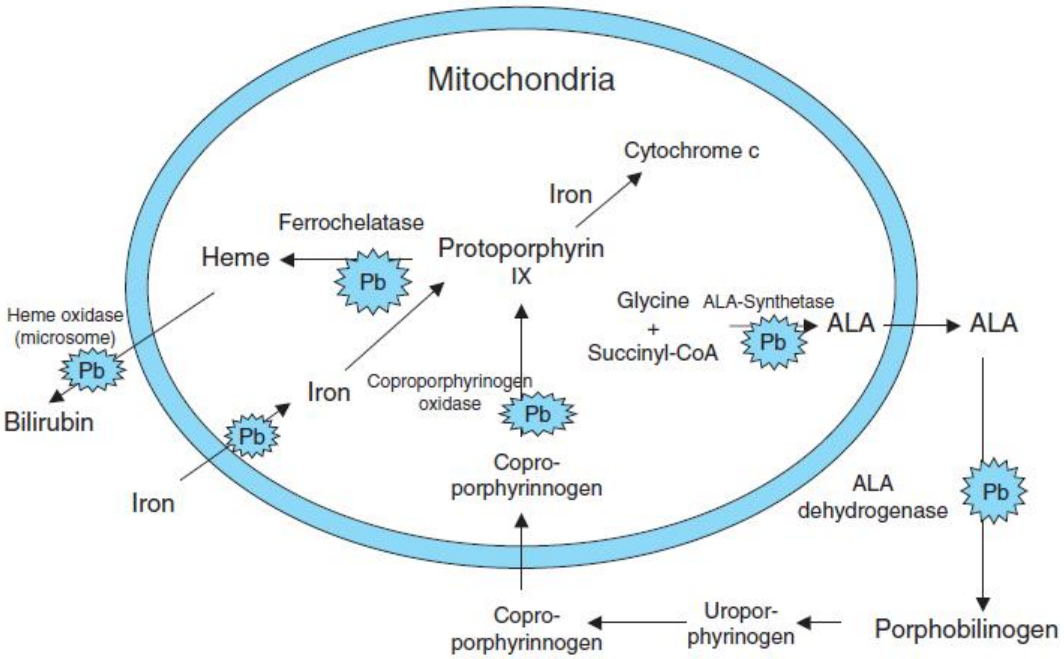

Figure 4. Lead interruption of heme biosynthesis.

Activity of another erythrocyte enzyme, pyrimidine-5′-nucleotidase (Py-5-N), is significantly reduced in lead-exposed individuals at blood levels of 30 µg/dL; reduced activity has been detected at lead levels of below 5 µg/dL, with no clear threshold. The consequence of reduced Py-5-N activity is thought to be accumulation of cellular nucleotides, reduced erythrocyte stability and survival, and reduced mRNA and protein synthesis related to production of the globulin chain. Furthermore, inhibition of erythrocyte Py-5-N activity may be indicative of a widespread impact on pyrimidine metabolism in other tissues besides blood. The possibility of generalized effects on pyrimidine metabolism and heme biosynthesis has serious implications regarding the health hazards of very low levels of lead, especially in children (Paglia et al., 1975, 1977; Buc and Kaplan, 1978; Angle et al., 1982).

1.4.3 Cardiovascular system effects

Although lead has been shown to produce various cardiovascular effects in animals (Vaziri and Sica, 2004), end points of greatest concern for humans at low exposures of lead and low blood lead levels are elevations in systemic blood pressure and decrements in glomerular filtration rate. Other cardiovascular changes have been noted in association with increasing lead body burdens and/or lead

exposures in humans include changes in cardiac conduction and rhythm (Kirkby and Gyntelberg, 1985; Cheng et al., 1998; Böckelmann et al., 2002). The association between lead exposure and blood pressure is established. Multiple studies in animal models and human populations show a causal relationship between low-level lead exposure and hypertension (ATSDR, 2007). Lead has been shown to produce various cardiovascular disorders including coronary artery disease, peripheral arterial disease, and stroke. Association between chronic lead exposure and hypertension in adult individuals has been irrefutably confirmed by numerous studies in experimental animal and human (Staessen et al., 1994; Schwartz, 1995; Gonick and Behari, 2002; ATSDR, 2007; Navas-Acien et al., 2007b). A cohort study of 590 men indicated an increase incidence of hypertension in individuals with elevated bone lead (Hu et al., 1996). Analysis of data from the National Health and Nutrition Examination Survey (NHANES II) of the U.S. population, with 5803 peoples demonstrated a correlation between blood lead level at relatively low levels and an increase in blood pressure (Harlan, 1988; Den Hond et al., 2002; Vupputuri et al., 2003). An epidemiology reappraisal using meta-analysis of 58,518 subjects from both the general population and occupationally exposed groups from 1980-2001 suggested a weak, but significant association between blood lead level and blood pressure (Nawrot et al., 2002). At higher levels of exposure in humans, lead produces cardiac lesions and electrocardiographic abnormalities. Several studies reported the associations between lead exposure and cardiovascular disease mortality and morbidity (Michaels et al., 1991; Schober et al., 2006). A variety of mechanisms may contribute to the increased blood pressure that is observed with chronic exposure to lead (Vaziri, 2008). Lead affects important hormonal and neural systems that contribute to the regulation of peripheral vascular resistance, heart rate and cardiac output (Khalil-Manesh et al., 1993; Carmignani et al., 2000; Ni et al., 2004; Vaziri and Sica, 2004), as well as depletion of nitric oxide (NO), which plays an important role in regulating of blood pressure. NO depletion induced by lead is thought to derive, at least in part, from oxidative stress and associated with increased activity of reactive oxygen species (ROS) and reactive nitrogen species (Vaziri et al., 1999a, 1999b; Ding et al., 2001). Lead may also disrupt the vasodilatory actions of NO by altering cell-signaling mechanisms in endothelial cells. Lead exerts direct constrictive effects on vascular smooth muscle, which are thought to be mediated by inhibition of Na^+-K^+ ATPase

activity and associated to elevation of intracellular Ca^{2+} levels, possibly through activation of PKC (Piccinini et al., 1977; Kramer et al., 1986; Watts et al., 1995; Hwang et al., 2001). These mechanisms include the ability of lead to inhibit or mimic the action of calcium and to interact with proteins. Regarding the interaction with proteins, lead binds with virtually available functional group, including sulfhydryl, amine, phosphate, and carboxyl groups, with the highest affinity to sulfhydryl group (ATSDR, 2007).

1.4.4 Hepatic effects

In children, exposure to lead was shown to inhibit formation of the heme-containing protein cytochrome P450, as reflected by decreased activity of hepatic mixed-function oxygenases. Two children with clinical manifestations of acute lead poisoning did not metabolize the test drug antipyrine as rapidly as did the controls (Alvares et al., 1975). Another study found a significant reduction in 6-hydroxylation of cortisol in children who had positive urinary excretion of lead upon ethylenediamine tetraacetic acid (EDTA) provocative tests as compared to the control group (Saenger et al., 1984). These biochemical transformations are mediated by hepatic mixed-function oxygenases. The association between lead exposure and serum lipid profile was examined in a study of Kristal-Boneh et al. (1999). The mean blood lead level of the 87 workers in a battery factory and a recycling factory was 42.3 ± 14.9 μg/dl and that of the 56 control subjects was 2.7 ± 3.6 μg/dl. The results showed that higher values of total cholesterol and the decrease of HDL-cholesterol were found in the workers as compared to the controls. No significant differences were seen for LDL-cholesterol and triglycerides. A study in rats administered with lead acetate for 7 weeks resulted in mean blood lead level of 17 and 32 μg/dl in lead exposure groups of 35 and 70 mg/kg, respectively. The results showed a dose-related increase in triglycerides and decrease in HDL-cholesterol in lead exposure group. An increase of serum triglycerides could be explained by an effect of lead-induced inhibition of hepatic lipoprotein lipase activity (Skoczynska et al., 1993). Liver function in industrial workers including serum aspartate aminotransferase and alanine aminotransferase activities, were not significant changed. However, significant increases in alkaline phosphatase and lactate dehydrogenase activities in the serum of those workers were observed (Al-Neamy et al., 2001).

1.4.5 Renal effects

Lead nephrotoxicity is characterized by proximal tubular nephropathy, glomerular sclerosis and interstitial fibrosis (Loghman-Adham, 1997; Goyer, 1989; Diamond, 2005). Functional deficits in humans that have been associated with excessive lead exposure include enzymuria, low- and high-molecular weight proteinuria, impaired transport of organic anions and glucose, and depressed glomerular filtration rate. A few studies have revealed histopathological features of renal injury in humans, including intranuclear inclusion bodies and cellular necrosis in the proximal tubule and interstitial fibrosis (Cramer et al., 1974; Biagini et al., 1977; Wedeen et al., 1975, 1979). Acute lead nephrotoxicity consists of proximal tubular dysfunction which can be reversed by treatment with chelating agents (Loghman-Adham, 1998). Acute exposure to lead is known to cause proximal renal tubular damage, characterized by generalized aminoaciduria, glycosuria and hyperphosphaturia with relative hypophosphatemia (Endo et al., 1993). Chronic lead nephrotoxicity consists of interstitial fibrosis and progressive nephron loss, azotaemia and renal failure (Goyer, 1989). Lead nephrotoxicity impairs the renal synthesis of heme-containing enzymes in the kidney, such as heme-containing hydroxylase involved in vitamin D metabolism causing bone effects (ATSDR, 2007). Hyperuricemia with gout occur more frequently in the presence of lead nephropathy (Batuman, 1993). Lead nephropathy is also a cause of hypertension (Gonick and Behari, 2002). On the other hand, irreversible chronic high-dose lead exposure can cause tubular nephropathy, hypertension, gout, and arteriosclerosis (Longman-Adham, 1998; Levin and Goldberg, 2000; Gurer-Orhan et al., 2004). Chronic interstitial nephropathy is mostly associated with prolonged exposure to lead, and blood lead levels greater than 40 μg/dl (Royce and Needelman, 1990).

1.4.6 Immunological and lymphoreticular effects

Lead is immunosuppressive. Studies of occupational exposure to lead suggested that lead inhibited proliferation of lymphocyte to phytoheamagglutinin (PHA) (Mishar et al., 2003) leading to decreased peripheral B lymphocyte (Bloom and Brandt, 2000). Kimber et al. (1986) reported that responses to PHA and NK cell activity were not altered in their study of workers whose mean blood lead level was 34.8 μg/dl, compared with an unexposed group with a mean blood lead levels of 11.8

µg/dl. Ündeger et al. (1996) and Basaran and Ündeger (2000) described a significant decrease in the number of CD4+ cells and C3 and C4 complement levels in workers with a mean blood lead levels of 74.8 µg/dl. A significant decrease in percentage and number of CD3+ and CD4+ cells also was observed in the study of firearm instructors, but other cell types including CD8+, B-lymphocytes, or NK cells were not significantly altered relative to controls (Fischbein et al., 1993).

1.4.7 Gastrointestinal effects

Gastrointestinal disturbance (colic) is a sign of acute lead intoxication, generally occurring at blood lead levels of 100–200 ug/dl in adults, but it may also occur at levels of 40–60 ug/dl. In children, gastrointestinal disturbances occur at levels greater than or equal to 60 ug/dl. Gastrointestinal symptoms include abdominal pain, constipation, cramps, nausea, vomiting, anorexia, and weight loss (WHO, 1995; ATSDR, 2007).

1.4.8 Reproductive system effects

Lead adversely affects the reproductive system both in male and female. In male population, blood lead has been correlated with reduced human semen quality (Alexander et al., 1996), which include asthenospermia, hypospermia, teratospermia and hypogonadism. This effect may result from chronic exposure to lead and blood lead levels of 40-50 µg/dl (WHO, 1995). Lead increases the frequency of spontaneous abortion (Lindbohm et al., 1999) and decreases length of gestation as observed in women who lived close to a smelter and had blood lead levels higher than 23 µg/dl. Adhikari and colleague (2001) reported that lead could induce apoptosis in the germ cells within the semineferous tubules.

1.4.9 Carcinogenic effects

The association of lead exposure with increased human cancer risk was strengthened by recent studies (ATSDR, 2007). Inorganic lead compounds were recently reclassified as probably carcinogenic to humans (IARC, 2004). A cohort study of 20,700 workers exposed to lead found a 1.4 fold increase in the overall cancer incidence and a 1.8 fold increase in lung cancer as compared to those who ever had elevated blood lead level (Anttila et al., 1995). Another epidemiological study of

27,060 brain cancer cases and 108,240 controls that died of nonmalignant disease in US from 1984 to 1992 provides evidence for a potential link between occupational exposure to lead and brain cancer (Cocco et al., 1998). A meta-analysis of published data on cancer incidence among workers in various industries with lead exposure indicates a significant excess of cancer deaths from stomach cancer, lung cancer, and bladder cancer (Fu and Boffetta, 1995). Several mechanisms have been proposed for lead-induced carcinogenesis, including inhibition of a regenerative repair, inhibition of DNA synthesis or repair, generation of ROS with oxidative damage to DNA, substitution of lead for zinc in transcriptional regulators, interaction with DNA-binding proteins, and aberrant gene expression (Silbergeld et al., 2000; Qu et al., 2002; Silbergeld, 2003).

1.5 Mechanism of lead induce reactive oxygen species production

In vitro studies found that production of ROS increased after lead treatment in aortic endothelial cell (Ding et al., 2000), coronary endothelial cell (Vaziri et al., 2001; Ni et al., 2004) and vascular smooth muscle cell (Ni et al., 2004). Also, *in vivo* studies suggested that lead exposure cause generation of ROS and alteration of antioxidant defense systems in animals (Lawton and Donaldson, 1991; Tandon et al., 2002; Burns et al., 2003; Fowler et al., 2004; Farmand et al., 2005), and in workers (Ye et al., 1999; Guillaume et al., 2004; Faruk et al., 2004). Furthermore, oxidative stress associated with the presence of lead in mammalian tissues and organs (predominately blood, liver, brain, and kidneys) appears to be one possible molecular mechanism for lead toxicity (Ercal et al., 1996). Lead-induced oxidative stress is mostly due to increases hydroxyl radical generation in both intact animal and cultured endothelial cell (Ding et al., 2000).

Lead is considered to be a strong hemolytic agent, and is able to cause erythrocyte destruction through the formation of lipid peroxides in red blood cells (RBC) membranes (Ribarov et al., 1981) leading to hemoglobin oxidation (Gurer et al., 2000). Lead can bind strongly to phosphatidylcholine in the RBC membrane, leading to a decrease in phospholipid levels (Shafiq-ur-Rehman et al., 1993), decrease of cell membrane fluidity, permeability and function and increase rate of erythrocyte hemolysis (Lawton et al., 1991). Mechanism for lead induced-membrane oxidative damage is the changes in fatty acid composition of membrane because fatty acid chain

length and unsaturation are associated with membrane susceptibility to peroxidation. Also, lead could affect membrane-related processes such as the activity of membrane enzyme, endocytosis and exocytosis, the transport of solutes across the bilayer, and signal transduction process (Adonaylo and Oteiza, 1999; Hsu and Guo, 2002). The interaction of lead with oxyhemoglobin has been suggested as an important source of superoxide radical formation in RBC. Lead inhibited ALAD is a well-known mechanism of lead toxicity. As a result, elevated levels of the ALA are found in blood and urine of lead-exposed subjects. These elevated levels of ALA enhance generation of hydrogen peroxide and superoxide radical anion, and also interact with oxyhemoglobin, resulting in the generation of hydroxyl radicals, which is the most reactive free radicals (Bechara et al., 1996).

Many evidences show that oxidative stress plays an important role in hypertension. Antioxidant supplementation has been shown to reduce the degree of hypertension in rats (Vaziri et al., 2000). In addition, Vaziri and colleagues (2004) described that high level of blood pressure in lead exposure is involving with NO metabolism, which plays an important role in blood pressure homeostasis. Normally, NO is necessary for endothelium-dependent vasodilation, inhibition of smooth muscle cell proliferation, platelet aggregation, and monocyte adhesion (Boger et al., 2005). NO is also known as an endothelium-relaxing factor. NO can be inactivated by ROS resulting in nitric oxide deficiency (Vaziri et al., 1998). Also, increased peroxynitrite levels, which are a highly active ROS, can damage macromolecule of cells (Halliwell et al., 1997).

Several studies have shown that lead induces oxidative stress via depleting of antioxidant defense system, one of which is glutathione metabolism. Glutathione, a cysteine-based molecule produced in the interior compartment of the lymphocyte, acts as an antioxidant substance for quenching ROS (Vaziri et al., 2000). Heavy metal (e.g. mercury, arsenic, lead, etc.) have high affinity with sulfhydryl groups. Binding of lead to the sulfhydryl group of glutathione causes glutathione losing its antioxidant functions (Christie et al., 2001). Concentrations of glutathione in blood have been shown to be significantly lower than the control levels in animals exposed to lead (Farmand et al., 2005), in lead-exposed children (Ahamed et al., 2005) and workers (Sung et al., 2005). Lead also binds to antioxidant enzymes, such as catalase (CAT), superoxide dismutase (SOD) that have sulfhydryl groups, leads to dysfunction of

antioxidant enzymes resulting in increased oxidative stress. Alternatively, lead can inhibit or mimic the actions of essential trace elements in antioxidant enzyme such as selenium, zinc, copper, calcium resulting in inactivating the enzymes such as SOD, CAT and glutathione peroxidase (GPx) (Guillaume et al., 2004; Faruk et al., 2004). Lead is known to inhibit heme synthesis, and CAT is a heme-containing enzyme, it causes CAT activity to decrease (Mylroie et al., 1984). Hence, PON1 is a potential target of lead toxicity because its activity depends on calcium and sulfhydryl groups. PON1 is the enzyme that plays an important role on detoxifying organophosphate and also convincingly believed to be an anti-atherosclerosis due to its antioxidant property in inhibiting the oxidation of LDL and HDL as well as decreasing oxidative stress. Previous studies have shown that lead and several other metal ions are able to inhibit PON1 activity *in vitro*. Effects of several metal ions [Co, Cu, Mn, Hg and *p*-hydroxymercurybenzoate (*p*OHMB)] on purified PONs (e.g. PON1 and PON3) from rat liver were studied. Among all the compounds tested, Hg and *p*OHMB were the most potent inhibitors of PON1. For PON3, mercurials and copper showed the highest inhibitory potency (Pla et al., 2007). Moreover, Pb^{2+}, Cr^{2+}, Fe^{2+}, and Zn^{2+} inhibited PON1 activity in purified PON1 in human serum (Ekinci and Beydemir, 2010). Li et al. (2006) demonstrated that lead exposure decreased serum PON1 activity. They conducted a cross-sectional study of workers from a lead battery manufactory and lead recycling plant. Blood samples were analyzed for whole-blood lead levels and serum PON1 activity. The mean blood lead level of the subjects this study was 27.1 ± 15 μg/dl. Multiple linear regression analysis showed that blood lead levels were significantly associated with the decreased serum PON1 activity in the workers.

1.6 Biological indices of lead exposure and body burden

1.6.1 Blood lead

Blood lead levels are the best indicator of recent lead exposure and used as the internal index for biological monitoring and epidemiological survey. Blood lead concentration increases with age, alcohol consumption, cigarette smoking and environmental pollution. Blood comprises <2% of the total lead burden; most of the lead burden resides in bone (Barry, 1975). The elimination half-time of lead in blood is approximately 30 days (Griffin et al., 1975; Chamberlain et al., 1978);

therefore, lead concentration in blood relatively reflects, mainly, the exposure history of the previous few months and does not necessarily reflect the larger burden and much slower elimination kinetics of lead in bone (Lyngbye et al., 1990; Graziano, 1994). Lead intake-blood lead relationships also vary with age as a result of age-dependency of gastrointestinal absorption of lead, and vary with diet and nutritional status (Mushak, 1991).

1.6.2 Bone and tooth lead

Lead in bone is considered a biomarker of cumulative exposure to lead because lead accumulates in bone over the lifetime and most of the lead body burden resides in bone. Lead is not distributed uniformly in bone. Lead will accumulate in those regions of bone undergoing the most active calcification at the time of exposure. During infancy and childhood, bone calcification is most active in trabecular bone, whereas in adulthood, calcification occurs at sites of remodeling in cortical and trabecular bone (Aufderheide and Wittmers, 1992). Lead levels in cortical bone may be a better indicator of long-term cumulative exposure than lead in trabecular bone, possibly because lead in trabecular bone may exchange more actively with lead in blood than does cortical bone. This is consistent with estimates of a longer elimination half-time of lead in cortical bone, compared to trabecular bone (Borjesson et al., 1997; Brito et al., 2005). Relationships between bone lead levels and health outcomes have been studied in several epidemiology studies, but not as extensively as have other biomarkers of exposure such as blood lead level. These studies suggest that bone lead levels may be predictors of certain health outcomes, including neurodevelopmental and behavioral outcomes in children and adolescents (Needleman et al., 1996; Campbell et al., 2000a), hypertension and declines in renal function in adults (Korrick et al., 1999; Cheng et al., 2001; Gerr et al., 2002; Tsaih et al., 2004).

Tooth lead has been considered a potential biomarker for measuring long-term exposure to lead (e.g., years) because lead that accumulates in tooth dentin and enamel appears to be retained until the tooth is shed or extracted (Ericson, 2001; Gomes et al., 2004).

2. Paraoxonases (PONs) family

PON gene family consists of three members, PON1, PON2, and PON3, located adjacent to each other on the long arm of chromosome 7 between q22.3 and q23.1 in humans (Primo-Parmo et al., 1996). These three members appear to be clustered together as shown in Figure 5 (Hong-Liang et al., 2003). Three human PON genes share approximately 65% similarity at the amino acid level and approximately 70% similarity at the nucleotide level (Mackness et al., 2002). From an evolutionary standpoint, PON2 appears to be the oldest member, followed by PON3, and then PON1 (Draganov et al., 2004).

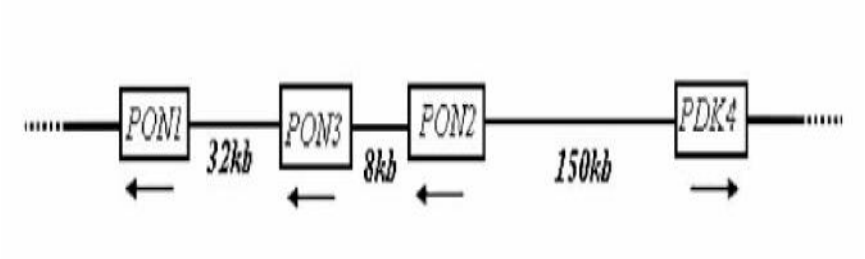

Figure 5. Paraoxonases family (Hong-Liang et al., 2003)

2.1 Paraoxonase 1

2.1.1 PON1 expression and structure

PON1 is a calcium-dependent esterase consisting of 354 amino acids with a molecular mass of approximately 45 kDa (Primo-Parmo et al., 1996). The name PON1 reflects its ability to hydrolyze paraoxon, a metabolite of the organophosphate insecticide, parathion. PON1 are expressed primarily in the liver (Figure 6) and excreted in the blood where they are associated with HDL particles.

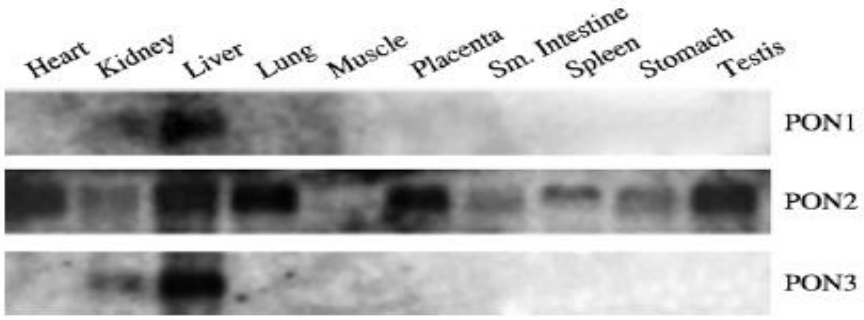

Figure 6. mRNA analysis showing tissue distribution of human PON1 family (Ng et al., 2005)

General structure of human PON1 contains as many as four carbohydrate chains, three cysteine (Cys) residues, two (Cys-42 and Cys-353) of which form a disulfide. A single free cysteine only at position 284 which has a free sulfhydryl group is needed for the action of arylesterase (ARE) activity and inhibition of LDL oxidation (Aviram et al., 1998). PON1 has an extremely hydrophobic N-terminal end that could anchor it to HDL lipids. PON1 is not present in LDL or very low density lipoprotein (VLDL), indicating a specific interaction with HDL by association with apo AI (Sorenson et al., 1999). The general structure of human serum PON1 is shown in Figure 7.

