THE ROLE OF LIVER X RECEPTORS (LXRs) IN CADMIUM-INDUCED NEPHROTOXICITY

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ABSTRACT

Liver X receptors (LXRs) including LXRα and LXRβ are members of the nuclear receptor superfamily of ligand-activated transcription factors, which are normally expressed in high metabolic organs such as liver, adipose tissues, and kidney. Recent evidence demonstrated that LXRs have anti-inflammatory and antioxidant effects in tissues of several organs induced by oxidative agents. Cadmium-induced cell death in several cell types is mediated by reactive oxidative species (ROS) generation and JNK activation. Therefore, this study was aimed to investigate the effects of LXR activation and the underlying mechanisms on the cadmium-induced cell death in human renal proximal tubular cells. The result showed that treatment of HK-2 cells with 20 μM $CdCl_2$ for 24 hours led to cell death via apoptosis but not necrosis. Interestingly, pretreatment of HK-2 cells with T0901317, an LXR agonist, significantly inhibited the apoptotic cell death induced by $CdCl_2$. The protective effect of T0901317 was eliminated by coincubation with fenofibrate, an LXR antagonist, indicating that the effect of T0901317 on cadmium-induced apoptotic cell death was mediated by LXR activation. In addition, the effect of $CdCl_2$ was attenuated by a reactive oxygen species (ROS) scavenger, N-acetyl-L-cysteine (NAC). It was found that an increase in ROS induced by $CdCl_2$ was mediated by inhibition of catalase (CAT) but not superoxide dismutase (SOD) which was attenuated by T0901317. In addition, the effect of $CdCl_2$ on cell death was involved the up-regulated NAD(P)H dehydrogenase/ NADH: quinone oxidoreductase-1 (NQO1) expression which was blocked by NAC. This indicated that NQO1 induction by cadmium was downstream of ROS generation. Western blot analysis revealed that $CdCl_2$ stimulated expression of c-jun N-terminal kinase (JNK) phosphorylation. The stimulation was inhibited by NAC, indicating the induction of JNK phosphorylation was downstream of ROS production. Moreover, the increases of ROS and JNK phosphorylation induced by $CdCl_2$ were attenuated by LXR activation.

In conclusion, this study provided the first evidence showing that LXR activation could reduce cadmium-induced apoptotic cell death of human renal proximal tubular cells by inhibition of ROS production and JNK activation.

KEY WORD: CADMIUM / LIVER X RECEPTORS / REACTIVE OXIDATIVE SPECIES / JNK SIGNALING / CATALASE /

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บทบาทของนิวเคลียร์รีเซพเตอร์ชนิดแอลเอกซ์อาร์ต่อการเกิดพิษของไตที่ถูกเหนี่ยวนำโดยสารแคดเมียม
THE ROLE OF LIVER X RECEPTORS (LXRs) IN CADMIUM-INDUCED NEPHROTOXICITY

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บทคัดย่อ

นิวเคลียร์รีเซพเตอร์ชนิดแอลเอกซ์อาร์ (LXRs) ซึ่งเป็นสมาชิกของกลุ่มสารนิวเคลียร์รีเซพเตอร์ที่ควบคุมกระบวนการเมตาบอลิซึมของเซลล์ ประกอบด้วยสมาชิก 2 ชนิดได้แก่ อัลฟาและเบต้า โดยพบมากในอวัยวะที่มีเมตาบอลิซึมสูงเช่น ตับ เนื้อเยื่อไขมันและไต ปัจจุบันพบว่านิวเคลียร์รีเซพเตอร์ชนิดนี้มีบทบาทที่เกี่ยวข้องกับการต่อต้านการอักเสบและต้านการเกิดอนุมูลอิสสระในเนื้อเยื่อของหลายอวัยวะ ในโรคไตที่เกิดจากพิษของสารแคดเมียมพบว่าแคดเมียมกระตุ้นการสร้างอนุมูลอิสระ (ROS) และ กระตุ้นการทำงานของโปรตีน JNK (JNK phosphorylation) ซึ่งจะทำให้เกิดการตายของเนื้อเยื่อ ดังนั้นในการศึกษาครั้งนี้จึงได้ทำการทดสอบบทบาทและกลไกการทำงานของแอลเอกซ์อาร์ ต่อการเกิดพิษของสารแคดเมียมในเนื้อเยื่อของเซลล์หลอดไตส่วนต้น จากการศึกษาการทำเกิดพิษของสารแคดเมียมต่อเซลล์ (cytotoxicity) โดยวิธี MTT assay และตรวจยืนยันด้วยวิธี flow cytometry พบว่าการเกิดพิษของสารแคดเมียมทำให้เซลล์ตายแบบ apoptosis แต่ไม่เกิดการตายแบบ necrosis และพบว่าการเกิดพิษนี้ถูกยับยั้งด้วยสารกระตุ้นแอลเอกซ์อาร์รีเซพเตอร์ T0901317 เมื่อเทียบกับกลุ่มควบคุม การป้องกันการเกิดพิษจากสารแคดเมียมของ T0901317 นี้จะถูกยับยั้งด้วยสาร fenofibrate ซึ่งมีฤทธิ์ในการต้านการทำงานของนิวเคลียร์รีเซพเตอร์ชนิดแอลเอกซ์อาร์ จึงแสดงว่าฤทธิ์ของ T0901317 ในการป้องกันการเกิดพิษของสารแคดเมียมอาศัยการกระตุ้นแอลเอกซ์อาร์ นอกจากนี้พบว่าสารแคดเมียมสามารถกระตุ้นการแสดงออกของโปรตีน p-JNK และถูกยับยั้งด้วยสาร N-acetyl-L-cysteine (NAC) ดังนั้นแสดงว่าการกระตุ้นการทำงานของ JNK โดยสารแคดเมียมเป็นผลจากการสร้าง ROS และพบว่าการยับยั้งการสร้าง ROS โดยสาร T0901317 เป็นผลมาจาก T0901317 ไปกระตุ้นการแสดงออกของเอนไซม์ catalase และยังพบว่า T0901317 สามารถลดการการกระตุ้นการแสดงออกของโปรตีน p-JNK ได้โดยตรง ดังนั้นจากการศึกษาจึงสรุปได้ว่าการกระตุ้นแอลเอกซ์อาร์รีเซพเตอร์สามารถลดการเกิดพิษของสารแคดเมียมต่อเซลล์ไตโดย 1) ลดการสร้าง ROS โดยกระตุ้นการแสดงออกของ CAT 2) ยับยั้งการแสดงออกของโปรตีน p-JNK และผลการศึกษาครั้งนี้เป็นการรายงานครั้งแรกที่แสดงให้เห็นถึงบทบาทแอลเอกซ์อาร์รีเซพเตอร์ต่อการพิษของไตที่ถูกเหนี่ยวนำโดยสาร โลหะหนัก อันจะเป็นแนวทางหนึ่งในการพัฒนา การรักษาพิษของสารแคดเมียมต่อไตได้ในอนาคต

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LIST OF ABBREVIATIONS

%	percentage
AA	atomic absorption
ANOVA	analysis of variance
BSA	bovine serum albumin
^{o}C	degree celsius
CAT	catalase
$CdCl_2$	cadmium chloride
cDNA	complementary deoxyribonucleic acid
CO_2	carbondioxide
dNTP	mixed deoxynucleotide
DMT-1	divalent metal transporter 1
DMSO	dimethyl sulfoxide
ERK1/2	dimethyl sulfoxide
FXRs	farnesoid x receptors
g	gram
GSH	reduced glutathione
HK-2	human proximal tubular cell
HCl	hydrochloric acid
H_2O_2	hydrogen peroxide
JNK	c-jun N-terminal kinase
KCl	potassium chloride
KH_2PO_4	potassium dihydrogen phosphate
LLC-PK1	porcine renal cells
LXRs	liver x receptors
MAPK	mitogen-actived protein kinase
$MgSO_4.7\ H_2O$	magnesium sulfate
min	Minute

LIST OF ABBREVIATION (cont.)

MT	metallothionein
mM	milimolar
μM	micromolar
mRNA	messenger ribonucleic acid
NAC	N-acetyl-L-cysteine
NaCl	sodium chloride
Na_2CO_3	sodium dicarbonate
$Na_2HPO_4H_2O$	sodium dihydrogen phosphate monohydrate
Na_2HPO_4	disidium hydrogen phosphate
NaOH	sodium hydroxide
NF-kB	nuclear factor kappa-light-chain-enhancer of activate B
NQO1	NAD(P)H dehydrogenase: quinone oxidoreductase-1
O_2	oxygen
O_2^{o-}	supper oxide
O.D.	optical density
^{-}OH	hydroxyl radical
PCR	polymerase chain reaction
PPARs	Peroxisome-priferator-activated receptors
PT	proximal tubules
PXRs	pregnane x receptors
22S-HC	22-(S)-hydroxycholesterol
rpm	revolution per minute
ROS	reactive oxygen species
RXRs	retinoic x receptors

LIST OFABBREVIATION (cont.)

SE	standard error
sec	second
SEM	standard error of mean
TEA	tetraethylammonium

CHAPTER I
INTRODUCTION

Cadmium (Cd) is a toxic heavy metal by which its biological function is not known. It is an environmental pollutant, which is accompanied by cadmium-related industry, contaminating to water, plants, and food stuffs, entering the food chain and eventually accumulating in animal and human body. Cadmium has a very long biological half-life, causing a cumulative toxic effect in various organs including liver, pancreas, testis and kidney, especially renal proximal tubular cells (1). It has been well established that chronic exposure to cadmium causes irreversible kidney damage and renal tubular dysfunction both *in vivo* and *in vitro* (2, 3). However, the exact molecular mechanisms underlying cadmium-induced nephrotoxicity and renal protective strategies have not been established.

As entering into the blood circulation, Cd binds to albumin, binding- low molecular weight proteins containing thiol groups or sulfhydryl molecules such as glutathione (GSH) or cysteine (Cys), and is distributed in the body but primarily accumulated in the liver where the production of detoxifying metal-binding proteins, metallothionein (MT), is induced. In the liver, Cd is found mainly as a complex binding with high affinity to MT (Cd-MT complexes). However, Cd in the liver may also be excreted with bile as a Cd-glutathione complex. After the Cd-MT complexes is formed, it is released into circulation and then is distributed to the kidney (4) and filtered in the glomerulus due to the small molecular weight of Cd-MT (~6-7 kDa) and may be reabsorbed by renal proximal tubular S1 ans S2 segments. Following glomerular filtration, the Cd-MT complexs are taken up by tubular endocytosis in the renal proximal tubular cells (PT) that involves receptor-mediated endocytosis via megalin/cubulin-medicated transports (5-7) which are highly expressed in the apical membrane of proximal tubule cells. In proximal tubular cells, Cd may be liberated from the MT moiety in late endosomes/ lysosomes producing free Cd in cytosol. The primary target of Cd-induced nephrotoxicity is the S1 segment of the proximal tubular because it is the first opportunistic site of reabsorption following filtration from the

glomerulus. In addition, proximal tubular cells of the S1 segment are abundant in a variety of transporters, metabolising enzymes and receptors that are essential for Cd uptake into the cells (8-10).

Cd-MT complexes are mainly taken up at apical membrane of renal proximal tubule cells via receptor-mediated endocytosis, whereas the exact uptake pathways for free Cd or Cd-thiol molecule complexes such as Cd-N-acetylcysteine or Cd-glutathione are not established. Possible mechanisms for free Cd may be ionic mimicry by cationic transporters including divalent metal transporter 1 (DMT1) which plays a important role in iron (Fe^{2+}) homeostasis and mediates transport of essential and toxic divalent metal ions, such as Zn^{2+}, Cu^{2+}, Mn^{2+}, Pb^{2+} and Cd^{2+}, or ZIP8 transporter regulating zinc and iron transports (11, 12), whereas Cd-N-acetylcysteine or Cd-glutathione may enter the cell by molecular mimicry via organic anion transporters such as OAT1 and OAT3(13, 14). Recent study, Soodvilai et al. have reported that renal organic cation transporters (OCTs) mediate cadmium transport across the basolateral membrane into the renal proximal tubular cells (15). Therefore, these transports, mentioned above, have been proposed to account for intracellular cadmium accumulation and contribution of cadmium-induced nephrotoxicity.

It has been reported that the Cd produces a variety of relatively non-specific toxic effects that result in the death of renal epithelial cells via necrotic or apoptotic mechanisms. However, low cadmium exposure (~5-20 μM) has shown predominated apoptotic cell death in S1 or S2 segment of a rat proximal tubule cell line (16). Several studies have shown mechanisms of cadmium-induced cell death including mitochondrial induction of ROS via complex I and complex III of electron transport chain (17, 18), increase of intracellular Ca^{2+} (19), alterations of sphingolipid metabolism (20), and depletion of ROS scavengers such as glutathione (21). In addition to inducing intracellular ROS generation, cadmium toxicity has been shown to activate phosphorylation of c-jun N-terminal kinase (p-JNK), a stress-activated protein kinase (SAPK), leading to apoptotic cell death (22, 23). All these mechanisms by which cadmium-induced toxicity occurs in renal proximal tubular cells have been required to find potential targets that mediate protective effects on the impaired renal

function induced by cadmium. Nowadays, the exact strategic treatment of cadmium toxicity has not been established.

Liver X receptors (LXRs) including LXRα and LXRβ isoforms are members of the nuclear receptor family of ligand-activated transcription factors (24, 25) that regulate the expression of genes involved cholesterol and fatty acid metabolism and are also emerging as key modulators of inflammation. Although both LXRα and LXRβ isoforns share almost 80 % structural homology, there is a much greater difference in their expression patterns. LXRα is highly expressed, mainly in the liver, adrenal glands, adipose tissue, intestine, kidney, and macrophages, while LXRβ is found in various tissues at a lower level (26, 27). LXRs regulate gene expression by forming heterodimers with the retinoid x receptor (RXR) and subsequently binding to LXR responsive elements (LXRE) of target gene promoters stimulating or inhibiting their target genes. These target genes of LXRs include ATP-binding cassette transporters (ABCA1, ABCG5, ABCG8), fatty acid synthase (FAS), and SREBP-1c, suggesting that LXRs play a crucial role in lipid and cholesterol metabolism. The synthetic LXR agonist such as T0901317 and GW 3965 can attenuated LPS inducing liver injury in a murine model of non-alcoholic fatty liver disease (NAFLD) via TNFα and iNOS expression through inhibiting JNK and the PI3K signaling pathway (28). T0901317 also alleviated ROS release induced by high glucose in endothelial progenitor cells (29). In addition, LXR agonist shows upreglation of antioxidant enzyme such as glutathione *S*-transferases (GST), superoxide dismutases (SOD), catalase (CAT) and metallothionin (MT) in lung tissue of mice induced by lipopolysaccharide (LPS) (30). Therefore, LXRs' effects on anti-inflammation processes and antioxidant make them attractive as potential targets for the treatment of cadmium-induced renal cell damage.

However, little is known about the role of LXR activation in renal cells except to stimulate lipid metabolis. Thus, this study aims to explore the effects of LXR activation on cadmium induced-cytoxicity in renal tubular cells. The involvement of LXRs in mediators of cell cytotoxicity including ROS generation and JNK signaling protein are investigated. The main objective of this study was to investigate the effects of LXR activation and underlying mechanisms on the cadmium–induced cytotoxicity in renal proximal tubular cells. In order to achieve this

objective, the following steps were performed: 1) determination of the effect of LXR agonists on cadmium-induced cytotoxicity; 2) determination of the mechanism of LXR agonists on cadmium-induced cytotoxicity, including reduction of ROS, and suppression of JNK signaling. The results obtained from this study revealed that the activation of LXR alleviates cadmium-induced nephrotoxicity, and this finding may provide a new insight and therapeutic targets for treatment of cadmium-induced nephrotoxicity.

CHAPTER II
LITERATURE REVIEW

2.1 Cadmium Toxicity

Cadmium (Cd) is a divalent heavy metal. It is usually found in form of inorganic salts including cadmium oxide, (CdO), cadmium chloride, ($CdCl_2$), or cadmium sulfate, ($CdSO_4$). Cd commonly used in cadmium-related industry including battery manufacturing, pigments, and plastic stabilizers, applications in alloys, and electroplating (31). It is released to an environment and contaminating in foodstuff. Its accumulation in the body can damage and cause renal tubular cell dysfunction which lead to in proteinuria, aminoaciduria and glucosuria (32). Humans are exposed to Cd via an ingestion of contaminated food or water and the inhalation of cigarette smoke or cadmium fume of work-place in cadmium-related industry. Major sources of dietary cadmium are fish, liver, grains, leafy vegetables, potatoes, and other root vegetables.

2.1.1 Cadmium absorption and distribution

Absorption and distribution of cadmium (Cd) in the body occur after exposure to cadmium by two major routes including respiratory and gastrointestinal (GI) tracts. With inhalation exposure, Cd that is absorbed from the lung approximately 40-60% of inhaled Cd got into the blood circulation (33, 34). For oral exposure, the absorption of Cd from the gastrointestinal tract is about 5-10 % of cadmium-contaminated diet (35). However, chronic exposure to low level of absorbed Cd in the gastrointestinal tract can lead to accumulation of Cd in target organs which subsequently cause cadmium toxicities (36). Following its absorption into the circulation via portal blood, Cd binds with low affinity to albumin, amino acids or the sulfhydryl compounds such as glutathione (GSH) or cysteine and is taken up into the liver where it induces the synthesis of metallothionein (MT) which binds Cd and neutralizes its toxic effects in the liver cell. In the liver, Cd, which binds

metallothionein with high affinity to form Cd-metallothionein complex (Cd-MT), can be released into the blood circulation, and may also be excreted into bile as a Cd-glutathione complex (37) or may be loosely bound with other proteins and thiol-containing molecules, such as albumin, glutathione, and cysteine (38, 39). The Cd-metallothionein (MT) complex is released from injured hepatocytes into the bloodstream (36, 37). This Cd-MT complex can be filtered at the glomeruli and taken up by the epithelial cells of the proximal tubule causing kidney injury and damage (Figure 2.1).

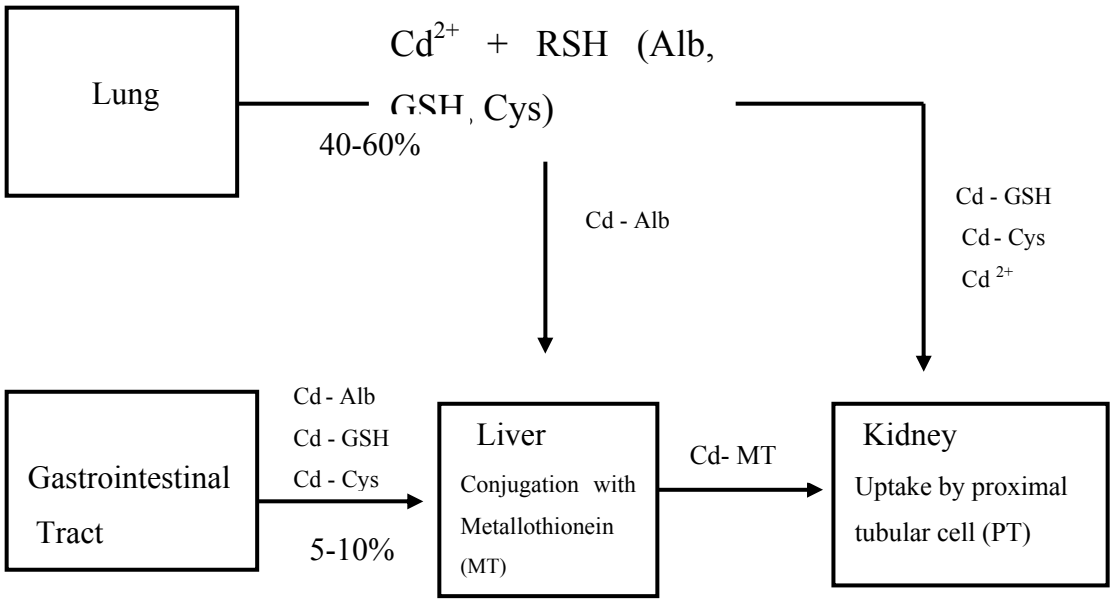


Figure 2.1 Diagram of cadmium absorption and distribution in the body. In plasma, Cd binds to proteins and low molecular weight thiols (RSH: Alb, GSH, Cys) that deliver Cd in the form of Cd-Albumin to the liver, whereas Cd in form of Cd-GSH, Cd-Cys or Cd^{2+} is delivered to the kidney and filtered at the glomreruli. In the liver, Cd binds metallothionein (Cd-MT) that is filtered at the glomeruli and then taken up by epithelial cells of the proximal tubule. In gastrointestinal tract (GI), $Cd2^{+}$ also binds to proteins and low molecular weight thiols (RSH: Alb, GSH, Cys) that delive in the form of Cd-complexs to the liver.

2.1.2 Transport of cadmium into the cells

After Cd is absorbed through gastrointestinal tract (GI) or lung into systemic circulation and then distributed into various tissues including liver, it binds various polypeptides or non-peptide thiols such as metallothionein (MT), reduced glutathione (GSH) and other thiol-proteins. The complexes of cadmium-metallothionein (Cd–MT), cadmium-glutathione (GS–Cd–SG), and cadmium-cysteine (Cys–S–Cd–S–Cys) in blood circulation are transported to targeted organs and then Cd is deposited primarily in liver and especially in the kidney. The kidney is recognized as a critical target organ of cadmium-induced toxicity. Thus, the document of cadmium transport in the kidney will give more understanding of cadmium-induced nephrotoxicity. In previous studies, the types of transport proteins have been identified that they play a pivotal role in renal cadmium accumulation leading to cadmium-induced cell apoptosis. These transport proteins include metallothioneins (MTs), zinc transporters (ZIP8), divalent metal-ion transporter-1 (DMT1), calcium transporter, and organic cation transporters (OCTs).

2.1.2.1 Metallothioneins (MTs)

Metallothioneins (MTs) are cysteine-abundant proteins with low molecular weights (MW) approximately 6 - 7 kDa, and act as metal-binding proteins. MTs consist of single chain polypeptides with 61-68 amino acids that contain 20 cysteine residues in a reduced form. Because of the abundant sulhydryl groups in their cysteine residues, leading to high affinity to bind with divalent metal cations including Zn^{2+}, Mn^{2+}, Co^{2+}, Pb^{2+}, $Cd^{2+,}$ and Cu^{2+}. MTs are known to play an important role not only in the homeostasis of physiological metal ions, such as zinc, copper, and selenium, but also in prevention from the toxicity of heavy metals, such as cadmium, mercury, lead, and arsenic. After exposure to cadmium, it is absorbed via respiratory and gastrointestinal tracts. In blood circulation, most cadmium molecules are formed to albumin and the metal-albumin complex is mainly taken up by the liver (Figure 2.1). In the liver, cadmium stimulates induction of metallothionein (MTs) synthesis and then it is bound to MTs as a defence mechanism to sequester toxic cadmium. Cadmium-metallothionein (Cd–MT) complex is then secreted from liver to the blood circulation and subsequently distributed to various tissues, especially kidney. Because of the small size of Cd-MT complex, it is readily filtered by the glomerulus and

reabsorbed by the renal proximal tubular (PT) epithelial cells in a process that involves megalin/cubilin-medicated transporter at the apical side of PT (37, 40) via endocytosis (41, 42). The evidence of cadmium delivery from the liver to the kidney was demonstrated by Chan and colleagues. They found that the rats were treated with cadmium chloride (3 mg/kg/day) for two weeks and the livers of cadmium-treated rats were transplanted to the control group, showing the levels of cadmium and MTs in the renal tissues of the transplanted rats increased significantly, whereas those in the liver decreased in a time-dependent manner (43). In addition, the involvement of MTs in renal cadmium accumulation has been reported by Liu and colleagues, demonstrated that the cadmium concentration in the kidney of MT-I/II knockout mice was decreased to compare with that of wild-type mice after treatment with cadmium chloride for six months (44). Moreover, Dorian and colleagues have reported that the Cd-MT complex was accumulated in the rat kidney after intravenous injection for fifteen minutes, and this finding showed that the Cd-MT complex was highly taken up into S1 and S2 segment of proximal tubules (45). After this complex get into the renal proximal tubular cells, it is rapidly degraded by lysosome. The free-cadmium is released to cytosolic compartment, and then bound to newly synthesized MTs (46). This binding of cadmium to MTs is a protective mechanism that is saturable at high concentration of cadmium for which subsequent renal toxicity may be occured. The data were mentioned above, suggesting that metallothioneines play a crucial role in cadmium accumulation in the kidney. It is implied that the role of metallothioneines supports cadmium-induced nephrotoxicity. Althougt, the previous study showed that cadmium accumulation in the kidney of MT-I/II knockout mice was less than ten percent that of the wide-type controls, the knockout mice were more susceptible to the cadmium-induced nephrotoxicity (44). However, metallothioneines have been still recognized as protective molecule rather than the contributor in cadmium-induced nephrotoxicity.

2.1.2.2 Calcium Transporters

Another route of cadmium get into the cell is the calcium transporter that is also an essential transporter for the body. The study of Wang and colleagues found that increasing calcium in the luminal side of renal tubules could decrease cadmium accumulation in rabbit tubular cells, suggesting the involvement of calcium transporters in renal cadmium uptake (47). The transient receptor potential

(TRP) channels are ubiquitously expressed in many tissues including intestine, kidney, and placenta. These channels are established as the major routes for calcium transport in the cells (48). The both types of transient receptor potential (TRP) channels, TRPV6 and TRPV5, are an important role in calcium absorption and re absorption. TRPV6 mainly expressed in duodenum and placenta, while TRPV5 is expressed in the distal convoluted tubules and the connected tubules of the kidney. In recent study, Kovacs et al. demonstrated that the intracellular accumulation of both calcium and cadmium increased significantly in the HEK293 cells transfected with TRPV6 as compared to the parent cell line, and the transport of calcium could be decreased by coincubation with cadmium. This finding suggested the competition between calcium and cadmium ions for the same transport system (49). Moreover, cadmium accumulation in cells over expressing human TRPV6 could be inhibited by2-aminoethoxydiphenyl borate (2-APB), the non-specific human TRPV6 inhibitor, indicating that cadmium uptake is mediated by TRPV6. In addition, the uptake of cadmium in human TRPV5-transfected cell was found to increase cytotoxicity (48). Taken together, calcium transporters or channels could mediate the cadmium accumulation leading to cell damage.

2.1.2.3 Divalent metal-ion transporter-1 (DMT1)

Divalent metal-ion transporter-1 (DMT1), also called SLC11A2, is a transporter mediating transport of divalent metal cations, such as Zn^{2+}, Mn^{2+}, Co^{2+}, Pb^{2+}, $Cd^{2+,}$ and Cu^{2+}.Since it highly expresses in proximal tubule S3 segment, distal convoluted tubule, and connected ducts (Figure 2.2), the role of DMT1 in renal accumulation of cadmium has been interested. The study of Barbier et al. by using microinjection technique found that accumulation of cadmium in distal tubule cells might involves the role of DMT1 (50). In addition, mouse embryonic cells that resist to cadmium-induced toxicity showed a significant decrease in the expression of DMT1 (51), and it was also reported that DMT1 had a higher affinity for Cd^{2+} than Fe^{2+} (52). Moreover, it has been reported that cadmium accumulation in mouse proximal tubule cells is significantly decreased following DMT1 knockdown by siRNA (53). As mention above, these result suggested that DMT1 had an important role in cadmium accumulation in the renal proximal tubular cell.

