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腺苷單磷酸活化蛋白質激酶誘發細胞自噬以保護缺血再灌流所引起之腎小管細胞凋亡

AMP-Activated Protein Kinase-Evoked Autophagy Protects Ischemia/Reperfusion-Induced Renal Tubular Cell Apoptosis

王麗婷

Li-Ting Wang

指導教授：劉興華博士

Advisor: Shing-Hwa Liu, Ph.D.

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CONTENTS

中文摘要

腎臟缺血再灌流 (ischemia/reperfusion, I/R)為常見造成急性腎衰竭的原因，其造成的傷害常發生於腎臟移植、腎臟動脈血管狹窄或是其他原因所導致。過去文獻指出，腎臟 I/R 會造成內皮細胞功能異常、引發嚴重的發炎反應、活化細胞死亡相關的蛋白及造成氧化壓力增加等而導致腎臟傷害。腎臟 I/R 所產生大量的過氧化物 (reactive oxygen species, ROS)被認為是造成腎臟傷害的主要原因，這些 ROS 會經由蛋白質氧化、脂質過氧化、DNA 的傷害而導致腎臟急性傷害以及細胞凋亡等。許多抗氧化劑像是 N-乙醯半胱胺酸 (N-acetyl-L-cysteine)、超氧化物歧化酶 (superoxide dismutase)等能藉由抑制 ROS 生成而對急性 I/R 腎傷害具有保護的作用。近期研究指出 I/R 會誘發細胞產生自噬作用 (autophagy)，而細胞可能會透過此機制走向死亡或存活。然而，I/R 引起腎臟細胞傷害的分子機制並不完全清楚，有進一步研究的必要。在本研究中，我們以模擬的 I/R 細胞模式探討腎臟近曲小管細胞受到傷害時所牽涉到的分子調控機制。我們將豬腎臟近曲小管上皮細胞株 (LLC-PK1)處理 1.5 個小時的抗黴素 A (antimycin A)及二去氧 2-去氧-D-葡萄糖 (2-deoxy-D-glucose)，並分別以干擾粒線體呼吸傳遞鏈及抑制糖解作用方式，模擬細胞化學性缺氧的情形，之後移除藥物模擬細胞再灌流情形。實驗結果顯示，細胞在缺氧時並不會發生嚴重損傷，但隨著再灌流時間延長會造成細胞凋亡。此外，由西方墨點法實驗結果發現，細胞處理化學性缺氧藥物時，會活化與能量調控相關的蛋白-腺苷單磷酸活化蛋白質激酶 (adenosine monophosphate-activated protein kinase, AMPK)磷酸化的表現，並且隨著再灌流時間延長，藉由 monodansylcadaverine 染色及轉染標記 LC3 綠螢光蛋白 (green fluorescent protein-labeled LC3)實驗都證實 I/R 會誘發腎臟細胞自噬作用的情形。在流式細胞儀實驗中，我們以細胞自噬作用專一性抑制劑-3-methylamphetamine 抑制細胞自噬作用的表現，觀察到細胞於處理 I/R 後 24 小時凋亡情形明顯增加。接著，為了確認 AMPK 在 I/R 中扮演之角色，我們利用干擾性核醣核酸抑制細胞 AMPK 的表現，結果顯示細胞於 I/R 處理後 mTOR 蛋白磷酸化情形增加、細胞自噬作用表現受抑制，並令細胞凋亡情形更嚴重；而處理 AMPK 專一性抑制劑-compound C 於細胞中也有相同結果。另外，利用 mTOR 專一性抑制劑-RAD001 抑制細胞 mTOR 磷酸化表現，我們發現細胞在 I/R 處理後會明顯活化

細胞自噬作用的表現，並且能保護細胞不走向凋亡。接著，我們探討抗氧化劑-槲黃素(quercetin)是否能減緩腎臟細胞所受到 I/R 之傷害，並探討其中分子調控機制。我們將細胞處理於化學性缺氧的過程同時合併給予 quercetin，觀察控制組與加藥組之間的不同。實驗結果發現，處理 quercetin 的細胞會造成 AMPK 蛋白磷酸化表現增加、mTOR 蛋白磷酸化表現下降、細胞自噬作用活化，並減低 I/R 誘發的細胞凋亡；利用干擾性核醣核酸抑制細胞 AMPK 的表現，我們發現 quercetin 能恢復原先受抑制的細胞自噬作用。綜合以上實驗結果，顯示 AMPK 能透過負調控 mTOR 以誘發細胞自噬作用來保護 I/R 引起腎臟細胞凋亡之傷害，而 quercetin 可能經由此調控機制來保護腎臟細胞在 I/R 時所造成的傷害。希望藉由此研究確認細胞自噬作用在腎臟 I/R 所扮演的角色與其分子調控機制，未來可望開發為藥物治療之新方向。

關鍵字：缺血再灌流、腺苷單磷酸活化蛋白質激酶、細胞自噬、細胞凋亡、槲黃素

Abstract

Renal ischemia/reperfusion (I/R) injury is the most common cause of acute kidney injury. Renal I/R injury occurs in many clinical conditions such as hypovolemic shock, thromboembolism, and renal transplantation. Several mechanisms participate in renal I/R injury including deleterious inflammatory responses, endothelial dysfunction, nitric oxide dysregulation, oxidative stress, and caspase activation. Among of them, oxidative stress is considered a major event. Moreover, the increasing lines of evidence also suggest that autophagy may participate in I/R injury, and will lead cell to death or survival. However, the detail molecular mechanisms of I/R injury on renal tubular cells still remain to be clarified. Therefore, the aim of study is to investigate the molecular mechanisms of I/R injury on renal proximal tubule epithelial cells. To mimic renal I/R injury *in vivo*, LLC-PK1 cells were incubated with antimycin A and 2-deoxy-D-glucose for 1.5 h to induce ischemia injury, which could disturb mitochondrial respiratory chain and inhibit energy generation via blocking glycolysis. The reperfusion was achieved by replacing the ischemic medium by a glucose-replete complete growth medium. We demonstrated I/R induced tubular cell apoptosis in a reperfusion time-dependent manner at first. By using western blotting analysis, transfection green fluorescent protein (GFP)-labeled LC3, and stainings of monodansylcadaverine, I/R induced the LC3-II forms protein expression and autophagosome formations in LLC-PK1 cells, which were obvious after 6 h of reperfusion. Analysed by flow cytometry, it revealed that inhibit autophagy by 3-methyladenine significantly enhanced I/R-induced renal tubular cell apoptosis. In addition, the

phosphorylation of adenosine monophosphate-activated protein kinase (AMPK) was also increased by I/R treatment in LLC-PK1 cells. Inhibition of AMPK by shRNA for AMPKα1 or compound C increased the phosphorylation of mammalian target of rapamycin (mTOR) protein and decreased induction of autophagy, and then enhanced I/R-induced renal tubular cell apoptosis. Moreover, RAD001, a mTOR inhibitor, could increase the autophagy activation and attenuate I/R-induced renal tubular cell apoptosis. On the other hand, we investigated the protective effect of antioxidant quercetin on I/R injury in renal tubular cells. We observed that quercetin significantly up-regulated the AMPK phosphorylation, down-regulated the mTOR phosphorylation, activated the autophagy, and decreased the I/R-induced renal cell apoptosis. In I/R-treated renal tubular cells, quercetin could also reverse the shRNA of AMPKα1-reduced renal tubular cells autophagy. Taken together, these findings suggest that autophagy protects renal tubular cells from I/R injury through an AMPK-regulated mTOR pathway. Quercetin may reduce the I/R-induced renal tubular cell injury by AMPK-regulated autophagy induction. These findings suggested that induction of autophagy by AMPK phosphorylation and mTOR reduction in renal tubular cells as a potential target for intervention renal I/R injury. Besides, quercetin may be as a potential treatment for I/R-induced renal cell injury.

Keywords: ischemia/reperfusion, monophosphate-activated protein kinase, autophagy, cell apoptosis, quercetin

Abbreviations

2DG: 2-Deoxy-D-Glucose

3MA: 3-Methylamphetamine

4E-BP1: Elongation Factor-4E Binding Protein 1

AKI: Acute Kidney Injury

AMPK: Adenosine Monophosphate-Activated Protein Kinase

BSA: Bovine Serum Albumin

CaMKK: Calmodulin-Dependent Protein Kinase Kinase

CHOP: C/EBP Homologous Protein

CO_2: Carbon Dioxide

ddH_2O: Deionized Distilled Water

DMSO: Dimethylsufoxide

eEF-2: Eukaryotic Elongation Factor-2

eIF2*α:* Eukaryotic Initiation Factor 2α

ER: Endoplasmic Reticulum

FBS: Fetal Bovine Serum

GFP: Green Fluorescent Protein

GRP: Glucose-Regulated Protein

HO-1: Heme Oxygenase-1

HRP: Horseradish Peroxidase

I/R: Ischemia/Reperfusion

KH: Krebs-Henseleit

LC3: Cytosolic Microtubule-Associated Protein Light Chain 3

LKB1: Tumor Suppressor-Liver Kinase B1

MDC: Monodansylcadaverine

M199: Medium 199

MAPK: Mitogen-Activated Protein Kinase

mTOR: Mammalian Target of Rapamycin

NAC: N-Acetyl-L-Cysteine

NO: Nitric Oxide

PAGE: Polyacrylamide Gel Electrophoresis

PBS: Phosphate Buffered Saline

PE: Phosphatidylethanolamine

PGE2: Prostaglandin E2

PI: Propidium Iodide

PI3K: Phosphatidylinositol 3-Kinase

PMSF: Phenylmethylsulfonyl Fluoride

PVDF: Polyvinylidene Difluoride

ROS: Reactive Oxygen Species

S6K1: Ribosomal Protein S6 Kinase 1

SDS: Sodium Dodecyl Sulfate

SOD: Superoxide Dismutase

TBST: Tris-Buffered Saline/Tween-20

CHAPTER I

Introduction

Renal ischemia/reperfusion (I/R) injury

Ischemia/reperfusion (I/R) injury, including arterial occlusion, shock, and organ transplantation, results in a common and important clinical problem in many different organ systems. It has common been seen in myocardial infarctions, strokes, acute kidney injury (AKI), shock liver, mesenteric ischemia, and systemic shock (Tilney & Guttmann, 1997). Ischemia is a unique process as short of the blood supply, and reperfusion injury is the effector phase of ischemic injury, which develops hours or days after the initial insult. I/R injury can directly or indirectly cause tissue injury through the deprivation of oxygen, ATP depletion (Venkatachalam et al, 1981), calcium overload (Kosieradzki & Rowinski, 2008), leukocyte infiltration (Solez et al, 1974), membrane lipid peroxidation (Erdogan et al, 2006), enzyme activities alteration (Davies et al, 1995), proteases activation (Schrier et al, 2004), activation of phospholipases messengers such as ceramide and phospholipase A2 (Fermin et al, 2008), production of free radicals such as reactive oxygen species (ROS) (Montagna et al, 1998), augmentation pro-inflammatory mediators reaction (Friedewald & Rabb, 2004), alteration in intracellular ion and pH homeostasis (Giaccia et al, 2004), induction of cell apoptosis (Eefting et al, 2004), and alteration in autophagy activation (Zhu et al, 2006). Repairing and regenerative processes take place in parallel to I/R-induced apoptosis, autophagy, or necrosis. It depends on whether cell death or regeneration prevails (Kosieradzki & Rowinski, 2008).

Renal I/R injury is the most common cause of AKI. It inevitably occurs in many clinical conditions such as hypovolemic shock, thromboembolism, and renal transplantation (Inal et al, 2002). AKI often conducts to renal cell death, delayed graft function, renal graft rejection, and permanent impairment of renal function (Lu et al, 1999; Perico et al, 2004). Moreover, AKI also has clinically deleterious effect associated with unacceptably high mortality rates (Hoste et al, 2006) and end-stage renal disease (Chertow et al, 2006; Xue et al, 2006). Renal I/R injury causes several pathological changes, including tubular cytoskeletal breakdown (Molitoris, 2004), loss of polarity (Woroniecki et al, 2003), cell death (Castaneda et al, 2003), desquamation of viable and necrotic cells, tubular obstruction (Kwon et al, 1998), and backleak. Tubular damages can induce microvascular events through inflammatory and vasoactive mediators (Bonventre & Weinberg, 2003). The vasomotor changes included increase of vasoconstriction (Okusa, 2002; Wolf et al, 1997), decrease of vasodilation (Conger & Weil, 1995), up-regulation of endothelial and vascular smooth muscle cell structural damage (Brodsky et al, 2002; Kwon et al, 2002), elevation of leukocyte-endothelial adhesion vascular obstruction (Meier-Kriesche et al, 2001; Solez et al, 1974), leukocyte activation (Meier-Kriesche et al, 2001), and inflammation (Park et al, 2002). Vasoconstrictors contributed to the regulation of I/R-caused microvascular events contain angiotensin II, thromboxane A2, leukotrienes C4 and D4, endothelin-1, adenosine, endothelium-derived prostaglandin H2, and sympathetic nerve stimulation. Vasodilators implicated in the regulation of I/R-caused microvascular events contain nitric oxide (NO), prostaglandin E2

(PGE2), and acetylcholine bradykinin. Renal I/R injury can induce the deleterious inflammatory responses, endothelial dysfunction, NO dysregulation, oxidative stress, and caspase activation (Fig. 1). Oxidative stress plays a pivotal role in the I/R-induced acute kidney injury.

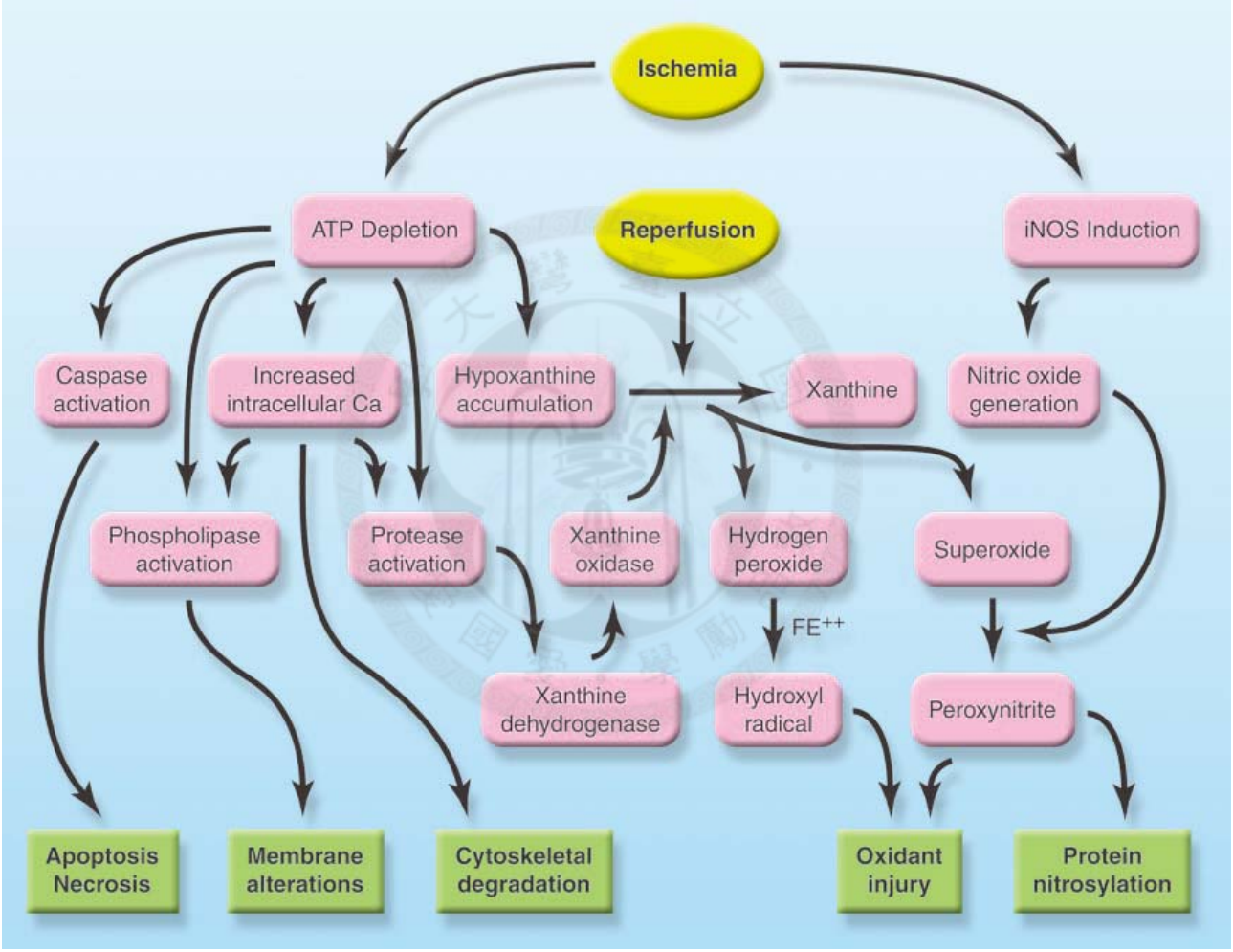


Fig. 1 Renal I/R injury induces several mechanisms (Devarajan, 2006)

Adenosine monophosphate-activated protein kinase (AMPK)

AMPK is a heterotrimeric complex consisting of a catalytic α-subunit and regulatory β- and γ-subunits with multiple isoforms (α1, α2, β1, β2, γ1, γ2, γ3) (Stapleton et al, 1996) (Fig. 2-1). AMPK was activated while the depletion of ATP caused by either inhibition or consumption. Thereby, AMPK acts as a cellular energy sensor. Activation of AMPK by calmodulin-dependent protein kinase kinase (CaMKK) or tumor suppressor-liver kinase B1 (LKB1) sensitive mechanism is dependent on the intracellular calcium and AMP/ATP ratio, respectively (Long & Zierath, 2006). Phosphorylation of Thr-172 in the catalytic domain of α subunit is required for AMPK activation (Hawley et al, 1995). AMPK activity can be up-regulated by widely metabolic stresses that deplete cellular ATP, including hypoxia, ischemia, oxidative stress and metabolic poisoning (Hardie, 2004). Furthermore, physiological stress such as exercise and glucose deprivation in skeletal muscle can also activate AMPK (Winder & Hardie, 1996). Several studies have suggested that AMPK is an important mediator of many metabolic responses to exercise. Generally, AMPK maintains energy homeostasis by activating catabolic pathways that produce ATP (e.g., fatty acid oxidation, glucose uptake, and glycolysis) while inhibiting biosynthetic pathways that consume energy (e.g., fatty acid and cholesterol biosynthesis) (Carling, 2004; Hardie, 2004).

