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糖化終產物引起腎腸細胞傷害之機制：內質網壓力、自噬、凋亡之角色探討

Involvement of Endoplasmic Reticulum Stress, Autophagy, and Apoptosis in Advanced Glycation End Products-Induced Mesangial Cells Injury

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本論文係陸天鳳君(R01447003)在國立台灣大學毒理所完成之碩士論文，於民國 103 年 7 月 11 日承下列考試委員審查通過及口試及格，特此證明

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中文摘要

糖尿病發病約 20-25 年之後，約有 25-40%的病人會併發糖尿病性腎病。糖尿病性腎病是造成末期腎病變的主因，並會導致糖尿病患者殘障或有較高之死亡率。過去報導指出，糖化終產物在有末期腎病變的糖尿病患者組織中，累積量約為無末期腎病變之糖尿病患者的兩倍。然而，糖化終產物對腎腸細胞的影響尚未明瞭。因此，本研究將探討內質網壓力(ER stress)、凋亡(apoptosis)以及自噬(autophagy)作用在糖化終產物累積於腎腸細胞(mesangial cells)所扮演的角色，以及相互間之作用機制。給予腎腸細胞不同濃度之糖化終產物(10, 20, 40, 80 and 160 μg/ml)後，細胞存活率顯著地隨劑量增加而下降，顯示糖化終產物對腎腸細胞具有毒性。而 beclin-1, Atg5, LC3-II, CHOP, p-eIF2α 以及 cleaved caspase-3 之蛋白表現顯著增加，顯示糖化終產物會誘發自噬、內質網壓力以及凋亡作用。另外，處理糖化終產物後，凋亡細胞之比例顯著上升，進一步指出大量累積之糖化終產物會導致腎腸細胞凋亡。共同給予 4-苯基丁酸(4PBA)抑制內質網壓力，發現 LC3-II 及 cleaved caspase-3 蛋白表現顯著下降，同時，凋亡細胞之比例顯著下降，顯示抑制內質網壓力可以進一步抑制自噬與凋亡作用。另外，細胞轉染(transfection) siAtg5 抑制自噬作用，發現 cleaved caspase-3 蛋白表現顯著增加，並且細胞凋亡比例也顯著增加，指出在糖化終產物導致之細胞凋亡中，自噬作用扮演保護細胞之角色。綜合以上研究結果，糖化終產物可經由誘發內質網壓力而導致腎腸細胞凋亡，以造成腎腸細胞存活率下降；同時，糖化終產物可經由誘發內質網壓力而活化自噬作用，以避免腎腸細胞凋亡而達到保護之作用。

關鍵字：糖尿病性腎病、糖化終產物、腎腸細胞、內質網壓力、凋亡、自噬

Abstract

25-40 % of diabetic patients develop diabetic nephropathy within 20-25 years after the onset of diabetes. Diabetic nephropathy could lead to disability and high mortality rate in diabetic patients. Also, diabetic nephropathy is the most common cause of end-stage renal disease. It has been reported that the amount of advanced glycation end products (AGEs) in tissue of diabetic patients with end-stage renal disease is twice as much as diabetic patients without end-stage renal disease. Still, the influence of AGEs on mesangial cells remains unclear. Therefore, we investigated the effects of ER stress, apoptosis and autophagy responses in mesangial cells cultured with AGEs.

Cells were cultured with BSA (160 μg/ml) and different concentrations of AGEs (10, 20, 40, 80 and 160 μg/ml), and the cell viability decreased by AGEs in a dose-dependent manner, suggesting that AGEs are cytotoxic to mesangial cells. Then, the induction of beclin-1, Atg5, LC3-II, CHOP, p-eIF2α and cleaved caspase-3 as well as the elevated apoptoic cell ratio indicated that AGEs could induce autophagy, ER stress and apoptosis response in mesangial cells. Further, mesangial cells were treated with siAtg5 and 4-phenylbutyric acid (4PBA) to study the role of autophagy and ER stress in AGEs-induced apoptosis. 4PBA, a chemical chaperone, significantly reduced the protein expression of LC3-II and cleaved caspase-3. Also, the ratio of apoptotic cell was

significantly decreased with 4PBA. These data suggested that AGEs might induce autophagy and apoptosis through ER stress. On the other hand, transfection of siAtg5 significantly aggravated expression of cleaved caspase-3 and exacerbated apoptotic cell ratio, suggesting that autophagy played a protective role in AGEs-induced mesangial cells apoptosis. In conclusion, AGEs could induce apoptosis and autophagy through ER stress in mesangial cells. And, autophagy played a protective role in AGEs-induced mesangial cell apoptosis.