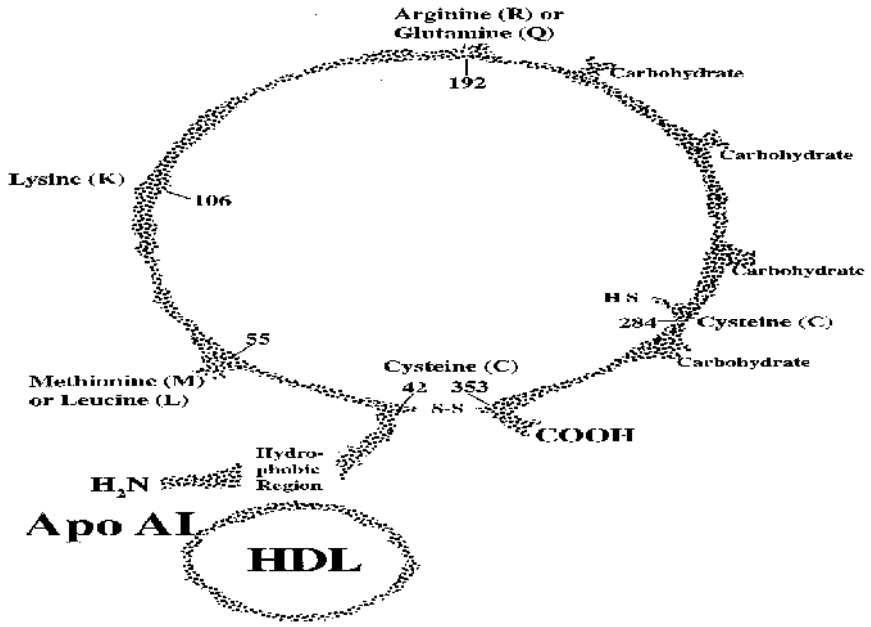

Figure 7. General structure of human serum PON1 (La Du et al., 1999)

PON1 consists of six bladed (1-6) beta propellers and each blade contains four strands (A, B, C and D). PON1 contains 2 calcium ions (Ca^{+2} 1 and Ca^{+2} 2) in the central of the structure which are required for its hydrolytic activity (Figure 8). Aviram and colleague demonstrated that chelating of calcium ion affected to PON1 stability and decreased its activity, but did not interfere with its ability to inhibit LDL oxidation (Aviram et al., 1998).

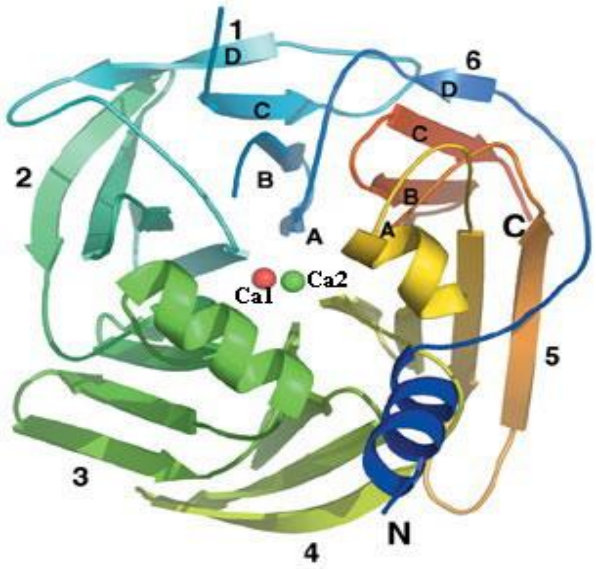

Figure 8. Overall structure of PON1 (Harel et al., 2004)

In addition to paraoxon, PON1 has been shown to hydrolyze metabolites of a number of other insecticides and also to detoxify various nerve agents (Costa et al., 2005). However, evidence gained in recent years suggests that the name PON may be a misnomer, as PON2 and PON3 lack any significant paraoxonase activity (Draganov et al., 2000; Ng et al., 2001; Reddy et al., 2001). PON1, PON2, and PON3 do, however, share an ability to hydrolyze aromatic and long-chain aliphatic lactones, and thus the term lactonase may be more appropriate (Draganov et al., 2004). Nonetheless, the physiological substrates for these proteins have not been identified and are currently under investigation. Interestingly, Draganov and colleagues (2005) characterized the enzymatic activities of the purified recombinant human PONs as shown in Table 1.

Table 1 Specific enzymatic activities of the purified recombinant human PONs (Draganov et al., 2005)

Substrate	PON1	PON2	PON3
Organophosphatase activity (U/mg)			
Paraoxon	1.94 ± 0.11	ND	0.205 ± 0.05
Chlorpyrifos oxon (0.32 mmol/L)	40.9 ± 0.9	ND	ND
Diazoxon	113 ± 5	ND	ND
Arylesterase activity (U/mg)			
Phenyl acetate	1,120 ± 50	0.086 ± 0.0013	4.1 ± 0.3
p-NO_2-acetate	15.0 ± 0.03	0.7 ± 0.07	39.0 ± 4.1
p-NO_2-propionate	13.6 ± 0.04	0.96 ± 0.06	20.7 ± 3.2
p-NO_2-butyrate	1.3 ± 0.015	1.4 ± 0.03	11.4 ± 0.7
Lactonase activity (U/mg)			
Dihydrocoumarin	129.9 ± 8.30	3.1 ± 0.2	126.1 ± 12
Homogentisic acid lactone	329.5 ± 13.1	ND	ND
γ-Butyrolactone	32.1 ± 2.73	ND	0.81 ± 0.1
γ-Valerolactone	45.0 ± 3.7	ND	6.2 ± 0.4
γ-Hexalactone	51.7 ± 4.2	ND	23.9 ± 3.2
γ-Heptalactone	57.2 ± 2.3	ND	27.7 ± 2.7
γ-Octalactone	69.2 ± 4.3	ND	25.6 ± 3.2
γ-Nonalactone	144.7 ± 11.3	ND	30.9 ± 2.7
-Decanolactone	173.8 ± 14.7	ND	45.6 ± 3.6
γ-Undecanolactone	127.6 ± 10.5	ND	71.4 ± 3.1
α-Angelica lactone	183.0 ± 16	ND	20.7 ± 3.2
γ-Phenyl-γ-butyrolactone (0.5 mmol/L)	63.0 ± 3.1	0.68 ± 0.08	11.4 ± 0.7
α-Valerolactone	671 ± 14	ND	14.5 ± 0.7
δ-Hexalactone	72 ± 2.3	ND	11.7 ± 1.2
δ-Nonalactone	150 ± 12.3	ND	11.1 ± 0.9
δ-Decanolactone	251 ± 13	ND	44.3 ± 3.2
δ-Undecanolactone	287 ± 17	ND	84.4 ± 2.7

Substrate	PON1	PON2	PON3
5-HETEL (10 μM)	75.4 ± 8.36	1.83 ± 0.08	27.5 ± 3.6
DL-3-Oxo-hexanoyl-HSL (250 μM)	0.0334 ± 0.0031	0.2683 ± 0.0384	ND
L-3-Oxo-hexanoyl-HSL (250 μM)		0.5080 ± 0.0661	
DL-Heptanoyl-HSL (25 μM)	0.0036 ± 0.0004	0.0311 ± 0.0026	0.0049 ± 0.0023
DL-Dodecanoyl-HSL (25 μM)	0.0167 ± 0.0005	0.4588 ± 0.0371	0.0877 ± 0.0014
DL-Tetradecanoyl-HSL (25 μM)	0.0035 ± 0.0013	0.4239 ± 0.0204	0.0255 ± 0.0003
Lovastatin (25 μM)	ND	ND	0.0266 ± 0.022
Spironolactone (25 μM)	ND	ND	0.011 ± 0.001
Canrenone (25 μM)	ND	ND	0.013 ± 0.001
Lactonizing activity (U/mg)			
Coumaric acid (100 μM)	0.047 ± 0.004	ND	0.013 ± 0.0007
4-HDoHE (10 μM)	1.51 ± 0.16	0.52 ± 0.03	13.7 ± 2.0

5-HETEL, (±)5-hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid 1,5-lactone; HSL, homoserine lactone; ND, not detectable under these assay conditions. Data are averages from two to four measurements ± SD or range. One unit = 1 μmol of substrate metabolized per minute. All substrates were at 1 mM final concentration unless indicated otherwise.

PON1 can be modulated by environmental chemicals, drugs, smoking, alcohol, diet, age, and disease condition. Age plays the most relevant role, as PON1 activity is very low before birth and gradually increases during the first or two years of life in human (Costa et al., 2005). An influence of gender has also been suggested with females displaying higher PON1 activity (Costa et al., 2005). Some studies have shown that PON1 activity was decreased in some diseases such as in diabetic patients (Boemi et al., 2001; Senti et al., 2003), vascular dementia (Dantoine et al.,2002), Alzheimer's disease (Scacchi et al., 2003), Parkinson disease (Liat et al.,

2005), liver cirrhosis and chronic hepatitis (Ferre et al., 2006) as well as patients with renal failure (Dantoine et al., 1998; Hasselwander et al., 1998; Paragh et al., 1998). However, PON1 activity was restored to normal levels after kidney transplantation. These suggesting that the effect on PON1 activity is a consequence of the disease (Dantoine et al., 1998).

Modulation of PON1 is attributed from several pharmaceutical drugs, such as lipid-lowering compounds including statins, fibrates as well as some other drugs. In human, treatments with simvastatin (Deakin et al., 2003; Mirdamadi et al., 2008), or atorvastatin (Mirdamadi et al., 2008; Harangi et al., 2009) have been shown to increase serum paraoxonase activity. In contrast, fluvastatin decreased serum and liver paraoxonase activity. A similar decrease of PON1 mRNA and activity levels induced by pravastatin, simvastatim and fluvastatin in a HuH7 human hepatoma cell line (Gouedard et al., 2003). In hepatocyte cells, simvastatin and pravastatin were found to upregulate the activity of the PON1 promoter by increasing a nuclear transcription factor, Sp1 and sterol regulatory element-binding protein-2 (SREBP-2) (Deakin et al., 2003; Deakin et al., 2007; Arii et al., 2009).

Regarding fibrates, PON1 activity was increased in patients with type 2 diabetes treated with gemfibrozil, in patients with coronary heart disease treated with micronized fenofibrate, and in patients with metabolic syndrome treated with ciprofibrate (Costa et al., 2005; Paragh et al., 2006). Additionally, Gouedard et al. (2003) found a 70% increase in PON1 activity and mRNA in HuH7 cells upon exposure to fenofibrate.

The cholesterol lowering drug probucol was found to up-regulate serum paraoxonase activity and PON1 expression in hepatocytes of hypercholesterolemic rabbits (Hong et al., 2006). Administration of rosiglitazone to diabetic patients was found to cause a small increase in fasting and post-prandial serum PON1 activity (van Wijk et al., 2006). The oral hypoglycemic agents, sulphonylureas including glimepiride and glibenclamide were reported to increase PON1 activity in the liver of control group and streptozotocin-treated group, diabetic rats (Wojcicka et al., 2006).

Several dietary polyphenols, particularly quercetin, have been shown to up-regulate PON1 (Gouedard et al., 2004a; Gouedard et al., 2004b). Low dose of polyphenols was also capable of reversing the decrease of plasma and hepatic PON1

activities and of liver mRNA levels present in hyperhomocysteinemic mice (Noll et al., 2009). Resveratrol, a polyphenolic phytoalexin found in grapes and wine, was shown to increase PON1 gene expression in human hepatocyte primary cultures and in HuH7 cells (Gouedard et al., 2004b; Boesch-Saadatmandi et al., 2010). A series of studies examined the effects of pomegranate juice (PJ) and extract, containing several polyphenolic compounds such as punicalagin, gallic acid and ellagic acid, on PON1 activity and expression. In HuH7 hepatoma cells, PJ and its major polyphenols, up-regulated PON1 expression and release by sequential activation of protein kinase A and peroxisome proliferator-activated receptor gamma (PPAR-γ) (Khateeb et al., 2010). Dietary lipids, consumption of olive oil increased postprandial serum PON1 activity in diabetic patients, particularly in females, while safflower oil had no effect (Wallace et al., 2001). As moderate doses of alcohol exert a protective role in cardiovascular disease by modulating HDL levels, some studies investigated modulation of PON1 by ethanol. An early in vitro study had indicated that several aliphatic alcohols, including ethanol, inhibited human serum PON1 activity (Costa et al., 2005). In contrast, two subsequent studies in humans showed that moderate alcohol consumption caused a small (5-10%) increase in serum PON1 activity (van de Gaag et al., 1999; Sierksma et al., 2002). It has been suggested that an effect of alcohol on PKC, which may phosphorylate Sp1 and regulate its binding to the Sp1 binding site in the promoter region of PON1 (Osaki et al., 2004), may explain the effect of alcohol on PON1 levels (Rao et al., 2003).

2.1.2 PON1 as anti-oxidative and anti-atherosclerosis

Physiological substrates for PON1 have not been identified and are currently under investigation. PON1 from both serum and liver hydrolyses the active metabolites of several organophosphorus (OP) insecticides, arylesters. PON1 may play an important role in diseases where oxidative damage is implicated such as atherosclerosis (Durrington et al., 2001). Mackness and colleagues (1991) found that purified PON1 might play an important physiological role in lipid metabolism and protect against the development of atherosclerosis. PON1 was shown to protect against oxidative stress in mouse peritoneal macrophage and human (Fuhrman et al., 2002; Rozenberg et al., 2003) as well as inhibit macrophage foam cell formation and atherogenesis in J774 A.1 murine macrophage cells (Rosenblat et al., 2006).

Throughout the past decade, effects of PON1 have been clarified especially the antioxidant properties that protect LDL against oxidation and reverse the biological effects of Oxidized-LDL (Ox-LDL) as well as preserve the function of HDL by inhibiting its oxidation (Aviram, 1999; Durrington et al., 2001). PON1 prevented production of reactive aldehydes resulting from lipid peroxidation through hydrolyzing oxidized lipids in a cohort study (Mackness et al., 2003). PON1 null mice were shown to develop atherosclerosis when fed with atherogenic diet, whereas the corresponding wild-type did not. In addition, their HDL failed to prevent LDL oxidation in cultured artery cells and these animals were more susceptible to organophosphate toxicity (Shih et al., 1998). Furthermore, when PON1 null mice were crossed with apoE null mice, PON1/apoE null mice developed significantly larger lesions of atherosclerosis than apoE null mice. LDL freshly isolated from PON1/apoE null mice had higher levels of biologically active phospholipids relative to LDL from apoE null mice, suggesting higher levels of oxidative stress in the double knockout mice. In addition, HDL from PON1/apoE null mice failed to protect LDL against oxidation (Shih et al., 2000; Gang-She et al., 2009). Results from these studies are correlated to the hypothesis that PON1 protects against atherosclerosis and it is importantly contributing HDL of the antioxidant capacity. Furthermore, a decreased lesion size of atherosclerosis was observed in human PON1 transgenic mice (Tward et al., 2002). This information of the *in vivo* studies underscores the potential of PON1 as a therapeutic role to prevent atheroma. Human PON1 attenuates Ox-LDL induced monocyte/endothelial cell interactions in a co-culture model, therefore retarding one of the initiating steps in the inflammatory response leading to atherosclerosis (Mackness et al., 2004). Conversely, macrophages of PON1 knockout mice showed increased oxidative stress, increased lipid peroxides, reduced glutathione and increased capacity to oxidize LDL resulting in macrophage foam cell formation and a marked increase in atherosclerosis development (Aviram and Rosenblat, 2004). Several studies in human showed an inverse linear relationship between the concentration of Ox-LDL in the circulation and PON1 activity, strongly implicating PON1 in the metabolism of Ox-LDL in vivo (Tsuzara et al., 2004; Sampson et al., 2005). Expressing human PON1 in human carotid plaques significantly reduced the total volume of plaque, the volume of macrophages in the plaques (i.e. macrophage size) and the volume of plaque associated Ox-LDL. Human

PON1 in this model inhibited the development of atherosclerosis, probably by reducing the amount of Ox-LDL in plaques, thereby preventing its pro-atherogenic effects and stabilizing the plaque (Tavori et al., 2009). In addition to protect atherosclerosis development, PON1 is shown to associate to various diseases induced by oxidative stress including liver diseases, neurological diseases (Ferre et al., 2001, 2005; He et al., 2006).

2.2 Paraoxonase 2

2.2.1 PON2 structure and expression

PON2 is the only member of paraoxonases that does not presence either in HDL or LDL but ubiquitously expresses in many different tissues. PON2 is first found in brain, liver, kidney, and testis (Mochizuki et al., 1998). Following investigation by Ng et al (2005) PON2 transcripts were detected in nearly all human tissues examined as shown in Figure 6 and highest expression of PON2 was found in heart, lung, liver, placenta, and testis. Interestingly, that studied also revealed that PON2 is expressed in primary and immortalized human endothelial cells, human arterial smooth muscle cells and macrophage (Ng et al., 2001). In the earlier time, PON2 is identified as intracellular protein. In contrast, using the confocal and biochemical cell fraction indicated the prominent enrichment of PON2 in the nuclear envelope and the endoplasmic reticulum (ER) of vascular cells, EA.hy 926. In addition, digestion of extracellular proteins of outermembrane of EA.hy 926 cells with proteinase K demonstrated that PON2 is exclusively intracellular localized not at plasma membrane (Horke et al., 2007).

PON2 gene consists of 9 exons and is encoded for 355 amino acids with an approximately 43 kDa protein. Several mRNA forms of PON2 have been identified which occurred by alternative splicing, or by the use of a second transcription start site (Mochizuki et al., 1998). Until now, there are still no data to support the cell type specific to PON2 isoform since only two isoforms of PON2 protein at 40 and 43 kDa are usually seen in the immunoblotting with specific anti-PON2 antibody in variety cell type. These two isoforms are found to be non-glycosylated and glycosylated protein and there are no reports on their different function (Horke et al., 2007).

PON2 acts as an antioxidant enzyme, thus investigation of PON2 expression in respond to environmental stimuli such as oxidative stress, lipid lowering agents, and polyphenol have been revealed. Both *in vitro* and *in vivo* study found that PON2 expression and enzymatic activity increased under oxidative stress. PON2 is up-regulated by oxidative stress in macrophages, mice fed high fat diets, apoE knockout mice, and hypercholesterolemic patients (Shih et al., 1996, 1998; Reddy et al., 2001; Forte et al., 2002; Rosenblat et al., 2003, 2004). Treatment of mouse peritoneal macrophage with various oxidative stress agents resulted in an increase in PON2 expression and lactonase activity (Rosenblat et al., 2003). Shiner and colleague (2004) found an increased in PON2 expression during monocyte differentiation via reduced nicotinamide adenine dinucleotide phosphate (NADPH), which correlated with increased cellular oxidative stress. In addition, PON2 is inactivated under the low levels of oxidative stress, whereas at the high levels of oxidative stress, cellular compensatory mechanism may act in order to up-regulate PON2 expression in macrophage (Shiner et al., 2006). Hepatic PON2 mRNA was increased in C57BL6 or apoE null mice feed a high-fat, high-cholesterol, cholate-containing diet (Ng et al., 2005) as well as lysophosphatidylcholine stimulated PON2 lactonase activity in J774A.1 macrophages cells (Rosenblat et al., 2006). In addition, macrophages from diabetic mice demonstrate increased oxidative stress associated with activation of NADPH oxidase and up-regulation of cellular PON2, as well as increased macrophages cholesterol uptake and biosynthesis (Hayek et al., 2007). Urokinase plasminogen activator (uPA) activates PON2 promoter through are extracellular signal-regulated kinase (ERK1/2), NADPH oxidase, phosphatidylinositol 3-kinase (PI3K), platelet-derived growth factor receptor-β (PDGFR-β), and tyrosine kinase cascade and SREBP-2 resulted in increase PON2 mRNA (Fuhrman et al., 2008, 2009).

In addition, PON2 has been found to response to exogenous stimuli such as lipid lowering drugs. Atorvastatin therapy up-regulated PON2 expression and activity in human monocyte-derived macrophages (Rosenblat et al., 2004).

PJ and the pomegranate extracts from the different parts resulted in a significant increment in mouse peritoneal macrophage PON2 lactonase activity in atherosclerotic apoE-deficient mice (Aviram et al., 2008). Macrophage PON2 expression (mRNA and protein) and activity are up-regulated dose-dependently by PJ

and PJ-derived phenolic (punicalagin and gallic acid). The transcription factors PPAR-γ and activated protein-1 (AP-1) were shown to play a role in PJ-mediated PON2 up-regulation. In addition, rosiglitazone induced an increase in PON2 expression through activation of PPAR-γ (Shiner et al., 2007). Quercetin supplementation up-regulates PON2 mRNA and protein levels in RAW264.7 murine macrophage cells. The methylated quercetin derivative isohamnetin enhanced PON2 gene expression. However, supplementation human volunteers with quercetin did not change PON2 mRNA levels in human monocytes (Boesch-Saadatmandi et al., 2009).

2.2.2 PON2 as anti-oxidative and anti-atherosclerosis

PON2 also possesses antioxidative properties despite lower than PON1 (Draganov et al., 2005). However, with the wide expression and location, PON2 is resent widely studied. PON2 is an intracellular protein, hence, most investigations are focused on decreased intracellular and local oxidative stress. Hela cells overexpression with PON2 exhibited an antioxidant property by prevent LDL lipid peroxidation, reverse the oxidation of minimally modified LDL (MM-LDL) and inhibit the ability of MM-LDL to induce monocyte chemotaxis (Ng et al., 2001). Over-expression of PON2 in HeLa cells reduced cellular oxidative status and decreased the ability of the cells to Ox-LDL. Furthermore, PON2 has been shown to reduce ROS in HeLa cells (Ng et al., 2001). Rosenblat and colleagues (2003) demonstrated that incubation of purified recombinant PON2 with mouse peritoneal macrophages (MPM) isolated from the atherosclerotic apo E deficient mice protected against LDL oxidation. Stably transfected cells over-expressing PON2 also exhibited significantly lower level of intracellular oxidative stress when exposed to hydrogen peroxide or oxidized phospholipids (Ng et al., 2001). Additionally, mouse peritoneal macrophage isolated from PON2 knockout mouse had increased in foam cells and lipid droplets. It had been also found that PON2 increased microsomal acyl-CoA:diacylglycerol acytranseferas 1 activity and decreased the accumulation of triglycerides (Rosenblat et al., 2009). The incubation of mouse peritoneal macrophage with PON2 or human-transfected PON2 cells had decreased in microsomal acyl-CoA:diacylglycerol acytranseferas 1 activity and accumulation of triglycerides under high glucose as diabetic conditions which had been demonstrated through decreased NADPH-oxidase activity (Meilin et al., 2010). Recently, the data on PON2 as anti-

apoptotic has been revealed that overexpression of PON2 protected against endoplasmic reticulum stress-induced apoptosis when the stress was induced by interference with protein modification but not when ER stress was induced by Ca^{2+} deregulation (Horke et al., 2008). A study in PON2-deficient mice showed that PON2 protected against atherogenesis *in vivo* by modulating lipoprotein properties, thereby reducing cellular oxidative stress and attenuating the inflammatory response (Ng et al., 2006a) whereas adenovirus-mediated PON2 overexpression exerted a protective effect by reducing the plaque area (Ng et al., 2006b). In hypercholesterolemic patients, lower level of PON2 mRNA was seen in monocyte differentiated macrophages relative to individuals with normal cholesterol levels (Rosenblat, et al., 2004). PON2 mRNA and protein expression in human carotids was shown to be decreased during the progression of atherosclerosis (Fortunato et al., 2008). Interestingly, administration of atorvastatin was able to reduce both cellular oxidative stress and cholesterol content leading to up-regulated macrophage PON2 expression and activity (Rosenblat et al., 2004). Recently, Horke and colleagues (2007) investigated the expression, regulation, and potential anti-oxidative functions of PON2 in 3 major cell types of the human vasculature. The results showed that PON2 expressed at similar levels in human endothelial cells, smooth muscle cells, and adventitial fibroblasts, which implied that PON2 represented an endogenous defense mechanism against vascular oxidative stress and unfolded protein response induced cell death, thereby contributing to the prevention of atherosclerosis. In contrast, PON2 expression increased in monocytes during differentiation into macrophages, and this effect was shown to be mediated via the transcription factor AP-1 (Shiner et al., 2007). Upregulation of macrophage PON2 was shown to occur via several mechanisms, including NADPH-oxidase activation (Shiner et al., 2004), unesterified cholesterol accumulation (Shiner et al., 2007), and uPA (Fuhrman et al., 2008, 2009). Macrophage atherogenicity was defined by increased cellular oxidative stress, as well as, lipid accumulation whereas macrophage PON2 reduced cellular oxidative stress (Furhman et al., 2008). Thus, one function of PON2 may act as a cellular antioxidant, protecting cells from oxidative stress, via inhibiting of LDL lipid peroxidation, reversion the oxidation of MM-LDL and inhibiting its ability to induce monocyte chemotaxis as shown in Figure 9. Although the physiological role of PON2 is not well

established, recent studies indicate that PON2 reduces macrophage oxidative stress and acts as a potent cellular anti-oxidant.