When AMPK is activated by stress signals, several downstream targets will be activated (Fig. 2-2). Although there is no single case let us completely understand the detailed mechanisms by which AMPK activation regulates the expression of a particular gene, it has many effects on

individual transcription factors and coactivators. For example, AMPK negatively regulates the mammalian target of rapamycin (mTOR) pathway (Bolster et al, 2002), which is a major positive stimulus for protein synthesis, cell growth, and cell size under a cellular stress. The activation of mTOR regulates two mechanisms including activation of ribosomal protein S6 kinase 1 (S6K1) and elevation phosphorylation of elongation factor-4E binding protein 1 (4E-BP1), which could stimulate the initiation step of translation (Carrera, 2004). Several studies have suggested that inhibition of the mTOR pathway by AMPK may occur through phosphorylation of TSC2 (tuberin) (Inoki et al, 2003). Since mTOR negatively regulates autophagy, the AMPK-mTOR pathway has been considered to be an important regulator of autophagy in the energy depletion (Herrero-Martin et al, 2009; Liang et al, 2007; Meley et al, 2006).

It has been known that AMPK is abundantly expressed in the kidney (Stapleton et al, 1996), and regulates gluconeogenesis, glycogen synthesis, and glycolysis (Weidemann & Krebs, 1969). Because sodium transport is a major energy-consuming process in the kidney, AMPK might lead to the coupling of ion transport and cellular energy regulation. Moreover, AMPK is involved in the multiple aspects of renal pathophysiology, including ion transport (Carattino et al, 2005), podocyte function (Sharma et al, 2008), renal hypertrophy (Lee et al, 2007), diabetes (Cammisotto et al, 2008; Lee et al, 2007), ischemia (Mount et al, 2005), inflammation (Peairs et al, 2009), and polycystic kidney disease (McCarty et al, 2009). Furthermore, recent researches also suggested that oxidative stress and aging (Jin et al, 2004), endothelial function (Fisslthaler & Fleming, 2009), and

gluconeogenesis (Sato et al, 2011) may influence AMPK expression in kidney as well.

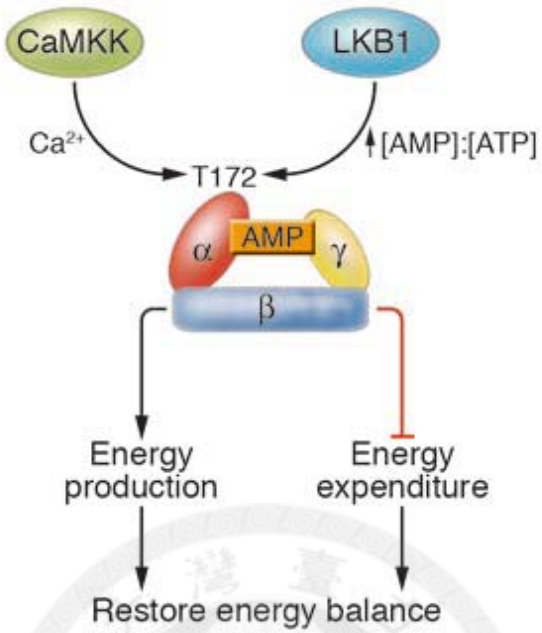


Fig. 2-1 Structure and regulation of AMPK (Long & Zierath, 2006)

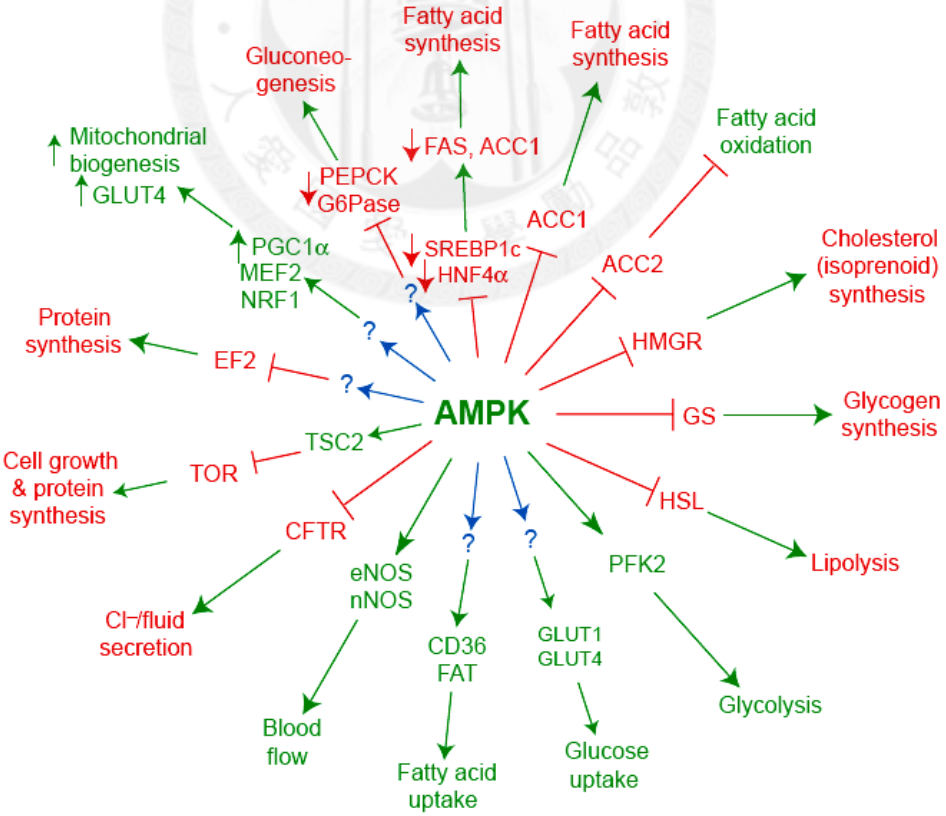


Fig. 2-2 Targets for AMPK (Carling, 2004)

Autophagy

Autophagy is a cellular digestive process of self-degradation when various cytoplasmic constituents such as cytosolic proteins, damaged or excess organelles, protein aggregates, and invasive microbes are broken down by resident hydrolases and recycled through the lysosomal degradation pathway (Levine & Klionsky, 2004). It is an evolutionarily conserved process that occurs in all eukaryotic cells, from yeast to mammals (Levine & Klionsky, 2004). Autophagy has a complex process that contains sequential steps including sequestration of cytoplasmic portions by isolation membrane to form autophagosome, fusion of the autophagosome with lysosome to form an autolysosome, and degradation of the engulfed material. More comprehensive processes were listed. First, the class III phosphatidylinositol 3-kinase (PI3K) mediates nucleation of the phagophore membrane. Two ubiquitin-like conjugation systems are required for the formation of the autophagosome. Autophagy-regulating protein (Atg) 12 and LC3/Atg8 are ubiquitin-like proteins which during autophagosome formation are conjugated to Atg5 and phosphatidylethanolamine (PE), respectively. Atg12 is activated and bound by E1-like Atg7, transferred to E2-like Atg10, and conjugated to Atg5. Homodimers of Atg16 bind a pair of Atg12-Atg5 conjugates and this complex transiently associates with the forming autophagosomal membrane. The membrane-bound Atg12-Atg5・Atg16 complex is a prerequisite for the recruitment of LC3-II, which is the product of the second ubiquitin-like conjugation. Initially, the cysteine protease Atg4 removes the C-terminal arginine residue of cytosolic LC3, generating LC3-I with a

revealed glycine residue. LC3-I is activated by E1-like Atg7, transferred to E2-like Atg3, and conjugated to PE. The resulting LC3-II is recruited into the forming autophagosomal membrane. After completion of autophagosomes formation, a second cleavage by Atg4 results in some removal of PE from LC3-II, reverting it back into LC3-I which is released from the membrane. The remaining membrane attached LC3-II is degraded by lysosomal proteases (Fig. 3-1). Degradation these materials usually generates free fatty acids and amino acids which can be reused to maintain mitochondrial ATP production, protein synthesis, and cell survival (Baehrecke, 2005).

Autophagy can be activated by a variety of stress stimuli, including nutrient and energy stress (Liang et al, 2007; Lum et al, 2005), ER stress (Ogata et al, 2006), pathogen infection (Wang et al, 2009), hypoxia (Azad et al, 2008; Zhang et al, 2008), oxidative stress (Chen et al, 2008), and mitochondrial damage (Zhang et al, 2008). Recently studies suggested that autophagy could be induced by various regulatory mechanism pathways (Fig. 3-2).

When cell stress induced at the early stage, autophagy is activated and is cytoprotective. However, as cell stress is too severe at late stage, excessive autophagy may trigger cell injury and death (Baehrecke, 2005; Periyasamy-Thandavan et al, 2009). Because autophagy has dual roles in cell survival and death, it may be involved in various physiological processes, and linked to the pathogenesis of a wide array of diseases, such as neurodegeneration (Hara et al, 2006; Komatsu et al, 2006), cancer (Gozuacik & Kimchi, 2004; Kondo et al, 2005), and heart disease (Nakai et al, 2007; Zhu et al, 2007). Moreover, emerging evidence has demonstrated that autophagy participates

in renal disease conditions including renal I/R and nephrotoxicity (Jiang et al, 2010). However, it remains largely unknown how autophagy makes the survival or death fate of a stressed cell.

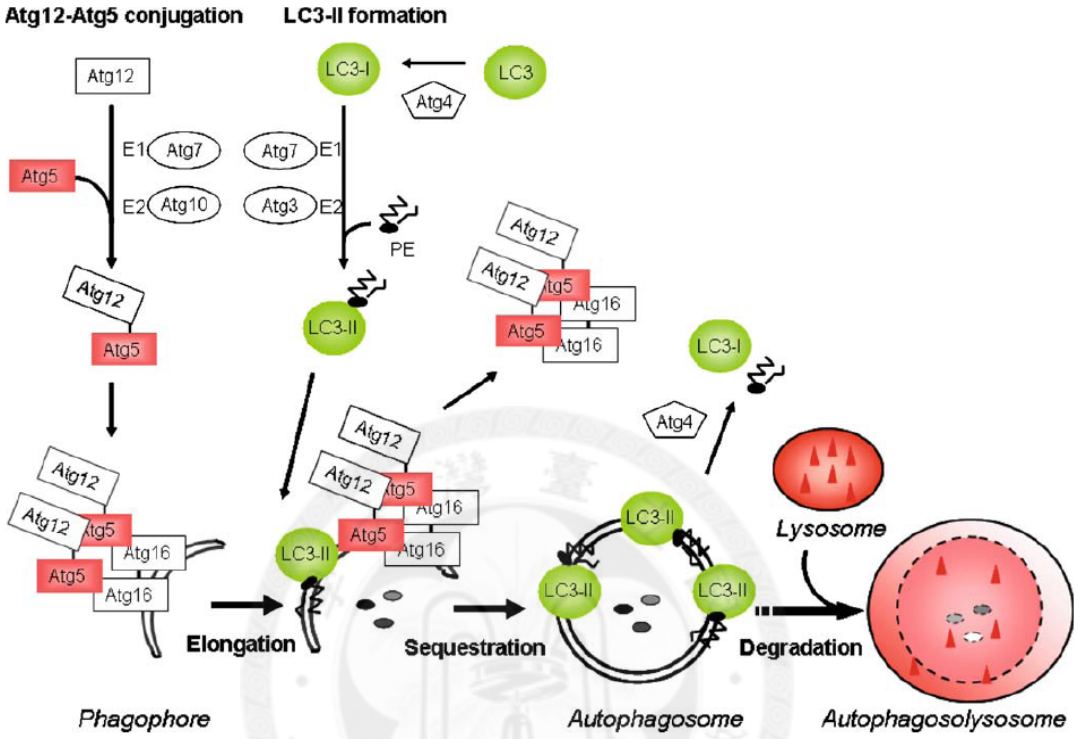


Fig. 3-1 Schematic model of autophagy (Hamacher-Brady et al, 2006)

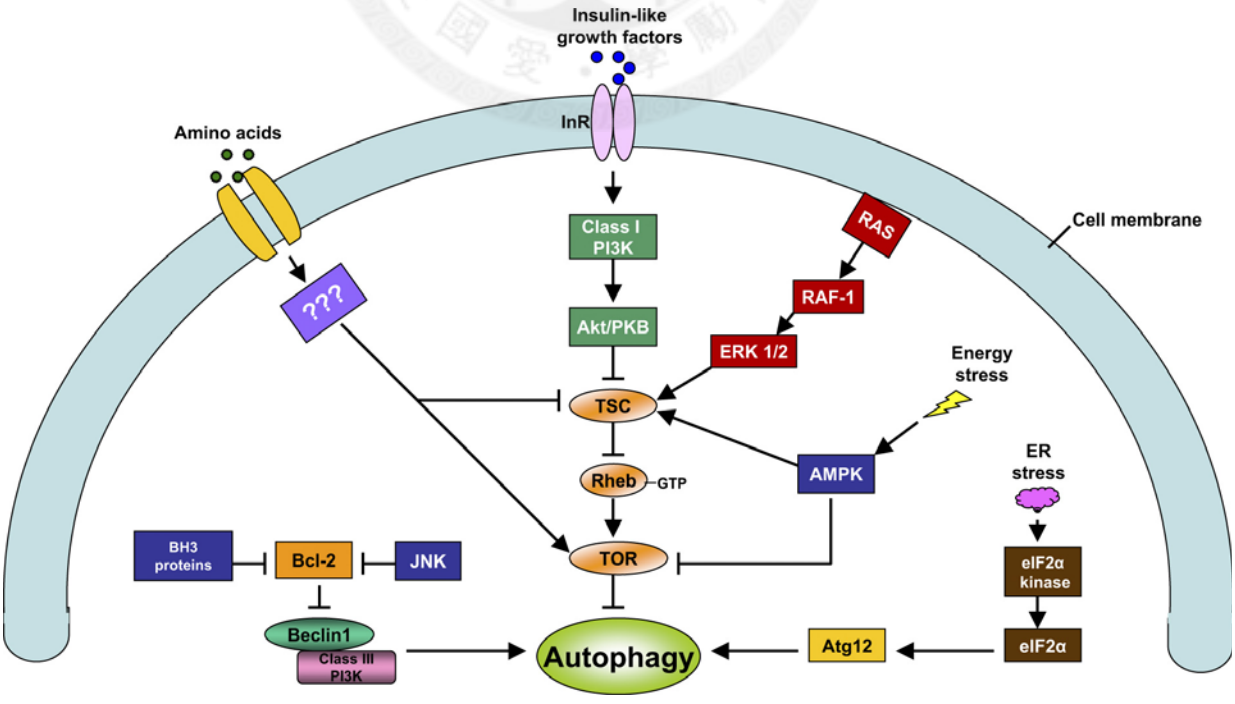


Fig. 3-2 Major regulatory mechanisms of autophagy (Periyasamy-Thandavan et al, 2009)