2.1.2.4 Zinc transporters (ZIP8)

Cadmium is a toxic heavy metal which its physiological function in the body has not been known, and it shows no a specific substrate to any metal-mediated transporters in the body. It is also recognized that cadmium can compete with other metals in entering into cell. Among metal-mediated transporters, zinc transporter by which it has the same divalent cation as cadmium obtained the most attention. Zinc/Iron-regulated transporters, also called ZIP proteins which were identified from the root plant that expresses an iron-deficiency, have been shown to play a pivotal role in zinc and iron transport across the cellular membrane in the intestine and renal proximal tubules (54) (Figure 2.2). Fujishiro and colleagues demonstrated that cadmium uptake in ZIP8 and ZIP14-knockdown mouse was significantly reduced on the apical side of renal proximal tubular cells. In addition, it was also found that the ZIP8-transfected HEK cell line protect cadmium-induced toxicity by reducing its uptake with knockdown of ZIP8 (55). Moreover, the cadmium transport has examinned in rat basophilic leukemia (RBL-2H3) cells which highly expressed both ZIP8 and ZIP14 protein (56, 57), and it was found that ZIP8 plays an more important role since the uptake of cadmium was increased following knockdown of ZIP14 by siRNA. The study of Barbier and colleagues (58) gave a more details in the role of zinc transporters on cadmium accumulation under physiological conditions by using the nephron microinjection technique. They showed that intracellular accumulation cadmium in distal convoluted tubules (DCT) could be significantly inhibited by co-injection with zinc ion, indicating that the cadmium uptake in distal convoluted tubules (DCT) could be mediated by zinc transporters. However, the inhibition effect of co-injection with with zinc ion on accumulation cadmium was not observed in proximal tubules (PT). Although DCT and PT highly express zinc transporters, cadmium may have a lower affinity for zinc transporters in DCT segments, the uptake of which could not be competatived by zinc ions. However, cadmium uptake which was mediated by zinc transporters has been demonstred by *in vitro* studies, the data of it *in vivo* studies are needed to investigate. The result from further *in vivo* study showed that the intracellulara cdmium accumulation was also mediated by zinc transporters, causing cadmium-induced toxicity (57). Since zinc transporter is located on the apical side of renal epithelia cells, and respossible for

cadmium uptake, suggesting that zinc transporters contribute cadmium accumulation and toxicity. Therefore, several studies showed that pre-treatment with zinc ions and dietary co-administration of cadmium chloride with zinc attenuated renal cadmium accumulation and significantly reduced nephrotoxicity.

2.1.1.5 Organic Cation Transporter (OCTs)

Organic cation transporters (OCTs) are integral transmembrane proteins including OCT1, OCT2 and OCT3. These transporters translocate and eliminate a variety of cationic drugs and toxins from the renal tubular cells (Figure 2.2). However, some cations are significantly different in affinity and kinetic rate between OCT1, OCT2 and OCT3. Renal proximal tubue (PT) uptakes of organic cations into renal cell on baolateral side are mediated by OCTs (15). Among these, OCT1 and OCT2 have shown to mainly translocate the organic cation ion through the renal tubular basolateral membrane. However, OCT2 highly expressed in the basolateral membrane of proximal tubular cells, leading to an crucial role in the elimination of an exogenous and endogenous compounds (59). Recently, the evidence for the involvement of OCTs in cadmium transport became apparent recently. Soodvilai *et al.* reported that the intracellular accumulation of cadmium was increased in Chinese hamster ovary (CHO-K1) cells with overexpression of either OCT1 or OCT2, and could be inhibited significantly by the OCT substrate tetraethylammonium (TEA). In order to examine the role of OCTs *in vivo*, they used a bilateral ureteral ligation rat model to determine the basolateral uptake of cadmium independently from glomerular filtration and tubular absorption. The result showed that cadmium accumulation in the kidney was reduced by over 80 percent when co-treated with TEA in this mode (15). In renal proximal tubular cells, organic cation (OCT) secretion involves two step processes. First, organic cations are taken up into the cell via organic cation transporters (OCTs) that located on apical site of renal tubular epithelium cells. The uptake of organic cations from the blood side is driven by an inside negative membrane potential (60). The exit step is mediated largely by electro-neutral H^+/OC exchanger across the luminal membrane.

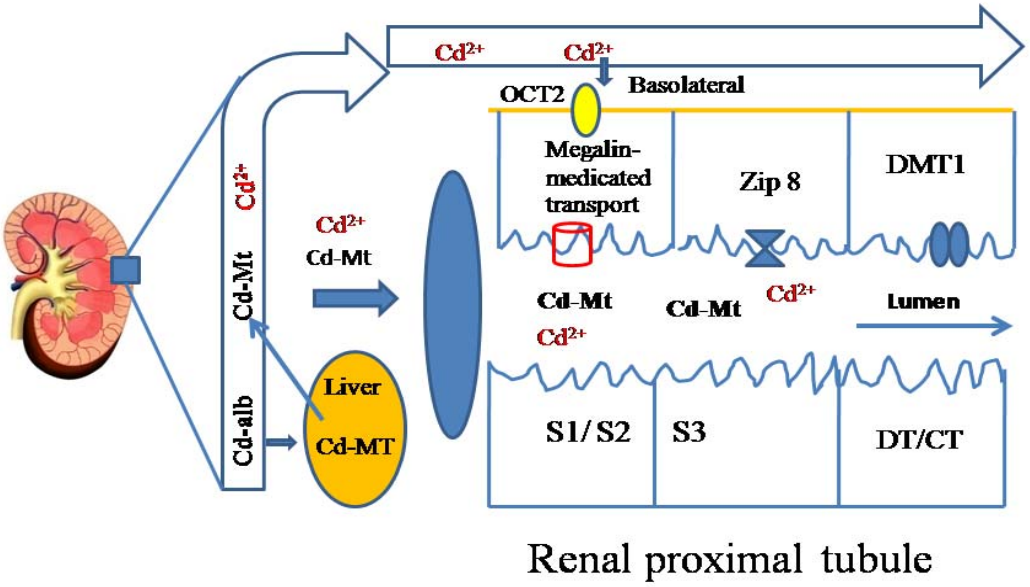


Figure 2.2 Diagram of cadmium secretion and reabsorption by renal tubular cells. (Modified from He et al., 2009). Cadmium (Cd) is conjugated to albumin (Cd-Alb) in the blood circulation following absorption and then taken up by the liver. In the liver, cadmium induces the synthesis of metallothioneins (MTs) and bind new MTs to form Cd–MT complex which is released into the blood circulation. Cd–MTs are filtered through the glomerulus and reabsorbed into PT segment 1 (S1) and segment 2 (S2). Divalent metal-ion transporter-1 (DMT1), organic cation transporter 2 (OCT2), and zinc/iron-regulated transporter 8 (ZIP8) mediate the transport of free cadmium ions in renal proximal tubule (PT), distal convoluted tubular (CT), and connected tubular.

2.1.3 Cadmium-induced renal proximal tubular cell damage.

Cd-induced cellular damage to a target organ is revealed after its uptake and accumulation by the cell (61). After Cd in blood circulation is present, it can distribute into the target organ, especially kidney, and enter the renal tubular epithelial cell by possible mechanisms as previously described. Cd deposition occurs largely in the renal proximal tubule epithelium cells. Higher concentrations of Cd which is reabsorped from the glomerular filtrate enter segment 1 (S1) of the proximal tubule, whereas reaborption at S2 and S3 are lower. There is an evidence showing that free Cd ions and Cd-thiol conjugates were transported from renal tubular capillaries to the

basolateral side of the proximal tubular epithelial cells, from where Cd can be taken up (38). After it is taken up by the renal proximal tubular cells, the Cd-metallothionein complex is accumulated in lysosomes, where it is degraded, resulting in the release of free Cd ion within the cells (37, 62). The free Cd ion rapidly binds with intracellular sulfhydryl groups on the proteins or low molecular weight compounds such as glutathione. The interactions of Cd with sulfhydryl groups on proteins can result in direct alterations in protein function. These interactions also cause disruptio in glutathione metabolism resulting in the induction of oxidative stress in the cell (63). In addition, the intracellular Cd can induce the synthesis of metallothionein to counteract its toxicity (37). Moreover, Cd can interfere with the function of essential metal ions (64) and disrupt various signaling pathways (65). In chronic exposure of cadmim, the accumulation of Cd in the proximal tubule cells continue to increase until reaching a critical threshold concentration of about 150-200 μg/g of tissue (66, 67) and subsequently inducing oxidative stress (ROS) that leads to injury and either necrotic or apoptotic cell death (8, 63). With the mean of oxidative stress (ROS) generation, Cd can destroy numerous enzymes and other proteins by forming disulfide bonds. Similarly, if NBC**,** sodium/ bicarbonate cotransporter-4 (SLC4A4), becomes nonfunctioning, HCO_3^- cannot cross the basolateral membrane and proximal tubular metabolic acidosis will be occurred. If 25-hydroxy-D3 1α-hydroxylase (CYP27B1), the most potent ligand for the vitamin D3 receptor, becomes nonfunctioning, 1α,25-dihydroxy-D3 would not be produced leading to bone disease. The combination of renal proximal tubular acidosis and osteomalacia can lead to renal Fanconi syndrome (39).

2.1.4 Cadmium-induced cell death in kidney cells.

Cell death is critical biological process that is important in normal histogenesis, organelle turnover and the pathogenesis of tissue injury and several diseases (68). Normally, physiological cell death or programmed cell death (PCD) occurs continuously in proliferating tissues to counter balancing of excessive cell proliferation during mitosis. PCD is mechanism of maintaining process for the normal functioning of the immune system and regurate the early embryonic development. Imbalance of apoptotic cell death can result in pathophysiological outcome and is

implicated in causing a variety of human diseases. Excessive PCD can lead to impaired growth and development, degenerative diseases, and acquired immunodeficiency syndrome (69). On the other hand, suppression of apoptosis can result in autoimmune diseases and develpement of cancer (70). In additinal apoptosis that occurs in normal and disease states, necrosis is induced only when cells or tissues are exposed to severe and acute injury (71, 72).

During chronic exposure, Cd is taken up to contenue in the renal proximal tubule leading to accumulation of Cd and toxicity. When the tissue level of Cd exceed a critical concentration of about 150 μg/g tissue, intracellular defenses such as metallothionein and glutathione are overwhelmed and then the cells are injured and begining to die (73, 74). Two major mechanisms of Cd-induced proximal tubular cell death involve apoptotic and necrotic processes. Each of these pathways has its unique sequence of pathophysiologic events (75). Apoptotic cell death is defined by mitochondrial depolarization, caspase activation, DNA fragmentation and cell shrinkage, followed by fragmentation of the cell into small, membrane-coated apoptotic bodies, whwreas necrosis is characterized by swelling of mitochondria and other organelles, breakdown of the cell membrane and the leakage of cytosolic contents into the surrounding environment. It has been recognized that high nephrotoxic doses of Cd can cause proximal tubule necrosis. However, several investigators reported that the early stages of Cd nephrotoxicity were associated with an increase in the number of apoptotic cell death in the renal proximal tubule (76, 77).

2.1.4.1 Cadmium-induced apoptotic cell death

Apoptotic cell death is a physiological process in the tissue. Perturbance of apoptosis execution could lead to a disruption in tissue homeostasis and cell function, resulting in several diseases such as cancer, neurodegenerative diseases and abnormal development (78, 79). Apoptotic signaling pathways can be subdivided into three pathways as followings: 1) the receptor mediated or extrinsic pathway (79); 2) the mitochondrial or intrinsic pathway (80), and 3) the endoplasmic reticulum (ER) pathway (81, 82). The receptor-mediated pathway involves binding of a death ligand to its receptor, which recruits death mediators, activates caspase-8 and subsequent caspase-3/6 leading to cleavage of intracellular substrates, DNA condensation and/or fragmentation, morphological changes. The mitochondrial pathway can be activated

by other injurious molecules such as reactive oxygen species (ROS) and Ca^{2+}. When it was activated, the mitochondria release proapoptotic factors such as cytochrome c, apoptosis inducing factor (AIF), and endonuclease G which reside in the intracellular space and in the cytosol. Cytochrome c can either activate caspase-9 or caspase-3 to induce apoptosis, causing apoptosis in a caspase-dependent manner, whereas apoptosis inducing factor (AIF), and endonuclease G causing apoptosis in a caspase-independent manner (80). Recently, the involvement of the ER in apoptosis has been emerging, in particular the roles of increased cytosolic Ca^{2+} (83, 84) and the unfolded protein response (85). Increased cytosolic Ca^{2+} causes toxicity to the cell by disrupting mitochondrial function and increasing the activity of enzymes, which are Ca^{2+}-dependent activation, such as the calpains. The unfolded protein response is activated through aggregation of misfolded proteins in the ER, which can be a result of cellular stresses such as hypoxia and heavy metal ions (86). The stresses of ER can upregulate a number of proteins, including chaperones and enzymes, leading to a commitment activation of apoptotic pathways and ultimately cell death (87).

Normally apoptosis can be induced by exogenous compounds, such as poisons or environmental toxins. Depending on the compound, apoptosis may be targeted to one particular organ or in the body. The environmental pollutant such as Cd found to affect several organs in the body and disrupt their function by inducing apoptosis, in particular the kidney proximal tubule. Following short-term exposure to Cd (up to 6 h), ROS, ceramides and cytosolic Ca^{2+} are increased, and calpains are activated (21). Whereas long-term exposure to Cd (24 h) results in a different signaling pathway activation such as MAP-Kinase including JNK, ERK, and p38 (65). Mitochondrial damage that induced by Cd cause the release of proapoptotic factors from the intermembrane space leading to apoptosis. Cross-talk between the two pathways for short- and long-term Cd exposures has been observed at the calpain-caspase level (21, 88). In addition, a number of studies have shown that cadmium induces apoptosis of the proximal tubular cells of experimental animals and LLC-PK1 porcine renal epithelium cell line (89).

A. Morphology of apoptosis

Apoptosis is the earliest characteristic change which occurs in the nucleus with chromatin condensation, pyknosis, and karyorrhexis (71, 90). The

condensed chromatin appears as crescents along the periphery of the nuclear membrane or as spherical bodies within the nucleus. The cytoplasmic condensation causes the cell to shrink and form numerous vacuoles within the cytoplasm (91). Subsequently, the nuclear and plasma membranes become convoluted, and small masses of condensed chromatin undergo fragmentation along with condensed cytoplasm to form apoptotic bodies. Apoptotic bodies are bound by plasma membrane, containing functional mitochondria and other organelles (92). The phosphatidyl serine residues that are normally localized to the inner membrane are relocated to the outside of the cell membrane before its fragmentation. The phosphatidyl serine residues on the apoptotic bodies serve as a signal to the surounding healthy cells to phagocytose and clear the cellular debris, thus avoiding an inflammatory response (93). *In vitro* experiment, in the absence of phagocytosis, apoptotic bodies eventurlly will be swell and lysis, and this terminal process of cell death has been termed "secondary necrosis". Secondary necrosis may occur *in vivo* in autoimmune disorders associated with defected clearance of apoptotic cells (94).

B. Molecular mechanism of apoptosis

Horvitz and colleagues reported that the existence of a genetically controlled program cell death in which at least three gene products including CED-3, CED-4, and CED-9, participate to cause selective programmed cell death (PCD) during *Caernohabditis elegans* development (95). The studies in other organisms revealed that several cysteine proteases that share homology to CED-3 are present in mammalian cells. Fourteen isoforms of cysteine proteases have been identified as caspase-1 to caspase-14 (96, 97). The caspases are the molecular executioners of apoptosis because they come about most of the morphological and biochemical change of apoptotic cell death. They are a family of constitutively expressed proenzymes that undergo proteolytic stimulating to generate their activated forms (98). The caspase family can be divided by their function into two major subfamilies. The first is the caspases-1,- 4, and -5 that their functions involve in the production of cytokines such as interleukin-1β and interleukin-18, and promotes proinflammatory functions. The second, the members of the family function are the part of the apoptotic pathway. They are divided into initiator caspases including caspases-2, -8, -9 and -10 , and effector caspases such as caspases-3, -6, and -7 (98).

The initiator caspases are activated by adapter-facilitated self cleavage in response to apoptotic stimuli whereas, the effector caspases are stimulated through cleavage by initiator caspases (99). In the apoptotic process, the active caspases mediated DNase to facilitate DNA degradation (100), cleavage of nuclear lamins to facilitate nuclear shrinkage and budding (101), and activation of p2-activated kinase 2 to cause blebbing in apoptotic cells (102).

2.1.4.2 Cadmium-induced necrotic cell death

The mediators contributing to necrosis are mostly extrinsic factors such as osmotic, thermal, toxic, hypoxic-ischemic, and traumatic insults (72, 103). Necrosis is defined by progressive loss of cytoplasmic membrane integrity, and influx of Na^{+}, $Ca^{2+,}$ and water, resulting in cytoplasmic swelling, nuclear pyknosis, and ultimately organel injury (104, 105). The next process leads to cellular fragmentation, and eventually release of lysosomal, and granular contents into the surrounding extracellular space, with subsequent causing an inflammation (106).

In humans and other mammals, Cd adversely affects a number of organs and tissues including lung, pancreas, testis, placenta, and bone, with kidney and liver being the two primary target organs (42). Cadmium exposure in humans and experimental animals produces renal cell damage characterized by degeneration and necrosis of tubular epithelial cells followed by interstitial inflammation of proximal tubular cells (107). The type of cell death is induced by Cd, depending on the concentration, exposure time and cell types. Generally, acute exposure to high concentration of Cd (>50 μM) leads to necrosis, whereas following exposure to low Cd concentration, apoptotic cell death predominates (89, 108).

A. Morphology of necrosis

The morphology of a necrotic cell death is different from that of a cell undergoing apoptosis, with ultrastructural changes occurring in both the cytoplasm and the nucleus. The features of necrosis are characterized by chromatin flocculation, swelling and degeneration of the entire cytoplasm and the mitochondrial matrix, blebbing of the plasma membrane, and eventually shedding of the cytoplasmic contents into the extracellular space (109). Unlike apoptotic cell death, the chromatin is not packed into separable membrane-bound particles, but it forms irregularly shaped clumps, a feature that is being used for differentiating between the apoptotic and

necrotic cell death (110). The mitochondria show inner membrane swelling, cristeolysis, and disintegration of inter space contents (111). The ribosomes are dissociated and dispersed throughout the cytoplasm, giving the cytoplasmic matrix dense and granular appearance. Dilation and fragmentation of the cisterns of rough endoplasmic reticulum and Golgi apparatus are frequently observed in necrotic cell death (112).

B. Molecular mechanism of necrosis

Necrosis is the noticeable mode of cell death that happens in various degenerative conditions. It is recognized to be a passive process occurring as a consequence of acute ATP depletion. Several ATP-dependent ion channels become ineffective to regurate ion exchange and biomolecule transport through cytoplasmic membrane, leading to ion dyshomeostasis, disruption of the actin cytoskeleton, cell swelling, membrane blebbing, and eventual collapse of the cell (104, 113, 114). However, recent reports suggest that in addition to the passive mechanisms, the "active" mechanisms may also contribute in the necrotic process.

The necrotic cell death is mediated by an influx of Na^+ and release of ATP molecules because of membrane leakage. The increased Na^+ level in the cytosol activates Na^+-K^+-ATPase, causing rapid depletion of ATP. In the beginning stages of the cell injury, a simultaneous efflux of K^+ maintains ion homeostasis. Severe depletion of ATP leads to loss fuction of the Na^+/K^+-pump mechanism, leading to retention of intracellular Na^+ and water that results in swelling and collapse of the cell. Therefore, the overloading of Na^+ accompanies with severe ATP depletion look like to be the major determinant of a necrotic outcome (115). There are the several ion channels such as the Na^+/K^+pump, Na^+/H^+ exchanger, Na^+/Ca^{2+} exchanger, and Na^+-K^+-$2Cl^-$ cotransporter have been reported to responsible for Na^+ influx in various cell lines (115-117). In addition to influx of Na^+, cytosolic Ca^{2+} seems to be involved in ATP depletion causing necrosis in some cell types (104), but in several other cell types including hepatocytes and renal tubular cells can undergo necrotic cell death in its absence (118). The oxidative stress condition (ROS) can mediate increasing of necrotic volume and Na^+ influx, suggesting to be initiated by the binding of the free radicals to ion channels including non-selective Ca^{2+} channels (119-121). The increased level of Na^+ activates Na^+- K^+-ATPase and

decreases ATP, which further activates non-selective Ca^{2+}channels, leading to massive cytosolic Ca^{2+} accumulation. In addition, the high level of Ca^{2+} can contribute ATP depletion by activating Ca^{2+}-ATPase and mitochondrial depolarization. The increased levels of Ca^{2+} also activate endonucleases to degrade DNA content and activate cellular proteases such as calpain to degrade several structural and signaling proteins in the cells (122). Recently, two novel Ca^{2+} permeable cation channels, which are transient receptor potential (TRP) channels including LTRPC2 and LTRPC7, are found to be mediated by a disturbanc of the oxidation-reduction status (123). LTRPC7 is an intracellular ligand-gated ion channel that can regulate both Ca^{2+} and Mg^{2+} transports and is suppressed by higher Mg^{2+} concentration (124). LTRPC2 permeates to both Na^{+} and Ca^{2+}. It is activated by binding of ADP ribose and increasing concentration of arachidonic acid and Ca^{2+} but suppressed by high Na^{+} levels. LTRPC2 is also activated by very low levels of H_2O_2 and agents that produce ROS such as Cd, thus anintrinsic mechanism that response to changes in oxidative stress mediates $Ca^{2+}$and Na^{+} overload. In addition, the moderate level of H_2O_2 production can increase cytocolic Ca^{2+} level by releasing from internal source such as the endoplasmic reticulum (ER) (123, 125).

2.2 Mechanisms of cadmium-induced toxicity

2.2.1 Cadmium induces reactive oxygen species (ROS) production

It was reported that the accumulation of cadmium (Cd) can induce oxidative stress in many organisms (124), resulting in physiological damage and diseases to various organs such as kidneys, liver, lung, pancreas, testes, placenta, and bone (126, 127). Cd is a divalent cation and unable to generate free radicals directly, but the production of reactive oxygen species (ROS) after Cd exposure has been reported in several studies (128-130). Because of complicated interactions between Cd and metabolism, a wide range of cellular responses is found in different organs after cadmium exposure. Several studies, the oxidative stress has been mentioned and demonstrated that it was a process of early biological responses that involve molecular changes in organ systems prior to the onset of clinical diseases or the development of

cancer (65, 131). Oxidative stress is a disturbance of the cellular redox balance in contribution of the pro-oxidants, leading to disruption of cellular macromolecules, including degradation of proteins, cross-links in DNA, and membrane fatty acid peroxidation. Nevertheless, elevated oxidative stress (ROS) concentrations can also react to signaling molecules in signal transduction (65). Cd shows a high affinity for thiols group in glutathione (GSH) that is highly abundant in cells, a primary target for free Cd-ions. Therefore, Cd-induced depletion of the reduced GSH pool results in a disturbance of the redox balance, leading to an oxidative stress status causing clinical pathological development. Under normal physiological conditions, ROS are generated in organelles with a highly oxidizing metabolic rate or those possessing electron transport chains, including peroxisomes and mitochondria. Cd is a non redox-active metal; it can not induce ROS production directly. However, it can induce ROS production indirectly by 3 major mechanisms including:

A. Replacement of redox-active elements

Cadmium is unable to catalyze redox reactions in biological systems under physiological conditions. However, it has been shown that Cd possibly increases the free Fe^{2+} by its replacement in various proteins and increases the cellular amount of free redox-active metals such as Cu^{2+} (132, 133). Free redox-active metals directly stimulate the production of $^{\bullet}OH$ (hydroxyl) radicals through the Fenton reaction. Reduction of the oxidized metal ion can be achieved by the Haber–Weiss reaction with superoxide radicals ($O2^{\bullet-}$) as a substrate (Figure 2.3).

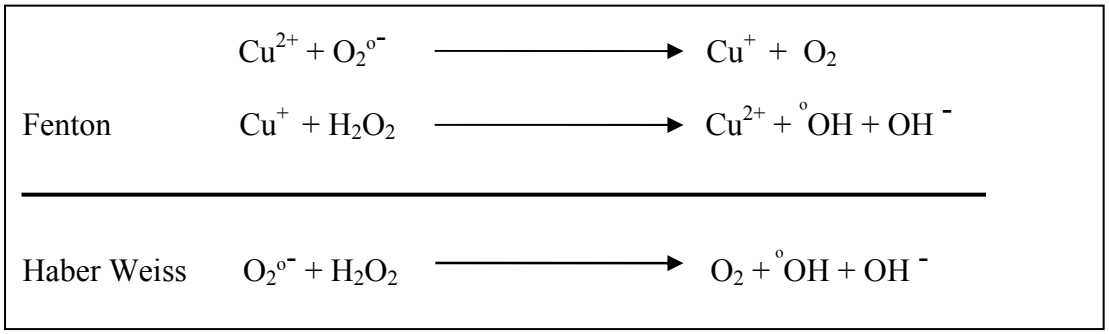


Figure 2.3 Fenton and Haber Weiss reaction with cupper as an example of a redox-active metal.

B. Mitochondrial ROS production

Mitochondria are a major cellular source of ROS production (134, 135). The physiological activity of the respiratory electron transport chain (complexes I to V) that occurs in the inner mitochondrial membrane produces ROS at complex I (NADH/ubiquinone oxidoreductase) and complex III (ubiquinol/cytochrome c oxidoreductase) sites (136-139). Although the production of free radicals (ROS) by mitochondria in normal tissues is rather low, ROS production substantially increases in the presence of xenobiotics or oxidative stress condition (139). As a result, these ROS might cause membrane lipid peroxidation, DNA cleavage and impaired ATP generation leading to mitochondrial damage and induction of apoptosis during oxidative stress status (140, 141). Several studies also demonstrated that the generation of oxidative stress associated with mitochondrial structural dysfunction during metal-induced cytotoxicity (142, 143).

Mustafa and Cross reported that Cd has affacted on electron and energy transfer reactions in the mitochondria of pulmonary alveolar macrophages (144). Moreover, recent studies confirmed that mitochondria is one of the primary cellular targets of toxic metals such as Cd and suggested the altered mitochondria function as a potential mechanism of Cd-induced cytotoxicity (145-148). In addition, Belyaeva et al. found that Cd could induce ROS generation, causing cell necrosis and/or apoptosis in rat ascitic hepatoma cells. This increased ROS generation is probably occured at the complex III and is related to the opening of the mitochondrial permeability transition (MPT) pore (149). Wang et al. showed that the individual complexes of electron transport chains were inhibited by Cd in mitochondria of guinea pig liver, brain and heart. They found that complex II and III are more sensitive to Cd than complexes I, IV and V in all three tissues, and also demonstrated that ROS generation induced by Cd at the level of complex III only. The possible mechanism of Cd-induced ROS generation in mitochondria was determined by kinetic studies and electron turnover experiments, resulting in the accumulation of unstable semiubiquinones prone to transfer one electron to molecular oxygen and thereby forming superoxide (150). In addition, the study of Poliandri et al. found that Cd-induced ROS generation in mitochondria of anterior pituitary cells was inhibited by rotenone, indicating that it is electron-transfer-chain-dependent (151). The

mitochondrial damage is mediated by ROS, leading to loss of the mitochondrial membrane potential causing liberation of cytochrom c to cytosol. This further leads to the activation of caspases and consequently induces cell death by apoptosis (152). In addition to Cd-induced ROS generation, it also directly interacts with specific protein thiols causing mitochondrial membrane lipid peroxidation leading to membrane permeability transition (133).

C. Induction of NADPH oxidases

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is recognized as a key component of the innate host defense in human body such as NADPH oxidase of neutrophils which is responsible for the bacterial infection (153, 154). NADPH oxidases function as multi-complex enzymes using electrons derived from intracellular NADPH to generate superoxide ($O^{2\bullet-}$) from oxygen (O_2). Superoxide generation is conversed to H_2O_2 spontaneously by its dismutation or through SOD activity.The NADPH oxidase (NOX) family contains seven members including NOX1-5, DUOX1 and DUOX2, and their physiological function is the generation of reactive oxygen species (ROS) (155). All members of NOC share a core structure consisting of six transmembrane domains and a long cytoplasmic C-terminus. In addition, N-terminal EF domains of NOX5, DUOX1, and DUOX2 bind with Ca^{2+} in order to their activation. They associate with important cellular processes, related to signaling, cell proliferation and apoptosis. Although ROS generated from NADPH oxidase plays a crucial role in physiological functioning, these molecules have also been implicated in oxidative stress and pathological conditions (156). Thijssen et al. found that NOX4 gene expression was upregulated in mice kidneys following chronic exposure to low level of Cd. Thus, increased NOX4 gene expression may have led to increased NOX activity in mice kidneys following Cd exposure in the kidney tissue. The exact role of NOX4 in Cd toxicity has not been described, but may be linked to the production of free radicals for signalmtransduction to activate the antioxidative defense system or adaptive mechanisms (131). This ROS production could mediate signaling pathway including STAT3 and ERK in HepG2 cells, leading to protective mechanisms that were reported by Souza et al.(157). Alternatively, generation of excess ROS could lead to Cd-induced toxicity in mouse

neuronal cells, as shown by Rockwell et al. (158). Therefore, potential NOX expressing cells could be targeted by Cd, and the influence of Cd on NADPH oxidase activity could lead to the cellular protection mechanisms or, alternatively, cell death.