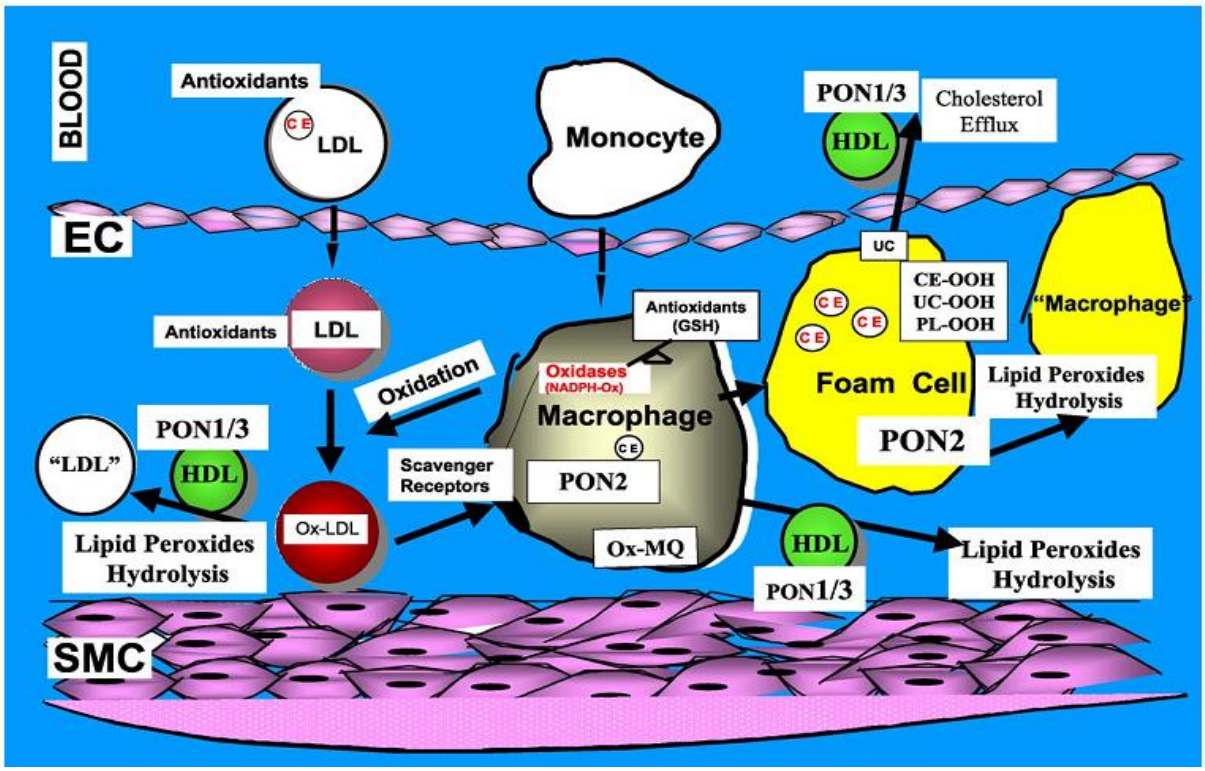

Figure 9. Paraoxonases (PONs) and macrophage foam cell formation (Aviram, 2004)

2.3 Paraoxonase 3

PON3 enzyme is a 40-kDa glycoprotein with calcium-dependent esterase activities. Thus, PON3 is able to catalyze the hydrolysis of a broad range of substrates including aryl-esters, lactones, and many pharmacological agents. PON3 shows high similarity in structure and functions with PON1. Similar to PON1, PON3 are expressed primarily in the liver and secreted into the serum where they are closely associated with HDL. However, significant PON3 mRNA level is also detectable in kidney (Figure 6) (Reddy et al., 2001) and various tissues including muscle and subcutaneous fat tissues (Labrecque et al., 2009). PON3, like PON1, contains the N-terminal hydrophobic peptide and share three conserved cysteine residues: Cys-42, Cys-284, and Cys-353, and possesses similar properties in structure and activities.

Many evidences suggested that PON3 is important in the prevention of atherosclerosis and seems to be a candidate in PONs family (Aviram and Rosenblat,

2004). Purified rabbit PON3 possesses the ability to protect LDL against *in vitro* copper-induced oxidation with approximately 100 times more potent than rabbit PON1 in protecting LDL against oxidation (Draganov et al., 2000). Recently, it was also found that over-expression of human PON3 in mice reduced atherosclerotic lesion (Ng et al., 2007). PON3 is capable of preventing the formation of MM-LDL as well as inactivating preformed MM-LDL. In contrast to PON1, PON3 has very limited ARE activity and no paraoxonase activity, but it can rapidly hydrolyze lactones as shown in Table 1 (Draganov et al., 2000; La Du et al., 2001; Ng et al., 2001). Under oxidative stress, the expression of PON1 is down-regulated while the expression of PON3 is unchanged (Reddy et al., 2001). Unlike PON2, PON3 message remains unchanged in MPM exposed to various oxidative stress inducing agents, although PON3 lactonase activity decreased whereas PON2 expression and enzymatic activity increased (Rosenblat et al., 2003). Draganov and colleagues (2005) reported the expression of PON1, PON2 and PON3 in human tissue using a baculovirus-mediated system. Insect cells used in conjunction with the baculovirus expression vector system are gaining ground rapidly as a platform for recombinant gene expression, especially for proteins that are insoluble in *Escherichia coli* or are covalently modified while Lu et al. (2005), who amplified PON3 cDNA from Human Fetal Liver Marathon-Ready cDNA and expressed it in baculovirus-mediated Sf9 cells at high level. The results showed that purified PON3 from Sf9 cells could inhibit LDL oxidation *in vitro*. Also, recombinant human PON3 was able to retard LDL oxidation, prevent macrophage oxidative stress, and promote macrophage cholesterol efflux (Liu et al., 2008). A recent study showed that elevated expression of human PON3 can reduce the atherosclerotic lesion and obesity in transgenically expressed male mice (Shin et al., 2007).

CHAPTER III

MATERIALS AND METHODS

Materials

1. Experimental cell culture

Human hepatocarcinoma (HepG2) cells were obtained from American Type Culture Collection (ATCC) and human monocytic (THP-1) cells were kindly provided by Assistant Professor Tanapat Palaga, Department of Microbiology, Faculty of Sciences, Chulalongkorn University.

2. Instrument

Instruments used in this study were as following: Adjustable pipette 1-10 µl, 2-20 µl, 10-100 µl, 20-200 µl, 100-1000 µl (Gilson, France), analytical balance (Precisa, Switzerland), blot electrophoresis (Bio Rad, USA), cell culture dish: diameter 100 mm, 6-well cell culture plate, 96- well cell culture plate (Costa, USA), centrifuge (Beckman Microfuge, Germany), CO_2 incubator (ThermoForma Scientific, USA), conical tube: 15, 50 ml (Corning Incorporation, USA), densitometry scanner (Canon, Japan) with program image J (HIH, Maryland), electrophoresis Cell & Mini Trans-Blot electrophoretic transfer cell (Bio-Rad, USA), ELISA microplate reader (Shimadzu, Japan), enhanced chemiluminescence (ThermoScientific, USA), freezer -80 ˚C (Thermoelectron, USA), hemocytometer (Resistant, Germany), high liquid performance chromatography (HPLC) (Shimadzu, Japan), hot plate (Labnet, USA), light microscope (Olympus, Japan), Mikro 22R centrifuge (Hettich, Germany), millipore filter 0.22 µm (Millipore, USA), multichannel pipettors (Gilson, France), pH meter (Therma, Canada), real time RT-PCR with iQ TM reagent optical system software version 2.0 (Bio Rad, USA), serological pipette: 5 ml & 10 ml (Costar, USA), timer, spectrofluorometer (Jassco Model FP-777, Germany), UV-spectrophotometer (Shimadzu, Japan), UV transluminator gel documentation (Syngene, UK), vortex mixer (CT Laboratory Clay Adams, USA), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio Rad, USA), power supply (Bio Rad, USA), and vertical laminar flow cabinet (Microflow, UK).

3. Chemicals

Chemicals used in this study were as following: Acrylamide, ammonium persulfate, bovine serum albumin (BSA), butylated hydroxytoluene, 2′,7′-dichlorofluorescein diacetate (DCFH-DA), dihydrocoumarin (DHC), dimethylsulfoxide (DMSO), ethylenediamine tetraacetic acid (EDTA), lead acetate, lovastatin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phorbol 12-myristate 13-acetate (PMA), ponceau S, sodium bicarbonate ($NaHCO_3$), sodium citrate, sodium dodecyl sulfate (SDS), sodium hydroxide (NaOH), sodium phosphate dibasic anhydrous, sodium phosphate dibasic heptahydrate, sodium phosphate monobasic anhydrous, tris [hydroxymethyl] aminomethane hydrochloride, triton X-100, and tween 20. These chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Calcium chloride ($CaCl_2$), chloroform, disodium hydrogen phosphate (Na_2HPO_4), ethanol, glycerol, hydrochloric acid (HCl), isopropanol, methanol, phenyl acetate, potassium chloride (KCl), sodium chloride (NaCl), and trichloroacetic acid were purchased from Merck Darmstadt, Germany.

Potassium dihydrogen phosphate (KH_2PO_4) and di-potassium hydrogen phosphate (K_2HPO_4) were purchased from Fluka, Switzerland.

Fetal bovine serum, L-glutamine, penicillin & streptomycin, protein kit assay and RPMI-1640 medium were purchased from GIBCO, USA.

Mouse monoclonal PON3 antibody and rabbit polyclonal PON2 antibody were purchased from Abcam, USA.

β-actin antibody, horseradish peroxidase (HRP)-conjugated secondary antibody, and mouse monoclonal were purchased from Santa Cruz, USA.

SuperSignal® West Pico chemilluminescent substrate was purchased from ThemoScientific, USA.

Taq DNA polymerase (5 U/ul) and Page Ruler™ Prestained Protein Ladder was purchased from Fermentus Life Sciences, USA.

SYBR green I sDNA-nucleic acid gel stain dye was purchased from Bio Basic Inc, Canada.

Hybond™-ECL™ nitrocellulose membranes were purchased from Amersham Biosciences UK Limited, UK.

Amersham Hyperfilm™ ECL high performance chemiluminescence film was purchased from GE Healthcare Limited, UK.

Carbon dioxide gas was purchased from Thai Industrial Gases Public Company, Ltd., Thailand.

All other chemicals and solvents used throughout this study were analytical grade reagents.

Methods

1. Cell Culture

Both HepG2 and THP-1 differentiated macrophage cells were cultured in complete RPMI1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine and 1% antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). Cells were incubated at 37 ºC and 5% CO_2 under 95% humidifier for 24 hours before exposed to lead acetate at various concentration. Before used, THP-1 cells were induced to be differentiated macrophage cells using 20 ng/ml of phorbol 12-myristate 13-acetate (PMA). In each experiment, cells were treated with lead acetate at concentrations of 0, 0.05, 0.1, 0.5, 1, 10, and 100 µg/ml (0, 0.132, 0.264, 1.32, 2.64, 26.36, and 263.62 µM, respectively).

2. Experimental process

2.1. Cytotoxicity of lead acetate on HepG2 cells and THP-1 differentiated macrophage cells

To obtain the optimal concentration of lead acetate for the subsequent experiment and to obtain the toxicity of lead on HepG2 and THP-1 differentiated macrophage cells, cytotoxicity test was performed using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenltetrazolium bromide (MTT) assay.

Lead acetate was dissolved in sterile distilled water as a stock solution (10 mg/ml) and directly added to the culture medium to attain final concentrations as indicated in the experiment.

HepG2 cells were harvested with 0.25% trypsin and resuspended to a final concentration of 3 x 10^4 cells/ml while THP-1 differentiated macrophage cells

were resuspended to a concentration of at 5 x 10^4 cells/ml. Two hundred microlitres of cell suspension were added into each well of 96-well cell culture plate. After 24 hours of incubation, cells were treated with lead acetate at concentrations of 0, 0.05, 0.1, 0.5, 1, 10, 100, and 1000 µg/ml and further incubated for 24, 48, and 72 hours. After each incubation period, cell viability was determined by MTT assay (Mossmann, 1983). Reduction of MTT is one of the most frequently used methods for measuring cytotoxicity. MTT reduction is an index of mitochondrial viability because it requires metalbolically active mitochondria. The MTT assay based on the reduction of yellow-colored MTT into a purple insoluble formazan product by mitochondrial reductase in living cells (Figure 10) (Mosman, 1983).

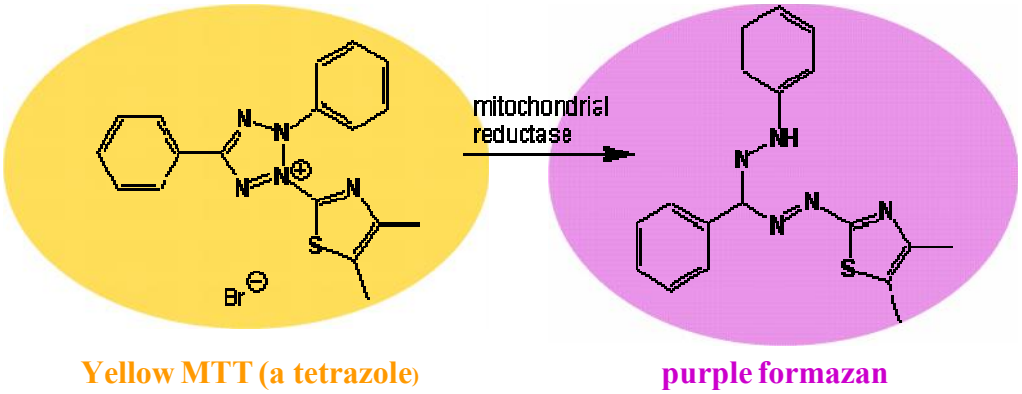

Figure 10 The conversion of yellow MTT to purple formazan (http://en.wikipedia.org/wiki/File:Mttscheme.png)

Briefly, MTT was dissolved in phosphate buffered saline (PBS), pH 7.4 at a concentration of 5 mg/ml. MTT solution was added to the cell culture to the final concentration of 0.5 mg/ml. After 2 hours of incubation, medium was removed and the remaining insoluble formazan was dissolved in 100 µl DMSO and the optical density was measured spectrophotometrically at 570 nm. It is widely assumed that MTT is mostly reduced by active mitochondria in living cells. A decrease in cellular MTT reduction could be an index of cell viability.

2.2. Determination of intracellular reactive oxygen species production in HepG2 cells and THP-1 differentiated macrophage cells

Intracellular ROS production in HepG2 cells and THP-1 differentiated macrophage cells was assayed through the oxidation of 2',7'-

dichlorodihydrofluoroscein diacetate (DCFH-DA). Normally, DCFH-DA was used to assess generation of intracellular ROS (Wang and Joseph, 1999). The nonfluorescent probe, DCFH-DA is nonpolar and diffusible into the cells. Intracellular esterases cleave the diacetate ester group resulting the polar, nonfluorescent 2′,7′-dichlorofluorescein (DCFH), which is able to interact with intracellular free radicals or peroxide to generate the highly fluorescent compound 2',7'-dichlorofluoroscin (DCF).

Briefly, HepG2 cells and THP-1 differentiated macrophage cells were cultured in 96-well plate at the density of 3 x 10^4 cells/ml and at 5 x 10^4 cells/ml, respectively. After 24 hours of incubation, cells were treated with lead acetate at concentrations of 0, 0.05, 0.1, 0.5, 1, 10, and 100 µg/ml and further incubated for 1 hour. Then, cells were washed three times with cold PBS, pH 7.4 and cells were incubated with 50 µM of DCFH-DA for 45 minutes, at 37 ºC in dark condition. After incubation, cells were washed three times with cool PBS and resuspened in 200 µl of PBS, pH 7.4. The fluorescence of DCF was measured using a fluorescence microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The fluorescence of the cell population is proportional to the levels of intracellular ROS generated. Intracellular ROS levels were expressed in term of the percentage of the control.

2.3 Determination of lipid peroxidation in $HepG_2$ cells and THP-1 differentiated macrophage cells

Thiobarbituric acid reactive substances (TBARS) assay is the method of choice for screening and monitoring lipid peroxidation, a major indicator of oxidative stress (Yagi et al., 1998). This assay measures the amount of malondialdehyde (MDA), an end product of polyunsaturated fatty acid oxygenation. One molecule of MDA reacts with molecules of thiobarbituric acid (TBA) to generate the pink pigment product which can be determined spectrofluorometrically (Gutteridge, 1989; Sattler et al., 1998) (Figure 11). Briefly, after 1 hour of the treatment, the media was aspirated from the well and washed three times with PBS. Two hundred microliters of 2% sodium lauryl sulfate was added for solubilization for 30 minutes. Then, 50 µl of 4% butylated hydroxytoluene and 1 ml of 15% trichloroacetic acid was added and mixed. Next, 1.5 ml of 0.7% thiobarbituric acid

was added and heated for 60 minutes at 95 ºC in a water bath. After incubation, the samples were left at room temperature. Then, 4 ml of n-butanol was added and mixed. The samples were centrifuged at 3,000 rpm for 10 minutes. The supernatant was measured using a spectrofluorometer with an excitation wavelength of 515 nm and emission wavelength of 553 nm. MDA level was calculated from a standard curve of 1,1,3,3-tetraethoxypropane (TEP). Lipid peroxidation was expressed in term of MDA equivalence (1M TEP = 1M MDA).

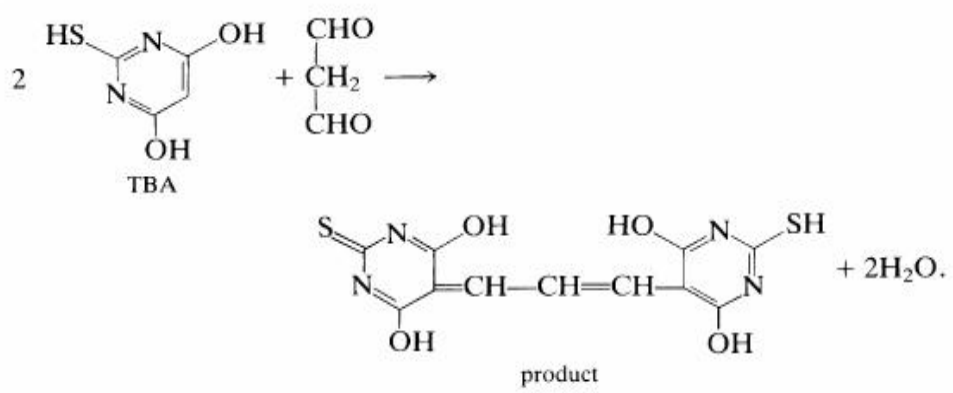

Figure 11. The reaction of TBA and MDA (Halliwell and Gutteridge, 1989).

2.4 Determination of protein assay

Total protein concentration in supernatant of cell lysate was determined using Bradford method (Bradford, 1976). This assay is a dye binding assay in which differential color change of dye occurs in response to various concentrations of proteins. Twenty microlitre of the sample was mixed with 1 mL of Bradford reagent and incubated at room temperature for 5 minutes. Total protein was determined at 595 nm using spectrophotometer. Bovine serum albumin (BSA) was used as a standard.

2.5 Determination of PON1 (arylesterase) activity in HepG2 cells

HepG2 cells were cultured in 100 mm cultured plate at the density of 5 x 10^6 cells/ml. After 24 hours of incubation, cells were treated with lead acetate at concentrations of 0, 0.05, 0.1, 0.5, 1, 10, and 100 µg/ml and further incubated for 24,

48, and 72 hours. After the treatment, cells were washed with cold PBS and scrapped and collected in a tube. Cells were washed three times with PBS by centrifugation at 5,000 ×*g* for 10 minutes at 4 °C. The cells pellets were used for measuring PON1 activity. PON1 activity was determined using phenylacetate as a substrate as previously described by Deakin et al. (2001). ARE activity was determined from an increase in the absorbance of phenol at 270 nm using spectrophotometer. The reaction was shown in Figure 12. Briefly, the reaction mixture of 1 ml contained 1.0 mM phenylacetate and 0.9 mM $CaCl_2$ in 10 mM Tris-HCl buffer pH 8.0. The reaction was initiated by adding HepG2 cells (about 5 x 10^6 cells/well of every final time of incubation) in 10 mM Tris-HCl buffer pH 7.4. The enzyme activity was calculated from the molar extinction coefficient of 1,310 M^{-1} cm^{-1}. Each sample was performed in triplicate. PON1 activity toward phenylacetate was determined from the amount of the product formed (phenol) per minute per milligram of protein. PON1 activity was expressed in term of percentage of the control.

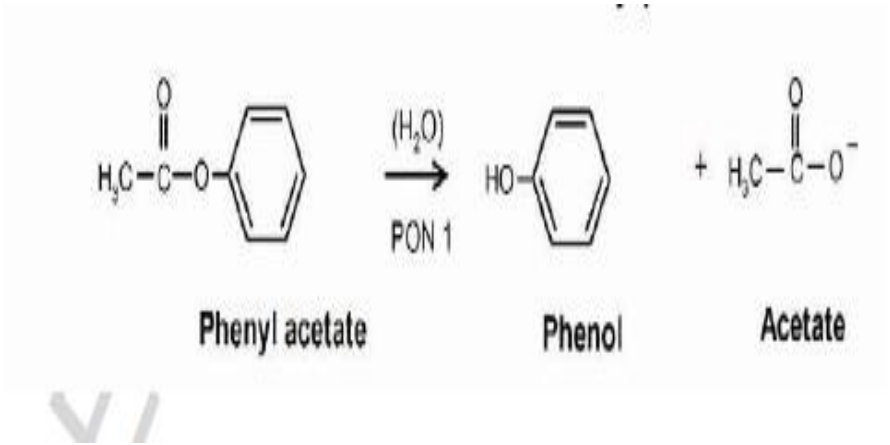

Figure 12. The reaction using to determine arylesterase activity which represents PON1 activity.