Quercetin

Quercetin, 3,3',4',5,7-pentahydroxyavone (Fig. 4), is a member of the flavonoids family ubiquitously present in fruit, vegetables, tea, wine as well as countless food supplements (Pietta, 2000). Flavonoids are categorized as flavonol, flavanol, flavanone, flavone, anthocyanidin, and isoflavone. The antioxidant activity of flavonoids has attracted much attention regarding to it chemoprevention in ischemic heart disease and diabetes (Hollman & Katan, 1997; Skibola & Smith, 2000). Within the flavonoids family, quercetin is one of the most potent scavenger of ROS (Hanasaki et al, 1994). Apples, tea, onions, and red wine contain high concentration of quercetin. Although quercetin is the major bioflavonoid in the human diet (Lamson & Brignall, 2000), US consumption of dietary quercetin is only about 30 mg/d (Weldin et al, 2003). In addition to diseases prevention, such as osteoporosis, diabetes, cancer, obesity, pulmonary, and cardiovascular diseases, and aging (Ahn et al, 2008), quercetin induces cell cycle arrest and apoptosis in human colon cancer cells (Psahoulia et al, 2007). Furthermore, quercetin is known to have strong anti-inflammatory properties (Orsolic et al, 2004). For example, several *in vitro* studies have shown that the quercetin is capable of inhibiting LPS-induced cytokine production (Geraets et al, 2007). Furthermore, there are some studies has been investigated *in vitro* that quercetin has anti-fibrotic (Lee et al, 2003), anti-coagulative (Bucki et al, 2003), anti-bacterial (Cushnie & Lamb, 2005), anti-atherogenic, anti-ischemic, anti-peroxidative, anti-hypertensive (Perez-Vizcaino et al, 2006), and anti-proliferative properties (Gulati et al, 2006). Altogether, quercetin may exert health-beneficial

capacities in many dimensions.

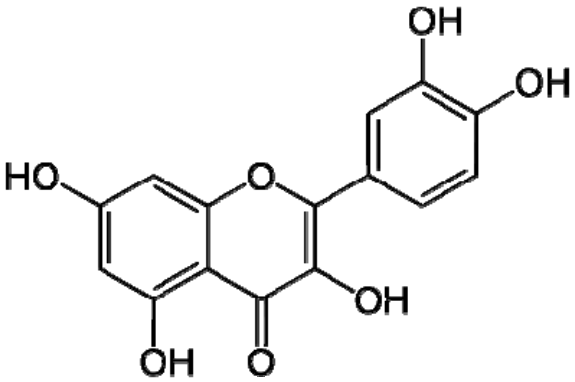
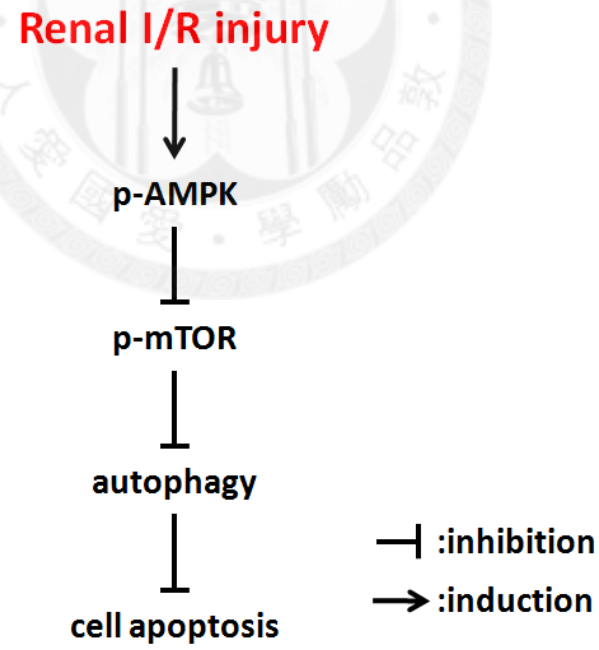


Fig. 4 Chemical structure of quercetin

Hypothesis and aims

Despite the emerging evidences suggest that autophagy participates in I/R injury and plays a protective role in ischemic heart disease, there is little known about the involvement of autophagy in the renal I/R injury. Previous studies suggest that phosphorylation and activation of AMPK were induced in I/R-induced kidney injury. Therefore, the aim of study is to clarify the links between AMPK-mTOR-autophagy signals in renal proximal tubule epithelial cells suffering I/R.

On the other hand, because oxidative stress is considered a major event in I/R-induced renal injury, we investigated the protective effect of quercetin on renal I/R injury and its possible molecular mechanisms.



Schematic representation of experimental model

CHAPTER II

Materials and Methods

Cell culture and chemical treatment

LLC-PK1 (renal proximal tubular epithelial kidney cell line) were purchased from American Type Culture Collection and cultured in growth medium consisting of medium 199 (M199; GIBCO, Grand Island, NY, USA) supplemented with 3% fetal bovine serum (FBS) and 1% antibiotics (100 IU/mL penicillin, 100 μg/mL streptomycin) at 37°C under 5% carbon dioxide (CO_2). Chemicals and compounds: antimycin A (Sigma-Aldrich, St. Louis, MO, USA), 2-deoxy-D-glucose (2DG; Sigma-Aldrich), compound C (AMPK inhibitor; Merck, Darmstadt, Germany), N-acetyl-L-cysteine (NAC; Sigma-Aldrich), RAD001 (mTOR inhibitor; Sigma-Aldrich), rapamycin (Calbiochem, Bad Soden, Germany), 3-methylamphetamine (3MA; autophagy specific inhibitor; Sigma-Aldrich), and quercetin **(**Alfa Aesar, Karlsruhe, Germany).

Ischemia/reperfusion (I/R) treatment of LLC-PK1 cells

To mimic *in vivo* renal I/R injury, a confluent monolayer of LLC-PK1 cells grown were incubated in a Krebs-Henseleit (KH) buffer (115 mM NaCl, 3.6 mM KCl, 1.3 mM/L KH_2PO_4, 25 mM/L $NaHCO_3$, 1 mM/L $CaCl_2$, 1 mM/L $MgCl_2$, pH =7.4) with antimycin A and 2DG (5mM) for 1.5 hour (h) to induce ischemia injury. The reperfusion was achieved by washing with KH buffer twice and replacing the ischemic culture medium by a glucose-replete complete growth medium for

various time courses. Antimycin A was dissolved in dimethylsufoxide (DMSO) (Sigma-Aldrich) and 2DG was dissolved in deionized distilled water (ddH_2O).

Sub-G1 analysis for fragmented DNA

Cell death can be identified as the cells containing fragmented DNA in the sub-G1 phase by propidium iodide (PI) staining. After exposure of LLC-PK1 cells to vehicle or various concentrations of antimycin A and 5 mM 2DG for 1.5 h and then followed by reperfusion for 24 h, the cells were washed with phosphate-buffered saline (PBS) twice, fixed with ice-cold 70% methanol, and stored at -20℃ for 24 h. Subsequently, the cells were pelleted, suspended into PBS, and incubated with 0.1 mg/ml of RNaseA (Invitrogen, Carlsbad, CA, USA) and 10 μg/ml of PI (Sigma-Aldrich). Flow cytometric analysis was performed using Becton Dickinson FACSCalibur cytometer with an argon ion laser (488 nm) as the excitation source and Cell Quest version 6.0 software for DNA content analysis. A total of 10,000 cells were analyzed per sample.

Annexin V and PI assays for apoptosis detection

For Annexin V and PI assays, the cells were stained with Annexin V–FITC and PI and evaluated for apoptosis by flow cytometry. Briefly, after I/R treatment, LLC-PK1 cells were collected in different reperfusion time points, then washed with PBS twice and stained with 5 μl of Annexin V–FITC (BD Biosciences, Heidelberg, Germany) and 5 μl of PI (0.5 μg/ml) (BD Biosciences) in 1×

binding buffer 〔10 mM HEPES (pH 7.4), 140 mM NaOH, 2.5 mM $CaCl_2$)〕for 15 min at room temperature in the dark. Flow cytometric analysis was performed using Becton Dickinson FACSCalibur cytometer with an argon ion laser (488 nm) as the excitation source and Cell Quest version 3.3 software for DNA content analysis. Both early (Annexin V-positive, PI-negative) apoptotic and late (Annexin V-positive and PI-positive) apoptotic cells were included in analysis. A total of 10,000 cells were analyzed per sample.

Analysis of autophagy by green fluorescent protein (GFP)-cytosolic microtubule-associated protein light chain 3 (LC3) distribution and monodansylcadaverine (MDC) staining

First, we performed transient transfections using a Lipofectamine 2000 reagent (Invitrogen) according to the manufacture's recommendations. After rinsing with serum-free and antibiotic-free medium, the cells were transfected separately with a control pcDNA6.2 empty vector or a GFP-LC3 fusion protein expression vector (pcDNA6.2-Em GFP-LC3), which was kindly provided by Dr. CK Chiang (National Taiwan University Hospital, College of Medicine, Taipei, Taiwan), and incubated at 37°C under 5% CO_2 for 4 h, and then replaced by complete growth medium for more than 14 h. After I/R treatment, the cells were washed with PBS followed by fixation for 20 min with 4% paraformaldehyde in PBS. Cells were then washed with 0.2% Triton X-100/PBS for 5 min twice at room temperature. Hoechst33258 nuclear dye was added at a final concentration of 1 μg/ml. Finally, cells were washed with PBS three times. For MDC staining, the cells were treated with 50 μM

MDC (Sigma-Aldrich) in the medium and incubated at 37°C under 5% CO_2 for 20 mins. The localization of LC3 and autophagosome formations was examined by fluorescence microscope.

Western blotting analysis

For western blotting analysis, cells were washed with PBS (pH 7.5) twice and lysed by RIPA buffer [10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM ethylene glycol tetraacetic acid, 0.1% sodium dodecyl sulfate (SDS), 1 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/mL aprotinin and 1 μg/mL leupeptin]. The cell lysate was kept on ice for 10 min and then centrifuged at 10,000×g for 20 min at 4℃. The protein concentration of the supernatant solution was determined by bicinchoninic acid protein assay reagent (Thermo Fisher Scientific, Dreieich, Germany) and bovine serum albumin (BSA) as the standard. Equal amounts of proteins (40 μg) from each sample preparation were added SDS dye to denature and incubated for 5 min at 95℃ and then separated by 6-15% SDS–polyacrylamide gel electrophoresis (PAGE). The proteins electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane using transfer buffer (192 mM glycine, 25 mM Tris, 20% methanol, pH 8.3). The membranes were blocked with 5% fat-free milk in Tris-buffered saline/Tween-20 (TBST) buffer (20 mM Tris, 150 mM NaCl, 0.01% Tween-20, pH 7.5) for 1 h and followed by immunoblotting with primary antibodies overnight at 4°C. After washing three times with TBST, membranes were reacted with secondary goat anti-rabbit or anti-mouse horseradish peroxidase (HRP)-conjugated antibodies

followed by three times washes. The signals were visualized by an enhanced chemiluminescence reagents (Millipore Corporation, Billerica, MA, USA) detection system. Exposures were recorded on X-film. The relative values of each protein were normalized with β-actin from the samples. The following primary antibodies were used in this study: caspase-3 (BD Biosciences), phospho-AMPKα (Cell Signaling Technology, Beverly, MA, USA), AMPKα1 (Cell Signaling Technology), phospho-mTOR (Cell Signaling Technology), LC3B (Cell Signaling Technology), Bax (GeneTex Inc., Irvine, CA, USA) and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Lentivirus infection of shRNA

The shRNAs were purchased from National RNAi core Facility in Academic Sinica, Taipei, Taiwan. The target sequences of AMPKα1 shRNA is (5'- CTTGAAATGTGTGCAAATCTAA -3'). Briefly, H293T cells were co-transfected using the calcium phosphate DNA precipitation method with shRNA-expressing vector, envelope plasmid pMD.G (VSV-G expressing), and packaging plasmid pCMV-△R8.91 (containing *gag, pol* and *rev* genes). Virus was harvested 72 hours post-transfection. Viral supernatants were harvested and used to infect cells with 8 μg/ml polybrene. Stable expressing cells were selected by 2 μl/mL puromycin.

Statistical analysis

Data are presented as means ± SDs. Statistical differences between control and treated groups were determined by Student's *t*-test. Differences were considered significant at $P<0.05$ or 0.01.

CHAPTER III

Results

Effects of renal ischemia/reperfusion (I/R) on cell viability

Several studies indicated that I/R can cause renal cell injury. In order to mimic *in vivo* renal I/R injury, LLC-PK1 cells were incubated with antimycin A and 2DG, which could disturb the function of mitochondria respiratory chain and inhibit energy generation via blocking glycolysis respectively (Li et al, 2008). To evaluate the effects of I/R on renal cell viability, LLC-PK1 cells were treated with different concentrations of antimycin A and 5 mM 2DG for 1.5 h to induce ischemia injury followed by reperfusion for 24 h and the survival rates were determined by PI staining. As presented in Figure 1A, the cell death percentages at reperfusion 24 h were obviously higher than ischemia period in a dose-dependent manner. When cells were treated with 50 μM of antimycin A and 5 mM 2DG for inducing ischemia, cells significantly caused approximately 50% cell death at reperfusion 24 h. We further investigated whether the I/R-induced renal cell death was apoptosis or necrosis. By using Annexin V and PI assays, the results showed that after I/R treatment, cells initially went to early apoptosis, we observed that the early apoptosis percentages were 20.21% at reperfusion 3 h. As reperfusion time increased, most cells went to late apoptosis eventually (Figure 1B). To investigate whether the apoptosis marker protein-caspase-3 could be activated during I/R, we examined the capase-3 cleavage form by western blotting analysis. As shown in Figure 1C, the I/R-treated cells caused an increase in capase-3 cleavage form compared to the control cells and

most obviously at 24 h of reperfusion. Figure 1D revealed the LLC-PK1 cells morphology at various reperfusion time points by microscope. Cells apparently shrunk as reperfusion time increased. These data indicated that renal cells caused cell apoptosis after I/R treatment in a time-dependent manner.

Induction of autophagy by I/R treatment in LLC-PK1 cells

To investigate whether autophagy takes a part in I/R-induced renal cell apoptosis, we examined autophagy induction in LLC-PK1 cells during I/R treatment. Accumulation of LC3 in autophagosome and lipidation of LC3 to form LC3-II are two hallmarks of autophagy and are commonly used for autophagy detection (Mizushima & Yoshimori, 2007). Thus, we initially examined the LC3-II forms protein expression by western blotting analysis. As shown in Figure 2A, we obviously observed that I/R activated the LC3-II forms protein expression (14 kD) in LLC-PK1 cells. The LC3-II forms protein expression was not obvious during the ischemia period, but was significantly enhanced during reperfusion period in a time-dependent manner and particularly at reperfusion 6 h. However, it decreased at reperfusion 24 h. To confirm the result, we used GFP-labeled LC3-transfected cells to examine the LC3 localization during I/R treatment. The results obviously showed GFP-LC3 green dots distribution in cytoplasm at reperfusion 6 h (Figure 2B). We further observed the autophagosome formations by MDC staining, an autofluorescent base that has been reported to accumulate in autophagic vacuoles (Munafo & Colombo, 2001), and

analyzed the results by fluorescence microscope. As shown in Figure 2C, LLC-PK1 cells apparently displayed most positive granules in cytoplasm at reperfusion 6 h. The statistical data also showed more than 50% MDC positive granules at reperfusion 6 h compared to the control group (Figure 2D). The positive control cells were treated with 1 μM rapamycin for 6 h. These data indicated that I/R caused autophagy induction in renal cells.