Keywords: diabetic nephropathy, AGEs, mesangial cells, ER stress, apoptosis, autophagy

Abbreviations

4PBA: 4-phenylbutyric acid

AGEs: advanced glycation end products

ATF: activating transcription factor

Atg: autophagy-related genes

BSA: bovine serum albumin

CHOP: CCAAT/enhancer-binding protein homologous protein

CO_2: carbon dioxide

EGTA: ethylene glycol tetraacetic acid

eIF2α: eukaryotic initiation factor 2α

ER: endoplasmic reticulum

ERK: extracellular signal-regulated kinase

FITC: fluoresceine isothiocyanate

GRP78: glucose-regulated protein 78

HEK293: human embryonic kidney cells

HMCs: human mesangial cells

HRP: horseradish peroxidase

hVps34: mammalian vacuolar protein sorting 34 homologue

IRE1: inositol requiring enzyme 1

LC3: microtubule-associated protein 1A/1B-light chain 3

MCP-1: monocyte chemoattractant protein-1

mTOR: mammalian target of rapamycin

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

PC: positive control

PERK: protein kinase r-like endoplasmic reticulum kinase

PI: propidium iodide

PI3K: class III phosphatidylinositol 3-kinase

PVDF: polyvinyl difluoride

RAGE: receptor for AGEs

RNA: ribonucleic acid

ROS: reactive oxygen species

S1P: site-1 protease

S2P: site-2 protease

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM: standard error of mean

siRNA: small interfering RNA

TBST: Tris-buffered saline/Tween-20

TRAF2: tumor necrosis factor receptor-associated factor-2

Ulk: unc-51-like kinase

UPR: unfolded protein response

VEGF: vascular endothelial growth factor

WHO: World Health Organization

1. Introduction

1.1. Diabetes mellitus

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia. The chronic hyperglycemia of diabetes is resulting from defects in insulin secretion and/or insulin action [1]. Recent estimates indicate the world prevalence of diabetes among adults will be 285 million people in 2010, and increase to 439 million people by 2030 [2]. As people around the world have lifestyles that lead to reducing physical activity, and increasing obesity, understanding diabetes mellitus gets more and more important nowadays.

According to the diagnostic criteria of World Health Organization (WHO) and recent studies [1, 4, 5], diabetes is diagnosed by observing any one of the following criterions:

a. Fasting plasma glucose level $\geq$ 126 mg/dl (7.0 mmol/l)

b. Plasma glucose level $\geq$ 200 mg/dl (11.1 m mol/l) 2 hours after ingestion of 75 g oral glucose load

c. Random plasma glucose level $\geq$ 200 mg/dl (11.1 mmol/l) in a patient with classic symptoms of hyperglycemia or hyperglycemic crisis

d. Glycated haemoglobin (HbA1c) $\geq$ 6.5%

Diabetes can be divided into two major forms: type 1 diabetes (insulin-dependent or childhood-onset diabetes) and type 2 diabetes (non-insulin-dependent or adult-onset diabetes). Type 1 diabetes mellitus accounts for only 5-10% of those with diabetes, and results from autoimmune destruction of the β-cells in the pancreatic islets of Langerhans [1, 6]. Type 2 diabetes mellitus, which is much more common than type 1 diabetes, accounts for 90-95% of those with diabetes, and results from insulin resistance in one's body and usually have relative insulin deficiency [1]. Under diabetic condition for a long time will cause lots of complications such as retinopathy, nephropathy, peripheral neuropathy, and autonomic neuropathy causing genitourinary, gastrointestinal, and cardiovascular symptoms and sexual dysfunction.