Calculations

PON1 activity toward phenyl acetate was determined from the concentration (U/L) of the product formed (phenol) per minute per milligram of protein. PON1 activity was expressed in term of percentage of the control. ARE activity was calculated as following;

$$\text{Activity (mole/min/mg protein)} = \frac{\text{(OD/min) x reaction volume (1 ml)}}{\varepsilon\ (M^{-1}cm^{-1}) \text{ x sample volume (ml) x mg protein (mg/ml) x path length (cm)}}$$

Where ε = Molar extinction coefficient (1310 $M^{-1}cm^{-1}$)

2.6 Determination of PON2 activity in HepG2 and THP-1 differentiated macrophage cells.

HepG2 cells and THP-1 differentiated macrophage cells were culture in 100 mm cultured plate at the density of 5 x 10^6 cells/ml. After 24 hours of incubation, cells were treated with lead acetate at concentrations of 0, 0.05, 0.1, 0.5, 1, 10, and 100 µg/ml and further incubated for 4, 8, 24, 48, and 72 hours on HepG2 cells and 24, 48, and 72 hours on THP-1 differentiated macrophage cells. After the treatment, cells were washed with cold PBS and scrapped and collected in a tube. Cells were washed three times with PBS by centrifugation at 5,000 ×*g* for 10 minutes at 4 °C. The cell pellet was suspended in 0.3 ml of 0.1 M potassium phosphate buffer, pH 7.4. Then, cells were lysated with hand homogenizer on ice. The lysate was viewed under microscope. The lysate suspension was separated by centrifugation at 10,000 ×*g* for 10 minutes at 4 °C, and the supernatant was transferred into a new tube and then stored at -80 °C until assays. This supernatant of cell lylate was used for analysis of PON2 activity. PON2 activity was determined using dihydrocumarin (DHC) as a substrate, with minor modification from the protocol previously described by Draganov et al. (2000). Briely, 20 µl of the cell lysate of HepG2 or THP-1 differentiated macrophage cells was added to 1 ml of 50 mM Tris-HCl buffer, pH 8.0 containing 1 mM $CaCl_2$ and 1 mM DHC. The hydrolysis rate of DHC was assessed by measuring the liberations of 3-(2-hydroxyphenyl)propionic acid (Figure 13) at 270 nm at 37 ˚C using spectrophotometer. The enzyme activity was calculated from the molar extinction coefficient of 1,295 $M^{-1}cm^{-1}$. Each sample was performed in triplicate. PON2 activity was determined from the amount of the product formed (3-(2-hydroxyphenyl) propionic acid) per minute per milligram of protein of the cell lysate.

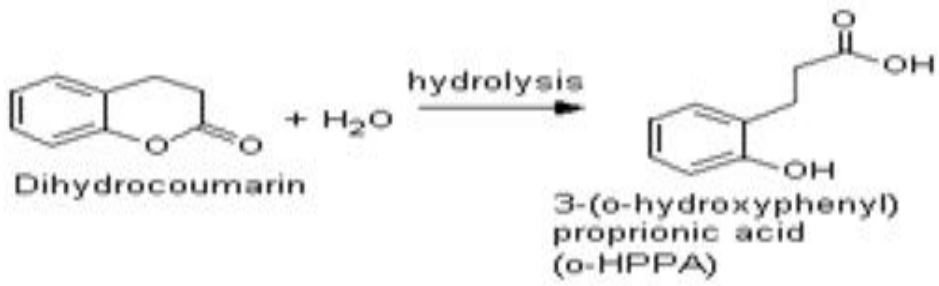

Figure 13. Lactonase activity representing PON2 activity

Calculations

PON2 activity was determined from the concentration (U/L) of the product formed (3-(2-hydroxyphenyl) propionic acid) per minute per milligram of protein concentration in supernatant of cell lysate. PON2 activity was expressed in term of the percentage of control. PON2 activity was calculated as following;

$$\text{Activity (mole/min/mg protein)} = \frac{\text{(OD/min) x reaction volume (1 ml)}}{\varepsilon\ (M^{-1}cm^{-1}) \text{ x sample volume (ml) x mg protein (mg/ml) x path length (cm)}}$$

Where ε = Molar extinction coefficient (1295 $M^{-1}cm^{-1}$)

2.7 Verification of the methods

2.7.1 Optimal condition variance (OCV)

Before performing the methods to determine PON1 and PON2 activity, the methods were verified using OCV. Because very small amount of PON1 was found in HepG2 cells, human serum which possesses large amount of this enzyme was used for verification of the method. PON2 which was found large amount in HepG2 cells, verification of method for PON2 activity was performed using HepG2 cells. Pooled human serum or HepG2 cells were used in the reaction as

mentioned earlier in 2.4 (for PON1) and 2.5 (for PON2) for 10 times (for HepG2 cells) or 20 times (for human serum). Then, OCV was calculated as following:

$$\text{OCV (\%)} = \frac{\text{SD}}{\text{Mean}} \times 100$$

The methods would be accepted if OCV was less than 5%. Data of method verification were shown in Appendix A.

2.7.2 Routine condition variance (RCV)

During performing the assay for PON1 and PON2 activities, the methods were verified using RCV. Pooled human serum or HepG2 cells were used to performed the reaction as mentioned earlier in 2.4 (for PON1) and 2.5 (for PON2) for 10 times (for HepG2 cells) or 20 times (for human serum) routinely during the experiments. RCV was calculated as following:

$$\text{RCV (\%)} = \frac{\text{SD}}{\text{Mean}} \times 100$$

The methods would be assured if RCV was within ± 2 SD. Data of method verification were shown in Appendix A.

2.8 Determination of PON3 activity in HepG2 cells

HepG2 cells were cultured in 100 mm cultured plate at the density of 5 x 10^6 cells/ml. After 24 hours of incubation, cells were treated with lead acetate at concentrations of 0, 0.05, 0.1, 0.5, 1, 10, and 100 µg/ml and further incubated for 24, 48, and 72 hours. After the treatment, cells were washed with cold PBS, scrapped and collected in a tube. Cells were washed three times with PBS by centrifugation at 5,000 ×*g* for 10 minutes at 4 °C. The cell pellet was suspended in 0.3 ml of 0.1 M potassium phosphate buffer, pH 7.4. Then, cells were lysated with hand homogenizer on ice. The lysate was viewed under microscope. The lysate suspension was centrifuged at 10,000 ×*g* for 10 minutes at 4 °C, and the supernatant was transferred into a new tube and

then stored at -80 °C until assays. This supernatant of cell lylate was used for analysis of PON3 activity using high performance liquid chromatography (HPLC).

HPLC chromatographic system

Apparatus: Shimazu® LC-10AD HPLC pump, a communication bus module (CBM-10A), an autoinjector (SIL-10A), a column oven (CTO-10A), a spectro UV-VIS detector (SPD-10A) and computerized integrator

Column: 5μ Migthsil RP-18 GP, stainless steel column, 250x4.6 mm (Waters Associates Pty-Ltd., Molford, MA, USA)

UV detector: 238 nm

Mobile phase: Acetonitrile:30 mM potassium-phosphate buffer, pH 4.5 (70:30, v/v)

Flow rate: 1.0 mL/min

Quantification of PON3 activity in HepG2 cells

PON3 activity was determined using lovastatin as a substrate, with minor modification from the protocol previously described by Draganov et al. (2000) and Suchocka et al. (2006). In the reaction, lovastatin was hydrolyzed to lovastatin acid (Figure 14). Briefly, 100 μl of cell lysate (except for the blank tube) was added to 1 ml of 50 mM Tris-HCl buffer, pH 8.0 containing 1 mM $CaCl_2$ and 5 μg of lovastatin. The reaction mixture was incubated at 37 ºC for 90 minutes. The enzymatic reaction was stopped by deproteinization with cold acetonitrile and placed in an ice-bath for 5 minutes. The reaction mixture was centrifuged at 10,000 ×*g*, 4 ºC for 10 minutes. The supernatant was transferred into a new tube, capped, and stored on ice until performing HPLC analysis. Twenty microliters of the filtered supernatant was injected into HPLC. PON3 activity was determined from rate of the increase of lovastatin acid. A standard curve was constructed from the concentrations of the reference standard (lovastatin acid) and the corresponding area under the peaks.

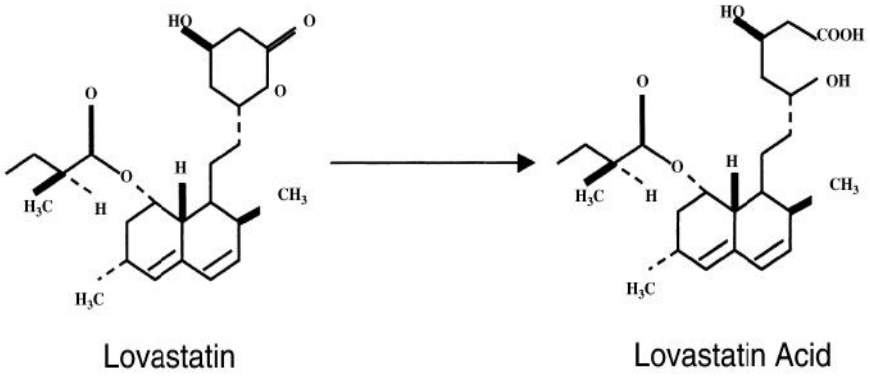

Figure 14. Statinase activity representing PON3 activity

Standard calibration curve

Lovastatin stock solution of 1 mg/ml was prepared in methanol. Working standard solution of 100 µg/ml of lovastatin was prepared from the stock solution. Serial dilution of lavastatin in methanol of 0, 0.5, 1, 2.5, 5, 10, 20, and 40 µg/ml was prepared by pipetting the working standard solution of 0, 2.5,5, 12.5, 25.5, 50, 100, and 200 µl, respectively and made the volume up to 500 µl. Each serial dilution of lovastation was added with 500 µl of 0.02 M NaOH. The mixture was incubated at 50 °C for 1 hour. After incubation, 1 ml of 0.02 M HCL was added for stabilization of the acid product. Twenty microlitre of each solution was injected into the HPLC system. The standard curve of lovastatin acid product was constructed between the concentration of lovastatin acid product (µg/ml) and the corresponding area under the peak.

2.9 Determination of the effect of calcium on PON2 activity after treatment HepG2 cells with lead acetate

The method was modified from the method of Gonzalvo et al. (1997) and Pla et al. (2007). Briefly, HepG2 cells were cultured in 100 mm cultured plate at the density of 5 x 10^6 cells/ml. After 24 hours of incubation, cells were washed with cold PBS buffer pH 7.4, scrapped and collected. Cells were washed three times with PBS by centrifugation at 5,000 ×*g* at 4 °C for 10 minutes. Then, cells were lysated with hand homogenizer on ice. The lysate suspension was separated by centrifugation

at 10,000 ×*g* for at 4 °C 10 minutes, and the supernatant was transferred into a new tube. The supernatants were preincubated with various concentration of lead acetate at room temperature for 30 minutes before adding 1 mM $CaCl_2$ and incubated for 30 minutes before starting the enzymatic reaction for determining as mentioned in 2.5 without 1 mM $CaCl_2$ in the buffer.

2.10 Determination of PON protein expression in HepG2 and THP-1 differentiated macrophage cells using Western blot analysis

HepG2 and THP-1 differentiated macrophage cells were cultured in 100 mm cultured plate at the density of 5 x 10^6 cells/ml. After 24 hours of incubation, cells were treated with lead acetate at concentrations of 0, 0.05, 0.1, 0.5, 1, 10, and 100 µg/ml and further incubated for 24 and 72 hours. After the treatment, cells were washed with cold PBS and scrapped and collected in a tube. Cells were washed three times with PBS by centrifugation at 5,000 ×*g* for 10 minutes at 4 °C. The cell pellet was suspended in 0.3 ml of 0.1 M potassium phosphate buffer, pH 7.4. Then, cells were lysated with hand homogenizer on ice. The lysate was viewed under microscope. The lysate suspension was separated by centrifugation at 10,000 ×*g* for 10 minutes at 4 °C, and the supernatant was transferred into a new tube and then stored at -80 °C until assays. This supernatant of cell lylate was used for analysis of PON2 and PON3 protein levels. The supernatant containing 10 and 30 µg protein for PON2 and PON3, respectively of HepG2 cells and 20 µg protein of THP-1 differentiated macrophage cells were mixed with equal volume of 2x sample buffer. The samples were boiled for 95 ºC 5 minutes and subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under constant voltage of 100 V for 1:30 hours. After electrophoresis, protein bands in the gel were transferred to a nitrocellulose membrane using Mini TransBlot electrophoretic Transfer Cell under constant voltage of 100 V for 2 hours. The membrane was blocked at 4 ºC overnight in blocking buffer containing 5% non-fat dry milk to prevent non-specific binding. The membrane was probed with specific primary antibodies. Primary antibody was diluted (1:1250 for anti PON2 rabbit polyclonal, 1:1000 for anti PON3 mouse monoclonal and 1:1000 for anti-actin mouse monoclonal) in blocking solution and added to the membrane for 2 hours at room temperature. After washing with 1x tris buffered saline-0.05% tween 20 (TBST), the membrane were incubated with HRP conjugated secondary antibody for

1 hour at room temperature. The membranes were washed three times in TBST and the bound antibodies were detected using enhanced chemiluminescence for 5 minutes. The immunoblots were exposed to X-ray film for radiographic detection of the bands. Actin bands were monitored on the same blot to verify the consistency of protein loading. Signal intensity of the bands were quantified by densitometry scanning and quantitated densitometrically by using the program Image J program (NIH, Maryland).

2.11 Determination of PON gene expression in HepG2 cells and THP-1 differentiated macrophage cells using real time RT-PCR

HepG2 and THP-1 differentiated macrophage cells were cultured in 100 mm cultured plate at the density of 5 x 10^6 cells/ml. After 24 hours of incubation, cells were treated with lead acetate at concentrations of 0, 0.05, 0.1, 0.5, 1, 10, and 100 µg/ml and further incubated for 6 hours on HepG2 cells. After the treatment, cells were washed with cold PBS and extracted the RNA.

RNA preparation: Cells were extracted with Trizol® reagent according to the supplier's instruction. RNA was quantitated by optical density measurements at 260/280 nm using a spectrophotometer.

Real time PCR analysis: One microgram of total RNA was reverse transcribed to cDNA using iScript™ Select cDNA Synthesis Kit. Real-time PCR was performed using the iQ™5 Multicolor Real-Time PCR Detection System. Amplification and detection of PON2 and PON3 gene were performed using Taq DNA Polymerase and SYBR green I sDNA-nucleic acid gel stain dye. Each reaction contained 0.05 U/µl Taq DNA polymerase, 0.2 mM dNTP mix, 1x reaction buffer, 2.08 mM $MgCl_2$, SYBR Green I sDNA acid gel stain dye, 1 pmol/µl of each specific primer (Table 2), and cDNA. The cDNA templates were probed with specific primers (Table 2) designed by PerlPrimer version 1.1.18 for PON2, PON3 and Actin. Actin was used as an internal control. Amplification programs was set as following: 35 cycle of 95°C for 30 seconds, an annealing step (55.6 °C for PON2 and 56.5 °C for PON3, 30 seconds) and, finally, an extension step (72°C for 30 seconds). Calculation of the threshold cycle (Ct) and normalization were performed using the iQ™ reagent

optical system software version 2.0. Specificity of the amplification was verified by melt curve analysis.

Table 2. Oligonucleotide primer for real time RT-PCR

	Primer sequence
PON2	Forward: 5′-CAGAGGTTCTCCGCATCCA-3′ Reverse: 5′-GAGCAGCTTCCCATCATACAC-3′
PON3	Forward: 5’-TGAAGGTGATACAGTTGG-3’ Reverse: 5’-CACAGAAGCCACAGAG-3’
Beta-actin	Forward: 5′-GATCATTGCTCCTCCTGAGC-3′ Reverse: 5′-ACTCCTGCTTGCTGATCCAC-3′.

3． Statistical analysis

Statistical analysis was performed using the SPSS version 16.0 for windows software. The normality of the sample distribution of each continuous parameter was tested with Kolmogorov-Smirnov test. All data were presented as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was used for testing the differences of the mean values among groups and if significant, pairwise comparison between groups was analyzed by Tukey post hoc test. Values of $P<0.05$ were considered to be statistically significant.

CHAPTER IV

RESULTS

1. Cytotoxicity of lead acetate on HepG2 cells and THP-1 differentiated macrophage cells

To examine cytotoxicity of lead acetate, HepG2 cells and THP-1 differentiated macrophage cells were cultured for 24 hours and then incubated with lead acetate at various concentrations (0, 0.05, 0.1, 0.5, 1, 10, 100, and 1000 µg/ml) and durations (24, 48, and 72 hours). Cells viability was determined using MTT assay. It was shown that lead acetate did not significantly decrease cell viability at concentration up to 100 µg/ml in HepG2 cells but significant decrease of cell viability was shown by lead acetate at 1000 µg/ml. (Figure 15).

Similarly, in THP-1 differentiated macrophage cells, lead acetate only at 1,000 µg/ml significantly decreased cell viability after 48 and 72 hours of lead exposure (Figure 16). However, significant decrease of cell viability by lead acetate was not shown at 24 hours of lead exposure the concentrations of 1000 µg/ml.

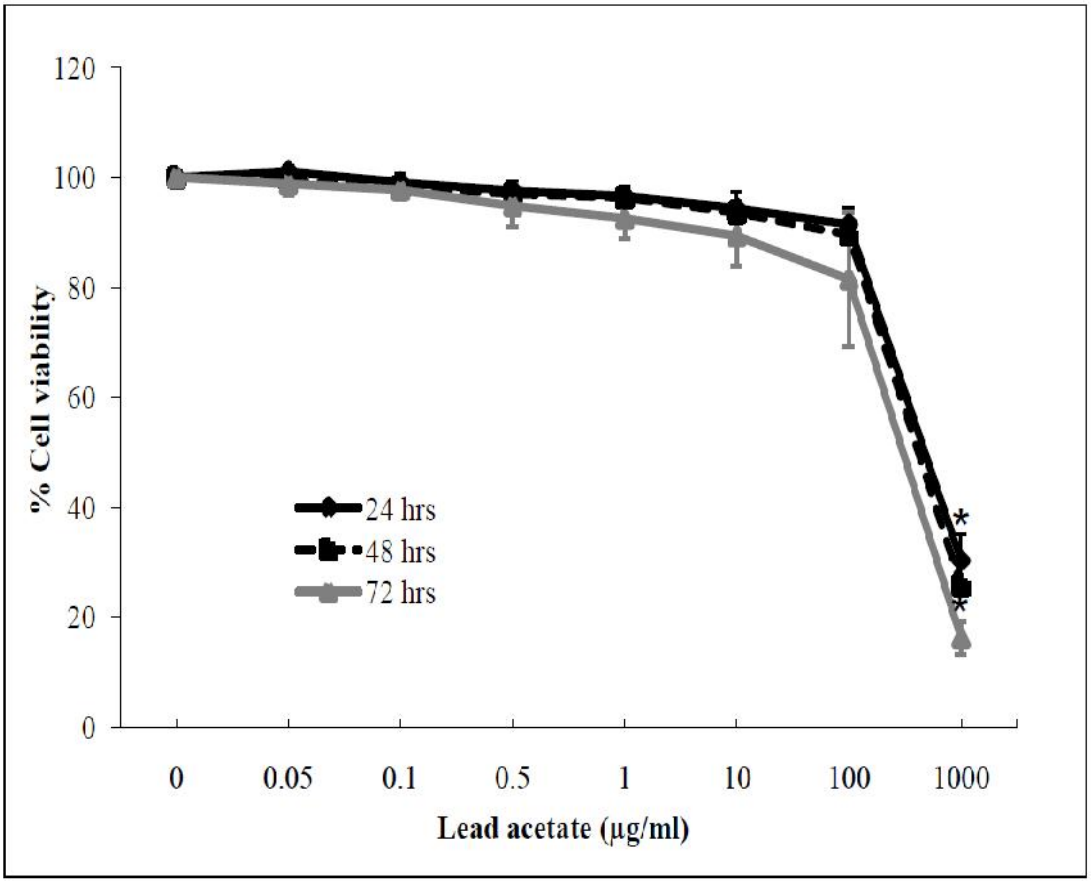

Figure 15. Cytotoxicity of lead acetate on HepG2 cells. HepG2 cells were treated with various concentrations (0.05–1000 µg/ml) of lead acetate for 24, 48, and 72 hours. Cell viability was determined by MTT assay. Data shown are mean ± SEM of four independent experiments. Differences among groups were evaluated by one-way ANOVA followed by Tukey's post hoc test.

* $P < 0.001$ compared to the control (0 µg/ml of lead acetate).

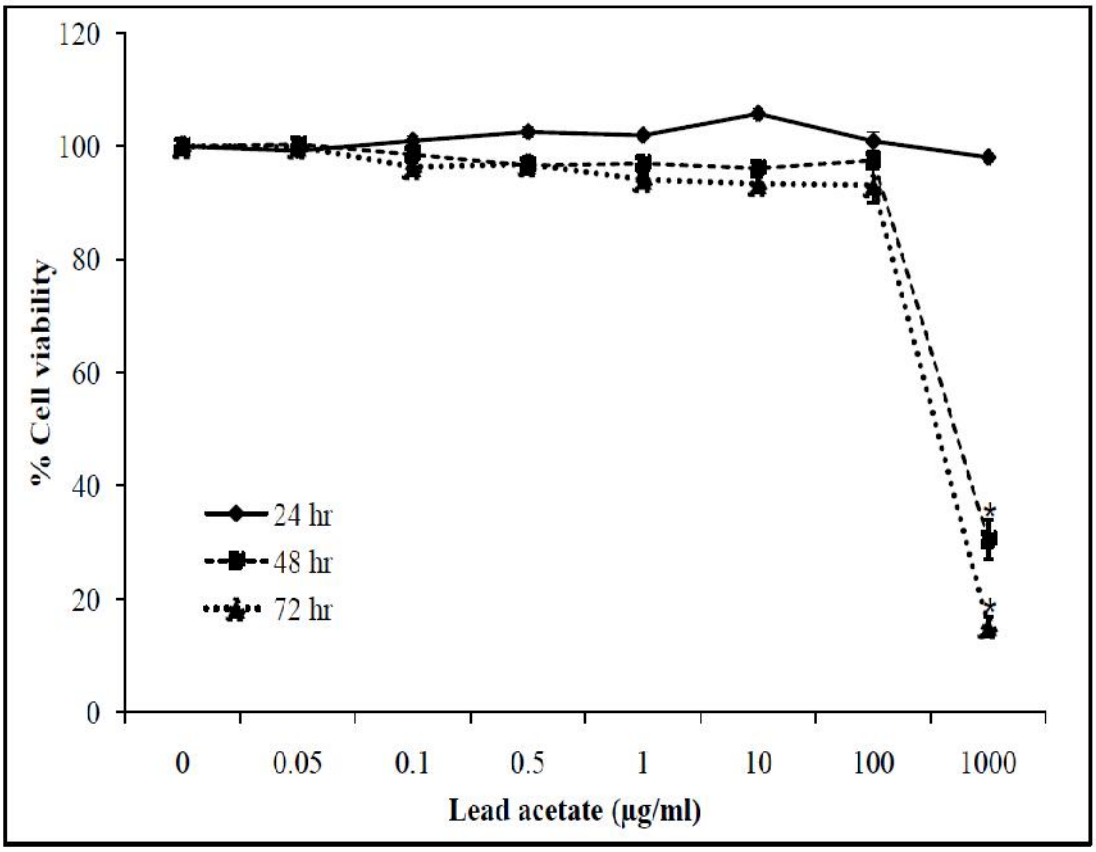

Figure 16. Cytotoxicity of lead acetate on THP-1 differentiated macrophage cells. Cells were treated with various concentrations (0.05–1000 µg/ml) of lead acetate for 24, 48, and 72 hours. Cell viability was determined by MTT reduction assay. Data shown are mean ± SEM of values from four independent experiments. Differences among groups were evaluated with one-way ANOVA followed by Tukey's post hoc test.

* $P < 0.001$ compared with control (0 µg/ml of lead acetate).