Autophagy protected renal cells from I/R-induced apoptosis

Several studies suggested that at the early stage of cell stress induction, autophagy is activated and is cytoprotective; however, as cell stress is too severe at late stage, excessive autophagy may trigger cell injury and death (Baehrecke, 2005; Periyasamy-Thandavan et al, 2009). In order to investigate the role of autophagy on renal I/R injury, we used 3MA, a pharmacological inhibitor of autophagy (Seglen & Gordon, 1982), to determine the effect of autophagy on cell apoptosis. As shown in Figure 3, when LLC-PK1 cells were pretreated with 5 mM of 3MA, the cell apoptosis percentages were elevated significantly compared non-treated 3MA cells at reperfusion 24 h by flow cytometry. The result indicated that inhibition of autophagy could increase I/R-induced renal cell apoptosis, suggesting that autophagy might be a cytoprotective mechanism on renal I/R injury.

I/R increased the phosphorylation of AMPK and decreased the phosphorylation of the mTOR in LLC-PK1 cells

Several studies have reported that the energy sensor-AMPK may regulate autophagy through different downstream signals, including inhibition of mTOR, phosphorylation of eukaryotic elongation factor-2 (eEF-2) kinase, phosphorylation of p27, and direct activation of autophagic genes (Liang et al, 2007; Takagi et al, 2007). Furthermore, mTOR signaling plays an important role in autophagy owing to its ability to sense nutrient, metabolic, stress, and hormone signals (Pattingre et al, 2008). Therefore, we examined the AMPK and mTOR protein expression during I/R treatment by western blotting analysis. As shown in Figure 4, the I/R-treated cells caused an increase in AMPKα (62 kD) phosphorylation and a decrease in mTOR (289 kD) phosphorylation compared to the control cells. However, while the reperfusion time increased, these two proteins phosphorylation level recovered afterward. These data suggested that I/R increased the phosphorylation of AMPK and decreased the phosphorylation of mTOR in LLC-PK1 cells.

AMPK negatively regulated the phosphorylation of mTOR and activated autophagy on renal I/R injury

Next, we proposed a hypothesis that is I/R might activate autophagy, moreover, recover renal cells from I/R-induced apoptosis through the AMPK negatively regulates mTOR pathway. First, we used shRNA to knockdown AMPKα1 level of LLC-PK1 cells, the western blotting showed that the

LC3-II forms protein expression decreased and the phosphorylation of mTOR increased significantly at reperfusion 6 h (Figure 5A). We further observed the autophagosome formations by MDC staining and analyzed the results by fluorescence microscope. As shown in Figure 5B, LLC-PK1 cells apparently displayed less positive granules in cytoplasm in knockdown AMPKα1 group compared with normal cells at reperfusion 6 h. The statistical data also showed that less than 50% MDC positive granules in knockdown AMPKα1 group compared to normal cells at reperfusion 6 h (Figure 5C).

AMPK protected renal cells from I/R-induced apoptosis

To investigate whether AMPK can protect renal cells from I/R-induced apoptosis, we examined by using shRNA for AMPKα1 and compound C to inhibit AMPK level of LLC-PK1 cells. As shown in Figure 6A, flow cytometry data showed cell apoptosis percentages significantly elevated at reperfusion 24 h compared to non-knockdown AMPKα1 group. Similar results were found in pretreating cells with 15 μM compound C, cell apoptosis percentages significantly elevated at reperfusion 24 h compared to the non-treated compound C group (Figure 6B). Together, these results demonstrated that AMPK protected renal cells from I/R-induced apoptosis by negatively regulating the phosphorylation of mTOR and activating autophagy.

Inhibition of mTOR could activate autophagy and protect renal cells from I/R-induced apoptosis

In order to identify the relationship between mTOR and autophagy on I/R-induced renal cell apoptosis, LLC-PK1 cells were treated with RAD001, a mTOR inhibitor, together with I/R treatment. As shown in Figure 7A, the western blotting demonstrated that the phosphorylation of mTOR was significantly suppressed and the LC3-II forms protein level was significantly activated at reperfusion 6 h in treating 10 nM RAD001 groups. Moreover, RAD001 treatment obviously reduced I/R-induced renal cell apoptosis at reperfusion 24 h compared with the non-treated RAD001 group (Figure 7B). These data suggested that inhibition of mTOR could decrease I/R-induced renal cell apoptosis by increasing autophagy induction.

Quercetin increased the phosphorylation of AMPK and activated autophagy on renal I/R injury rather than NAC

Owing to oxidative stress is considered a major event on I/R injury, therefore, we would like to investigate the protective effect of antioxidant on I/R injury in renal cells. In addition, there is an evidence corroborated that quercetin reduces renal injury by preventing the oxidative stress dependent on I/R *in vivo* (Inal et al, 2002), however, its other protective molecular mechanisms are still unknown. On the other hand, several studies suggested that quercetin could regulate downstream signals through activating AMPK protein expression (Ahn et al, 2008; Jung et al, 2010). Therefore, we attempted to examine the antioxidant-caused possible molecular mechanisms

on renal I/R injury. First, we used two kinds of antioxidants-quercetin and NAC to investigate. As shown in Figure 8, by using western blotting, LLC-PK1 cells were exposed to 30 μM quecetin or 2 mM NAC during I/R treatment. The results showed that quercetin increased the phosphorylation of AMPK and the LC3-II forms protein expression at reperfusion 6 h. However, NAC didn't alter these protein levels. The result indicated that quercetin might participate in activating the AMPK-regulated autophagy induction pathway rather NAC on renal I/R injury.

Quercetin increased the phosphorylation of AMPK, decreased the phosphorylation of mTOR, and activated autophagy on renal I/R injury

To ascertain if quercetin increased the phosphorylation of AMPK and activated the LC3-II forms protein expression, by using western blotting, we found that quercetin affected these protein expressions in a dose-dependent manner at reperfusion 6 h during I/R treatment (Figure 9A). Next, as shown in Figure 9B, the western blotting significantly showed that the phosphorylation of AMPK was increased, the phosphorylation of mTOR was suppressed, and the LC3-II forms protein level was activated in treating 30 μM quecetin groups during I/R treatment.

Quercetin protected renal cells from I/R-induced apoptosis

To investigate whether quercetin could protect renal cells from I/R-induced apoptosis, we examined apoptosis markers after treating quercetin during I/R treatment. As shown in Figure 10A, quercetin protected renal cells from I/R-induced apoptosis at reperfusion 24 h in a dose-dependent manner by flow cytometry. In addition, western blotting showed that the proapoptotic protein-bax (21 kD) expression was obviously reduced in treating with 30 μM quecetin cells at reperfusin 6 and 24 h (Figure 10B).

Quercetin recovered cells from downregulation of AMPK-induced mTOR phosphorylation, autophagy inactivation, and cell apoptosis on renal I/R injury

To elucidate if quercetin protected renal cells from I/R-induced apoptosis is through the AMPK activation pathway. By using shRNA to knockdown AMPKα1, the western blotting showed that the phosphorylation of AMPK protein and the LC3-II forms protein expression, which at first were inhibited by shRNA for AMPKα1, were enhanced in treating with 30 μM quecetin compared with non-treated quercetin cells at reperfusin 1 and 6 h. However, the phosphorylation of mTOR protein expressions had opposite results (Figure 11A). Moreover, by using flow cytometry, in I/R-treated renal cells, quercetin reversed the renal cell apoptosis, which induced by shRNA for AMPKα1 (Figure 11B). These results suggested that quercetin may reduce the I/R-induced renal cell injury through an AMPK-regulated autophagy induction pathway.

CHAPTER IV

Discussion

AKI refers to a complex disorder including multiple causative factors and occurs in a variety of settings with varied clinical manifestations. I/R is most common cause of AKI. It inevitably occurs in many clinical conditions such as hypovolemic shock, thromboembolism, and renal transplantation and results in a clinical problem (Inal et al, 2002). It can induce several mechanisms to cause repair and regeneration processes take place together with I/R-induced cellular apoptosis, autophagy, or necrosis depends on whether cell death or regeneration prevails (Kosieradzki & Rowinski, 2008). In this study, we investigated the role of autophagy on renal I/R injury, because there are increasing lines of evidence suggested that autophagy participates in I/R injury and plays a protective role in heart and kidney (Jiang et al, 2010; Takagi et al, 2007). Consistent with previous reports, our data revealed that I/R certainly induced autophagy in renal cells, and the induction of autophagy plays a protective role on renal I/R injury (Figure 2 and 3). However, very little is known about the molecular mechanisms of autophagy induction on renal I/R injury. Therefore, the aim of this study is to clarify the molecular mechanisms of autophagy on I/R injury in renal proximal tubular epithelial cells. In the recent years, several studies revealed that the energy sensor-AMPK regulates autophagy through different downstream signals, including inhibition of mTOR, phosphorylation of eEF-2 kinase, phosphorylation of p27, and direct activation of autophagic genes. (Liang et al, 2007; Takagi et al, 2007). Besides, the mTOR pathway is a major positive stimulus for

protein synthesis, cell growth, and cell size under a cellular stress (Bolster et al, 2002). Since mTOR negatively regulates autophagy, the AMPK negatively regulates mTOR pathway has been considered to be an important regulator of autophagy in response to energy depletion (Herrero-Martin et al, 2009; Liang et al, 2007; Meley et al, 2006). Many studies found that I/R-induced injury could activate AMPK protein expression, which is considered a major event on I/R-induced injury. Despite the recent advances, there remain significant gaps in the present understanding of both the upstream regulating pathways and the downstream substrates for AMPK on renal I/R injury. Therefore, we examined the AMPK protein and its downstream signals protein expression on renal I/R injury. Our data revealed that autophagy could protect the renal cells from I/R-induced apoptosis through the AMPK negatively regulated mTOR pathway (Figure 4, 5, and 6).

However, previous studies suggested that autophagy may be a mechanism for cell survival but may also induce cell death. Autophagy and its related signaling seem to be integrated with that of cell death (Periyasamy-Thandavan et al, 2009). Several studies also demonstrated that when cell stress induced at the early stage, autophagy is activated and is cytoprotective. However, as cell stress is too severe at late stage, excessive autophagy may trigger cell injury and death (Baehrecke, 2005). Nevertheless, it remains largely to clarify how autophagy protects or kills a cell. Obviously, many signaling pathways activated by environmental stress play roles in the regulation of autophagy and cell death. These include mTOR, eIF2*α,* PI3K/Akt, Bcl-2 family protein, AMPK, and mitogen-activated protein kinase (MAPK). Even at the level of core machineries, autophagy

and cell death share important molecules, which may determine the fate of the cells. Thus, the cross-regulation between autophagy and apoptosis raise an interesting possibility that signaling activated during autophagy interfering cell death pathways (Periyasamy-Thandavan et al, 2009). Therefore, the mechanisms of autophagy induction and regulation in renal pathophysiology should be further explored.

Recently, causing of endoplasmic reticulum (ER) stress proteins activation have been characterized on renal I/R injury (Paschen, 2001; Wouters & Koritzinsky, 2008). In addition, ER stress through unfolded protein response and intracellular calcium has been implicated in autophagy regulation (Hoyer-Hansen & Jaattela, 2007). Previous study also showed that ER stress induced autophagy in renal tubular cells (Kawakami et al, 2009). One of the mechanisms connecting calcium release from the ER and autophagy is the stimulation of AMPK (Hoyer-Hansen et al, 2007). Further, there is a study indicated that increases in cytosolic calcium concentration upon treatment with different ER stress inducers stimulate CaMKKβ in response to an increase in the cytosolic-free calcium, leading in turn to AMPK activation, inhibition of mTORC1, and autophagy stimulation (Hoyer-Hansen et al, 2007). In this study, our data also indicated that ER stress markers, such as eukaryotic initiation factor 2α (eIF2α), C/EBP homologous protein (CHOP), glucose-regulated protein (GRP) 78, could be activated following I/R treatment in renal cells (data not shown). Owing to previous studies illustrated about these signaling relationships, it would be important to investigate the ER stress-regulated AMPK pathway as a mechanism of autophagy induction under

the I/R condition.

On the other hand, because oxidative stress is considered a hallmark on I/R-induced renal injury, we investigated the antioxidants effects on renal I/R injury and its possible molecular mechanisms. One of antioxidants, quercetin, which is a member of the flavonoids and is the most potent scavenger of ROS, is successfully employed in the prevention and treatment in variety of diseases (Ahn et al, 2008). Many studies have demonstrated that quercetin possesses a lot of activities including anti-oxidative activity (Abdel-Raheem et al, 2009), anti-inflammation (Morales et al, 2006), and anti-apoptosis (Ishikawa & Kitamura, 2000). The antioxidant efficacy of quercetin may be owing to its higher diffusion into the membranes (Moridani et al, 2003), allowing it to scavenge oxyradicals through the lipid bilayer. Quercetin also could inhibit oxidative enzymes such as xanthine oxidase, lipoxygenase, and NADPH oxidase (Day et al, 2000). Further, it has been demonstrated that quercetin metabolites can also inhibit peroxynitrite-mediated oxidation, similar to free quercetin (Klotz & Sies, 2003). There is a study also demonstrated that quercetin induced heme oxygenase-1 (HO-1), which resulted in reducing ethanol-derived oxidative damage in human hepatocytes *in vitro* (Yao et al, 2007). Besides, increasing evidences demonstrated that quercetin has the potential to reduce renal injury and help the improvement of kidney functions. For example, quercetin could reduce cisplatin and lead-induced nephrotoxicity or prevent chronic cadmium nephrotoxicity in rats based on the anti-oxidant properties of quercetin (Abdel-Raheem et al, 2009; Behling et al, 2006; Mishra & Flora, 2008; Morales et al, 2006; Renugadevi & Prabu, 2010). Also,

there is a study suggested that renal protection exerted by quercetin seems to be based on its ability to increase metallothionein in kidneys (Morales et al, 2006). Furthermore, an *in vivo* study showed that quercetin could protect kidney by significantly reducing the serum levels of creatinine and urea nitrogen in a model of renal I/R in rats (Singh et al, 2004). However, the molecular mechanisms of the nephroprotective effects of quercetin on I/R-induced injury are not yet completely understood. Therefore, the aim of this study was to investigate the possible nephroprotective mechanisms of quercetin against I/R-induced injury in renal cells. In the aspect of protective mechanisms of quercetin, there is a study indicated that quercetin could induce protective autophagy in cancer cells (Wang et al, 2011). On the other hand, recent studies demonstrated that quercetin could significantly accelerate the phosphorylation of AMPK protein expression (Ahn et al, 2008; Jung et al, 2010). Thus, in an attempt to elucidate the molecular mechanisms underlying quercetin protected renal cells from I/R-induced apoptosis by the AMPK-regulated autophagy induction pathway, we measured the protein levels of the phosphorylated AMPK, phosphorylated mTOR, and LC3 following I/R treatment. First, we observed that quercetin could obviously attenuate the ROS production on I//R injury in renal cells (data not shown). Consistent with hypothesis, our data revealed that quercetin activates the phosphorylation of AMPK, inactivated the phosphorylation of mTOR, and induced the LC3-II forms protein expression (Figure 9B). Unexpectedly, NAC, an antioxidant, didn't alter the protein expressions in this study. These results indicated that quercetin may specifically induce the AMPK-regulated pathway to protect renal cells from the I/R-induced

injury (Figure 8). Moreover, in I/R-treated renal cells, quercetin could also reverse the renal cell autophagy and cell apoptosis, which inhibited and induced by shRNA for AMPKα1 respectively (Figure 11A and 11B). These results suggested that quercetin might reduce the I/R-induced renal cell injury by the AMPK-regulated autophagy induction pathway. However, the further studies need to be investigated in the future: the relationship between mTOR, autophagy, and cell apoptosis in quercetin-treated cells on renal I/R injury.

