ผลของแคดเมียมต่อโปรตีนจีเอพีดีเอชและการแสดงออกของยีนนีโมในส่วนที่มีความสัมพันธ์กับยีนของเอ็นไซม์จี-6-พีดีในเซลล์เฮปจี2

นาย ชัชช์กุญช์ เตชะกิตติโรจน์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีวเวชเคมี ภาควิชาชีวเคมีและจุลชีววิทยา
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2553

EFFECTS OF CADMIUM ON GAPDH PROTEIN AND *NEMO* GENE EXPRESSION RELATING TO *G-6-PD* GENE IN HEPG2 CELLS

Mr. Chatkul Techakitiroj

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biomedicinal Chemistry
Department of Biochemistry and Microbiology
Faculty of Pharmaceutical Sciences
Chulalongkorn University
Academic Year 2010

Thesis Title	EFFECTS OF CADMIUM ON GAPDH PROTEIN AND *NEMO* GENE EXPRESSION RELATING TO *G-6-PD* GENE IN HEPG2 CELLS
By	Mr. Chatkul Techakitiroj
Field of Study	Biomedicinal Chemistry
Thesis Advisor	Associate Professor Palarp Sinhaseni, Ph.D.
Thesis Co-Advisor	Assistant Professor Wisit Tangkeangsirisin, 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 Pintip Pongpech, Ph.D.)

THESIS COMMITTEE

..... Chairman

(Associate Professor Nongluksna Sriubolmas, Ph.D.)

..... Thesis Advisor

(Associate Professor Palarp Sinhaseni, Ph.D.)

..... Thesis Co-Advisor

(Assistant Professor Wisit Tangkeangsirisin, Ph.D.)

..... Examiner

(Associate Professor Vimolmas Lipipun, Ph.D.)

..... Examiner

(Assistant Professor Maneewan Suksomtip, Ph.D.)

..... External Examiner

(Witaya Swaddiwudhipong, M.D.)

ชัชช์กุญช์ เตชะกิตติโรจน์: ผลของแคดเมียมต่อโปรตีนจีเอพีดีเอชและการแสดงออกของยีนนีโมในส่วนที่มีความสัมพันธ์กับยีนของเอ็นไซม์จี-6-พีดีในเซลล์เฮปจี2 (EFFECT OF CADMIUM ON GAPDH PROTEIN AND *NEMO* GENE EXPRESSION RELATING TO *G-6-PD* GENE IN HEPG2 CELLS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. ดร.พาลาภ สิงหเสนี ,อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ. ดร. วิสิฐ ตั้งเคียงศิริสิน 125 หน้า.

แคดเมียมเป็นสารปนเปื้อนที่มีการแพร่หลายทั่วไปโดยพบทั้งในสิ่งแวดล้อม อาหาร และยาสมุนไพร โดยมีรายงานของการศึกษาก่อนหน้านี้พบว่าแคดเมียมลดการทำงานของทั้งเอ็นไซม์จี-6-พีดี และ เอ็นไซม์จีเอพีดีเอช แต่ผลของแคดเมียมต่อการแสดงออกของทั้งยีนจี-6-พีดี และยีนจีเอพีดีเอชนั้นยังไม่เป็นเข้าใจอย่างชัดเจน

ในวิทยานิพนธ์นี้ ผู้วิจัยได้พบว่าการแสดงออกของจี-6-พีดี เมซเซ็นเจอะอาร์เอ็นถูกยับยั้งโดยแคดเมียม นอกจากนั้นทั้งโปรตีนจีเอพีดีเอชและโปรตีนแอลดีเอชนั้นถูกลดลงมาพร้อมกันภายหลังได้รับแคดเมียม อย่างไรก็ตามแคดเมียมยังเพิ่มการแตกหักของโปรตีนฮีสโตน เอช2บี ในเซลล์เฮปจี2 ซึ่งผลดังกล่าวข้างต้นนั้นมีสัมพันธ์กับกลไกการตายของเซลล์แบบเนโครซิสเนื่องจากแคดเมียมชักนำให้โปรตีนพีเออาร์พี-1แตกหักเป็นโปรตีนขนาด 55 กิโลดัลตัน โดยการตอบสนองนี้ขึ้นกับขนาดของแคดเมียมที่ได้รับ นอกจากนี้การแสดงออกของโปรตีนจีเอพีดีเอชที่ลดลงในเซลล์เฮปจี2นั้นถูกทำให้กลับคืนมาได้โดยการได้รับอินซูลิน

การเปลี่ยนแปลงของรีดอกซ์โปรตีนนั้นอาจช่วยในการเข้าใจถึงกลไกการเกิดพิษของแคดเมียมในระดับโมเลกุลและการแตกหักของโปรตีนฮีสโตน เอช2บีนั้นอาจช่วยในการที่แคดเมียมชักนำให้เกิดเซลล์ตายโดยผ่านทางกลไกของอีพิเจเนติก โดยผลทั้งหลายเหล่านี้นำไปสู่การเข้าใจที่ดีขึ้นต่อการเกิดการบาดเจ็บของเซลล์ตับซึ่งสัมพันธ์กับการปรับตัวต่อการเกิดพิษของแคดเมียม

ภาควิชา ชีวเคมีและจุลชีววิทยา ลายมือชื่อนิสิต .....

สาขาวิชา ชีวเคมี ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

4876956433 : MAJOR BIOMEDICINAL CHEMISTRY

KEYWORDS : CADMINM / GAPDH / HISTONE / HEPG2 / HEAVY METAL

CHATKUL TECHAKITIROJ : EFFECT OF CADMIUM ON GAPDH PROTEIN AND *NEMO* GENE EXPRESSION RELATING TO *G-6-PD* GENE IN HEPG2 CELLS. THESIS ADVISOR : ASSOCIATE PROFESSOR PALARP SINHASENI, Ph.D., THESIS CO-ADVISOR: ASSISTANT PROFESSOR WISIT TANGKEANGSIRISIN, Ph.D., 125 pp.

Cadmium is an ubiquitous environmental contaminants in environmental medium, food and herbal medicines. Previous studies reported that cadmium decreased the activity of G-6-PD and GAPDH but the effect of cadmium to *G-6-PD* and *GAPDH* gene expression are not yet clearly understood.

In this thesis, we found that *G-6-PD* mRNA expression was inhibited by cadmium. Moreover, GAPDH and LDHA proteins are concomitantly decreased after cadmium exposure. However it increased histone H2B truncation in HepG2 cells. These results are related to the mechanisms of cell necrosis because cadmium induced 55 kDa PARP-1 cleavage in a dose-response manner. In HepG2 cells, reduced GAPDH protein expression is recovered by insulin treatment.

The alteration of redox system protein may assist in understand molecular mechanism of cadmium toxicity and the truncation of histone H2B may assist in cadmium induced cell death through epigenetic mechanisms. These results bright about to better the understanding in hepatocellular injury in relation to adaptive coping of cadmium toxicity.

Department : Biochemistry and Microbiology Student's Signature Chatkul T.

Field of Study : Biomedicinal Chemistry Advisor's Signature

Academic Year : 2010 Co-Advisor's Signature

ACKNOWLEDGEMENT

First and foremost I would like to deeply thank you my dear advisor, Associate Professor Dr. Palarp Sinhaseni, for her enthusiastic support, and encouragement in the development of this work. She also gives me invaluable encouragement and inspires me to be a good toxicologist with her witty guidance, total support, and avid motivation. I am extremely thankful for her endless effort to provide invaluable guidance and inspired ideas.

I would like to deeply thank my dear co-advisor, Assistant Professor Dr. Wisit Tangkeangsirisin, in Department of Biopharmacy, Faculty of Pharmacy, Silpakorn University for providing me with wonderful facilities in their laboratory, and for their kindness, great help and good advice me. This thesis would not be successful without the kindness, guidance and assistance from him.

I wish to thank Associate Professor Dr. Duangdeun Meksuriyen, Assistant Professor Dr. Maneewan Suksomtip and Associate Professor Dr. Thitima Pengsuparp for kindness and providing me great comment.

I would like to express my thanks to the Faculty of Pharmaceutical Sciences, Chulalongkorn University, especially the Department of Biochemistry and Microbiology staffs, for providing me good facilities and comments.

I would like to acknowledge the help of my lovely colleagues at the Faculty of Pharmaceutical Sciences, Chulalongkorn University and Faculty of Pharmacy, Silpakorn University. Ms .Nopparat Nuntharatanapong, Ms Sasithorn Chamthaworn, and Miss Sutharuthai Chenkwuanma, who always stayed with me and cheers up me. I am also grateful to them for their useful advice. I would like to thank my friends in the Dr. Wisit's Laboratory staffs, Faculty of Pharmacy, Silpakorn University, for their generous support and friendship.

This work was supported in part by a Grant-in-Aid for Scientific Research from Graduate School, Chulalongkorn University.

CONTENTS

CONTENTS

PAGE

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES

ABBREVIATIONS

ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
BSA	Bovine serum albumin
Cys	Cysteine
DMSO	Dimethyl sulfoxide
DNA-PK	DNA-activated protein kinase
FBS	Fetal bovine serum
G-6-PD	Glucose-6-phosphate dehydrogenase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
$GdCl_3$	Gadolinium (III) chloride
GLUT1	Glucose transporters type 1
GLUT2	Glucose transporters type 2
GLUT4	Glucose transporters type 4
GSH	Glutathione
HhomoCys	Homocysteine
HNE	4-hydroxy-2-nonenal
IKK	IκB kinase
IRE-A	Insulin responsive element type A
IRE-B	Insulin responsive element type B
LDHA	Lactate Dehydrogenase A
MEM	Minimal essential medium
MT	Metallothionein
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) - 2- (4-sulfophenyl)-2H-tetrazolium
NAC	N-acetylcysteine
NEMO	NFkappaB essential modulator

NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PARPs	Poly(ADP-ribose) polymerases
OCA-S	Oct-1 co-activator in S-phase
Oct-1	Octamer binding factor 1
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline-Tween® 20
PCR	Polymerase chain reaction
PMS	Phenazine methosulfate
TAE	Tris-Acetate-EDTA
TRIS	Tris (hydroxymethyl) aminomethane

CHAPTER I
INTRODUCTION

Problem Statement

Metals play important biological roles. They are widely used and found in our environment. Dysfunctions of organs are known to be their toxicity.

Cadmium is a widely, but sparsely, distributed element found in the earth's crust at concentrations ranging from 0.1 to 1 ppm, primarily in association with zinc areas. As a by-product of zinc processing, cadmium production has closely followed the demand for zinc. The use of cadmium compounds falls into five categories: active electrode materials in nickel-cadmium batteries (70% of total cadmium use); pigments used mainly in plastics, ceramics, and glasses (12%); stabilizers for polyvinyl chloride (PVC) against heat and light (17%); engineering coatings on steel and some nonferrous metals (8%); and components of various specialized alloys (2%) [1]. It is a toxic metal occurring in the environment naturally and as a pollutant emanating from industrial and agricultural sources. The diet is the main source of cadmium intake in the non-smoking population. Cadmium is present in virtually all foods, but the concentrations vary to a great extent, depending on type of food and level of environmental contamination. High concentrations of cadmium are present in oysters and crabs. High levels are also found in offal products such as liver and kidney, especially from older animals. Plants contain higher concentrations of cadmium than meat, egg, milk and fish. Among food from plants, cereals such as rice, green leafy vegetables, and root vegetables contain higher concentration than other food from plants. Cadmium exposure in Asia is commonly much higher than in Europe and in USA, because of the fast evolution of polluting industries and high intake of rice locally grown on contaminated soil [2].

Adult population living in cadmium-contaminated areas in Mae Sot District, Tak Province, Thailand. The overall prevalence rates of hypertension, diabetes, and urinary stones are 33.3%, 6.2%, and 8.9% respectively. The prevalence rates of hypertension, diabetes, and urinary stones do not significantly increase with increasing urinary cadmium levels. Hypertension, diabetes, and urinary stones are also significant

predictors of impaired renal function [3]. The residents living in these contaminated areas consume rice grown locally. They were at risk of chronic cadmium toxicity [4]. Furthermore, cadmium contamination about 80% of Indian medicinal plants exceeded the maximum permissible level designated by the World Health Organization [5]. A safe intake limit of 7 μM cadmium/week/ kg body weight (PTWI) was set, based on the critical renal cadmium concentration of between 100 and 200 μg/g wet weight that corresponds to a urinary threshold limit of 5-10 μg/g creatinine. However, numerous studies have revealed adverse kidney effects at urinary cadmium levels below 0.5 μg/g creatinine [1, 6].

Cadmium disposition relates to chronic effects. Cadmium is initially distributed to the liver, where it can bind to glutathione and/or induce the synthesis of the cadmium-binding protein metallothionein, which are both thought to serve as intracellular lines of defense against cadmium toxicity. Under these conditions, the liver is usually not injured [7]. Metallothionein plays an important role in cadmium tolerance and cadmium-induced hepatotoxicity. Metallothionein binds cadmium in the hepatic cytosol and renders it "inert." Therefore, metallothionein is beneficial to the liver [8]. In contrast, with the acute, higher levels of exposure that are often used in animal studies, the liver rapidly accumulates high levels of cadmium that overcomes these defense mechanisms and the liver becomes one of the primary sites of injury [7]. *In vivo* studies demonstrate that cadmium-induced cytotoxicity in mouse and rat livers is intimately related to apoptosis, and *in vitro* studies have also demonstrated that cadmium induces DNA fragmentation, a characteristic feature of apoptosis, in HepG2 cells [9]. It is the most toxic nonessential heavy metal. It has an adverse effect on numerous vital processes in cells. It influences body growth and development, physiology, biochemical compositions, morphology, and cytology. It is well known that cadmium binds to and activates calmodulin which plays an important role in the calcium-dependent regulatory pathways of many cellular processes [10].

Cadmium content in kidney cortex of *protein*-calorie malnutrition group is higher than normal nutrition group. This indicated cadmium is not bound to metallothionein in

significant degree in malnutrition group [11]. In 2002, Thai Ministry of Public Health reported that preschool children in the North region are more likely to be malnutrition than in others. In particular, preschool children who reside in the North region have been found about 8 times to have malnutrition than those in Bangkok Metropolitan Areas [12]. Furthermore, glucose-6-phosphate dehydrogenase (G-6-PD) deficiency is the most common human enzyme defect, being present in more than 400 million people worldwide. The highest prevalence of glucose-6-phosphate dehydrogenase deficiency is reported in Southeast Asia, especially Thailand has prevalence about 12% [13]. All previous reports shown that preschool children in North region of Thailand maybe at greater risk population to adverse effects of cadmium.

The non-genotoxic mechanisms upregulating intracellular signaling pathways leading to increased mitogenesis as major mechanisms for the interpretation of the carcinogenic activity by chronic cadmium exposure. Low concentrations, 1 μM, of cadmium stimulates DNA synthesis and cell proliferation in various cell lines, whereas more elevated concentrations are inhibitory. Cadmium enhances the expression of several classes of genes at concentrations of a few micromoles. It stimulates the expression of immediate early genes (*c-fos*, *c-jun*, and *c-myc*), of the tumor suppressor gene p53, and of genes coding for the synthesis of protective molecules, including metallothioneins, glutathione, and stress (heat shock) proteins [14].

Modulation of gene activity by cadmium are thought to be by interfering with cellular signaling at the levels of cell surface receptors, cellular calcium homeostasis, protein phosphorylation, and modification of transcription factors [14]. Furthermore, the protein level of glucose-6-phosphate dehydrogenase (G-6-PD) is increased by cadmium acetate (5 mg/kg) in male rat [15]. The activity of glucose-6-phosphate dehydrogenase (G-6-PD) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) enzymes are significantly decreased by cadmium [16]. Glucose-6-phosphate dehydrogenase (G-6-PD) is essential in all cells. As the first and rate-determining step of the pentose phosphate pathway, its most important function is the production of NADPH for protection against oxidative agents in all cells and in the liver and adipose tissues for the

synthesis of fatty acids. Deficiency of G-6-PD causes hemolytic anemia in response to consumption of fava beans, viral illnesses, and drugs such as antimalarial agents, sulfonamides, nonsteroidal anti-inflammatory agents, and even aspirin [17]. The *G-6-PD* gene is located at the telomeric region of the long arm of the X chromosome (band Xq28). The gene consists of 13 exons and 12 introns, spanning nearly 20 kb in total; it encodes 515 amino acids, and a GC-rich (more than 70%) promoter region. G-6-PD deficiency is an X-linked, hereditary genetic defect caused by mutations in the *G-6-PD* gene, resulting in protein variants with different levels of enzyme activity, that are associated with a wide range of biochemical and clinical phenotypes. About 140 mutations have been described; most are single base changes, leading to amino acid substitutions [13]. The human *G-6-PD* gene maps to Xq28 and is arranged head to head with the NFkappaB essential modulator (*NEMO*) gene [18]. NEMO, the regulatory subunit of the IkappaB kinase (IKK) complex that controls the activation of the transcription factor NF-kappaB, is required for IKK function in most situations [19]. NF-kappaB is maintained in the cytosol bound by Inhibitory-kappaB proteins (IκB). NF-kappaB activity is regulated by phosphorylation of the IκB proteins. External and internal stimuli promote the phosphorylation of IκB proteins *via* the IκB kinase (IKK) complex. Consequently, rapid polyubiquitination of IκBα on two adjacent lysine residues targets it for degradation by the proteosomes [20]. Although NEMO is the only subunit absolutely essential for activation of the IKK complex by diverse stimuli, very little is known about its mechanism of action [21].

In most tissues 80-90% of glucose oxidation is via glycolysis pathway [22]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an important ATP-generating enzyme in the glycolytic pathway [23]. GAPDH gene expression is increased in hepatocarcinoma cell line [24]. Cadmium can affect to GAPDH and glucose-6-phosphate dehydrogenase (G-6-PD) activity in endothelial cell [16, 25]. Redox-acting metals, such as iron and copper, undergo redox recycling to produce free radicals. In contrast, cadmium is redox inactive metals that challenge antioxidant defenses by

binding to thiols in the cell [26]. Reduced glutathione is an antioxidant with –SH group. G-6-PD is a thiol antioxidant enzyme that subject to modification during oxidative stress.

In Endothelial cells, cadmium concentrations greater than 3-5 μM show depletion of glutathione and ATP and almost complete inhibition of the activity of key thiol enzymes such as glucose-6-phosphate dehydrogenase (G-6-PD) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In contrast, cadmium concentrations less than 1–2 μM induced increases in glutathione and thiol-enzyme activities with minimal changes in LDH leakage, barrier function and ATP content. The cytotoxicity is the result of GSH depletion and thiol protein modification, but that at low doses of the cadmium ions, there is a significant compensatory antioxidant response, characterized by an increase in GSH concentration and an increase, possibly an induction, of the thiol enzymes, G-6-PD and GAPDH [16]. Another important thiol enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is involved in ATP production from carbohydrate precursors during glycolysis. Yu *et al.* [27] has proposed GAPDH as a link to NAD^+/NADH status, thus link redox status to cell metabolism. Jiang *et al.* [28] reported that low concentration of cadmium induces the ERK and JNK pathways and promotes human embryo lung fibroblast cell proliferation; while high concentration of cadmium induces p38 pathway and inhibits human embryo lung fibroblast cell proliferation. Furthermore, Hao *et al.* [29] reported that cadmium can stimulate human embryonic kidney cell proliferation at lower concentrations (0.05 and 0.5 μM) but inhibit it at higher concentrations (50 and 500 μM). Apoptosis increases at higher concentrations (50 and 500 μM) of cadmium. While 0.5 μM cadmium decreases the JNK phosphorylation, 50 μM cadmium increases the JNK and P38 phosphorylation. A dose-response increase in metallothionein produced by $CdCl_2$ indicated a biphasic response, with low doses producing relatively more hepatic metallothionein than higher doses [30]. These previous data are supported that cadmium has biphasic effect on cell proliferation, MAPK pathways and metallothionein expression.

Cadmium as a well-recognized human carcinogen, pulmonary cancer. It has been classified as a human carcinogen by IARC on the basis of inhalation studies in

rodents and epidemiological studies in humans. lung is another major target organ for cadmium toxicity and for carcinogenicity. Cadmium-induced oxidative stress appears to play a major role in mediating the negative effects of cadmium in the lung in relation to both asthma and pulmonary fibrosis. The development of lung cancer has also been shown to follow inhalation of cadmium in experimental animals [31].

Cadmium has long been considered a non-genotoxic carcinogen and thought to act through epigenetic mechanisms. Long-term exposure to low-dose cadmium in vitro disrupted growth dynamics and stimulates cell proliferation. Thus cadmium-induced errors in DNA methylation (epigenetic modification), increase the level of genomic DNA methylation and DNA methyltransferases activity, can be an early molecular lesion with the potential for impacting malignant transformation and may be the possible underlying carcinogenic mechanism of cadmium [32].

GAPDH exists in the nucleus and related with in gene transcription, DNA replication, DNA repair, and nuclear RNA export. GAPDH is a necessary component for the proper functioning of octamer binding factor 1 (Oct-1) and Oct-1 co-activator in S-phase (OCA-S), that is essential for S-phase-dependent histone H2B transcription [33]. However, Karaczyn *et al.* [34] reported that the histone H2B truncation do not concur with apoptosis. Cadmium induces either apoptosis or necrosis depending on the cadmium concentration [35, 36].

The setting of standards for individual substances is an important tool in the protection of human health. However, Humans are exposed to very complex environmental mixtures. Assessment of the cumulative risk posed by exposure to multiple chemicals is a problem the USEPA's, FDA's and EU's (Registration, Evaluation, Authorization and Restriction of Chemicals: REACH) program discuss regularly. Mixtures of chemicals produced toxicity only for those chemicals that showed the same toxic effect on the same target organ when given singly at dose above the LOAEL values [37, 38]. Basic concepts of "dose/concentration additively" and "response addition/independence" may be applied to evaluate chemical mixtures in human toxicology, as well as in ecotoxicology. In the case of compounds that cause the same

toxicological effect by the same mode of action, 'dose addition' is a more plausible form of joint action than 'response addition' [39]. A primary goal of the hazard identification and dose–response assessment components of the risk assessment paradigm should include development of a biologically plausible weight of evidence description of the key events in the mode(s) of action, and the dose–response and temporal relationships among key events in each mode of action [40]. Changes in the developmental program by some form of induced epigenetic reprogramming could result in better risk assessment and therefore, management and communication [41].

Epigenetic regulation of mammalian gene expression has profound effects in controlling cell growth, differentiation and cancer development. In general terms, these epigenetic alterations can include covalent modifications of DNA and histones as well as non-covalent changes in nucleosome positioning. The N-termini of histone polypeptides are extensively modified by more than 60 different posttranslational modifications such as methylation, acetylation, phosphorylation and ubiquitination [42]. Moreover, DNA and histone synthesis are coupled and ongoing replication is required to maintain histone gene expression. DNA replication and histone protein synthesis are essential, finely balanced S-phase events. DNA synthesis inhibition causes rapid histone mRNA destabilization that results in histone synthesis shutdown, which raises the possibility that checkpoints regulate histone mRNA levels. A key mechanism in regulating the delivery of histone protein to newly synthesized DNA occurs via control of histone mRNA transcription and degradation. DNA-activated protein kinase (DNA-PK) is activated during replication stress and DNA-PK signaling is enhanced when ataxia telangiectasia and Rad3 related (ATR)/ataxia telangiectasia mutated (ATM) signaling is abrogated. Caffeine (ATR–Chk1 pathway inhibitor) has a substantial effect in stabilizing the bulk of histone mRNA. Histone mRNA stability may be directly controlled by ATR/ATM- and DNA-PK-mediated phosphorylation of UPF1 [43]. Cadmium inhibits DNA-PK activity that accumulation of unrepaired breaks leading to genotoxicity [44] that cause to decrease histone mRNA decay. The epigenetic modification is involved with cadmium by it can inhibit DNA methyltransferase activity in short-term exposure, but prolonged exposure to

cadmium cause neoplastic transformation and attend increases in DNA methylation and DNA methyltransferase activity [45]. However, the mechanisms by which cadmium induces cancer related with epigenetic modifications are poorly understood. Furthermore, metal toxicant such as cadmium, vanadium, and manganese induced apoptosis of varies models [46-49].

Some metals, such as vanadium (IV) oxo-bis(maltolato) (BMOV), an organovanadium compound, is a protein phosphotyrosine phosphatase inhibitor, induced apoptosis in a B-cell line [50]. IGF-1R and PKCδ are required to stimulate PKB phosphorylation in response to BMOV in HepG2 cells [51]. Moreover, vanadium pentoxide (V_2O_5) is neurotoxic to dopaminergic neuronal cells, increase reactive oxygen species generation, followed by release of mitochondrial cytochrome c into cytoplasm and subsequent activation of caspase-9 and caspase-3. Vanadium exposure induced proteolytic cleavage of native PKCδ to catalytically active fragment resulting in a persistent increase in PKCδ kinase activity [52]. This data is supported that vanadium activates caspase-3 to increase PKCδ activity [51] as same as manganese-induced dopaminergic neuronal cell death by caspase-3-dependent proteolytic activation of PKCδ [53]. This process may be explained the mechanisms of PKCδ activation leads to apoptotic cell death. The catalytically active PKCδ fragment can regulate the activity of a variety of cell signaling molecules associated with apoptotic cell death, such as Histone 2B [53]. Moreover, Ajiro *et al.* [54] reported that histone H2B phosphorylation at Serine 14 (phosS14) has been proposed as an epigenetic marker of apoptotic cells and H2B phosphorylated at Ser14 is associated with soluble, cleaved DNA in apoptotic nuclei. This data suggest chromatin post-translational modifications are associated with DNA degradation during apoptosis.

From previous study, cadmium is initially distributed to the liver and accumulates at high levels. When defense mechanisms are overloaded and cells death is related to the liver becomes one of the primary site of injury in human hepatocyte. However, mechanisms are not yet clearly understood. Yet, cadmium decreases both NADH and NADPH, the mechanisms have not been well understood. Therefore, we will investigate

GAPDH and *G-6-PD* gene expression, as two key enzymes to produce NADH and NADPH. On the over hand, cadmium is carcinogen but it is not mutagen effect, which epigenetic modification may assist cadmium-induced cancer that we will to investigate the cadmium effect on nucleosomes, by determination of some core histone, especially histone H2B. Furthermore, determine possible pathway to reverse toxicity effect of cadmium. We suspect some growth factors are reverse cadmium toxicity; the decrement toxicity effect of cadmium may bright to reduce cadmium toxicity in Mae Sot populations.

Objectives

1. To study the effect of cadmium on *NEMO* gene expression related to *G-6-PD* gene in HepG2 cells.
2. To study the effect of cadmium on GAPDH protein expression in HepG2 cells.
3. To study the effect of cadmium on nucleosome component via histone H2B stability in HepG2 cells.
4. To study the effect of cadmium on cell injury relating to apoptosis and/or necrosis in HepG2 cells.
5. To study the effect of insulin to prevent cadmium toxicity in HepG2 cells.

Hypotheses

Cadmium is accumulated at high levels in liver. Acute toxicity on cadmium induces liver cell death. Its effect may be related to redox system in the liver cells. GAPDH and G-6-PD are two enzymes to maintain of redox system in living cell. Furthermore, cadmium is a carcinogen but not mutagen. These effects maybe related to epigenetic modification.

We hypothesizes that

i) Cadmium affect to *NEMO* gene expression related to *G-6-PD* gene expression in HepG2 cells.

ii) Cadmium affect to GAPDH protein expression in HepG2 cells.

iii) Cadmium affects to the nucleosome via histone H2B stability in HepG2 cells.

iv) Cadmium is induced to the cell injury by apoptosis and/or necrosis in HepG2 cells.

v) Insulin could reverse the cadmium toxicity in HepG2 cells.

Contributions of the Study

1. Understand molecular mechanism of cadmium toxicity to better assessment.
2. Better assessment of hepatotoxicity and identified the possible pathway to develop reversible hepatotoxicity in relation to adaptive coping of cadmium toxicity in setting threshold level of metal toxicity.

CHAPTER II
LITERATURE REVIEW

Cadmium

Recently, environmental pollution by cadmium has been discovered in Mae Sot District, Tak Province, Northwestern Thailand. Of 524 rice samples analyzed, their cadmium content ranges between 0.05 and 7.7 mg/kg, with over 95% of the samples contained cadmium above the level considered to be “safe” for staple food (0.1 mg/kg). Mae Sot District is the first reported case of environmental cadmium pollution in Thailand although the exact duration of such extensive pollution remains unknown. In response to the report of cadmium pollution, rice farming in the affected area has been suspended, while rice for household consumption has been supplied to farmers by Thailand officials. In addition, a surveillance program for cadmium toxicity was launched in 2004 by the Department of Community and Social Medicine, the Mae Sot General Hospital and the Bureau of Occupational and Environmental Disease, Ministry of Public Health [3]. The paddy fields receiving irrigation from the two creeks (Mae Tao and Mae Ku) were found to contain markedly elevated cadmium levels during the surveys in 2001-2004. Both creeks passed through a zinc rich area where the zinc mine had been actively operated for more than 20 years [55]. Since most of the residents living in these contaminated areas consumed rice grown locally, they were at risk of chronic cadmium toxicity. Of the 7,697 cadmium-exposed surveyed in 2004 about 7.2% (554 cases) had urinary cadmium levels much more 5 μg/g creatinine [13] and 69.2% of the sediment samples of the creeks exceeded the maximum permissible level of 3.0 mg/kg. Cadmium concentrations were low in the samples collected from the creeks before reaching the zinc area, became much higher when passing through this area, and then declined according to the distance. About 85.0% of the paddy soil samples receiving irrigation from both creeks contained cadmium content above the acceptable level. Rice grain and soybean grown in the areas were also detected to have elevated cadmium content compared with the normal values. In 2004 the cadmium-contaminated areas were estimated to be about 13,200 rais (x 1,600 m^2) of paddy fields affecting 12 villages with

a total population of 12,075. Since the majority of residents consumed rice grown locally, they were at risk of chronic cadmium toxicity. Health risk assessment among these exposed people was launched in 2004 [55].

Cadmium was discovered as an element by the German chemist F. Strohmeier in 1817. The earliest observations of toxic effects from cadmium exposure are from clinical medicine [56]. Cadmium is a metal that may give rise to adverse health effects in humans e.g. renal tubular dysfunction and bone effects [57]. Agency for Toxic Substances and Disease Registry reported that cadmium is an element that occurs naturally in the earth's crust. Pure cadmium is a soft, silver white metal. Cadmium is not usually present in the environment as a pure metal, but as a mineral combined with other elements such as oxygen (cadmium oxide), chlorine (cadmium chloride), or sulfur (cadmium sulfate, cadmium sulfide). Cadmium is often present in nature as complex oxides, sulfides, and carbonates in zinc, lead, and copper ores. It is rarely present in large quantities as the chlorides and sulfates. These different forms of cadmium compounds are solids that dissolve in water to varying degrees. The chlorides and sulfates are the forms that most easily dissolve in water. Cadmium may change forms, but the cadmium metal itself does not disappear from the environment. Knowing the particular form of cadmium, however, is very important when determining the risk of potential adverse health effects. Cadmium compounds are often found in or attached to small particles present in air. Most people can not tell by smell or taste that cadmium is present in air or water because it does not have any recognizable taste or odor. Soils and rocks contain varying amounts of cadmium, generally in small amounts but sometimes in larger amounts (for example in some fossil fuels or fertilizers) [1].