1.2. Diabetic nephropathy

1.2.1. Pathophysiology of diabetic nephropathy

Diabetic nephropathy is the leading cause of end stage renal disease and a serious complication that accounts for disabilities and the high mortality rate in patients with diabetes [7]. There are studies indicate that about 25-40% of diabetic patients develop diabetic nephropathy within 20-25 years after the onset of diabetes [8]. If untreated, about 20-50% of patients with diabetic nephropathy will develop end stage renal disease over the next 10-20 years [7, 9]. Diabetic nephropathy is a chronic disease characterized

clinically by glomerular hypertrophy, microalbuminuria, a steady decrease glomerular filtration rate, renal fibrosis and elevation of systemic blood pressure [10-13]. In the kidney of patients with diabetic nephropathy, we can observe morphological and ultrastructural changes including glomerular basement membranes thickening, mesangial matrix expansion, arteriolar accumulation of hyaline, nodular sclerosis (Kimmelstiel-Wilson lesion) and interstitial fibrosis [7-13]. Diabetic nephropathy ultimately progresses to glomerular sclerosis associated with renal dysfunction. In glomerulus, different types of cells such as endothelial, tubular, mesangial cells, and podocytes suffer pressures and injuries in a long-term diabetic environment.

1.2.2. Role of mesangial cells in diabetic nephropathy

Mesangial cells play a central role in the pathogenesis of diabetic nephropathy. These cells and their matrix form the central stalk of the glomerulus, and these cells comprise one third of decapsulated glomerular cell population. Mesangial cells play a crucial role in maintaining structural integrity and function of glomerular tufts, modulating glomerular filtration by their smooth muscle activity, maintaining mesangial matrix homeostasis and providing structural support for capillary loops [7, 13-15]. These cells also contribute to phagocytose apoptotic cells or immune-complex formed at or delivered to the glomerular capillaries [14].

1.2.3. Implication of mesangial cells apoptosis in diabetic nephropathy

There are numerous in vitro and in vivo studies demonstrate that mesangial cells are intimately involved in glomerular sclerosis, a characteristic pathogenesis of end stage renal disease in diabetic nephropathy. Mesangiolysis/apoptosis is a concenquence of exposure of mesangial cells to soluble mediators and metabolites in diabetes and in particular hyperglycemia. It remains unclear why and how mesangial cells respond differentially to a diabetic environment. Facing the metabolic, hemodynamic or immunologic injury, some mesangial cells respond by undergoing apoptosis and other cells respond by acquiring an activated phenotype and undergoing hypertrophy, proliferation with excessive production of matrix proteins, growth factors, chemokines and cytokines [14]. There are studies indicated that apoptosis is involved in the course of various renal disease, including diabetic nephropathy. Apoptosis may play a pathologic role during progressive glomerular sclerosis by reducing the number of mesangial cells. Moreover, mesangial cells apoptosis has been correlated with the increasing severity of albuminuria in mice [13]. Also, recent study indicate that advanced glycation end products (AGEs) disturbed glomerular homeostasis by inducing human mesangial cells apoptosis, and elicited hyperfiltration and microalbuminuria by stimulating secretion of vascular endothelial growth factor (VEGF) and monocyte

chemoattractant protein-1 (MCP-1) proteins, thereby being involved in the pathogenesis of the early phase of diabetic nephropathy [16]. Furthermore the apoptosis of mesangial cells is implicated in the late stages of diabetic nephropathy, suggesting that apoptosis may be a homeostatic mechanism which regulates the population of glomerular cells [13].

1.3. Advanced glycation end products (AGEs)

1.3.1 Role of advanced glycation end products (AGEs) in diabetic nephropathy

AGEs are reactive derivatives from non-enzymatic glucose-protein condensation reactions (the Maillard reaction), as well as lipids and nucleic acids exposed to reducing sugars, from a heterogeneous group of irreversible adducts (Figure 1). Studies have demonstrated that serum and tissue AGEs levels were significantly increased in type 1 and type 2 diabetic patients compared with non-diabetic control subjects. Also, the amount of AGEs in tissues in diabetic patients with end-stage renal disease had almost twice as much as in diabetic patients without renal disease [8]. Therefore, there are increasing evidence demonstrate that AGEs play a pivotal role in the development and progression of diabetic vascular damage [8, 10].

Both enhanced formation and decreased clearance are responsible for the accumulation of AGEs in patients with diabetic nephropathy [10]. Accumulation of

AGEs in kidney may contribute to the progressive alteration in renal architecture and loss of renal function in patients and rodents via various mechanisms, including their cross-linking (β-sheets or cross-β structure) properties of matrix proteins and activation of the downstream signaling. The formation of AGEs on various types of matrix proteins impairs their degradation by matrix metalloproteinase, contributing to basement membrane thickening and mesangial expansion, hallmarks of diabetic nephropathy [8, 10].