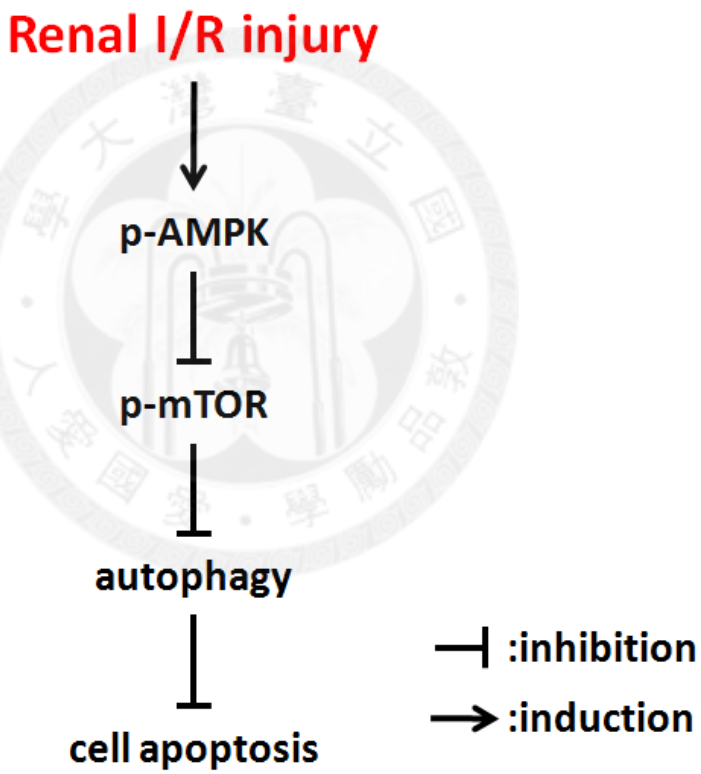
However, all of our data didn't demonstrate the molecular mechanisms on I/R-induced renal injury *in vivo*. This part remains further to investigate, thus, the molecular mechanisms of autophagy on renal I/R injury will be clear.

In conclusion, the present study provides the evidence that autophagy protects renal cells from I/R injury through an AMPK negatively regulated mTOR pathway. Moreover, quercetin may reduce I/R-induced renal cell injury via the AMPK-regulated autophagy induction pathway. Taken together, we demonstrate for the first time that an AMPK-regulated autophagy induction pathway plays an important protective role on renal I/R injury.

CHAPTER V

Conclusion

Taken together, these findings suggest that autophagy protects renal cells from I/R injury through an AMPK negatively regulated mTOR pathway. On the other hand, quercetin may reduce I/R-induced renal cell injury via an AMPK-regulated autophagy induction pathway.



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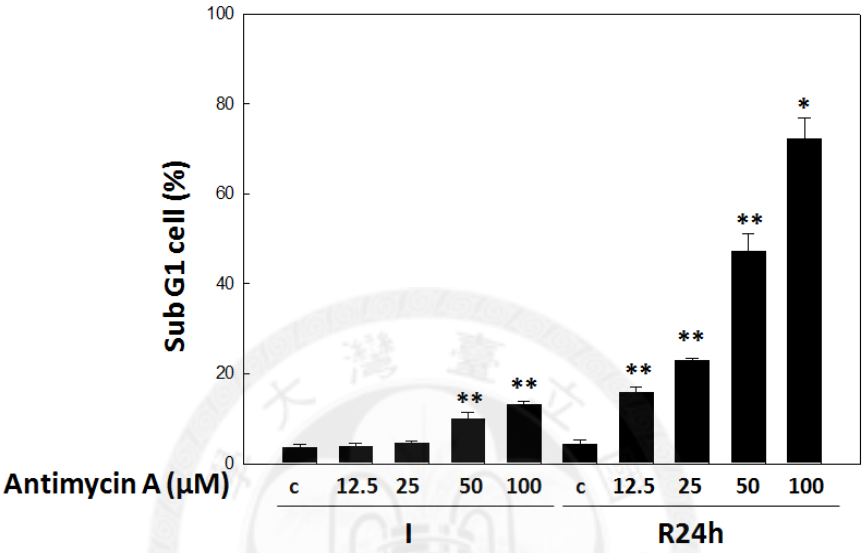
Figures and Figure legends

Figure 1

(A)



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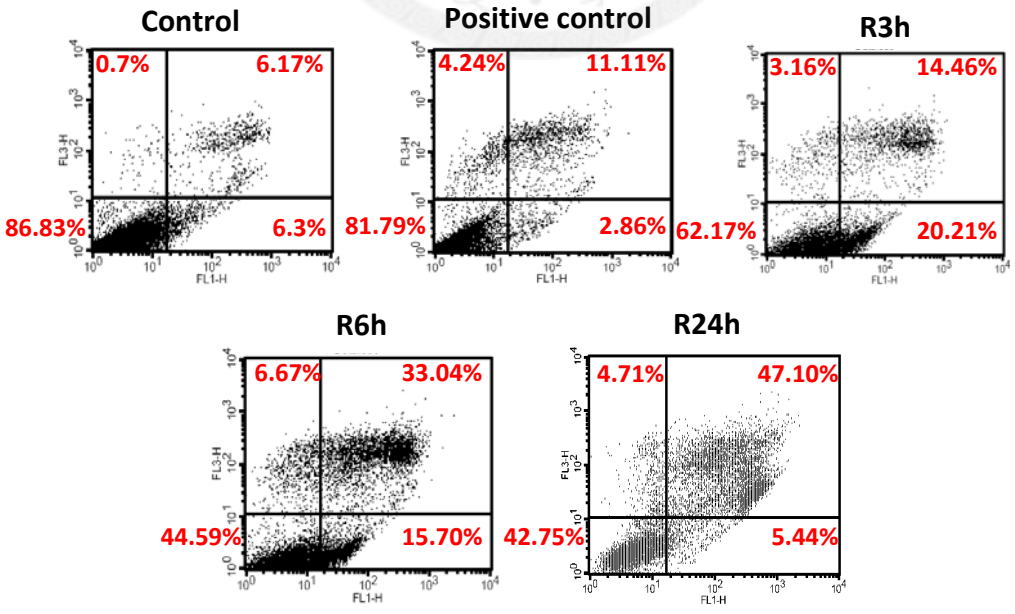

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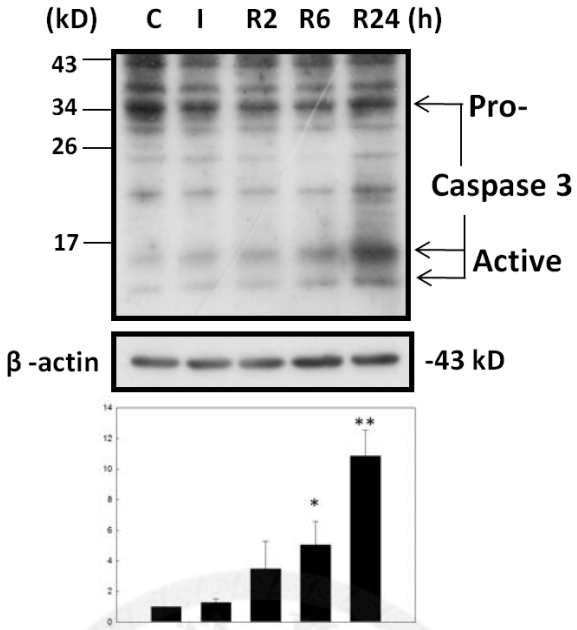
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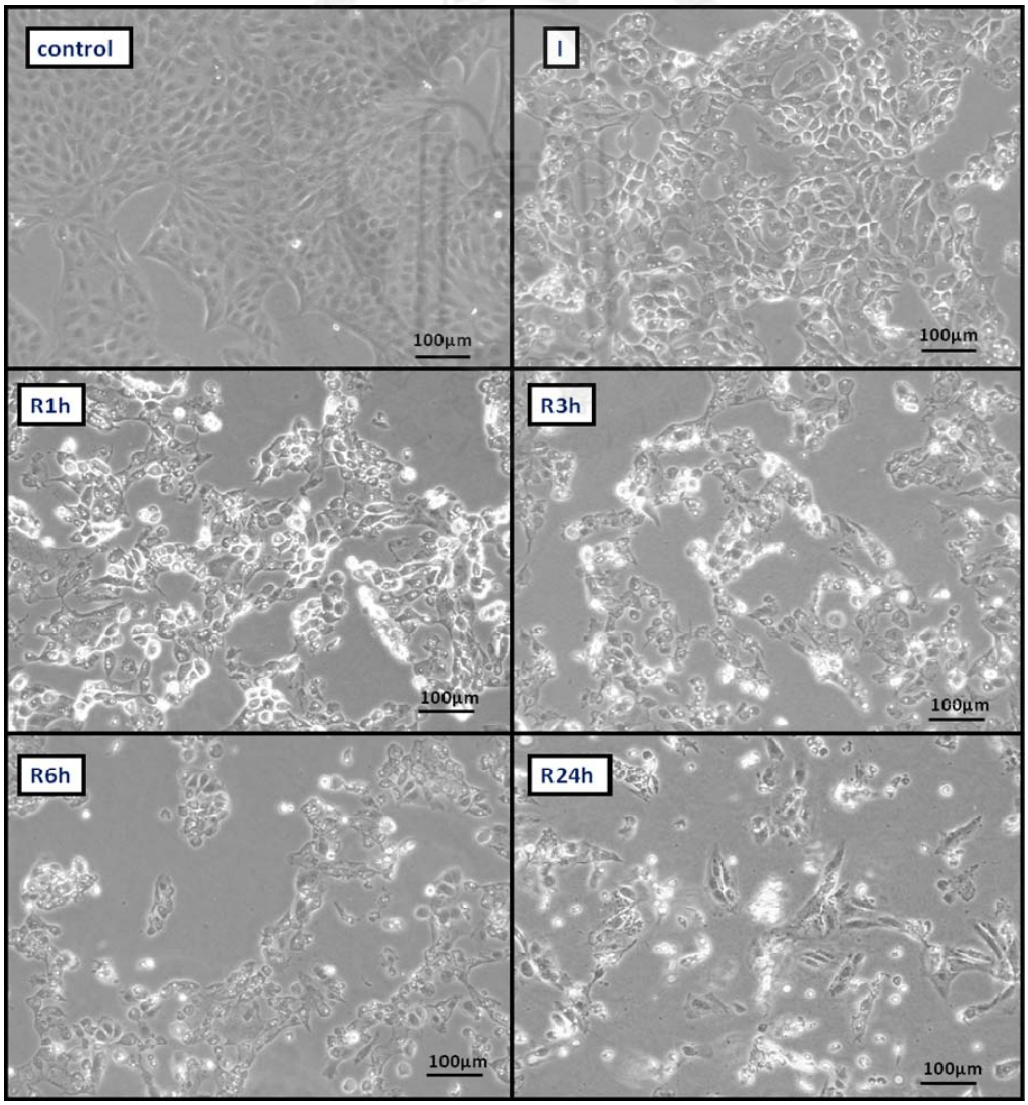
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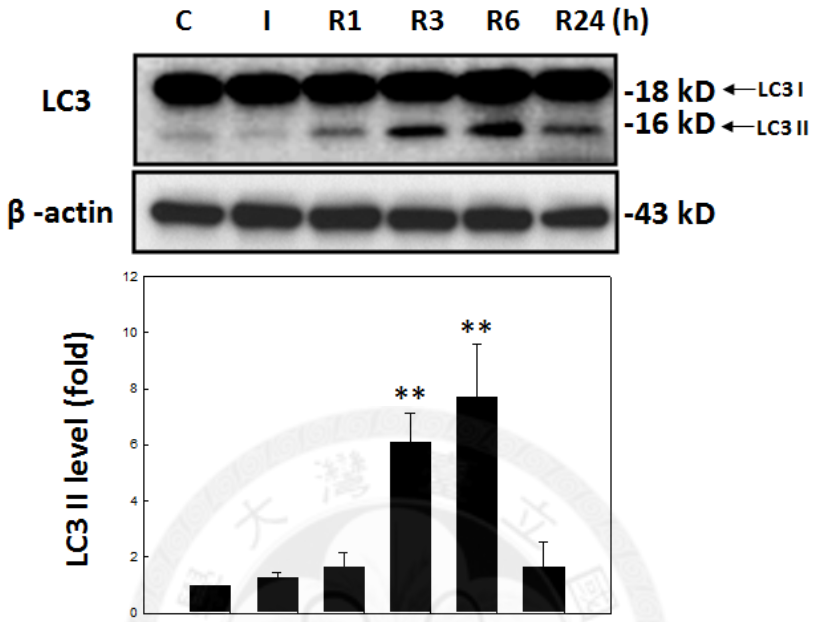
Figure 1. Effects of renal I/R on cell viability. (A) LLC-PK1 cells were treated with different concentrations of antimycin A (12.5, 25, 50, and 100 μM) and 5 mM 2DG for 1.5 h to induce ischemia injury followed by reperfusion for 24 h. Then the cells were fixed and stained with PI. Percentages of cells with the hypodiploid DNA content were determined by flow cytometry. Results are means ± SDs of three separate experiments. *, Significant difference compared to the vehicle-treated group (*P<0.05, **P<0.01). (B) LLC-PK1 cells were treated with 50 μM antimycin A and 5 mM 2DG for 1.5 h followed by reperfusion for 3, 6, and 24 h. Then the cells were stained with Annexin V and PI. Percentages of cells with different cell death patterns were determined by flow cytometry. Apoptosis positive control cells were treated with 3% formaldehyde for 1 h. Upper left panel means necrosis cells; upper right panel means late apoptosis cells; lower left panel means normal cells; lower right panel means early apoptosis cells. (C) LLC-PK1 cells were treated with 50 μM antimycin A and 5 mM 2DG for 1.5 h followed by reperfusion for 2, 6, and 24 h. Whole cell extracts were prepared and protein expression level was estimated by western blotting using antibodies that recognized of caspase-3. β-actin was used to an internal control. The relative level of caspase-3 cleavage form were densitometric analyses of three independent experiments and expressed as the means ± SDs. *, Significant difference compared to the vehicle-treated group (*P<0.05, **P<0.01). (D) LLC-PK1 cells were treated with 50 μM antimycin A and 5 mM 2DG for 1.5 h followed by reperfusion for 1, 3, 6, and 24 h. Cell morphology after I/R treatment was examined by the microscope. I: ischemia; R: reperfusion; h: hour.