Most cadmium used in the United States is extracted as a by-product during the production of other metals such as zinc, lead, or copper. Cadmium has many uses in industry and consumer products, mainly in batteries, pigments, metal coatings, plastics, and some metal alloys. Release of cadmium from human activities is estimated at from 4,000 to 13,000 tons per year, with major contributions from mining activities, and burning of fossil fuels. The cadmium metal itself does not break down in the

environment, but it can change into different forms. Most forms of cadmium stay for a long time in the same place where they first entered the environment. Some forms of the cadmium that goes into the water will bind to soil, but some will remain in the water. Some forms of cadmium in soil can enter water or be taken up by plants[1].

Fish, plants, and animals can take some forms of cadmium into their bodies from air, water, or food. Cadmium can change forms in the body, but it also stays in the body for a very long time (years). Food and cigarette smoke are the biggest sources of cadmium exposure for people in the general population. Average cadmium levels in U.S. foods range from 2 to 40 parts of cadmium per billion parts of food. Lowest levels are in fruits and beverages, and highest levels are in leafy vegetables and potatoes. Most of the cadmium that enters body goes to kidney and liver and can remain there for many years [1]. It has a long biological half-life of around 20 years in humans [57]. A small portion of the cadmium that enters body leaves slowly in urine and feces. Body can change most cadmium to a form that is not harmful, but too much cadmium can overload the ability of liver and kidney to change the cadmium to a harmless form and the harmful form may damage health [1].

Liver damage is not usually associated with oral cadmium exposure, except at very high levels of exposure. In humans, a fatal dose of cadmium can cause pronounced liver damage. Hepatic effects have been found in rats, mice, and rabbits after oral cadmium exposure. Acute exposure via gavages at doses of 30-138 mg/kg/day causes liver necrosis in most studies. Depletion of liver glutathione by fasting increases the liver necrosis following acute oral exposure to cadmium in rats [1]. Cadmium produces extensive liver injury after acute and chronic exposure in animals. Unlike most hepatotoxicants, cadmium produces liver damage without biotransformation as it does not undergo enzymatic conjugation, and there is no possibility of degradation. Endothelial cells are thought to be the initial target of cadmium in the liver. Following acute cadmium administration, hepatic congestion, ischemia, and hypoxia occur very rapidly. The resultant ischemic hypoxia leads to neutrophil infiltration, Kupffer cell activation, and inflammation, which could potentially contribute to the widespread

hepatocellular apoptosis and necrosis observed with cadmium [58]. The studies referred to in the foregoing sections demonstrated that after uptake from lungs or GI-tract, cadmium is transported in blood plasma bound to albumin. This binding form of cadmium is predominantly taken up by the liver. A part of liver cadmium is excreted in the bile bound to glutathione o However, total biliary excretion and the proportion bound to glutathione decreases in long term exposure (when a larger proportion of liver-cadmium is bound to MT) and the dominating route for gastrointestinal excretion of cadmium is not via bile or pancreatic fluid, but via shedding of gastrointestinal mucosal cells. It was shown in animal experiments that within 24 hour after a single dose of cadmium chloride, an increased proportion of liver cadmium will be bound to metallothionein in liver tissue. A small proportion of metallothionein-bound cadmium will be released into blood plasma. Metallothionein-bound cadmium in plasma is filtered through the renal glomeruli and reabsorbed in the proximal renal tubules. This transport mechanism between the liver and kidney was demonstrated by transplantation of livers from cadmium-exposed animals to non-exposed ones. There was a selective transport of cadmium from the liver and uptake of cadmium in the kidneys of the transplanted animals. Reabsorption of the cadmium -metallothionein complex occurs in the renal tubules by adsorptive endocytosis mediated by the ZIP8 transporter on the apical surface of renal tubular cells. After uptake, cadmium enters the lyzosomes in the tubular cells where cadmium is released from metallothionein and the released cadmium ions may cause tissue damage if protective metallothionein is not present. In long-term exposure, the unbound cadmium stimulates new metallothionein production in renal tubular cells and metallothionein binds cadmium and protects the cells. This is the situation in animals and humans with intact metallothionein synthesis. These mechanisms are summarized in the scheme shown in Figure 1 [56].

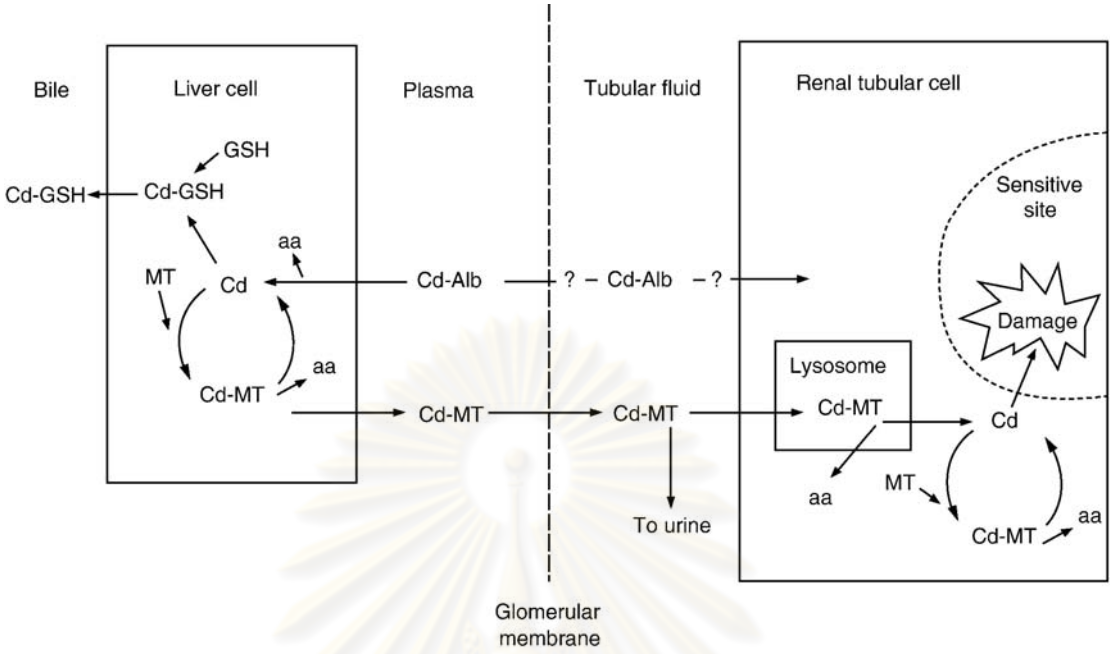

Figure 1. Basic flow scheme of cadmium in the body demonstrating the role of binding forms in blood and metallothionein synthesis and degradation in liver and kidney tissue. GSH, glutathione; MT, metallothionein; aa, amino acids; Alb, albumin [56].

Cellular injury induced by cadmium is dependent on a number of factors, including dose, route of exposure, and duration of exposure. In order to understand how cadmium intoxicates target cells, it is necessary to gain a detailed understanding of the mechanisms involved in the transport of cadmium into those cells and the subsequent bonding interactions that occur with cadmium before and after it has entered into the intracellular milieu. In particular, it is important to gain a thorough understanding of the bonding interactions that occur between cadmium and the abundant low-molecular-weight, intracellular thiols (such as glutathione and metallothionein) and critical nucleophilic binding sites on proteins that play vital roles in maintaining normal homeostasis in cells [59].

Humans are generally exposed to cadmium by two main routes, inhalation and ingestion. The body burden of cadmium is derived primarily from ingestion of food and drinking water contaminated with cadmium. $CdCl_2$ is the principal form of cadmium associated with oral exposure, as it is highly soluble in water. The efficiency of gastrointestinal absorption of cadmium has been reported to be approximately 3–8% of the ingested load. The bioavailability of cadmium from a single dietary load has been shown to be almost three times greater in women than in men. Intestinal absorption of cadmium is characterized by a rapid rate of accumulation of cadmium within the intestinal mucosa and a low rate of diffusive transfer into systemic circulation. The intestinal uptake of cadmium and its subsequent distribution to target tissues is greatly dependent on the chemical form of cadmium presented to the intestinal epithelium. Cadmium bound to metallothionein (Cd-MT) or cysteine (Cd-(Cys) is in a form that permits cadmium ions to be delivered to, and taken up more readily by, the target epithelial cells in the kidneys. The complex nature of the handling of cadmium in the body after oral/gastrointestinal and pulmonary exposure to cadmium and the potential species of cadmium that may be involved in the uptake, accumulation, and elimination of cadmium in the primary target organs are summarized in the scheme shown in Figure 2 [59].

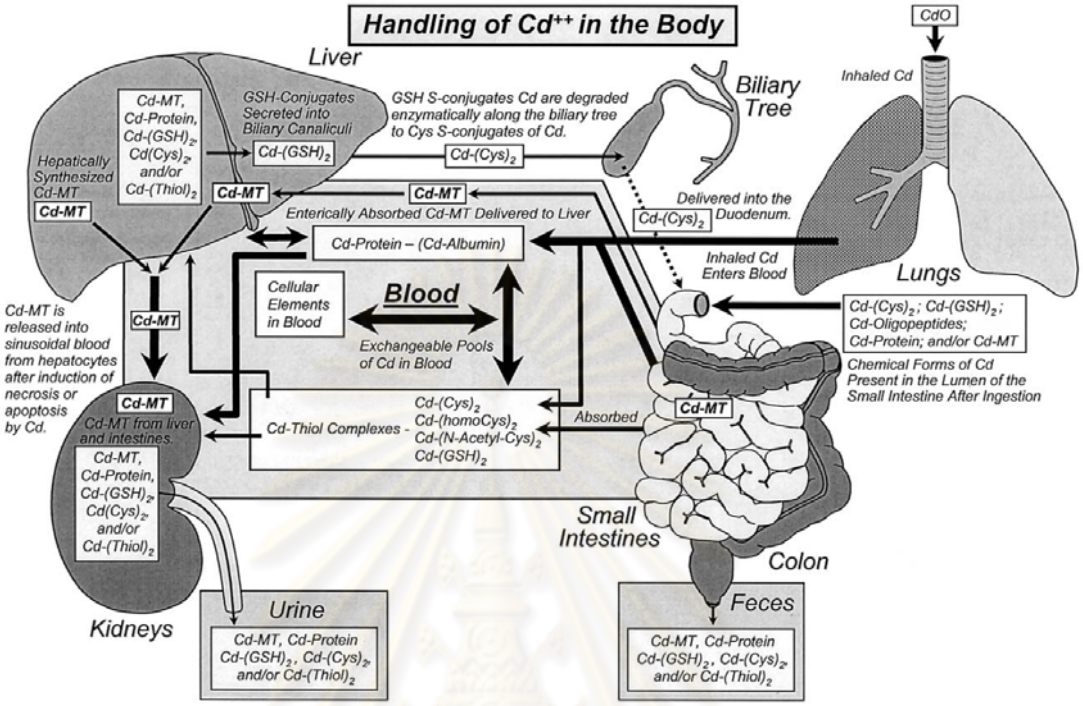

Figure 2. A summary of the potential forms of cadmium present in the small and large intestines, liver, biliary tree, and kidneys after oral or pulmonary exposure to cadmium salts or cadmium-protein complexes. This figure provides a schematic presentation of the potential pathways involved in the handling and excretion of the different forms of cadmium that may be present in the relevant compartments of the body. MT, metallothionein; Cys, cysteine; GSH, glutathione; *N*-Acetyl-Cys, *N*-acetylcysteine; homoCys, homocysteine [59].

Regardless of oral, pulmonary, or parenteral exposure, the liver is by far the primary organ that takes up the greatest quantity of cadmium during the initial hours after exposure, especially after parenteral exposure. The liver is so effective in clearing the blood of cadmium that less than 1% of the dose was remaining in the total blood volume 1 hour after exposure. The amounts of cadmium taken up by the liver accounted for as much as 50–60% of the administered dose. The liver accumulates substantial amounts of cadmium after either acute or chronic exposure to hepatotoxic doses of cadmium. Numerous investigators believe that, when hepatocellular necrosis and/or apoptosis is/are induced by cadmium, a significant amount of the metal in the necrotic and/or apoptotic hepatocyte is released into hepatic circulation in the form of Cd-MT [59].

The mechanisms of cadmium-induced hepatotoxic effects have been reported, in experimental animal models following elevated in vivo exposure conditions and following in vitro exposures and cadmium produced DNA damage, elevated expression of the proto-oncogenes *c-myc*, *c-fos*, and *c-jun* and apoptosis in a dose related manner in liver cells of rats injected with cadmium chloride at doses of 5, 10, 20 μmol/kg. In vitro studies on human liver cells and rat liver cells have reported similar findings with regard to cadmium-induced oxidative stress and dose-related cellular stress protein responses and apoptosis. Normal and immortalized human liver cells exposed to cadmium over a concentration range of 1–100 μmol/L [23]. They observed DNA damage and loss of mitochondrial membrane potential with activation of caspase-9 and caspase-3 which was associated with the development of apoptosis. In addition, they also observed increased expression of p53 and phosphorylation of the serine 15 residue on this protein which is important for its biological activity [23].

These studies provide useful mechanistic information on the central roles of oxidative stress in mediating cadmium toxicity in human liver cells which are consistent with the results of in vivo exposure studies. Normal rat liver cells exposed to cadmium and focused on roles of altered expression of heat shock proteins such as heme oxygenase-1 (HO-1) and the major stress protein families in mediating cadmium-induced oxidative stress related toxicity. They found that these protective cellular systems were able to maintain cell viability over short exposure periods but with longer exposures, down regulation of fundamental cellular protein systems occurred resulting in cell death. Consistent with possible indirect linkages and extensive anti-oxidant protection systems in the liver are the results from in vitro gene expression. These studies also confirmed the up-regulation of antioxidant systems in rat liver hepatocytes [23].

Cadmium toxicity often implicate with reactive oxygen species (ROS), either in a variety of cell culture systems or in intact animals through all routes of exposure. It has been suggested that the mechanisms of acute cadmium toxicity involve the depletion of glutathione and protein-bound sulfhydryl groups, resulting in enhanced production of reactive oxygen species such as superoxide ion, hydrogen peroxide, and hydroxyl radicals. Cadmium can induce reactive oxygen species and overwhelm the antioxidant defenses, leading to increased lipid peroxidation and oxidative DNA damage. Acute cadmium overload can generated free radicals, including superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$) and lipid radicals ($^{\cdot}L$) [60].

Cadmium is not a redox active metal, and free radical generation by cadmium must be mediated through indirect mechanisms, including glutathione depletion, Kupffer cell activation, inflammation, and involvement of iron for the Fenton reaction. However, during long-term exposure to cadmium at low doses, reactive oxygen species accumulation may not be the only mechanisms associated with cadmium-induced chronic toxicity and carcinogenesis. Adaptive mechanisms including induction of metallothionein, glutathione, and cellular antioxidants. The initiation of oxidative DNA damage and subsequent apoptotic resistance, epigenetic DNA methylation status

changes, and aberrant gene expressions, all of which could play integrated roles in cadmium carcinogenesis (Figure 3) [60].

Occupational exposure to cadmium has been associated with occurrence of increased oxidative stress and cancer such as human pancreatic cancer and renal carcinoma. Cadmium itself is unable to generate free radials directly, however, via indirect mechanisms; it can cause free radical-induced damage to the gene expression. It has been reported that cadmium can cause activation of cellular protein kinases, which result in enhanced phosphorylation of transcription factors and consequently lead to the transcriptional activation of target gene expression [61].

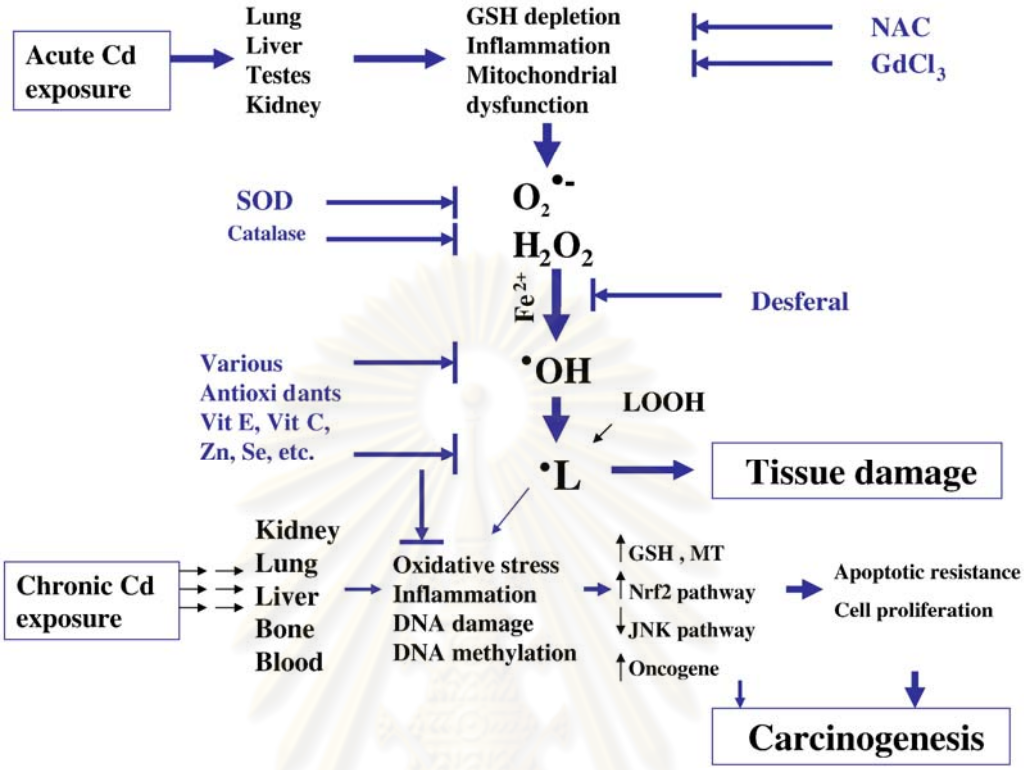

Figure 3. Proposed pathways for reactive oxygen species in cadmium toxicology and carcinogenesis following acute and chronic exposures. MT, metallothionein; SOD, superoxide dismutase; GSH, glutathione; NAC, *N*-acetylcysteine; $GdCl_3$, gadolinium (III) chloride [60].

Reactive Oxygen Species

Oxygen free radicals or, more generally, reactive oxygen species (ROS), as well as reactive nitrogen species (RNS), are products of normal cellular metabolism. ROS and RNS are well recognized for playing a dual role as both deleterious and beneficial species, since they can be either harmful or beneficial to living systems. Beneficial effects of ROS occur at low/moderate concentrations and involve physiological roles in cellular responses to normoxia. The harmful effect of free radicals causing potential biological damage is termed oxidative stress and nitrosative stress [61]. Reactive oxygen species (ROS) are highly reactive molecules or molecular fragments that are continuously produced in all aerobic organisms, mostly as a consequence of aerobic respiration. The term covers several types of reactive oxygen metabolites, including free radicals, which are defined as a molecule containing one or more un-paired electrons on its outermost orbital, for example, superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($HO^{\cdot}$) and singlet oxygen. The term ROS also encompasses some non-radicals such as hydrogen peroxide (H_2O_2). The life-span of different ROS varies considerably, from less than 1 ns of hydroxyl radical to even hours of H_2O_2, these figures depending on numerous cellular environment factors. Besides the high reactivity, another important feature of ROS is that their reactions with non-radicals tend to result in the formation of new radicals [62].

At low levels, they are known to act as important signaling molecules. For instance, in plants, ROS are used to facilitate an array of essential biological processes, including immune defence, growth and development, seed germination and the alleviation of seed dormancy, programmed cell death and stress acclimation. Yet, the effects of ROS are dose dependent, and at high levels these highly reactive molecules will exert oxidative stress on the cell, and invoke profound changes in gene expression. If left unchecked, oxidative stress will result in cumulative oxidative damage to DNA, RNA and proteins within the cell [63]. Multiple pathways involved in ROS-induced cell death have been proposed. ROS can cause direct injury to proteins, lipids, and nucleic acids, leading to cell death. Protein oxidation can impair a wide variety of enzymatic processes and growth factors that can result in marked cellular dysfunction [64].

Mechanisms involved in the oxidation of proteins by ROS were elucidated by studies in which amino acids, simple peptides and proteins were exposed to ionizing radiations under conditions where hydroxyl radicals or a mixture of hydroxyl/superoxide radicals are formed. The side chains of all amino acid residues of proteins, in particular cysteine and methionine residues of proteins are susceptible to oxidation by the action of ROS. Oxidation of cysteine residues may lead to the reversible formation of mixed disulphide between protein thiol groups (–SH) and low molecular weight thiols, in particular GSH (*S*-glutathiolation). The concentration of carbonyl groups, generated by many different mechanisms is a good measure of ROS-mediated protein oxidation [61]. Lipid peroxidation has been linked to cell death through effects on cellular phospholipids through activation of sphingomyelinase and release of ceramide, which activates apoptosis. Nucleic acid oxidation has been linked with physiologic and premature aging as well as DNA strand breaks, leading to necrosis and/or maladaptive apoptosis [64].

Oxidative stress has been implicated in various pathological conditions involving cardiovascular disease, cancer, neurological disorders, diabetes, ischemia/reperfusion, other diseases and ageing. In addition to ROS, various redox metals, due to their ability to generate free radicals, or non-redox metals, due to their ability to bind to critical thiols, have been implicated in the mechanisms of carcinogenesis and ageing. These diseases fall into two groups: (i) the first group involves diseases characterized by pro-oxidants shifting the thiol/disulphide redox state and impairing glucose tolerance—the so-called “mitochondrial oxidative stress” conditions (cancer and diabetes mellitus); (ii) the second group involves disease characterized by “inflammatory oxidative conditions” and enhanced activity of either NAD(P)H oxidase (leading to atherosclerosis and chronic inflammation) or xanthine oxidase-induced formation of ROS (implicated in ischemia and reperfusion injury). The process of ageing is to a large extent due to the damaging consequence of free radical action (lipid peroxidation, DNA damage, protein oxidation), as illustrated in Figure 4 [61].

Oxygen has a unique molecular structure and is abundant within cells. It readily accepts free electrons generated by normal oxidative metabolism within the cell, producing ROS, such as superoxide anion and hydroxyl radical, as well as the oxidant H_2O_2. Processes causing uncoupling of electron transport can enhance the production of ROS, with mitochondria being a major source [64]. The production of superoxide occurs mostly within the mitochondria of a cell. The mitochondrial electron transport chain is the main source of ATP in the mammalian cell and thus is essential for life. During energy transduction, a small number of electrons “leak” to oxygen prematurely, forming the oxygen free radical superoxide, which has been implicated in the pathophysiology of a variety of diseases [61].

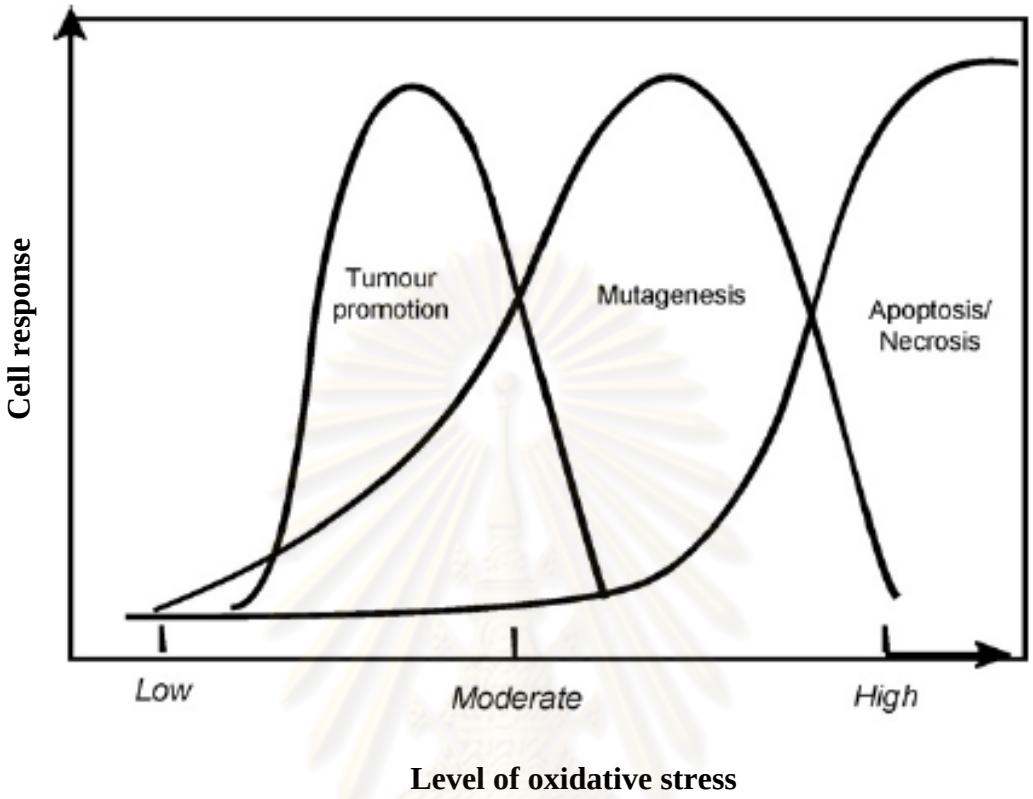

Figure 4. The dose-dependent effect of relationship between level of oxidative stress and the process of tumor promotion, mutagenesis, and apoptosis/necrosis [61].

Antioxidant and other cell redox state modulating enzyme systems act as the first-line defense against ROS in all cellular compartments and also extracellularly. The most important of these enzymes include superoxide dismutases, glutathione peroxidases, catalase and peroxiredoxins. The specific role of cell redox state modulating enzymes in carcinogenesis is still unambiguous since their roles in ROS detoxification are to a certain extent overlapping and they are also involved in many physiological processes [62]. The cellular, subcellular, and tissue-specific expression of antioxidant enzymes, such as superoxide dismutases (SOD), catalase, glutathione peroxidases, and peroxiredoxin largely determine the relative vulnerabilities of tissues and cells to ROS-mediated injury. However, oxidative stress may actually regulate antioxidant capacity, with newborn rats demonstrating upregulation of glutathione peroxidase catalase, and CuZn (cytosolic) SOD expression and activity in response to hyperoxia [64]. Furthermore, bioenergetics by mitochondria and redox state are determined by intracellular Ca^{2+} levels. Ca^{2+} may increase mitochondrial ROS formation by many mechanisms, including enhancing citric acid cycle activity and NADH formation, promoting the loss of cytochrome c due to the mitochondrial permeability transition and activating ROS-generating enzymes such as glycerol phosphate, α-ketoglutarate dehydrogenase. An early event in Ca^{2+}-induced mitochondrial ROS generation may be an increase in lipid packing in the mitochondrial inner membrane [65]. Cadmium depletes glutathione and protein bound sulfhydryl groups, resulting in enhanced production of ROS such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals [60]. Furthermore, previous study reported that cadmium chloride can induce hepatic toxicity in rodents. ROS formations are increased with LD_{50} concentrations of cadmium (20 μM of cadmium chloride) which are released by proton ionophores that uncouple oxidative phosphorylation. Mitochondria are major contributors to endogenous ROS formation. Glycolytic substrates (Fructose, xylitol, glutamine) inhibit cadmium-induced ROS formation and mitochondria are the site of ROS formation. Mitochondria are the ROS sites for non-redox or poor redox cycling transition metals such as cadmium [66]. The targets of reactive oxygen species for

modification protein are containing with thiol groups of cysteine residues. Such modification can lead to important cellular signaling processes that ultimately result in modification of the physiology of the organism [67].

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GAPD)

In most tissues 80-90% of glucose oxidation is via glycolysis, and the remaining 10-20% occurs via the pentose phosphate pathway [22]. The glycolysis is ancient metabolic machinery in which one mole of glucose is catabolized to two moles of each pyruvate, NADH and ATP. Under aerobic conditions, pyruvate is further oxidized by mitochondrial enzymes to CO_2 and H_2O. Glycolysis can be separated into two steps, the first step uses two moles of ATP to convert glucose to fructose-1, 6-bisphosphate. In the second step, fructose-1,6-bisphosphate is converted stepwise into pyruvate with the production of four moles of ATP and two moles of NADH, as illustrated in Figure 5 [68].

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH;GAPD) (EC1.2.1.13) has been considered to be primarily a housekeeping enzyme involved in the glycolytic pathway, catalyzing the NAD-dependent conversion of glyceraldehyde-3-phosphate into 1,3-diphosphoglycerate [69], using NAD^+ as the electron acceptor [70]. GAPDH comprises a polypeptide chain of 335 amino acids. Structural studies identified two regions, namely the glyceraldehyde-3-phosphate catalytic site and the NAD^+ binding site, a primary structure known as the Rossmann fold, which is also required for the activity of other dehydrogenases [70]. One of the consequences of oxidative stress is a drop in cellular ATP levels and blocked glycolysis, largely due to the inactivation of the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH catalyses the oxidative phosphorylation of glyceraldehyde-3-phosphate to 1,3 bisphosphoglycerate by converting NAD^+ to the high-energy electron carrier NADH [71]. which GAPDH is an important ATP-generating enzyme in the glycolytic pathway [23].

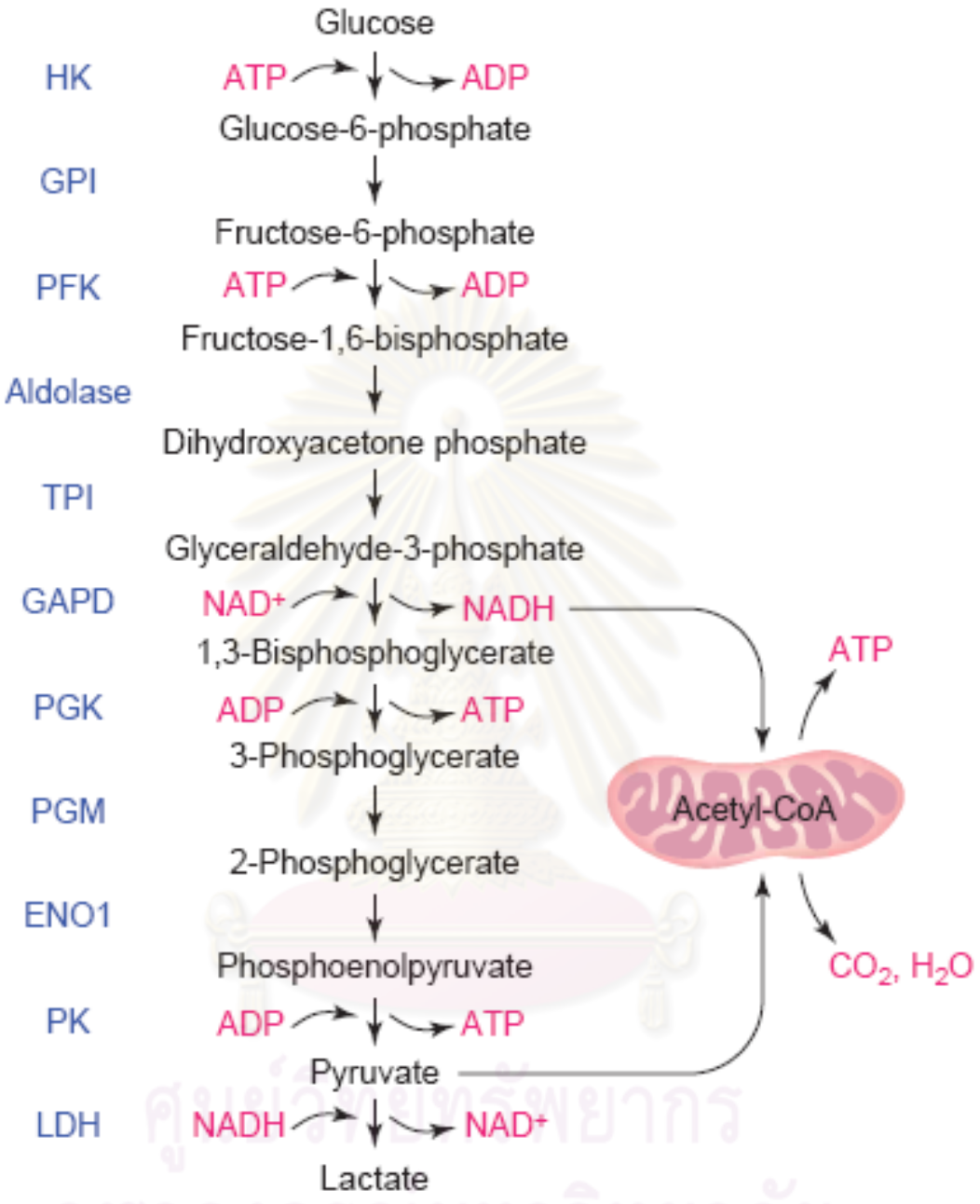

Figure 5. The glycolytic pathway [68].