2. Effect of lead acetate on ROS production in HepG2 cells and THP-1 differentiated macrophage cells

After incubation with lead acetate for 1 hour, it was found that lead acetate at 0.1, 0.5, 1, 10, and 100 µg/ml significantly increased intracellular ROS to 135.39±6.57, 139.16±7.19, 142.08±9.66, 149.06±12.13, and 130.46±3.14% of the control, respectively. Lead acetate at 0.05 µg/ml did not significantly increase ROS in HepG2 cells (Figure 17).

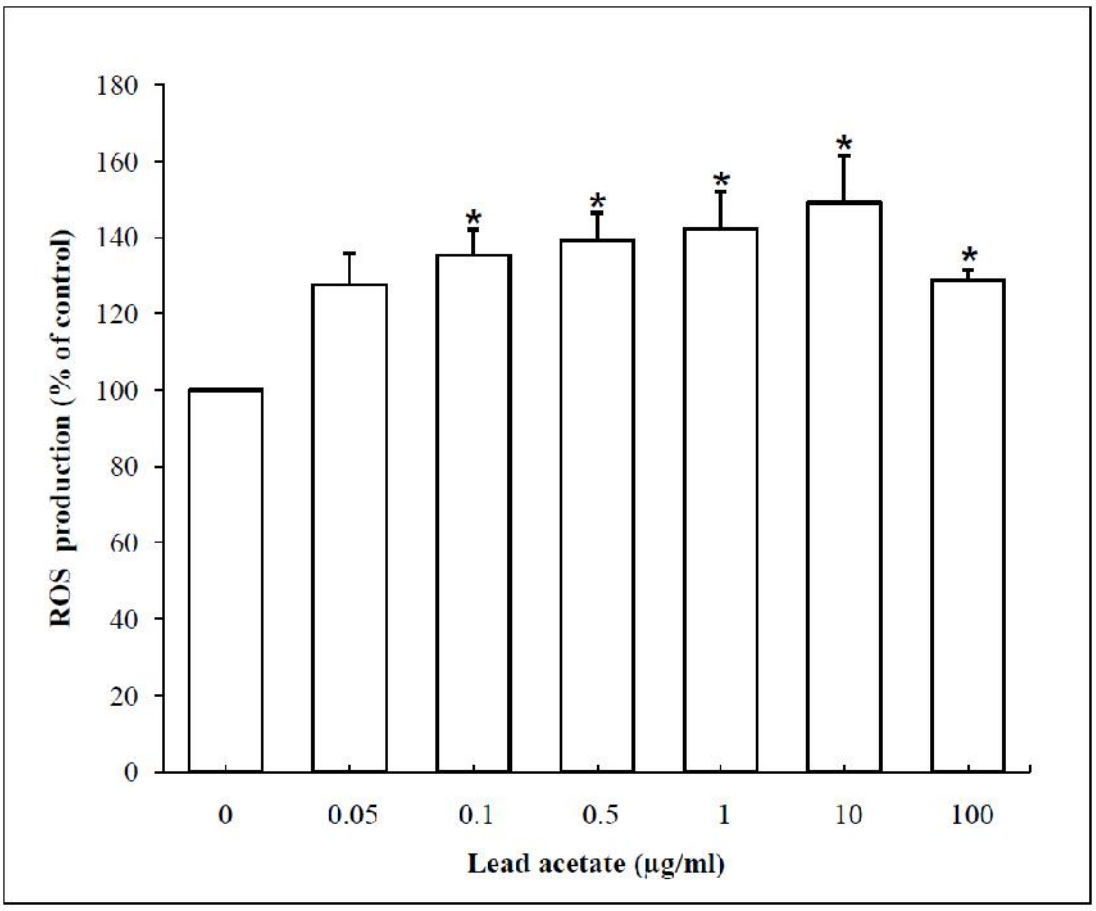

Figure 17. Effect of lead acetate on intracellular ROS in HepG2 cells. Cells were treated with various concentrations (0.05–100 µg/ml) of lead acetate for 1 hour. ROS production was determined by DCFH-DA assay. Data shown are mean ± SEM of four independent experiments. Differences among groups were evaluated by one-way ANOVA followed by Tukey's post hoc test.

* $P<0.05$ compared to the control (0 µg/ml of lead acetate).

In THP-1 differentiated macrophage cells, lead acetate also significantly increased ROS at the concentration of 0.1, 0.5, 1, 10, and 100 μg/ml by 129.41±4.54, 135.35±8.83, 149.70±8.82, 151.16±10.09, and 135.08±5.22% of the control (0 μg/ml of lead acetate), respectively. However, lead acetate at 0.05 μg/ml did not significantly increase intracellular ROS (Figure 18).

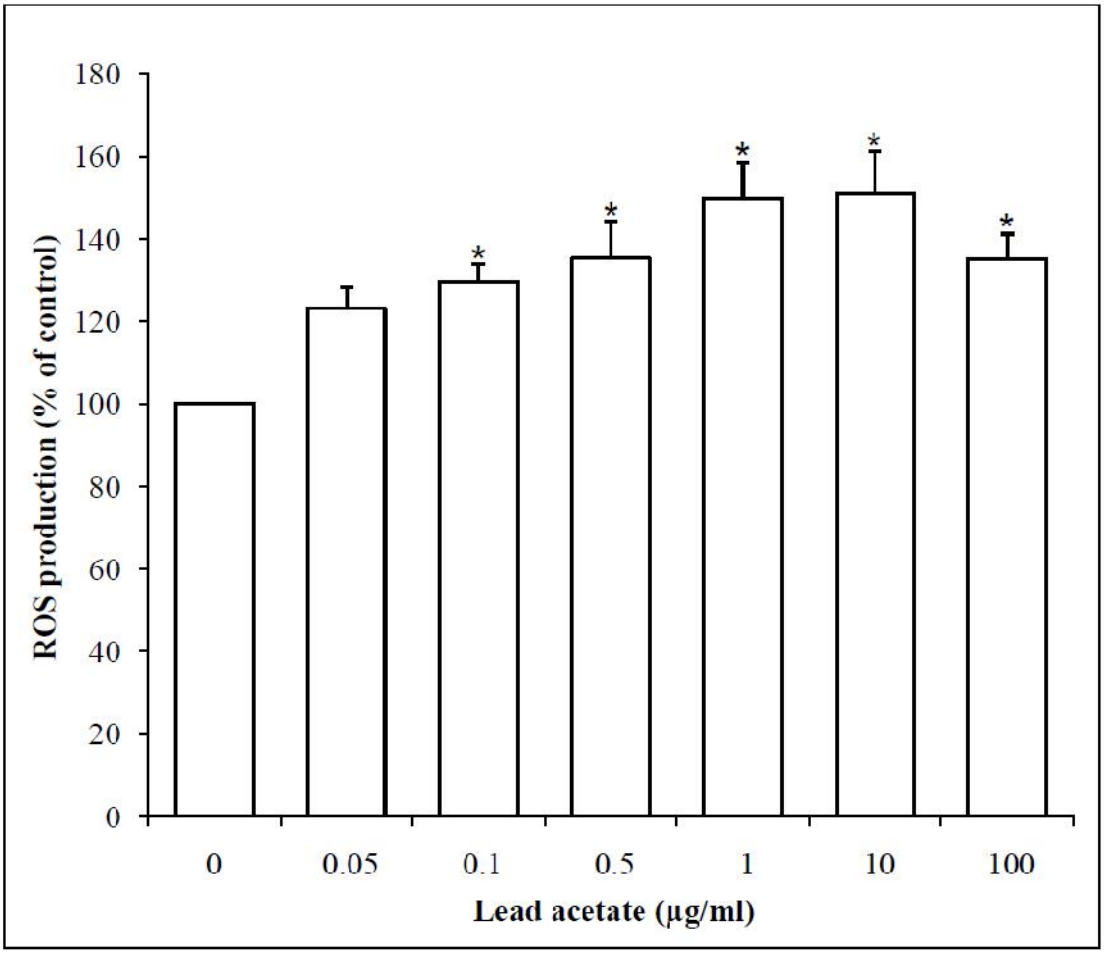

Figure 18. Effect of lead acetate on intracellular ROS in THP-1 differentiated macrophage cells. Cells were treated with various concentrations (0.05–100 μg/ml) of lead acetate for 1 hour. ROS production was determined by DCFH-DA assay. Data shown are mean ± SEM of from four independent experiments. Differences among groups were evaluated by one-way ANOVA followed by Tukey's post hoc test.

* $P<0.05$ compared with control (0 μg/ml of lead acetate).

3. Effect of lead acetate on lipid peroxidation in HepG2 cells and THP-1 differentiated macrophage cells

Effects of lead acetate on lipid peroxidation were determined using TBARS assay. It was found that lead acetate did not signicantly induce lipid peroxidation in HepG2 cells (Figure 19) as well as in THP-1 differentiated macrophage cells (Figure 20).

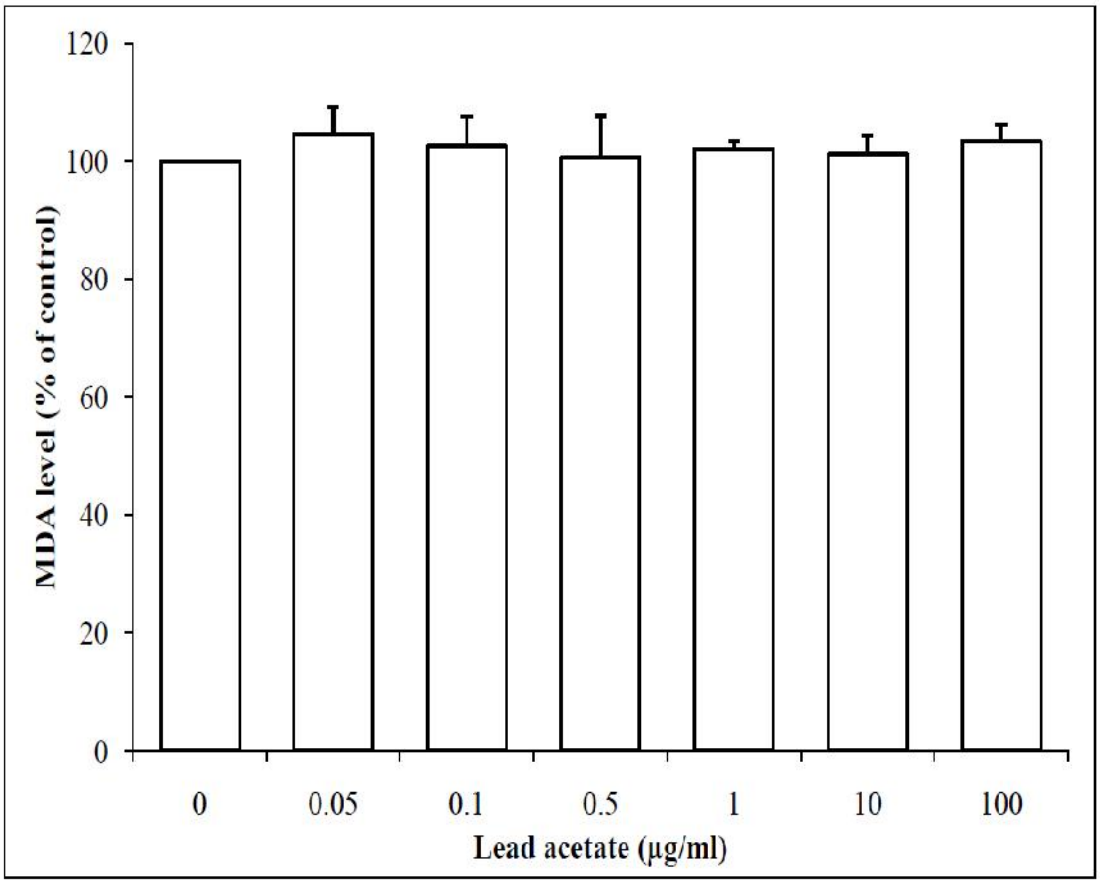

Figure 19. Effect of lead acetate on lipid peroxidation in HepG2 cells**.** Cells were treated with various concentrations (0.05–100 µg/ml) of lead acetate for 1 hour. The production of lipid peroxidation was performed by TBARS assay. Data shown are mean ± SEM of four independent experiments. Differences among groups were evaluated by one-way ANOVA followed by Tukey's post hoc test.

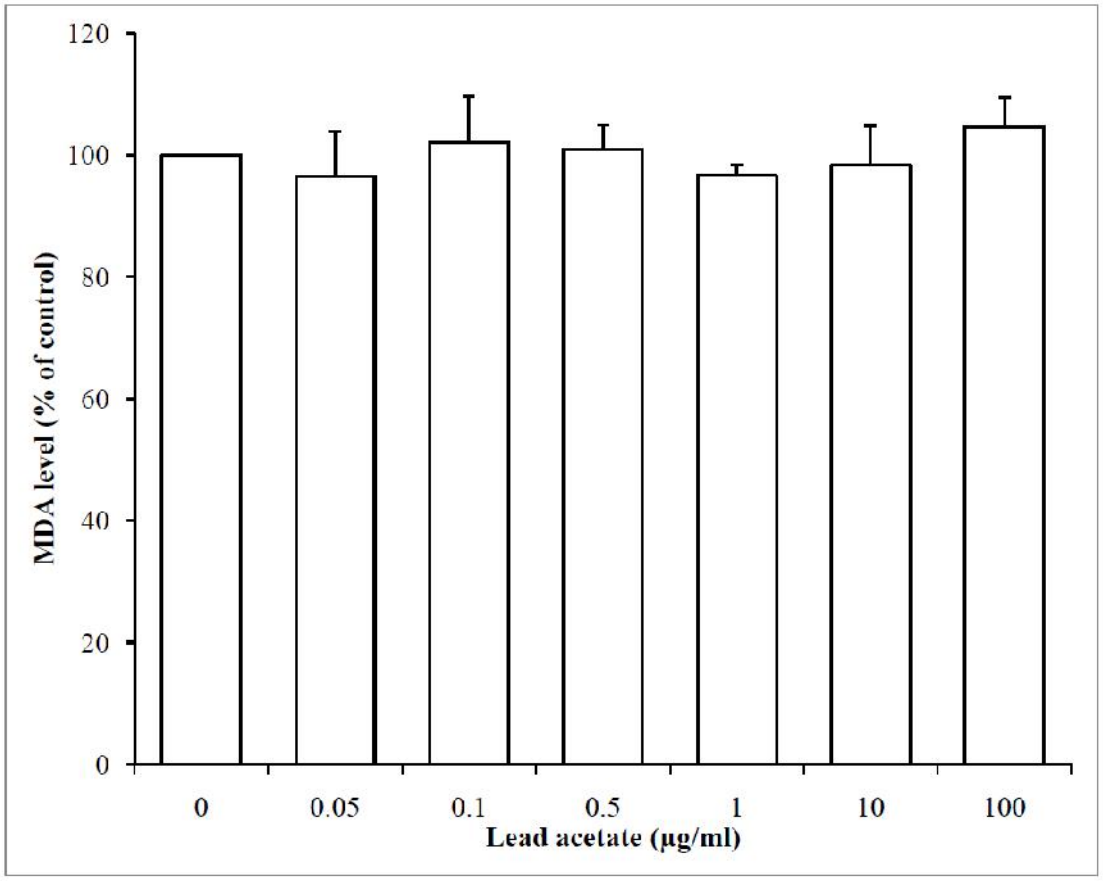

Figure 20. Effect of lead acetate on lipid peroxidation in THP-1 differentiated macrophage cells. Cells were treated with various concentrations (0.05–100 µg/ml) of lead acetate for 1 hour. The production of lipid peroxidation was performed by TBARS assay. Data shown are mean ± SEM of four independent experiments. Differences among groups were evaluated by one-way ANOVA followed by Tukey's post hoc test.

4. Effect of lead acetate on PON1 (arylesterase) activity in HepG2 cells

To determine effect of lead acetate on PON1 (arylesterase) activity in HepG2 cells, lead acetate at various concentrations (0.05-100 μg/ml) were incubated with the cells for 24, 48, and 72 hours. No significant changes of arylesterase activity toward phenylacetate were shown as compared to the control at all concentrations and all durations of lead acetate (Figure 21).

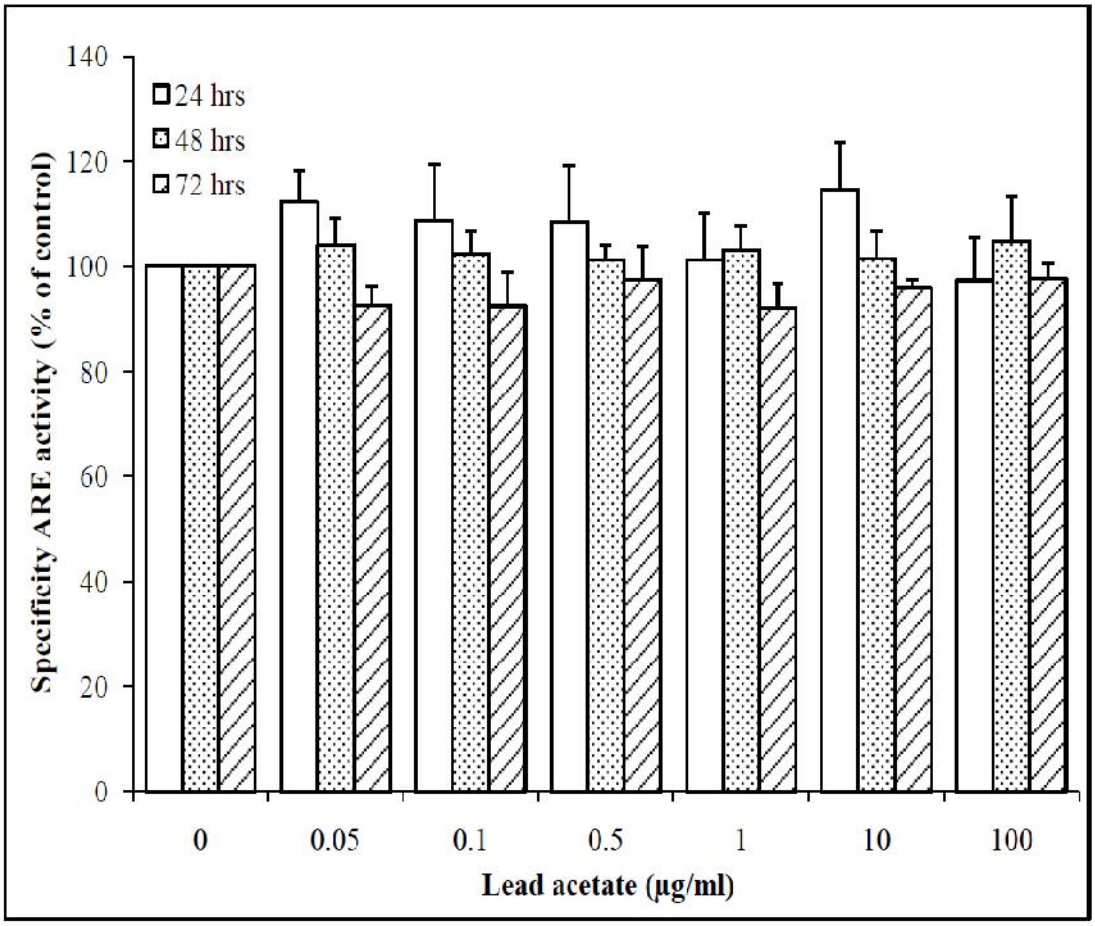

Figure 21. Effect of lead acetate on PON1 activity in HepG2 cells. Cells were treated with lead acetate (0.05-100 μg/ml) for 24, 48, and 72 hours. After exposure, PON1 activity toward phenylacetate was determined. Data shown are mean ± SEM of three independent experiments. Differences among groups were evaluated by one-way ANOVA followed by Tukey's post hoc testing.

5. Effect of lead acetate on PON2 activity and expression in HepG2 cells

Effect of lead acetate on PON2 activity in HepG2 cells is shown in Figure 22. It was found that lead acetate at 0.05-100 μg/ml significantly decreased PON2 activity in a concentration- and time-dependent manner at 24, 48 and 72 hours of exposure. Lead acetate at 0.5, 1, 10, and 100 μg/ml significantly decreased PON2 activity earlier than the lower concentration (0.05 and 0.1 μg/ml) of lead acetate. The decreases of PON2 activities at 0.5, 1, 10, and 100 μg/ml were demonstrated at 4, 8, 24, 48, and 72 hours while at lower concentrations (0.05 and 0.1 μg/ml) of lead acetate, significantly decrease of PON2 activity was shown starting at 24 hours of exposure (Figure 22).

Because lead acetate significantly decreased PON2 activities in HepG2 cells, PON2 protein and transcript were further determined using Western blot analysis and real time RT-PCR, respectively. As shown in Figure 23, lead acetate at all concentrations (0.05-100 μg/ml) did not significantly affect PON2 protein at either 24 or 72 hours of exposure. These results indicated that the reduction of PON2 activity by lead acetate was not associated with the amount of PON2. In contrast, all concentrations (0.05, 0.1, 0.5, 1, 10, and 100 μg/ml) of lead acetate were significantly increased of PON2 mRNA (Figure 24).

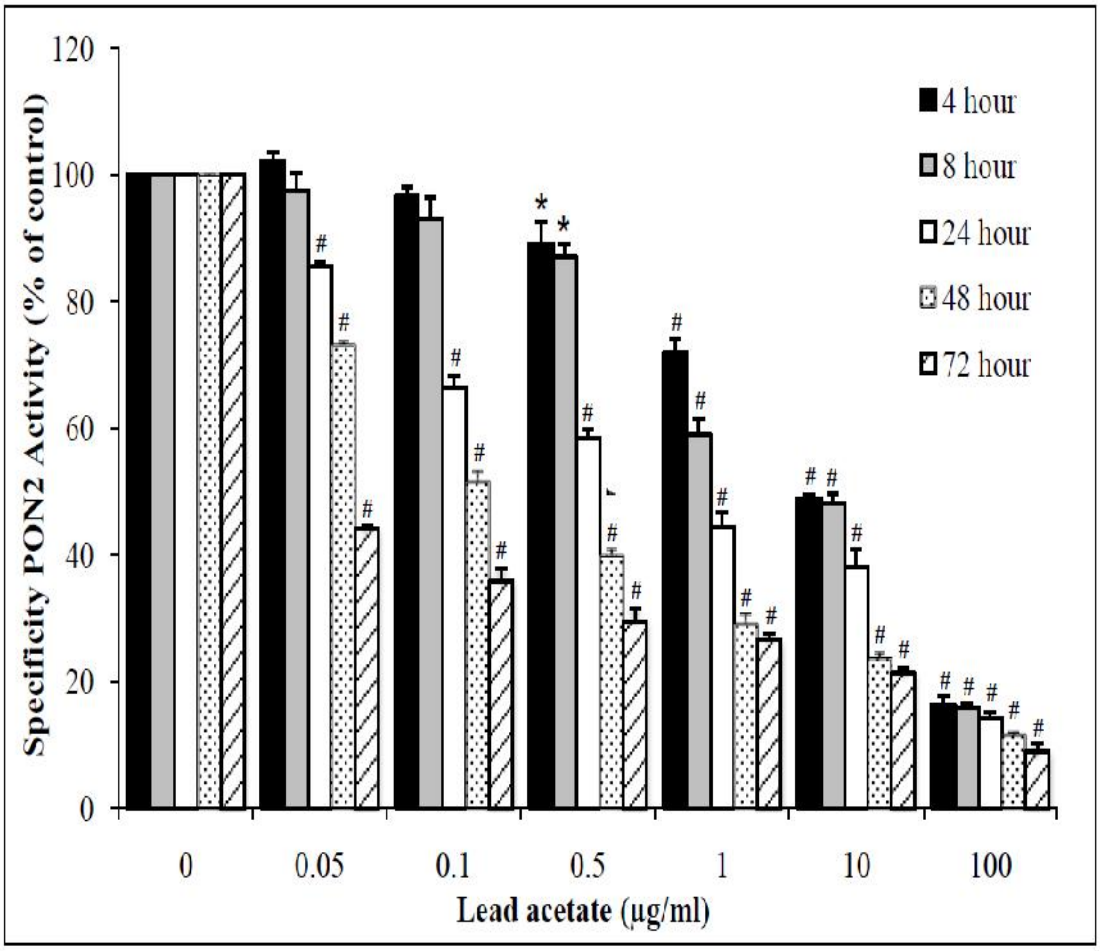

Figure 22. Effect of lead acetate on PON2 activity in HepG2 cells. Cells were treated with lead acetate (0.05-100 µg/ml) for 4, 8, 24, 48, and 72 hours. PON2 activity toward DHC was measured using spectrophotometer. Data shown are mean ± SEM of three independent experiments. Differences among groups were evaluated by one-way ANOVA followed by Tukey's post hoc testing.