2.2.2 Induction of cellular signaling by cadmium

The effects of cadmium (Cd) on the cellular function are multiple in signaling pathways. The cellular signaling cascades are mediated by Cd, affecting on the large number of the cellular reactions leading to signaling dysregulation. The regulation of transducing modules disturbed by Cd resulting in altered levels of second messengers, which overwhelm the control mechanisms of signaling leading to signaling dysregulation. Furthermore, the signaling dysregulation can alter physiological cellular functions and gene transcription and regulation causing cell death, stress-induced adaptation and survival. It was reported that Cd affects on Ca^{2+}, cAMP, NO, ROS, MAP-kinase (P38 and JNK), PKB/Akt, nuclear factor-kappa B (NF-κβ), and developmental signaling (65). However, these impacts of Cd are well understood. The cascades of these signaling molecules activated by Cd are illustrated in the renal proximal tubule, a major target of Cd toxicity. The variety of the complicated mechanisms of Cd toxicity in target tissues could be revealed and eventually lead to rationales and strategies for prevention and therapy of Cd toxicity.

2.2.2.1 Cadmium and Ca^{2+} signaling

Cadmium causes increased cytosolic Ca^{2+} through 2 major critical mechanisms that were reported by many studies: The first, inhibition of Cd on SERCA pump on ER membrane via inhibition of SERCA-type Ca^{2+}ATPases (19, 159, 160), and Ca^{2+}-ATPase (PMCA) pump on the plasma membrane (161). Others, activation of cadmium on G-protein coupled receptor (162, 163) and, directly on calcium channels such as voltage-dependent calcium channels (VDCCs) on the plasma membrane (164-166) and non-voltage-gated Ca^{2+} channels (167, 168). The consequence of an increasing cytosolic Ca^{2+} induced by Cd may be resulting in cell death and transcription of regulatory protein (65, 169).

2.2.2.2 Cadmium - activated MAP kinase

Oxidative stress (ROS) causes a potential threat for humans, which it might be a causative role in many disease processes. Oxidants and oxidative

stress can trigger the activation of multiple signaling pathways that influence the cytotoxicity observed in affected cells. These include the phosphorylation cascades leading to the activation of mitogen-activated protein kinases (MAPKs), which include extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 subfamilies, which are important regulatory proteins through which various extracellular signals are transduced into intracellular events (170, 171).

Accumulated cadmium in the cells leads to an increased intracellular reactive oxygen species (ROS) level (172, 173), lipid peroxidation, and an alteration in glutathione (GSH) level in many cell types (174, 175), suggesting that cadmium-induced apoptosis may be involved with oxidative stress. Moreover, a number of studies have shown that cadmium induces apoptosis of the proximal tubular cells in experimental animal (176), LLC-PK1 porcine renal epithelium cell line (177) and other types of cells such as CEM-C12 human T cell line (178) , rat testis (179) and mouse liver (180).

The involvement of mitogen-activated protein kinases (MAPKs) and oxidative stress (ROS) in Cd-induced cytotoxicity has been reported. For example, Yokouchi et al. (181) demonstrated that superoxide anion (O_2^{-o}) is the important reactive oxygen species (ROS) that induces JNK phosphorylation and eventually cause apoptotic cell death in LLC-PK1 cells exposed to Cd. The evidence for the involvement of JNK in Cd-induced apoptosis has been also reported by Papadakis et al. (182), who found inhibition of the mitochondrial apoptotic in jnk1 and jnk2 null (−/−) fibroblasts pathway following exposure to Cd for 15–25 h in low serum medium. Accordingly jnk −/− fibroblasts did not exhibit decreased cytosolic cytochrome c, increased caspase activity and DNA fragmentation following cell incubation with 10 μM Cd exposure. In response to environmental stresses including heavy metals such as cadmium, the c-jun N-terminal kinase (JNK) is activated by dual phosphorylation on Thr183 and Thr185 (22, 183). Curent report suggests that JNK activity may play an important role in triggering apoptotic signaling (184, 185). In addition, recent studies also indicated that the JNK/c-jun signaling cascade plays a crucial role in cadmium-induced apoptosis in neuronal cell and CL3 human lung adenocarcinoma cells (186, 187). Considering the above studies, JNK signaling

pathway may be responsible target for protective mechanism of cadmium-induced kidney damage.

2.2.2.3 Induction of NF-κB signaling by cadmium

The sinaling pathway that involves the gene regulatory protein nuclear factor-kappa B (NF-κB) regulates many physiological processes including apoptosis, cell adhesion, proliferation, the innate-and adaptive-immune responses, inflammation, the cellular-stress response, and tissue remodeling (188). Therefore, disturbance of NF-κB function contributes to many human diseases including cancer. NF-κB can induce and repress gene expression by binding to DNA sequences, known as κB elements, in promoter and enhancer regions. In various cell types, NF-κB complexes are resided in the cytoplasm by a set of inhibitory proteins known as inhibitors of NF-κB (IκBs). Activation of NF-κB typically involves the phosphorylation of IκB by the IκB kinase (IKK) complex, which results in IκB degradation. This releases NF-κB and allows it to translocate freely to the nucleus to transactivate target genes. There are several distinct NF-κB-activation pathways. The most frequently observed is classical pathway, which is induced in by various cellular stresses and stimuli including the pro-inflammatory cytokines tumor necrosis factor-α (TNFα), interleukin-1(IL-1), and bacterial products such as lipopolysaccharide (LPS). This pathway is stimulated by the rapid phosphorylation of IκBα at Ser32 and Ser36 by activation of the IKK complex and subsequent ubiquitin-induced degradation by the 26S proteasome (188). NF-κB is a prominent factor to balance cell death and cell survival. Some cellular stresses and stimuli that induce ROS promote activation of mitogen-activated-protein kinase kinase-7 (MKK7) and JNK, and eventually leads to apoptosis. But these stimuli also activate IKK and the canonical NF-κB pathway to induce the expression of antioxidizing enzymes such as MnSOD, which counteract ROS generation. In response to inflammatory stimuli, NF-κB induces anti-apoptotic and/ or survival genes. Other inducers of NF-κB, such as genotoxic stress, result in repression of anti-apoptotic genes and induction of proapoptotic genes. Which of these pathways will be dominate one probably depend on the cell type and the nature of the inducing stimulus. Whereas Cd has been shown to inhibit NF-κB binding to DNA in vitro on gel mobility shift assays (EMSA) (189), the toxicological relevance of this observation in intact cells is doubtful, also considering these effects were observed at

Cd concentration of 200-500 μM. Most subsequent studies, which were performed in a variety of cultured cells and in vivo, have demonstrated association of apoptosis induced by low micromolar concentrations of Cd with NF-κB activation, trans-activation of NF-κB oxidative stress-related target genes (MTs, GST or HO-1) (129, 190, 191), anti-apoptotic and/or survival genes (e.g. the ATP-binding cassette xenobiotic transporter Abcb1 and caspase inhibitory protein-2) (192), intercellular adhesion molecule 1 (ICAM-1) (193) and the inflammatory cytokine IL-8 (194). Interestingly, NF-κB activation was also demonstrated by EMSA of lung tissues following in *in vivo* (mouse) intranasal instillation of Cd (195). However, only one study demonstrated that 20 μM Cd induced apoptosis of rat renal proximal tubular cells (NRK-52E) and decreased NF-κB activity (196). In summary, the majority of studies suggests that Cd induced ROS formation triggers NF-κB dependent trans-activation of oxidative stress genes, anti-apoptotic and protective target genes in various cell lines and in vivo to counteract apoptosis (65).

2.2.2.4 Induction of ceramide formation by cadmium

Although the cellular processes underlying Cd-induced nephrotoxicity usually culminate the triggering of cell death by either apoptosis or necrosis (18, 197) involving generation of reactive oxygen species (16), the proximal tubular (PT) apoptosis induced by Cd has been reported to associate with ceramide production in *in vivo* and *in vitro* experiment (18, 198).

Ceramides are important regulators of cell proliferation, differentiation, inflammation, and apoptosis (199-201). They are either synthesized from serine and palmitate in a de novo pathway involving ceramide synthase or generated from the hydrolysis of the plasma membrane sphingolipid sphingomyelin (SM) by sphingomyelinases (SMases) (Figure 2.4). There are several types of SM; the most important in ceramide generation are the neutral and acidic forms (202, 203). The generation of ceramides is triggered by a variety of stimuli such as cytokines, heat, UV radiation, hypoxia-reperfusion, or various cytotoxic agents and may involve oxidative stress (203, 204). The activity of neutral SMase is thought to be regulated by glutathione, because high level of this reducing agent inactivates SMase. When high level of ROS overwhelm the cell's antioxidant systems, SMase is activated and subsequently hydrolyzes SM to generate ceramides that go on to act as downstream

signaling molecules (205). Ceramides have long been known to be intimately involved in apoptotic signaling pathways (206, 207).

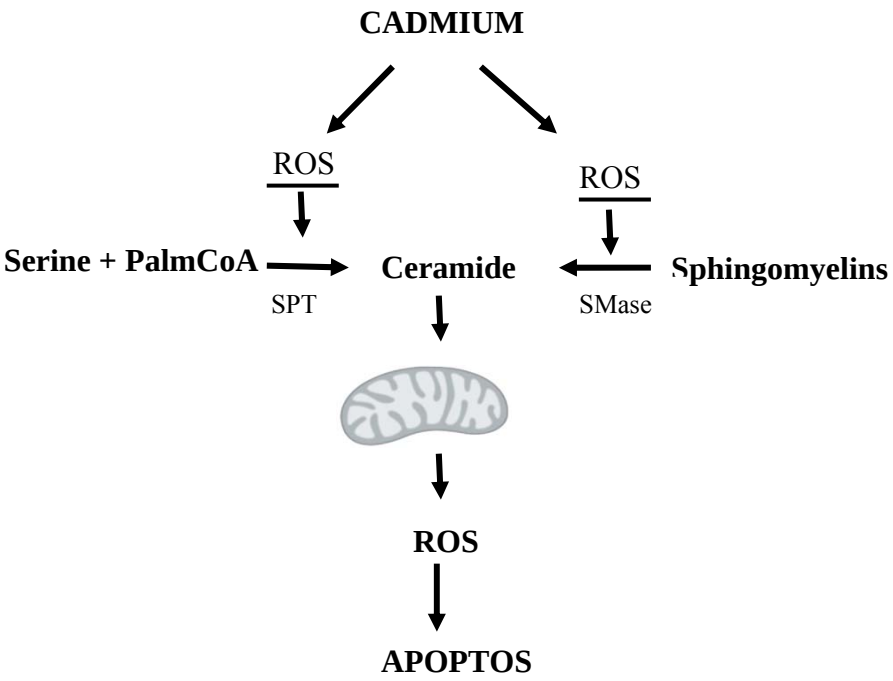


Figure 2.4 Pathways of metabolism of sphingomyelin, ceramide, and cholesterol, their modulation by oxidative stress. (Adapted from Cutler et al., 2004) (205).

There is substantial evidence for ceramides acting at the mitochondrial level to induce cytochrome *c* release leading to activation of executioner caspases without the requirement of initiator caspases (208-211). The study by Kim et al showed that C6-ceramide induced cytochrome *c* release, which was mediated by a Bax-dependent and an initiator caspase-independent mechanism (209). Furthermore, Siskind et al. provided evidence for a ceramide-induced increased outer membrane permeability mechanism by which ceramides could trigger apoptosis independently of initiator caspases via the activation of calpains (210). Two reports so far associate ceramides with calpain-dependent cell death. These studies were performed in neuronal cells and showed that calpain activation required a mitochondrial step (211, 212), although cell death could also occur without activation of executioner caspases (211). In addition, a few studies in cultured PT cells have

implicated ceramides as an acute renal stress reactant that increases in response to diverse ischemic or toxic insults (213, 214). Moreover, Apoptotic cell death caused by ceramides (Cer) has been shown to involve Ca^{2+}- activated proteases, calpains (20), as well as mitochondrial dysfunction (215), and is thought to be regulated by reactive oxygen species (ROS) (216). Inorganic cadmium can induce nephrotoxicity and particularly affects the proximal tubular S1 segment. In the cell, Cd generates ROS and increases Cer formation (20), could lead to cell death by apoptosis if the ROS-mediated stress events are not sufficiently balanced by repair processes (16).

2.3 Liver X receptors (LXRs)

2.3.1 Structure of LXRs and their ligands

Liver X receptors (LXRs), which are classified as α and β isoforms encoded by the genes NR1H3 and NR1H2, respectively, are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors. LXRα and LXRβ share a structural identity of almost 80%, but there is much difference in their patterns of expression (217). LXRα is expressed mainly in the tissue known to play an important role in lipid metabolism, such as liver, adipose tissue, intestine, kidney, spleen, and macrophages, while LXRβ is expressed everywhere in the body (26). Human LXRα and LXRβ genes are located on chromosome 11p11.2 and 19q13.3, respectively, they share an amino acid identity in their DNA-binding and ligand binding domain (218). LXR has a highly conserved structure comprising a hydrophobic ligand-binding domain, a DNA-binding domain, an N-terminal ligand-independent activation function domain and a C-terminal ligand-dependent activation function domain (Figure 2.5). LXRs resides in the nucleus and binds to DNA in an inactive state in the absence of a ligand, to form a complex with co-repressors, such as the silencing mediator of retinoic acid, thyroid hormone receptor and the nuclear receptor co-repressor (219). In active state, both isoforms which bind with their ligands form dimer with the retinoid X receptor (RXR) as LXR/retinoid X receptor(RXR) heterodimers resulting in activating target genes by binding to the LXR response element (LXRE) which located in their promoter regions (220). LXRE

contains two AGGTCA hexametric sequences separated by four nucleotides (DR-4) (Figure 2.6). Additional types of LXR-responsive elements, such as IR-0 and ER-8, have also been reported (221, 222). A PPAR response element has been identified in LXR gene promoter; therefore it seems likely that PPAR regulate ABCA1 gene expression through the LXR pathway. Similarly to peroxisome proliferator-activated receptors (PPARs) to some extent, the ligand activation of LXRs may also suppress the transcription of certain genes without LXRE, which was described as transrepression (223, 224). Numerous oxysterols have been demonstrated to be potential endogenous ligands for LXRs, such as 24-(S),25-epoxycholesterol, 24-(S)-hydroxycholesterol, 22-(R)-hydroxycholesterol, 25 hydroxycholesterol and 27-hydroxycholesterol. These oxysterols are shown to be bound to LXRs and stimulate transcriptional activity of LXRs at physiological concentrations (225, 226).

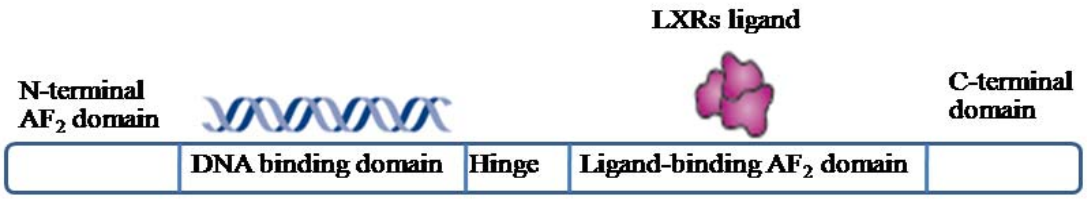


Figure 2.5 Schematic basis of LXRs of structure (Adapted from Anna C. et al., 2012)

In addition, several synthetic agonists have also been identified to have aided in the characterization of the function of LXRs, including T0901317 and GW3965, which are non-steroid LXR activators. LXR activator, T0901317, activates both LXR-α and LXR-β with a half maximal effective concentration (EC50) at very low concentrations. However, T0901317 is not a completely selective LXR agonist because it also activates farnesoid X receptor (FXR) and pregnane X receptor (PXR). However, the affinity of T0901317 for LXRs is much higher than that of PXR and FXR (227, 228). In addition, LXR activation showed stimulating expression of target gene such as SREBP1c, whereas this target gene expression was suppressed by FXR in renal tissue (229)

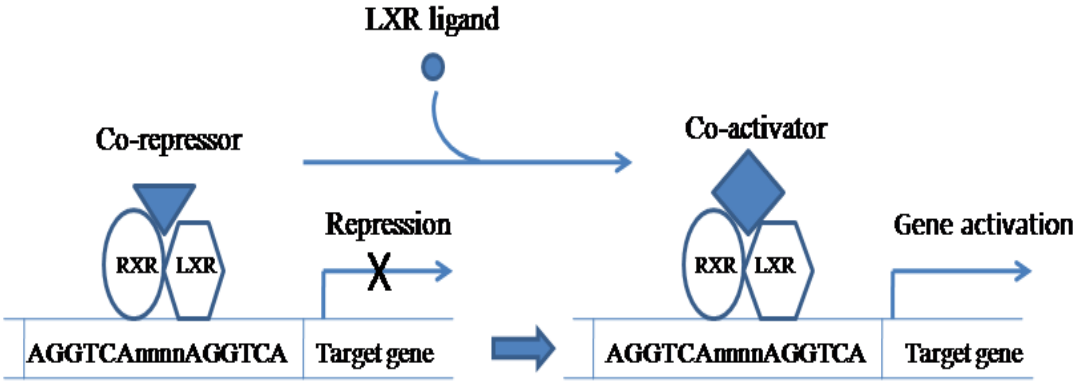


Figure 2.6 Schematic of LXRs activation (Adapted from Noam Zelcer and Peter Tontonoz, 2006**)**

2.3.2 LXRs regulate lipid metabolism

LXRs were classified as cholesterol sensors that regulate the expression of genes involved in lipid metabolism in response to certain oxygenated cholesterol-derivatives (oxysterol) ligands (26). Previously, regulation of hepatic cholesterol homeostasis has been shown to be mediated by the liver X receptors, both LXRα and LXRβ (230), but LXRα is likely to be the majority of isoform in the liver (26). The LXRs are activated by oxysterols, which are endogenous metabolites of cholesterol (25). These receptors form obligate heterodimers with retinoid X receptors (RXRs) to govern target gene transcription (217). Numerous studies have established that LXRs regulate gene expression linked to cholesterol metabolism in specific tissues including liver, adipose tissue, macropharges, intestine, kidney and heart. Activation of the gene transport cholesterol from peripheral tissues to the liver for catabolism and excretion. LXR target genes that involve transport of cholesterol and metabolism include ABC transporters such as ABCA1 and ABCG1 regulating reverse cholesterol transport, thereby stimulating the efflux of cholesterol from the peripheral tissue to the liver. Whereas ABCG5 and ABCG8 regulate intestinal reabsorption of cholesterol (231-233), SREBP-1c and fatty acid synthase (FAS) (234) stimulate triglyceride and fatty acid synthesis leading to hyper-triglyceridemia (235), and cholesterol 7α–

hydroxylase (CYP7A1) control the rate-limiting step in the catabolism of hepatic cholesterol to bile acids (236)

2.3.3 LXRs and anti-inflammation

Previously, LXRs were only classified as lipid sensors that regulate the expression of genes involved in lipid metabolism in response to oxysterol ligands. Recently, LXRs have also emerged as essential regulators of inflammatory signaling (237), this makes them potentially attractive targets for the modulation of inflammation responses to various stimuli.

Activation of inflammatory signaling pathways and release of inflammatory mediators are defense mechanisms to the immune functions of macrophages. LXRs can influence macrophage not only via regulation of lipid metabolism but also via effects on innate immunity. The release of cytokines from macrophages results in recruitment of monocytes, cross-talk with T-lymphocytic cells, perpetuates cellular activation and further contributes atherosclerotic lesion development and inflammation (238).

The anti-inflammatory effect of LXRs was reported showing that LXR activation attenuated lipopolysaccharide (LPS)-induced expression of pro-inflammatory molecules, including interleukin (IL)-6, inflammatory nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in macrophages from wild type but not LXR null mice (239). Moreover, LXRα/β-null macrophages reveal enhanced apoptosis after microbial infection due to defects of LXR dependent target gene expression, implying that LXR would be important for macrophage survival and innate immune response (240). In addition, mice deficient of LXR molecules exhibit increased atherosclerosis, suggesting that the potential anti-inflammatory effects of LXRs may contribute to their anti-atherosclerotic effects. Mechanistically, the anti-inflammatory effects of LXRs have been attributed to nuclear inhibition of NF-kB signaling (224, 241). Subsequent studies demonstrated that LXR also attenuates expression of the NF-kB target gene, MMP-9, both *in vivo* and *in vitro* (242). MMP-9 has been shown to be localized to macrophage-rich areas within atherosclerotic lesions and is associated with enhanced extracellular matrix degradation, influencing smooth muscle cell migration, neointima formation and plaque instability.

Involvement of lipid metabolism and immunity via the LXR pathway was further demonstrated. The study by Castrillo *et al* who demonstrated that bacterial pathogens such as E.coli and influenza A, which signal via the TLR-3/4 pathway, can downregulate LXR signaling, resulting in reduced ABC transporter expression and efflux of cholesterol, an effect known to exacerbate atherosclerotic lesions formation (243). More studies demonstrate that LXRs can also modulate the TLR2/TLR4/MyD88 pathway (244). *C.pneumoniae*-induced atherosclerosis, which can be attenuated by TRL2, TLR4 or MyD88 deficiency, was accelerated in the absence of LXRα. Transfected LXRα−/−and apoE−/−mice exhibited increased atherosclerosis with lesions rich in dendritic cells and cholesterol as well as higher plasma IL-6 levels compared to LXR+/+ and apoE−/− mice or uninfected LXRα−/− and apoE−/−mice (245). Phagocytosis of apoptotic cells results in LXR activation due to cholesterol loading, leading to an increase in the LXR target gene and apoptotic cell receptor, Mer (246). LXR activation by apoptotic cells was shown to promote further clearance of apoptotic cells and to concomitantly suppress inflammatory pathways. In contrast, LXR null macrophages were defective in their ability to induce Mer expression and phagocytose apoptotic cells, and exhibited an induction of the pro-inflammatory mediators IL-1β and MCP. Interestingly, losses of Mer expression and defective apoptotic cell clearance have both previously been linked to accelerated atherosclerosis (247, 248)

In addition, activation of LXRs suppresses collagen-induced arthritis in mice and reduces inflammation and joint destruction. GW3965 treatment significantly reduced the arthritis incidence and attenuated the clinical and histological severity, compared with vehicle-treated mice. GW3965 treatment also significantly reduced inflammatory mediator production in joint sections and serum pro-inflammatory cytokine levels in a dose-dependent manner (249). Furthermore, administration of LXR ligands to mice inhibits tissue factor expression, which is expressed by macrophages and other types of cell within injury lesions, induced by LPS in the kidney and lung (250). Resent study demonstrated that LXR agonist T0901317 attenuated LPS induced liver injury in a murine model of non-alcoholic fatty liver disease (NAFLD), reflected by reduced serum alanine aminotransferase and aspartate aminotransferase levels, and reduced liver histology changes. In addition, activation

of LXRs also reduced TNF-α and iNOS expression through inhibiting JNK signaling pathway (28). All together, these studies illustrate that LXR regulates a number of immune and inflammatory pathways that have the potential to modulate inflammation in cell injury.

2.3.4 LXRs and antioxidants

Reactive oxygen species (ROS), such as hydroxyl radicals ($^{\circ}OH$), superoxide ($O_2^{\circ-}$), and hydrogen peroxide (H_2O_2), are highly reactive molecules produced during normal cellular processes involving oxygen, as well as during pathological responses by leukocyte enzymes. A variety of pro-inflammatory compounds, such as lipopolysaccharide (LPS), cytokines, chemokines, and lipid mediators, are capable of activating leukocytes to generate ROS. ROS causes cellular damages by reacting with macromolecules, resulting in dearangements, such as mutations in DNA, alteration in protein function, and membrane damage. (251-253). The imbalance of oxidants and antioxidants plays an important role in the development of various diseases, such as acute respiratory distress syndrome and chronic obstructive pulmonary disease (254, 255). Oxidative stress also affects inflammatory responses and alters the balance of cytokines (256). To neutralize free radicals and counteract the detrimental effect of ROS, cells express a wide array of endogenous antioxidant enzymes. These include direct antioxidants, such as metallothioneins (MT), superoxide dismutases (SODs), catalase (CAT), and glutathione peroxidase (GS-Px) , as well as indirect antioxidant enzymes, such as glutathione *S*-transferases (GSTs), and NADPH: quinine oxidoreductase (NQO1) (257-259). Proper regulation of these antioxidant enzymes is essential for mammals to maintain balances between oxidants and antioxidants among antioxidant enzymes. For example, GSTs are a family of phase II enzymes that catalyze the conjugation of the tripeptide GSH to a variety of hydrophobic, electrophilic, and cytotoxic substrates. The majority of GST substrates are either xenobiotics or products of oxidative stress that are toxic to cells. The formation of a thioether bond between electrophiles and GSH almost always yields a conjugate that is less reactive than the parent compounds, and therefore the GST mediated conjugation generally results in xeno-and endobiotic detoxification (260).

It is widely accepted that LXRs play an important role in cholesterol metabolism and triglyceride synthesis in various tissues (261, 262). LXRs have also been shown to inhibit inflammatory gene expression and prevent either bacterial or LPS-triggered inflammatory responses in macrophages. In addition, Haibiao Gong et. al. revealed a role of LXR in regulating antioxidant enzymes such as GST and SOD in the bronchoalveolar lavage (BAL) fluids of mice treated with LPS of LPS-induced lung injury (30). However, the hepatointestinal functions of LXRs have been well documented, the role of LXRs in the pathophysiology of the kidney remains unknown. Therefore, the role for LXRs in preventing cadmium- induced kidney injury needs to be investigated.

2.3.5 LXRs and MAPK signaling

Previously, LXRs were mentioned to involve in regulation proinflammatory and antioxidant genes, as well as apoptosis induced by LPS in macrophage (263, 264). In addition, resent study reported that activation of LXR attenuates endotoxin-induced liver injury in mice with nonalcoholicfatty liver disease through inhibiting the JNK phosphorylation which contributes to apoptosis (28, 265). Although, a few studies on the effect of LXR activation on MAPK signaling has been reported, their effects on JNK phosphorylation linking apoptosis make them attractive as potential targets for the treatment of cadmium toxicity.

CHAPTER III
MATERIALS AND METHODS

3.1 Cell culture procedure

Renal proximal tubular cell lines, porcine renal epithelial cell (LLC-PK1 cell) and human renal proximal tubular cells (HK-2 cell), were cultured in low glucose - DMEM (Dulbecco's Modified Eagle Medium) supplemented with 5-10 % heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin G, and 100 µg/ml streptomycin (Gibco, Invitrogen Corp., Carlsbad, CA) and incubated in a humidified incubator under 5 % CO_2/95 % O_2 at 37˚C. For the experiment, an appropriate number of LLC-PK1 and HK-2 cells were seeded in 6 or 96 well plates for 24 h until the cell growth was approximately 70-80 % confluent.

3.2 Chemicals, antibodies, and reagents

3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide (MTT), dichorofluorescein diacetate ($DCFH_2$-DA), N-acetyl-L-cysteine (NAC), T0901317 (T0), fenofibrate, and GW6471 were purchased from Sigma (MO, USA). Protease inhibitor cocktail was obtained from Roche Diagnostics (Mannheim, Germany). iScript Select cDNA synthesis kit was obtained from Bio-Rad Laboratories (Hercules,CA). KAPA SYBR FAST qPCR kit was obtained from KAPA Biosysthems (Wilmington, MA). Anti-p-JNK and anti-SREBP-1c antibodies were from Novas biologicals (Littleton, CO). Anti-total-JNK antibody was purchased from Cell Signaling (Beverly, MA). Anti-LXRα anti-LXRβ antibodies were purchased from Abcam (Burlingame, CA). Annexin V-FITC Apoptosis Detection Kit was purchased from BD Pharmingen (San Jose, CA). Penicillin-Streptomycin solution and fetal bovine serum (FBS) were purchased from Gibco BRL (Rockville, MD). In addition, other chemicals used in this study are analytical grade and purchased from commercial sources.