GAPDH also has a role in mediating cell death under oxidative stress. GAPDH nuclear translocation is triggered in response to low-to-moderate oxidative stresses, whereas increased levels of oxidative stress promote robust formation of GAPDH aggregates, resulting in more extensive cell death [72]. GAPDH is a major target protein in oxidative stresses and becomes thiolated in its active site. It is extremely sensitive to the modification of the cysteine residue (Cys152) located in its active site. The active site cysteine to be S-thiolated by hydrogen peroxide, S-nitrosilated by nitric oxide, and nitroalkylated by nitroalkene derivatives. GAPDH exerts other functions beyond glycolysis, and that oxidatively modified glyceraldehyde-3-phosphate dehydrogenase regulates its cellular functions by changing its interacting proteins. Thus GAPDH is a major target of oxidative stress [71].

A number of studies, accelerating in the last decade, have indicated that GAPDH is not an uncomplicated, simple glycolytic protein [73]. GAPDH is a multifunctional protein such as GAPDH binds to microtubules and modulates microtubule bundling, contributes to membrane transport and in membrane fusion, binding with the inositol 1,4,5-trisphosphate receptor (IP3R), modulating calcium flux, protein phosphotransferase/kinase reactions, the translational control of gene expression [73]. Furthermore, GAPDH exists in the nucleus and related with in gene transcription, DNA replication, DNA repair, and nuclear RNA export, as illustrated in Figure 6, each activity appears to be distinct from its glycolytic function [9].

At S-phase progression requires cyclin E/cdk2 signaling, which orchestrates coupled DNA replication and histone expression. This is mediated by NPAT, nuclear protein, the ataxia-telangiectasia locus, a cyclin E/cdk2 substrate. Transcription of histone genes is mediated by subtype-specific promoter elements and associated transcription factors and/or co-activators, and the overall histone expression levels are regulated post-transcriptionally as well. In addition, the histone expression is highly coordinated and is tightly coupled to S-phase progression [74]. Transcription from the histone H2B (H2B) gene requires octamer binding factor 1 (Oct-1) and Oct-1 co-activator in S-phase (OCA-S), a multi-subunit complex that contains the

NAD(H)-linked classical glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase (p38/GADPH) and lactate dehydrogenase (p36/LDH) along with other components. During inter-phase, the essential octamer (ATTTGCAT) element in the histone H2B gene promoter is constitutively bound by Oct-1 that in turn recruits the OCA-S complex via a direct p38/GAPDH-Oct-1 interaction, which is S-phase specific hence accounting for S-phase induction of the histone H2B gene [27].

The GAPDH gene and protein are actively regulated on cell proliferation. GAPDH is the target of different transcription factors, and various control regions have been identified in its promoter, including hypoxia and insulin responsive elements [70]. GAPDH is a necessary component for the proper functioning of Oct-1 coactivator and OCA-S, that is essential for S-phase-dependent histone H2B transcription in HeLa cells [33]. GAPDH appears to protect cells against oxidative stress. This protective action involves generation of the reduced pyridine nucleotides and NADH, from NAD^+. The generation of these reducing equivalents depends upon an adequate supply of NAD^+. NAD^+ precursors protect against oxidative stress and DNA damage by up-regulating the stress response genes GAPDH [75]. GAPDH mRNA and protein levels vary in response to various stimuli [70] such as 1,25-dihydroxyvitamin D3 (1,25-(OH)2D3) [76], fudenine [77], oestradiol [78], bacterial endotoxin or lipopolysaccharide [79], hypoxia [80], insulin [81] and manganese [82].

GAPDH is a redox sensitive protein that is inactivated by ROS. Therefore, in conditions that induce a mild ROS production (lymphocyte activation), GAPDH upregulation may be protective for the cell. However, in the presence of higher amounts of ROS, such as those observed under conditions of mitochondrial dysfunction, oxidation of Cys152 may lead to a GAPDH inhibition, participating in cell death [70].

The strong depletion of ATP may be the consequence of the depletion of intracellular NAD^+ via PARP activation and/or the inactivation of GAPDH by S-nitrosylation and formation of sulfenic acid. Activation PARP consumes NAD^+, and because NAD^+ is required for glycolysis. Therefore, when an oxidative stress induces the activation of PARP, including GAPDH, but since NAD+ continues to be depleted,

one of the main consequences for such a drop in NAD^+ is an arrest of the glycolysis. Thus, when glycolysis is finally block, ATP depletion will follow, culminating 8-12 hour later in cell death [83].

The Nucleosome

In 1974, a revolution occurred in molecular biology with the elucidation of the "nucleosomal" subunit structure in chromatin [84]. The eukaryotic cell stores its genetic information in DNA molecules that can be over 1 m in length. The DNA is hierarchically packed in the nucleus (up to ~2×10^{-5}-times smaller in length) with the aid of proteins to form a complex called chromatin. The nucleosome core particle represents the first level of chromatin organization and is composed of two copies of each of histones H2A, H2B, H3 and H4, assembled in an octameric core with 146-147 bp of DNA tightly wrapped around it. Nucleosome cores are separated by linker DNA of variable length and are associated with the linker histone H1 [85]. The DNA most tightly associated with the nucleosome called the core DNA, is wound approximately 1.65 times around the outside of the histone octamer like thread around a spool. Histones are by far the most abundant proteins associated with eukaryotic DNA [86].

Eukaryotic cells commonly contain five abundant histones: H1, H2A, H2B, H3 and H4. Histones H2A, H2B, H3 and H4 are the core histones and form the protein core around which nucleosomal DNA is wrapped. Histone H1 is not part of the nucleosome core particle. Instead, it binds to the linker DNA and is referred to as linker histone [86].

Core histones play structural roles in chromatin assembly and compaction by forming the nucleosome. Nucleosomes are heterotypic tetramer $(H3–H4)_2$ with two associated dimers (H2A–H2B) in the form ([H2A–H2B] [$(H3\text{-}H4)_2$] [H2A–H2B]). Associated with this octamer are about 147 bp of DNA wrapped in 1.7 superhelical turns [85]. Nucleosomes are connected by a DNA linker of variable length that forms a 10-nm beads-on-a-string array. Structural details of the nucleosome core particle are obtained from a 7 Å resolution crystallographic analysis, with an $(H3)_2(H4)_2$ tetramer and two H2A-H2B dimers at the core, and the DNA wrapped around it.

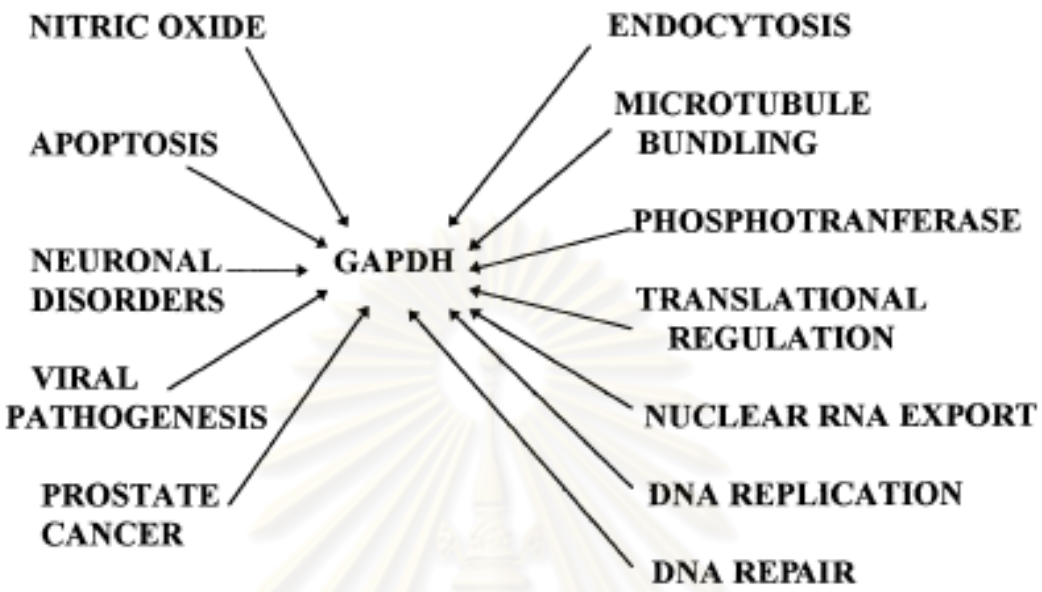

Figure 6. New roles for GAPDH in mammalian cells [73].

The core histone tails play important roles in nucleosome stability, and may contribute to define the condensed state of the chromatin fiber and higher order structures by facilitating nucleosome assembly or disassembly. Histone H2B and H2A are found to be the most variable pair, whereas H4 and H3 were the most conserved. Interestingly a large fraction of the positions where sequence variation in the histone fold domain was observed had similar physicochemical properties (i.e., changes in sequence were mostly to physicochemically conserved amino adds; e.g., leucine to isoleucine or valine, or lysine to arginine) [85].

Eukaryotic chromatin is a highly dynamic macromolecular assembly. Nucleosome particles can be modified in their composition, structure and location by chromatin remodeling complexes that introduce posttranslational modifications (PTMs) to the core histones and leads to nucleosome assembly and reassembly. Additionally, the chromatin fiber can be modified by the incorporation of histone variants. This would change local chromatin structure by promoting nucleosome subunit exchange to facilitate cellular processes such as transcription or development. Most eukaryotic organisms have multiple copies of histone genes. The major histone protein genes are mostly present in gene clusters and are expressed primarily during the S phase of the cell cycle. These histones are used for nucleosome assembly and packing of newly synthesized DNA. In contrast, some histone variants are expressed throughout the cell cycle and their expression is not restricted to the S phase [85].

In human cells, each passage through S-phase requires the synthesis and carefully orchestrated assembly of about 30 million nucleosomes. Nucleosome assembly during DNA replication represents a considerable challenge for eukaryotic cells. Part of the problem lies in the fact that cells normally deposit newly synthesized histones behind the replication fork almost as soon enough DNA has emerged from the replication machinery to allow the formation of nucleosomes (~150–300 base pairs). The passage through S-phase in the absence of histone synthesis results in a severe loss of cell viability in budding yeast. The nucleosome assembly challenge is that histones are highly basic proteins that bind very avidly and non-specifically to nucleic acids.

Because of this, free histones and DNA do not spontaneously associate into nucleosomes at physiological ionic strength, but rather form insoluble aggregates. Therefore, S-phase cells need to maintain a very delicate balance between histone and DNA synthesis to avoid making either too little or too much newly synthesized histones [87].

Core histone variants

Eukaryotic cells commonly contain four of core histones: H2A, H2B, H3 and H4 form the protein core around which nucleosomal DNA is wrapped.

Histone H2A

H2A is the core histone with the largest number of variants. The histone H2A variants include H2AZ and H2AX, which are found in most eukaryotes, and H2A. The serine residue in the consensus is phosphorylated in response to double-strand DNA breaks. Additionally, phosphorylation of H2AX has been found to aid in the recruitment of proteins involved in DNA repair [85].

Histone H2B

Histone H2B variants are few in number and those that have been documented have specialized roles in chromatin compaction during gametogenesis. The H2B variant documented in sea urchin has an N-terminal tail with a characteristic pentapeptide repeat that is highly charged. Additional H2B variants have also been found in male gametic cells from lily and, more recently, in bovine and human spermatozoa, however, their specific roles remain to be elucidated [85].

Histone H3

Histone H3 variants include H3.3, CenH3 and H3.4. H3.3 is a histone variant that is not S-phase regulated and is found in transcriptionally active chromatin. H3.4 is a testis-specific H3 variant found in primary spermatocytes [54, 55]. The CenH3 variant is localized in centromeric chromatin; their N-terminal tails are extremely divergent and share no sequence similarity with canonical H3 [85].

Histone H4

Histone H4 is the most highly conserved histone. H4 makes extensive contacts with the other three core histones in the nucleosome core particle and is thus constrained in its sequence variability. H4 has no known sequence variants; indeed, there are even identical sequence variants that are expressed in a cell cycle-independent manner as opposed to the predominant synthesis period for histones in the S phase of the cell cycle [85].

Greater than 20% of the residues in each histone are either lysine or arginine. The core histone are also relatively small proteins ranging in size from 11-15 kilo Daltons (kDa) whereas histone H1 is about 20 kDa (Table 1) [86].

Table 1. General Properties of the Histones [86].

Histone type	Histone	Molecular weight (MW)	% of Lysine and Arginine
Core Histone	H2A	14,000	20
	H2B	13,900	22
	H3	15,400	23
	H4	11,400	24
Linker Histone	H1	20,800	32

The duplication of a chromosome requires replication of the DNA and the reassembly of the associated proteins on each daughter DNA molecule. The latter process is tightly linked to DNA replication to ensure that the newly replication DNA is rapidly packaged into nucleosomes. To duplicate a chromosome, at least half of the nucleosomes on the daughter chromosomes must be newly synthesized. In experiments that differentially labeled old and new histones, it was found that the old histones are present on both of the daughter chromosomes. Mixing is not entirely random, however. H3-H4 tetramers and H2A-H2B dimers are composed of either all new or all old histones. Thus, as the replication fork passes, nucleosomes are break down into their component subassemblies. H3-H4 tetramers appear to remain bound to one of the two daughter duplexes at random and are never released from DNA into the free pool. In contrast, the H2A-H2B dimmers are released and enter the local pool available for new nucleosome assembly. On average, each new DNA molecule receives half old and half new histones [86].

The Epigenetics

Epigenetics is derived from epi- (meaning upon) and genetics. The term “epigenetics” broadly refers to changes in gene function that occur in the absence of any change in DNA sequence. Epigenetic regulation of mammalian gene expression has profound effects in controlling cell growth, differentiation and cancer development. It has been proposed that “perturbations of epigenetic gene regulation may play a critical role in the genesis of most, if not all, cancers”. In general terms, these epigenetic alterations can include covalent modifications of DNA and histones as well as non-covalent changes in nucleosome positioning. The important epigenetic mechanisms include DNA cytosine methylation; covalent histone modifications (acetylation and methylation of lysines and arginines, phosphorylation of serine and threonines, ubiquitination, sumolyation and biotinylation of lysines as well as ADP ribosylation) (Figure 7); noncovalent histone protein modifications such as incorporation of histone variants and nucleosome remodeling; and non-coding RNAs, functional RNA molecules that are not translated into proteins. All histone proteins are modified inside the nucleus

of the cell [41, 88-90]. Epigenetic inheritance is a key element in the adaptation of organisms to a rapidly changing environment without stably changing their DNA sequence [91].

In eukaryotic cells epigenetic modifications are encoded via two primary modes which differ dramatically in their information content. DNA methylation, the most characterized epigenetic modification, is binary in nature and its information content is related to local density within a genomic region. Even minor disruptions of methylation density can be lethal during development, and a broad range of developmental abnormalities and diseases have been linked to abnormal methylation patterning. The second mode of epigenetic encoding is through covalent modification of polypeptides which package DNA within the cell. The nucleosome is the fundamental unit of the packaged DNA and it is composed of two copies of each of the four core histones (H3, H4, H2A and H2B) around which 146 base pairs of DNA are wrapped. Histones are evolutionarily highly conserved proteins characterized by an accessible amino-terminal tail and a histone fold domain that mediates interactions between histones to form the nucleosome scaffold. The N-termini of histone polypeptides are extensively modified by more than 60 different posttranslational modifications including methylation, acetylation, phosphorylation and ubiquitination [42].

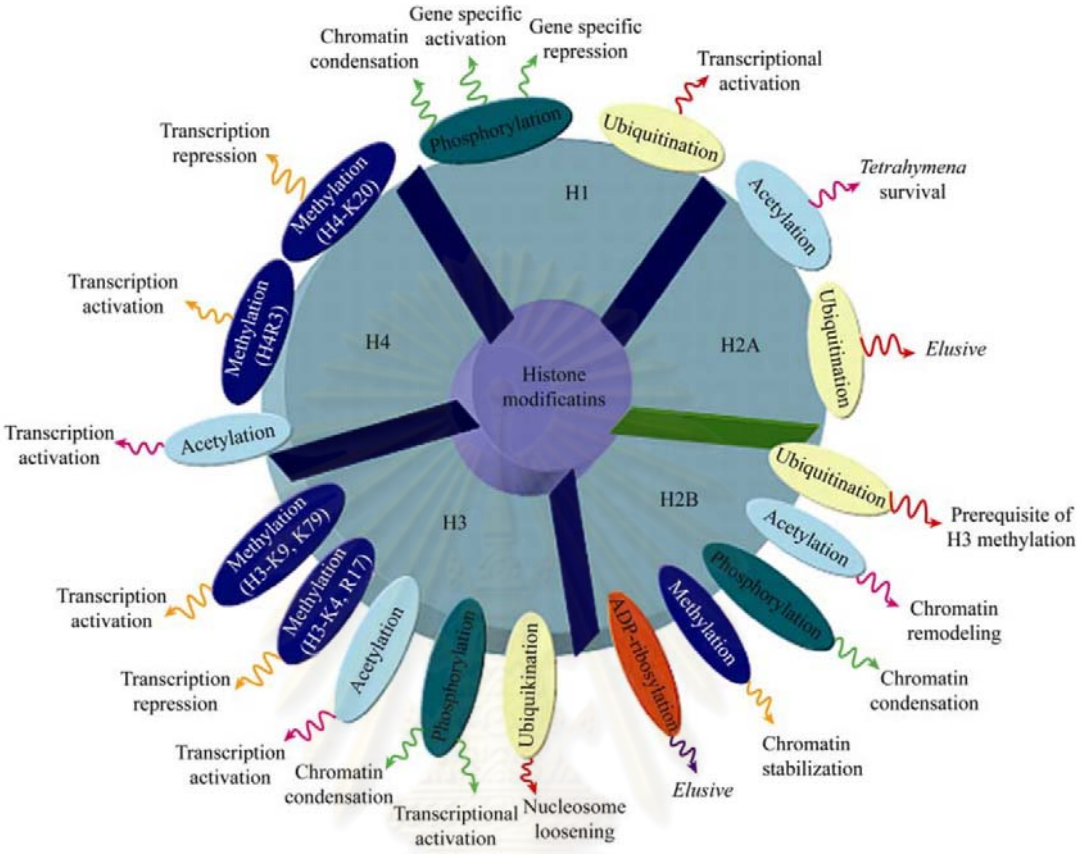

Figure 7. The pictorial representation of the histone modifications and their biological roles [89].

Epigenetic abnormalities in cancer have an important biological and clinical impact. The aberrant epigenetic mechanisms affect the transcription of genes involved in the regulation of cellular growth, differentiation, apoptosis, transformation, and tumor progression. Epigenetically inactivated genes include tumor suppressor genes, suggesting that epigenetic silencing of gene expression plays an important role in tumorigenesis. Reexpression of epigenetically silenced genes may result in the suppression of tumor growth [92]. HDAC complexes are operated by HSP70, where it may play a role in the ATP-dependent deacetylation of chromatin substrates. Butyric acid changes the structure of chromatin by posttranslational modifications, the most important of which are the acetylation and phosphorylation of nuclear histones. High levels of butyrate may also act in synergy with folate deficiency to impede the formation of inactive chromatin. Micronutrients whose deficiency or excess may perturb epigenetic processes include zinc, selenium, arsenic, nickel, iron, vitamin C and niacin, a precursor of NAD^+. Arsenic and alcohol deplete the methyl donor pool [76]. Short-term exposure to cadmium inhibited DNA methyltransferase activity, but prolonged exposure to cadmium caused neoplastic transformation and attended increases in DNA methylation and DNA methyltransferase activity [45].

The U.S. Environmental Protection Agency's *Guidelines for Carcinogen Risk Assessment* encourages the use of mechanistic data in the assessment of human cancer risk at low (environmental) exposure levels. The key events that define a particular mode of action for tumor formation have been concentrated to date more on mutational responses that are broadly the result of induced DNA damage and enhanced cell proliferation. While it is clear that these processes are important in terms of tumor induction, other modes that fall under the umbrella of epigenetic responses are increasingly being considered to play an important role in susceptibility to tumor induction by environmental chemicals and as significant modifiers of tumor responses [41].

Programmed Cell Death

Programmed cell death (PCD) is a basic biological phenomenon that plays an important role during development, preservation of tissue homeostasis, and elimination of damaged cells. Several nomenclature systems have been proposed to classify PCD. One widely accepted system describes three major morphologies of programmed cell death, and classifies PCD as type I, apoptosis; type II, autophagy; and type III, programmed necrosis [93].

Apoptosis

Apoptosis is the best-described form of programmed cell death, and plays a major role in both embryonic development and organismal aging. It involves the controlled activation of proteases and other hydrolyses that rapidly degrade all cellular structures. At the morphological level, the classic hallmarks of apoptosis are the condensation of chromatin (pyknosis), nuclear fragmentation (karyorrhexis), shrinkage of the cell and plasma membrane blabbing. Two major pathways initiate apoptosis: the intrinsic (or mitochondrial) and the extrinsic pathways (Figure 8) [94].

The apoptosis cascade can be initiated via two major pathways, involving either activation of death receptors in response to ligand binding (extrinsic or death receptor pathway), or the release of proapoptotic proteins, such as cytochrome c, from mitochondria to cytosol (intrinsic or mitochondrial pathway). The death receptor pathway is activated through the tumor necrosis factor (TNF) family of cytokine receptors, and has a fundamental role in maintaining tissue homeostasis, particularly in immune recognition [93].

The extrinsic pathway is initiated at the plasma membrane level by activation of death receptors from the TNFR (tumor necrosis factor receptor) family that include Fas/CD95 and the TRAIL (TNF-related apoptosis inducing ligand) receptors. Ligand-induced death receptor trimerization results in the recruitment and activation of caspase-8 via adaptor proteins such as FADD/TRADD (Fas-associated death domain/TNFR1-associated death domain) to form a death-inducing signaling complex, which further propagates death signals in at least 3 ways: (1) by direct proteolysis and

activation of the effector caspases, (2) by proteolysis of the BH3-only protein Bid, translocation of tBid to mitochondria and consequent mitochondrial outer-membrane permeabilization (MOMP) or (3) by activation of the kinases RIP1 (receptor-interacting protein 1) and JNK (c-Jun N-terminal kinase), that mediate the translocation of tBid to the lysosome and Bax-dependent lysosomal membrane permeabilization, resulting in cathepsin B/D-mediated general proteolysis and MOMP [94]. Furthermore, extrinsic pathway is triggered at the cell surface through cytokine induced death by receptor mediated activation of caspase-8 or caspase-10, followed by activation of caspase-3 and caspase-7 [93]. It propagates death signals in three directions: to lysosome membrane permeabilization (LMP), to caspase-8-dependent activation of effector caspases, or to BH3-only-dependent MOMP [94].

In the intrinsic pathway, multiple sensors, including the BH3-only proteins and p53, react in response to different stress conditions or to DNA damage and activate a signaling cascade conducive to MOMP. The release of intermembrane space proteins from permeabilized mitochondria allows for the assembly of the apoptosome, a caspase-activating complex formed by APAF-1 (apoptotic protease activating factor 1), caspase- 9 and cytochrome *c*, resulting in the activation of effector caspases that are responsible for dismantling the cell's structures. Apoptosis initiated at the mitochondrial level is tightly regulated by the Bcl-2 family of proteins, which is subclassified into 3 groups: (1) anti-apoptotic multidomain members (Bcl-2, Bcl-X_L and Mcl-1), which contain four Bcl-2 homology domains (BH1, BH2, BH3 and BH4), (2) pro-apoptotic multidomain members (such as Bax and Bak), lacking the BH4 domain and (3) proapoptotic BH3-only proteins (such as Bid, Bim and Bad). Intrinsic and extrinsic stimuli can induce the proteolytic cleavage of Bid and translocation of truncated Bid (tBid) to the mitochondrial membranes where it stimulates MOMP, presumably through the activation of Bax/Bak channels and other mechanisms [94].

The multiple internal interactions among the Bcl-2 protein family members, summed to the many pathways that modulate the levels and activity of these proteins, induce or avoid the execution of mitochondrial apoptosis. Furthermore, intrinsic pathway

is stimulated by multiple signal transducers and noxious agents that frequently result in the activation of pro-apoptotic BH3-only proteins from the Bcl-2 family. The rate-limiting step of the intrinsic pathway is mitochondrial membrane permeabilization resulting in the release of caspase activators [94] (caspase-9 followed by caspases-3 and -7) [93] and caspase-independent death effectors from mitochondria [94]. The central players in both pathways are the caspases (the cysteine dependent, aspartate specific family of proteases), which also function as the executioner in apoptotic cell death. Regulated at the post-translational level, caspases are synthesized as pro-caspases. Under stimulation of pro-apoptotic signals from different sources, pro-caspases are digested by protease to become active caspases. Mitochondrial dynamics contribute substantially to apoptotic pathways by stimulation of caspases, and by chromosomal fragmentation [93].

Necrosis

Necrotic cell death or necrosis is morphologically characterized by a gain in cell volume (oncosis), swelling of organelles, plasma membrane rupture and subsequent loss of intracellular contents. For a long time, necrosis has been considered merely as an accidental uncontrolled form of cell death, but evidence is accumulating that the execution of necrotic cell death may be finely regulated by a set of signal transduction pathways and catabolic mechanisms [95].

Under extreme conditions tissues and cells die through unregulated processes of destruction of membranes and cytosol. Among the agents that can induce necrosis are various viruses, bacteria, and protozoa. Necrosis can be activated by bacterial toxins and components of immune defense, such as complement, activated natural killers, and peritoneal macrophages. The pathogen-induced necrotic programs in cells of immunological barriers (e.g., intestine mucosa) may alleviate invasion of pathogens through the surfaces affected by inflammation and, in the case of intracellular pathogens, to avoid “altruistic” apoptotic suicide that can prevent pathogen propagation. Recent cytological data indicate that necrotic death occurs not only during

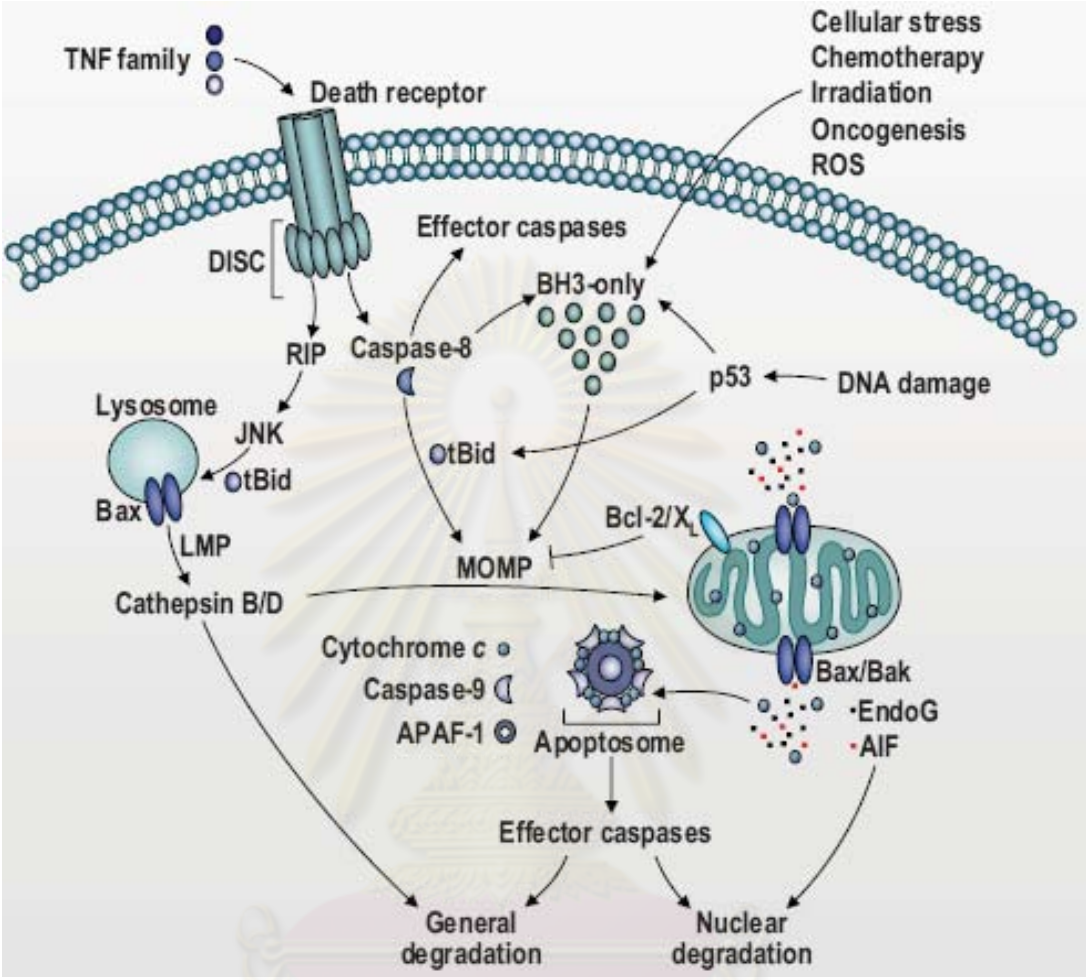

Figure 8. Two major pathways lead to the execution of apoptosis [94].

pathological events, but it is also a component of some physiological processes. For example, during renewal of the small intestine, both apoptosis and necrosis of enterocytes contribute to cell loss. Follicular maturation during oogenesis involves, along with apoptosis, necrotic cell death. Activation-induced death of primary T lymphocytes, an important constituent of negative selection in immune response, is caspase-independent and necrotic by morphology [96].

Recent studies have demonstrated that in response to a given death stimulus, there is often a continuum of apoptosis and necrosis. Many insults induce apoptosis at lower doses and necrosis at higher doses. Even in response to a certain dose of death-inducing agent, features of both apoptosis and necrosis may coexist in the same cell. In addition, if not engulfed by neighboring cells or in cell culture, where phagocytosis does not usually happen, dead cells in the late stages of apoptosis may present necrotic features due to the loss of cellular energy and plasma membrane integrity. This process is called “apoptotic necrosis” or “secondary necrosis”. Despite the idea that necrosis is an uncontrolled or default form of cell death, accumulating studies have suggested that this may not be true. Rather, it appears that necrotic cell death can be a regulated event that contributes to development and to the maintenance of organismal homeostasis [97].