Figure 2

(A)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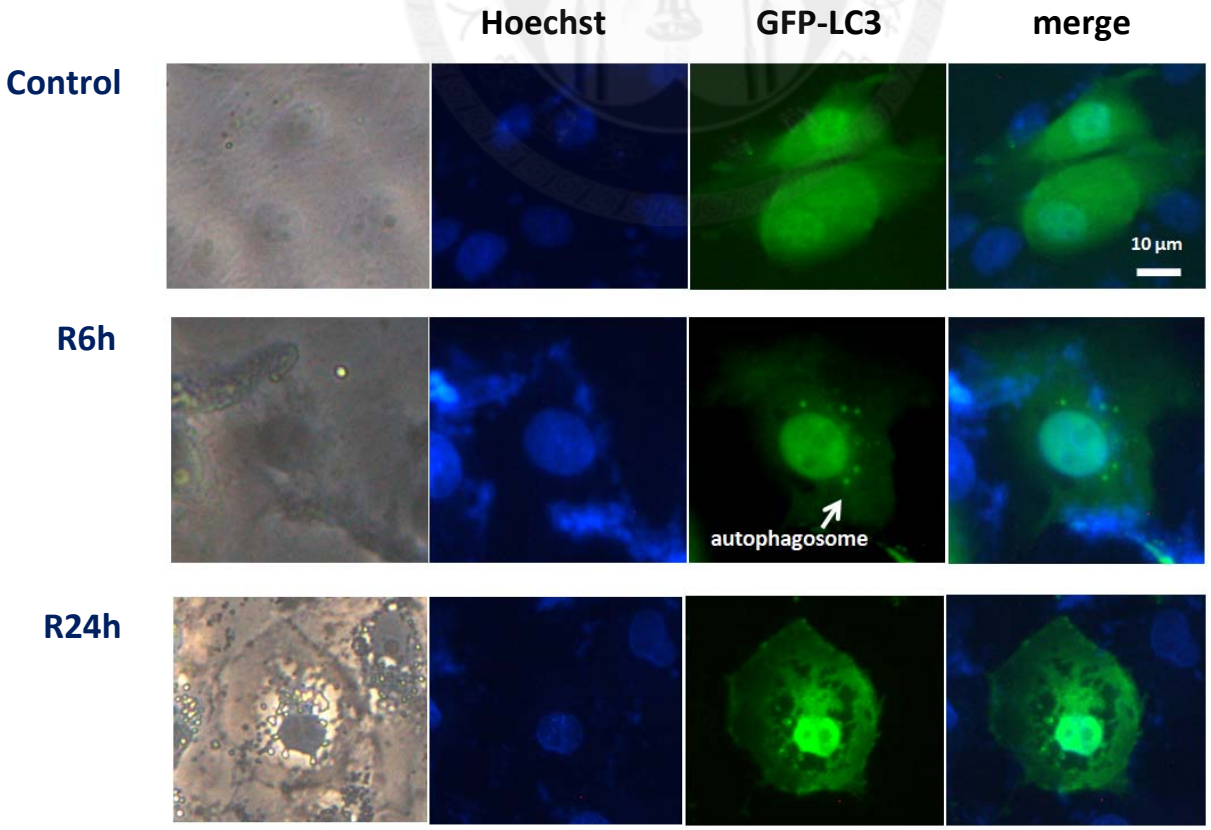
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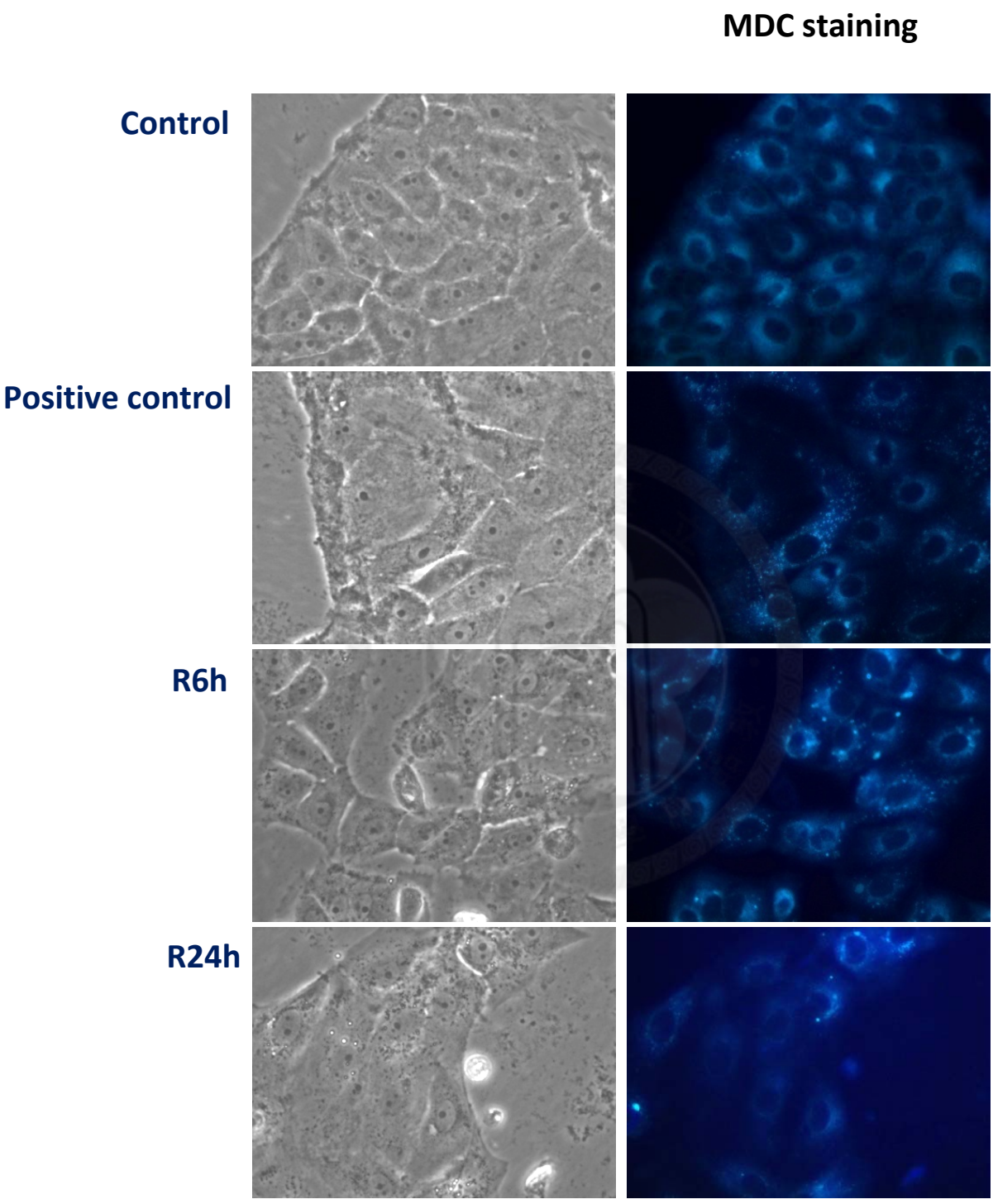
(C)



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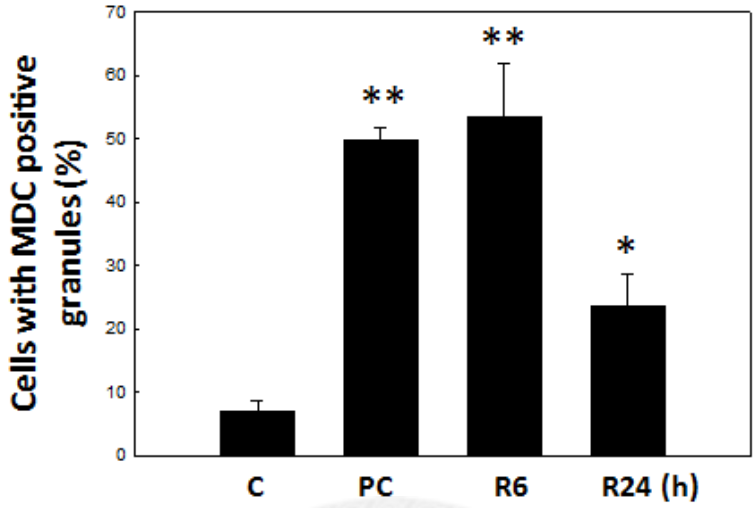


Figure 2. Induction of autophagy by I/R treatment in LLC-PK1 cells. (A) LLC-PK1 cells were treated with 50 μM antimycin A and 5 mM 2DG for 1.5 h followed by reperfusion for 1, 3, 6, and 24 h. Whole cell extracts were prepared and autophagy activation was estimated by western blotting using antibodies that recognized of LC3. β-actin (43 kD) was used to an internal control. The relative level of LC3-II forms (14 kD) were densitometric analyses of three independent experiments and expressed as the means ± SDs. *, Significant difference compared to the vehicle-treated group (**P<0.01). (B) LLC-PK1 cells were transiently transfected with GFP-LC3 for 4 h before I/R treatment. The transfected cells were then treated with 50 μM antimycin A and 5 mM 2DG for 1.5 h followed by reperfusion for 6 and 24 h. After I/R treatment, the cells were stained with 1 μg/ml Hoechst33258 nuclear dye. The formation of GFP-LC3 puncta was examined by fluorescence microscopy immediately after reperfusion, arrow indicates autophagosome

formations. (C) LLC-PK1 cells were treated with 50 μM antimycin A and 5 mM 2DG for 1.5 h followed by reperfusion for 6 and 24 h before MDC staining. Cells were treated with 1 μM rapamycin for 6 h as positive control for autophagosome formations. The granular positive MDC staining cells were examined by fluorescence microscope immediately after reperfusion. (D) The number of cells with a granular positive MDC staining was counted (a minimum of 100 cells per sample). The MDC staining percentage values represent means ± SDs of three independent experiments. *, Significant difference compared to the vehicle-treated group (*P<0.05, **P<0.01). I: ischemia; R: reperfusion; h: hour; PC: positive control.

Figure 3

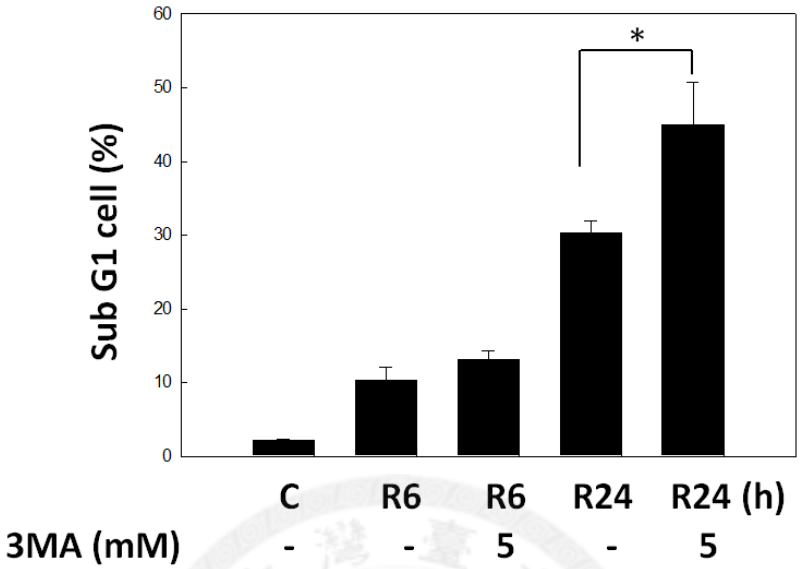


Figure 3. Autophagy protected renal cells from I/R-induced apoptosis. LLC-PK1 cells were treated with 50 μM antimycin A and 5 mM 2DG for 1.5 h followed by reperfusion for 6 and 24 h in the absence or presence of 5 mM 3MA. Then the cells were fixed and stained with PI. Percentages of cells with the hypodiploid DNA content were determined by flow cytometry. Results are means ± SDs of three separate experiments. *, Significant difference compared to respective study group (*P<0.05). R: reperfusion; h: hour.

Figure 4

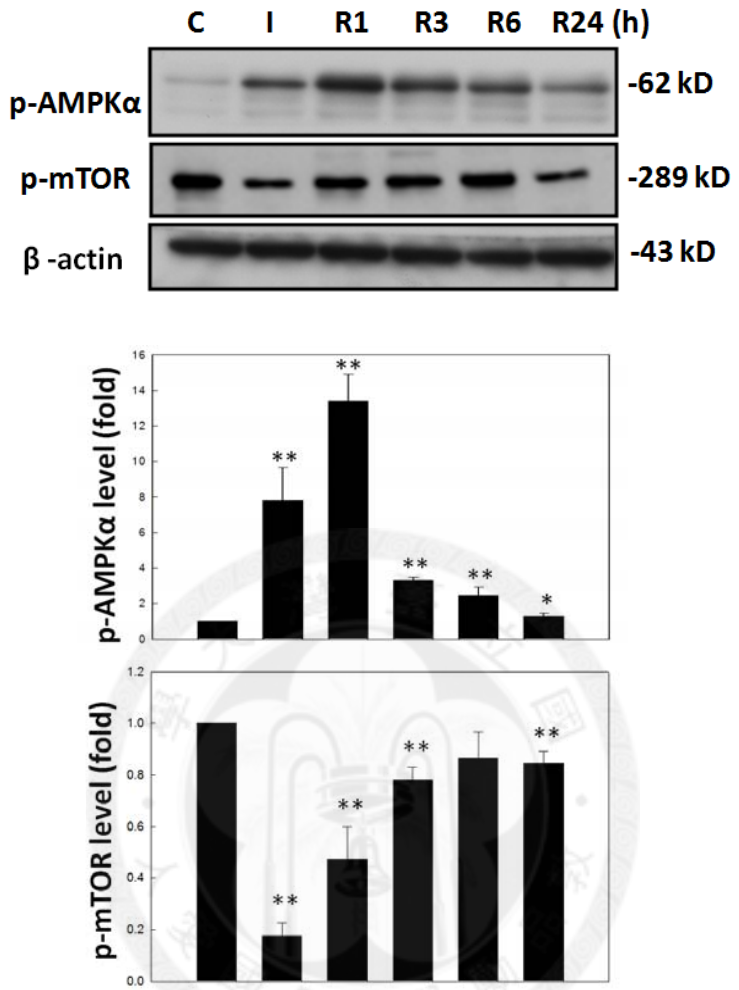
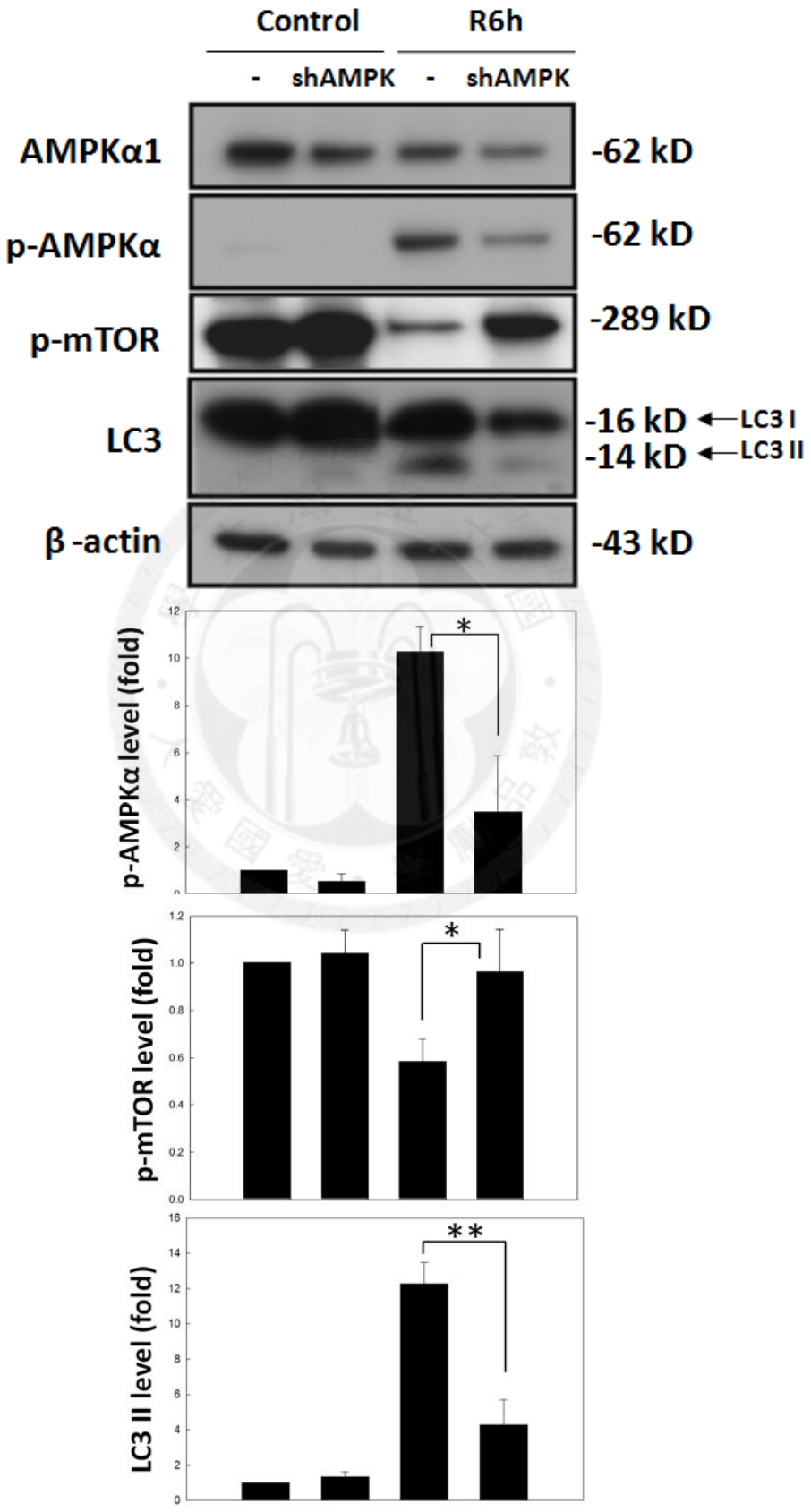