3.3 The instruments

The instruments used in this study include the followings:

1. The CO_2 incubator (Mid Atlatic Diagnostic Inc. , Marton, NJ) was used for incubation of mammalian cell line culture.

2. The PCR thermo cycles (Major Science, Philadelphia, PA) was used for conversion of RNA to cDNA products.

3. The NanoDrop (NanoDrop Technologyies, Wimington, DE) was used to measure the quantity and purity of RNA samples.

4. The Applied Biosystem 7500 Real-Time PCR System (Applied Biosystem, Foster City, CA) was used for detection and quantification of PCR products.

5. The BD FACScanto flow cytometer (BD Biosciences, San Jose, CA) was used to determine apoptotic cell death.

6. EL 312 Bio Kinetics Microplate Reader (Bio-Tek Instruments Inc., Helsiki, Finland) was used for determination of cell viability by MTT assay, and determination of intracellular ROS generation by fluorescent probe, $DCFH_2$-DA.

7. Microplate BCA protein assay kit (Thermo Scientific, Pierce Biotechnology) was used for determination of cellular protein.

3.4 Experimental procedures

1. Cytotoxicity assay

Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) assay as described previously (266). For the assay, LLC-PK1 or HK-2 cells in 100 µl medium were seeded into 96-well microplates and incubated in the incubator under condition with 5 % CO_2 / 95 % O_2 at 37 °C for 24 h. The cells were incubated in serum-free medium containing various concentrations of toxic agent including cadmium (0-100 µM) for further 24 h. At the end of incubation period, the cells were washed with phosphate buffer (PBS), and then 20 µl of MTT solution (5 mg/ml in phosphate buffered saline, PBS) was added to each well and incubated further for another 4 h. At the end of incubation period, the

solution was replaced by 150 µl of DMSO and shaking with 180 rpm rotation for 15-20 min to dissolve the formazan crystals. The color intensity of the formazan solution was measured on a microplate spectrophotometer at 540 nm (Bio-Kinetics Reader; Bio-Tek Instruments Inc., Helsinki, Finland). Cell viability was calculated as percent of control (vehicle-treated groups).

2. Determination of cell death by flow cytometry

Apoptosis and necrosis were assessed by using the annexinV-FITC apoptosis detection kit (BD Biosciences) according to the protocol outlined by the manufacturer. The assay is based on the binding properties of annexin V to phosphatidylserine and on DNA-intercalating capabilities of propidium iodide (PI). Briefly, after treated HK-2 cells were harvested and washed twice with cold PBS, they were re-suspended in a binding buffer solution. An aliquot of 100 µl cell suspension was incubated with annexin V-FITC and PI for 15 min in dark at room temperature, and 400 µl binding buffer solution was added to each sample. The stained cells were analyzed directly by flow cytometer (Model: FACS Canto, Becton Dickinson, USA) using the Cell Quest program. Cell apoptosis and necrosis were identified and quantified as the percent of the total cells.

3. Determination of intracellular reactive oxygen species (ROS)

The production of ROS was measured by ROS-responsive fluorescent probe, $DCFH_2$-DA. HK-2 cells were grown in 96 well plates, then washed twice with DPBS buffer, followed by loading with 20 µM $DCFH_2$-DA in DPBS for 40 min. After washing twice with DPBS, the cells were incubated with several treatments of toxic agents including cadmium for 3 h. When acetate esters of $DCFH_2$-DA get into the cell, esterases will cleave it to non-flourescent $DCFH_2$, which can no longer pass out of the cell. After oxidation by intracellular ROS, predominantly hydrogen peroxide (H_2O_2), $DCFH_2$ is converted to green-flourescing dichloro-fluoresein (DCF). At the end of incubation, the DCF fluorescence intensity (267) was determined as indicated times by microplate reader spectrofluorometer (Model Max M3) at the wavelength pairs of 488/530 nm. The relative DCF fluorescence intensity was expressed as a percentage of control (vehicle-treated cells).

4. Western blot analysis

The protein expressions of LXRs, SREBP-1c, ERK, and JNK in HK-2 cells were determined by Western blot analysis. HK-2 cells were seeded in 6-well plates at the confluence of 70-80 % and then exposed to various conditions for indicated times. At the end of incubation period, cells were washed twice with ice-cold PBS buffer and incubated with 150 µl lysis buffer / well. Subsequently, the whole cellular protein extracts were prepared in ice-cold lysis buffer containing:150 mM NaCl, 1 mM EDTA, 1 % triton X-100 , and 50 mM Tris-Cl, pH 7.4, 1 mM PMSF, and protease inhibitor cocktail. The cell lysate was centrifuged at 12,000 rpm for 20 min at 4 ˚C, and then the supernatants containing proteins were kept at -80 ˚C. The supernatants were assayed for total protein content using BCA protein assay kit (Thermo Scientific, Pierce biotechnology) according to the manufacture's protocols. Equal amounts of proteins (20 µg) were separated using 10 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins on SDS-PAGE were transferred to a hybond-ECL nitrocellulose membrane (Amersham Biosciences). The membrane was incubated in 5 % nonfat dry milk solution at room temperature for 1 h and then blotted with primary antibodies (1:1,000 dilution) at 4 ˚C for overnight. After incubation with the primary antibody, the membrane was washed three times with TBST buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.02 % Tween-20) for 15 min / time. And then it was incubated with a 1:4,000 dilution of secondary antibody horseradish peroxidase-conjugated goat anti-rabbit IgG at room temperature for 1 h. The blots were determined by an enhanced chemiluminescence detection system (ECL) and visualized by exposure to sensitive film (Amersham Biosciences). The beta-actin or GAPDH was used as control loading of proteins.

5. RNA isolation and Real-time PCR

Total RNA was isolated from treated HK-2 cells using TRIzol RNA Isolation kit (Invitrogen, Carlsbad, CA). Reverse transcription was performed by using the iScript select cDNA synthesis kit (Bio-Rad) according to the manufacture's protocols. Briefly, 1 µg RNA was denatured by incubation at 65°C for 5 min. The cDNA was generated by reverse transcriptase at 42˚C for 60 minutes. The reverse transcription products were then diluted 1:2 in distilled water and subjected to Real-

Time PCR amplification using specific primers. These primers were designed by using primer sequences as the followings; SOD primers (268): (F) 5-CTGAAGGCCTGCA-TGGATTC-3 and (R) 5-CCAAGTCTCCAACATGCCTCTC-3, catalase primers (269): (F) 5-CCATTATAAGACTGACCAGGGC-3 and (R) 5-AGTCCAGGAGGGG-TACTTTCC-3, quinine oxidoreductase (NQO1) primers (270): (F) 5-GGGATCCAC-GGGGACATGAATG-3 and (R) 5-ATTTGAATTCGGGCGTCTGCTG-3, and GAPDH primers (271) (F): 5-AGCCTTCTCCATGGTGGTGAAGAC-3 and (R) 5-CGGAGTCAACGGATTTGGTCG-3. The amount of RNA was quantified by Real-time PCR amplification using the SYBR Green master mix (KAPA-Biosystem, Wobun, MA) and the Prism 7500 real-time PCR Detection System (Applied Biosystems according to the manufacture's protocols). Briefly, the total volume of reaction consists of 16 µl of KAPA SYBR FAST ABI Prism 2X qPCR master mix. The cycling conditions of reaction were conducted in 96 well plates at 95 °C for 10 min, followed by 40 repeats of 95 °C for 15 s and 60 °C for 1 min. The cycling threshold (C_t) values were obtained from the 7500 System Sequence Detection Software (Version 2.0) (Forster City, CA). The mean of Ct values from three transcript reactions was used to calculate the relative expression levels of RNA by using the delta-delta method ($2^{-\Delta\Delta Ct}$) from threshold cycle number (272). Relative expression levels of mRNA were normalized by mRNA of GAPDH used as an internal control.

6. Statistic analysis

The data were presented as mean and the standard error of mean (mean ± S.E.). Each data point was generated from at least three independent experiments. One-way analysis of variance (One-way ANOVA) and unpaired t-test were used to determine the statistical significance of the group of experiments. Difference were considered statistically significance when $p \leq 0.05$.

3.5 Experimental protocols

1. Verification of cell models used in the study

The aim of this experiment was to determine whether cell models used in this study express LXRs including LXRα and LXRβ. The expressions and activations of LXRs were determined by detection of LXRα or LXRβ and SREBP-1c expressions, respectively, by Western blot analysis.

1.1 The expression of LXRα and LXRβ in cell models

The protein expression of LXRα and LXRβ in cell models used, LLC-PK1 and HK-2 cells, were verified. These cells were cultured in low glucose DMEM containing 10 % FBS, 100 U/ml penicillin and 100 µg/ml streptomycin until the cell growth were reached 70-80 % confluent. Subsequently, the cells were incubated in serum-free medium for 24 h and then were harvested in lysis buffer solution containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor (PI) cocktail. The cell lysates were centrifuged at 12,000 rpm, 4 ˚C for 20 min. The supernatants were assayed for total protein content using BCA Protein kit assay according to the manufacture's protocols. Equal amounts of proteins (30 µg) were separated using 10 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred to a hybond-ECL nitrocellulose membrane. The membrane was incubated in 5 % nonfat dry milk at room temperature for 1 h and then blotted with specific anti-LXRα or LXRβ antibodies at 4 °C for overnight. After incubation with anti-LXRα or LXRβ antibodies, the membranes were washed three times with TBST buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.02 % Tween-20) for 15 min. and then incubated with a 1:4,000 dilution of secondary antibody horseradish peroxidase-conjugated goat anti-rabbit IgG for further 1 h at room temperature. The blots were detected by an enhanced chemiluminescence detection system (ECL) and visualized by exposure to sensitive film.

1.2 The activation of LXRα and LXRβ following treatment with LXR agonist in cell model.

This experiment was set up to determine whether LXR agonist, T0901317, used in this study stimulated LXR activation. The expression of LXR-targeted protein, SREBP-1c, was determined following treatment with T0901317. The LLC-PK1 or HK-2 cells were divided into 2 groups including vehicle- and LXR agonist-treated group. For vehicle group, the cells were incubated in serum-free medium, whereas another group, the cells were incubated in serum-free medium containing 10 μM T0901317 for 24 h. The SREBP-1c protein expression was determined by Western blotting with anti-SREBP-1c antibody as described in the experimental procedure section.

2. Effects of LXR activation on cadmium-induced cytotoxicity in renal proximal tubular cell.

The effect of LXR activation in cadmium-induced renal proximal tubular cell damage was determined in LLC-PK1 and HK-2 cells using two independent techniques including MTT assay and flow cytometry.

2.1 Cadmium induces cytotoxicity in renal proximal tubular cells

The purpose of this experiment was to determine dose-and time-dependent effects of cadmium in renal proximal tubular cells, LLC-PK1 and HK-2 cells.

2.1.1 Dose-dependent effect of cadmium exposure on cell viability in renal proximal tubular cells.

LLC-PK1 and HK-2 cells were seeded in the medium supplement with 5-10 % FBS for 24 h. Cells were incubated with vehicle or cadmium chloride ($CdCl_2$) at several concentrations (0, 3, 5, 10, 20, 50, 100 μM) in serum-free medium for 24 h. At the end of incubation period, the cell viability was examined by MTT assay as described in experimental procedure section.

2.1.2 Time-dependent effect of cadmium exposure on cell viability in renal tubular cells.

LLC-PK1 and HK-2cells were seeded in the medium supplement with 10 % FBS maintained for 24 h. Cells were divided into 2 groups including vehicle and $CdCl_2$ treated group. Both cell types were incubated with

vehicle (serum-free medium) or $CdCl_2$ at optimal dose of cadmium (obtained from 2.1.1) for 24, 48, and 72 h, and then the cell viability was examined by MTT assay.

2.1.3 Cadmium induces cell death in human renal proximal tubular cells.

To further determine type of cadmium-induced human renal proximal tubular cell death, flow cytometry was performed**.** HK-2 cells were seeded in the medium supplement with 10 % FBS and maintained until cells reached 70-80 % confluence. Cells were divided into 2 groups including vehicle and $CdCl_2$-treated groups. The cells were stained with AnnexinV/FITC and propidium iodide (PI) and then measured by Flow cytometry. Cell apoptosis and necrosis were expressed as percent of total cells.

2.1.4 Cadmium induces caspase-independent apoptotic cell death in human renal proximal tubular cells**.**

To determine whether $CdCl_2$-induced apoptotic cell death was non-caspase dependent pathway, HK-2 cells were incubated in vehicle or 10, 20 μM $CdCl_2$ for 24 h. At the end of incubation time, the cells were harvested in lysis buffer. The protein extract was determined for procaspase-3 expression using Western blot analysis with anti-procaspase-3 antibody.

2.2 Effect of LXR activation on cadmium-induced cytotoxicity in human renal proximal tubular cells.

To investigate whether the effect of T0901317 on cadmium-induced cytotoxicity was required activation of LXR**,** HK-2cells were seeded in the medium supplement with 10 % FBS maintained until cells reached 70-80 % confluence. HK-2 cells were incubated with serum-free medium under 6 conditions: (1) Vehicle for 24 h; (2) medium containing 20 μM $CdCl_2$ for 24 h; (3) medium containing 10 μM T0901317 for 24 h, (4) medium containing 10 μM T0901317 for 24 h and then further incubated in medium containing 20 μM $CdCl_2$ and 10 μM T0901317 for another 24 h; (5) medium containing 10 μM fenofibrate, a LXR antagonist for 24 h; (6) medium containing 10 μM T0901317 and 10 μM fenofibrate for 24 h and then incubated in medium containing 10 μM T0901317, 10 μM fenofibrate, and 20 μM $CdCl_2$ for another 24 h. At the end of treatment period, the

cadmium-induced cytotoxicity was determined by MTT assay and flow cytometry as described in experimental procedure section.

2.3 Effect of LXR activation on bound forms of cadmium-induced cytotoxicity in HK-2 cells.

2.3.1 Effect of bound forms of cadmium on cadmium-induced cytotoxicity in HK-2 cells.

This experiment was set to determine whether by bound forms of cadmium including Cd-N-acetyl-L-cysteine (Cd-NAC) or Cd-albumin (Cd-Alb) could induce cell death differently from that induced by $CdCl_2$. The Cd-NAC and Cd-Alb were prepared by mixing of $CdCl_2$ and N-acetyl-L-cysteine (NAC) with mole ratio of 1:2 in serum-free medium at various concentrations of Cd (5, 10, 20, 40, 80 mM), and adding $CdCl_2$ into serum-free medium containing 5 g/dl albumin at various concentrations of $CdCl_2$ (5, 10, 20, 40, 80 mM), respectively. HK-2 cells were seeded in the medium supplement with 10 % FBS for 24 h until cells reached 70-80 % confluence. The cells were incubated under four conditions for 24 h: (1) vehicle; (2) medium containing Cd-NAC at various concentrations of $CdCl_2$ (5, 10, 20, 40, 80 μM); (3) medium containing Cd-Alb at several concentrations of $CdCl_2$ (5, 10, 20, 40, 80 μM). At the end of incubation period, cell viability was determined by MTT assay as described in experimental procedure section.

2.3.2 Effect of LXR agonist on cell viability induced by bound found of cadmium.

To determine the effects of T0901317 on bound forms of cadmium-induced reduction of cell viability, HK-2 cells were seeded in the medium supplement with 10 % FBS until cells reached 70-80 % confluence. The cells were incubated under six conditions: (1) vehicle for 24 h; (2) medium containing 10 μM T0901317 for 24 h; (3) medium containing 20 μM Cd-NAC for 24 h; (4) medium containing 10 μM T0901317 for 24 h and then further incubated in medium containing 10 μM T0901317 plus 20 μM Cd-NAC for another 24 h; (5) medium containing 20 μM Cd-Alb for 24 h; (6) medium containing 10 μM T0901317 for 24 h and then further incubated in medium containing 10 μM T0901317 plus 20 μM Cd-Alb for another 24 h; At the end of treatment period, the cytotoxicity was determined by MTT assay as described in experimental procedure section.

2.4 Effect of PPARα antagonist on protective effect of LXRs in cadmium induced cytotoxicity in human renal tubular cells.

Since fenofibrate had other activities such as activation of PPARα receptor which may counter balancing the LXR protection by changing availability or signaling of the heterodimeric partner, RXR alpha, leading to elimination the effect of LXR activation. Therefore, the selective effect of fenofibrate on LXR was determined by inhibition of PPARα with specific antagonist, GW 6471. HK-2 cells were represented and seeded in the medium supplement with 10 % FBS until cells reached 70-80 % confluence. HK-2 cells were incubated under ten conditions: (1) vehicle for 24 h; (2) medium containing 20 μM $CdCl_2$ for 24 h; (3) medium containing 10 μM T0901317 for 24 h; (4) medium containing 10 μM T0901317 for 24 h and then further incubated in medium containing 10 μM T0901317 and 20 μM $CdCl_2$ for another 24 h; (5) Medium containing 10 μM fenofibrate for 24 h; (6) Medium containing 10 μM fenofibrate for 24 h and then further incubated in medium containing 10 μM fenofibrate and 20 μM $CdCl_2$ for another 24 h; (7) medium containing 0.5 μM GW 6471 for 24 h; (8) medium containing 0.5 μM GW 6471 for 24 h and then further incubated in medium containing 0.5 μM GW 6471 and 20 μM $CdCl_2$ for another 24 h; (9) medium containing 10 μM T0901317, 10 μM fenofibrate and 20 μM $CdCl_2$ for 24 h and then 10 μM T0901317, 10 μM fenofibrate , and 20 μM $CdCl_2$ for another 24 h; (10) medium containing 10 μM T0901317, 10 μM fenofibrate, and 0.5 μM GW 6471 for 24 h and then further incubated in medium containing 10 μM T0901317, 10 μM fenofibrate, 0.5 μM GW 6471, and 20 μM $CdCl_2$ for another 24 h. At the end of treatment period, the cytotoxicity was determined by MTT assay as described in experimental procedure section.

2.5 Effect of pregnane X receptor (PXR) on cadmium induced cytotoxicity in HK-2 cells.

Since synthetic LXR agonist used in this study, T0901317, had been reported to activate PXRs, effect of PXR activation on cadmium-induced cytotoxicity was determined. To test this hypothesis, HK-2 cells were incubated under four conditions as following; (1) vehicle for 24 h; (2) medium containing 20 μM $CdCl_2$ for 24 h; (3) medium containing 20 μM rifampicin (PXR agonist) for 24 h; (4) medium containing 20 μM rifampicin for 24 h and then incubated in medium

containing 20 μM rifampicin plus 20 μM $CdCl_2$ for another 24 h. At the end of incubation period, cell viability was determined by MTT assay as described in experimental procedure section.

3. Investigation of mechanisms responsible for the protective effect of LXR activation on cadmium-induced cytotoxicity.

The potential mechanisms of for the protective effect of LXR activation on cadmium-induced cytotoxicity were proposed including; 1) inhibition of cadmium-induced ROS generation; 2) inhibition of cadmium-induced MAPK signaling pathway responsible for cell death. Therefore, the detailed underlying mechanisms of LXRs responsible for the cadmium-induced cytotoxicity were determined.

3.1 Effect of LXR activation on intracellular ROS generation induced by cadmium.

This experiment was set up to determine whether LXR activation could decrease intracellular ROS generation.

3.1.1 Effect of cadmium on cytotoxicity via intracellular ROS generation.

To determine whether cadmium-induced apoptosis is mediated by inducing ROS production, HK-2 cells with 70-80 % confluence were incubated in 4 conditions as following; (1) vehicle for 24 h; (2) 20 mM $CdCl_2$ for 24 h; (3) 1 mM NAC for 24 h; and (4) 20 mM $CdCl_2$ plus 20 mM $CdCl_2$ for 24 h. At the end of incubation period, cell viability was determined by MTT assay as described in experimental procedure section.

3.1.2 Effect of LXR activation on intracellular ROS generation.

To investigated whether the protective effect of LXR activation is due to the reduced the cellular oxidative state, HK-2 cells with 70-80 % confluence were divided into six groups as following;

Group 1. Vehicle

Group 2. 20 μM $CdCl_2$

Group 3. 10 μM T0901317

Group 4. 10 μM fenofibrate

Group 5. 10 μM T0901317 plus 20 μM $CdCl_2$

Group 6. 10 μM fenofibrate, 10 μM T0901317 and 20 μM $CdCl_2$.

Group 5 and 6 were pretreated with T0901317 alone or combination of 10 μM T0901317 and 10 μM fenofibrate for 12 h and then further incubated for 1, 2, and 3 h as indicated. For group 1-4, the cells were incubated in serum-free medium for 24 h and then incubated in DPBS for 1, 2, and 3 h as indicated. At the end of incubation time, intracellular ROS was determined by using DCHF-DA dye as described in experimental procedure section.

3.1.3 Effect of LXR on enzymes responsible for ROS generation and antioxidant enzymes.

To investigate the mechanisms by which LXR abolished $CdCl_2$-induced ROS generation, HK-2 cells were divided into four groups as the following;

Group 1. Vehicle

Group 2. 20 μM $CdCl_2$

Group 3. 10 μM T0901317

Group 4. 20 μM T0901317 plus 20 μM $CdCl_2$

Group 4 was pretreated with 10 μM T0901317 for 12 h and then further incubated with the combination of 10 μM T0901317 and 20 μM $CdCl_2$ for another 4 h. For group 1-3, the cells were incubated as described for 4 h. At the end of incubation time, mRNA of enzymes responsible for ROS production including NQO1, SOD, CAT, and GAPDH were determined by real-time PCR with primers as followings: SOD primers (268): (F) 5-CTGAAGGCCTGCATGGATTC-3 and (R) 5-CCAAGTCTCCAACATGCCTCTC-3, CAT primers (269): (F) 5-CCATTA TAAGACTGACCAGGGC-3 and (R) 5-AGTCCAGGAGGGGTACTTTCC-3, NQO1 primers (270): (F) 5-GGGATCCACGGGGACATGAATG-3 and (R) 5-ATTTGAAT-TCGGGCGTCTGCTG-3, and GAPDH primers (271) (F): 5-AGCCTTCTCCATGGT-GGTGAAGAC-3 and (R) 5-CGGAGTCAACGGATTTGGTCG-3. The amount of RNA was quantified by Real-time PCR amplification using the SYBR Green master mix (KAPA Biosystem, Wobun, MA) and the Prism 7500 real-time PCR Detection

System (Applied Biosystems according to the manufacture's protocols) as described in experimental procedure section.

3.1.4 Effect of N-acetyl-L-cysteine (NAC) on NQO1 expression induced by cadmium in human proximal tubular cells.

To investigate whether NQO1 expression was mediated by ROS, HK-2 cells with 70-80 % confluence were divided into 4 groups as the followings;

Group 1. Vehicle

Group 2. 20 μM $CdCl_2$

Group 3. 1 mM N-acetyl-L-cysteine (NAC)

Group 4. 20 μM $CdCl_2$ plus 1 μM NAC

Cells of group 4 were pretreated with NAC for 12 h followed by incubation with NAC plus $CdCl_2$ for further 1, 4, and 8 h. For group 1-3, the cells were incubated as indicated for 1, 4, and 8 h. At the end of incubation time, cells were quantified for mRNA expression of NQO1 by real-time PCR as described in experimental procedure section.

3.2 Effect of LXR activation on MAPK signaling in cadmium-induced apoptotic cell death in renal proximal tubular cells.

Since mitogen-activated protein kinase (MAPK) signaling pathways involved apoptosis, this experiment was set to determine the protective effect of LXR activation on cadmium-induced JNK-and ERK phosphorylation.

3.2.1 Effect of LXR activation on JNK signaling induced by cadmium.

The aim of this experiment was to determine whether LXR activation inhibited JNK phosphorylation induced by cadmium. HK-2 cells were treated with several conditions as following; (1) vehicle for 12 h; (2) medium containing 20 μM $CdCl_2$ for 12 h; (3) medium containing 10 μM T0901317 for 12 h; (4) medium containing 10 μM fenofibrate for 12 h; (5) medium containing 10 μM T0901317 for 24 h and then further treated with medium containing 10 μM T0901317 and 20 μM $CdCl_2$ for another 12 h; (6) medium containing 10 μM T0901317 and 10 μM fenofibrate for 24 h and then further treated with medium containing T0901317, 10 μM fenofibrate, and 20 μM $CdCl_2$ for another 12 h. At the

end of incubation time, cells are harvested in lysis buffer. The protein extract was determined for JNK expression using Western blot analysis with specific antibodies including anti-total-JNK antibody and anti-p-JNK antibody as described in the experimental procedure section.

3.2.2 Effect of LXR activation on ERK signaling induced by cadmium.

To investigate whether LXR activation affected an expression of ERK signaling induced by $CdCl_2$, HK-2 cells that reached 70-80 % confluence were divided into six groups as followings;

Group 1. Vehicle

Group 2. 20 μM $CdCl_2$

Group 3. 10 μM T0901317

Group 4. 10 μM fenofibrate

Group 5. T0901317 plus 20 μM $CdCl_2$

Group 6. T0901317 plus 20 μM $CdCl_2$ and 10 μM fenofibrate

HK-2 cells of group 1-4 were incubated for 24 h, whereas those of group 5 and 6 were pretreated with 10 μM T0901317 and 10 μM T0901317 plus 10 μM fenofibrate, respectively, for 12 h followed by incubation with the conditions as described. At the end of incubation time, the cells were harvested in lysis buffer. The protein extract was determined for ERK expression by Western blot analysis with specific antibodies including anti-total-ERK antibody and anti-p-ERK antibody.

3.2.3 Cadmium induces JNK phosphorylation through ROS generation in renal tubular cells.

The experiment was set to determined whether cadmium-induced JNK phosphorylation was down-stream of ROS generation. The cells were incubated in four conditions: (1) vehicle for 12 h, (2) 20 μM $CdCl_2$ for 12 h, (3) 1 mM NAC for 12 h, and (4) N-acetylcysteine (0.5 or 1 mM) for 4 h followed by medium containing NAC (0.5 and 1 mM) plus 20 μM $CdCl_2$ for 12 h. At the end of incubation period, JNK protein expression was determined by Western blot analysis.

3.2.4 Effect of LXR on intra-signaling cascade of cadmium-induced cytotoxicity.

The aim of this experiment was to investigate the primary target of LXR activation on $CdCl_2$-induced cytotoxicity. HK-2 cells were divided into four groups as shown below.

Group 1. Vehicle

Group 2. 100, 200, or 500 μM H_2O_2

Group 3. 10 μM T0901317

Group 4. 100, 200, or 500 μM H_2O_2 plus T0901317

The cells of group 4 were pretreated with 10 μM T0901317 for 12 h prior to incubated with combination of 10 μM T0901317 and 100, 200, or 500 μM H_2O_2 for 24 h. For group 1-3, the cells were incubated as indicated for 24 h. At the end of incubation period, JNK expression are determined by Western blot analysis as described in the experimental procedure section.

CHAPTER IV
RESULTS

4.1 Expression of LXRs in LLC-PK1 and HK-2 cells

Although it is well recognized that LXRα and LXRβ express in renal proximal tubular cells, there are no data concerning the expression of these receptors in the cell models used in this study. This experiment was set up to determine whether the cells used in this study express LXRα and LXRβ. The expressions of both LXRα and LXRβ isoforms in renal proximal tubular cells, LLC-PK-1 and HK-2 cells, were determined by Western blot analysis. The results showed that the expressions of LXRα and LXRβ proteins in both LLC-PK1 (Figure 4.1A) and HK-2 cells (Figure 4.1B) were clearly detected at 50 and 52 kD, respectively.