The programmed cell necrosis can be a consequence of extracellular signaling or can be initiated as a form of cellular suicide in response to intracellular perturbations. Cell suicide by necrosis appears to have evolved to allow multicellular organisms to have an early warning system to recognize and adapt to events that might compromise the integrity of the organism as a whole. As such programmed cell necrosis plays a role in a number of disease processes including vascular-occlusive disease, neurodegenerative diseases, infection, inflammatory diseases, exposures to toxins, and cancer [97]. Furthermore, multiple lines of evidence indicate that necrosis can be a programmed event, both in its occurrence and in its mechanism: (1) cell death with a necrotic appearance can contribute to embryonic development and adult tissue homeostasis, (2) necrotic cell death can be induced by ligands that bind to specific plasma membrane receptors, and (3) necrosis can be regulated by genetic, epigenetic,

and pharmacological. Moreover, the inactivation of caspases causes a shift from apoptosis either to cell death morphologies with mixed necrotic and apoptotic features or to full-blown necrosis [98].

Recently, the term necroptosis has been used to designate one particular type of programmed necrosis that depends on the serine/threonine kinase activity of RIP1. The pharmacological or genetic inhibition of several key enzymes has been shown to deeply affect the execution of programmed necrosis such as RIP1, cyclophilin D, poly(ADP-ribose) polymerase-1 (PARP-1), and apoptosis inducing factor (AIF) [98].

The interface between apoptosis and programmed necrosis (Figure 9), activation of the serine/threonine kinase RIP1 (receptor-interacting protein kinase 1), which can be triggered in L929 murine fibrosarcoma cells by ligation of the tumor necrosis factor receptor (TNFR) or inhibition of caspases and NIH 3T3 murine fibroblasts, TNFR activation ignites the extrinsic apoptotic pathway, which depends on caspase-8. Caspase-8-mediated degradation of RIP1 may represent one of the major molecular switches between apoptosis and necroptosis. Apoptosis and necroptosis may preferentially involve mitochondrial outer membrane permeabilization (MOMP) and the mitochondrial permeability transition (MPT) [98]. Kroemer *et al*. [95] and Amaravadi and Thompson [99] summarize the specific morphological characteristics, key regulators to apoptosis, and necrosis (Table 2). Furthermore, Core histones (H2A, H2B, H3, H4) are detected in peripheral blood mononuclear cells lysates significant increase when deprivation of interleukin-2 and correlated significantly with signs of early apoptosis cell death [100].

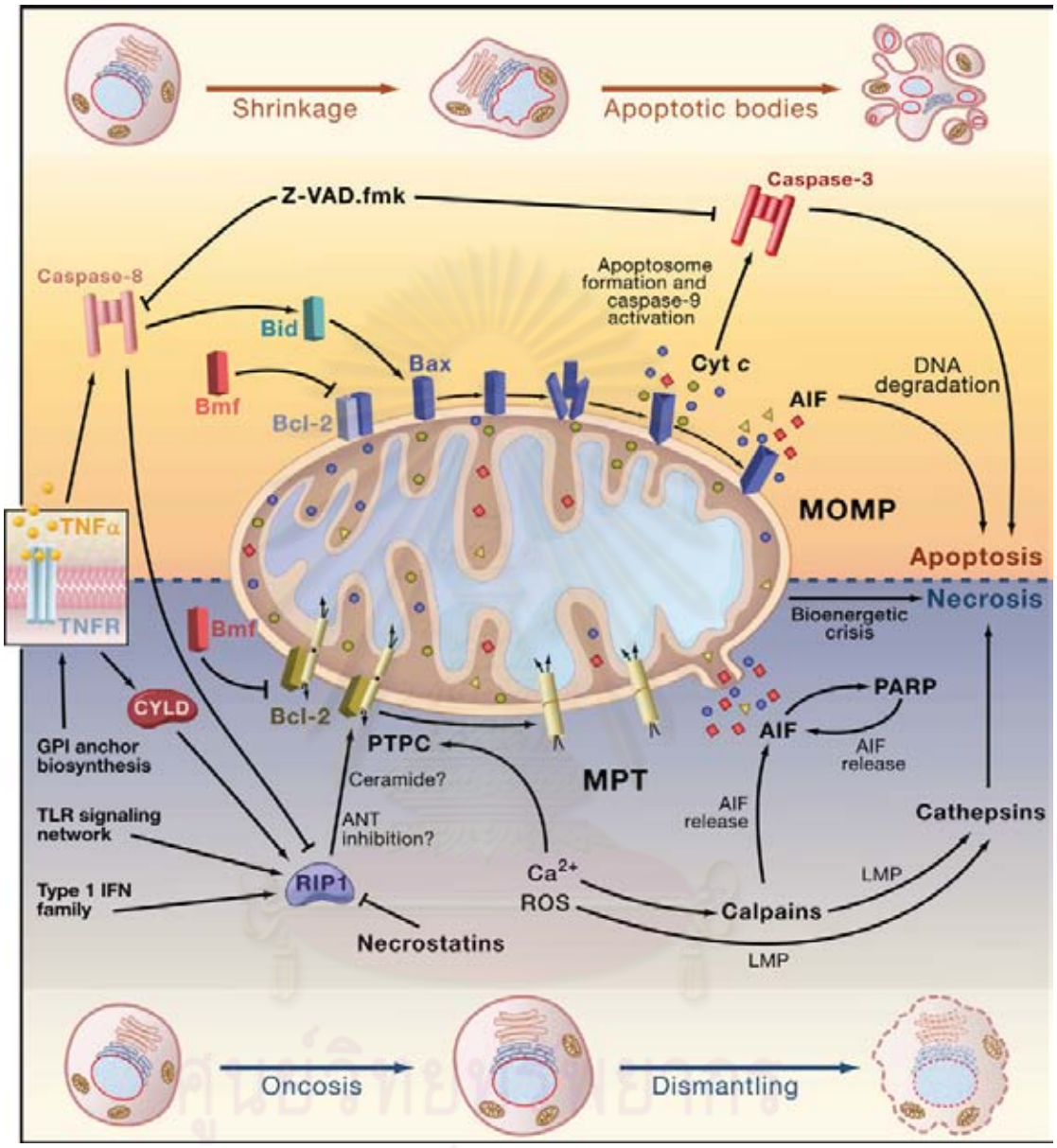

Figure 9. The interface between apoptosis and programmed necrosis [98].

Table 2. Characteristics of apoptosis, and necrotic cell death [95, 99].

	Apoptosis	Necrotic cell death
Morphology	• Retraction of pseudopods • Rounding-up of the cell • Reduction of cellular and nuclear volume (pyknosis) • Nuclear fragmentation (karyorrhexis) • Plasma membrane blabbing • Engulfment by resident phagocytes, *in vivo*	• Cytoplasmic swelling (oncosis) • Rupture of plasma membrane • Swelling of cytoplasmic organelles • Moderate chromatin condensation
Key regulators	• Caspases • cytochome *c* • Bcl-2 family members	• RIPK1 • TRAF2 • PARP • Calpains

Poly(ADP-ribose) polymerases (PARPs)

Poly(ADP-ribose) (PAR), the reaction product of the large family of poly(ADP-ribose) polymerases (PARPs), has been discovered in the 1960s. PARPs use NAD^+ as a substrate from which they cleave off nicotinamide and form, via repeated reaction cycles, a polymer of ADP-ribose units, which can be branched [101]. NAD^+/NADH is among the most versatile biomolecules, as it can be used not only as a coenzyme for a large number of oxidoreduction reactions, but in its oxidized version can also serve as substrate for several different of ADP-ribosyl transfer reactions [102]. The main target proteins ('acceptors') are PARPs themselves, which is referred to as PARP automodification. Cellular PAR formation is dramatically stimulated after exposure to DNA-damaging agents that induce single- or double-strand breaks in DNA. This is mediated by facilitating and regulating DNA strand break repair. This kind of induced PAR formation depends on the activation of two abundantly expressed members of the PARP family (PARP-1; PARP-2) [101]. Under DNA damage as induced by ionizing radiation, alkylating agents and oxidants about 90% of poly(ADP-ribose) is synthesized by PARP-1 [102]. Under conditions of genotoxic exposure poly(ADP-ribose) undergoes a rapid turnover, since, in parallel with the synthesis of polymer, its enzymatic catabolism is highly active [101].

PARP-1, a 113-kDa protein, is an abundant nuclear protein that is present in all nucleated cells of multi-cellular eukaryote organisms. On average, approximately one molecule of this enzyme is present per 1000 base pairs of DNA. PARP-1 is involved in a variety of physiological and pathological events such as DNA replication, DNA repair, gene expression, cellular differentiation, chromatin decondensation, malignant transformation, inflammation, developmental aspect and apoptosis [103]. Oxidative-stress-induced DNA strand breakage triggers the activation of PARP-1, leading to AIF release from the mitochondria and AIF-mediated, caspase-independent apoptotic cell death NAD^+/ATP consumption and consequent necrotic cell death [104].

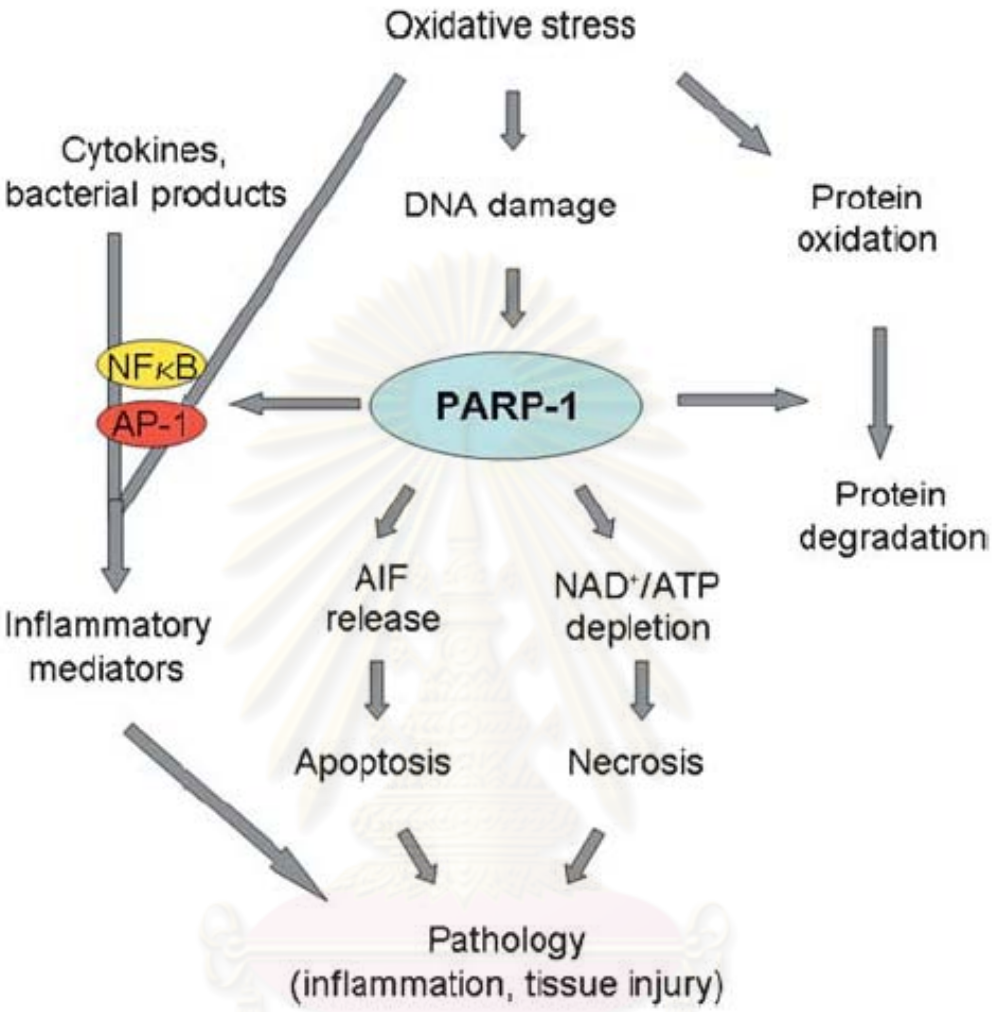

Figure 10. The central role of PARP-1 in oxidative stress-related pathology [104].

Oxidative stress also stimulates activation of redox-sensitive transcription factors such as NFκB and AP-1, key regulators of inflammatory cytokines and chemokines. Poly(ADP-ribosyl)ation is also required for the proteasomal degradation of oxidatively damaged proteins (Figure 10) [104].

PARP is one of the favorite substrates for a large number of suicidal proteases like caspase, calpain, cathepsin, and granzyme-b. Calpains and cathepsins are known to mediate both apoptotic and necrotic cell deaths, whereas caspases are implicated only in apoptotic cell deaths The 89- and 21-kDa signature fragments correlated with the activation of caspase-3 and the presence of apoptotic cell deaths and presence of 50-kDa fragment correlated with cathepsin-b and necrotic cell death and the presence of 64-kDa fragment correlated with the elevated levels of granzyme-b [103]. The major cleavage fragment form of PARP-1 in necrosis cell is represented by about 55 kDa [105, 106].

NF-κB Essential Modulator (NEMO, IKKγ)

NF-κB transcription factors are rapidly activated in response to various stimuli, allowing quick activation of target genes that encode cytokines such as interleukin-1, interleukin-12, interleukin-2, and interferon-β; membrane proteins such as major histocompatibility complex classes I and II, ICAM-1 (intracellular adhesion molecule 1), and E-selectin; transcription factors such as c-Myc and IRF4 (interferon-regulatory factor); inhibitors of apoptosis such as c-FLIP (cellular FLICE-like inhibitory protein) and Bcl-XL. This rapid response system depends on the sequestration of NF-κB dimmers in the cytoplasm through interaction with inhibitory IκB proteins. Cell stimulation leads to IκB phosphorylation, thereby creating a recognition signal for ubiquitinating enzymes, which mark the IκBs for rapid proteasomal degradation. Degradation of IκBs liberates NF-κB dimers to translocate to the nucleus and activate transcription of target genes (Figure 11). Phosphorylation is accomplished by protein kinases, whose activity is tightly controlled and represents the primary mode of NF-κB regulation. Two protein kinases

with high sequence similarity, IκB kinase alpha (IKKα) and IKKβ, are the most important IκB kinases [107, 108].

Both kinases were purified and cloned on the basis of their ability to phosphorylate IκB proteins in response to cell stimulation with tumor necrosis factor α (TNFα) and are present in cells as part of a high molecular weight complex that also contains a regulatory subunit termed IKKγ or NEMO (NF-κB essential modulator). This basic trimolecular complex, which may contain an additional substrate-targeting subunit named ELKS, is referred to as the IKK complex. Despite their similarities and presence in a common protein complex, IKKα and IKKβ have largely nonoverlapping functions, due to different substrate specificities and other factors. IKKβ is the major IKK catalytic subunit for NF-κB activation by pro-inflammatory stimuli, such as TNFα, IL-1, and "Toll-like" receptor (TLR) agonists, such as lipopolysaccharide (LPS). In contrast, kinase activity of IKKα is primarily required for activation of a specific type of NF-κB dimer (p52:RelB) in response to a subset of TNF family members, such as BAFF (B cell–activating factor), CD40-ligand, and lymphotoxin α (LTα)–LTβ heterotrimers. Whereas the IKKβ-dependent pathway is essential for activation of innate immunity, the IKKα-dependent pathway is more important for regulation of adaptive immunity and lymphoid organogenesis [107].

NEMO is a 23 kb gene composed of 10 exons. The 48 kDa NEMO protein has two coiled-coil motifs and a leucine zipper which are required for dimerization and protein–protein interactions, and a zinc finger at the C-terminus that appears to be necessary for post-translational stability. It has also been shown that the C-terminus of NEMO is indispensable for function. NEMO is the regulatory component of IκB kinase (IKK), a central activator of the NF-κB transcriptional signaling pathway. In response to various cytokines, IKK phosphorylates the inhibitory IκB molecules, which sequester NF-κB in the cytoplasm. The removal of IκB allows NF-κB to translocate into the nucleus and activate transcription of various genes. Through this mechanism, NF-κB regulates immune and inflammatory responses, and prevents apoptosis in response to TNF-α. Therefore, *NEMO* mutations eliminate NF-κB activity and cause potentially widespread

disruption of downstream cellular responses, although the exact downstream effects are only now being elucidated. With respect to IP, loss-of-function mutations in *NEMO* create a susceptibility to cellular apoptosis in response to TNF-α. This phenomenon explains the male lethality and skewing of X-inactivation in female patients [109].

NEMO interacts with IKK subunits through the N-terminal part of its CC1, whereas the C-terminal part of this domain provides a binding site for Tax or RIP. In addition, the CC2/LZ part of NEMO represents the minimal oligomerization domain of the molecule. Finally, the NEMO ZF appears required for IKK activation in response to TNF, LPS or IL-110 although its exact function remains unclear. The gene encoding NEMO is located on the X chromosome, at Xq28, where G-6-PD and Haemophilia Factor VIII genes are also present.11 Such X-linkage is not observed with any other genes encoding known molecules of the NF-κB pathway and, as will be discussed below in more details, it has a major impact on human pathology. Besides the NEMO-dependent pathway of IKK activation, it has been recently demonstrated that IKK activation can also be triggered by an alternative pathway that requires the upstream kinase NIK and IKK-1 but neither NEMO nor IKK-2. This pathway, which plays an important role in B cell development and homeostasis, does not target IκBs but induces instead p100 processing to release active p50/relB dimmers [110]. The human *NEMO* gene maps to Xq28 and is arranged head to head with the proximal *G-6-PD* gene [18].

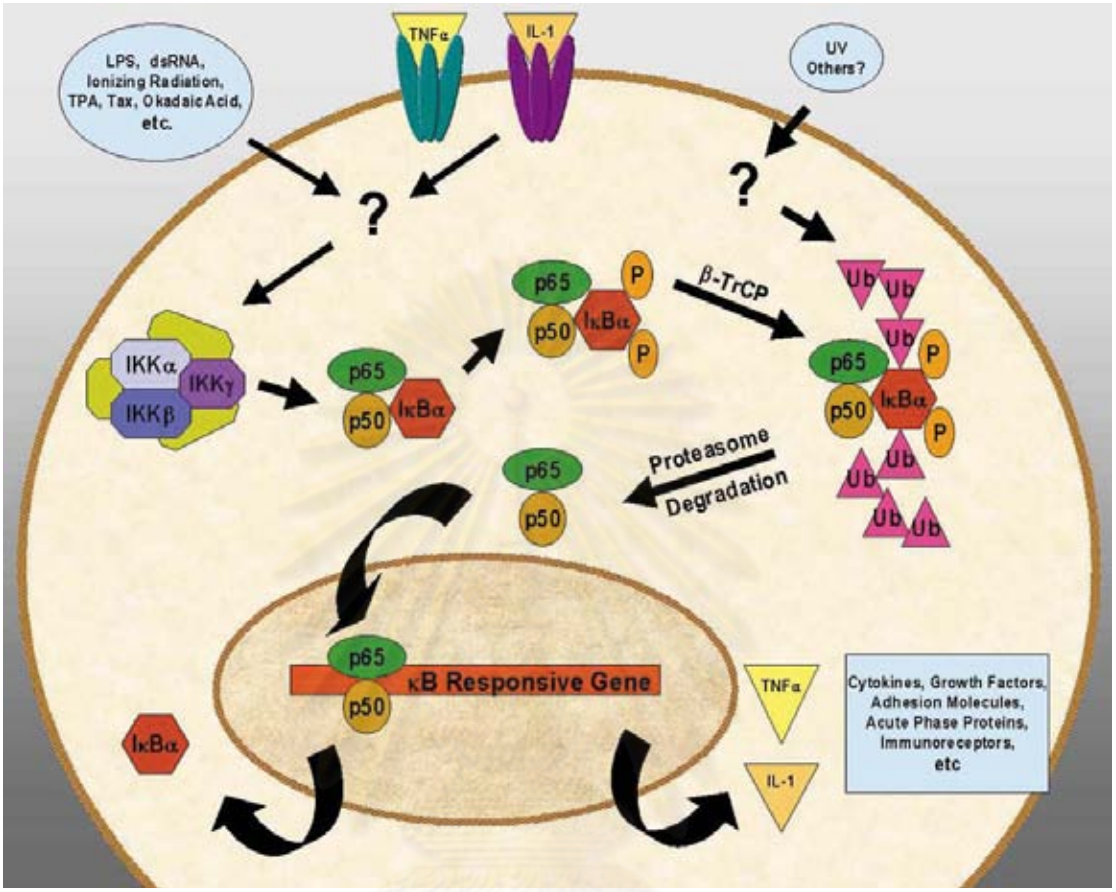

Figure 11. A schematic model of NF-κB activation [108].

Glucose-6-phosphate dehydrogenase (G-6-PD)

G-6-PD catalyses the first reaction in the pentose phosphate pathway, in which glucose is converted into the pentose sugars required for glycolysis and for various biosynthetic reactions. The pentose phosphate pathway also provides reducing power in the form of NADPH (Figure 12), by the action of G-6-PD and 6-phosphogluconate dehydrogenase. NADPH serves as an electron donor for many enzymatic reactions essential in biosynthetic pathways, and its production is crucial to the protection of cells from oxidative stress [13]. G-6-PD is also necessary to regenerate the reduced form of glutathione that is produced with one molecule of NADPH. The reduced form of glutathione is essential for the reduction of hydrogen peroxide and oxygen radicals and the maintenance of haemoglobin and other red-blood-cell proteins in the reduced state. G-6-PD is present in all cells; however, its concentration varies in different tissues. Since red blood cells do not contain mitochondria, the pentose phosphate pathway is their only source of NADPH; therefore, defence against oxidative damage is dependent on G-6-PD. In healthy red blood cells, the enzyme operates at only 1–2% of its maximum potential (even under oxidative stress generated by methylene blue): a large reserve of reductive potential exists, which is substantially decreased in G-6-PD-deficient red-blood cells, leading to pathophysiological features. After G-6-PD deficiency was established as a clinical disorder, its phenotypic expression was noted to be heterogeneous. More than 140 mutations of the *G-6-PD* gene have been identified, suggesting genetic heterogeneity [13].

G-6-PD deficiency is an X-linked, hereditary genetic defect caused by mutations in the *G-6-PD* gene, resulting in protein variants with different levels of enzyme activity, that are associated with a wide range of biochemical and clinical phenotypes. The most common clinical manifestations are neonatal jaundice and acute haemolytic anaemia, which in most patients is triggered by an exogenous agent. The striking similarity between the areas where G-6-PD deficiency is common and *Plasmodium falciparum* malaria is endemic provides circumstantial evidence that G-6-PD deficiency confers resistance against malaria [13].

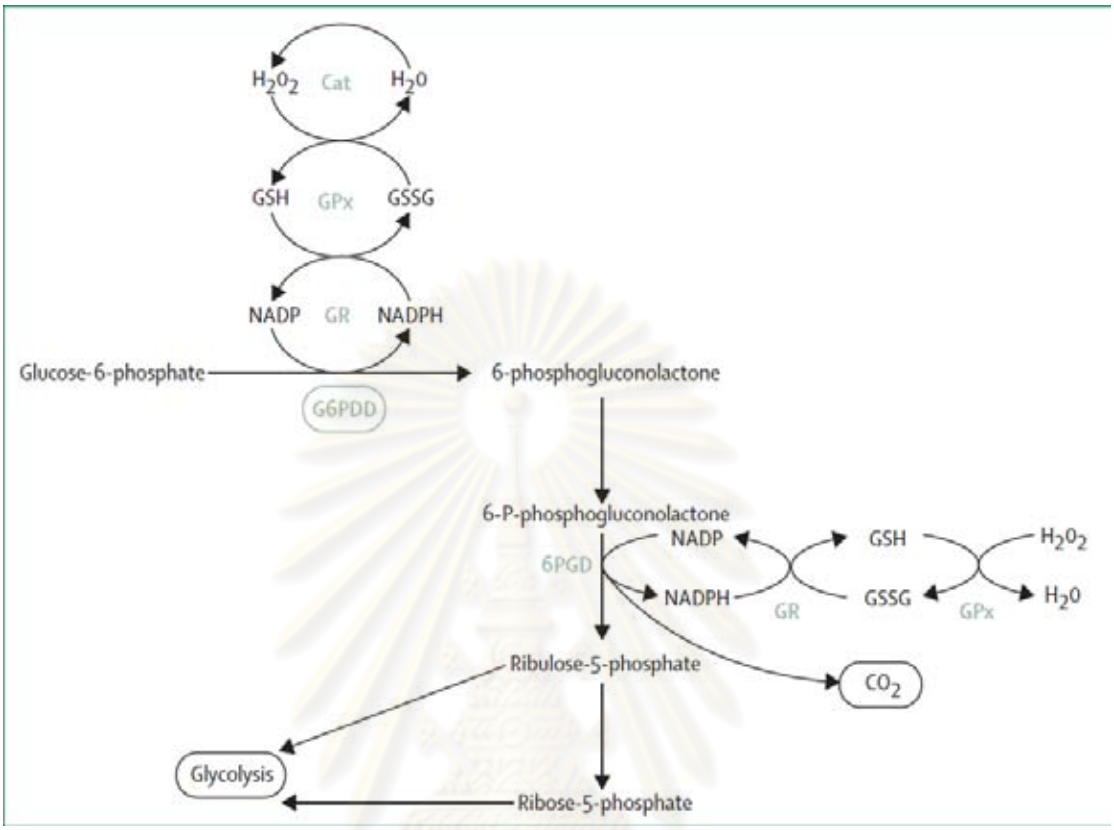

Figure 12. Pentose phosphate pathway [13].

The highest frequencies are detected in Africa, Asia, the Mediterranean region, and in the middle east; owing to recent migrations, however, the disorder is also found in North and South America and in northern European countries. G-6-PD deficiency is the most common human enzyme defect, present in more than 400 million people worldwide [13].

The relationship of NAD^+ / NADH and $NADP^+$ / NADPH

NAD, including nicotinamide adenine dinucleotide (NAD^+) and reduced nicotinamide adenine dinucleotide (NADH), have been long known as key molecules in energy metabolism and mitochondrial functions. NADP, including nicotinamide adenine dinucleotide phosphate ($NADP^+$) and reduced nicotinamide adenine dinucleotide phosphate (NADPH), have been known as critical molecules in antioxidation and reductive biosynthesis. However, a rapidly growing body of information has suggested that NAD and NADP also play crucial roles in various biological processes, including calcium homeostasis, gene expression, immunological functions and aging [111].

There are several potential mechanisms underlying the roles of NAD in apoptosis: 1) NADH / NAD^+ ratio is a major index of cellular reducing power that affects mitochondrial permeability transition (MPT), which mediates apoptosis under many conditions; 2) NAD plays a key role in energy metabolism that is a key factor determining cell death modes; 3) NAD^+-dependent sirtuins may mediate apoptosis; and 4) NAD^+ levels significantly affect the activity of caspase-dependent endonuclease [111].

Due to the critical roles of oxidative stress in cell death, NAD may affect cell survival by influencing oxidative stress and antioxidation systems in cells: 1) NAD^+ can be converted by NAD kinases to $NADP^+$, the precursor for NADPH formation; 2) NADH / NAD^+ ratio is an index of cellular reducing potential, since the redox couple plays key roles in numerous redox reactions; 3) some studies have suggested that NADH can produce direct antioxidation effects; 4) NAD^+ can inhibit ROS generation from α-ketoglutarate dehydrogenase and pyruvate dehydrogenase. It is noteworthy that

excessive intracellular NADH can produce “reductive stress”, which may result from the capacity of xanthine oxidase / xanthine dehydrogenase to generate ROS by oxidizing NADH, or from the capacity of NADH to induce release of ferrous iron from ferritin [111].

NADPH is a key factor in cellular antioxidation through the following mechanisms: 1) GSH is essential for the functions of such key antioxidation enzymes as glutathione peroxidase, while NADPH is required for regeneration of GSH from GSSG; 2) NADPH is a key component in another important antioxidation system such as thioredoxin system; 3) in some cell types such as red cells NADPH binds the important H_2O_2-disposing enzyme catalase, which can reactivate catalase when catalase is inactivated by H_2O_2. NAD and NADP play critical roles in cell death, which can affect cell survival through several pathways (Figure 13).

NAD^+ biosynthesis plays a central role in the metabolism of NAD and NADP, because NAD^+ is necessary for the generation of NADH, $NADP^+$, and NADPH. There are two major mechanisms by which $NADP^+$ can be formed: $NADP^+$ can be generated *de novo* from NAD^+ through the action of NAD^+ kinases (NADKs); and $NADP^+$ can also be formed from NADPH by multiple NADPH-dependent enzymes such as glutathione reductase. There are also two major mechanisms by which NADPH can be formed: The first is that NADPH is generated from NADH and $NADP^+$ by mitochondrial transhydrogenase; and the second is that NADPH is generated from $NADP^+$ by multiple $NADP^+$-dependent enzymes. In cytosol, $NADP^+$ is generated from NAD^+ by NAD^+ kinase (NADK). NADPH can be generated from $NADP^+$ by glucose-6-phosphate dehydrogenase (G-6-PD), 6-glyconate phosphate dehydrogenase (6-GPD), cytosolic $NADP^+$-dependent isocitrate dehydrogenase (IDPc), or cytosolic $NADP^+$-dependent malic enzymes (MEPc). In mitochondria, $NADP^+$ is generated from NAD^+ by NAD^+ kinase, and NADPH can be generated from $NADP^+$ by mitochondrial $NADP^+$-dependent isocitrate dehydrogenase (IDPm), mitochondrial $NADP^+$-dependent malic enzymes (MEPm), or mitochondrial transhydrogenase (TH) (Figure 14) [112].

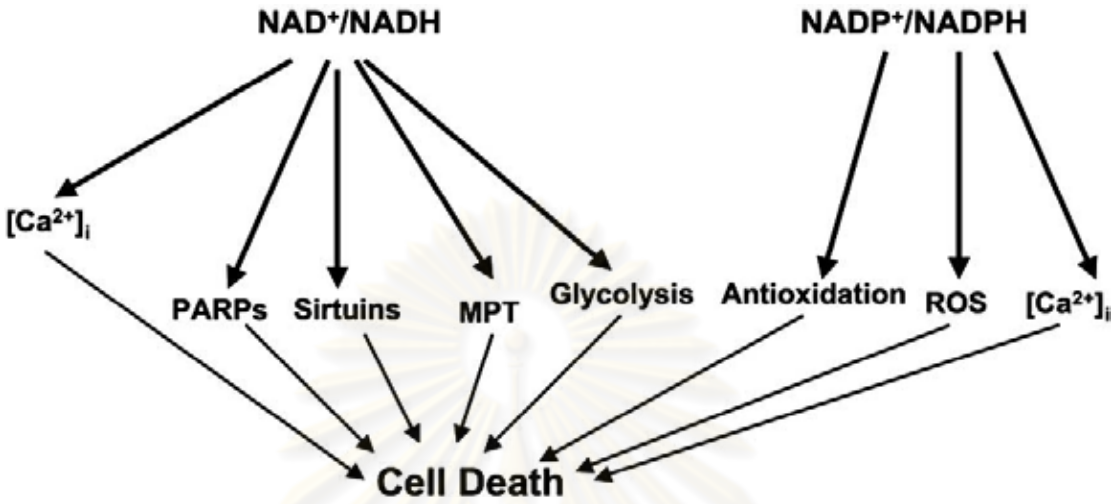

Figure 13. The pathways from NAD isoforms to cell death are shown [111].