* $P< 0.05$ compared to the control (0 µg/ml of lead acetate).

# $P< 0.001$ compared to the control (0 µg/ml of lead acetate).

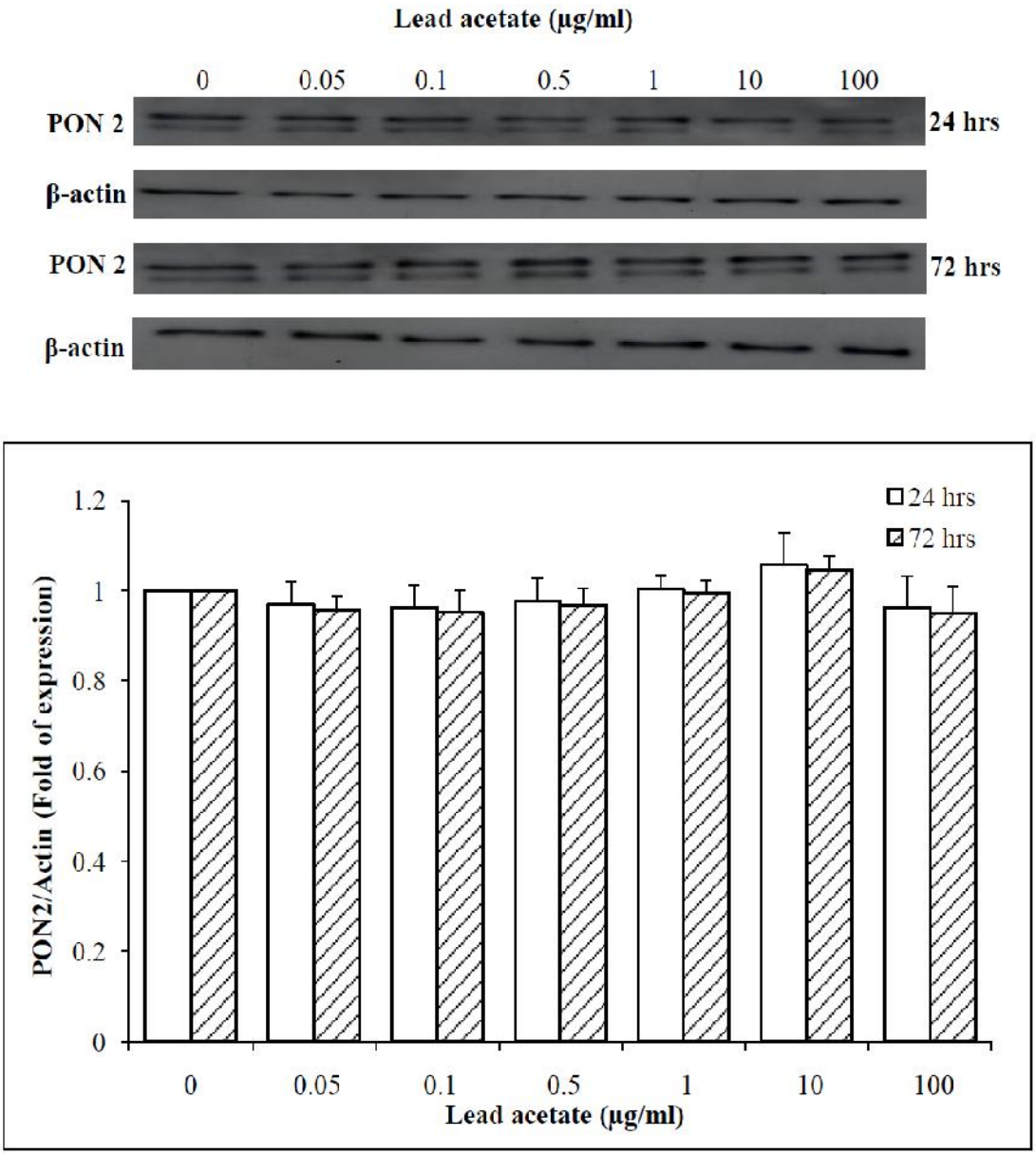

Figure 23. Effect of lead acetate on PON2 protein in HepG2 cells. HepG2 cells were treated with various concentrations of lead acetate (0.05-100 µg/ml) for 24 and 72 hours. The cell supernatant was separated on 10% SDS-PAGE. **A**, Representative immunoblot of PON2: lane 1, control; lane 2-7, lead acetate at 0.05, 0.1, 0.5, 1, 10, and 100 µg/ml, respectively. **B**, Data form densitometric analysis of the immunoblots of PON2 shown are mean ± SEM of four independent experiments. Differences among groups were evaluated by one-way ANOVA followed by Tukey's post hoc test.

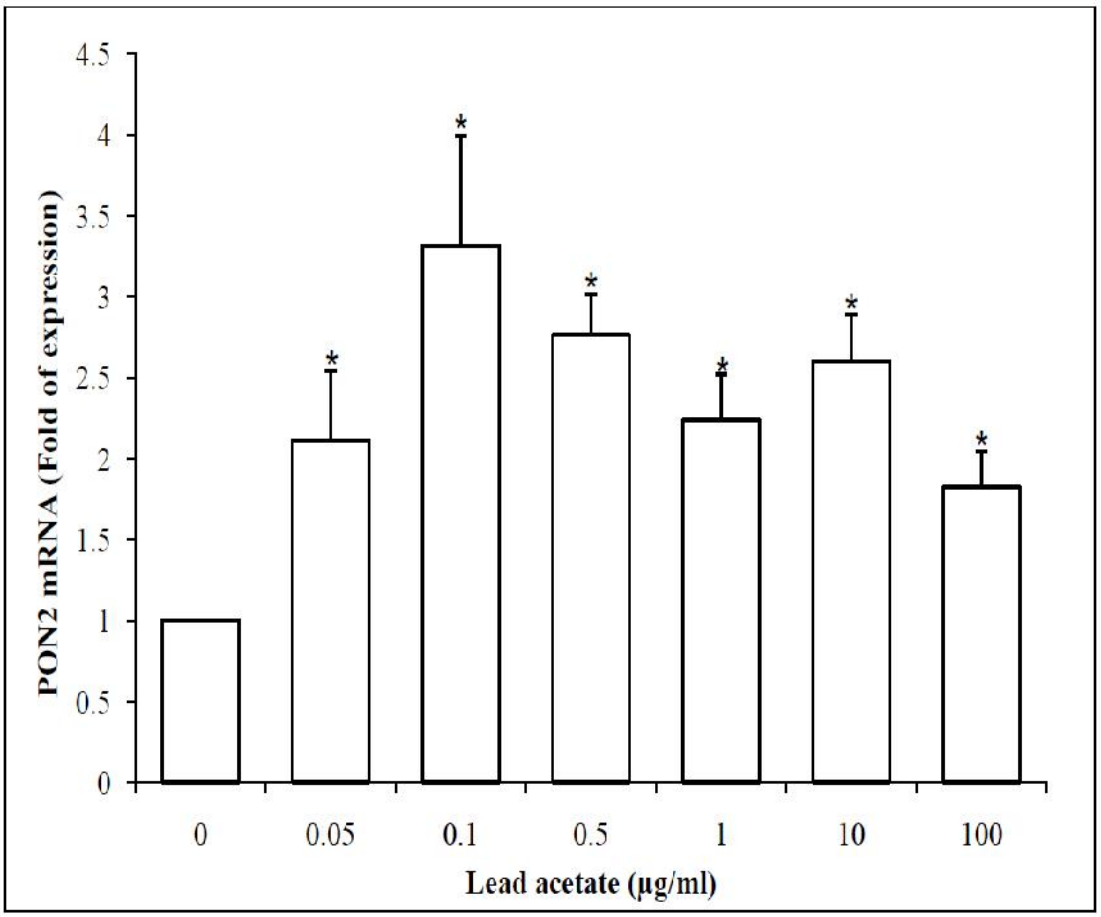

Figure 24. Effect of lead acetate on PON2 mRNA in HepG2 cells. HepG2 cells were treated with various concentrations of lead acetate (0.05-100 µg/ml) for 6 hours. mRNA levels were determined using real time RT-PCR with β-actin as the internal control. Data shown are mean ± SEM of four independent experiments. Differences among groups were evaluated by one-way ANOVA followed by Tukey's post hoc test. * $P< 0.05$ compared to the control (0 µg/ml of lead acetate).

6. Effect of lead acetate on PON2 activity and expression in THP-1 differentiated macrophage cells

In THP-1 differentiated macrophage cells, lead acetate only at 0.1 μg/ml significantly increased PON2 activity by 159.13±9.09% of the control after 72 hours of lead exposure while other concentration of lead acetate (0.05, 0.5, 1, 10, and 100 μg/ml) did not significantly affect PON2 activity (Figure 25).

Effect of lead acetate on PON2 protein and transcript were performed using Western blot analysis and real time RT-PCR, respectively. The results showed that lead acetate at all concentrations used in this study (0.05-100 μg/ml) did not significantly affect PON2 protein at both 24 and 72 hours of exposure (Figure 26) and PON2 mRNA at 6 hours of lead exposure (Figure 27).

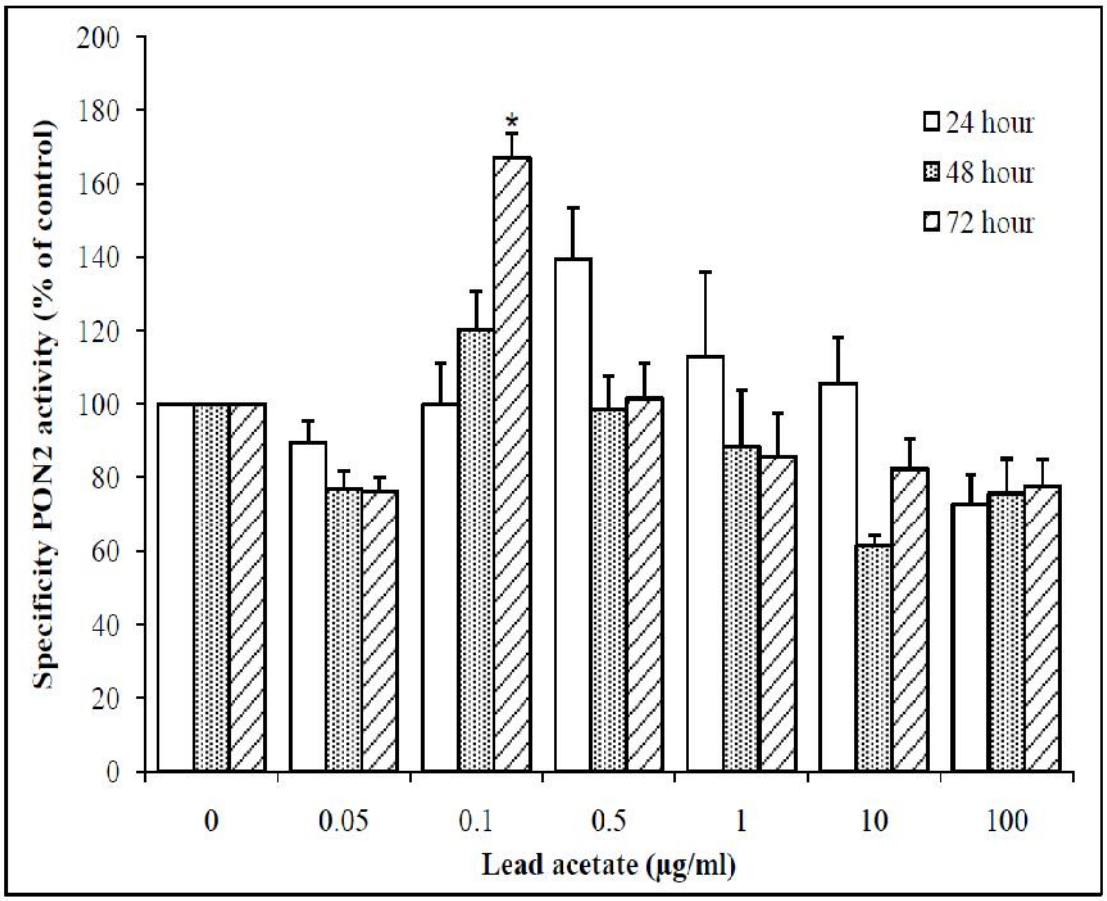

Figure 25. Effect of lead acetate on PON2 activity in THP-1 differentiated macrophage cells. Cells were treated with lead acetate (0.05-100 µg/ml) for 24, 48, and 72 hours. After exposure, PON2 activity toward DHC was measured using spectrophotometer. Data shown are mean ± SEM of three independent experiments. Differences among groups were evaluated by one-way ANOVA followed by Tukey's post hoc test.

* $P < 0.01$ compared to the control (0 µg/ml of lead acetate).

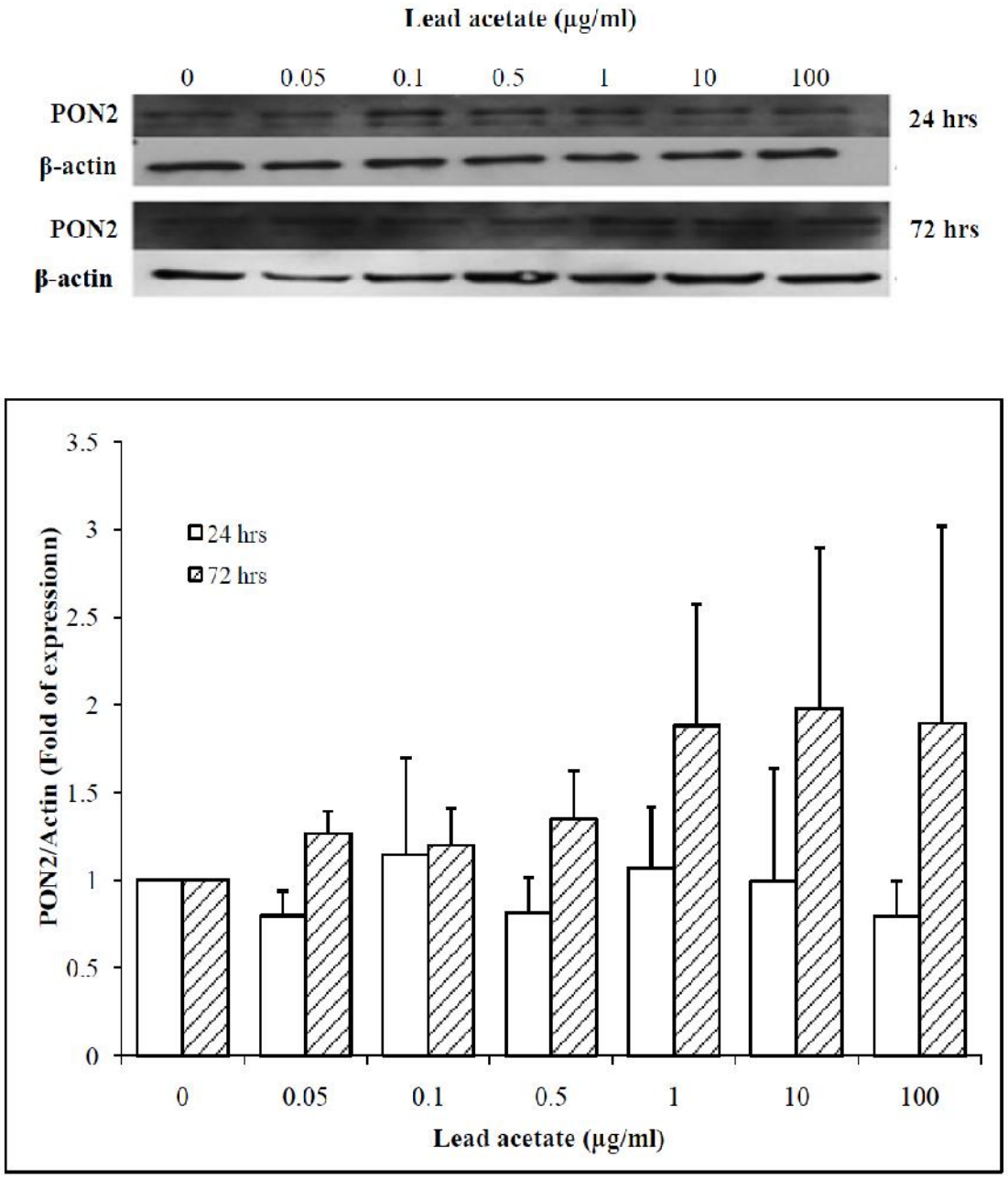

Figure 26. Effect of lead acetate on PON2 protein in THP-1 differentiated macrophage cells. THP-1 differentiated macrophage cells were treated with various concentrations of lead acetate (0.05-100 µg/ml) for 24 and 72 hours. The cell supernatant was separated on 10% SDS-PAGE. **A**, Representative immunoblot of PON2: lane 1, control; lane 2-7, lead acetate at 0.05, 0.1, 0.5, 1, 10, and 100 µg/ml, respectively. **B**, Data from densitometric analysis of the immunoblots of PON2 shown are mean ± SEM of three independent experiments. Differences among groups were evaluated by one-way ANOVA followed by Tukry's post hoc test.

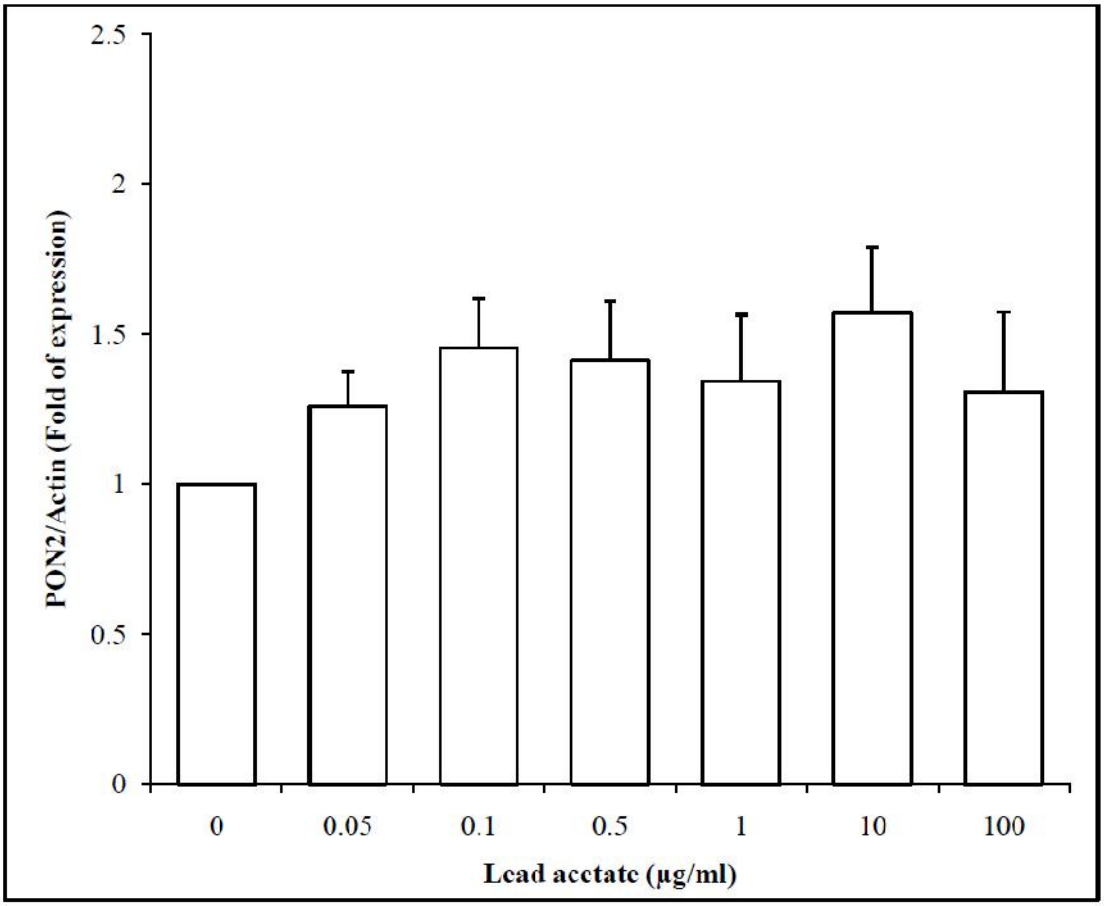

Figure 27. Effect of lead acetate on PON2 mRNA in THP-1 differentiated macrophage cells. THP-1 differentiated macrophage cells were treated with various concentrations of lead acetate (0.05-100 µg/ml) for 6 hours. mRNA levels were determined using real time RT-PCR with β-actin as the internal control. Data shown are mean ± SEM of four independent experiments. Differences among groups were evaluated by one-way ANOVA followed by Tukey's post hoc test.

7. Effect of lead acetate on PON3 activity and expression in HepG2 cells

Lead acetate at 0.1, 0.5, 1, 10, and 100 μg/ml significantly decreased PON3 activity at 24 hours of exposure. In contrast, at 48 and 72 hours of exposure, lead acetate at all concentrations did not significantly affect PON3 activity (Figure 28).

As shown in Figure 29, lead acetate at 10 and 100 μg/ml decreased PON3 protein by 0.51 and 0.48 fold of the control, respectively at 24 hours as well as significant decrease PON3 protein to 0.52 and 0.23 fold of the control, respectively at 72 hours. Lead acetate at 10 and 100 μg/ml significantly decrease PON3 mRNA level in HepG2 cells at 6 hours of exposure (Figure 30).

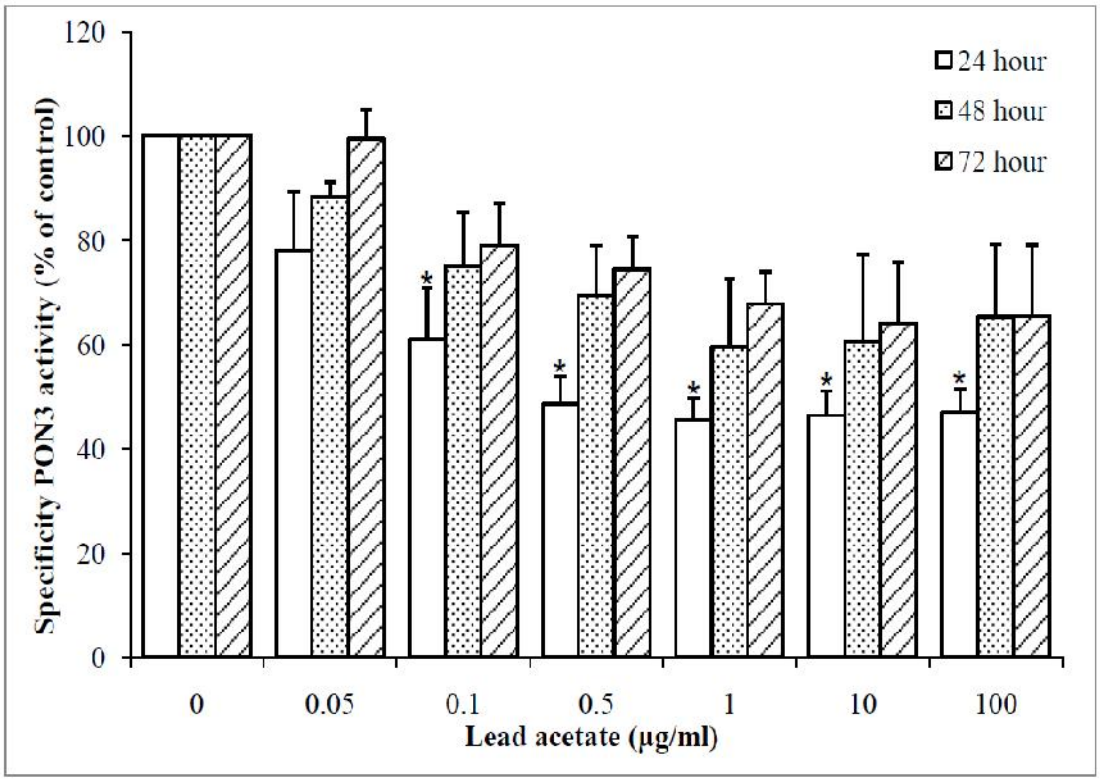

Figure 28. Effect of lead acetate on PON3 activity in HepG2 cells. Cells were treated with lead acetate (0.05-100 μg/ml) for 24, 48, and 72 hour. PON3 activity toward lovastatin was measured using high performance liquid chromatography. Data shown are mean ± SEM of three independent experiments. Differences among groups were evaluated with one-way ANOVA followed by Tukey's post hoc test.