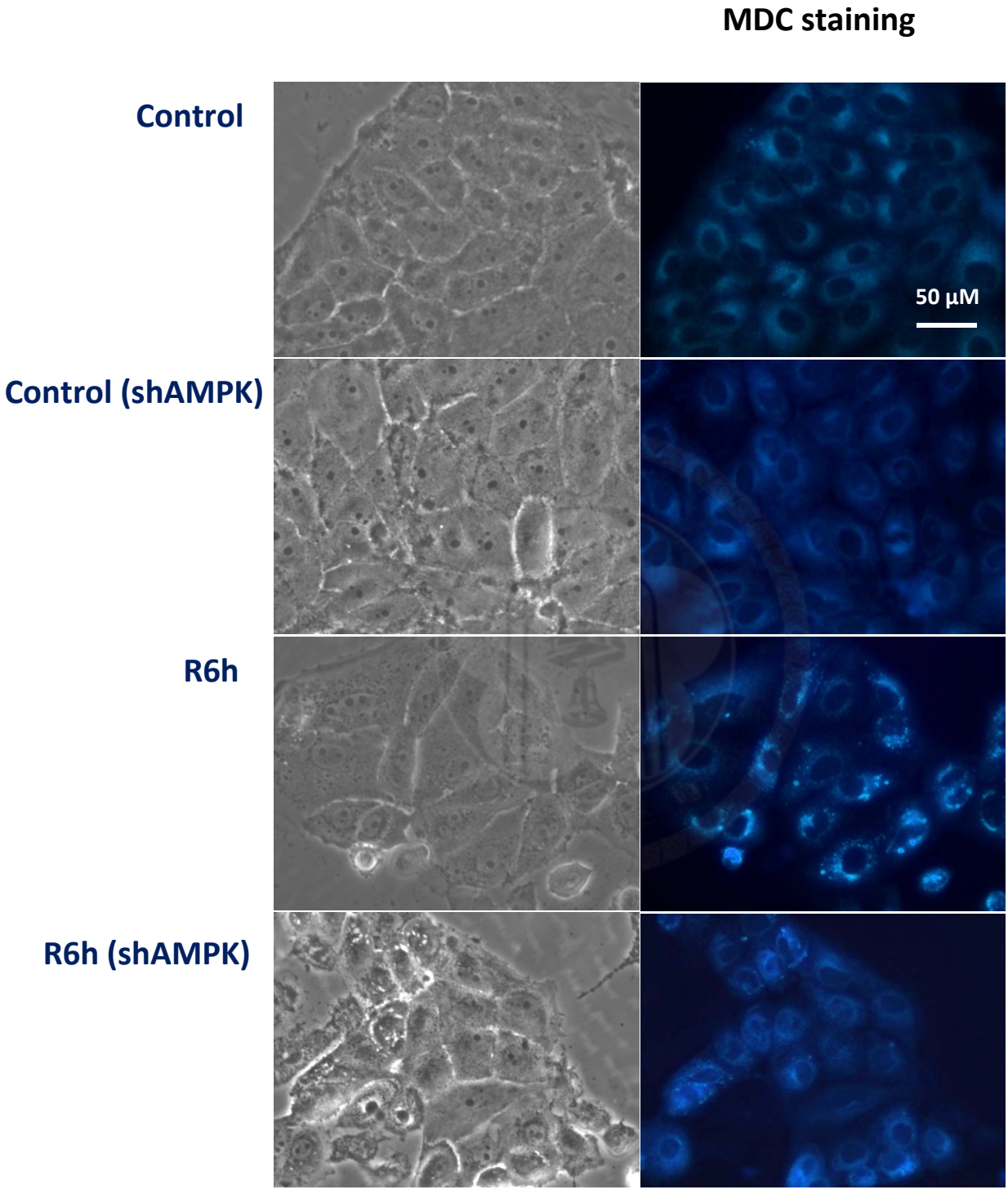
Figure 4. I/R increased the phosphorylation of AMPKα and decreased the phosphorylation of mTOR in LLC-PK1 cells. LLC-PK1 cells were treated with 50 μM antimycin A and 5 mM 2DG for 1.5 h followed by reperfusion for 1, 3, 6, and 24 h. Whole cell extracts were prepared and protein expression level was estimated by western blotting using antibodies that recognized of phospho-AMPKα (62 kD) and phospho-mTOR (289 kD). β-actin was used to an internal control. The relative level of phospho-AMPKα and phospho-mTOR were densitometric analyses of three independent experiments and expressed as the means ± SDs. *, Significant difference compared to the vehicle-treated group (*P<0.05, **P<0.01). I: ischemia; R: reperfusion; h: hour.

Figure 5

(A)


Control
R6h
- shAMPK - shAMPK
AMPKα1
-62 kD
p-AMPKα
-62 kD
p-mTOR
-289 kD
LC3
-16 kD ←LC3 I
-14 kD ←LC3 II
β -actin
-43 kD
p-AMPKα level (fold)
p-mTOR level (fold)
LC3 II level (fold)
*
*
**

(B)



(C)

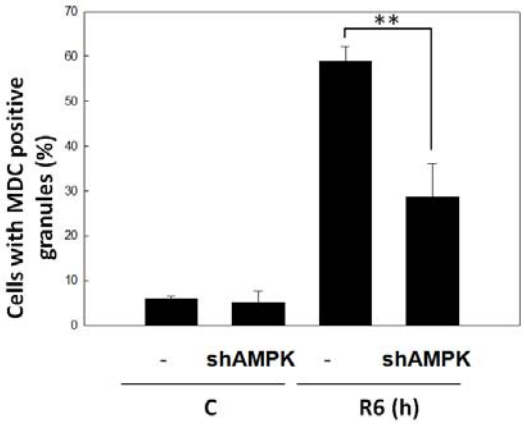


Figure 5. AMPK negatively regulated the phosphorylation of mTOR and activated autophagy on renal I/R injury. (A) LLC-PK1 cells which were infected with scramble shRNA or shRNA for AMPKα1 were treated with 50 μM antimycin A and 5 mM 2DG for 1.5 h followed by reperfusion for 6 h. Whole cell extracts were prepared and protein expression level was estimated by western blotting using antibodies that recognized of AMPKα1, phospho-AMPKα, phospho-mTOR and LC3. β-actin was used to an internal control. The relative level of phospho-AMPKα, phospho-mTOR, and LC3-II forms were densitometric analyses of three independent experiments and expressed as the means ± SDs. (B) LLC-PK1 cells which were infected with scramble shRNA or shRNA for AMPKα1 were treated with 50 μM antimycin A and 5 mM 2DG for 1.5 h followed by reperfusion for 6 h before MDC staining. The granular positive MDC staining cells were examined by fluorescence microscope immediately after reperfusion 6 h. (C) The number of cells with a granular positive MDC staining was counted (a minimum of 100 cells per sample). The MDC staining percentage values represent means ± SDs of three independent experiments. *, Significant difference compared to respective study group (*P<0.05, **P<0.01). R: reperfusion; h: hour.

Figure 6

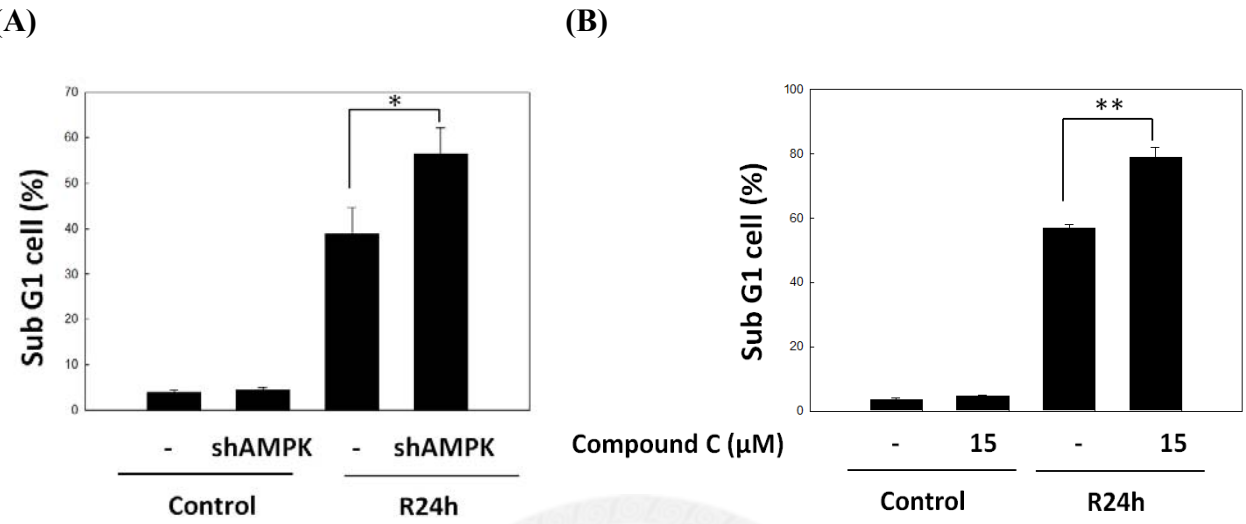
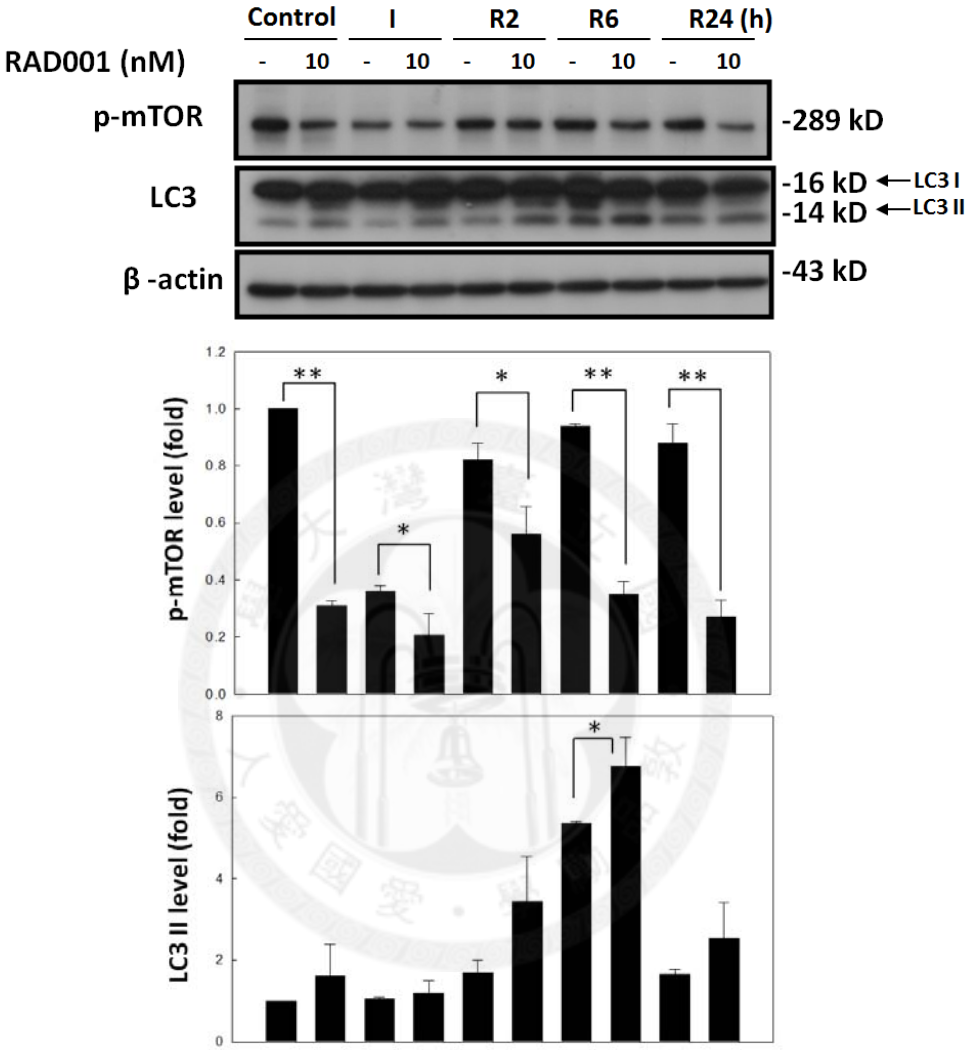


Figure 6. AMPK protected renal cells from I/R-induced apoptosis. (A) LLC-PK1 cells which were infected with scramble shRNA or shRNA for AMPKα1 were treated with 50 μM antimycin A and 5 mM 2DG for 1.5 h followed by reperfusion for 24 h. Then the cells were fixed and stained with PI. (B) LLC-PK1 cells were treated with 50 μM antimycin A and 5 mM 2DG for 1.5 h followed by reperfusion for 24 h in the absence or presence of 15 μM compound C. Then the cells were fixed and stained with PI. Percentages of cells with the hypodiploid DNA content were determined by flow cytometry. Results are means ± SDs of three separate experiments. *, Significant difference compared to respective study group (**P<0.01). R: reperfusion; h: hour.

Figure 7

(A)


Control
I
R2
R6
R24 (h)
RAD001 (nM)
- 10 - 10 - 10 - 10 - 10
p-mTOR
-289 kD
LC3
-16 kD ←LC3 I
-14 kD ←LC3 II
β -actin
-43 kD
p-mTOR level (fold)
LC3 II level (fold)

(B)

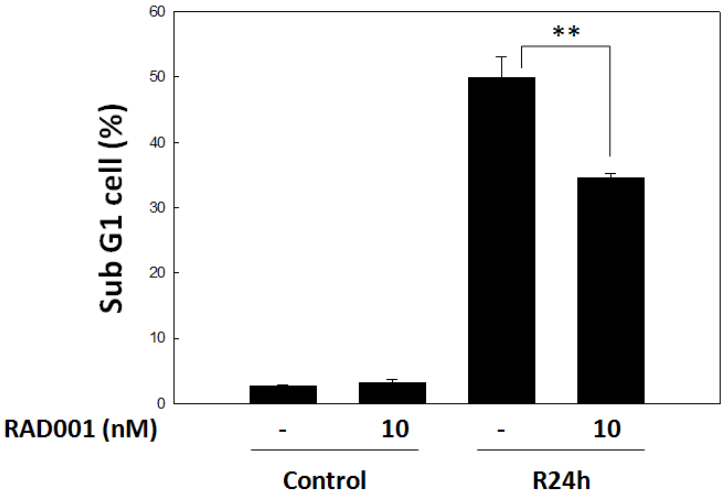

Sub G1 cell (%)
RAD001 (nM)
- 10 - 10
Control
R24h

Figure 7. Inhibition of mTOR could activate autophagy and protect renal cells from I/R-induced apoptosis. (A) LLC-PK1 cells were treated with 50 μM antimycin A and 5 mM 2DG for 1.5 h followed by reperfusion for 2, 6, and 24 h in the absence or presence of 10 nM RAD001. Whole cell extracts were prepared and protein expression level was estimated by western blotting using antibodies that recognized of phospho-mTOR and LC3. β-actin was used to an internal control. The relative level of phospho-mTOR and LC3-II forms were densitometric analyses of three independent experiments and expressed as the means ± SDs. (B) LLC-PK1 cells were treated with 50 μM antimycin A and 5 mM 2DG for 1.5 h followed by reperfusion for 24 h in the absence or presence of 10 nM RAD001. Then the cells were fixed and stained with PI. Percentages of cells with the hypodiploid DNA content were determined by flow cytometry. Results are means ± SDs of three separate experiments. *, Significant difference compared to respective study group (*P<0.05, **P<0.01). I: ischemia; R: reperfusion; h: hour.