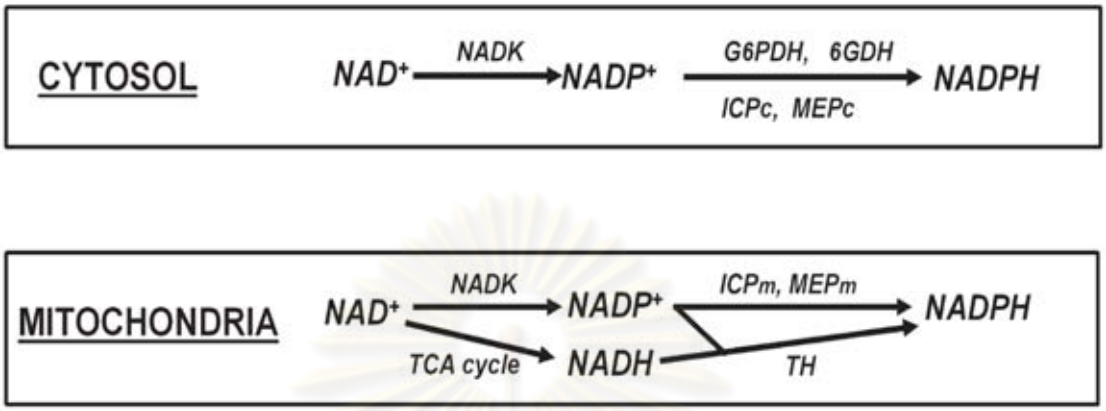

Figure 14. Pathways by which NADPH is generated in cytosol and mitochondria [112].

Furthermore, novel paradigm for the metabolism of NAD and NADP, which consists of the following major concepts: 1) NAD and NADP can be metabolized by many enzymes to generate multiple bioactive molecules, such as cyclic ADP-ribose, ADP-ribose, poly (ADP-ribose), NAADP, and O-acetyl-ADP-ribose; 2) there are NAD^+-synthesizing and NAD^+-catabolizing enzymes in not only the nucleus, but also in other subcellular organelles including the Golgi complex and mitochondria; third, extracellular NAD^+ can be metabolized by such ecto-enzymes as CD38 to produce biological effects; and NAD^+-synthesizing processes might also be catalyzed extracellular; 4) NAD can be transported across the plasma membranes of certain cell types; 5) there are close interactions among the key enzymes in NAD and NADP metabolism,such as the interactions between NMNAT-1 and PARP-1; and sixth, at least in yeast there are such novel metabolic pathways of NAD and NADP as the pathways mediated by NADH kinase and acetaldehyde dehydrogenase [112].

NAD^+ can be generated from the salvage pathway using nicotinic acid mononucleotide (NaMN) or nicotinamide mononucleotide (NMN) as precursors, or from the *de novo* pathway using quinolinic acid (QA) as the precursor. Through NAD^+-dependent dehydrogenases, poly(ADP-ribose) polymerases (PARPs), sirtuins, ADP-ribosyl cyclases (ARCs), and mono(ADP-ribosyl)transferases (ARTs), NAD^+ can significantly affect mitochondrial function, energy metabolism, calcium homeostasis, gene expression, aging, and cell death. NADH can be generated from NAD^+ by NAD-dependent dehydrogenases, which is used by the electron transport chain (ETC) or NADH oxidases. NADH can significantly affect mitochondrial function, energy metabolism, oxidative stress, calcium homeostasis, and gene expression. $NADP^+$ can be generated from NAD^+ by NAD^+ kinases (NADK), which can be used for NADPH generation through glucose-6-phosphate dehydrogenase (G-6-PD), 6-glyconate phosphate dehydrogenase (6GPDH), $NADP^+$-dependent isocitrate dehydrogenases (IDPs), $NADP^+$-dependent malic enzymes (MEPs), and transhydrogenase (TH). NADPH can be used by glutathione reductase, NADPH oxidase, and other NADPH-dependent enzymes to mediate antioxidation, ROS generation, and reductive synthesis.

$NADP^+$could also be used by ADP-ribosyl cyclases (ARCs) to generate NAADP that can mobilize intracellular Ca^{2+} stores (Figure 15) [112].

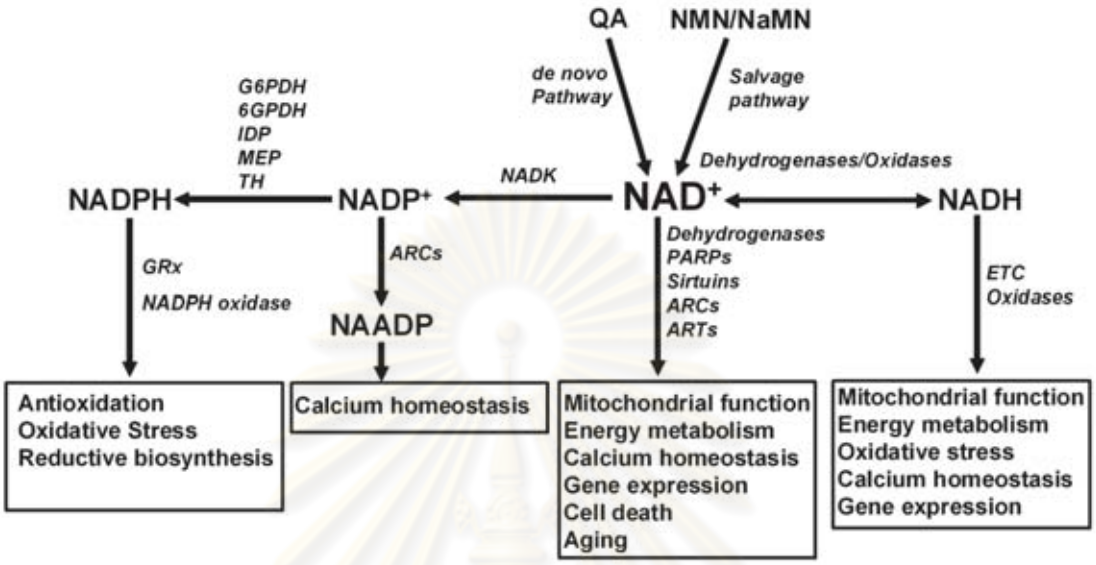

Figure 15. Metabolism and biological activities of NAD and NADP [112].

CHAPTER III
MATERIALS AND METHODS

1. Cell line isolation and Maintenance

The human hepatocellular cancer cell line HepG2 was obtained from the American Type Culture collection (Manassas, VA). HepG2 cells were isolated as previously described [113]. Minimal essential medium (MEM) was obtained from Invitrogen (Carlsbad, CA). HepG2 cells were maintained in MEM supplemented with 5% FBS from Invitrogen (Carlsbad, CA), 1% Non-Essential Amino Acid from Invitrogen (Carlsbad, CA) and 0.5% L-Glutamine from Invitrogen (Carlsbad, CA). All cells used for experiments were less than 20 passage numbers, due to concern for phenotypic changes. The 100-mm stock plates were washed once with 10 ml phosphate buffer saline (PBS). One ml of 1x trypsin-EDTA solution (0.05% trypsin, 0.53 mM EDTA .4Na, Invitrogen, Carlsbad, CA) was adding in the plate for the brief coverage on the surface. The solution was removed and plate was incubated at 37°C for 10 minutes. Five ml of complete medium was added to stop the trypsin-EDTA reaction. The stock plates were seeded in 100-mm dishes at density about 3×10^6 cells. Typically, cells were sub-cultured when reached 70-80% confluence and subcultured at about initial density.

2. Chemical treatments

The cells were then seeded at a density of 4.5×10^6 cells on 60-mm dishes, and they were utilized for chemical exposure after culturing overnight. Cadmium chloride hemi (pentahydrate) ($CdCl_2 \cdot 2.5H_2O$) from Sigma (St.Luis, MO) stock solutions were dissolved in water.

3. Measurement the effect of insulin to cadmium effect

The cells were then seeded at a density of 4.5×10^6 cells on 60-mm dishes, and they were utilized for chemical exposure after culturing overnight. The cells were then washed with PBS, and replacement with serum-free medium 48 hours. The cells were then washed with PBS and replacement with serum-free medium and pre-treated with

insulin 20 mU/ml and 2,000 mU/ml (Eli lilly, Indianapolis, IN) 24 hours following with cadmium 24 hours.

4. Total RNA isolation

All RNA samples were purified by the Trizol™ reagent from Invitrogen (Carlsbad, CA). For attached cells, Trizol™ reagent was added directly to the aspirated tissue 6-well plate (0.5 ml per well). The solution was mixed by pipetting for several times and incubated at ambient temperature for 5 minutes. The mixture was transferred to a 1.5-ml microcentrifuge tube. Chloroform (0.2ml) was added and mixture was shaken vigorously for 15 seconds and rested at room temperature for 3 minutes. To separate the $CHCl_3/H_2O$ phases, the tube was centrifuged in refrigerated centrifuge at 12,000 g for 15 minutes at 4°C. The upper phase (water phase or clear phase) was transferred into a new tube. Isopropanol (0.5 ml) was added to precipitate RNA. The tube was kept at -20°C for 20 minute before centrifuged at 12,000 g for 15 minutes at 4°C. At this point, RNA pellet can be observed. The supernatant was removed. The pellet was washed once with one ml of 70% ethanol. Ethanol solution was removed by pipetting and pellet was air-dried for 10 minutes at ambient temperature. RNA pellet was dissolved in RNase-free DNase-free DEPC-treated water (20-40 μl). Concentration of RNA was determined by UV-spectrophotometry (1 OD of RNA solution = 40 μg/ml). All RNA sample were stored at -80°C.

5. Reverse transcription

Five micrograms of total RNA was reverse transcribed by random hexamer with Super-scrip II reverse transcriptase (Life Technologies, Inc.). For cDNA synthesis, the reactions were carried out by adding the following components;

Random hexamer (250 ng/μl)	1	μl
Total RNA (50 μg)	a	μl
H_2O	11-a	μl

The mixture was incubated at 70°C in a PCR machine for 10 minutes and chilled on ice immediately for 3 minutes. The following component was added into the tube:

5 x first strand buffer (Gibco, BRL)	4	μl
0.1 M DTT	2	μl
10 mM dNTP mix	1	μl

After the mixture was incubated at room temperature for 10 minutes. It was further incubated in PCR machine at 42°C for two minutes. Super-script II reverse transcriptase from Invitrogen (200 units) was added and the reactions were carried on at 42°C for 50 minutes, followed by a 15-minutes heat inactivation step at 70°C. The DNA was stored at -20°C.

6. Polymerase chain reaction (PCR)

Polymerase chain reactions were performed by the protocol following the manufacturer's recommendation (Invitrogen). Briefly, each reaction contains:

10 x PCR buffer (mixing Mg)	5	μl
2 mM dNTP mixture	5	μl
50 mM $MgCl_2$	2	μl
Primer mix (10 μM each)	2	μl
Template DNA (cDNA from RT)	1	μl
Taq DNA polymerase (5 U/μl)	0.5	μl
H_2O to	50	μl

Reactions were performed in the 0.5-ml thin wall tubes (Corning Inc., Corning, NY) by the "hot-start" fashion. Tubes were incubated in a thermal cycler (Biometra T-Gradient. Germany) at 94°C for 2 minutes to completely denature the template. The reactions were performed 25-28 cycles of PCR amplification as follows, depended on primers used:

Denature	94°C for 20 s
Annealing	63°C for 20 s
Extension	72°C for 20 s

The reactions were carried out for an additional 10 minutes at 72°C and maintained at 4°C. PCR products were dissolved in 1.5% agarose in 1 x TAE buffer and detected by

ethidium bromide staining. Intensity of bands were determined by densitometric analysis and normalized to GAPDH bands.

The specific primer pairs used were:
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH): product size 958 bp;
forward primer, 5'- TGA AGG TCG GAG TCA ACG GAT TTG GT -3' and
reverse primer, 5'- CAT GTG GGC CAT GAG GTC CAC CAC -3' ;

Glucose-6-phosphate dehydrogenase (G-6-PD): product size 187 bp;
forward primer, 5'- GAT GCC TTC CAT CAG TCG GA -3' and
reverse primer, 5'- GCT CAC TCT GTT TGC GGA TG -3'.

NF-κB essential modulator (NEMO): product size 370 bp;
forward primer, 5'- ACG TAC TGG GCG AAG AGT CTC C-3' and
reverse primer, 5'- GAC GTC ACC TGG GCT TTC AC-3'.

7. Measurement of cell viability

Cell viability was measured by the MTS colorimetric assay (Promega, Madison, WI), which is based on the conversion of the terazolium salt to the colored product, formazan. A solution of 3-(4,5-dimethylthiazol-2-yl)-5- (3-carboxymethoxyphenyl) -2-(4-sulfophenyl)-2H-tetrazolium, inert salt (MTS) was prepared (2mg/ml in PBS 0.02 M, pH 7.2) and stored at -20°C. Phenazine methosulfate (PMS, 0.92 mg/ml) was similarly prepared in PBS (0.02 M, pH 7.2) and stored at -20°C. Cells were suspended in MEM at a 2 x 10^5 cells/ml. A 100 μl of HepG2 cells suspension were then seeded on to two 96-well plates. After being cultured overnight. At the end of designated exposure time, the medium was removed and add PBS 100 μl. The entire plate was incubated at 37°C for 1 hour and A490 nm was measured by microplate reader (Biohit, Finland). Treatments were performed in triplicate. Percent cell viability was expressed as follows: $A_{exp\ group} / A_{control}$ x100 [114].

8. Protein quatification by MicroBCA

The assay was performed by MicroBCA assay kit (Pierce Biotechnology, Rockford, IL). A standard curve was calculated by the following protocol. Bovine serum albumin (BSA) standard solution (2 mg/ml) was diluted with water to 200, 400, 600, 800 and 1000 μg/ml. Each dilution 10 μl was pipetted into a flat-bottom well microtitre plate (Corning, Lowell, MA) containing 90 μl of dH_2O in duplicate. For sample determination, 10 μl of protein sample was added in duplicate into the plate with 90 μl of dH_2O. At least two dilutions of samples were including ensuring the sample is in the range of standard curve. Typically, 1:2 and 1:10 were used. The working reagent was prepared by mixing 50 parts of Reagent A, 48 parts of Reagent B and 2 part of Reagent C 100 μl of the working solution was added to each well and the plate was sealed by Saran Wrap and incubated at 60°C for 60 minutes in the hybridization oven. Plate was cooled down at room temperature for 10 minutes and the A562 nm was measured by a microplate reader (Biohit, Finland).

9. Western Blotting

After treatment with cadmium, cells were washed twice with cold PBS and lysed with Lysis buffer (30 mM Tris HCl, pH 6.8; 10 mg/ml SDS; 1mM EDTA; 1μg/ml leupeptin; 1μg/ml aprotinin; 1mM PMSF; 1μg/ml pepstatin A; 1mM Na_3VO_4; 1mM NaF; 1mM Benzamidine) (250 μl for 60 mm dish) on ice for 5 minutes. After sonication five times at 130 W, 40% output for 5 sec each and wait 5 sec each, the insoluble material was removed by centrifugation at 12,000 x g for 10 minutes on ice. The protein concentration of clear lysate was determined by MicroBCA method (Pierce Biotechnology, Rockford, IL). Sample resolved on 15% polyacrylamide gel at 70 volts were subjected to electro-transferred by wet transfer technique onto Immobilon-P membrane (Millipore, Bedford, MA) in 1 x transfer buffer (25 mM Tris HCl; 192 mM glycine; 10% methanol; 0.01% SDS). The process was carried out for 90 minutes on ice, using constant voltage at 100 V. For immunodetection of the proteins, membrane was blocked by 3% BSA (PAA Laboratories) in PBST buffer (10mM sodium phosphate pH 7.2; 0.9% NaCl; 0.05%

Tween®-20) for overnight at 4°C. The blot was incubated for two hours at room temperature or overnight at 4°C with primary antibody (Table 3) diluted in 1% BSA in PBST buffer. The membrane was washed tree times with PBST for five minutes each and incubated for two hours at room temperature with a Cy3-Linked goat anti-mouse IgG and Cy5-Linked goat anti-rabbit IgG (GE Healthcare, Buckinghamshire, UK) were used as secondary antibody diluted in PBST buffer. The blot was washed five times with PBST for five minutes each. The fluorescence bands of proteins by Ettan DIGE Imager (GE Healthcare, Buckinghamshire, UK).

In some experiment, the membranes were needed to be re-probed. The membrane was briefly washed with PBST and incubated in Restore Plus Western Blot Stripping Buffer (Pierce Biotechnology, Rockford, IL) for 5-15 minutes at room temperature. The membrane was washed five times with PBS for five minutes each. After that, the membrane was blocked with 3% BSA in PBST buffer and subjected to incubate with the primary antibody.

Table 3. Name, concentration, and company of primary antibody.

Antibody	Concentration	Type of antibody	Company	Product No.
GAPDH (MW 38 kDa)	0.2 μg/ml	mouse monoclonal	Santa Cruz Biotech (Santa Cruz, CA)	sc-32233
β-actin (MW 43 kDa)	1.0 μg/ml	mouse monoclonal	Sigma (St.Luis, MO)	A-1978
LDHA (MW 36 kDa)	1:1000	rabbit monoclonal	Signaling Technology (Danvers, MA)	#3582
Histone H2B (MW 14, 12 kDa)	1:1000	mouse monoclonal	Signaling Technology (Danvers, MA)	#2934
PARP-1 (MW 118, 85, ~55 kDa)	0.2 μg/ml	rabbit polyclonal	Santa Cruz Biotech (Santa Cruz, CA).	sc-7150
IKKγ (MW 48 kDa)	0.2 μg/ml	rabbit polyclonal	Santa Cruz Biotech (Santa Cruz, CA).	sc-8330

10. Determination of PARP cleavage

To determine apoptosis and necrosis induced by cadmium, the extent of poly (ADP-ribose) polymerase-1 (PARP-1) cleavage was investigated. Cell lysates were prepared in lysis buffer contain 6 M urea. Protein concentrations were determined by MicroBCA method, according to the manufacturer's recommendation. Lysates equivalent to 90 μg proteins were separated in 10% SDS-PAGE and transferred onto Immobilon-P membrane (Millipore). Protein expression of PARP-1 cleavage was incubated for overnight at 4°C with 0.2 μg/ml rabbit polyclonal anti-PARP (sc-7150) from Santa Cruz Biotech (Santa Cruz, CA) and β-actin (use as internal control) was incubated for overnight at 4°C with 1.0 μg/ml mouse monoclonal anti-β-actin (A-1978) from Sigma (St.Luis, MO) diluted in 1% BSA in PBST buffer. The membrane was washed tree times with PBST for five minutes each and incubated for two hours at room temperature with a Cy3-Linked goat anti-mouse IgG and Cy5-Linked goat anti-rabbit IgG (GE Healthcare, Buckinghamshire, UK) were used as secondary antibody diluted in PBST buffer. The blot was washed five times with PBST for five minutes each. The fluorescence bands of proteins by Ettan DIGE Imager (GE Healthcare, Buckinghamshire, UK). Full-length PARP-1 bands 118 kDa, cleavage PARP-1 bands 85, 55 kDa.

11. Statistical analysis and Data presentation

Experiments were performed in at least triplicate experiment manner and repeated at least twice. The results were showed as mean ± SEM as indicated. Student two-tailed t-test was used to test the significance. $P<0.05$ was determined as the significance. All statistical analyses were performed using SPSS version 11.5 and graphic illustration was performed using Microsoft Excel 2003®. Densitometric analyses of protein expression performed by western blot were determined by Scion Image Software for window v. 4.0.3.2.

CHAPTER IV
RESULTS

1. The dose effect of cadmium on HepG2 cells.

1.1 Determination of *G-6-PD* mRNA expression by cadmium

To investigate the role of cadmium to *G-6-PD* mRNA expression. The *G-6-PD* mRNA expression level was investigated in HepG2 cells-treated with cadmium. The *G-6-PD* mRNA expression in HepG2 cells cultivated in the presence of cadmium become lower than in the absence of cadmium (2-4 fold) (Figure 16). This result indicated that *G-6-PD* mRNA expression was reduced by cadmium.

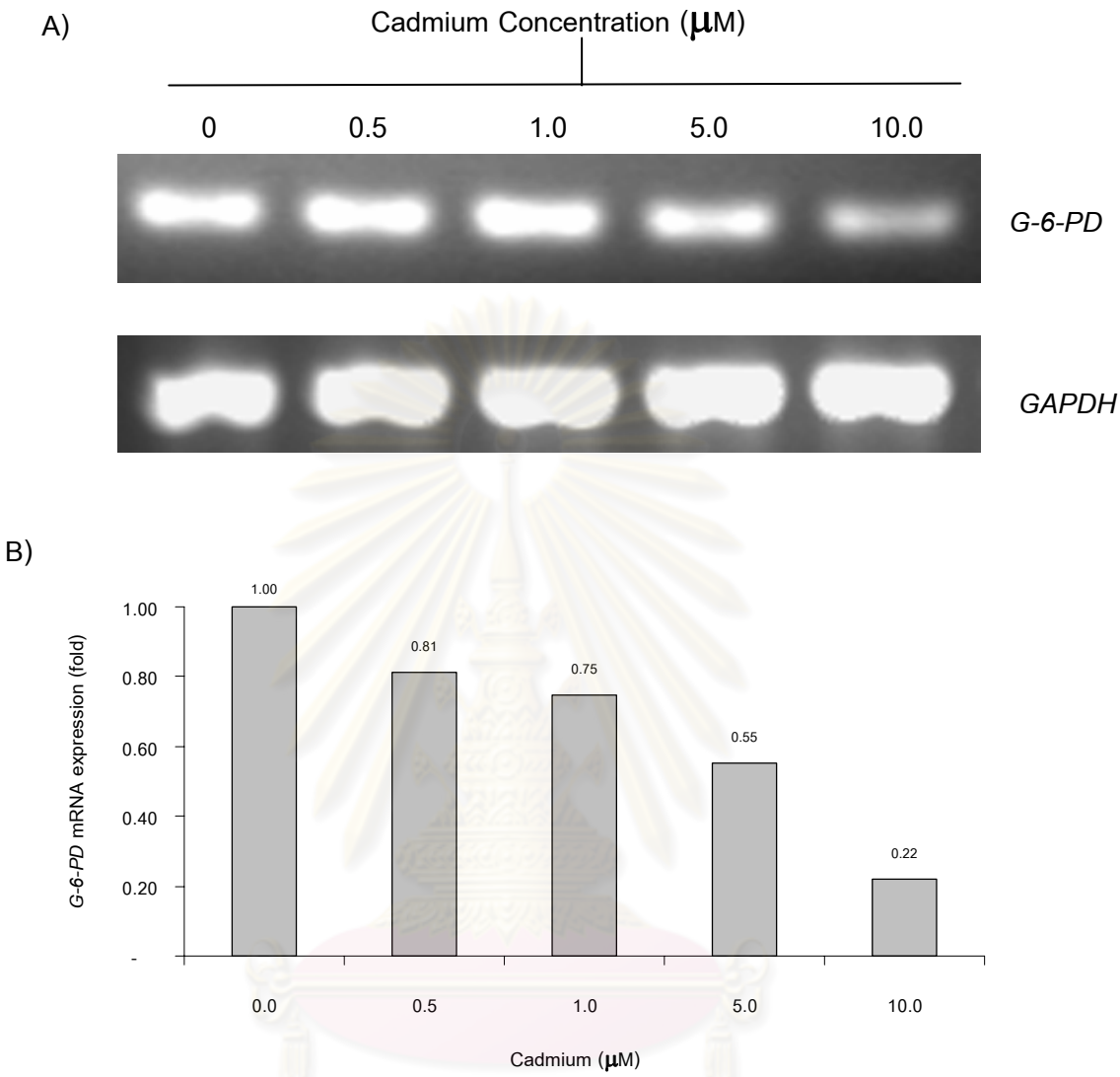

Figure 16. Effect of cadmium on *G-6-PD* mRNA expression in HepG2 cells.

HepG2 cell was cultivated in MEM for overnight, as described in materials and methods. Cell was treated with cadmium 0.5, 1.0, 5.0, and 10.0 μM for 24 hours. Total mRNA was isolated and *G-6-PD* mRNA expression was determined by reverse transcriptase PCR. A) Upper panel shows the *G-6-PD* mRNA expression by reverse transcriptase PCR analysis. Lower panel show *GAPDH* mRNA expression as an internal control. B) Relative density of *G-6-PD* mRNA normalized to *GAPDH* mRNA.

1.2 Determination of *NEMO* mRNA expression by cadmium

To investigate the role of cadmium to *NEMO* mRNA expression. The *NEMO* mRNA expression level was investigated in HepG2 cells-treated with cadmium. The *NEMO* mRNA expression in HepG2 cells cultivated in the presence of cadmium become lower than in the absence of cadmium (Figure 17). This result indicated that *NEMO* mRNA expression was reduced by cadmium.

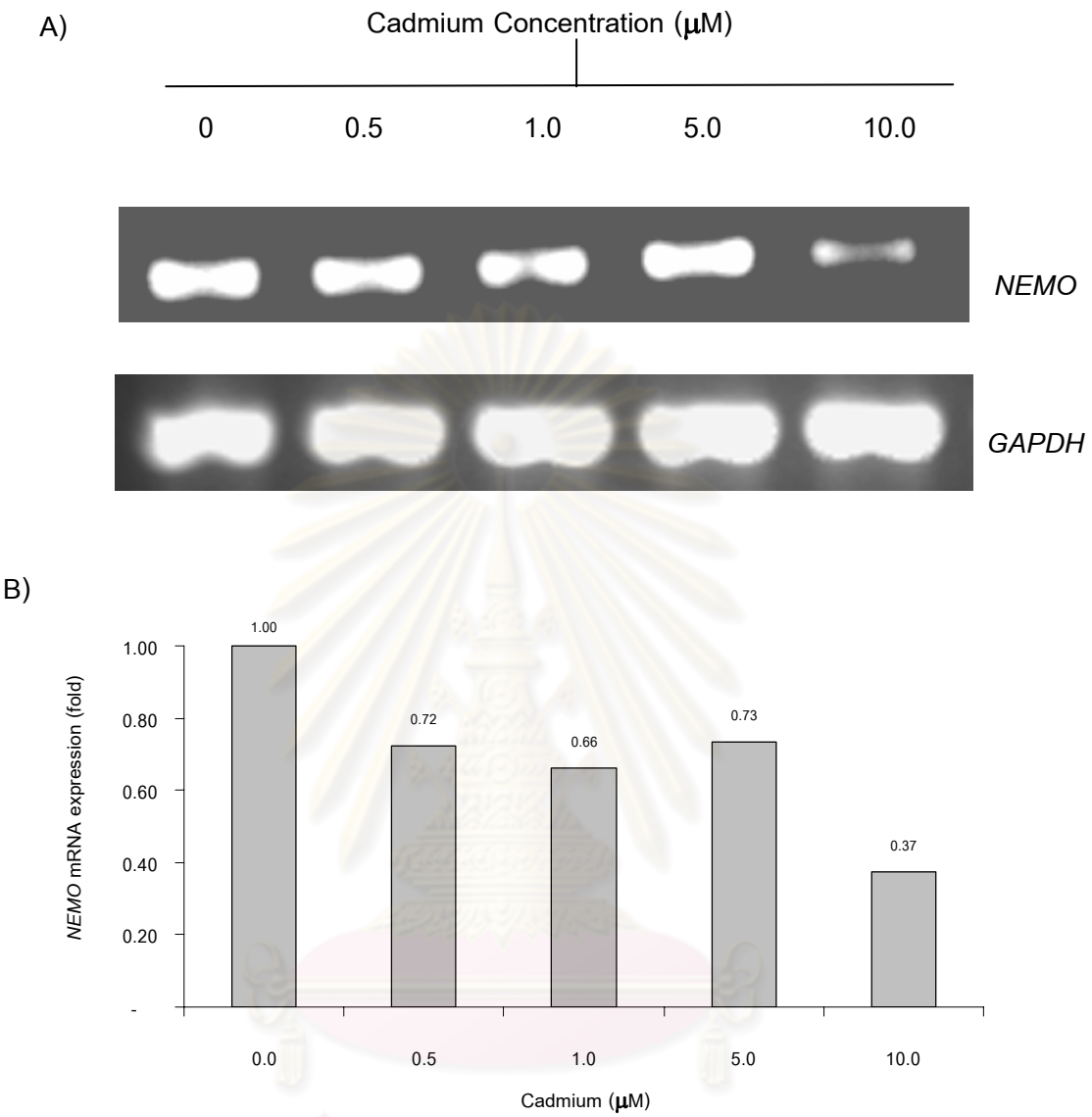

Figure 17. Effect of cadmium on *NEMO* mRNA expression in HepG2 cells.

HepG2 cell was cultivated in MEM for overnight, as described in materials and methods. Cell was treated with cadmium 0.5, 1.0, 5.0, and 10.0 μM for 24 hours. Total mRNA was isolated and *NEMO* mRNA expression was determined by reverse transcriptase PCR. A) Upper panel shows the *NEMO* mRNA expression by reverse transcriptase PCR analysis. Lower panel show *GAPDH* mRNA expression as an internal control. B) Relative density of *G-6-PD* mRNA normalized to *GAPDH* mRNA.

1.3 Determination of HepG2 cells viability by cadmium

Cadmium induced hepatic cell death depend on dose [59]. IC_{50} (inhibitory concentration 50) is the concentration of the concerned drug that will inhibit the concerned activity of a biological system. The IC_{50} of cadmium was determined by examining the HepG2 cell viability, the MTS assay was a measured of mitochondrial dehydrogenase enzymes activity in HepG2 cells. The cells viability was decreased when increasing cadmium dose (from 0-200 μM). IC_{50} has calculated as 15 μM. (Figure 18).

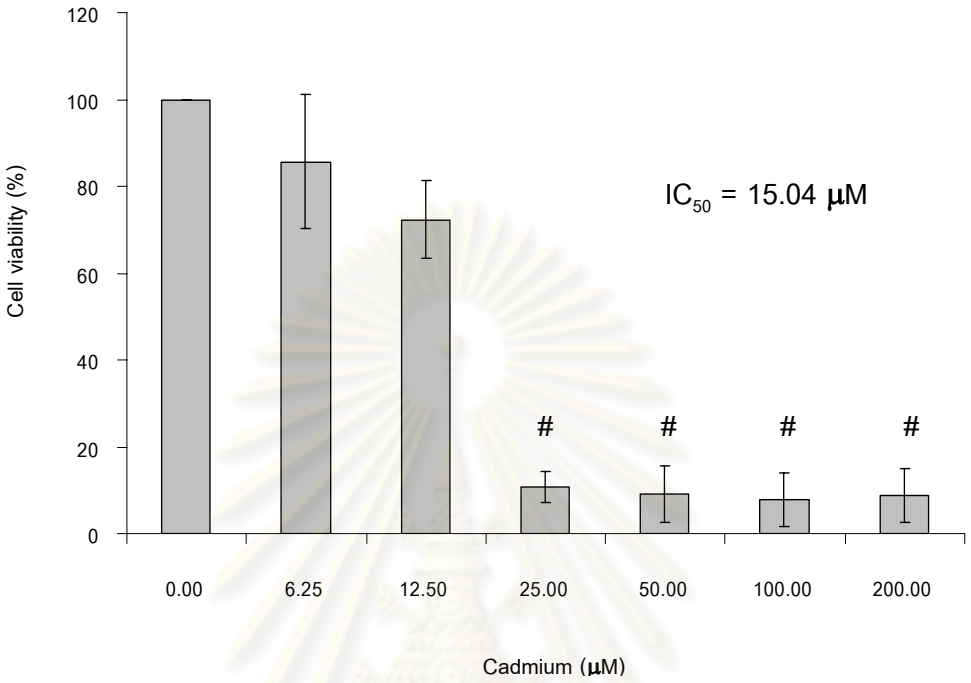

Figure 18. Effect of cadmium on HepG2 cells viability

HepG2 cell was cultivated in MEM for overnight, as described in materials and methods. Cell was treated with cadmium (ranging from 0-200 μM) for 24 hours. The cell viability was determined by MTS assay. Relative cell viability taking control as 100 percent;

$^{\#}p<0.01$ when compared with control.