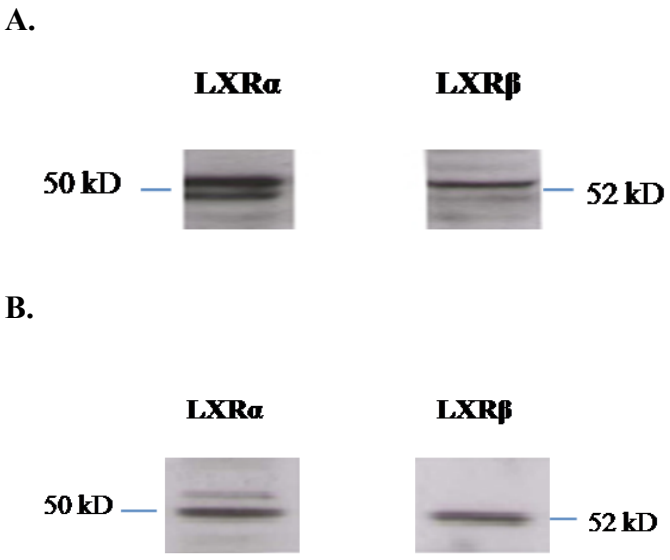


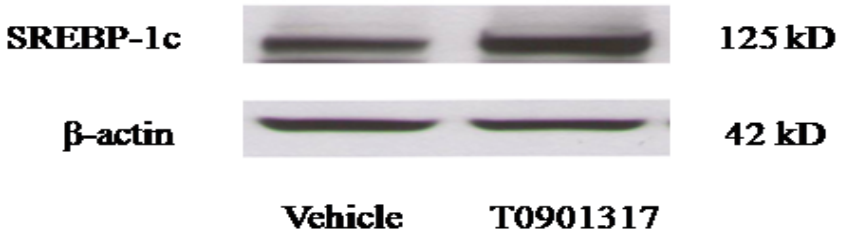
Figure 4.1 Expression of LXRα and LXRβ isoforms in LLC-PK1 and HK-2 cells. The protein lysates of LLC-PK1 and HK-2 cells were prepared using lysis buffer. The proteins (50 μg) were separated on 12 % SDS-PAGE and analyzed using Western blotting with antibodies against LXRα or LXRβ protein. Expression of LXRα and

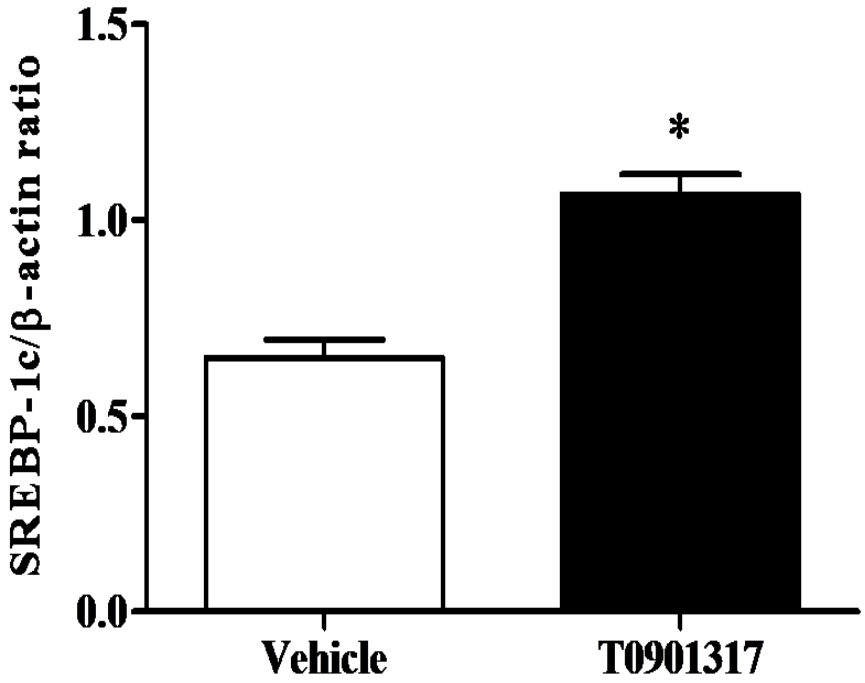
LXRβ proteins of LLC-PK1 cells (A) and HK-2 cells (B) were detected at 50 and 52 kD, respectively.

4.2 Activation of liver x receptors (LXRs) in renal proximal tubular cells.

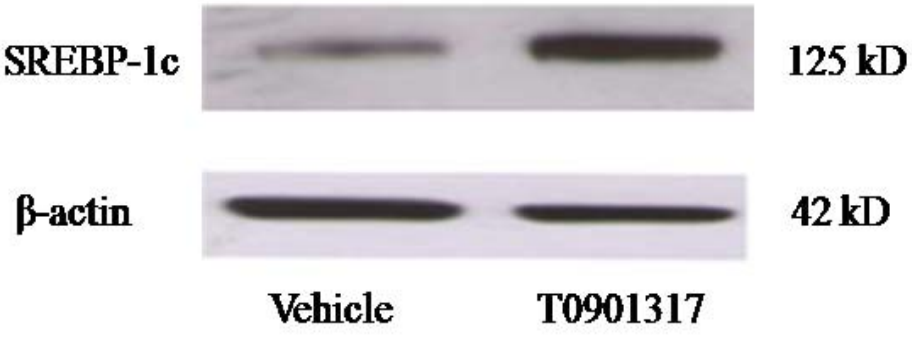
In order to demonstrate that LXR agonist, T0901317, used in this study exerts LXR activation, the expression of LXR-targeted protein, SREBP-1c, was determined following treatment of the cells with T0901317. After LLC-PK1 and HK-2 cells were cultured and fasted with serum-free medium for 4 h, these cells were treated with vehicle or 10 μM T0901317 for further 24 h. At the end of treatment, the protein lysates were extracted and followed by determination of SREBP-1c protein expression using Western blot analysis. The expression of SREBP-1c protein in treated cells was compared to control. As shown in figure 4.2, the cells that treated with T0901317 for 24 h significantly stimulated SREBP-1c protein expression compared with that of cells treated with vehicle in both LLC-PK1 (A) and HK-2 cell (B), indicating that T0901317 exerts LXR activation in the cell models. Therefore, these cells were suitable to use for determination of LXR activation in cadmium-induced nephrotoxicity.

A. LLC-PK1 cell




SREBP-1c/β-actin ratio
1.5
1.0
0.5
0.0
*
Vehicle
T0901317

B. HK-2 cell


SREBP-1c
125 kD
β-actin
42 kD
Vehicle
T0901317

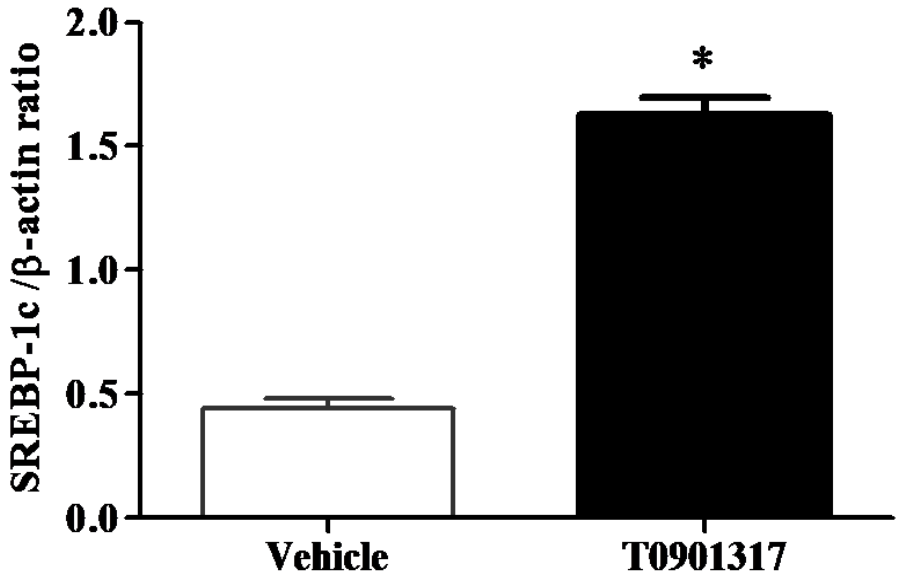


Figure 4.2 Activation of LXRs in LLC-PK1 and HK-2 cells by T0901317. The protein lysates, which were extracted from LLC-PK1 or HK-2 cells treated with vehicle or 10 μM T0901317 for 24 h, were separated by 10 % SDS-PAGE. The expression of SREBP-1c was determined by Western blot analysis with antibody against SREBP-1c protein while β-actin was used as a loading control. The expressions of SREBP-1c in both LLC-PK1 (A) and HK-2 cells (B) were detected at 125 kD. The band intensities from three independent experiments were normalized and expressed as ratio of SEBP-1c and β-actin. *$p < 0.05$ compared to control.

4.3 Cadmium-induced cytotoxicity in renal proximal tubular cells

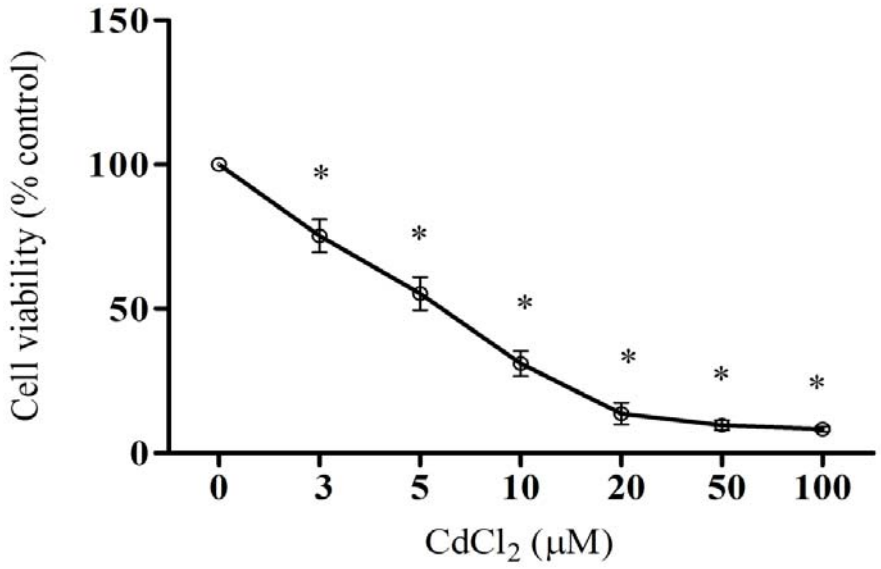
To determine whether cadmium induced cytotoxicity in renal proximal tubular cells including LLC-PK1 and HK-2 cells.

4.3.1 Dose-dependent effect of cadmium exposure on cell viability in LLC-PK1 and HK-2 cells.

The toxic effects of $CdCl_2$ on renal tubular cells, LLC-PK1 and HK-2 cells, were determined by incubation of the cells with several concentrations of $CdCl_2$

(0-100 μM) followed by measurement of cell viability using MTT assay. The data revealed that $CdCl_2$-induced cytotoxicity was in dose-dependent manner in both cells. The concentration of $CdCl_2$ at 5 and 20 μM, which reduced cell viability, was estimated 50% inhibitory concentration (IC_{50}) for LLC-PK1 cell (Figure 4.3A) and HK-2 cell (Figure 4.3B), respectively, suggesting that cadmium-induced cytotoxicity was dependent on cell types. Therefore, these concentrations were selected for next experiments.

A. LLC-PK1 cell



B. HK-2 cell

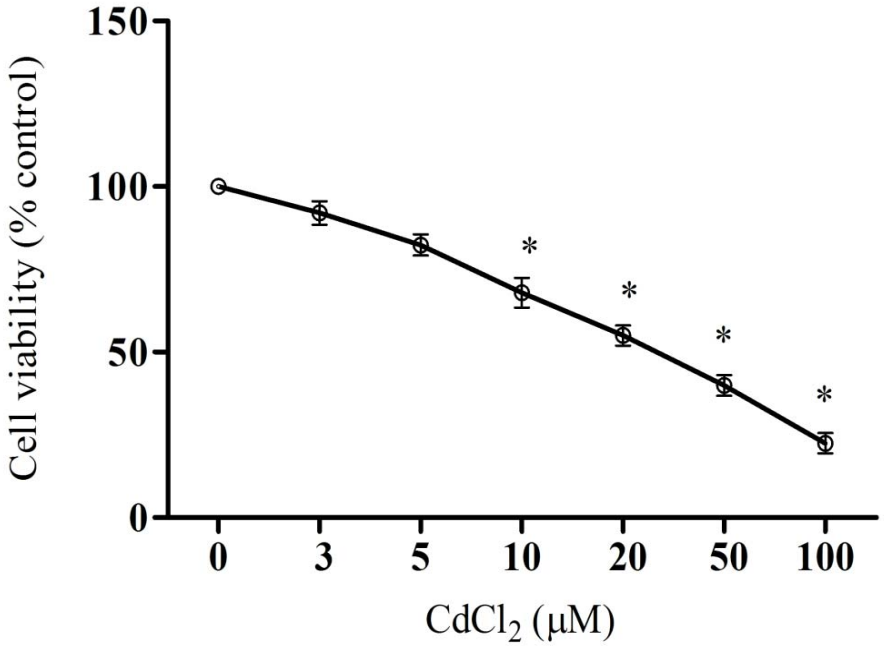


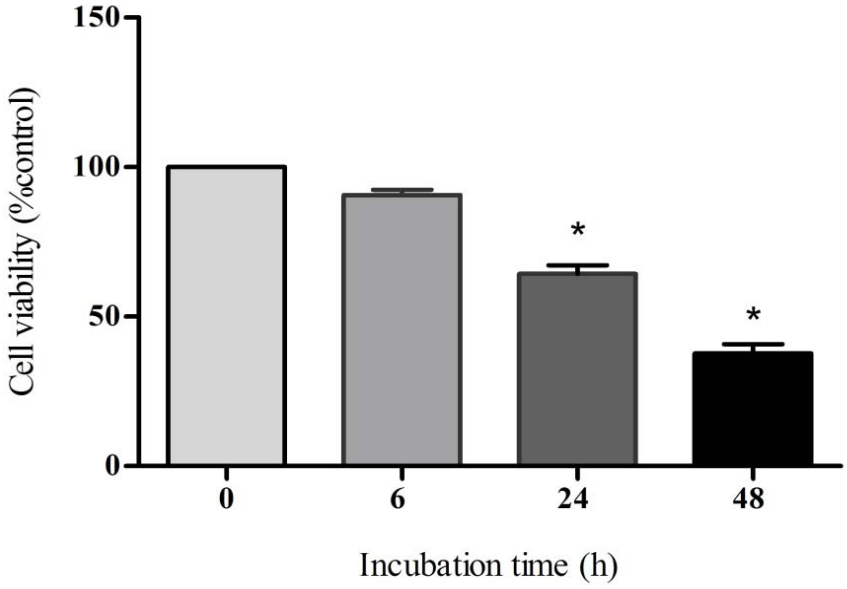
Figure 4.3 Dose-dependent effect of $CdCl_2$ on cell viability in renal proximal tubular cells, LLC-PK1 and HK-2 cells. LLC-PK1 and HK-2 cells were incubated with various concentrations of $CdCl_2$ (0-100 μM) in serum-free medium for 24 h and then the cell viability was determined by MTT assay. The cell viability of LLC-PK1 (A) and HK-2 (B) cells were expressed as the mean ± S.E. of three independent experiments. *$p < 0.05$ compared to control.

4.3.2 Time course-dependent effect of cadmium exposure on LLC-PK1 cell and HK-2 cell.

The time course dependent-effect of $CdCl_2$ was determined by incubation of LLC-PK1 and HK-2 cells with 5 and 20 μM $CdCl_2$, respectively, for 6-48 h followed by determination of cell viability using MTT assay. The data showed that effect of $CdCl_2$, which reduced cell viability of both cells, was in the time-dependent manner (Figure 4.4 A and 4.4 B). Incubation of the cells with $CdCl_2$ for 24 h significantly reduced cell viability compared with non-treated cells in both cell types; therefore, this time point was selected for determining types of cell death by flow cytometry. Moreover, increasing exposure time upto 48 h led to increase in its effect.

Since both cell types responded to $CdCl_2$ in the same pattern, human renal proximal tubular cell, HK-2, was selected for the cell model used in further studies.

A. LLC-PK1 cell



B. HK-2 cell

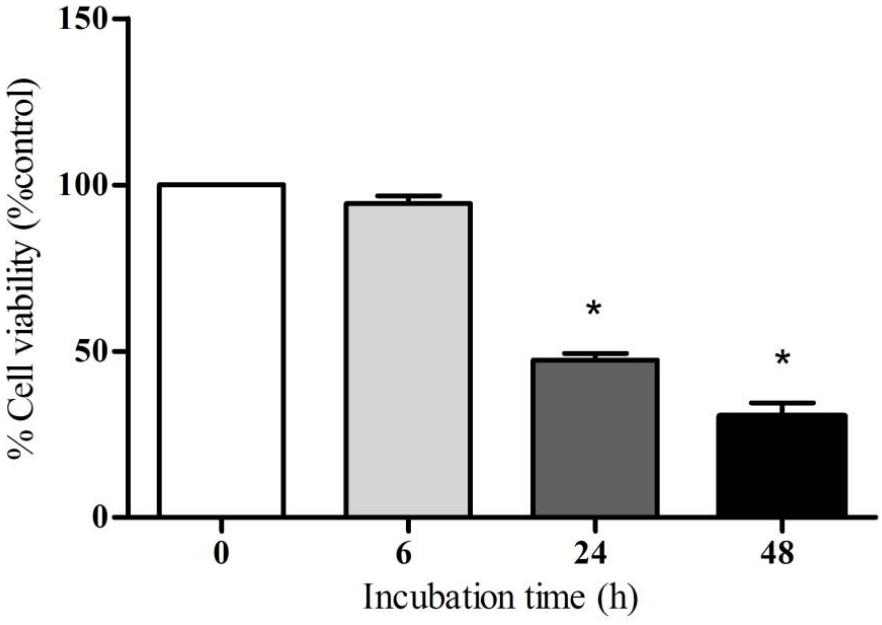


Figure 4.4 Time course effects of $CdCl_2$ on cell viability in LLC-PK1 and HK-2 cells. LLC-PK1 cells were incubated in serum-free medium containing vehicle or 5 μM $CdCl_2$ whereas HK-2 cells were incubated with serum-free medium containing vehicle or 20 μM $CdCl_2$ for 6, 24, and 48 h. At the indicated incubation periods, the cell viability was determined by MTT assay. The cell viability of LLC-PK1 cells (A) or HK-2 cells (B) was expressed as the mean ± S.E. of three independent experiments. *$p < 0.01$ compared to control.

4.3.3 Type of cell death induced by $CdCl_2$ in human renal proximal tubular cells, HK-2 cells.

The types of cell death induced by $CdCl_2$ in human renal proximal tubular cells were determined. Since both cells responded to $CdCl_2$ in the same pattern, HK-2 cells were used as a cell model for this experiment. They were treated under two conditions; 1) medium containing vehicle (control) for 24 h. 2) medium containing 20 μM $CdCl_2$ for 24 h. At the end of incubation period, types of cell death were determined by annexin-V/FITC (apoptotic marker) and propidium iodide (PI) staining measured by flow cytrometry. The data showed that the cells treated with 20 μM $CdCl_2$ for 24 h significantly increased cell apoptosis, but not necrosis (Figure 4.5). These data indicated that $CdCl_2$ predominately induced apoptotic cell death in human renal proximal tubular cells.

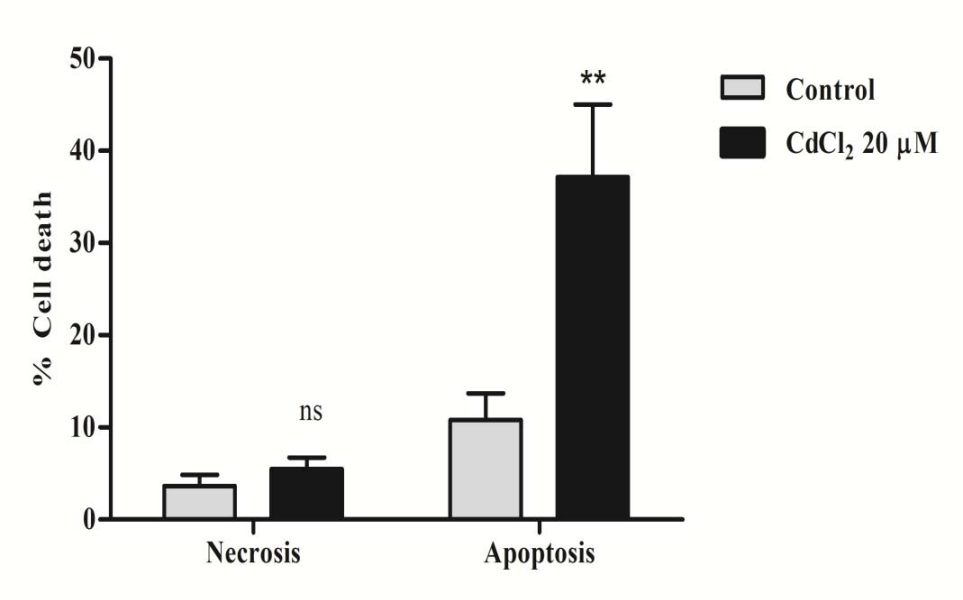
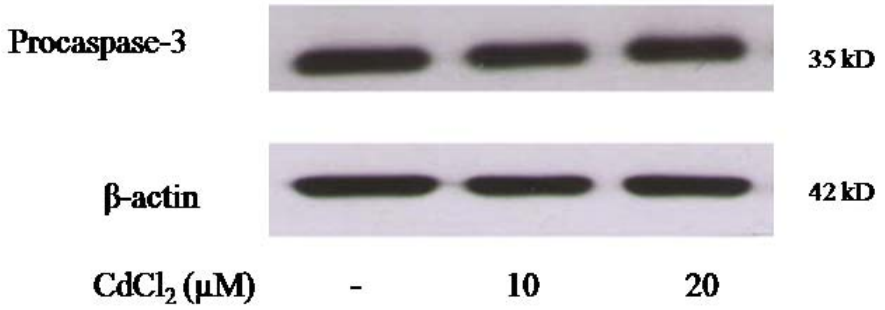


Figure 4.5 $CdCl_2$-induced cell death in human renal proximal tubular cells**.** HK-2 cells were treated with vehicle or 20 µM of $CdCl_2$ in serum-free medium for 24 h, and stained with annexin V/FITC and propidium iodide (PI). The types of cell death were determined by FITC and PI fluorescence measurement using flow cytometry. The data were shown as the percentage of stained cell of total cells. Values are mean ± S.E. for 3 independent experiments. **$p < 0.01$ compared with the vehicle-treated cells as determined by student's unpaired t-test and "ns" represents non significance.

4.3.4 Cadmium induces caspase-independent apoptotic cell death.

Since previous studies reported that cadmium induced apoptotic cell death by either caspase-dependent or caspase-independent pathway in cultured kidney proximal tubular cells (1), procaspases-3 expression in HK-2 cells induced by cadmium was determined by Western blotting. As show in figure 4.6, the HK-2 cells treated with 20 µM $CdCl_2$ for 12 h had no effect on procaspases-3 expression, indicating that cadmium-induced apoptotic cell death should be caspase-independent pathway.

A.



B.

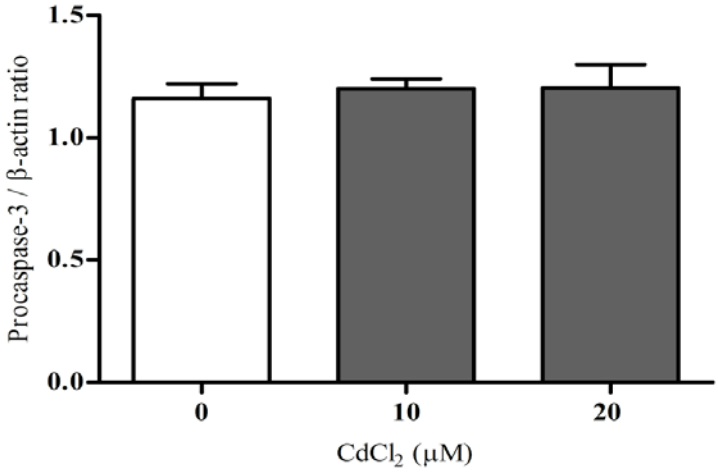


Figure 4.6 Effect of $CdCl_2$ on procaspase-3 expression in HK-2 cells. (A) HK-2 cells were treated in serum-free media with vehicle or, 10, 20 μM $CdCl_2$ for 12 h. Subsequently, equal amounts of total proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane in preparation for Western blot analysis. Proteins were probed with anti-procaspase-3 antibody. (B) The density of protein expressions were measured by image J analysis. Values are means ± S.E. of three independent experiments.

4.4 Effect of LXR activation on cadmium-induced cytotoxicity in human renal proximal tubular cells, HK-2 Cell.

The LXRs have been reported to have anti-oxidant effect, anti-inflamation and protection of cell death in endotoxin-induced mice through inhibition of pro-

apoptotic protein including JNK signaling (2). Therefore, the hypothesis was set that LXR activation attenuate cadmium-induced cytoxicity in human renal tubular cell. To investigate whether the effects of LXR activation protected cadmium-induced cytotoxicity in renal proximal tubular cell, following experiments were performed.

4.4.1 The dose-dependent effect of T0901317 on cadmium-induced cell death.

To investigate whether LXR activation decreased cell death induced by cadmium, the dose-dependent effect of T0901317, LXR agonist, on $CdCl_2$-induced cell death was determined. HK-2 cells were incubated with vehicle, 20 µM $CdCl_2$, 1-10 µM T0901317 (T0), or combination of $CdCl_2$ plus T0901317 for 24 h. As shown in figure 4.7A, the concentration of T0901317 at 10 µM but not 1 µM and 5 µM significantly attenuated $CdCl_2$-induced reduction of cell viability. Therefore, 10 µM T0901317 was selected to study the effects and underlying mechanism of LXR activation on $CdCl_2$-induced cell death.

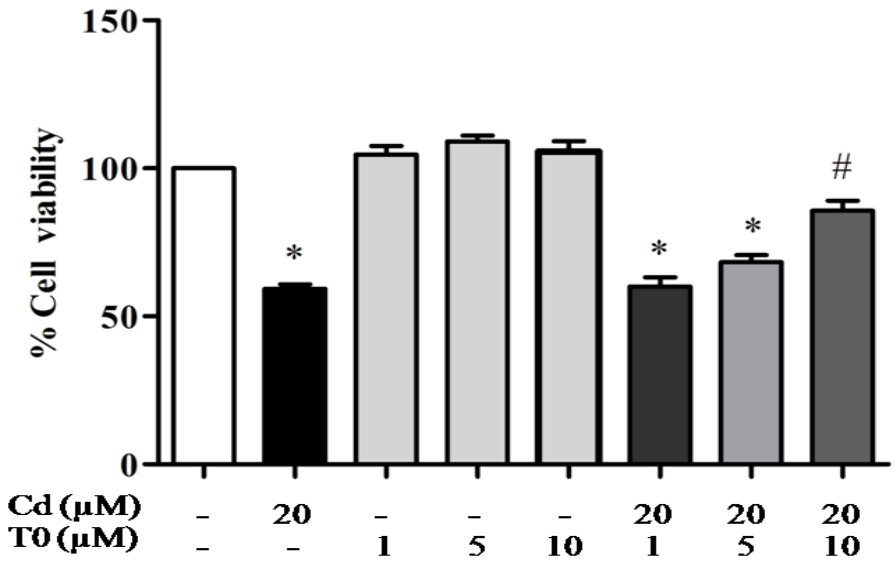
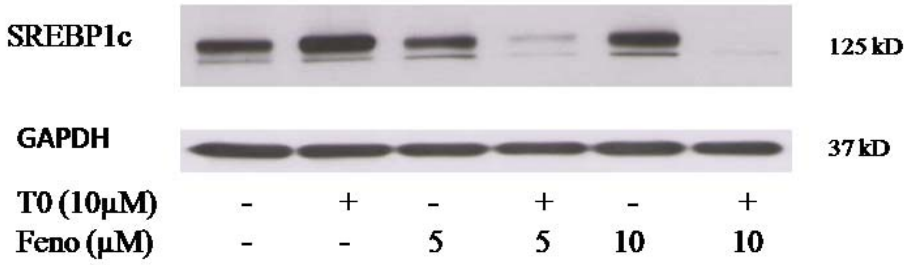


Figure 4.7 The dose-dependent effect of LXR agonist on cadmium-induced cell viability. HK-2 cells were incubated with vehicle, 20 μM $CdCl_2$ for 24 h, (1–10 μM) T0901317 for 24 h, or combination of $CdCl_2$ plus T0901317 for 24 h as indicated and then followed by determination of cell viability using MTT assay. The bar graphs represented percent of HK-2 cell viability of 3 independent experiments. *$p < 0.05$ compared with control, #$p < 0.05$ compared with $CdCl_2$-treated cell.