Figure 8

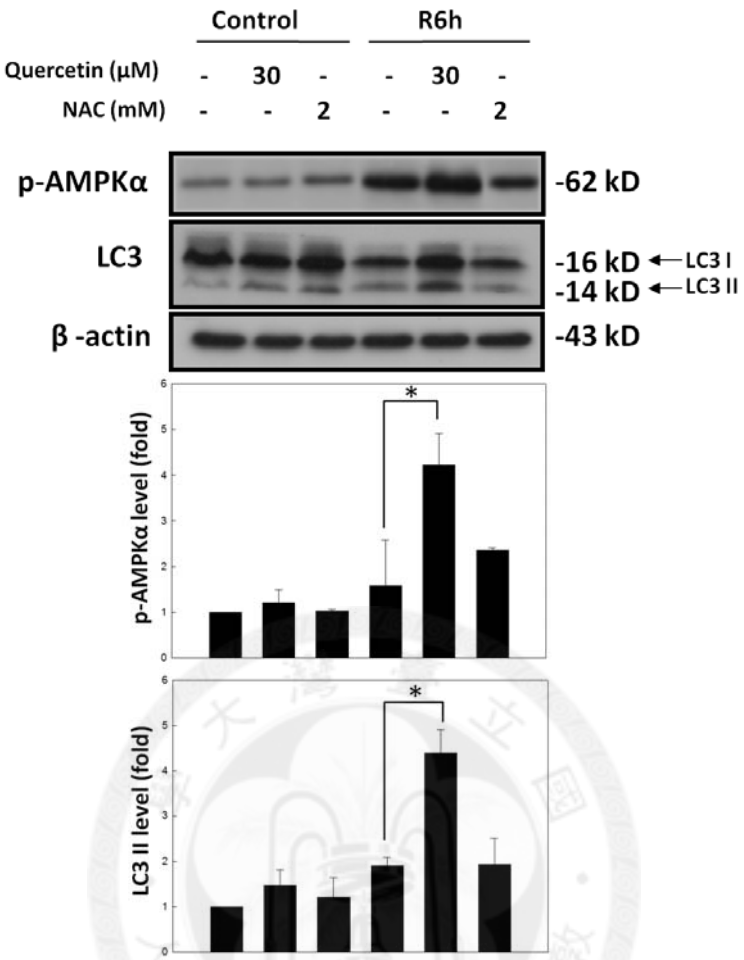
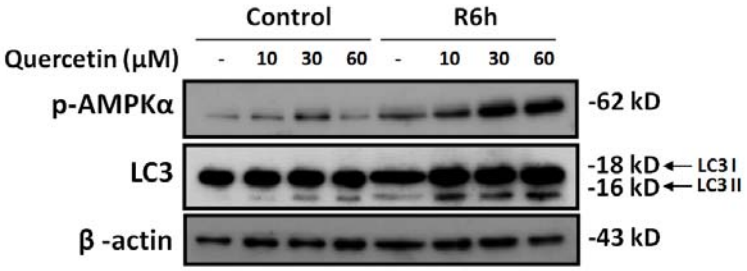


Figure 8. Quercetin increased the phosphorylation of AMPK and activated autophagy on renal I/R injury rather than NAC. LLC-PK1 cells were treated with 50 μM antimycin A and 5 mM 2DG for 1.5 h followed by reperfusion for 6 h in the absence or presence of 30 μM of quercetin and/or 2 mM NAC. Whole cell extracts were prepared and protein expression level was estimated by western blotting using antibodies that recognized of phospho-AMPKα and LC3. β-actin was used to an internal control. The relative level of phospho-AMPKα and LC3-II forms were densitometric analyses of three independent experiments and expressed as the means ± SDs. *, Significant difference compared to respective study group (*P<0.05). R: reperfusion; h: hour.

Figure 9

(A)


Control
R6h
Quercetin (μM)
- 10 30 60 - 10 30 60
p-AMPKα
-62 kD
LC3
-18 kD ← LC3 I
-16 kD ← LC3 II
β -actin
-43 kD

(B)

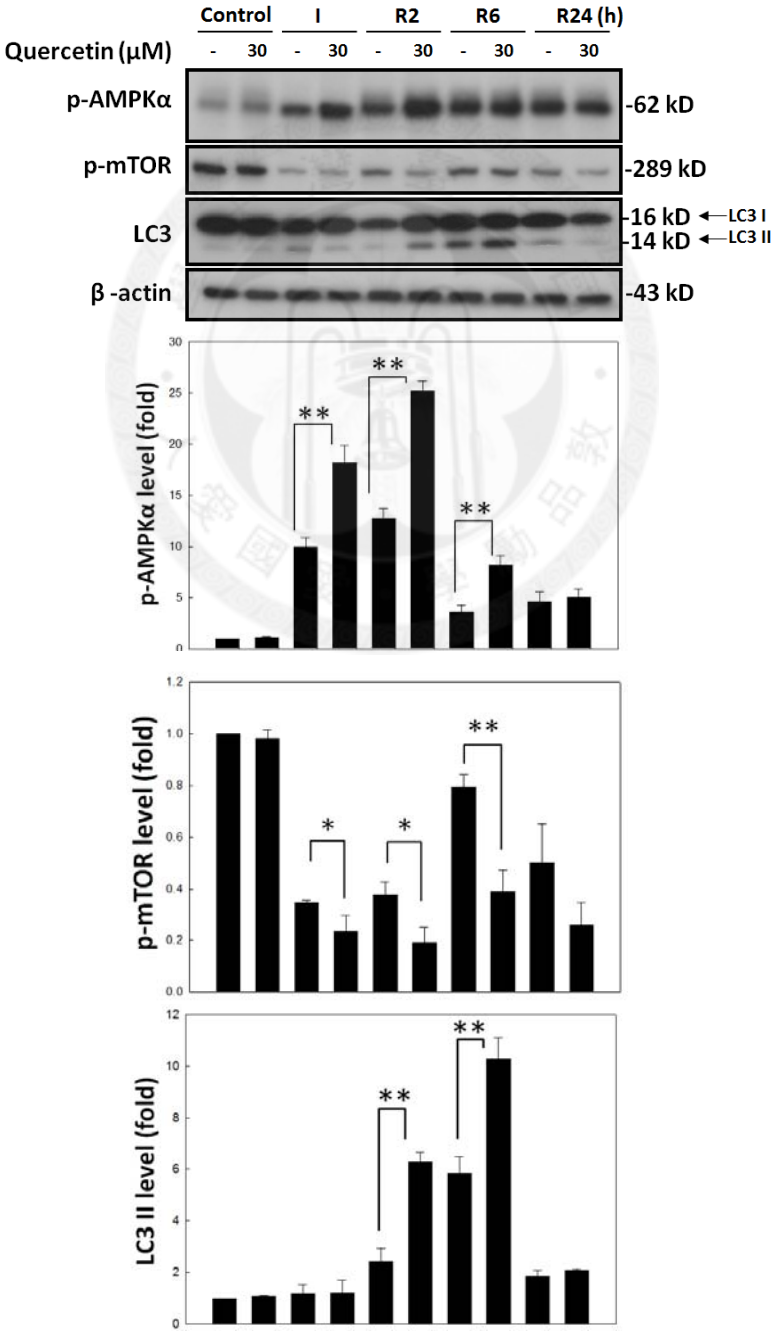
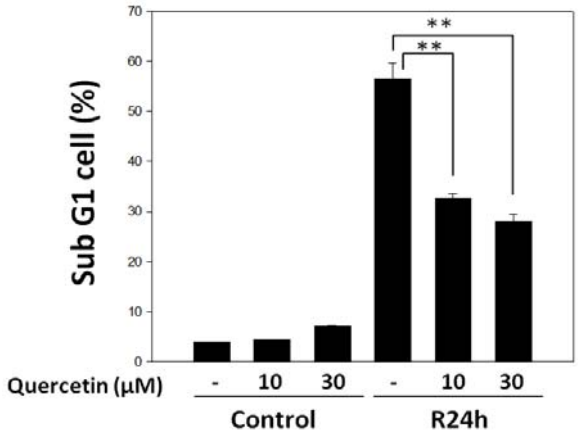

Control
I
R2
R6
R24 (h)
Quercetin (μM)
- 30 - 30 - 30 - 30 - 30
p-AMPKα
-62 kD
p-mTOR
-289 kD
LC3
-16 kD ← LC3 I
-14 kD ← LC3 II
β -actin
-43 kD
p-AMPKα level (fold)
p-mTOR level (fold)
LC3 II level (fold)

Figure 9. Quercetin increased the phosphorylation of AMPK, decreased the phosphorylation of mTOR, and activated autophagy on renal I/R injury. (A) LLC-PK1 cells were treated with 50 μM antimycin A and 5 mM 2DG for 1.5 h followed by reperfusion for 6 h in the absence or presence of 10, 30, or 60 μM of quercetin. Whole cell extracts were prepared and protein expression level was estimated by western blotting using antibodies that recognized of phospho-AMPKα and LC3. β-actin was used to an internal control. (B) LLC-PK1 cells were treated with 50 μM antimycin A and 5 mM 2DG for 1.5 h followed by reperfusion for 2, 6, 24 h in the absence or presence of 30 μM of quercetin. Whole cell extracts were prepared and protein expression level was estimated by western blotting using antibodies that recognized of phospho-AMPKα, phospho-mTOR, and LC3. β-actin was used to an internal control. The relative level of phospho-AMPKα, phospho-mTOR, and LC3-II forms were densitometric analyses of three independent experiments and expressed as the means ± SDs. *, Significant difference compared to respective study group (*P<0.05, **P<0.01). I: ischemia; R: reperfusion; h: hour.

Figure 10

(A)



(B)

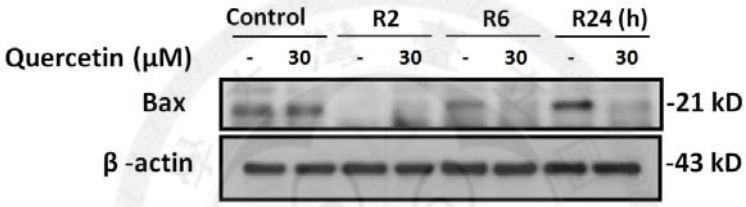
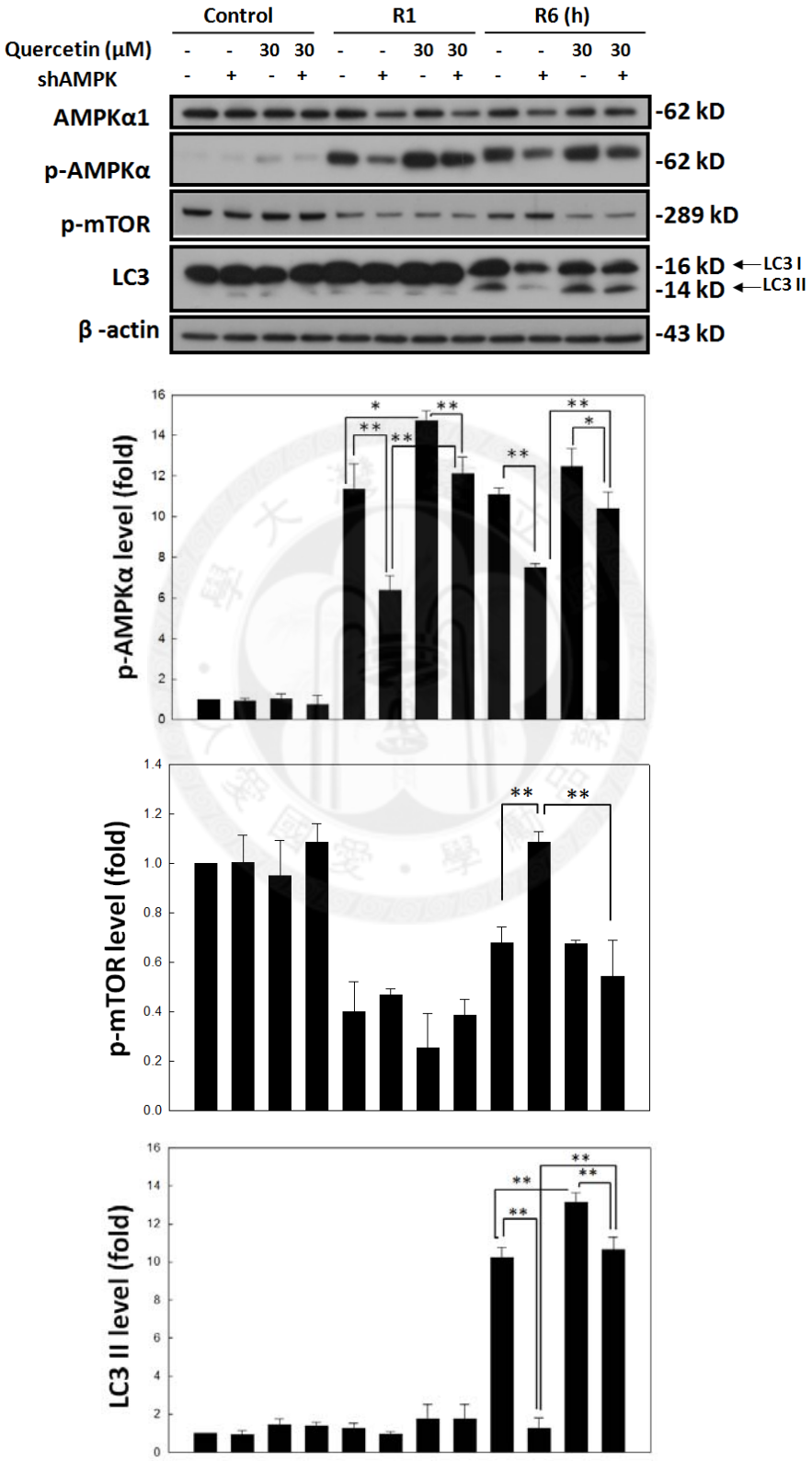


Figure 10. Quercetin protected renal cells from I/R-induced apoptosis. (A) LLC-PK1 cells were treated with 50 μM antimycin A and 5 mM 2DG for 1.5 h followed by reperfusion for 24 h in the absence or presence of 10 or 30 μM of quercetin. Then the cells were fixed and stained with PI. Percentages of cells with the hypodiploid DNA content were determined by flow cytometry. Results are means ± SDs of three separate experiments. *, Significant difference compared to respective study group (**P<0.01). (B) LLC-PK1 cells were treated with 50 μM antimycin A and 5 mM 2DG for 1.5 h followed by reperfusion for 2, 6, 24 h in the absence or presence of 30 μM of quercetin. Whole cell extracts were prepared and protein expression level was estimated by western blotting using antibodies that recognized of Bax. β-actin was used to an internal control. R: reperfusion; h: hour.

Figure 11

(A)


Control
R1
R6 (h)
Quercetin (μM) - - 30 30 - - 30 30 - - 30 30
shAMPK - + - + - + - + - + - +
AMPKα1
p-AMPKα
p-mTOR
LC3
β -actin
-62 kD
-62 kD
-289 kD
-16 kD ←LC3 I
-14 kD ←LC3 II
-43 kD
p-AMPKα level (fold)
p-mTOR level (fold)
LC3 II level (fold)

(B)

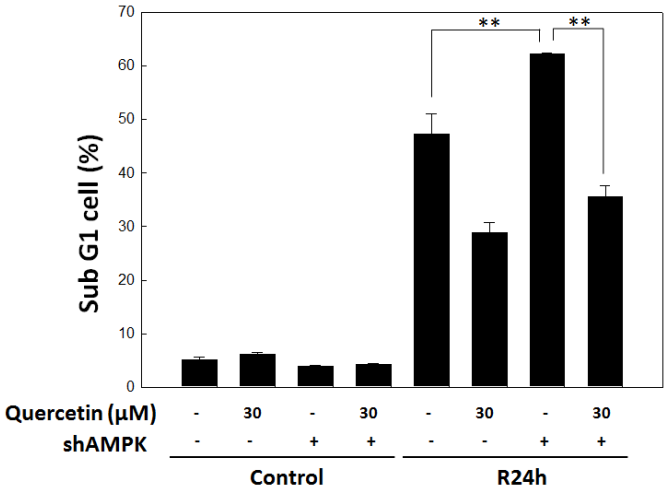


Figure 11. mTOR phosphorylation, autophagy inactivation, and cell apoptosis on renal I/R injury. (A) LLC-PK1 cells which were infected with scramble shRNA or shRNA for AMPKα1 were treated with 50 μM antimycin A and 5 mM 2DG for 1.5 h followed by reperfusion for 1 and 6 h in the absence or presence of 30 μM of quercetin. Whole cell extracts were prepared and protein expression level was estimated by western blotting using antibodies that recognized of AMPKα1, phospho-AMPKα, phospho-mTOR, and LC3. β-actin was used to an internal control. The relative level of phospho-AMPKα, phospho-mTOR, and LC3-II forms were densitometric analyses of three independent experiments and expressed as the means ± SDs. (B) LLC-PK1 cells which were infected with scramble shRNA or shRNA for AMPKα1 were treated with 50 μM antimycin A and 5 mM 2DG for 1.5 h followed by reperfusion for 24 h in the absence or presence of 30 μM of quercetin. Then the cells were fixed and stained with PI. Percentages of cells with the hypodiploid DNA content were determined by flow cytometry. Results are means ± SDs of three separate experiments. *, Significant difference compared to respective study group (*P<0.05, **P<0.01). R: reperfusion; h: hour.