1.4 Determination of NEMO protein expression by cadmium

After cell viability experiment, cadmium induced cell death in dose dependent manner. To investigate the role of cadmium to NEMO protein (48 kDa) expression. NEMO protein expression was investigated HepG2 cells-treated with cadmium. The NEMO protein expression in HepG2 cells cultivated in the presence of cadmium become similar in absence of cadmium (Figure 19). This result indicated that NEMO protein expression was not affected by cadmium. However, the GAPDH protein (38 kDa) expression in HepG2 cells cultivated in the presence of cadmium become lower than in the absence of cadmium. This result indicated that GAPDH protein expression may was reduced by cadmium.

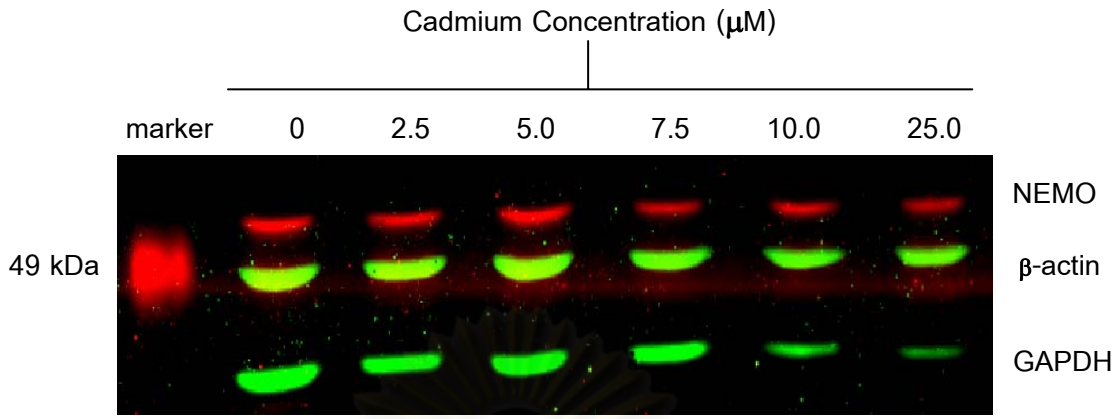

Figure 19. Effect of cadmium on NEMO protein expression in HepG2 cells.

HepG2 cell was cultivated in MEM for overnight, as described in materials and methods. Cell was treated with cadmium 2.5, 5.0, 7.5, 10.0, and 25.0 μM for 48 hours. Total protein was isolated and GAPDH protein expression was determined by western blot analysis. A) Upper panel show NEMO protein (48 kDa) expression and lower panel shows the GAPDH protein (38 kDa) expression by western blot analysis. Medial panel show β-actin protein (43 kDa) expression as an internal control.

1.5 Determination of GAPDH protein expression by cadmium

Cadmium induced cell death in dose dependent manner and may reduced GAPDH protein expression. In most tissues 80-90% of glucose oxidation is via glycolysis pathway [22]. NADH is reduced by cadmium [35]. Therefore, GAPDH protein expression level was investigated HepG2 cells-treated with cadmium. The GAPDH protein (38 kDa) expression was decreased when increasing cadmium dose (from 0-200 μM) (Figure 20A). The mean GAPDH protein expression in control group was significantly higher than in cadmium-treated groups ($p<0.01$) (Figure 20B). This result indicated that GAPDH protein expression was reduced by cadmium in a dose-dependent manner.

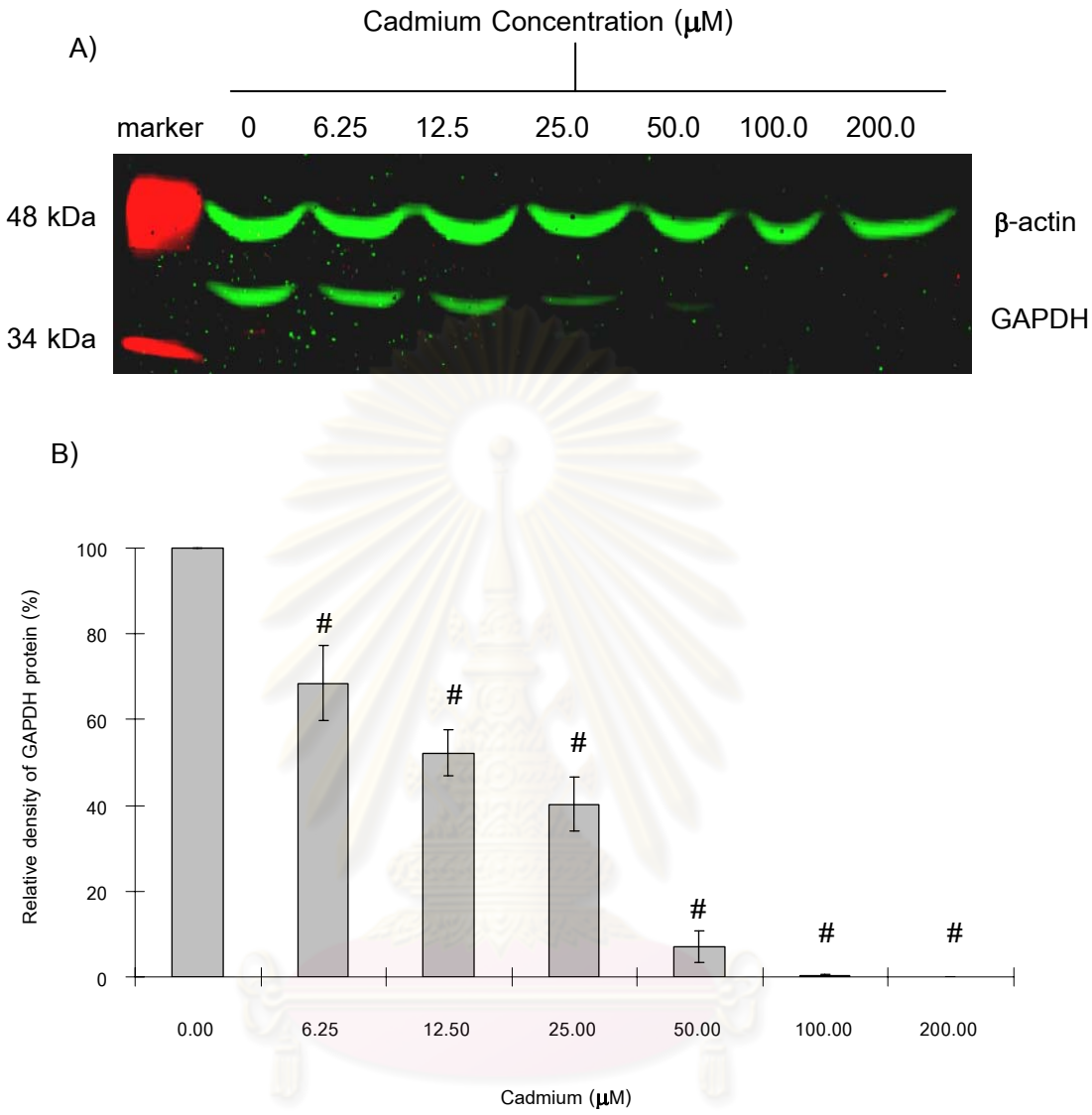

Figure 20. Effect of cadmium on GAPDH protein expression in HepG2 cells.

HepG2 cell was cultivated in MEM for overnight, as described in materials and methods. Cell was treated with cadmium (ranging from 0-200 μM) for 24 hours. Total protein was isolated and GAPDH protein expression was determined by western blot analysis. A) Lower panel shows the GAPDH protein (38 kDa) expression by western blot analysis. Upper panel show β-actin protein (43 kDa) expression as an internal control. B) Relative density of GAPDH protein normalized with β-actin.

# $p<0.01$ when compared with control.

1.6 Determination of LDHA protein expression by cadmium

GAPDH and LDH have functional interaction that can affect NAD^+/NADH and glycolysis in living cells [115]. The LDHA protein expression was investigated HepG2 cells-treated with cadmium. The LDHA protein (36 kDa) expression was decreased when increasing cadmium dose (from 0-200 μM) (Figure 21A). The mean LDHA protein expression in control group was significantly higher than in cadmium-treated groups ($p<0.01$) (Figure 21B). This result indicated that LDHA protein expression was reduced by cadmium in a dose-dependent manner.

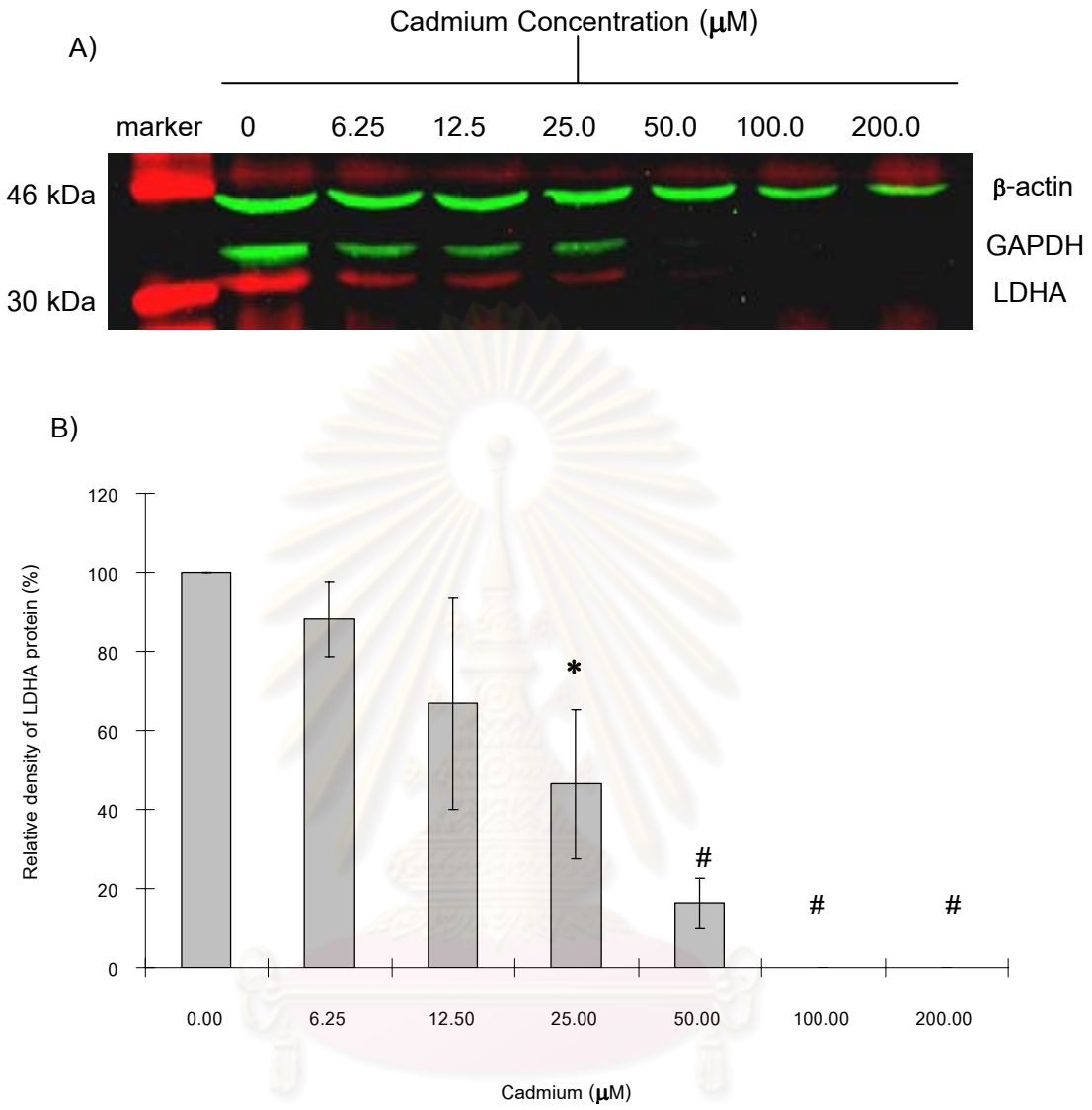

Figure 21. Effect of cadmium on LDHA protein expression in HepG2 cells.

Experiment was carried out as described in Figure 20. Total protein was isolated and LDHA protein expression was determined by western blot analysis. A) Lower panel shows the LDHA protein (red bands, 36 kDa) and medial panel shows the GAPDH protein (38 kDa) expression by western blot analysis. Upper panel show β-actin protein (green bands, 43 kDa) expression as an internal control. B) Relative density of LDHA protein normalized with β-actin.

$^{*}p<0.05$, $^{\#}p<0.01$ when compare with control.

1.7 Determination of histone H2B truncation by cadmium

GAPDH and LDHA are a necessary component for the proper functioning of Oct-1 coactivator and OCA-S, that is essential for S-phase-dependent histone H2B transcription in cells [33]. Transcription from the *histone H2B* gene requires Oct-1 and OCA-S, a multi-subunit complex that contains the NAD(H)-linked classical GAPDH and LDH [81]. Cleavage histone H2B form (q-H2B; ~12 kDa) and full length histone H2B protein (H2B; 14 kDa) expression level was investigated HepG2 cells-treated with cadmium. The ratio of cleavage histone H2B with full length histone H2B protein level (q-H2B:H2B) was increased when increasing cadmium dose (from 0-200 μM) (Figure 22A). The mean ratio of cleavage histone H2B and full length histone H2B protein level (q-H2B:H2B) in control group was significantly lower than in cadmium-treated groups ($p<0.01$) (Figure 22B). These result indicated that cadmium induced histone H2B truncation in a dose-dependent manner.

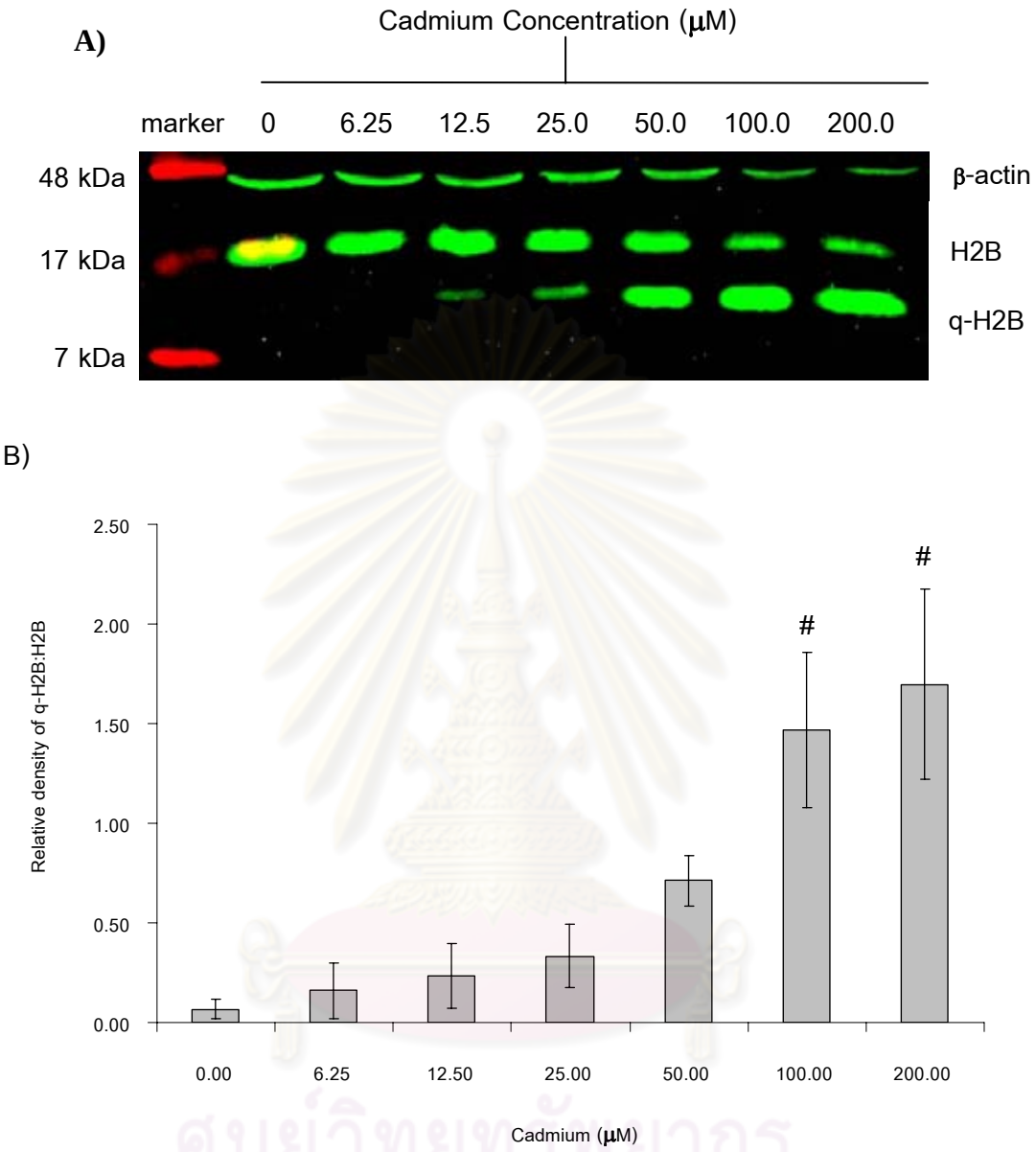

Figure 22. Cadmium-induced histone H2B truncation

Experiment was carried out as described in Figure 20. Total protein was isolated and both of cleavage histone H2B (q-H2B) and full length histone H2B (H2B) protein level protein expression was determined by western blot analysis. A) Lowest panel shows the q-H2B (12 kDa) and medial panel shows the H2B (14 kDa) expressions by western blot analysis. Upper panel show β-actin protein (43 kDa) expression as an internal control. B) Ratio density of q-H2B and H2B.

$^{\#}p<0.01$ when compared with control.

1.8 Determination of type of cells death by cadmium

Cadmium induces either apoptosis or necrosis depending on the cadmium concentration [116, 117]. High levels of GAPDH in cancer cells are at least partially a reflection of elevated glycolysis. *GAPDH* gene expression is increased in all human cancer tissues such as hepatocarcinoma cell line [24]. Furthermore, Karaczyn *et al.* [34] reported that the histone H2B truncation do not concur with apoptosis. This experiment was to investigate the type of cell death in the response to cadmium on HepG2 cells. At high-dose of cadmium treatment induced HepG2 cells necrosis, as seen of cleaved PARP form (55 kDa). Similarly, previous study of the major cleavage fragment form of PARP-1 in necrosis cell is represented by about 55 kDa [105, 106]. This data indicated high dose of cadmium could produce HepG2 cells death by necrosis (Figure 23).

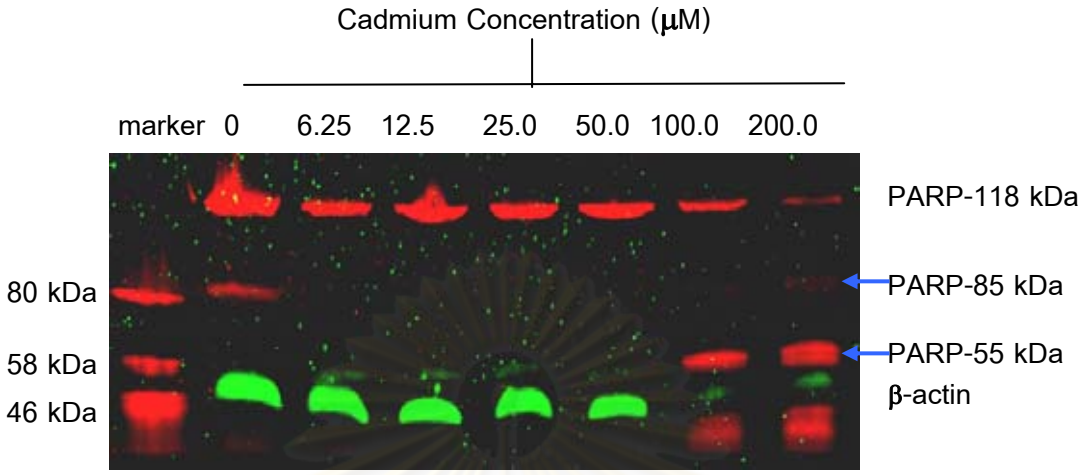

Figure 23. Cadmium-induced PARP-1 cleavage

Experiment was carried out as described in material and methods. Total protein was isolated and both of cleavage PARP-1 and full length PARP-1 protein level protein expression was determined by western blot analysis. Highest panel shows the full length PARP-1 (red bands, 118 kDa), second panel shows the cleavage PARP-1 (red bands, 85 kDa), and third panel shows the cleavage PARP-1 (red bands, 55 kDa) expressions by western blot analysis. Lowest panel show β-actin protein (green bands, 43 kDa) expression as an internal control.

2. Time course of necrosis induction by cadmium.

2.1 Determination of exposure times of cadmium on HepG2 cells viability

Cadmium-induced cell death by necrotic show a biphasic elevation at 12 hour and peaked at 48 hour [118]. The MTS assay was a measured of mitochondrial dehydrogenase activity in HepG2 cells. Cell viability was investigated HepG2 cells-treated with cadmium 25 μM for various exposure times. The cells viability was decreased when increasing exposure times (from 0-60 hours) (Figure 24). This result indicated that cell viability was reduced by cadmium in a time-exposure dependent manner when compared with control.

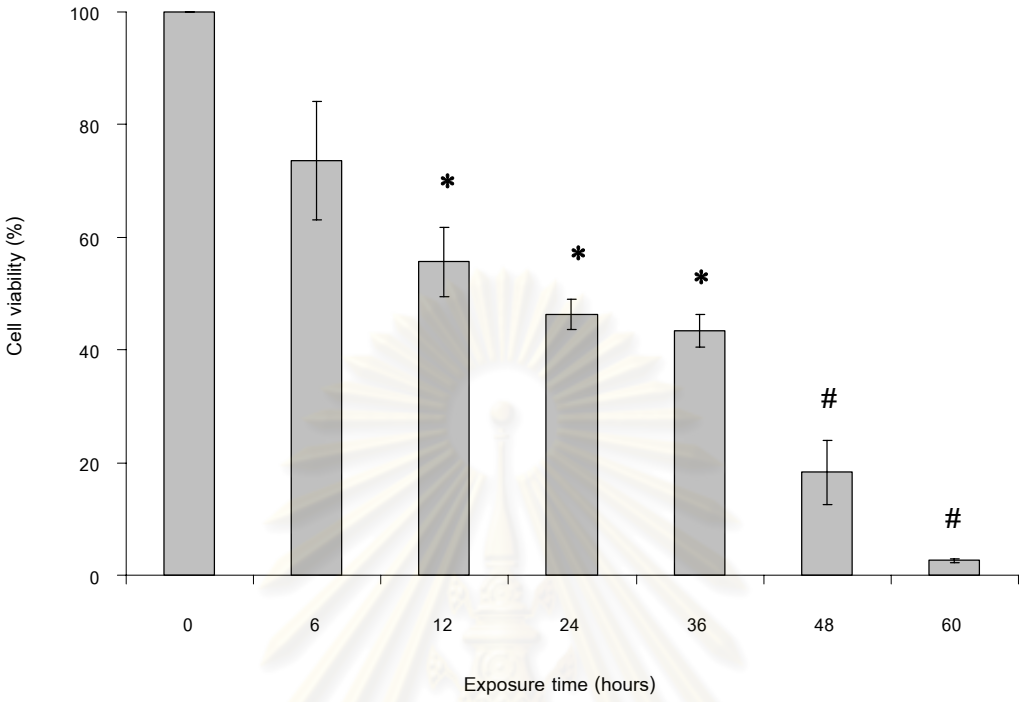

Figure 24. Effect of various exposure time of cadmium on HepG2 cells viability

HepG2 cell was cultivated in MEM for overnight, as described in materials and methods. Cell was treated with cadmium 25 μM for exposure times 0-60 hours. The cell viability was determined by MTS assay. Relative cell viability taking control as 100 percent; * $p<0.05$, # $p<0.01$ when compared with control.

2.2 Determination of exposure times of cadmium on GAPDH protein expression

In most tissues 80-90% of glucose oxidation is via glycolysis pathway [22]. NADH is reduced by cadmium [35]. Therefore, GAPDH protein expression was investigated HepG2 cells-treated with cadmium 25 μM for various exposure times. The GAPDH protein (38 kDa) expression was decreased when increasing exposure times (from 0-60 hours) (Figure 25A). The mean GAPDH protein expression in control group was significantly higher in 12 hours of exposure times ($p<0.05$) and 24-60 hours of exposure times ($p<0.01$) (Figure 25B). This result indicated that GAPDH protein expression was reduced by cadmium in exposure time dependent manner.

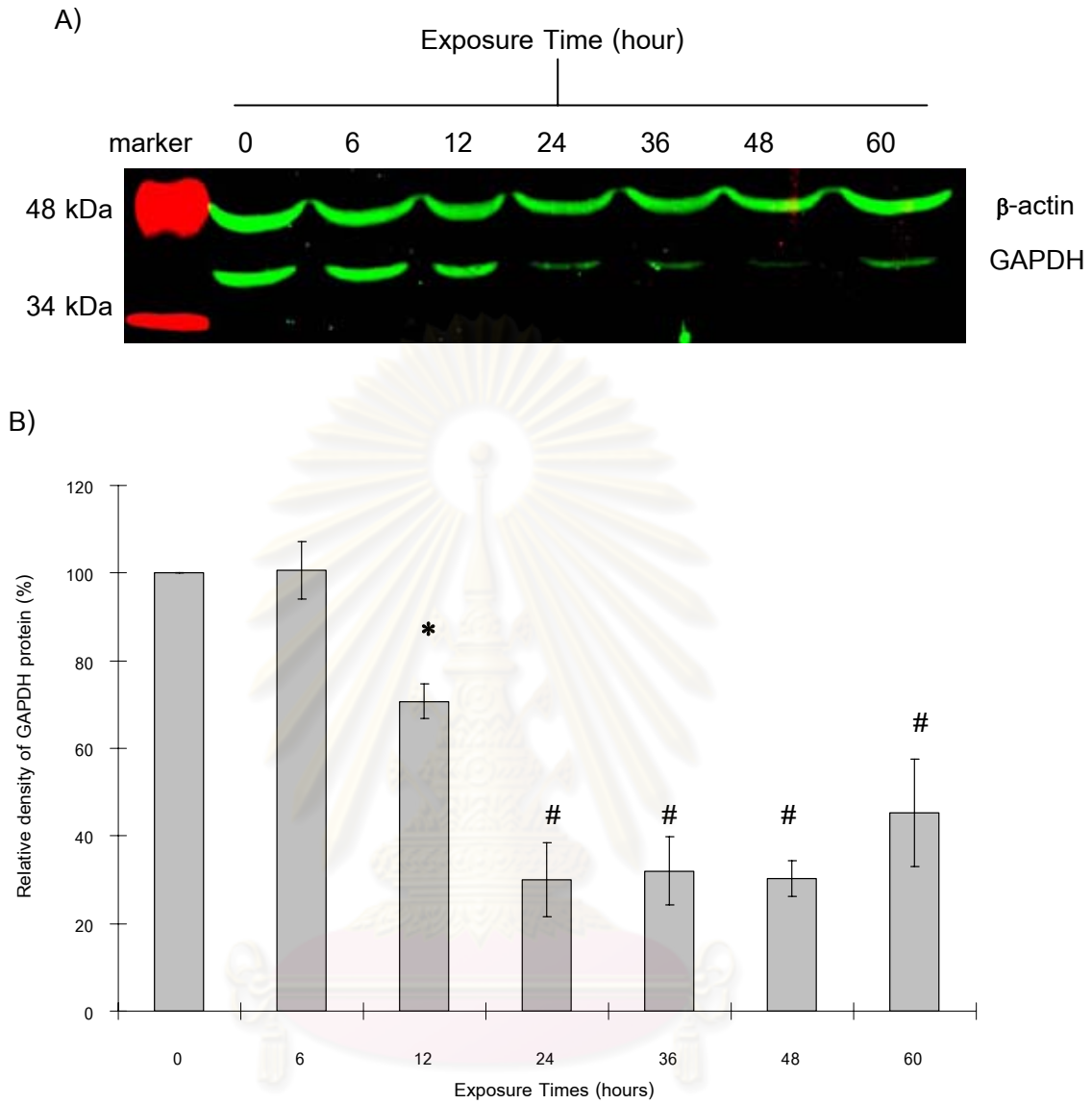

Figure 25. Effect of various exposure time of cadmium on GAPDH protein expression in HepG2 cells.

HepG2 cell was cultivated in MEM for overnight, as described in materials and methods. After overnight of incubation, cells were treated with cadmium 25 μM for various exposure times (0-60 hours). Total protein was isolated and GAPDH protein expression was determined by western blot analysis. A) Lower panel shows the GAPDH protein (38 kDa) expression by western blot analysis. Upper panel show β-actin protein (43 kDa) expression as an internal control. B) Relative density of GAPDH protein normalized with β-actin.

* $p<0.05$, # $p<0.01$ when compared with control.

2.3 Determination of exposure times to cadmium on LDHA protein expression

GAPDH and LDH have functional interaction that can affect NAD^+/NADH and glycolysis in living cells [115] and transcription from the histone H2B gene requires octamer binding factor 1 (Oct-1) and Oct-1 co-activator in S-phase (OCA-S), a multi-subunit complex that contains the NAD(H)-linked classical glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase (p38/GADPH) and lactate dehydrogenase (p36/LDH) along with other compared [27]. In previous study we found GAPDH protein expression was reduced by cadmium in exposure time dependent manner and may reduced LDHA protein expression. Therefore, LDHA protein expression was investigated HepG2 cells-treated with cadmium 25 μM for various exposure times. The LDHA protein (36 kDa) expression was decreased when increasing exposure times (from 0-60 hours) (Figure 26A). The mean LDHA protein expression in control group was significantly higher in 24-60 hours of exposure times ($p<0.01$) (Figure 26B). This result indicated that LDHA protein expression was reduced by cadmium in exposure time dependent manner.