4.4.2 The antagonized effect of fenofibrate on LXR activation

Since the antagonized effect of the fenofibrate on LXRs has been reported, fenofibrate was used to investigate the protective effect of LXR activation on cadmium-induced cell death. To confirm whether the antagonized effect of fenofibrate decreased LXR activation, the inhibition effect of fenofibrate on LXR activation was determined. HK-2 cells were incubated with vehicle, 10 μM T0901317, 5-10 μM fenofibrate (Feno), or combination of T0901317 plus fenofibrate for 24 h. At the end of incubation period, SREBP-1c protein expression, LXR-targeted gene product was determined by Western blotting. As shown in figure 4.8, the concentration of fenofinrate at 10 μM significantly decreased SREBP-1c protein expression, indicating that fenofibrate can antagonize LXR. Therefore, 10 μM fenofibrate was selected to use as LXR antagonist.

A



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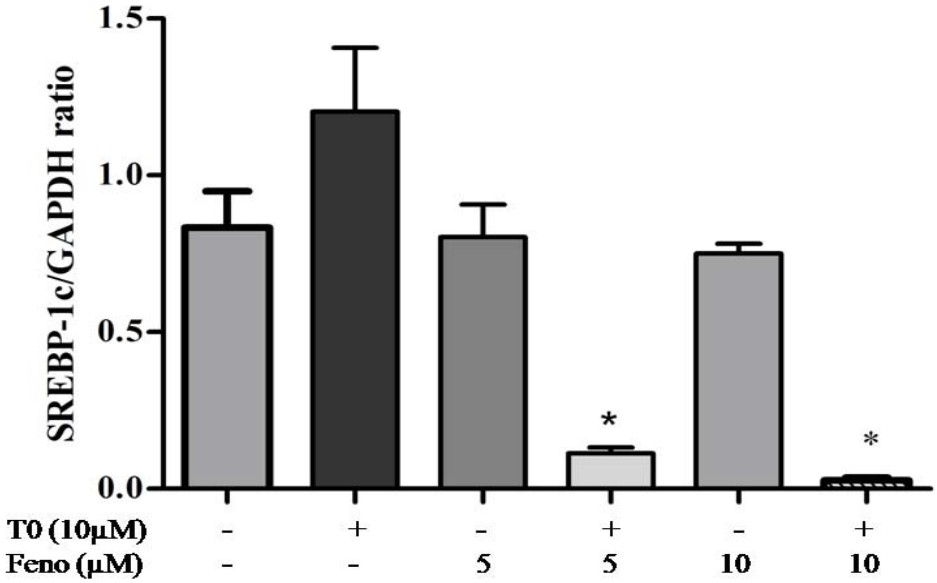


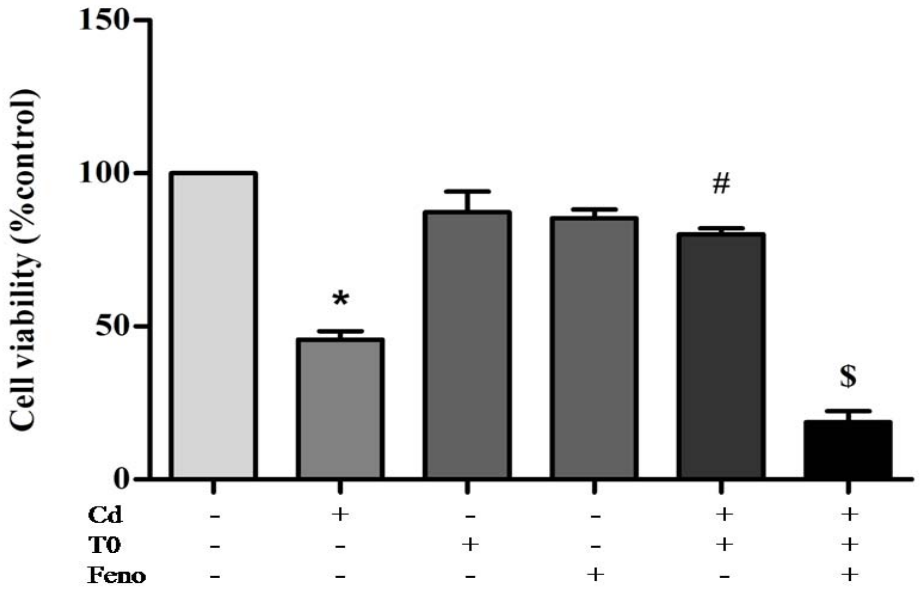
Figure 4.8 The antagonized effect of the fenofibrate on LXR activation**.** (A) HK-2 cells were incubated with vehicle, 10 μM T0901317 (T0), 5-10 μM fenofibrate (Feno), or combinations 10 μM T0 and 5-10 μM feno of for 24 h followed by determination of SREBP-1c protein expression. (B) The density of protein expressions were measured by image J analysis. Values are means ± S.E. of three independent experiments. *p < 0.05 compared to T0901317 alone**.**

4.4.3 The protective effect of LXR activation on cadmium – induced apoptotic cell death

To determined whether the protective effect of LXR activation on admium toxicity required activation of LXR, HK-2 cells were incubated under six conditions: 1) control medium for 24 h, 2) medium containing 10 μM T0901317 for 24 h, 3) medium containing 20 μM $CdCl_2$ for 24 h, 4) medium containing 10 μM fenofibrate, a LXR antagonist, for 24 h, 5) medium containing 10 μM T0901317 for 24 h before changing the medium to one containing 10 μM T0901317 and 20 μM $CdCl_2$, and incubated for further 24 h, and 6) medium containing 10 μM T0901317 and 10 μM fenofibrate for 24 h and then further incubated in the medium containing 10 μM

T0901317, 10 μM fenofibrate, and 20 μM $CdCl_2$ for further 24 h. At the end of incubation, cell viability and apoptotic cell death were determined using MTT assay and flow cytometry, respectively. As shown in figure 4.9 A, the reduction of cell viability induced by $CdCl_2$ was attenuated by pretreatment with T0901317. Moreover, cell damage was not observed in HK-2 cells treated with cadmium plus T0901317 compared with HK-2 cells treated with cadmium alone as shown in figure 4.9 B (cell morphology), indicating that T0 901317 protected $CdCl_2$-induced cell damage. In addition, the protective effect of T0901317 on $CdCl_2$-induced cell viability was eliminated by the fenofibrate, indicating that the protective effect of T0901317 was through LXR-dependent mechanism. Furthermore, the protective effect of T0901317 on cadmium-induced apoptotic cell death was also abolished by fenofibrate (figure 4.9 C, D).

A



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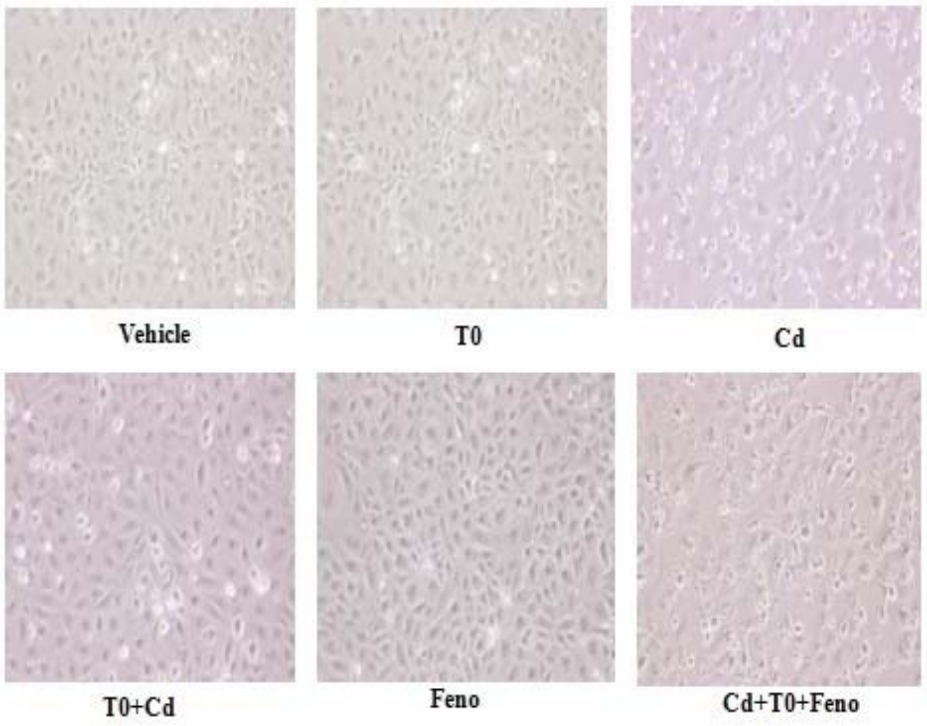
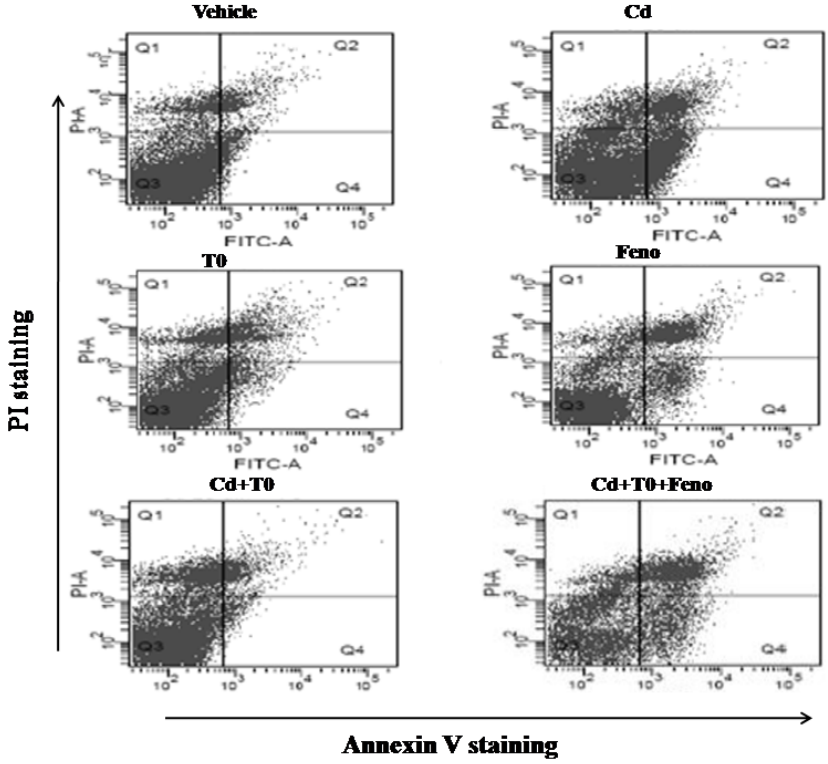

Vehicle
T0
Cd
T0+Cd
Feno
Cd+T0+Feno

C


Vehicle
Cd
T0
Feno
Cd+T0
Cd+T0+Feno
PI staining
Annexin V staining
FITC-A
PHA
Q1
Q2
Q3
Q4

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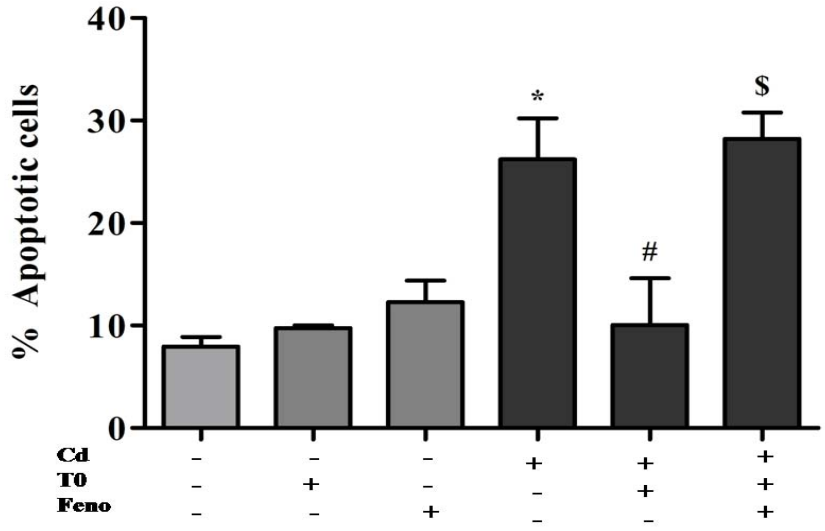
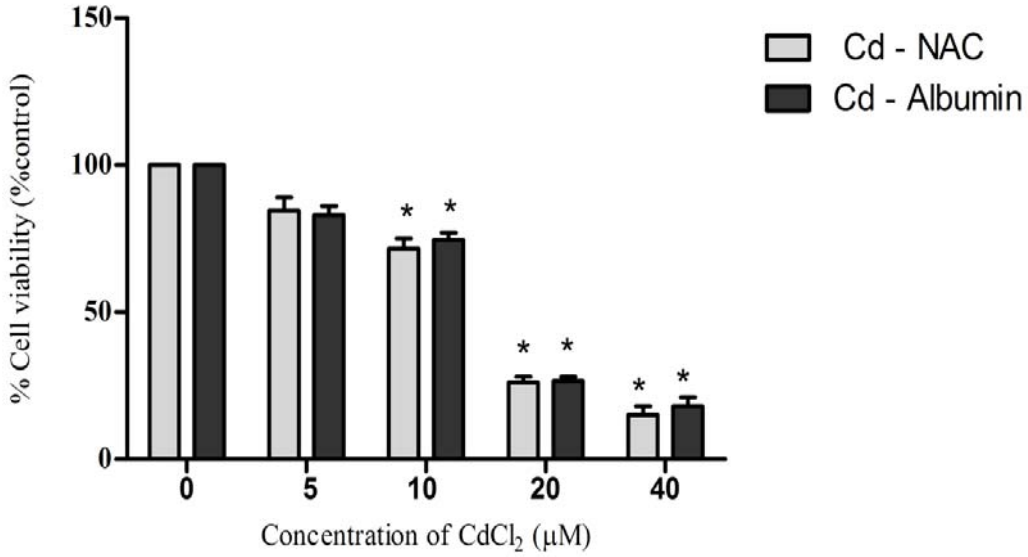


Figure 4.9 Effect of LXR activation on cadmium-induced apoptotic cell death of HK-2 cells. (A) The effects of fenofibrate (feno) on the protective effect of T0901317; HK-2 cells were incubated with vehicle, 20 μM $CdCl_2$ (Cd), 10 μM T0, 10 μM Feno, or combinations for 24 h as indicated (see detail in result) followed by determination of cell viability and observation of cell morphological change. (B) Cell morphology was observed by phase-contrast microscopy following the cells treated with vehicle, 20 μM $CdCl_2$ (Cd), 10 μM T0, 10 μM Feno, or combinations for 24 h. The data are expressed as a percentage of control (mean $\pm$ S.E.). (C) The effects of fenofibrate on the protective effect of T0. HK-2 cells were incubated with the same condition in C, and then the HK-2 cells stained with annexin-V/FITC and PI were determined by flow cytometry. Apoptotic cells were localized in the lower right (early apoptosis) and upper right (late apoptosis) quadrants of the dot-pot graph using propidium iodide vs annexin V. (D) The bar graphs represented percent of HK-2 cell apoptosis of 3-independent experiments. *$p < 0.05$ compared with control, #$p < 0.05$ compared with $CdCl_2$-treated cell, and $p < 0.05$ compared with $CdCl_2$ plus T0-treated cells.

4.5 Effect of LXRs on bound forms of cadmium-induced cell death in HK-2 cells

Since HK-2 cells were exposed directly to CdCl2, which is not a physiological form of cadmium. Within biological systems, Cd is usually bound to a thiol-containing molecule such as albumin, cysteine, glutathione (GSH), or N-acetylcysteine. Therefore, the experiments had been carried out in order to determine if Cd-induced toxicity is different in cells exposure to an albumin or NAC conjugate of Cd. The bound forms of cadmium including $CdCl_2$-N-acetyl-Lcysteine (NAC) and $CdCl_2$-albumin (Cd-Abl) were prepared by mixing of $CdCl_2$ and NAC with mole ratio of 1:2 in serum-free medium and adding of $CdCl_2$ into serum-free medium containing 5 mg/dl albumin, respectively. To determine the effects of bound forms of cadmium on cell viability, HK-2 cells were incubated in medium containing several concentrations of bound forms of cadmium for 24 h. As shown in figure 4.10 A, both $CdCl_2$-NAC and $CdCl_2$-albumin reduced cell viability of HK-2 cells in concentration-dependent manner. The effects of T0901317 on reduction of cell viability induced by $CdCl_2$-NAC and $CdCl_2$-albumin were also investigated. HK-2 cells were incubated with vehicle, 10 μM T0901317, 20 μM of cadmium in the form of $CdCl_2$-NAC or $CdCl_2$-albumin, and T0901317 plus $CdCl_2$-NAC or $CdCl_2$-albumin for 24 h. The results showed that the reduction of cell viability induced by bound form of cadmium was also attenuated by T0901317 (figure 4.10 B).

A.



B.

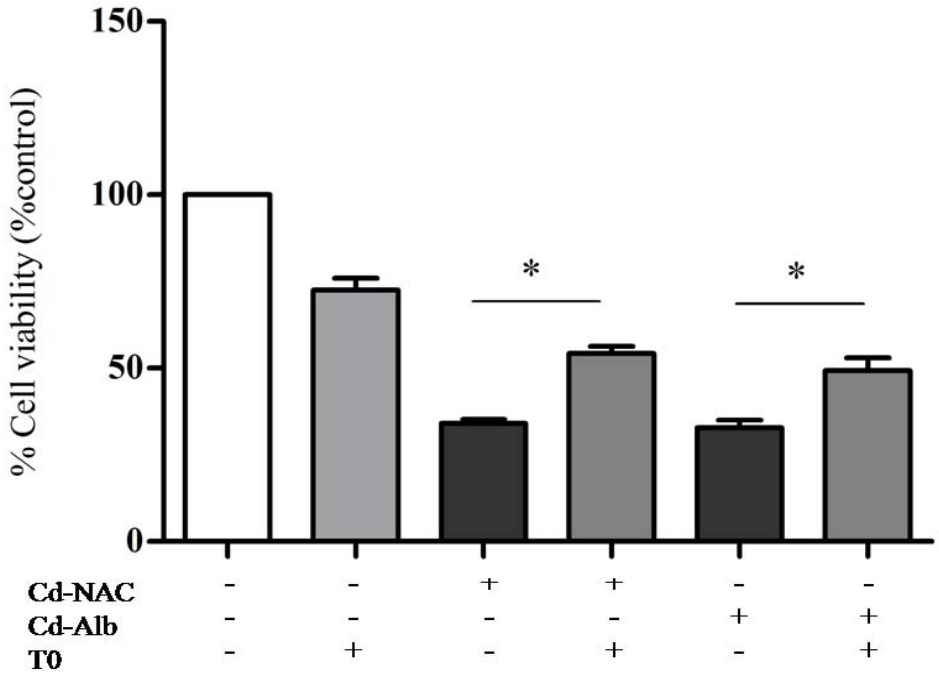


Figure 4.10 Effect of LXR agonist on reduction of cell viability induced by bound forms of cadmium. (A) Effect of bound forms of cadmium on cell viability; HK-2

cells were incubated in medium containing several concentrations of bound forms of cadmium ($CdCl_2$–NAC and $CdCl_2$–albumin) for 24 h prior determination of the cell viability by MTT assay. (B) Effect of T0901317 on bound forms of cadmium-induced reduction of cell viability; HK-2 cells were incubated with vehicle, 10 μM T0901317 (T0) for 24 h, 20 μM of cadmium in the form of $CdCl_2$–NAC or $CdCl_2$–albumin (Alb) alone for 24 h, and T0 plus $CdCl_2$–NAC or $CdCl_2$–Alb for further 24 h. Data expressed as mean ±S.E. from 3 experiments. *$p < 0.05$ compared with Cd-NAC lone.

4.6 Effect of PPARα activation on protective effect of LXR activation in cadmium-induced cytotoxicity.

Although fenofibrate, used as LXR antagonist, prevents SREBP-1c induction by T0901317, it has also been reported to posses other activities including activation of PPARα receptors that could be counter balancing LXR protection by changing availability or signaling of the heterodimeric partner, RXR. Therefore, this hypothesis was determined by PPARα antagonist. To determine whether the PPARα activation involved the effect of LXR activation, the effect of PPARα antagonist on effect of LXR activation was revealed. HK-2 cells were treated under seven conditions: (1) vehicle for 24 h; (2) medium containing 20 μM $CdCl_2$ for 24 h; (3) medium containing 10 μM T0901317 for 24 h; (4) medium containing 10 μM fenofibrate for 24 h; (5) medium containing 0.5 μM GW 6471 for 24 h; (6) medium containing 10 μM T0901317 and 10 μM fenofibrate for 24 h and then further medium containing 10 μM T0901317, 10 μM fenofibrate , and 20 μM $CdCl_2$ for another 24 h; (7) medium containing 10 μM T0901317, 10 μM fenofibrate, and 0.5 μM GW 6471 for 24 h and then further incubated in medium containing 10 μM T0901317, 10 μM fenofibrate, 0.5 μM GW 6471, and 20 μM $CdCl_2$ for another 24 h. At the end of incubation, cell viability was determined using MTT assay. As show in figure 4.11, the result showed that the antagonized effect of fenofibrate on protection of LXR activation T0901317 was not affected by GW 6471, a PPARα antagonist, indicating that the protective effect of T0901317 in cadmium-induced toxicity was not involved with reduction of RXR, a heterodimer binding protein, in PPARα activation.

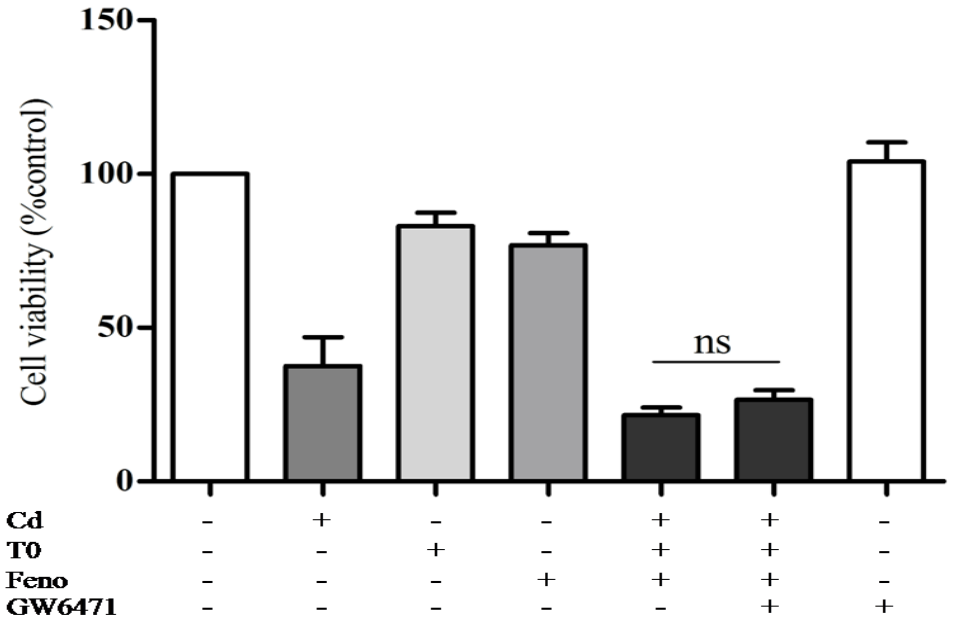


Figure 4.11 Effect of PPARα activation on protective effect of LXRs in cadmium-induced cytotoxicity. HK-2 cells were incubated with vehicle, 20 μM $CdCl_2$ (Cd), 10 μM T0901317 (T0), 10 μM fenofibrate (Feno), 0.5 μM GW6471, or combination for 24 h, and then followed by determination of cell viability using MTT assay. The bar graphs represented percent of control (mean ± S.E.) from 3 experiments. "ns" represents non significance.

4.7 Effect of pregnane X receptor (PXR) on cadmium-induced cytotoxicity in HK-2 cells.

Since synthetic LXR agonist, T0901317, had reported to cross talk with PXR. The purpose of this experiment was to determine whether the PXR activation inhibited cadmium-induced cytotoxicity. HK-2 cells were incubated under six conditions: 1) control medium for 24 h, 2) medium containing 20 μM rifampicin for 24 h, 3) medium containing 20 μM $CdCl_2$ for 24 h, and 4) medium containing 20 μM rifampicin for 24 h before changing the medium to one containing 20 μM rifampicin and 20 μM $CdCl_2$, and incubated for another 24 h. At the end of incubation, cell viability was determined by MTT assay. As shown figure 4.12, PXR activation by

rifampicin had no effect on cadmium-induced cytotoxicity in renal proximal tubular cells.

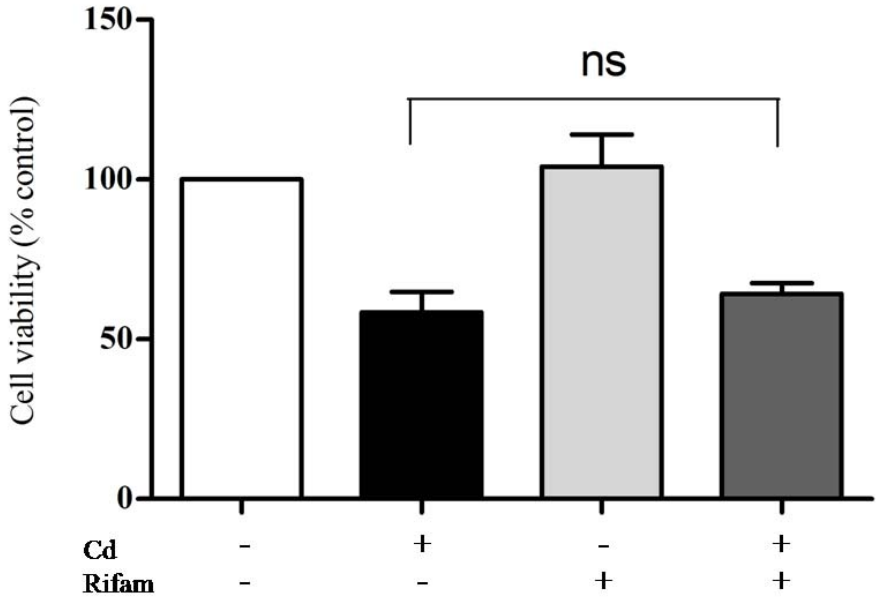


Figure 4.12 Effect PXR activation on cadmium-induced cytotoxicity in human renal tubular cells. HK-2 cells were incubated with vehicle, 20 μM $CdCl_2$ (Cd), 20 μM rifampicin (Rifam), or combinations for 24 h as indicated (see detail in text) followed by determination of cell viability. Cell viability was determined by MTT assay. The data were expressed as a percentage of control (mean $\pm$ S.E.) from 3 experiments. "ns" represents non significance

4.8 Effect of LXRs on cadmium-induced intracellular ROS generation in renal proximal tubular cell.

It has been known that cadmium induces intracellular ROS generation leading to increase cell death. Therefore, it is possible that mechanism responsible for LXR activation attenuating toxicity of cadmium may be mediated by reduction of ROS.

4.8.1 Cadmium induces cytotoxicity through ROS generation.

Since it has been shown that cadmium toxicity is mediated by induction of ROS generation, this study investigated whether a ROS scavenger, N-acetyl-L-cysteine (NAC), inhibited cadmium toxicity. HK-2 cells were incubated in four conditions: 1) medium containing vehicle for 24 h, 2) medium containing 20 μM $CdCl_2$ for 24 h, 3) medium containing 0.5 or 1 mM NAC for 24 h, and 4) medium containing 1 mM NAC plus 20 μM $CdCl_2$ for 24 h after preincubation with 1 mM NAC for 4 h. At the end incubation, cell viability was determined by MTT assay. As shown in figure 4.13, NAC treatment significantly inhibited $CdCl_2$ toxicity, indicating that cadmium induced cytotoxicity via ROS generation.