* $P < 0.01$ compared to the control (0 μg/ml of lead acetate).

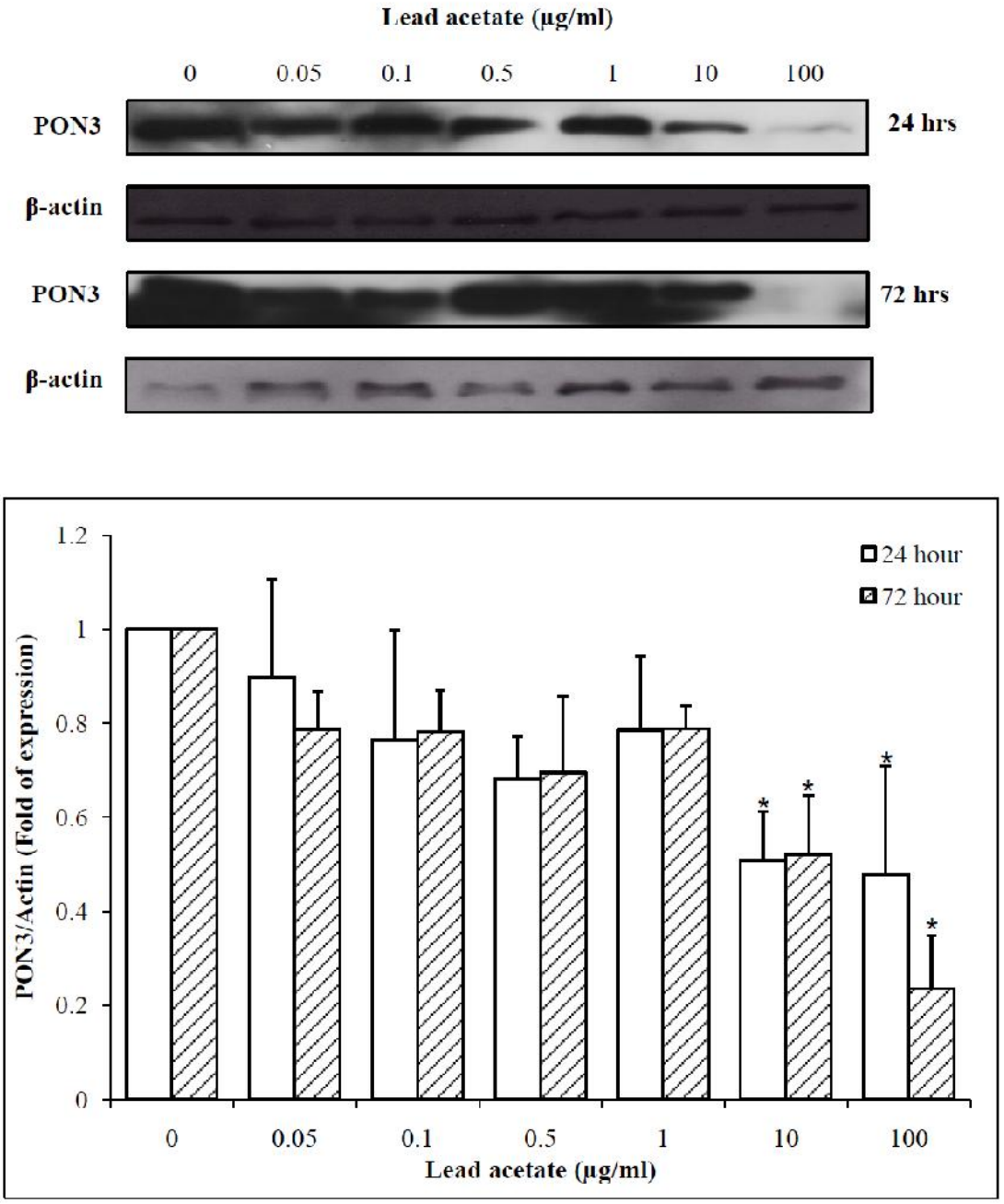

Figure 29. Effect of lead acetate on PON3 protein in HepG2 cells. HepG2 cells were treated with various concentrations of lead acetate (0.05-100 µg/ml) for 24 and 72 hours. The cell supernatant was separated on 10% SDS-PAGE. **A**, Representative immunoblot of PON2: lane 1, control; lane 2-7 lead acetate at 0.05, 0.1, 0.5, 1, 10, and 100 µg/ml, respectively. **B**, Data from densitometric analysis of the immunoblots of PON3 shown are as mean ± SEM of four independent experiments. Differences among groups were evaluated by one-way ANOVA followed by Tukey's post hoc test. * $P< 0.01$ compared to the control (0 µg/ml of lead acetate).

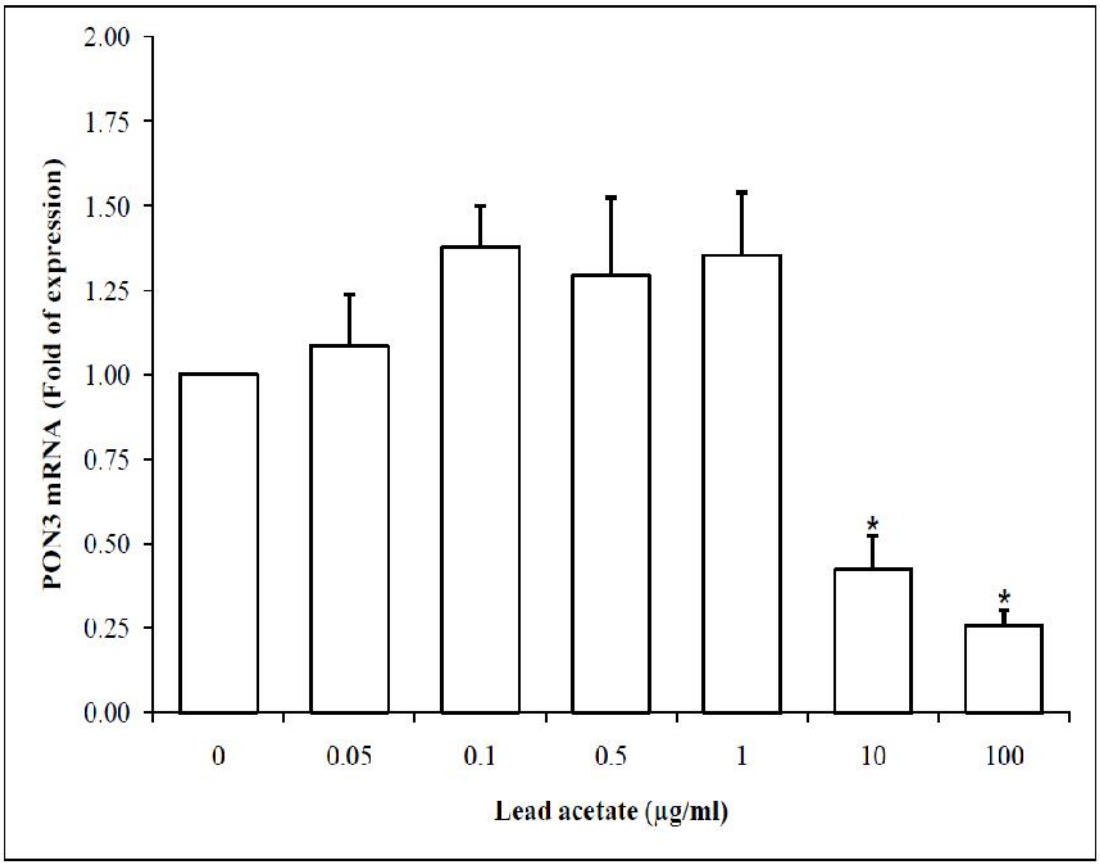

Figure 30. Effect of lead acetate on PON3 mRNA expression in HepG2 cells. HepG2 cells were treated with various concentrations of lead acetate (0.05-100 µg/ml) for 6 hours. mRNA levels were determined using real time RT-PCR, with β-actin as on internal control. Data shown are mean ± SEM of four independent experiments. Differences among groups were evaluated with one-way ANOVA followed by Tukey's post hoc test.

* $P< 0.05$ compared to the control (0 µg/ml of lead acetate).

8. Restoration of lead-inhibited PON2 activity by calcium

Because lead acetate decreased PON2 activities without affecting PON2 protein and mRNA, the decrease of only enzyme activity might be mediated through the effect of lead on Ca^{2+} atom in PON2 structure. To determine whether Ca^{2+} can restore PON2 activity, HepG2 cells lysates were pre-incubated with various concentrations of lead acetate for 30 minutes followed by incubation with 1 mM $CaCl_2$ for 30 minutes before detecting PON2 enzymatic activity. The result showed that Ca^{2+} could significantly restore PON2 activity to approximately 114.39, 108.03, 100.90, 101.03, and 108.57% of the control at 0.05, 0.1, 0.5, 1, and 10 μg/ml of lead acetate exposure, respectively. However at the highest concentration (100 μg/ml) of lead acetate, Ca^{2+} could not restore PON2 activity but if when the 1 mM $CaCl_2$ incubation time was changed from 30 minutes to 45 minutes, PON2 activity could be restored to normal level (Figure 31).

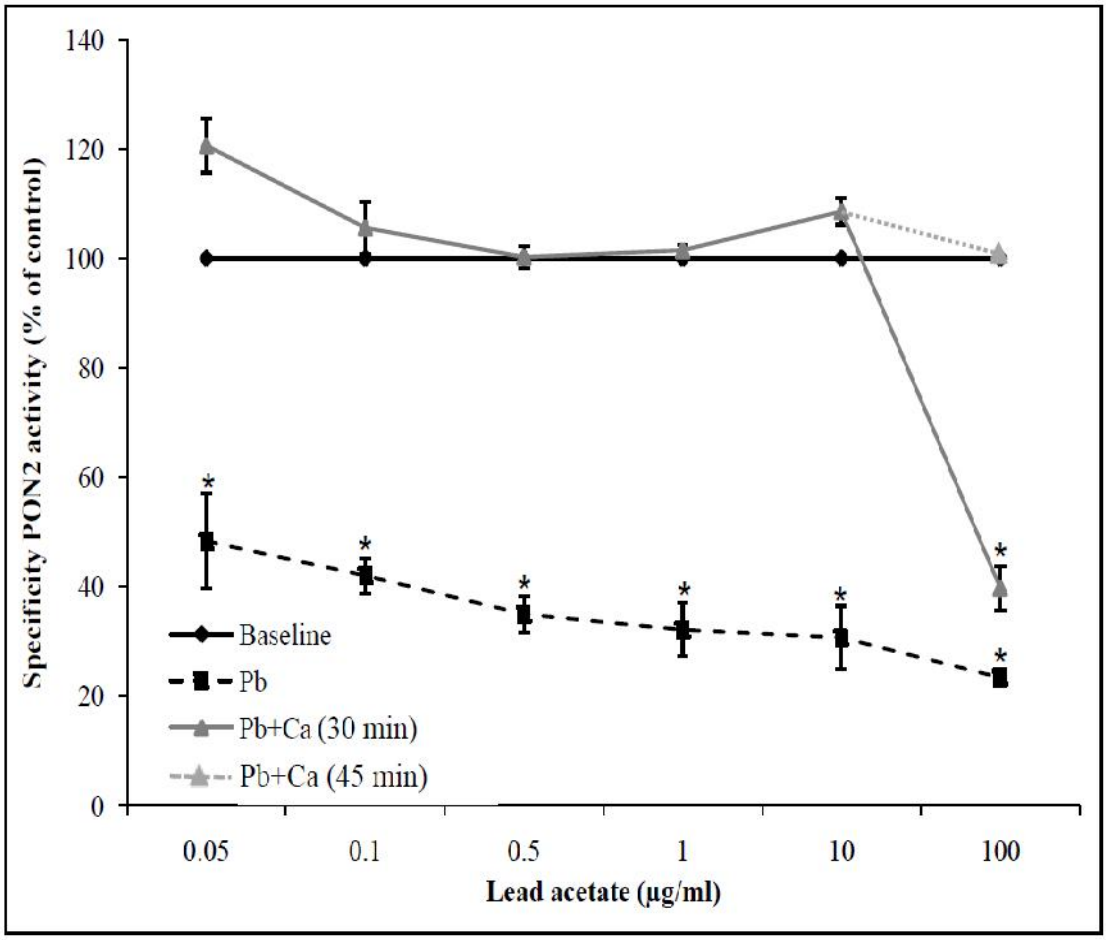

Figure 31. Restoration effect of calcium on lead-inhibited PON2 activity in HepG2 cells. HepG2 cell lysate was added with lead acetate (0.05-100 µg/ml) for 30 minutes followed by incubation with or without 1 mM $CaCl_2$ for 30 minutes before detecting PON2 activity. Data shown are mean ± SEM of three independent experiments.

* $P < 0.001$ compared to the control.

CHAPTER V

DISCUSSION AND CONCLUSION

This study was performed primarily to investigate effects of lead on PON1, PON2 and PON3 activities and investigate the possible mechanism of lead to modulate PON1, PON2 and PON3. HepG2 cells were used in this study because PONs are primarily expressed in the liver (Ng et al., 2005). THP-1 differentiated macrophage cells were additionally used to explore effect of lead on PON2 because this enzyme is also expressed in macrophage (Ng et al., 2001). First of all, effects of lead on cell viability of both cell types were assessed using MTT assay. The results showed that lead acetate did not cause significant cytotoxicity to both HepG2 cells and THP-1 differentiated macrophage cells at the range concentrations of lead acetate between 0.05-100 μg/ml. Significant decrease of cell viability in both cell types was shown at 1000 μg/ml of lead acetate at 24, 48, and 72 hours. Thus, concentrations of lead acetate in the range of 0.05-100 μg/ml were used in the subsequent study so as to evaluate effect of lead on PON while cells were alive. Lead is known to induce oxidative stress and alteration of antioxidant defense systems (Burns et al., 2003; Guillaume et al., 2004; Faruk et al., 2004). PON1, PON2 and PON3 have been proposed as anti-oxidative enzymes (Draganov and La Du, 2004; Teilber et al., 2003; Draganov et al., 2005). Thus, ROS was assessed following exposure to lead acetate at the concentrations between 0.05-100 μg/ml, the concentrations which would be used for further exploration the effect of lead on PON enzymes. It was shown that lead acetate significantly increased ROS in both HepG2 cells and THP-1 differentiated macrophage cells at 0.1-100 μg/ml. In this study, lead-induced ROS similar to the observations reported by other studies *in vitro* and *in vivo*. *In vitro* studies demonstrated an increase of ROS after lead exposure in aortic endothelial cells (Ding et al., 2001), coronary endothelial cells (Vaziri and Ding, 2001; Ni et al., 2004), vascular smooth muscle cells (Ni et al., 2004), neuronal cells, hepatocytes, and kupfer cells (Mudipalli, 2007). In an *in vivo* study in rats given lead also demonstrated an increase of ROS in various organ tissues (Skocznska et al., 1993). However, lead acetate at the concentration between 0.05-100 μg/ml did not change MDA level in both HepG2 cells and THP-1 differentiated macrophage cells. This is consistent to the

results that lead at the concentration between 0.05-100 µg/ml did not cause cell injury (as shown by cell viability result) despite ROS was increased.

Lead-induced oxidative stress may be explained by the reduction effect of lead on antioxidant defense systems. Heavy metals (e.g. mercury, arsenic and lead) have high affinity to sulfhydryl (-SH) groups of proteins. Lead binds to antioxidant enzymes, such as catalase, superoxide dismutase which possess sulfhydryl groups, resulting in dysfunction of antioxidant enzymes then increase of oxidative stress (Hsu, 1981; McGowan and Donaldson, 1986; Chiba et al., 1996; Christie et al., 2001). Furthermore, lead can inhibit or mimic the actions of essential trace elements in antioxidant enzymes such as zinc, copper, calcium etc. resulting in inactivated the enzymes such as catalase, superoxide dismutase and glutathione peroxidase, (Oktem et al., 2004; Jin et al., 2006). PON enzymes have been reported by many studies that possess antioxidative properties by inhibiting oxidized LDL resulting in anti-atherosclerosis (Draganov and La Du, 2004; Mackness et al., 2006; Ng et al., 2006a; Shin et al., 2007; Ng et al., 2007). Thus, the information of effect of lead on PON enzymes may partly explain the atherosclerotic toxicity of lead. The range of lead concentration between 0.05-100 µg/ml which covered the range concentration of lead that significantly increased ROS were used to assess its effect on PON enzymes which have been known as an antioxidant enzymes (Draganov and La Du, 2004; Mackness et al., 2006; Ng et al., 2006a; Shin et al., 2007; Ng et al., 2007).

In this study, lead at all concentrations and exposure times did not cause any significant effects on cell-associated PON1 activity toward phenylacetate. This result is not consistent to previous *in vitro* studies that demonstrated the inactivation of purified human liver/serum PON1 and PON1 rat liver by various metals (lead, cobalt and mercury) using paraoxon as a specific substrate (Gonzalvo et al., 1997; Debord et al., 2003; Pla et al., 2007; Ekinci and Beydemir, 2010). In the *in vivo* studies, lead was found to decrease PON1 activity toward paraoxon (Li et al., 2006) and phenylacetate (Permpongpaiboon et al., 2011) in serum of workers. Modulation of serum PON1 activity in human may involve several other exogenous factors such as PON1 polymorphism, environmental chemicals, drugs, smoking, alcohol, diet, age, and disease conditions which can influence PON1 activity (Costa et al., 2005; Hernandez et al., 2009). Specific substrate using for measurement of PON1 activity is also one of the factor of different sensitivity to detect PON inactivation (Draganov et al., 2005;

Hernandez et al., 2009). In addition, the effect of lead may be different between cell-associated and secreted PON1. Even though, no evidence demonstrates a physical or biological difference between cell-associated and secreted forms of PON1, a study on the structure of PON1 revealed that two helices of PON1 form a unique lid over the pocket of the active site, tunnel of calcium residing, which may play a role in the function of PON1 and the anchoring of PON1 to the cell membrane (Harel et al., 2004). This difference in structure may be, in part, involved in lead to the interaction between lead and calcium at the active site.

There is little known about PON2 until now. However, the similarity in the structure of PON2 to PON1, an antioxidant property, and the highly expression in the liver and other crucial organs triggered the speculation about the effect of lead on PON2. In contrast to PON1, lead significantly decreased PON2 activity toward DHC at all concentrations (0.05-100 μg/ml) and all durations (24, 48, and 72 hours) of lead exposure in HepG2 cells. Considering from this result, lead acetate was more effective at inhibiting PON2 activity than PON1 activity in liver cells. This discrepancy may be due to the difference in the structure and cell localization of the enzymes (Harel et al., 2004). The three PON genes share approximately 65% similarity of amino acids (Mackness et al., 2002). Although the PON2 shares highly homologous in amino acid sequence to PON1, the study on its active site revealed that the two activities, esterase and lactonase, share the same active site but different residues in the active site are involved in its hydrolysis (Harel et al., 2004; Yeung et al., 2005). Hence, it is possible that structural differences between PON1 and PON2 may affect the different inhibitory effects of lead observed in this study. PON2 contains a larger pocket of active site than PON1 (Draganov et al., 2000; Harel et al., 2004) and this may result in an increase the probability of an interaction between lead and the active site of PON2. Regarding cell localization, PON1 is localized at plasma membrane whereas PON2 is exclusively intracellular localized. This intracellular PON2 is not anchor to the membrane (Ng et al., 2001), so these may not be a chance of hindrance of the active site. Since lead decreased PON2 activities in HepG2 cells, PON2 protein and transcript were further assessed. It was shown that lead did not decrease amount of PON2 protein throughout the duration of exposure (24 and 72 hours) and all concentrations of lead exposure. This result implied that inhibitory effect of lead on PON2 activity may be independent of in the amount of PON2

protein. However, lead acetate at all concentration significantly increased PON2 transcript while PON2 activity was decreased at this concentration of lead exposure. This could be explained by the adaptive response of the tissue so as to replenish the enzyme available for performing the reaction (Morel et al., 1999). In addition, PON2 has been found to up-regulate in response to oxidative stress in macrophages (Rosenblat et al., 2003; Shiner et al., 2004, 2006). However, the strong or biphase U-shape response to oxidative stress as previously reported in macrophages did not observed in this study. These may be due to the different cell types (Rosenblat et al., 2003; Shiner et al., 2004, 2006) or lead may induce a lesser oxidative stress than previous model.

Lead may inhibit or mimic the action of calcium as shown in several enzymes such as superoxide dismutase, catalase, and glutathione peroxidase (Gurer and Ercal, 2000). PON2 protein structure contains two calcium atoms, a structural calcium and a catalytic calcium at a pocket of active site, a central tunnel of the propeller (Harel et al., 2004). Thus, it is speculative that lead may decrease PON2 activity via displacement of calcium atom in the structure of PON2 protein. To clarify this issue, this study investigated whether Ca^{2+} could restore PON2 activity from the inhibitory effect of lead on this enzyme. The result showed that Ca^{2+} did restore PON2 activities to normal level except at lead concentration of 100 μg/ml. This implied that lead inhibited PON2 by replacing calcium at the requisite structure or catalytic site of PON2. As previously reported, both plasma and liver paraoxonases exhibited a requirement of Ca^{2+} for catalytic activity (Gil et al., 1994; Gonzalvo et al., 1997; Pla et al., 2007). Previous studies had shown that lead and several other metal ions (lead, cobalt, mercury) were able to inhibit PON1 activity and the enzyme activity could be restored by the addition of free calcium (Gil et al., 1994; Gonzalvo et al., 1997; Pla et al., 2007). Even though Ca^{2+} could not restore PON2 activity at lead concentration of 100 μg/ml, the activity was restored to normal level when the incubation was longer (the duration of Ca^{2+} incubation was changed from 30 minutes to 45 minutes). This simply explained that high concentration of lead exposure may need more duration for the Ca^{2+} to displace lead at the enzyme.

In THP-1 differentiated macrophage cells, lead acetate did not consistently affect PON2 activity. Lead acetate at 0.05, 0.5, 1, 10, and 100 μg/ml did not significantly affect PON2 activity at 24, 48, and 72 hours of exposure while caused

significant increase of PON2 activity at 0.1 μg/ml only at 72 hours. In this study, exposure to higher levels of lead acetate had no influence on PON2 activity. It is possible that different doses of lead may influence PON2 activity differently, an effect which has also been observed in conflicting results obtained using other antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase. One study found that a low dose of lead inhibited superoxide dismutase, catalase, and glutathione peroxidase activity, but a high dose of lead increased the activities of those enzymes in another study (Patra and Swarup, 2001, Sivaprasad et al., 2003, Farmand et al., 2005). The results of the present study may explain the previous contradictory responses of these antioxidant enzymes to different doses of lead. ROS, hydrogen peroxide and superoxide anions have been found to modulate PON2 expression in THP-1 differentiated macrophages (Rosenblat et al., 2003; Shiner et al., 2004). However, in this study, PON2 protein and mRNA were not affected by lead exposure at all concentrations used in this study. Lead acetate seemed not to affect PON2 in THP-1 differentiated macrophage cells. Lead seemed to be less toxic to THP-1 differentiated macrophage cells than HepG2 cells as shown by the lower decrease of cell viability and no toxic was found to 1000 μg/ml at 24 hours of lead exposure in THP-1 differentiated macrophage cells. The different response of lead on PON2 activity in THP-1 differentiated macrophage cells and HepG2 cells seen in this study may be due to different cell types. Hence, the data obtained here could not be explained, the requirement of further investigation is necessary.