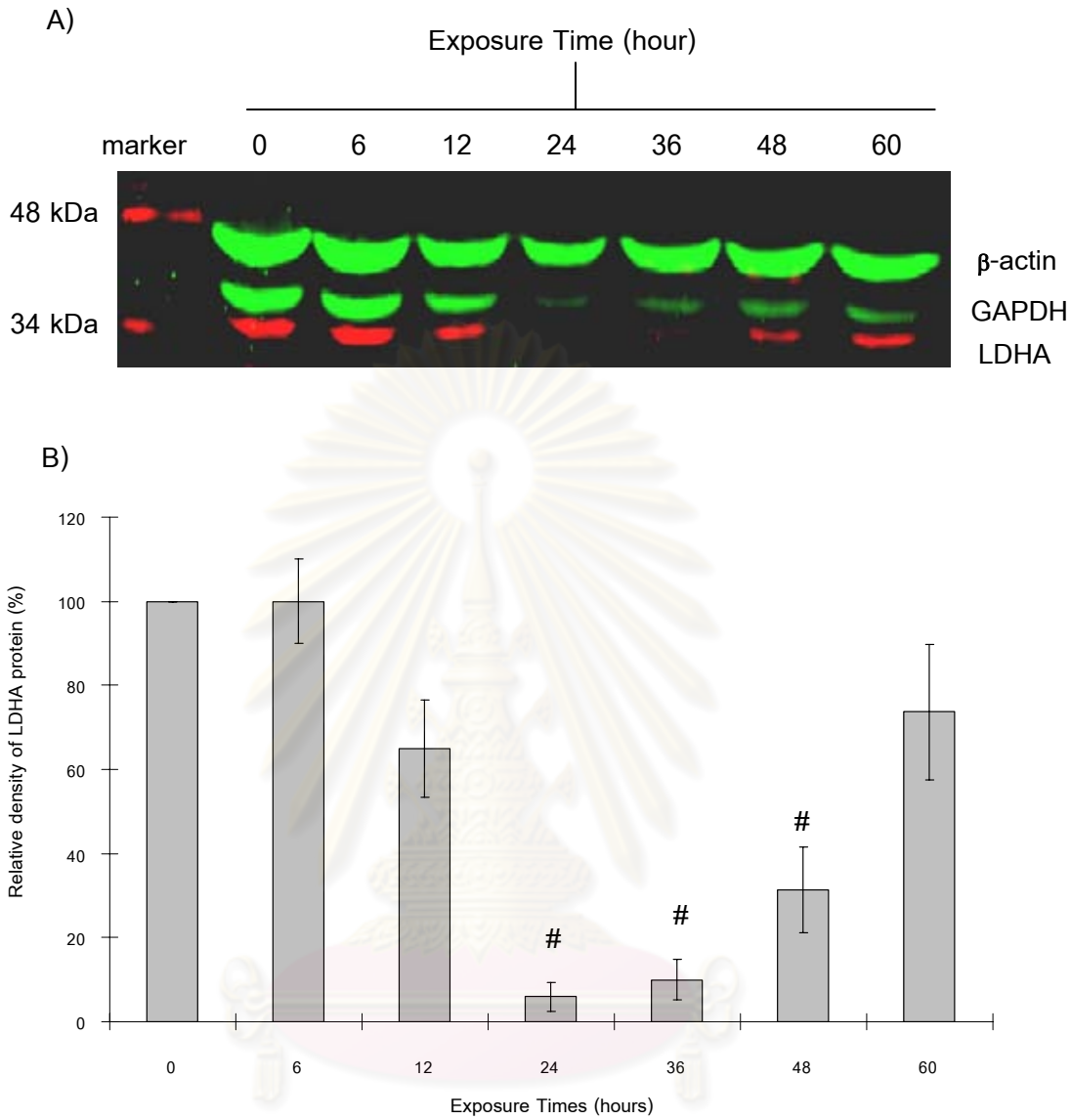

Figure 26. Effect of various exposure time of cadmium on LDHA protein expression in HepG2 cells.

HepG2 cell was cultivated in MEM for overnight, as described in materials and methods. After overnight of incubation, cells were treated with cadmium 25 μM for various exposure times (0-60 hours). Total protein was isolated and LDHA protein expression was determined by western blot analysis. A) Lower panel shows the LDHA protein (36 kDa) expression by western blot analysis. Upper panel show β-actin protein (43 kDa) expression as an internal control. B) Relative density of LDHA protein normalized with β-actin.

# $p<0.01$ when compare with control.

2.4 Determination of exposure times of cadmium on histone H2B truncation

GAPDH and LDH is a necessary component for the proper functioning of Oct-1 coactivator and OCA-S, that is essential for S-phase-dependent histone H2B transcription in cells [33]. Transcription from the histone H2B gene requires Oct-1 and OCA-S, a multi-subunit complex that contains the NAD(H)-linked classical GAPDH and LDH [106]. Therefore, cleavage histone H2B form (q-H2B; 12 kDa) and full length histone H2B protein (H2B; 14 kDa) expression level was investigated HepG2 cells-treated with various exposure times. The ratio of cleavage histone H2B with full length histone H2B protein level (q-H2B:H2B) were increase when increasing exposure times (from 0-60 hours) (Figure 27A). The mean ratio of cleavage histone H2B and full length histone H2B protein level (q-H2B:H2B) in control group was significantly lower in 36-48 hours of exposure times ($p<0.05$) (Figure 27B). This result indicated that histone H2B truncation was induced by cadmium in exposure time dependent manner.

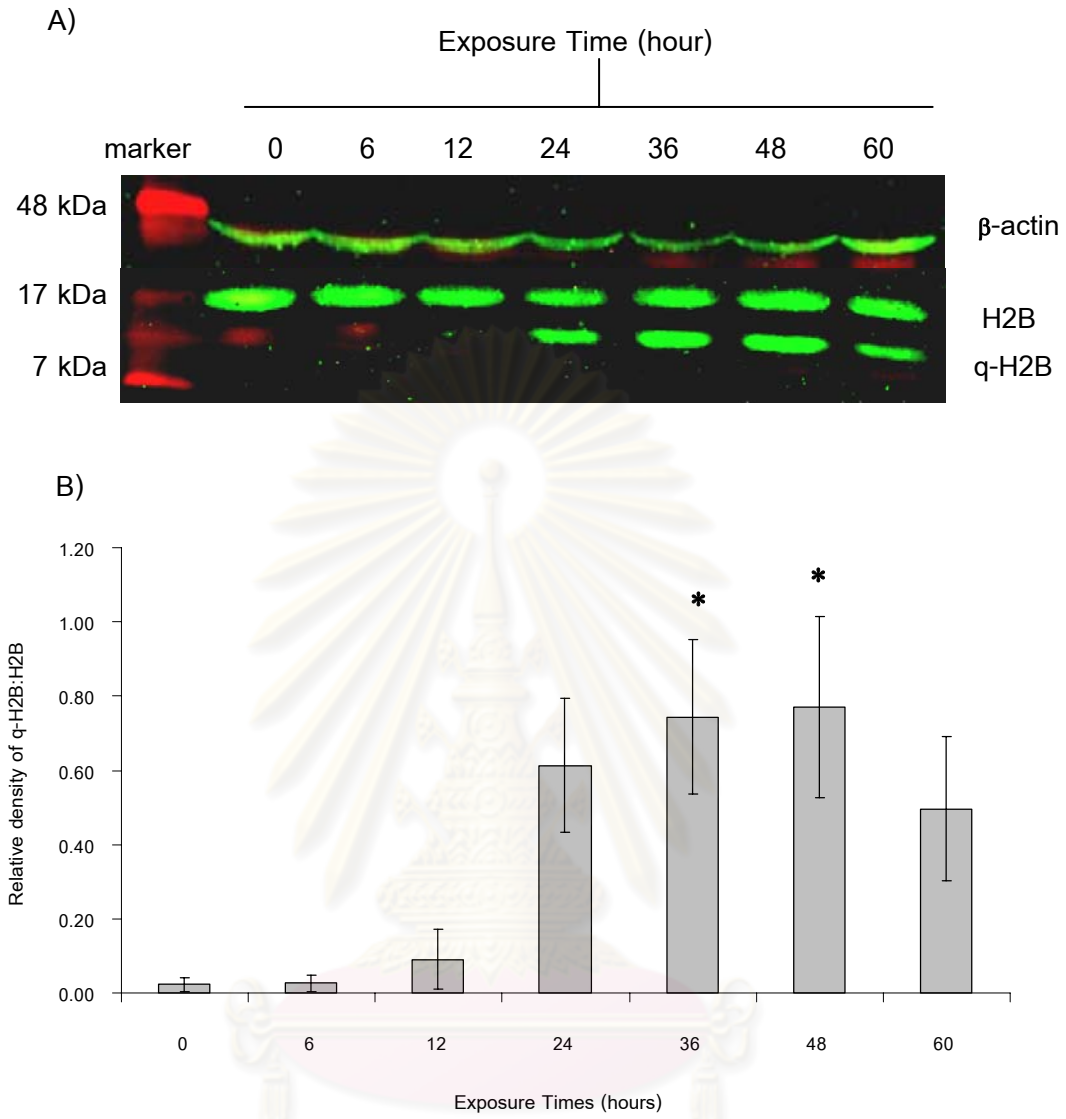

Figure 27. Effect of various exposure time of cadmium-induced histone H2B truncation in HepG2 cells.

Experiment was carried out as described in Figure 25. Total protein was isolated and both of cleavage histone H2B (q-H2B) protein and full length histone H2B (H2B) protein expression were determined by western blot analysis. A) Lowest panel shows the q-H2B (12 kDa) and medial panel shows the H2B protein (14 kDa) expressions by western blot analysis. Upper panel show β-actin protein (43 kDa) expression as an internal control. B) Ratio density of q-H2B and H2B protein level (q-H2B:H2B).

* $p<0.05$ when compared with control.

2.5 Determination of exposure times of cadmium on type of cell death

Cadmium induced both a time- and dose-dependent increase in apoptotic, and necrosis [116]. Furthermore, Karaczyn *et al.* [34] reported that the histone H2B truncation do not concur with apoptosis. Type of cell death was investigated by HepG2 cells-treated with cadmium 25 μM for various exposure times. HepG2 cells necrosis was increased in exposure time 24-48 hours, as seen of cleaved PARP form (55 kDa), but was decreased in exposure time 60 hour. The major cleavage fragment form of PARP-1 in necrosis cell is represented by about 55 kDa. These results indicated that cadmium induced necrosis in exposure time dependent manner (Figure 28).

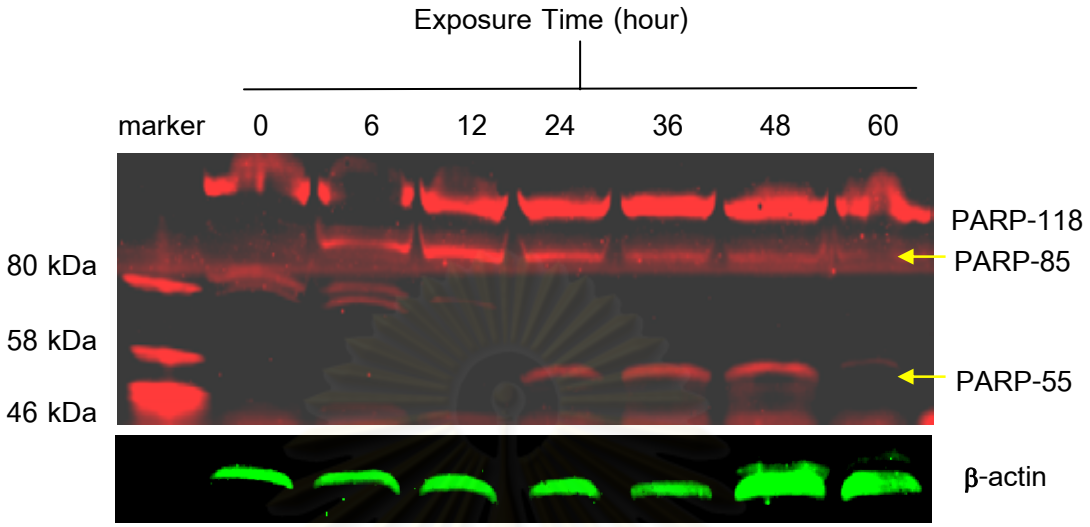

Figure 28. Effect of various exposure time of cadmium-induced PARP-1 cleavage in HepG2 cells.

Experiment was carried out as described in material and methods. Total protein was isolated. Both cleavages PARP-1 and full length PARP-1 protein level protein expression was determined by western blot analysis. Highest panel shows the full length PARP-1 (red bands, 118 kDa), second panel shows the cleavage PARP-1 (red bands; 85 kDa; represent to apoptosis), and third panel shows the cleavage PARP-1 (red bands; 55 kDa; represent to necrosis). Lowest panel show β-actin protein (green bands, 43 kDa) expression as an internal control.

3. Determination the effect of insulin in cadmium-reduced GAPDH protein expression.

Cadmium reduced GAPDH and LDHA protein expression and induced cell death. Insulin is growth factor and glycolysis regulator. This experiment was to investigate the effect of insulin on cadmium toxicity to HepG2 cells. We found insulin can prevent the effect of cadmium on reduction of GAPDH and LDHA protein expression in dose dependent (Figure 29). This result indicated that GAPDH protein expression was recovered by insulin treatment.

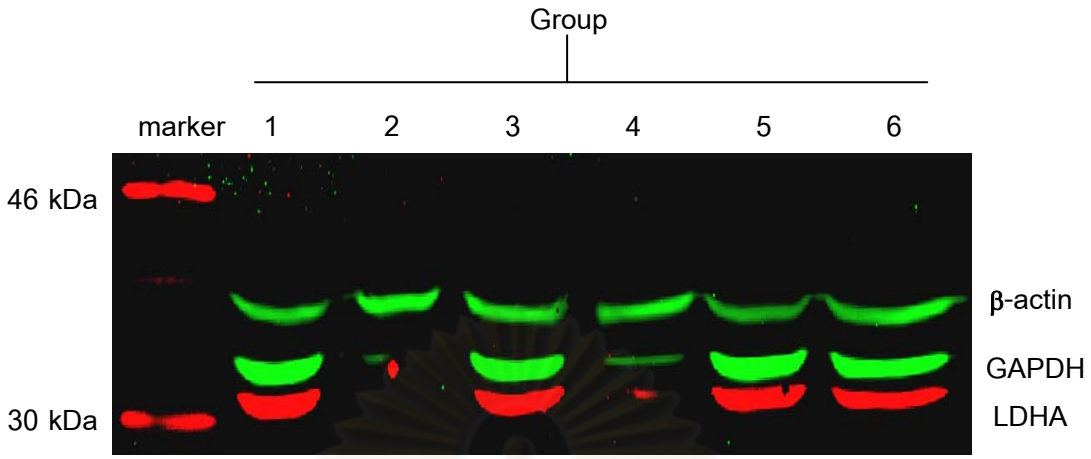

Figure 29. Effect of insulin in cadmium-reduced GAPDH protein expression.

Experiment was carried out as described in material and method. Total protein was isolated and proteins were determined by western blot analysis. Lower panel show the intracellular LDHA protein (red bands, 36 kDa) by western blot analysis. Medial panel show GAPDH protein (green bands, 38 kDa) expression by western blot analysis. Upper panel show β-actin protein (green bands, 43 kDa) expression as an internal control (N=2). (gr.1 = H_2O as control; gr.2 = cadmium 25 μM; gr.3 = insulin 20 mU/ml; gr.4 = insulin 20 mU/ml plus cadmium 25 μM; gr.5 = insulin 2,000 mU/ml; gr.6 = insulin 2,000 mU/ml plus cadmium 25 μM)

CHAPTER V
DISCUSSION

1. Cadmium decreases HepG2 cells viability

Cadmium is a widespread industrial environmental pollutant that affects adversely a number of organs in human such as kidneys, liver, and pancreas. Liver and kidneys are especially sensitive to the toxic effects of cadmium. Cadmium induced hepatic cell death depend on dose [59, 119]. The purpose of this study is to investigate the concentrations of cadmium induced HepG2 cell death. The maximum concentration of cadmium (200 μM) in this study was higher than previous studies (0-100 μM) [119, 120]. However, cadmium concentration for this time-course experiments (25 μM) was in the range of cadmium concentrations in previous studies (0-100 μM) [119, 120]. The IC_{50} of cadmium was determined by examining the HepG2 cell viability. The MTS assay was a measured of mitochondrial dehydrogenase enzymes activity in HepG2 cells. The cells viability was decreased by increasing the dose of cadmium and IC_{50} was calculated as 15 μM. The effect of cadmium on the viability of HepG2 cell has been investigated previously, Lawel and Ellis [120] indicated that the human hepatoma cell line HepG2 is the most sensitive to cadmium toxicity when compared with a human astrocytoma cell line 1321N1 and human embryonic kidney cell line HEK293. Moreover, cadmium depleted of ATP production in HepG2 cells in a dose-dependent manner. The data obtained from the present work indicate that cadmium is highly cytotoxic to human hepatoma cells (HepG2).

2. Cadmium decreases GAPDH and LDHA protein expression and increases 55 kDa PARP-1 cleavage

Cadmium induced cell death by increase of cytosolic calcium and intracellular ROS generation, but depletion of glutathione and intracellular ATP [36, 117]. In most tissues about 80-90% of glucose oxidation is via glycolytic pathway [22]. GAPDH is an important ATP-generating enzyme in the glycolytic pathway [23]. We found that cadmium decreases intracellular GAPDH protein but has no affect on *GAPDH* mRNA

expression. This result proposed that cadmium may reduce GAPDH protein translation and/or induce GAPDH protein degradation which will decrease the NADH production. Aniento *et al*. [121] postulated that the degradation mechanism of GAPDH protein is via a lysosomal pathway. The postulated pathway involves binding of GAPDH to the lysosomal membrane, entering into the lysosomal matrix, and degradation. Furthermore, the presence of higher amount of reactive oxygen species, oxidation at the cys152 of GAPDH may lead to the inhibition of GAPDH activity. The activity of GAPDH is largely affected by covalent modifications of oxidants at its highly reactive of the cys152 residue, participating in cell death [70]. Oxidation of this cysteine residue on GAPDH to sulfonic acid, the end result of which is degradation [122]. Tsuchiya *et al*. [123] studied in human leukemia U937 cell line treated with 4-hydroxy-2-nonenal (HNE), a lipid peroxidation product. Their results suggested that GAPDH modified by HNE was degraded by a chymotrypsin-like serine protease, not proteasomes, tripeptidyl peptidase II, and lysosomal enzymes. Recently, it has been reported that the cathepsin G is able to degrade HNE-modified GAPDH. Cathepsin G plays an important role in the degradation of HNE-modified proteins formed during the exposure to oxidative stress [124]. This result suggested that cadmium may reduce GAPDH protein translation and/or induce GAPDH protein degradation, resulting in the decrease of NADH production. The GAPDH protein may degrade via cathepsin pathway.

Furthermore, we found that cadmium increases PARP-1 cleavage 55 kDa related to cell necrosis. Gobeil *et al*. [125] reported that PARP-1 was cleaved to give two major active fragments 55 and 42 kDa, representing cell necrosis. Necrotic neuronal death mediated by Ca^{2+}-dependent cysteine proteases such as calpain, cathepsin [126]. PARP-1 is cleaved to 55 and 42 kDa by cathepsin G [125]. Moreover, PARP was activated by the oxidative stress. Activated PARP utilizes cytosolic NAD^+ and NAD^+ was essential in glycolysis. Therefore, when an oxidative stress induced PARP activation, several proteins such as GAPDH are poly-ADP-ribosylated but since NAD^+ continues to be depleted. One of the main consequences for such a drop in NAD^+ is an arrest of the glycolysis. Thus, when glycolysis is finally blocked, consequently with the ATP depletion,

the cell death will occurs after 8-12 hour [83]. Cadmium induces apoptosis and necrosis on yeast cells in a dose and time related with intracellular NADH level [35]. Moreover, we proposed that the decreased intracellular LDHA protein after cadmium exposure causes by the decreased LDHA expression and/or the increased LDHA secretion from HepG2 cells. Lopez at al. [36] reported that cadmium induced rat cortical neurons cell death and LDH released in a dose dependent manner. Their result also suggested a necrotic process. LDH reaction oxidizes NADH and replenishes NAD^+, which is essential for the glycolytic conversion of glucose to pyruvate [68]. LDHA is opposed to the function with GAPDH to regulate the intracellular redox stage (NAD^+/NADH).

Taken together, these findings shows the significant role of cadmium on cell necrosis, concomitant with the reduction of intracellular GAPDH and LDHA proteins, causing the alteration of the redox stage (NAD^+/NADH) in human hepatoma cells.

3. Cadmium induced histone H2B truncation.

GAPDH and LDH are the necessary components for the proper functioning of octamer binding factor 1 (Oct-1) and Oct-1 co-activator in S-phase (OCA-S), that is essential for S-phase-dependent histone H2B transcription in cells [33]. This study provides evidence that cadmium effects to histone H2B protein in HepG2 cells. We found that cadmium increased histone H2B protein truncation. Similarly, Karaczyn *et al*. [34] reported that treating cell with nickel showed time- and dose-dependent appearance of a new small band of histone H2B in a human pulmonary cell line. The small band was described as histone H2B which, in addition to being shorten at the N-terminus, have nine amino acids deleted from its C-terminus. The truncation occurs between lysine and alanine in the two identical -KAVTK- repeats of histone H2B. The truncation did not concur with apoptosis. Nickel-mediated histone H2B truncation is executed by calcium-activated proteases such as calpains. It seems possible that the truncated H2B (q-H2B) is originated from the pool of mono-ubiquitinate histone H2B; the ubiquitin tag could make histone H2B sensitive to calpain attack. Histone H2B truncation is via μ-calpain [127]. Calpain is a calcium-dependent cysteine protease. It is

distributed widely in animal cells [128]. Calpains are over activated by elevated intracellular calcium [129, 130]. There are two types of ubiquitous calpains, μ-calpain and m-calpain, which differ in their calcium ions requirements *in vitro*. μ-calpain is activated by μM levels of calcium ions, whereas m-calpain requires mM levels for activation *in vitro* [128]. Cadmium increases cytosolic free calcium concentration subsequently mediated calpain activation to cell necrosis [117, 131]. The epigenetic inheritance is found even in several organisms such as flies and yeast that lack of DNA methylation [132]. Over the past decade, it has become evident that epigenetic states can be mediated by histones, the architectural proteins that package DNA into nucleosomal particles [133]. In yeast cells, repression of histone H2B causes a cell-cycle arrest at G2 phase and reduces the number of intact nucleosomes [134]. Furthermore, in transformed human kidney cells (293 cells); apoptotic cell death was induced satisfactorily at cadmium 12.5 to 37.5 μM for 48 hours show apoptosis as confirmed by characteristic electron microscopic features and a ladder on gel electrophoresis of extracted DNA, and fragmentation of nucleosomes as detected by ELISA. Cadmium at a low concentration (below 37.5 μM) might induce apoptosis in transformed human kidney cells (293 cells) [135]. Caspases are crucial mediators of apoptosis. Caspase-3 is a frequently activated death protease, catalyzing the specific cleavage of many key cellular proteins [136]. Our result shows that caspase-3 protein expression is not changed when treated with 25 μM cadmium for 24 hours (data not show). This result indicated that HepG2 apoptosis was not induced by cadmium. On the other hand, Hamasa *et al*. [135] reported that cadmium at high concentration (above 37.5 μM) might induce transformed human kidney cells (293 cells) death through a different mechanism, perhaps necrosis. Our result shows that HepG2 cell is necrosis when treated with 25 μM cadmium for 24 hours. Similarly, Lawel and Ellis [120] reported that the human hepatoma cell line most sensitive to cadmium toxicity when compare with a human astrocytoma cell line and human embryonic kidney cell line. Moreover, the necrotic initiation may involve intracellular Ca^{2+} mobilization through the uptake of extracellular Ca^{2+} and/or release from internal stores. Ca^{2+} mobilization increases the

calpain activity, subsequently induced lysosomal rupture presumably with the aid of reactive oxygen species. The lysosomal rupture release cathepsin proteases degrade cell constitutive protein, such as core histone proteins, leading to cell death [126]. Our result shows that histone H2B truncation affects to histone H2B dysfunction when treated with 25 μM cadmium for 24 hours. These results correlate with our previous GAPDH results related with calpain-cathepsin cascade.

These findings showed the significant role of cadmium to cell necrosis from histone H2B protein truncation in human hepatoma cell and these results explain the cadmium-induced cell injury related to epigenetic mechanism.

4. Determination the effect of insulin in cadmium-reduced GAPDH protein expression.

Cadmium affects glucose metabolism by acting on a variety of different organs such as liver, and adipose tissues. Cadmium reduces insulin levels and has direct cytotoxic effects on the pancreas. Cadmium induces the elevation of fasting blood glucose levels which in fact contribute to the etiology of cadmium induces renal dysfunction. Elevated blood glucose levels couples with the direct effects of cadmium on renal tissue eventually leads to kidney dysfunction and damage [137]. Moreover, cadmium influences the biosynthesis of insulin in rat (1.0 and 2.0 mg/kg bw) [138] and cadmium (20 μM) significantly diminished the rate of insulin release [137]. Cadmium induces impaired glucose tolerance (IGT) in rats by selectively down-regulating glucose transporters type 4 (GULT4) expression in rat adipocytes [139]. Insulin hormone affects not only regulates glucose metabolism, but also activates protein synthesis, and cell growth [140, 141]. Their results indicates that cadmium can develop both type of diabetes mellitus and insulin may recover effect of cadmium-induces diabetes mellitus.

Our results show that insulin could recover cadmium-reduced GAPDH protein and LDHA protein expression. Insulin induces a maximum upregulation of the *GAPDH* mRNA level and GAPDH promoter activity is significantly increased (2.5-fold) in HepG2 cell when treated with insulin 20mU/ml for 48 hours [142]. Their results are not consistent with our results. We found that insulin has no effect on increasing GAPDH protein

expressions. We detected GAPDH protein expression, involved translation and degradation of GAPDH protein. Lemaigre *et al*. [143] detected *GAPDH* mRNA expression and GAPDH promoter activity assay. Furthermore, insulin could recover the effect of cadmium-reduced GAPDH and LDHA protein expression. The promoter site of GAPDH contains both of insulin responsive element (IRE-A and IRE-B). This is best described in the context of the insulin regulation of *GAPDH* gene transcription [143] by increase *GAPDH* mRNA transcription, may be related to upregulation of GAPDH protein levels. Moreover, insulin decrease the rate of protein degradation in skeletal muscle through a decrease in the activity of the ubiquitin proteasome-dependent pathway [144] and insulin increases the rate of protein synthesis in liver tissues [145]. In previous our results we found GAPDH protein is degraded by cadmium may be related to cathepsin G protein expression and/or activity of cathepsin G, however Jefferson *et al*. [146] reported that insulin has not any effect on total cathepsin D activity. This result may indicate that insulin recover the effect of cadmium-reduced GAPDH protein expression did not related with cathepsin D (aspartic proteases). Han *et al*. [139] reported that cadmium induces impaired glucose tolerance (IGT) and hyperglycemia in rats. Cadmium specifically and selectively down-regulates GLUT4 by reducing synthesis or stability of *GLUT4* mRNA in rat adipocytes. However, cadmium does not affect in GLUT1 expression and *GLUT1* mRNA level in the rat muscle and cadmium does not affect in GLUT2 expression and *GLUT2* mRNA levels in the rat liver. The insulin-induced GLUT4 translocation in rat adipocytes is not affected by the cadmium treatment. However, the correlation of cellular and molecular mechanisms of cadmium, insulin and glucose transporters in the liver is not clear. Thus it should be investigated in the next studies.

These findings show the significant role of insulin which can recover the effect of cadmium reduces GAPDH protein expression in HepG2 cells. These results may be related to i) increase *GAPDH* gene expression by insulin binding to insulin responsive element on GAPDH gene promoter site, ii) decrease ubiquitin proteasome-dependent pathway and/or calpain-cathepsin pathway, and iii) increase protein synthesis.

5. Cadmium decrease *Glucose-6-phosphate dehydrogenase* mRNA expression.

G-6-PD protein is an enzyme that catalyses the first reaction in the pentose phosphate pathway, in which glucose is converted into the pentose sugars required for glycolysis and for various biosynthetic reactions. G-6-PD protein is also essential in regenerating the reduced form of glutathione. Reduced form of glutathione is produced with one molecule of NADPH. The reduced form of glutathione is essential in the reduction of hydrogen peroxide and oxygen radicals [13]. *In vivo* study, cadmium induces oxidative stress in rat kidney tissue is indicated by the decreased level both of G-6-PD protein and reduced from of glutathione protein. These effects cause kidney tubular cell necrosis [147]. *In vitro* studies, cadmium increase of *G-6-PD* mRNA expression in hepatocyte culture from silver sea bream [148]. Furthermore, Xu *et al*. [149] reported that cadmium (4 μM) increases both of *G-6-PD* mRNA expression and enzyme activity in primary rat hepatocytes. We found that cadmium decreased *G-6-PD* mRNA expression in dose dependent manner. Their reports is opposed to result with our result because cadmium has hormetic effect, a biphasic dose–response phenomenon [28], the high concentrations of cadmium has cytotoxic effects by decrease *G-6-PD* mRNA expression but lower concentrations may induce a compensatory protective response by increases *G-6-PD* mRNA expression and cell type are difference.

These findings showed the significant role of cadmium on *G-6-PD* gene expression which may disturb GSH/GSSG cycle, and cause alternate of the antioxidant defense ($NADP^+$/NADPH) in human hepatoma cell. These results may be due to the fact that NADPH is a key factor in cellular antioxidation relate with glutathione pathways.

6. Cadmium decrease *NEMO* mRNA expression but not affect to NEMO protein expression.

The human *G-6-PD* gene maps to Xq28 and is arranged head to head with the *NEMO* gene [150]. *G-6-PD* gene may be a possible promoter site of *NEMO* gene. The loss of NEMO protein mediate to hepatic cell damage [151]. Furthermore, NEMO protein is essential in protecting hepatocytes from tumor necrosis factor induced cell death and NF-κB–mediated anti-apoptotic response are inhibited by the lack of NEMO protein in

hepatocytes [152]. NEMO-mediated NF-κB activation in mice hepatocytes has an essential physiological function to prevent the spontaneous development of steatohepatitis and hepatocellular carcinoma, NEMO as a tumor suppressor in the liver [153]. In treated rats, steatosis is induced by cadmium [154]. Their results indicated NEMO may reduce cadmium-steatosis.

We found cadmium decreased *NEMO* mRNA expression but not affected to NEMO protein expression. This result suggested that HepG2 cell death may related to other factors such as oxidative stress and protein thiol groups (such as GAPDH protein) but no relation with NEMO protein in HepG2 cells.

7. Conclusion

The studies presented here demonstrate the role of cadmium-induced HepG2 cells necrosis. Cadmium decreases intracellular GAPDH and LDHA protein in dose- and time-dependent manner for human hepatoma cell. In addition, cadmium induces HepG2 cells necrosis by increases histone H2B truncation in dose- and time-dependent manner on human hepatoma cell. These results indicated that cadmium induced cell necrosis related to alteration of intracellular redox state and epigenetic mechanism. Furthermore, insulin may recover cadmium-induced HepG2 toxicity. This potential therapeutic strategy will be beneficial in the treatment of cadmium toxicity.