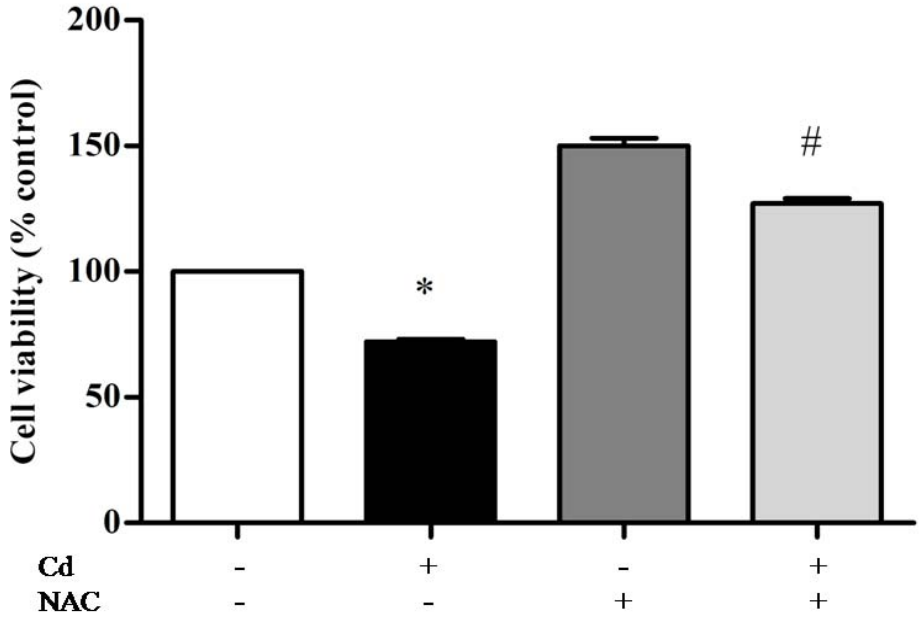


Figure 4.13 Effect of ROS scavenger on cadmium-induced cytotoxicity in human renal tubular cell. HK-2 cells were treated with vehicle, 20 μM $CdCl_2$ (Cd), 1 mM NAC, and 20 μM $CdCl_2$ plus 1 mM NAC for 24 h, and followed by measurement of cell viability using MTT assay. The data are expressed as a percentage of control (mean $\pm$ S.E.). *$p < 0.05$ compared with the vehicle-treated cells as determined by ANOVA. #$p < 0.05$ compared with cadmium-treated cells.

4.8.2 Effect of LXR agonist on cadmium-induced ROS generation in human proximal tubular cells.

The purpose of this experiment was to determine whether T0901317 reduced ROS generation induced by $CdCl_2$. Intracellular ROS level in renal proximal tubular, HK2-cells, was measured after the cells were pre-treated with 10 μM T0901317 for 24 h and then incubated with $CdCl_2$ for 3 h. As shown in figure 4.14**,** exposure the cells to $CdCl_2$ significantly increased intracellular ROS levels and the induction of ROS was significantly eliminated by T0901317 treatment. This result indicated that LXR activation restored cellular oxidative state induced by $CdCl_2$, leading to reduction of cell viability.

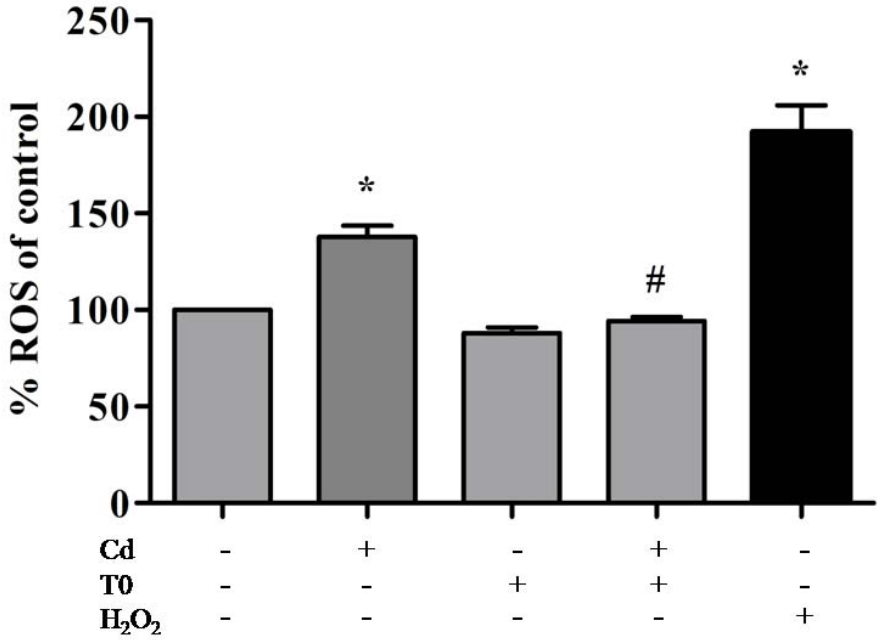


Figure 4.14 Effect of LXR agonist on cadmium induced-intracellular ROS generation in HK-2cells. HK-cells were preincubated with 10 μM T0901317 (T0) or serum-free media for 24 h prior loading with 10 μM DCHF-DA in DPBS for 30 min. After that cells were incubated with 100 μM $CdCl_2$ (Cd) for 3 h followed by movement of DCHF fluorescence**.** The cells incubated with 200 μM of H_2O_2 for 1 h as positive control. The intracellular ROS are expressed as a percentage of vehicle-treated cells. Each bar represents the mean ± S.E. of three independent experiments. *$p < 0.05$

compared with the vehicle-treated cells. #$p < 0.05$ compared with the $CdCl_2$-treated cells**.**

4.8.3 Effect of LXR agonist on expression of enzyme involving ROS production in human proximal tubular cells.

Intracellular ROS induced by cadmium is regulated by ROS production and antioxidant enzymes. Therefore, it is possible that mechanism responsible for LXR activation decreases toxicity of cadmium is mediated by inhibition of enzymes in ROS production and stimulating of antioxidant enzymes**.** The mechanisms by which LXRs abolished $CdCl_2$-induced ROS generation were investigated. HK-2 cells were incubated with vehicle or 20 μM $CdCl_2$ for 4 h. At the end of incubation period, mRNA expression of catalase (CAT), superoxide dismutases (SOD) and NAD(P)H dehydrogenase/NADH: quinone oxidoreductase-1 (NQO1) was determined by real-time PCR. Real-time PCR data revealed that $CdCl_2$ treatment for 4 h significantly increased mRNA of NQO1 expression, but decreased mRNA expression of CAT. However, $CdCl_2$ had no effect on mRNA expression of SOD (Figure 4.15A). Next, the effects of T0901317 on change of CAT and NQO1 mRNA expressions induced by $CdCl_2$ were investigated. The real-time PCR showed that preincubation of cells with T0901317 for 24 h reversed the inhibitory effect of $CdCl_2$ on CAT and NQO1 mRNA expressions as shown in figure 4.15B**.**

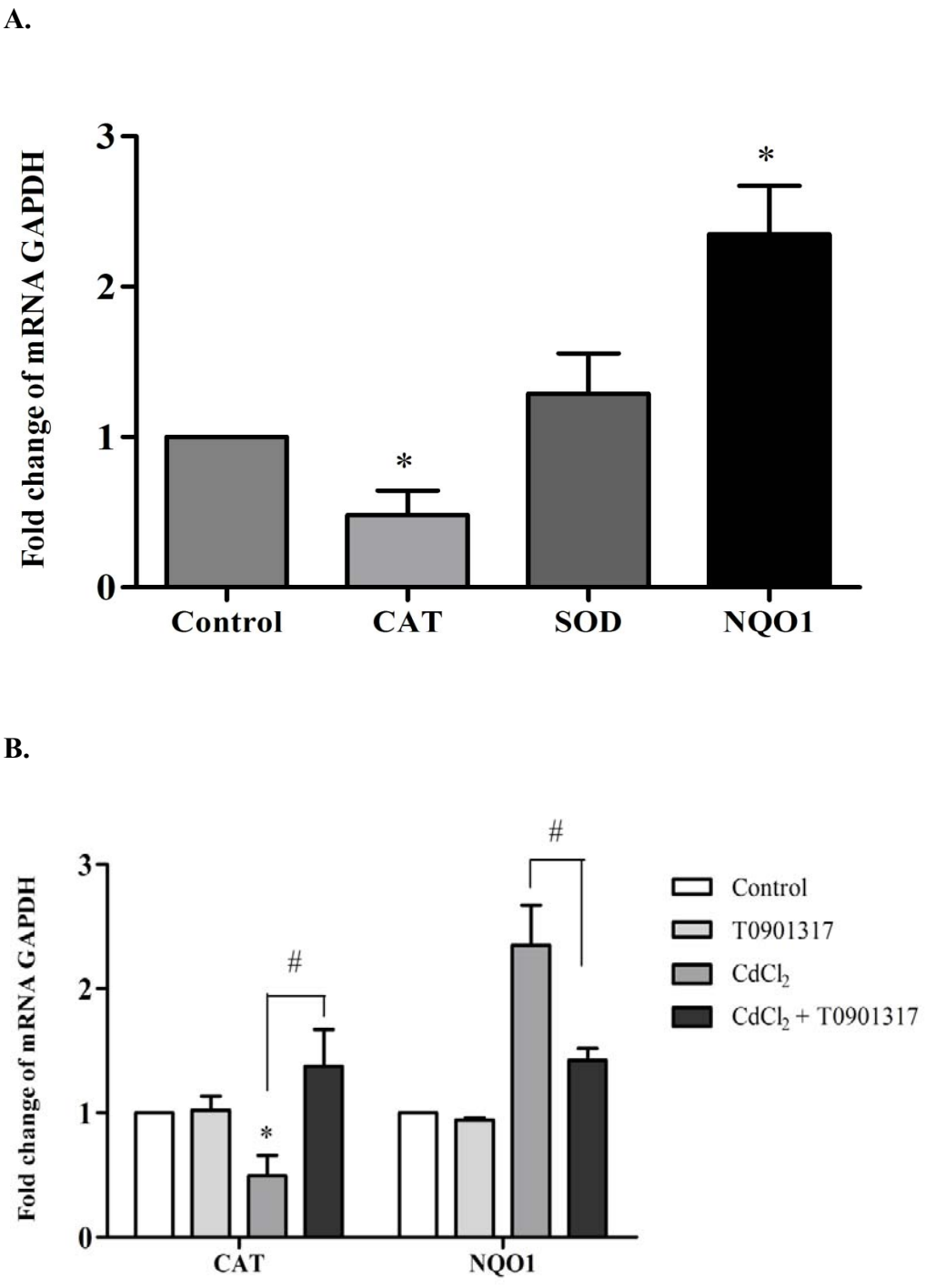


Figure 4.15 Effect of T0901317 on antioxidant enzymes in renal proximal tubular cells. (A) Effect of cadmium on mRNA of antioxidant enzymes in HK-2 cells. Expressions of mRNA of antioxidant enzymes including CAT and NQO1, were determined by real-time PCR. HK2-cells were incubated with 20 μM $CdCl_2$ for 4 h. The expression of mRNA was expressed as fold change of mRNA GAPDH. (B) HK-2

cells were treated with vehicle, 20 μM $CdCl_2$, 10 μM T0901317, and 20 μM $CdCl_2$ plus 10 μM T0901317 for 4 h. The data expressed were mean ± S.E. of transcriptional accumulation index (TAI) values of three independent experiments. mRNA expression of GAPDH was used as internal control. Each bar represents the mean ± S.E. of three independent experiments. *$p < 0.05$ compared to the vehicle-treated cells. #$p < 0.05$ compared with the cadmium-treated cells.

4.8.4 Effect of N-acetyl-L-cysteine (NAC) on NQO1 expression induced by cadmium in human proximal tubular cells.

NQO1 is recognized as an antioxidant enzyme, however, it also has been reported as an enzyme producing ROS as evidence showing that reduction of NQO1 expression using siRNA leads to decreased ROS production. This experiment was set up to determine whether an increase in mRNA expression of NQO1 was a sequence of ROS generation induced by $CdCl_2$. As shown in figure 4.16, mRNA expression of NQO1 induced by $CdCl_2$ was in time-dependent manner, and significantly inhibited by NAC. These data implied that elevated mRNA expression of NQO1 was occurred after intracellular ROS was generated.

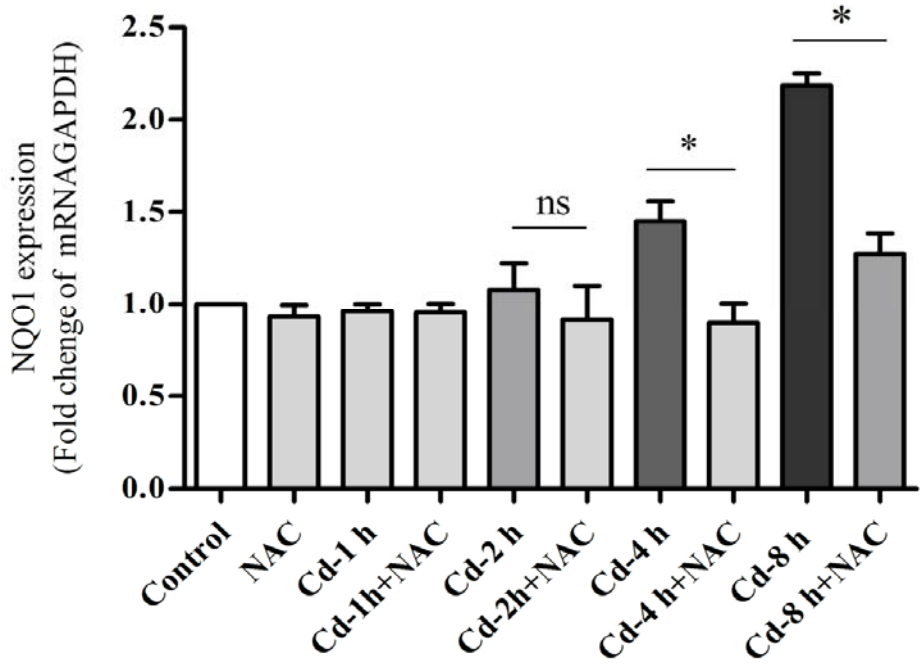


Figure 4.16 Effect of NAC on $CdCl_2$-induced mRNA expression of NQO1 in HK-2 cells. HK-2 cells were treated with medium containing (1) vehicle, (2) 1 mM NAC, (3) 20 μM $CdCl_2$, and (4) combination of NAC and $CdCl_2$ for several time points followed by determination of mRNA by real-time PCR. The data expressed were mean ± S.E. of transcriptional accumulation index values of three independent experiments. GAPDH was used as internal control.*$p < 0.05$ compared to $CdCl_2$-treated cells.

4.9 Effect of LXR activation on cadmium-activated MAPK signaling in human renal proximal tubular cells.

It has been reported that mitogen-activated protein kinase (MAPK) signaling pathways play a role in apoptosis. In addition, previous study demonstrated that cadmium induces apoptotic cell death via caspase-independent pathway through JNK phosphorylation (p-JNK) (3). Therefore, it was possible that mechanism responsible for LXR activation attenuating toxicity of cadmium was mediated by inhibition of p-JNK.

4.9.1 Effect of LXR activation on cadmium-induced JNK expression in HK-2 cells.

The purpose of this experiment was to investigate whether LXR inhibited cadmium induced JNK phosphorylation (p-JNK). HK-2 cells were incubated in six conditions: 1) control medium for 12 h, 2) medium containing 20 μM $CdCl_2$ for 12 h, 3) medium containing 10 μM T0901317 (T0) for 12 h, 4) medium containing 10 μM T0901317 (Cd) for 24 h and followed by treatment with 20 μM $CdCl_2$ plus 10 μM T0901317 for further 12 h. 5) medium containing 10 μM fenofibrate (Feno) for 12 h, and 6) medium containing 10 μM fenofibrate and 10 μM T0901317 for 24 h and then medium containing 10 μM Feno, 10 μM T0, and 20 μM Cd for 12 h. As shown in figure 4.17, Western blot data revealed that treatment of the cells with 20 μM $CdCl_2$ for 12 h significantly stimulated phosphorylated JNK but not total JNK. The effect of $CdCl_2$ on p-JNK expression was abolished by pretreatment the cells with 10 μM T0901317. In addition, the effect of T0901317 on JNK expression was attenuated by

10 μM fenofibrate, LXR antagonist. These data indicated that LXR activation inhibited cadmium-induced JNK phosphorylation.

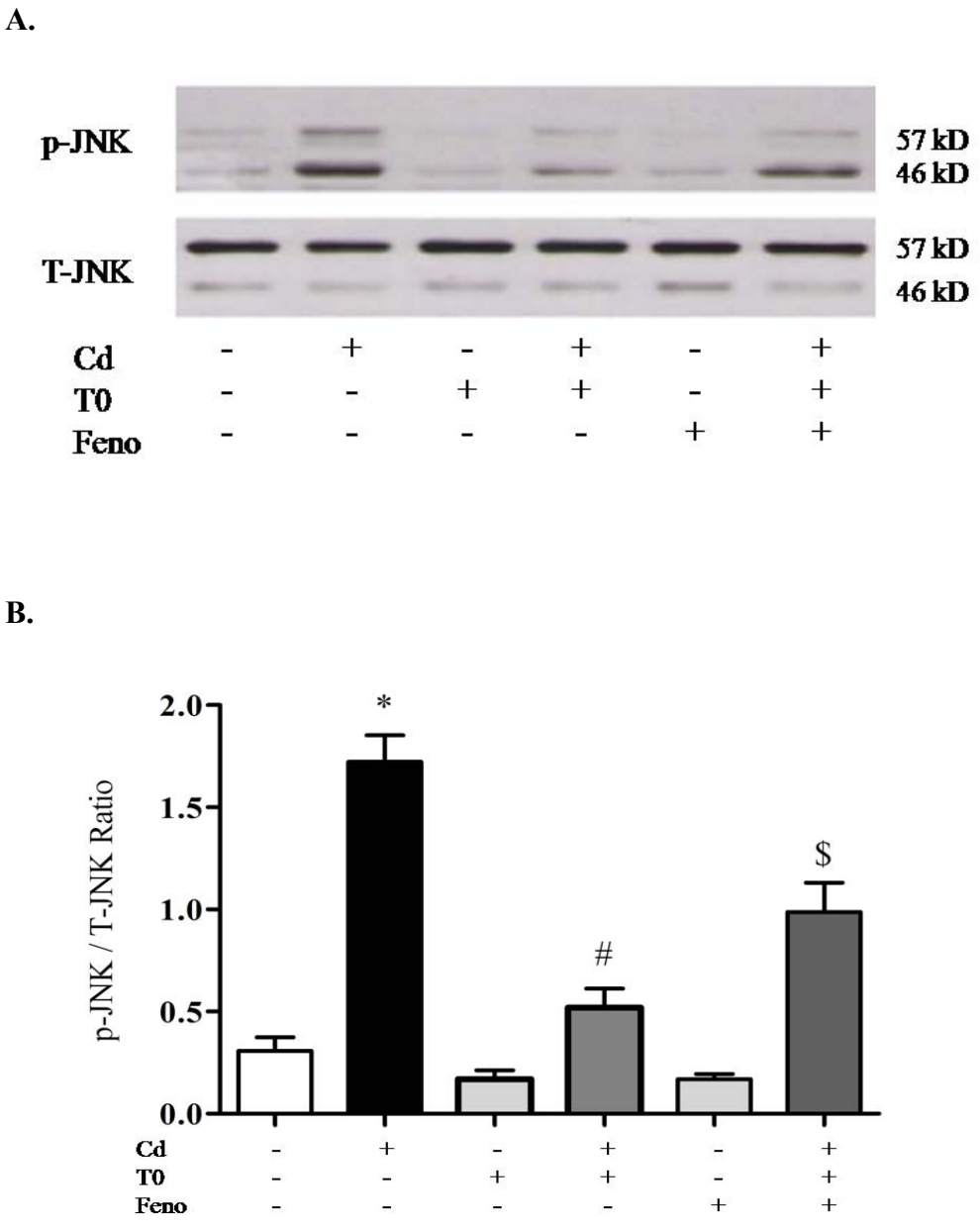


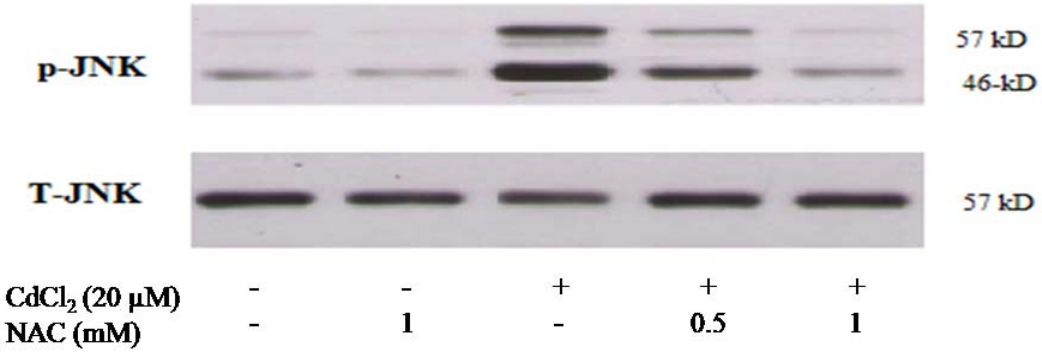
Figure 4.17 Effect of LXR activation on $CdCl_2$-induced JNK protein expression in renal proximal tubular cells. (A) HK-2 cells were pretreated in serum-free media with vehicle, CdCl2, 10 μM T0901317, 10 μM fenofibrate, or combination. Subsequently, equal amounts of total proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane in preparation for Western blot analysis. Proteins were

probed with phosphorylated JNK (p-JNK) and total JNK (T-JNK). (B) The density of protein expressions were measured by Image J analysis. Values are means ± S.E. of three independent experiment. *p < 0.05 compared with the vehicle-treated cells, #p < 0.05 compared with the CdCl2-treated cells, and $p < 0.05 compared with the cells treated with CdCl2 plus T0901317 as determined by one-way ANOVA.

4.9.2 Cadmium induces p-JNK signaling through ROS generation in renal proximal tubular cells.

Since cadmium ($CdCl_2$) induction of ROS generation and activation of JNK signaling in HK-2 cells have been shown, this experiment was set to investigate whether cadmium induced phosphorylated-JNK (p-JNK) expression required ROS generation. HK-2 cells were pretreated with 0.5 or 1 mM NAC for 2 h and then incubated with 20 µM $CdCl_2$ for 9 h. Western blot analysis revealed that phosphorylation of JNK induced by $CdCl_2$ was abolished by NAC treatment as shown in figure 4.18. These results indicated that p-JNK is downstream of ROS generation.

A.



B.

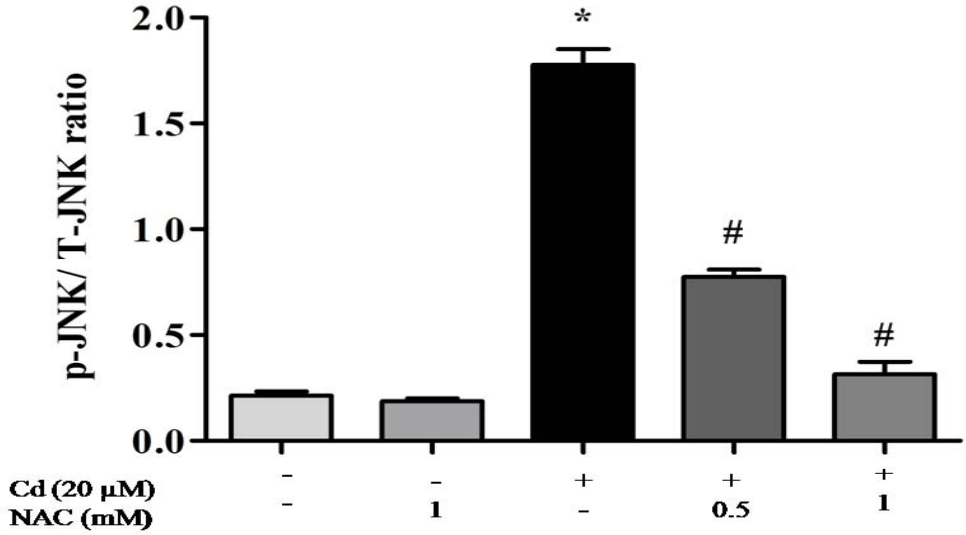


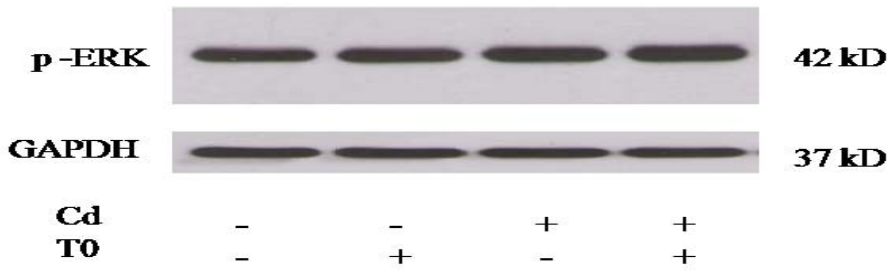
Figure 4.18 $CdCl_2$ induced JNK phosphorylation (p-JNK) via ROS production in HK-2 cells. (A) HK-2 cells were treated with vehicle or NAC (0.5-1 mM) for 2 h and then incubated with 20 µM $CdCl_2$ (Cd) alone or NAG (0.5-1 mM) plus 20 µM Cd for 12 h. At the end of incubation period, p-JNK and total JNK (T-JNK) proteins were determined by Western blotting. (B) The density of protein expressions were measured by Image J analysis. The data were expressed as the ratio of p-JNK/T-JNK expression. Values are means ± S.E. of 3 experiments. *$p < 0.05$ compared to control whereas, #$p < 0.05$ compared to cadmium-treated cells.

4.9.3 Effect of LXR activation on cadmium-induced ERK expression in HK-2 cells.

The ERK signaling which was early mediated by cadmium exposure in renal proximal tubular cell has been reported (3). This experiment was set to determine whether LXR activation attenuated ERK signaling protein induced by $CdCl_2$. HK-2 cells were incubated in four conditions: 1) vehicle medium for 6 h, 2) medium containing 20 µM $CdCl_2$ for 6 h, 3) medium containing 10 µM T0901317 for 24 h, 4) medium containing 10 µM T0901317 for 24 h and followed by treatment with 20 µM $CdCl_2$ plus 10 µM T0901317 for further 6 h. As shown in figure 4.19, Western blot data revealed that the treatment of cells with $CdCl_2$ significantly stimulated phosphorylated ERK (p-ERK). This effect of $CdCl_2$ on p-ERK expression was not

attenuated by pretreatment the cells with T0901317, indicating that LXR activation did not inhibit cadmium-induced ERK phosphorylation in HK-2 cell.

A.



B.