In this study, PON3 activity was decreased by lead exposure at 0.1-100 μg/ml only at 24 hours of exposure. This result is consistent to a previous *in vitro* study that demonstrated the inactivation of purified rat liver PON3 with many heavy metals including mercury, copper etc (Pla et al., 2007). In contrast, at 48 and 72 hours of exposure, lead acetate at all concentrations did not significantly affect PON3 activity. Effects of lead acetate on PON3 protein and mRNA level were then further assessed. PON3 protein was decreased only at high concentrations (10 and 100 μg/ml) of lead acetate at both 24 and 72 hours of exposure. PON3 mRNA expression was consistently decreased at 10 and 100 μg/ml of lead acetate. Modulation of PON3 by various substances or conditions was previously studied and the results were shown differently. Copper and mercury had been reported to inhibit purified rat liver PON3 activity but effect of these metals on PON3 protein and mRNA was not assessed in

that study (Pla et al., 2007). ROS was shown to inhibit PON3 activity in mouse peritoneal macrophage without change of PON3 protein and mRNA (Rosenblat et al., 2003) while lead-induced oxidative stress was found to inhibit PON3 activity and expression in this study. However, the inhibition of lead on PON3 activity found in this study was not clearly consistent to the protein and mRNA. The precise mechanism of the lead-induced effects on PON3 activity and expression levels could not be discerned in this study and require further investigation.

Taken together, lead acetate increased ROS and alteration of antioxidant defense system including the activity of PONs particularly of PON2 and partly of PON3 in HepG2 cells. Modulation of PON2 activity by lead was not occurred from modulation of protein or gene expression but possibly from the replacement of lead to Ca^{2+} in the structure of the enzyme.

In conclusion, this study demonstrated that lead significantly increased ROS in both HepG2 cells and THP-1 differentiated macrophage cells. Significant decrease of PON2 activity was observed by lead exposure in a concentration- and time-dependent manner in HepG2 cells but not in THP-1 differentiated macrophage cells. No effect of lead was observed on PON1 activity while partly decrease of PON3 activity was observed in HepG2 cells. Modulation of PON2 activity by lead exposure was not associated to the modulation of both PON2 protein and PON2 mRNA expression. Calcium could restore the inhibitory effect of lead on PON2 activity suggesting that lead decreased PON2 activity via replacement of Ca^{2+} in the structure of PON2 enzyme.

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APPENDICES

Appendix A

Precision assay: Optimal condition variance (OCV) and routine condition variance (RCV)

Table A1 Optimal condition variance (OCV) precisions of the assay for determination of PON1 activity

OCV (No.)	Initial reading	mAbs/Min	Activity (µmole/min/ml)
1	0.657	480.45	374.27
2	0.625	433.70	337.85
3	0.660	477.63	372.08
4	0.660	477.39	371.89
5	0.540	477.30	371.82
6	0.523	465.29	362.46
7	0.509	455.36	354.72
8	0.496	448.06	349.04
9	0.505	458.54	357.20
10	0.684	480.15	374.03
11	0.670	461.46	359.47
12	0.668	467.77	364.39
13	0.662	472.40	368.00
14	0.674	474.52	369.65
15	0.494	439.42	342.31
16	0.510	450.04	350.58
17	0.506	445.08	346.71
18	0.491	440.23	342.94
19	0.467	417.41	325.16
20	0.465	415.25	323.48
Mean			355.90
SD			15.79
OCV (%)			4.44

Figure A2 Routine condition variance (RCV) precisions of the assay for determination of PON1 activity

RCV (No.)	Initial reading	mAbs/Min	Activity (µmole/min/ml)
1	0.693	477.36	371.87
2	0.678	481.75	375.29
3	0.682	487.26	379.57
4	0.704	484.41	377.35
5	0.720	482.67	376.00
6	0.588	445.44	346.99
7	0.586	440.13	362.86
8	0.579	435.09	368.94
9	0.600	435.37	369.15
10	0.596	438.55	341.63
11	0.510	450.04	350.58
12	0.506	445.08	346.71
13	0.491	440.23	342.94
14	0.467	417.41	325.16
15	0.465	415.25	353.48
16	0.465	411.20	360.32
17	0.641	494.26	385.03
18	0.656	501.34	390.55
19	0.631	485.32	378.07
20	0.626	489.51	381.33

The data were shown as mean = 364.19 mmol/min/L, SD = 17.46, RCV = 4.79 and ±2SD = 329.27-399.11 mmol/min/L

Table A3 Optimal condition variance (OCV) precisions of the assay for determination of PON2 activity (using HepG2 sample)

No.	Initial reading	mAbs/Min	Activity (µmole/min/ml)
1	0.135	33.89	1334.88
2	0.082	34.24	1348.52
3	0.350	34.37	1353.53
4	0.309	35.24	1387.85
5	0.350	34.37	1353.53
6	0.242	34.75	1368.52
7	0.135	33.90	1334.88
8	0.350	34.37	1353.53
9	0.240	34.33	1352.14
10	0.350	34.37	1353.53
Mean			1354.093
SD			15.35357
OCV (%)			1.133864

Figure A4 Routine condition variance (RCV) precisions of the assay for determination of PON2 activity (using HepG2 sample)

RCV (No.)	Initial reading	mAbs/Min	Activity (µmole/min/ml)
1	0.186	35.91	1414.32
2	0.226	34.98	1377.76
3	0.125	35.85	1411.89
4	0.102	35.87	1412.61
5	0.085	34.99	1377.92
6	0.089	33.85	1333.12
7	0.123	35.47	1396.72
8	0.138	33.8	1331.11
9	0.133	34.61	1362.93
10	0.051	36.1	1421.64

The data were shown as mean = 1384.00 µmol/min/L, SD = 33.33, RCV = 2.41 and ±2SD = 1317.4-1450.6 µmol/min/L.

.

Appendix B

TABLES OF EXPERIMEMT RESULTS

Table B1. Percentage of cell viability following exposure to lead acetate in HepG2 cells

Lead acetate (µg/ml)	% Cell viability		
	24 hrs of incubation	48 hrs of incubation	72 hrs of incubation
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
0.05	101.08±0.56	99.49±0.44	98.77±1.06
0.1	99.05±0.86	98.17±0.47	97.70±0.79
0.5	97.56±0.53	97.00±1.09	94.80±1.88
1	96.59±0.50	96.30±0.94	92.53±1.84
10	94.32±0.61	93.52±1.96	89.33±2.73
100	91.43±1.47	89.51±0.68	81.45±6.10
1000	30.28±2.44*	25.71±0.55*	15.49±1.50*

Data shown are mean of triplicated experiments.

*P <0.001 as compared to the control (0 µg/ml of lead acetate)

Table B2. Percentage of cell viability following exposure to lead acetate in THP-1 differentiated macrophage cells

Lead acetate (µg/ml)	% Cell viability		
	24 hrs of incubation	48 hrs of incubation	72 hrs of incubation
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
0.05	99.21±0.73	100.32±0.42	99.94±1.08
0.1	100.96±0.94	98.55±0.61	96.34±0.96
0.5	102.52±0.89	96.60±1.43	96.83±0.85
1	101.92±0.60	96.93±0.77	94.09±0.89
10	105.75±0.85	96.09±1.18	93.36±1.58
100	100.88±1.67	97.51±1.43	93.11±3.11
1000	98.03±0.76	30.46±3.49*	15.17±1.70*

Data shown are mean of triplicated experiments.

*P <0.001 as compared to the control (0 µg/ml of lead acetate)

Table B3. Percentage of intracellular ROS production following exposure to lead acetate in HepG2 cells

Lead acetate (µg/ml)	ROS production (% of control)
	1 hr of incubation
0	100.00 ± 0.00
0.05	119.69±8.31*
0.1	135.39±6.57*
0.5	139.16±7.19*
1	142.08±9.66*
10	149.06±12.13*
100	130.46±3.14*

Data shown are mean of triplicated experiments.

*P <0.05 as compared to the control (0 µg/ml of lead acetate)

Table B4. Percentage of intracellular ROS production following exposure to lead acetate in THP-1 differentiated macrophage cells

Lead acetate (µg/ml)	ROS production (% of control)
	1 hr of incubation
0	100.00 ± 0.00
0.05	123.16±5.07*
0.1	129.41±4.54*
0.5	135.35±8.83*
1	149.70±8.82*
10	151.16±10.09*
100	135.21±5.22*

Data shown are mean of triplicated experiments.

*P <0.05 as compared to the control (0 µg/ml of lead acetate)

Table B5. Percentage of MDA level following exposure to lead acetate in HepG2 cells

Lead acetate (µg/ml)	MDA level (% of control)
	1 hr of incubation
0	100.00 ± 0.00
0.05	104.58±4.49
0.1	102.54±4.99
0.5	100.50±7.17
1	102.02±1.34
10	101.19±3.06
100	103.33±2.74

Data shown are mean of triplicated experiments.

Table B6. Percentage of MDA level following exposure to lead acetate in THP-1 differentiated macrophage cells

Lead acetate (µg/ml)	MDA level (% of control)
	1 hr of incubation
0	100.00 ± 0.00
0.05	96.44±7.35
0.1	101.98±7.67
0.5	100.94±4.05
1	96.67±1.61
10	98.27±6.56
100	104.65±4.77

Data shown are mean of triplicated experiments.

Table B7. Effect of lead acetate on PON1 activities in HepG2 cells

Lead acetate (µg/ml)	Specificity PON1 activity (% of control)		
	24 hrs of incubation	48 hrs of incubation	72 hrs of incubation
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
0.05	112.15±6.16	104.04±5.09	92.49±3.64
0.1	108.58±10.79	102.34±4.39	92.43±6.42
0.5	108.29±10.83	101.31±2.67	97.42±6.44
1	101.08±8.91	103.04±4.52	92.10±4.57
10	114.60±8.98	101.50±5.21	95.91±1.53
100	97.83±8.26	104.87±8.32	97.50±2.99

Data shown are mean of triplicated experiments.

Table B8. Effect of lead acetate on PON2 activities in HepG2 cells

Lead acetate (µg/ml)	Specificity PON2 activity (% of control)				
	4 hrs of incubation	8 hrs of incubation	24 hrs of incubation	48 hrs of incubation	72 hrs of incubation
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
0.05	102.24±1.41	97.52±2.76	85.63±0.67#	73.18±0.43#	44.11±0.54#
0.1	96.67±1.38	93.07±3.38	66.39±1.78#	51.51±1.66#	35.87±1.90#
0.5	89.14±3.36*	87.02±2.11*	58.50±1.25#	39.88±0.98#	29.50±2.09#
1	71.89±2.26#	59.01±2.46#	44.37±2.29#	29.07±1.57#	26.51±1.12#
10	48.87±0.57#	48.21±1.45#	38.05±2.78#	23.62±0.88#	21.36±0.84#
100	16.38±1.31#	15.82±0.61#	14.16±1.00#	11.55±0.47#	8.96±1.26#

Data shown are mean of triplicated experiments.

*P <0.05 as compared to the control (0 µg/ml of lead acetate)

#P <0.001 as compared to the control (0 µg/ml of lead acetate)

Table B9. Effect of lead acetate on PON2 protein in HepG2 cells

Lead acetate (µg/ml)	Fold of PON2 protein expression (PON2/Actin)	
	24 hrs of incubation	72 hrs of incubation
0	1.00±0.00	1.00±0.00
0.05	1.00±0.06	0.95±0.02
0.1	0.94±0.09	0.95±0.03
0.5	0.92±0.13	0.92±0.02
1	0.88±0.12	0.92±0.02
10	0.86±0.15	0.93±0.06
100	0.86±0.15	0.92±0.11

Table B10. Effect of lead acetate on PON2 mRNA in HepG2 cells

Lead acetate (µg/ml)	Fold of PON2 mRNA expression (PON2/Actin)
	6 hrs of incubation
0	1.00±0.00
0.05	2.11±0.43*
0.1	3.32±0.68*
0.5	2.76±0.25*
1	2.24±0.28*
10	2.60±0.29*
100	1.82±0.22*

Data shown are mean of triplicated experiments.

*P <0.05 as compared to the control (0 µg/ml of lead acetate)

Table B11. Effect of lead acetate on PON2 activities in THP-1 differentiated macrophage cells

Lead acetate (µg/ml)	Specificity PON2 activity (% of control)		
	24 hrs of incubation	48 hrs of incubation	72 hrs of incubation
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
0.05	89.36±5.97	76.96±4.81	76.27±3.78
0.1	99.78±11.25	120.23±10.54	166.94±6.58*
0.5	139.28±14.13	98.36±9.13	101.53±9.53
1	112.73±23.21	88.31±15.51	85.55±11.96
10	105.66±12.34	61.35±2.95	82.31±8.12
100	72.55±8.22	75.64±9.41	77.46±7.37

Data shown are mean of triplicated experiments.

*P <0.01 as compared to the control (0 µg/ml of lead acetate)

Table B12. Effect of lead acetate on PON2 protein in THP-1 differentiated macrophage cells

Lead acetate (µg/ml)	Fold of PON2 protein expression (PON2/Actin)	
	24 hrs of incubation	72 hrs of incubation
0	1.00±0.00	1.00±0.00
0.05	0.80±0.14	1.26±0.13
0.1	1.15±0.55	1.20±0.21
0.5	0.82±0.20	1.35±0.28
1	1.07±0.35	1.88±0.69
10	0.99±0.64	1.98±0.91
100	0.79±0.19	1.90±1.12

Table B13. Effect of lead acetate on PON2 mRNA in THP-1 differentiated macrophage cells

Lead acetate (µg/ml)	Fold of PON2 mRNA expression (PON2/Actin)
	6 hrs of incubation
0	1.00±0.00
0.05	1.26±0.11
0.1	1.45±0.16
0.5	1.16±0.19
1	1.34±0.22
10	1.57±0.22
100	1.30±0.26

Data shown are mean of triplicated experiments.

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Table B14. Effect of lead acetate on PON3 activities in HepG2 cells

Lead acetate (µg/ml)	Specificity PON3 activity (% of control)		
	24 hrs of incubation	48 hrs of incubation	72 hrs of incubation
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
0.05	77.97±15.22	88.36±3.11	99.46±4.37
0.1	61.12±7.71*	75.05±14.03	78.91±11.49
0.5	48.67±7.40*	69.54±11.21	74.45±8.65
1	45.66±5.37*	59.65±17.09	67.89±6.21
10	46.49±3.82*	60.73±14.67	64.16±6.11
100	47.19±2.20*	65.37±15.14	65.59±10.73

Data shown are mean of triplicated experiments.

*P <0.01 as compared to the control (0 µg/ml of lead acetate)

Table B15. Effect of lead acetate on PON3 protein in HepG2 cells

Lead acetate (µg/ml)	Fold of PON3 protein expression (PON3/Actin)	
	24 hrs of incubation	72 hrs of incubation
0	1.00±0.00	1.00±0.00
0.05	0.90±0.10	0.78±0.08
0.1	0.76±0.12	0.78±0.09
0.5	0.68±0.04	0.69±0.16
1	0.78±0.08	0.79±0.05
10	0.51±0.05*	0.52±0.13*
100	0.48±0.11*	0.23±0.11*

*P <0.01 as compared to the control (0 µg/ml of lead acetate)

Table B16. Effect of lead acetate on PON3 mRNA in HepG2 cells

Lead acetate (µg/ml)	Fold of PON3 mRNA expression (PON3/Actin)
	6 hrs of incubation
0	1.00±0.00
0.05	1.08±0.15
0.1	1.38±0.12
0.5	1.29±0.23
1	1.35±0.18
10	0.42±0.10
100	0.25±0.05

Data shown are mean of triplicated experiments.

*P <0.05 as compared to the control (0 µg/ml of lead acetate)

Table B17. Restoration of PON2 activity by calcium after lead acetate exposure in HepG2 cells

Lead acetate (µg/ml)	Specificity PON2 activity (% of control)			
	Baseline	Pb preincubation	30 min of $CaCl_2$ incubation	45 min of $CaCl_2$ incubation
0.05	100.00 ± 0.00	48.29±8.67*	114.39±4.90	
0.1	100.00 ± 0.00	43.28±3.19*	108.03±4.79	
0.5	100.00 ± 0.00	38.21±3.37*	100.90±2.05	
1	100.00 ± 0.00	35.38±4.87*	101.03±0.98	
10	100.00 ± 0.00	30.72±5.77*	108.57±2.43	
100	100.00 ± 0.00	24.87±1.49*	38.87±4.02	105.60±5.33

Data shown are mean of triplicate

*P <0.001 as compared to the control (0 µg/ml of lead acetate)

Appendix C
Preparation of reagents

1. Medium and buffer

1.1 RPMI1640 medium 1,000 ml

1. These following chemicals were dissolved with distilled water to make 1,000 ml of the medium.

RPMI1640	13.1 g
$NaHCO_3$	2.0 g

2. The pH of the solution was adjusted to 7.3 with 1 N HCl or 1 N NaOH.
3. The solution was steriled by filtration through 0.22 μm Millipore filter.
4. The solution was stored in a refrigerator at 4°C (stable for 1 month).
5. The solution was mixed with 10% FBS before use.
   (90 ml of medium + 10 ml of FBS+ 1 ml of penicilin-streptomycin).

1.2 Phosphate buffer saline (PBS) solution 1000 ml

1. These following chemicals were dissolved with distilled water to make 1000 ml of PBS.

Potassium chloride	0.200 g
Sodium chloride	8.000 g
Potassium phosphate monobasic (anhydrous)	0.240 g
Sodium phosphate dibasic (anhydrous)	1.440 g

2. The pH of the solution was adjusted to 7.2-7.4 with 1 N HCl or 1 N NaOH.
3. The solution was steriled by autoclaving.
4. The solution was stored in a refrigerator at 4°C.

1.3 Potassium phosphate buffer saline 1000 ml

1. The following chemicals were dissolved with distilled water to make 1000 ml of potassium phosphate buffer saline.

KH_2PO_4	4.27 g
K_2HPO_4	15.43 g

2. The pH of the solution was adjusted to 7.2-7.4 with 1 N HCl or 1 N NaOH.

3. The solution was steriled by autoclaving.
4. The solution was stored in a refrigerator at 4°C

1.4 10 mmol/L Tris HCl buffer, 0.9 mmol/L $CaCl_2$, pH 8.0 500 ml

1. The following chemicals were dissolved with distilled water to make 500 ml of 10 mmol/L Tris HCl buffer, 0.9 mmol/L $CaCl_2$.

1 mol/L Trise base	5.0 ml
2 mmol/L $CaCl_2$	0.9 ml
6 mol/L HCL	5.5 ml

2. The pH of the solution was adjusted to 8.0 with 1 N NaOH.
3. The solution was steriled by autoclaving.
4. The solution was stored in a refrigerator at 4°C.

1.5 50 mmol/L Tris HCl buffer, 1 mmol/L $CaCl_2$, pH 8.0 500 ml

1. The following chemical were dissolved with distilled water to make 500 ml of 50 mmol/L Tris HCl buffer, 1 mmol/L $CaCl_2$.

1 mol/L Trise base	25.0 ml
2 mmol/L $CaCl_2$	1.0 ml
6 mol/L HCL	5.5 ml

2. The pH of the solution was adjusted to 8.0 with 1 N NaOH.
3. The solution was steriled by autoclaving.
4. The solution was stored in a refrigerator at 4°C.

2. Reagents for Western blot analysis

2.1 Acrylamide gel

These following solutions were prepared.

Solution A comprised 0.8 % methylene bis acrylamide, 30% acrylamide

Solution B is 5X Separating buffer (Main gel) which was prepared by dissolving these following chemicals with distilled water to make 100 ml of 5X Separating buffer.

2M Tris HCl, pH 8.8	75 ml
10% SDS	4 ml
Distilled water	21 ml

Solution C is 5X Stacking buffer (Top gel) which was prepared by dissolving these following chemicals with distilled water to make 100 ml of 5X Stacking buffer.

2M Tris HCl, pH 6.8	25 ml
10% SDS	4 ml
Distilled water	71 ml

The solutions were stored in a refrigerator at 4°C.

To prepare 10% SDS-PAGE, these following gels were prepared.

Preparation of separating gel

The following solutions were prepared to make 35 ml of 10 % acrylamide gel

Solution A	11.7 ml
Solution B	7.0 ml
Distilled water	16.1 ml

All of the solutions were thoroughly mixed. The mixture was added with 150 µl of 10% ammonium persulfate (APS) and 50 µl of N, N, N, N-tetramethylenediamine (TEMED,) then thoroughly mixed and immediately poured the gel between the glass plates. Before gel polymerization was complete, 0.1% SDS was layered on the top of the separating gel (5 mm thick). The separating gel was completely polymerized at approximately 20-30 minutes.

Preparating of stacking gel

Once the separating gel was completely polymerized, 0.1% SDS was removed from the top of the polymerized gel. Stacking gel comprised these following solutions.

Solution A	2.0 ml
Solution C	2.0 ml
Distilled water	6.0 ml

All of the solutions were thoroughly mixed. The mixture was added with 50 µl of 10% APS and 5 µl of TEMED, then thoroughly mixed and immediately poured the gel between the glass plates. The combs were inserted between the two glass

plates of two sets of gel apparatus. The gels were standed for approximately 30 minutes to polymerize.

2.2 Application of the samples

Once the stacking gel was completely polymerized, the comb was gently removed. The wells were washed with running buffer. The clips and sealing tapes were removed and the gel chamber was set up. Running buffer was filled in both inner and outer chamber. Before loading samples and protein marker, all air bubbles between layers were removed by gentle shaking the chamber.

Running buffer

These following chemicals were used to prepare 1000 ml of 1X running buffer, pH 8.3.

Tris base	3.0 g
Glycine	14.4 g
SDS	1.0 g

All chemicals were dissolved in distilled water to make 1000 ml.

Transfer buffer

These following chemicals were used to prepare 1000 ml of 1X transfer buffer, pH 8.1-8.4.

Tris base	1.93 g
Glycine	9.00 g

All chemicals were dissolved in distilled water to make 800 ml. Before use, the solution was added with 200 ml of methanol.

Tris-buffer saline. 0.05% Tween 20 (TBST)

These following chemicals were used to prepare 500 ml of 10X TBS.

2M Tris-HCl, pH 7.5	25.0 ml
NaCl	43.8 g

All chemicals were dissolved in distilled water to make 500 ml. Before use, 100 ml of 10X TBS was added with 900 ml of distilled water to make 1X TBS and added with 0.5 ml of Tween 20.

Sample buffer

To prepare 2X sample buffer, these following chemicals were used.

0.5 M Tris HCl, pH 6.8	22.5 ml
SDS	3.0 g
Glycerol	10.0 ml
2% bromphenol blue	225.0 µl

All the chemical were dissolved in distilled water to make 50 ml of 2X sample buffer. Before use, 2-mercaptoethanol was added to make 9% of 2-mercaptoethanol in 2X sample buffer and then this solution was diluted to 1X sample buffer with cell lysate.

VITAE

Miss Wanida Sukketsiri was born in December 24, 1980 in Nakorn Sri Tharmmarat, Thailand. She graduated with a Bachelor Degree of Nursing from Faculty of Nursing, Prince of Songkla University in 2004. She completed her Master's Degree of Sciences (Pharmacology) from the Graduate School, Chulalongkorn University in 2006. After her graduation, she worked as clinical research assistance at Siriraj Clinical Research Center, Faculty of Medicine Siriraj Hospital for one year. She has studied for the Doctorral degree of Philosophy in Biopharmaceutical Sciences at Faculty of Pharmaceutical Sciences, Chulalongkorn University since 2008.