REFERENCES

1. ATSDR. Darft Toxicological Profile for Cadmium. [Online]. 2008. Available from: http://www.atsdr.cdc.gov/toxprofiles/tp5.html [2010, April 10]
2. Jarup, L., and Akesson, A. Current status of cadmium as an environmental health problem. Toxicol Appl Pharmacol 238 (2009): 201-8.
3. Swaddiwudhipong, W., et al. Cadmium-exposed population in Mae Sot district, Tak province: 3. Associations between urinary cadmium and renal dysfunction, hypertension, diabetes, and urinary stones. J Med Assoc Thai 93 (2010): 231-8.
4. Limpatanachote, P., Swaddiwudhipong, W., Mahasakpan, P., and Krintratun, S. Cadmium-exposed population in Mae Sot District, Tak Province: 2. Prevalence of renal dysfunction in the adults. J Med Assoc Thai 92 (2009): 1345-53.
5. Dey, S., Saxena, A., Dan, A., and Swarup, D. Indian medicinal herb: a source of lead and cadmium for humans and animals. Arch Environ Occup Health 64 (2009): 164-7.
6. Satarug, S., Garrett, S.H., Sens, M.A., and Sens, D.A. Cadmium, environmental exposure, and health outcomes. Environ Health Perspect 118 (2010): 182-90.
7. Prozialeck, W.C., Edwards, J.R., and Woods, J.M. The vascular endothelium as a target of cadmium toxicity. Life Sci 79 (2006): 1493-506.
8. Klaassen, C.D., and Liu, J. Role of metallothionein in cadmium-induced hepatotoxicity and nephrotoxicity. Drug Metab Rev 29 (1997): 79-102.
9. Oh, S.H., Choi, J.E., and Lim, S.C. Protection of betulin against cadmium-induced apoptosis in hepatoma cells. Toxicology 220 (2006): 1-12.
10. Pribyl, P., Cepak, V., and Zachleder, V. Cytoskeletal alterations in interphase cells of the green alga Spirogyra decimina in response to heavy metals exposure: I. The effect of cadmium. Protoplasma 226 (2005): 231-40.
11. Prasad, R., and Nath, R. Cadmium-induced nephrotoxicity in rhesus monkeys

(Macaca mulatta) in relation to protein calorie malnutrition. Toxicology 100 (1995): 89-100.

12. Wibulpolprasert, S., et al. Health Status and Healt Problems of the Thai People. in Thailand Health Profile 1999-2000, pp.192-193. Nonthaburi: Ministry of Public Health, 2002.

13. Cappellini, M.D., and Fiorelli, G. Glucose-6-phosphate dehydrogenase deficiency. Lancet 371 (2008): 64-74.

14. Beyersmann, D., and Hechtenberg, S. Cadmium, gene regulation, and cellular signalling in mammalian cells. Toxicol Appl Pharmacol 144 (1997): 247-61.

15. Salovsky, P., Shopova, V., Dancheva, V., and Marev, R. Changes in antioxidant lung protection after single intra-tracheal cadmium acetate instillation in rats. Hum Exp Toxicol 11 (1992): 217-22.

16. Wolf, M.B., and Baynes, J.W. Cadmium and mercury cause an oxidative stress-induced endothelial dysfunction. Biometals 20 (2007): 73-81.

17. Salati, L.M., et al. Nutritional regulation of mRNA processing. J Nutr 134 (2004): 2437S-2443S.

18. Galgoczy, P., Rosenthal, A., and Platzer, M. Human-mouse comparative sequence analysis of the NEMO gene reveals an alternative promoter within the neighboring G6PD gene. Gene 271 (2001): 93-8.

19. Sebban, H., Yamaoka, S., and Courtois, G. Posttranslational modifications of NEMO and its partners in NF-kappaB signaling. Trends Cell Biol 16 (2006): 569-77.

20. Yates, L.L., and Gorecki, D.C. The nuclear factor-kappaB (NF-kappaB): from a versatile transcription factor to a ubiquitous therapeutic target. Acta Biochim Pol 53 (2006): 651-62.

21. Makris, C., Roberts, J.L., and Karin, M. The carboxyl-terminal region of IkappaB kinase gamma (IKKgamma) is required for full IKK activation. Mol Cell Biol 22 (2002): 6573-81.

22. Wamelink, M.M., Struys, E.A., and Jakobs, C. The biochemistry, metabolism and inherited defects of the pentose phosphate pathway: a review. J Inherit Metab Dis 31 (2008): 703-17.

23. Choi, D.S., Kim, Y.B., Lee, Y.H., Cha, S.H., and Sok, D.E. Glyceraldehyde-3-phosphate dehydrogenase as a biochemical marker of cytotoxicity by vinyl sulfones in cultured murine spleen lymphocytes. Cell Biol Toxicol 11 (1995): 23-8.

24. Kim, J.W., et al. Increased glyceraldehyde-3-phosphate dehydrogenase gene expression in human cervical cancers. Gynecol Oncol 71 (1998): 266-9.

25. Sugawara, C., and Sugawara, N. The effect of cadmium on soluble proteins, enzymes, and essential metals of the duodenal mucosa. Arch Environ Contam Toxicol 6 (1977): 121-8.

26. Stohs, S.J., and Bagchi, D. Oxidative mechanisms in the toxicity of metal ions. Free Radic Biol Med 18 (1995): 321-36.

27. Yu, F.X., Dai, R.P., Goh, S.R., Zheng, L., and Luo, Y. Logic of a mammalian metabolic cycle: an oscillated NAD+/NADH redox signaling regulates coordinated histone expression and S-phase progression. Cell Cycle 8 (2009): 773-9.

28. Jiang, G., et al. Biphasic effect of cadmium on cell proliferation in human embryo lung fibroblast cells and its molecular mechanism. Toxicol In Vitro 23 (2009): 973-8.

29. Hao, C., et al. The role of MAPK in the biphasic dose-response phenomenon induced by cadmium and mercury in HEK293 cells. Toxicol In Vitro 23 (2009): 660-6.

30. McKim, J.M., Jr., Liu, J., Liu, Y.P., and Klaassen, C.D. Induction of metallothionein by cadmium-metallothionein in rat liver: a proposed mechanism. Toxicol Appl Pharmacol 112 (1992): 318-23.

31. Fowler, B.A. Monitoring of human populations for early markers of cadmium toxicity: a review. Toxicol Appl Pharmacol 238 (2009): 294-300.

32. Jiang, G., et al. Effects of long-term low-dose cadmium exposure on genomic DNA methylation in human embryo lung fibroblast cells. Toxicology 244 (2008): 49-55.

33. Hara, M.R., Cascio, M.B., and Sawa, A. GAPDH as a sensor of NO stress. Biochim Biophys Acta 1762 (2006): 502-9.

34. Karaczyn, A.A., Golebiowski, F., and Kasprzak, K.S. Truncation, deamidation, and oxidation of histone H2B in cells cultured with nickel(II). Chem Res Toxicol 18 (2005): 1934-42.

35. Ju, L., Ruxiau, C., Wenlan, W., and Zhihong, L. Cell Toxicity Effect of Cadmium on Yeast Cells by Detecting NADH Autofluorescence. Wuhan University Journal of Natural Sciences 12 (2007): 333-336.

36. Lopez, E., Figueroa, S., Oset-Gasque, M.J., and Gonzalez, M.P. Apoptosis and necrosis: two distinct events induced by cadmium in cortical neurons in culture. Br J Pharmacol 138 (2003): 901-11.

37. Van Zorge, J.A. Exposure to mixtures of chemical substances: is there a need for regulations? Food Chem Toxicol 34 (1996): 1033-6.

38. Galli, C.L., and Marinovich, M. Assessment of cumulative risk of mixtures with multiple target organs. G Ital Med Lav Ergon 25 (2003): 328-9.

39. Bolt, H.M., and Mumtaz, M.M. Risk assessment of mixtures and standard setting: working towards practical compromises. Food Chem Toxicol 34 (1996): 1179-81.

40. Lambert, J.C., and Lipscomb, J.C. Mode of action as a determining factor in additivity models for chemical mixture risk assessment. Regul Toxicol Pharmacol 49 (2007): 183-94.

41. Preston, R.J. Epigenetic processes and cancer risk assessment. Mutat Res 616 (2007): 7-10.

42. Hirst, M., and Marra, M.A. Epigenetics and human disease. Int J Biochem Cell Biol 41 (2009): 136-46.

43. Muller, B., Blackburn, J., Feijoo, C., Zhao, X., and Smythe, C. DNA-activated

protein kinase functions in a newly observed S phase checkpoint that links histone mRNA abundance with DNA replication. J Cell Biol 179 (2007): 1385-98.

44. Viau, M., Gastaldo, J., Bencokova, Z., Joubert, A., and Foray, N. Cadmium inhibits non-homologous end-joining and over-activates the MRE11-dependent repair pathway. Mutat Res 654 (2008): 13-21.

45. Tang, W.Y., and Ho, S.M. Epigenetic reprogramming and imprinting in origins of disease. Rev Endocr Metab Disord 8 (2007): 173-82.

46. Caicedo, M., Jacobs, J.J., Reddy, A., and Hallab, N.J. Analysis of metal ion-induced DNA damage, apoptosis, and necrosis in human (Jurkat) T-cells demonstrates Ni2+ and V3+ are more toxic than other metals: Al3+, Be2+, Co2+, Cr3+, Cu2+, Fe3+, Mo5+, Nb5+, Zr2+. J Biomed Mater Res A 86 (2008): 905-13.

47. Lasfer, M., et al. Cadmium induces mitochondria-dependent apoptosis of normal human hepatocytes. Cell Biol Toxicol 24 (2008): 55-62.

48. Tamm, C., Sabri, F., and Ceccatelli, S. Mitochondrial-mediated apoptosis in neural stem cells exposed to manganese. Toxicol Sci 101 (2008): 310-20.

49. Au, A., et al. Nickel and vanadium metal ions induce apoptosis of T-lymphocyte Jurkat cells. J Biomed Mater Res A 79 (2006): 512-21.

50. Dawson, G., Kilkus, J., and Schieven, G.L. Selective phosphotyrosine phosphatase inhibition and increased ceramide formation is associated with B-cell death by apoptosis. FEBS Lett 478 (2000): 233-6.

51. Mehdi, M.Z., Vardatsikos, G., Pandey, S.K., and Srivastava, A.K. Involvement of insulin-like growth factor type 1 receptor and protein kinase Cdelta in bis(maltolato)oxovanadium(IV)-induced phosphorylation of protein kinase B in HepG2 cells. Biochemistry 45 (2006): 11605-15.

52. Afeseh Ngwa, H., et al. Vanadium induces dopaminergic neurotoxicity via

protein kinase Cdelta dependent oxidative signaling mechanisms: relevance to etiopathogenesis of Parkinson's disease. Toxicol Appl Pharmacol 240 (2009): 273-85.

53. Latchoumycandane, C., et al. Protein kinase Cdelta is a key downstream mediator of manganese-induced apoptosis in dopaminergic neuronal cells. J Pharmacol Exp Ther 313 (2005): 46-55.

54. Ajiro, K., Scoltock, A.B., Smith, L.K., Ashasima, M., and Cidlowski, J.A. Reciprocal epigenetic modification of histone H2B occurs in chromatin during apoptosis in vitro and in vivo. Cell Death Differ 17 (2010): 984-93.

55. Swaddiwudhipong, W., Limpatanachote, P., Mahasakpan, P., Krintratun, S., and Padungtod, C. Cadmium-exposed population in Mae Sot District, Tak Province: 1. Prevalence of high urinary cadmium levels in the adults. J Med Assoc Thai 90 (2007): 143-8.

56. Nordberg, G.F. Historical perspectives on cadmium toxicology. Toxicol Appl Pharmacol 238 (2009): 192-200.

57. Nordberg, M. Environmental exposure and preventive measures in Sweden and EU. Biometals 17 (2004): 589-92.

58. Liu, J., Qu, W., Saavedra, J.E., and Waalkes, M.P. The nitric oxide donor, O2-vinyl 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (V-PYRRO/NO), protects against cadmium-induced hepatotoxicity in mice. J Pharmacol Exp Ther 310 (2004): 18-24.

59. Zalups, R.K., and Ahmad, S. Molecular handling of cadmium in transporting epithelia. Toxicol Appl Pharmacol 186 (2003): 163-88.

60. Liu, J., Qu, W., and Kadiiska, M.B. Role of oxidative stress in cadmium toxicity and carcinogenesis. Toxicol Appl Pharmacol 238 (2009): 209-14.

61. Valko, M., et al. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 39 (2007): 44-84.

62. Karihtala, P., and Soini, Y. Reactive oxygen species and antioxidant

mechanisms in human tissues and their relation to malignancies. APMIS 115 (2007): 81-103.

63. Dowling, D.K., and Simmons, L.W. Reactive oxygen species as universal constraints in life-history evolution. Proc Biol Sci 276 (2009): 1737-45.

64. Auten, R.L., and Davis, J.M. Oxygen toxicity and reactive oxygen species: the devil is in the details. Pediatr Res 66 (2009): 121-7.

65. Kowaltowski, A.J., de Souza-Pinto, N.C., Castilho, R.F., and Vercesi, A.E. Mitochondria and reactive oxygen species. Free Radic Biol Med 47 (2009): 333-43.

66. Pourahmad, J., O'Brien, P.J., Jokar, F., and Daraei, B. Carcinogenic metal induced sites of reactive oxygen species formation in hepatocytes. Toxicol In Vitro 17 (2003): 803-10.

67. Desikan, R., Neill, S.J., Slinn, J., and Hancock, J.T. Tools to investigate reaction oxygen species-sensitive signaling proteins. Methods Mol Biol 476 (2008): 87-99.

68. Kim, J.W., and Dang, C.V. Multifaceted roles of glycolytic enzymes. Trends Biochem Sci 30 (2005): 142-50.

69. Baek, D., et al. Suppression of reactive oxygen species by glyceraldehyde-3-phosphate dehydrogenase. Phytochemistry 69 (2008): 333-8.

70. Colell, A., Green, D.R., and Ricci, J.E. Novel roles for GAPDH in cell death and carcinogenesis. Cell Death Differ 16 (2009): 1573-81.

71. Hwang, N.R., et al. Oxidative modifications of glyceraldehyde-3-phosphate dehydrogenase play a key role in its multiple cellular functions. Biochem J 423 (2009): 253-64.

72. Nakajima, H., et al. An aggregate-prone mutant of human glyceraldehyde-3-phosphate dehydrogenase augments oxidative stress-induced cell death in SH-SY5Y cells. Biochem Biophys Res Commun 390 (2009): 1066-71.

73. Sirover, M.A. New insights into an old protein: the functional diversity of

mammalian glyceraldehyde-3-phosphate dehydrogenase. Biochim Biophys Acta 1432 (1999): 159-84.

74. Dai, R.P., et al. Histone 2B (H2B) expression is confined to a proper NAD+/NADH redox status. J Biol Chem 283 (2008): 26894-901.

75. Yan, Q., et al. The NAD+ precursors, nicotinic acid and nicotinamide upregulate glyceraldehyde-3-phosphate dehydrogenase and glucose-6-phosphate dehydrogenase mRNA in Jurkat cells. Biochem Biophys Res Commun 255 (1999): 133-6.

76. Desprez, P.Y., Poujol, D., and Saez, S. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, E.C. 1.2.1.12.) gene expression in two malignant human mammary epithelial cell lines: BT-20 and MCF-7. Regulation of gene expression by 1,25-dihydroxyvitamin D3 (1,25-(OH)2D3). Cancer Lett 64 (1992): 219-24.

77. Zhu, G., et al. Fudenine, a C-terminal truncated rat homologue of mouse prominin, is blood glucose-regulated and can up-regulate the expression of GAPDH. Biochem Biophys Res Commun 281 (2001): 951-6.

78. Zou, K., and Ing, N.H. Oestradiol up-regulates oestrogen receptor, cyclophilin, and glyceraldehyde phosphate dehydrogenase mRNA concentrations in endometrium, but down-regulates them in liver. J Steroid Biochem Mol Biol 64 (1998): 231-7.

79. Xie, W., et al. Bacterial endotoxin lipopolysaccharide induces up-regulation of glyceraldehyde-3-phosphate dehydrogenase in rat liver and lungs. Life Sci 79 (2006): 1820-7.

80. Yamaji, R., et al. Hypoxia up-regulates glyceraldehyde-3-phosphate dehydrogenase in mouse brain capillary endothelial cells: involvement of Na+/Ca2+ exchanger. Biochim Biophys Acta 1593 (2003): 269-76.

81. Alexander, M.C., Lomanto, M., Nasrin, N., and Ramaika, C. Insulin stimulates glyceraldehyde-3-phosphate dehydrogenase gene expression through cis-acting DNA sequences. Proc Natl Acad Sci U S A 85 (1988): 5092-6.

82. Hazell, A.S., Desjardins, P., and Butterworth, R.F. Increased expression of glyceraldehyde-3-phosphate dehydrogenase in cultured astrocytes following exposure to manganese. Neurochem Int 35 (1999): 11-7.

83. Verrax, J., Vanbever, S., Stockis, J., Taper, H., and Calderon, P.B. Role of glycolysis inhibition and poly(ADP-ribose) polymerase activation in necrotic-like cell death caused by ascorbate/menadione-induced oxidative stress in K562 human chronic myelogenous leukemic cells. Int J Cancer 120 (2007): 1192-7.

84. Zlatanova, J., Bishop, T.C., Victor, J.M., Jackson, V., and van Holde, K. The nucleosome family: dynamic and growing. Structure 17 (2009): 160-71.

85. Marino-Ramirez, L., Kann, M.G., Shoemaker, B.A., and Landsman, D. Histone structure and nucleosome stability. Expert Rev Proteomics 2 (2005): 719-29.

86. Watson, J.D. The Nucleosome. in Molecular Biology of the Gene, pp.151-159. San Francisco: Cold Spring Harbor Laboratory Press, 2004.

87. Gunjan, A., Paik, J., and Verreault, A. Regulation of histone synthesis and nucleosome assembly. Biochimie 87 (2005): 625-35.

88. Yu, H. Epigenetics: advances of non-coding RNAs regulation in mammalian cells. Yi Chuan 31 (2009): 1077-86.

89. Munshi, A., Shafi, G., Aliya, N., and Jyothy, A. Histone modifications dictate specific biological readouts. J Genet Genomics 36 (2009): 75-88.

90. Meshi, T., Taoka, K.I., and Iwabuchi, M. Regulation of histone gene expression during the cell cycle. Plant Mol Biol 43 (2000): 643-57.

91. Imhof, A. Epigenetic regulators and histone modification. Brief Funct Genomic Proteomic 5 (2006): 222-7.

92. Kalebic, T. Epigenetic changes: potential therapeutic targets. Ann N Y Acad Sci 983 (2003): 278-85.

93. Sun, Y., and Peng, Z.L. Programmed cell death and cancer. Postgrad Med J 85 (2009): 134-40.

94. Vicencio, J.M., et al. Senescence, apoptosis or autophagy? When a damaged cell must decide its path--a mini-review. Gerontology 54 (2008): 92-9.

95. Kroemer, G., et al. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell Death Differ 16 (2009): 3-11.

96. Proskuryakov, S.Y., Konoplyannikov, A.G., and Gabai, V.L. Necrosis: a specific form of programmed cell death? Exp Cell Res 283 (2003): 1-16.

97. Zong, W.X., and Thompson, C.B. Necrotic death as a cell fate. Genes Dev 20 (2006): 1-15.

98. Galluzzi, L., and Kroemer, G. Necroptosis: a specialized pathway of programmed necrosis. Cell 135 (2008): 1161-3.

99. Amaravadi, R.K., and Thompson, C.B. The roles of therapy-induced autophagy and necrosis in cancer treatment. Clin Cancer Res 13 (2007): 7271-9.

100. Gabler, C., et al. Extranuclear detection of histones and nucleosomes in activated human lymphoblasts as an early event in apoptosis. Ann Rheum Dis 63 (2004): 1135-44.

101. Beneke, S., and Burkle, A. Poly(ADP-ribosyl)ation in mammalian ageing. Nucleic Acids Res 35 (2007): 7456-65.

102. Burkle, A. Poly(ADP-ribose). The most elaborate metabolite of NAD+. FEBS J 272 (2005): 4576-89.

103. Chaitanya, G.V., and Babu, P.P. Differential PARP cleavage: an indication of heterogeneous forms of cell death and involvement of multiple proteases in the infarct of focal cerebral ischemia in rat. Cell Mol Neurobiol 29 (2009): 563-73.

104. Erdelyi, K., Bakondi, E., Gergely, P., Szabo, C., and Virag, L. Pathophysiologic role of oxidative stress-induced poly(ADP-ribose) polymerase-1 activation: focus on cell death and transcriptional regulation. Cell Mol Life Sci 62 (2005): 751-9.

105. Casiano, C.A., Ochs, R.L., and Tan, E.M. Distinct cleavage products of nuclear

proteins in apoptosis and necrosis revealed by autoantibody probes. Cell Death Differ 5 (1998): 183-90.

106. Zhu, P., et al. The cytotoxic T lymphocyte protease granzyme A cleaves and inactivates poly(adenosine 5'-diphosphate-ribose) polymerase-1. Blood 114 (2009): 1205-16.

107. Hacker, H., and Karin, M. Regulation and function of IKK and IKK-related kinases. Sci STKE 2006 (2006): 1-19.

108. Rothwarf, D.M., and Karin, M. The NF-kappa B activation pathway: a paradigm in information transfer from membrane to nucleus. Sci STKE 1999 (1999): 1-16.

109. Aradhya, S., et al. A recurrent deletion in the ubiquitously expressed NEMO (IKK-gamma) gene accounts for the vast majority of incontinentia pigmenti mutations. Hum Mol Genet 10 (2001): 2171-9.

110. Courtois, G., and Smahi, A. NF-kappaB-related genetic diseases. Cell Death Differ 13 (2006): 843-51.

111. Xia, W., et al. Roles of NAD(+) / NADH and NADP(+) / NADPH in cell death. Curr Pharm Des 15 (2009): 12-9.

112. Ying, W. NAD+/NADH and NADP+/NADPH in cellular functions and cell death: regulation and biological consequences. Antioxid Redox Signal 10 (2008): 179-206.

113. Tangkeangsirisin, W., and Serrero, G. PC cell-derived growth factor (PCDGF/GP88, progranulin) stimulates migration, invasiveness and VEGF expression in breast cancer cells. Carcinogenesis 25 (2004): 1587-92.

114. Mabuchi, S., et al. Maintenance treatment with bevacizumab prolongs survival in an in vivo ovarian cancer model. Clin Cancer Res 14 (2008): 7781-9.

115. Svedruzic, Z.M., and Spivey, H.O. Interaction between mammalian glyceraldehyde-3-phosphate dehydrogenase and L-lactate dehydrogenase from heart and muscle. Proteins 63 (2006): 501-11.

116. Habeebu, S.S., Liu, J., and Klaassen, C.D. Cadmium-induced apoptosis in mouse liver. Toxicol Appl Pharmacol 149 (1998): 203-9.

117. Wang, S.S., Chen, L., and Xia, S.K. Cadmium is acutely toxic for murine hepatocytes: effects on intracellular free Ca(2+) homeostasis. Physiol Res 56 (2007): 193-201.

118. Tzirogiannis, K.N., et al. Time-course of cadmium-induced acute hepatotoxicity in the rat liver: the role of apoptosis. Arch Toxicol 77 (2003): 694-701.

119. Urani, C., Melchioretto, P., Canevali, C., and Crosta, G.F. Cytotoxicity and induction of protective mechanisms in HepG2 cells exposed to cadmium. Toxicol In Vitro 19 (2005): 887-92.

120. Lawal, A.O., and Ellis, E. Differential sensitivity and responsiveness of three human cell lines HepG2, 1321N1 and HEK 293 to cadmium. J Toxicol Sci 35 (2010): 465-78.

121. Aniento, F., Roche, E., Cuervo, A.M., and Knecht, E. Uptake and degradation of glyceraldehyde-3-phosphate dehydrogenase by rat liver lysosomes. J Biol Chem 268 (1993): 10463-70.

122. Findlay, V.J., Tapiero, H., and Townsend, D.M. Sulfiredoxin: a potential therapeutic agent? Biomed Pharmacother 59 (2005): 374-9.

123. Tsuchiya, Y., Yamaguchi, M., Chikuma, T., and Hojo, H. Degradation of glyceraldehyde-3-phosphate dehydrogenase triggered by 4-hydroxy-2-nonenal and 4-hydroxy-2-hexenal. Arch Biochem Biophys 438 (2005): 217-22.

124. Tsuchiya, Y., et al. 4-Hydroxy-2-nonenal-modified glyceraldehyde-3-phosphate dehydrogenase is degraded by cathepsin G. Free Radic Biol Med 43 (2007): 1604-15.

125. Gobeil, S., Boucher, C.C., Nadeau, D., and Poirier, G.G. Characterization of the necrotic cleavage of poly(ADP-ribose) polymerase (PARP-1): implication of lysosomal proteases. Cell Death Differ 8 (2001): 588-94.

126. Yamashima, T. Ca2+-dependent proteases in ischemic neuronal death: a

conserved 'calpain-cathepsin cascade' from nematodes to primates. Cell Calcium 36 (2004): 285-93.

127. Karaczyn, A.A., Golebiowski, F., and Kasprzak, K.S. Ni(II) affects ubiquitination of core histones H2B and H2A. Exp Cell Res 312 (2006): 3252-9.

128. Kumagai, K., et al. Role of mu-calpain in human decidua for recurrent miscarriage. Am J Reprod Immunol 59 (2008): 339-46.

129. Azuma, M., and Shearer, T.R. The role of calcium-activated protease calpain in experimental retinal pathology. Surv Ophthalmol 53 (2008): 150-63.

130. Kim, M.J., et al. Hypoxia-induced cell death of HepG2 cells involves a necrotic cell death mediated by calpain. Apoptosis 12 (2007): 707-18.

131. Yang, P.M., Chen, H.C., Tsai, J.S., and Lin, L.Y. Cadmium induces Ca2+-dependent necrotic cell death through calpain-triggered mitochondrial depolarization and reactive oxygen species-mediated inhibition of nuclear factor-kappaB activity. Chem Res Toxicol 20 (2007): 406-15.

132. Kundu, S., and Peterson, C.L. Role of chromatin states in transcriptional memory. Biochim Biophys Acta 1790 (2009): 445-55.

133. Henikoff, S., Furuyama, T., and Ahmad, K. Histone variants, nucleosome assembly and epigenetic inheritance. Trends Genet 20 (2004): 320-6.

134. Han, M., Chang, M., Kim, U.J., and Grunstein, M. Histone H2B repression causes cell-cycle-specific arrest in yeast: effects on chromosomal segregation, replication, and transcription. Cell 48 (1987): 589-97.

135. Hamada, T., et al. Apoptosis of human kidney 293 cells is promoted by polymerized cadmium-metallothionein. Biochem Biophys Res Commun 219 (1996): 829-34.

136. Porter, A.G., and Janicke, R.U. Emerging roles of caspase-3 in apoptosis. Cell Death Differ 6 (1999): 99-104.

137. Edwards, J.R., and Prozialeck, W.C. Cadmium, diabetes and chronic kidney disease. Toxicol Appl Pharmacol 238 (2009): 289-93.

138. Lei, L.J., Jin, T.Y., and Zhou, Y.F. Insulin expression in rats exposed to cadmium.

Biomed Environ Sci 20 (2007): 295-301.

139. Han, J.C., et al. Cadmium induces impaired glucose tolerance in rat by down-regulating GLUT4 expression in adipocytes. Arch Biochem Biophys 413 (2003): 213-20.

140. Hyakukoku, M., et al. Tissue-specific impairment of insulin signaling in vasculature and skeletal muscle of fructose-fed rats. Hypertens Res 26 (2003): 169-76.

141. Papagianni, M., et al. Insulin causes a transient induction of proliferation via activation of the PI3-kinase pathway in airway smooth muscle cells. Exp Clin Endocrinol Diabetes 115 (2007): 118-23.

142. Claeyssens, S., et al. Amino acid control of the human glyceraldehyde 3-phosphate dehydrogenase gene transcription in hepatocyte. Am J Physiol Gastrointest Liver Physiol 285 (2003): G840-9.

143. Lemaigre, F.P., and Rousseau, G.G. Transcriptional control of genes that regulate glycolysis and gluconeogenesis in adult liver. Biochem J 303 (1994): 1-14.

144. Prod'homme, M., Rieu, I., Balage, M., Dardevet, D., and Grizard, J. Insulin and amino acids both strongly participate to the regulation of protein metabolism. Curr Opin Clin Nutr Metab Care 7 (2004): 71-7.

145. Newsholme, E.A., and Dimitriadis, G. Integration of biochemical and physiologic effects of insulin on glucose metabolism. Exp Clin Endocrinol Diabetes 109 Suppl 2 (2001): S122-34.

146. Jefferson, L.S., Li, J.B., and Rannels, S.R. Regulation by insulin of amino acid release and protein turnover in the perfused rat hemicorpus. J Biol Chem 252 (1977): 1476-83.

147. Renugadevi, J., and Milton Prabu, S. Quercetin protects against oxidative stress-related renal dysfunction by cadmium in rats. Exp Toxicol Pathol (2009).

148. Man, A.K., and Woo, N.Y. Upregulation of metallothionein and glucose-6-

phosphate dehydrogenase expression in silver sea bream, Sparus sarba exposed to sublethal levels of cadmium. Aquat Toxicol 89 (2008): 214-21.

149. Xu, J., Maki, D., and Stapleton, S.R. Mediation of cadmium-induced oxidative damage and glucose-6-phosphate dehydrogenase expression through glutathione depletion. J Biochem Mol Toxicol 17 (2003): 67-75.

150. Fusco, F., Mercadante, V., Miano, M.G., and Ursini, M.V. Multiple regulatory regions and tissue-specific transcription initiation mediate the expression of NEMO/IKKgamma gene. Gene 383 (2006): 99-107.

151. Seki, E., and Brenner, D.A. The role of NF-kappaB in hepatocarcinogenesis: promoter or suppressor? J Hepatol 47 (2007): 307-9.

152. Beraza, N., et al. Hepatocyte-specific IKK gamma/NEMO expression determines the degree of liver injury. Gastroenterology 132 (2007): 2504-17.

153. Luedde, T., et al. Deletion of NEMO/IKKgamma in liver parenchymal cells causes steatohepatitis and hepatocellular carcinoma. Cancer Cell 11 (2007): 119-32.

154. Jeyaprakash, K., and Chinnaswamy, P. Effect of spirulina and Liv-52 on cadmium induced toxicity in albino rats. Indian J Exp Biol 43 (2005): 773-81.

VITAE

Chatkul Techakitiroj

Personal Data

Date of Birth	June 28, 1972
Place of Birth	Bangkok, Thailand
Gender	Male

Address

88 M.18 Suwinthawong Road, Minburee, Bangkok 10510, Thailand.

Education

1985-1987	Debsirin High School, Bangkok, Thailand.
1988-1990	Amnuay Silapa High School, Bangkok, Thailand.
1991-1996	Bachelor of Science in Pharmacy, Faculty of Pharmaceutical sciences, Prince of Songkla University, Songkhla, Thailand.
2001-2004	Master of Science in Pharmacy (Pharmacology), Department of Pharmacology, Faculty of Pharmaceutical sciences, Chulalongkorn University.
2007	Certificate of International Workshop on Toxicology towards Diplomate of American Board of Toxicology (DABT).

Experiences

1996-1998	Hospital Pharmacist, Sriratana Hospital, Srisakate Thailand
1998-now	Regulatory Pharmacist, Food and Drug Administration, Ministry of Public Health, Thailand