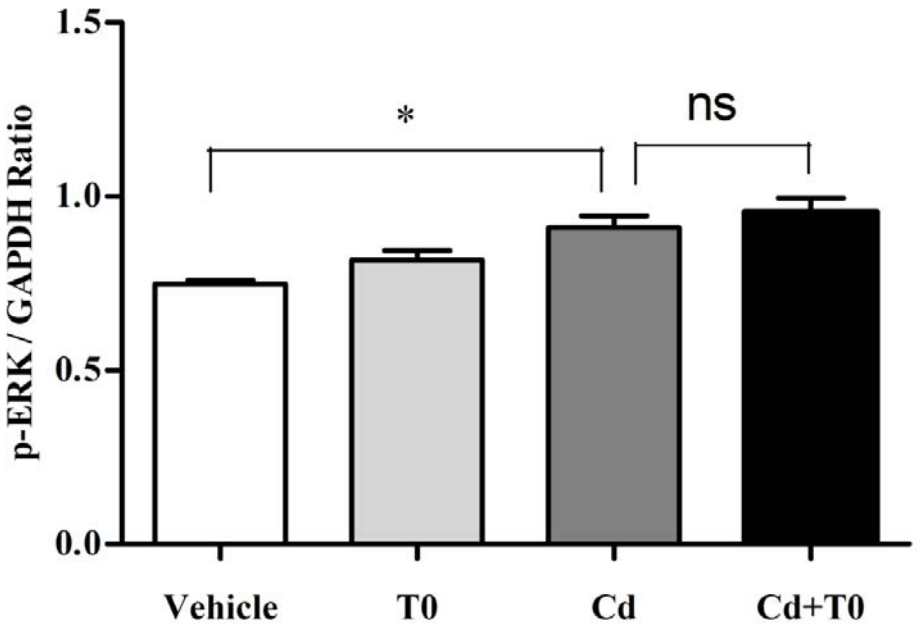


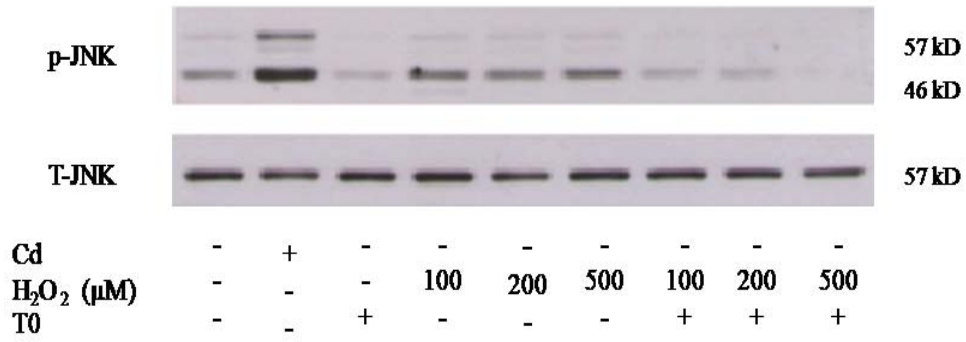
Figure 4.19 Effect of LXR activation on $CdCl_2$-induced ERK protein expression in HK-2 cells. (A) K-2 cells were treated with different conditions as indicated (see detail in text). Subsequently, equal amount of total proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane in preparation for Western blot analysis. Proteins were probed with antibody against phosphorylated ERK (p-ERK) and GAPDH. (B) The density of protein expressions were measured by Image J analysis.

Values are means ± S.E. of 3 independent experiment. *p < 0.05 compared to control. "ns" represents no significance.

4.10 Direct affect of LXR activation on cadmium-induced JNK expression in HK-2 cells.

The present data have shown that LXR activation decreases ROS generation leading to decreasing of JNK phosphorylation (p-JNK). It is interesting whether LXR activation directly inhibits p-JNK expression. HK-2 cells were incubated in 5 conditions: 1) vehicle for 24 h, 2) 10 μM T0901317 for 24 h, 3) several concentration of H_2O_2 (100, 200, and 500 μM) for 9 h, 4) pretreatment with 10 μM T0901317 for 24 h followed by incubation with 10 μM T0901317 plus H_2O_2 at several concentrations (100, 200, and 500 μM) for another 9 h, and 5) 20 μM $CdCl_2$ as positive control. As shown in figure 4.18, JNK phosphorylations (p-JNK) stimulated by loading of H_2O_2 (200, and 500 μM) were still attenuated by T0901317, indicating that LXRs activation directly inhibited p-JNK expression.

A.



B.

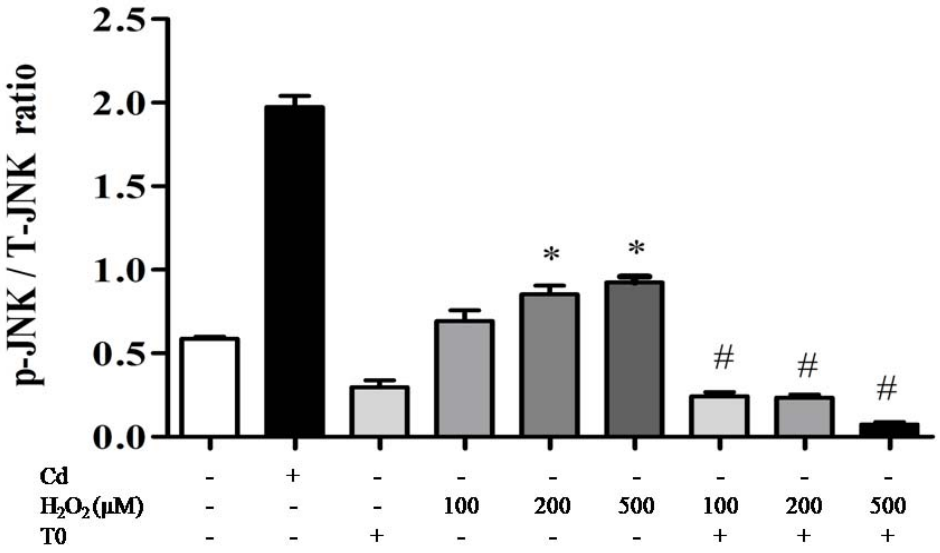


Figure 4.20 Direct effect of LXR activation on JNK phosphorylation in renal proximal tubular cells. (A) HK-2 Cells were treated with 20 μM $CdCl_2$ (Cd), 10 μM T0901317 (T0), 200-500 μM H_2O_2, or combination of T0901317 (T0) and H_2O_2. At the end of treatment, cells were harvested by lysis buffer and protein expressions were determined by Western blot with specific anti-p-JNK and T-JNK antibodies. (B) The density of protein expressions were measured by Image J analysis**.** Values are means ± S.E. of 3 independent experiments. *$p < 0.05$ compared with the vehicle-treated cells, $^{\#}p < 0.05$ compared with the H_2O_2-treated cells.

CHAPTER V
DISCUSSION

Nephrotoxicity, via exposure to cadmium, has been recognized and renal proximal tubular cells of the kidney are found to be a major target of cadmium toxicity (274). However, its precisely molecular mechanisms for nephrotoxicity have not been proposed. Several studies demonstrated that cadmium induces apoptosis in various cell types including renal proximal tubular cells through reactive oxygen species (ROS) production and induction of phosphorylation of JNK pathway (130, 275). Furthermore, it has been revealed that the renal proximal cell damage induced by cadmium is suppressed by selenium, an antioxidant nutrient (275). In addition, Boyce showed that induction of phosphorylation of JNK in renal proximal tubular cells treated by cadmium was reversed by salubrinal, a selective inhibitor of cellular complexes that dephosphorylate eukaryotic translation initiation factor 2 subunit a (eIF2a), resulting in reduction of apoptotic cell death (273).

The role of LXRs in high metabolic tissues including kidney is recognized. It regulates cholesterol and triglyceride metabolisms (261, 262). LXRs regulate lipogenesis through stimulation of their target genes such as SREBP-1c and fatty acid synthase (FAS) (25, 276). In addition, LXR activation has been shown to suppress inflammatory gene expressions in macrophages induced by bacterial infection or lipopolysaccharide (LPS) stimulation (239). They also activate anti-oxidant gene expressions including glutathione peroxidase (Gpx), catalase (CAT), and metallothioneins (MT) in LPS-induced lung injury (30), and prevent bacterial-induced macropharge apoptosis (263). Moreover, the role of LXRs was found to involve oxidative stress and apoptotic signal transduction pathways such as JNK phosphorylation (28, 273). However, there is no data concerning the effect of LXR activation on JNK phosporylation and ROS production induced by cadmium. Therefore, the purpose of this study is to investigate whether LXR activation could attenuate cadmium-induced cytotxicity of renal proximal tubular cell via oxidative

stress, JNK signaling pathway, and apoptosis. This study showed a novel role of LXRs in inhibition of cadmium-induced cytotoxity in human renal proximal tubular cells.

At first, the LXR expression and activation in the cell models used were verified in renal proximal tubular cells including LLC-PK1 and HK-2 cells. Expression of both LXR isoforms, LXRα and LXRβ, was clearly demonstrated in both LLC-PK1 and HK-2 cells. The obtained results were similar to that found in mice kidney (30), indicating that these cells endogenously expressed both isoforms of LXR. To be certain that LXR is activated by LXR ligand used in this experiment, their target protein, SREBP-1c, is determined following treatment of the cells with T0901317 for 24 h. The results showed that SREBP-1c expression was markedly stimulated by T0901317, LXR agonist. Therefore, these cells are appropriated as a model for determination the role of LXRs in cadmium-induced nephrotoxicity.

Dose and time effects of cadmium on cell death in human renal proximal tubular cell.

Although several studies have reported that cadmium induces cytototoxicity in dose-and time-dependent mannes, the dose- and time-effects of cadmium on cells are wide ranges and depending on the cell types. In porcine renal proximal tubular cell, LLC-PK1 showed the significant dose that induces cell viability at 20 μM $CdCl_2$ (IC_{50}) after 5 h exposure (181), whereas an immortalized S1 segment PT cell line from Wistar-Kyoto rat (WKPT-0293) showed significant cell damage at 10 μM $CdCl_2$ (IC_{50}) at 3 h (20). Furthermore, human renal proximal tubular cell, HK-2 cells, was significantly damaged at 10 μM $CdCl_2$ (IC_{50}) after 24 h exposure (273). Addition to renal proximal tubular cell, the dose-and time effects of cadmium on skin epidermal cell line and immortalized rat hepatocytes (BRL3A) were significantly induced by 20 μM cadmium exposure (IC_{50}) for 24 and 12 h, respectively (265, 277). In present study found that cadmium exposure in renal proximal tubular cells, HK-2 cells and LLC-PK1 cells, also induced cytotoxicity in dose- and time-dependent manners and showed significantly effective dose at 20 μM and 5 $CdCl_2$ (IC_{50}) at 24 h, respectively. These effects are different in various cell types because of several factors including transporters that contribute entering of cadmium into the cells and reaching rapidly high intracellular cadmium accumulation leading to cytotoxicity. For

examples, metallothioneins (MTs), zinc transporters (ZIP8), divalent metal-ion transporter-1 (DMT1), organic anion transporters (OATs), and organic cation transporters (OCT_s) were proposed to play a critical role in cadmium accumulation leading to cadmium-induced nephrotoxicity (13-15, 44, 53, 278). However, HK-2 cells was reported to show no OAT and OCT expression (279). Lacking an expression of OATs and OCTs causes HK-2 cell cells to be less sensitive to cadmium-induced toxicity than that of LLC-PK1 cells. Therefore, to eliminate the affect of transporters on cadmium-induced toxicity, HK-2 cell was selected to use as a model in this study.

Cadmium induces cell death in renal proximal tubular cells.

Cadmium-induced cell death in renal proximal tubular cells has been reported as apoptosis or necrosis in both *in vivo* (198, 280) and *in vitro* (16, 130), depending on dose of exposure. For example, in an immortalized cell line from the S1 segment of rat PT (WKPT0293), 5-10 μM $CdCl_2$ induced major apoptotic cell death, but not necrosis, whereas 50 μM $CdCl_2$ induced markedly increasing of necrotic cell death during 6-24 h (89). In this study, 20 μM $CdCl_2$ exposure for 24 h induced mainly apoptotic cell death in human renal proximal tubular cells. These finding suggested that cadmium induces cell death via apoptosis or necrosis depending on dose and time of exposure.

Effect of LXR activation on cadmium-induced cytotoxicity in human renal tubular cells.

The key finding of this study is a novel pharmacological effects of LXRs on cadmium-induced toxicity in human renal proximal tubular cells. Although, LXR activation was demonstrated to associate with anti-inflammation, antioxidant, and implicated in oxidative stress responses in various stress conditions, the protective effect of LXRs on apoptotic cell death induced by cadmium has not been documented. To investigate whether LXR activation decreases apoptotic cell death induced by cadmium, the effect of T0901317, LXR agonist, on $CdCl_2$-induced cell death was determined. The result revealed that pre-incubation of HK-2 cells with T0901317 significantly reduced cadmium-induced cytotoxicity at 10 μM T0901317. Furthermore, the treatment of cell with 10 μM T0901317 markedly suppressed $CdCl_2$-

induced cellular damage and significantly reduced apoptotic cell death (Figure 4.9). These protective effects of T0901317 were abolished by fenofibrate, a reported LXR antagonist (281). In addition, this study also demonstrated that the antagonized effect of fenofibrate attenuated T0901317-induced SREBP-1c expression in dose-dependent manner. These data suggested that protective effect of LXR agonist, T0901317, on cadmium-induced apoptosis is depended on LXR activation. However, the other functions of fenofibrate including PPARα activation by binding with heterodimeric RXR may counter balancing LXR protection, leading to eliminate protective effect of LXR activation on cadmium-induced cytotoxicity. To demonstrate whether the competitive binding of fenofibrate with heterodimeric RXR has no antagonized effect on LXR activation, this effect of fenofibrate on LXR activation was determined by using specific PPARα antagonist, GW 6471. It was found that fenofibrate did not affect on the protective effect of LXR activation in cadmium-induced toxicity after pretreatment with GW 6471, indicating that the effect of fenofibrate was directly mediated via LXR antagonist as previously reported (281). However, it has also been claimed that T0901317, synthetic LXR agonist, has an effect on PXR activation (227). The effect of PXR activation on cadmium-induced cytotoxicity in human renal proximal tubular cells was determined by stimulating the cells with PXR agonist, rifampicin. The results showed that rifampicin produced no effect of cadmium-induced cytotoxicity, implying that PXR activation by T0901317 could not reduced cadmium-induced cytotoxicity. Therefore, the involvement of PXR activation on cadmium-induced cytotoxicity should be excluded. Taken together, LXR activation prevented apoptotic cell death induced by cadmium in human renal proximal tubular cells. However, it has been reported that LXR activation down-regulates organic anion transporter 1 (OAT1) (282), a potential transporter for uptake of bound form of cadmium including Cd-GSH and Cd-NAC leading to intracellular cadmium accumulation (13, 278). In addition, organic cation transporter 2 (OCT2) mediates uptake of free form of cadmium into the renal proximal tubular cell, and is also regulated by LXR activation (15). Although the protective effect of LXR activation on cadmium-induced toxicity may be mediated by down-regulation of OAT1 and or OCT2, HK-2 cell shows no expression of both OAT1 and OCT2 (279). These evidences prompt me to rule out the possibility that the protective effect of LXR

activation on cadmium-induced toxicity in HK-2 cell is mediated by LXR-induced reduction of transporter expression and subsequent reduction of intracellular cadmium accumulation. At present, the precise mechanism responsible for cadmium uptake into HK-2 cells is still unknown.

Effect of LXR activation on cadmium-induced ROS production.

Cadmium stimulating intracellular ROS generation in renal proximal tubular cells has been reported (16). In addition, cadmium-induced apoptotic cell death was demonstrated to relate with ROS generation in porcine renal proximal tubular cell line, LLC-PK1 (181, 275). The present study showed that ROS production was detected by $DCFH_2$-DA dye following incubation of HK-2 cells with 20 μM $CdCl_2$ at 3 h. These data was confirmed by showing that NAC, a ROS scavenger, inhibited cadmium-induced cell death of human renal proximal tubular cells, HK-2 cells. Since NAC provides cysteine molecules which are used as the precursors of substrates in synthesis of reduced glutathione (GSH) (283), treatment of the cell with GSH may provide the similar result obtained from the NAC treatment. Therefore, it was hypothesized that a protective effect of LXR activation might be mediated by reduction of intracellular ROS. The present study also found that intracellular ROS induced by cadmium was suppressed by LXR activation in HK-2 cells. This result is the first evidence showing the effect of LXRs in reducing intracellular ROS generation induced by toxic heavy metal in human renal tubular cells. In line with previous findings, this study also supports evidence that cadmium increases intracellular ROS level in renal proximal tubular cells by disturbing enzymes responsible for ROS generation (172-174). Since intracellular ROS generation is regulated by antioxidant enzyme system including catalases (CAT), superoxide dismutases (SOD) and NAD(P)H: dehydrogenase 1 (NQO1) (284-286), the involvement of LXRs in these systems was further investigated. The present data were demonstrated that cadmium exposure reduced mRNA expression of CAT, an antioxidant enzyme responsible for converting hydrogen peroxide (H_2O_2) to water leading to detoxification. Interestingly, the inhibition of catalase expression induced by cadmium was restored by LXR activation. Therefore, the mechanism which is responsible for the protective effect of LXR activation on cadmium toxicity might be mediated by LXR attenuating

cadmium-induced inhibition of catalase (30), leading to reduction of intracellular ROS. However, LXR activation that showed reduction of intracellular ROS production may inhibit the enzyme involving mitochondrial ROS production in complex I or complex III of electron transport chain (287, 288), leading to attenuating cadmium-induced inhibition of mRNA of catalase expression. This hypothesis need to be further investigated.

Although NAD(P)H:dehydrogenase (NQO1) is recognized as an antioxidant enzyme (270, 289), it also has been reported as an enzyme producing ROS. This notion has been shown by the finding that reduction of NQO1 expression using siRNA leads to a decrease in production of ROS (270). The present data showed that mRNA of NQO1 expression was up-regulated by cadmium exposure following incubation HK-2 cells with cadmium for 4 h (Figure 4.15 A), and increasing of ROS production at 3 h. This finding raised the question of whether the induction of NQO1 induced by $CdCl_2$ causes ROS generation or it is in response to ROS. This result urged to examine how $CdCl_2$ increases expression of NQO1. The present data demonstrated that the stimulation of $CdCl_2$ on NQO1expression was blocked by NAC, indicating that the stimulatory effect of $CdCl_2$ on NQO1 required ROS generation. In addition, up-regulation of NQO1 mRNA expression was markedly increased after 8 h exposure to $CdCl_2$, whereas increasing of ROS production was occurred after 3 h $CdCl_2$ treatment. These data imply that induction of NQO1 might be occurred following $CdCl_2$-induced ROS generation. Therefore, it was speculated that induction of NQO1 expression following $CdCl_2$ treatment is a defended mechanism of the cells in response to an increase in intracellular ROS. Although the up-regulation of NQO1 mRNA expression induced by cadmium was also suppressed by T0901317, the restoration of NQO1 mRNA expression by T0901317 could be a result of decreasing ROS generation via up-regulation of CAT expression which was mediated by LXR activation.

Effect of LXR activation on cadmium-induced MAPK signaling in human renal proximal tubular cells.

In addition to induction of ROS production, cadmium has been shown to activate c-Jun NH_2-terminal kinas (JNK) (290, 291), a member of the mitogen-

activated protein kinases (MAPK) superfamily. JNK is also known as stress-activated protein kinase (SAPK), which is activated by phosphorylation leading to apoptotic cell death in renal proximal tubular cell (23, 185, 275). To investigate whether ROS activates JNK-phosphorylation, the effect of NAC on cadmium-induced JNK was determined in HK-2 cells. The result showed that p-JNK induced by $CdCl_2$ was blocked by NAC pretreatment, indicating that induction of p-JNK expression was mediated by ROS generation. Since present data showed that LXR activation attenuated cadmium-induced ROS production in HK-2 cells, it was possible that LXR activation might prevent JNK activation. As it was expected, the results show that the induction of p-JNK expression by cadmium was eliminated by T0901317 pretreatment. The effect of T0901317 was also attenuated by a LXR antagonist, supporting the notion that LXR activation prevented cadmium-induced JNK activation in human renal proximal tubular cells. As LXR activation suppressed p-JNK, phosphorylation of p-ERK was not affected, suggesting that LXR activation selectively attenuated JNK signaling. In addition to inhibitory effect of LXRs on JNK phosphorylation through reduction of intracellular ROS production via up-regulate CAT expression, the direct effect of LXR activation on p-JNK may be occurred. To evaluate the direct effect of LXR activation on JNK induced by cadmium, H_2O_2 loading was used to replace cadmium treatment in HK-2 cells. Surprisingly, the results showed that LXR activation could inhibit H_2O_2-induced JNK posphorylation, suggesting that LXR activation also produced a direct inhibition on p-JNK expression in renal proximal tubular cell.

In summary, the present results revealed the novel protective effects of LXR activation on cadmium-induced apoptotic cell death in renal proximal tubular cells. The proposed mechanism which is responsible for protective effects of LXR activation is as follows: LXR activation inhibits cadmium-induced ROS generation via up-regulation of catalase (CAT) and, subsequently, inhibits p-JNK, and directly inhibits p-JNK leading to alleviation of apoptotic cell death of renal proximal tubular cells as shown in figure 21. This study provides the first evidence to show LXR activation reduces cadmium-induced cell death in human renal proximal tubular cells.

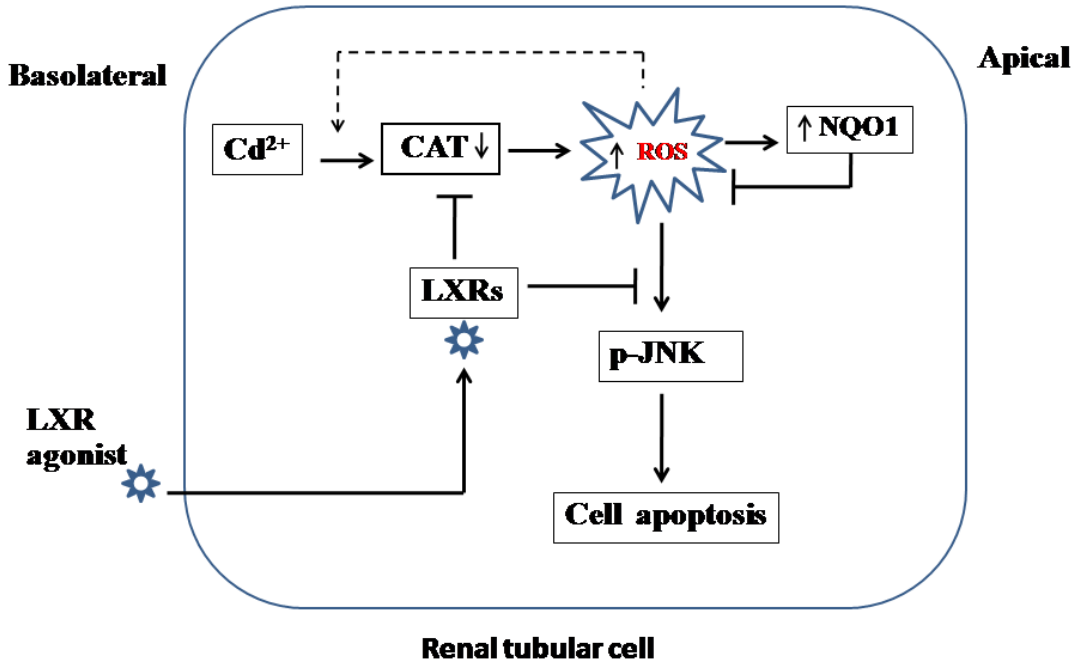


Figure 4.21 Proposed model for the protective effect of LXR activation on cadmiu-induced apoptosis of human renal proximal tubular cell. Cadmium (Cd^{2+}) increases ROS production by inhibition of catalase (CAT), subsequently stimulating phosphorylation of JNK and then leads to cell apoptosis. LXR activation reduces $CdCl_2$-induced renal proximal tubular cell apoptosis by inhibition of ROS generation via restoring catalase expression, and direct inhibition of JNK phosphorylation.

CHAPTER VI
CONCLUSION

Although LXRs are widely accepted to play an important role in cholesterol metabolism and triglyceride synthesis in various tissues, emerging studies also have interested in their anti-inflammatory and antioxidant effects which induced by infection, toxin and various stress condition in both *in vivo* and *in vitro* models. This study was designed to investigate the effects of LXR activation and underlying mechanism on the cadmium–induced cytotoxicity in renal proximal tubular cells.

The key finding of this study is that LXR activation attenuated cadmium-induced cytotoxicity of renal proximal tubular cells. The protective effect of LAR activation is mediated by reduction of ROS via upregulation of antioxidant enzyme expression, actalase, and suppression of JNK signaling.

This is the first study provides evidence showing that LXR activation reduced cadmium-induced cytotoxicity in human renal proximal tubular cells. LXR agonist could be developed as the effective treatment of cadmium-induced nephrotoxicity.

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APPENDICES

APPENDIX A
SOLUTIONS FOR PREPERATION

1. Phosphate Buffer Saline (PBS)

Phosphate Buffer Saline used for tissue culture experiment was prepared by dissolving of these chemicals in 900 ml of tissue culture grade water with stirring.

Components	Final concentration (mM)	Amount (g/L)
1. NACl	137	8.0
2. KCl	2.68	0.2
3. Na2PO4	10.14	1.44
KH2PO4	1.47	0.24

This solution was corrected pH to 7.4 with 1 M NaOH. Subsequently, the volume of this buffer was added to 1 liter with tissue culture grade water and then sterilized by autoclaving for 20 min at 121 $^{\circ}$C and under the pressure of 15 ib/sq. inch. Satirized buffer was stored at room temperature and used in 1 month.

2. Dulbecco's Modifiled Posphate-Buffer saline (DPBS) solution

Components	Final concentration	Amount (g/L)
1.NaCl	137	8.0
2. KCl	3	0.2
3.Na2HPO4. 7H2O	8	2.14
4. KH2PO4	1	0.1
5. MgCl2. 6H2O	0.5	0.4
6. CaCl2.2H2O	1	0.15
7. D-glucose	5.6	1.008

DPBS solution for ROS determination, these chemical were dissolved in 900 ml of cell culture grade water. The pH of this solution was adjusted to7.4 with 1 M NaOH. Subsequently , the total volume of this buffer was corrected to 1,000 ml with cell culture grade water and then sterilized sterilized by autoclaving for 20 min. at 121 $^{\circ}$C and under the pressure of 15 ib/sq. inch. Satirized buffer was stored at room temperature and used in 1 month.

APPENDIX B
DETERMINATION OF POTEIN CONCENTRATION

This assay was flowed by modified Lowry method (Lowry et al. 1951).

Reagents

1. 2% Na_2CO_3 in 1.0 N NaOH, 250 ml (Solution A)

- 0.1 N NaOH; 1 g of NaOH was dissolved in distilled water and made the final volume of 250 ml.

- 2 % Na_2CO_3; 5 g of Na_2CO_3 was dissolved in 0.1 N NaOH and made the final volume of 250 ml. This solution was stored at 4 $^{\circ}$C.

2. 4 % Sodium potassium tartate solution, 100 ml

- 4 g of NaK tartate was dissolved in distilled water and made the final volume of 100 ml. This solution was kept at 4°C.

3. 2 % $CuSO_4.5H_2O$, 100 ml

- 2 g of $CuSO_4.5H_2O$ was dissolved in distilled water and made the final volume of 100 ml. This solution was kept at 4°C.

4. 1 N Folin phenol reagent (Solution B)

- Commercial 2 N Folin Ciocalte Phenol reagent was diluted to 1 N Folin working solution with distilled water and immediately used.

5. Stock standard protein solution (100 mg%, w/v)

- 10 mg of bovine serum albumin (BSA) was dissolved in 10 ml of distilled water and immediately used. The stand curve of protein was prepared by stock standard BSA solution diluted at least four concentrations in range of 10-100 mg %.

6. Lowry working solution. This solution was freshly prepared before use as following

- 200 parts of 2% Na_2CO_3 in 1.0 N NaOH
- 1 part of 4 % Sodium potassium tartate solution
- 1 part of 2 % $CuSO_4.5H_2O$

Procedure

Each of Tests was performed in duplicate.

Reagent (µl)	Blank	Standard	Unknown	Remarks
DW.	50	-	-	
Standard	-	50	-	
Sample	-	-	50	
Solution A	150	150	150	
Solution B	1,000	1,000	1,000	
1 N Folin reagent	100	100	100	
Mixed well and kept at room temperature for 30 minutes in dark				
Read the optical density (OD) at 770 nm by spectrophotometer				

Calculation

The protein concentration was calculated by standard curve or formulation as following:

$$\text{Protein concentration (mg\%)} = \frac{\text{OD of Unknown x 100 mg of standard x dilution factor}}{\text{OD of 100 mg of standard}}$$

BIOGRAPHY

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