1.3.2 Involvement of AGEs in mesangial cells dysfunction

AGEs can be identified in many renal structures. An ultrastructural study carried out in rats with diabetic nephropathy demonstrated the presence of AGEs in the glomerular basement membrane, mesangial matrix, podocytes, tubule, endothelial and mesangial cells. Lots of observations suggested the pathological role for the AGE-RAGE (receptor for AGEs) pathway in glomerular sclerosis and proteinuria, hallmarks of diabetic nephropathy (Figure 2). Activation of RAGE on mesangial cells has effects on the cell cycle and maintains mesangial cells in a quiescent state. In turn, this inhibition of cell proliferation promotes mesangial cells apoptosis and hypertrophy. AGEs increase mesangial cells synthesis of fibronectin as well as collagen types I and IV. Following RAGE activation, mesangial cells secrete MCP-1, which participates in

the inflammatory process. Mesangial cells also express other receptors for AGEs, such as AGE-R1 and AGE-R2, the activation of which may contribute to the damaging effects of AGEs by promoting mesangial cells apoptosis [17].

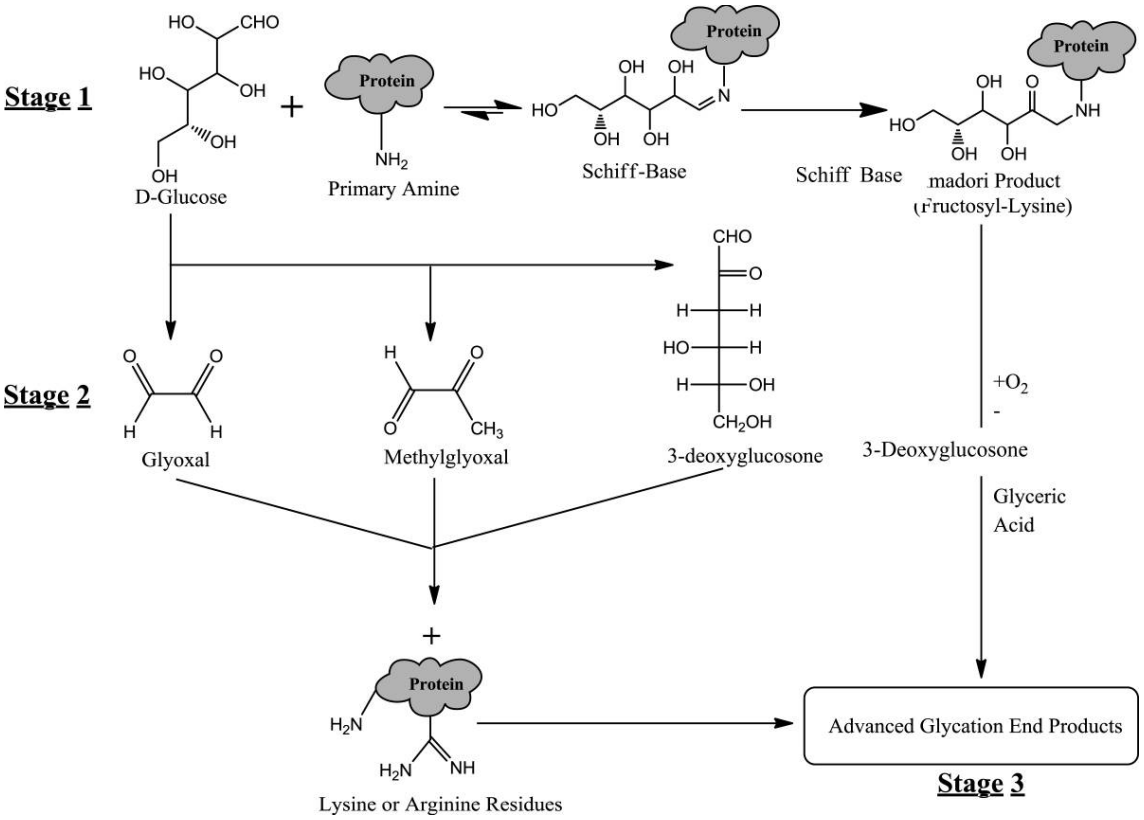


Figure 1. Formation of AGEs

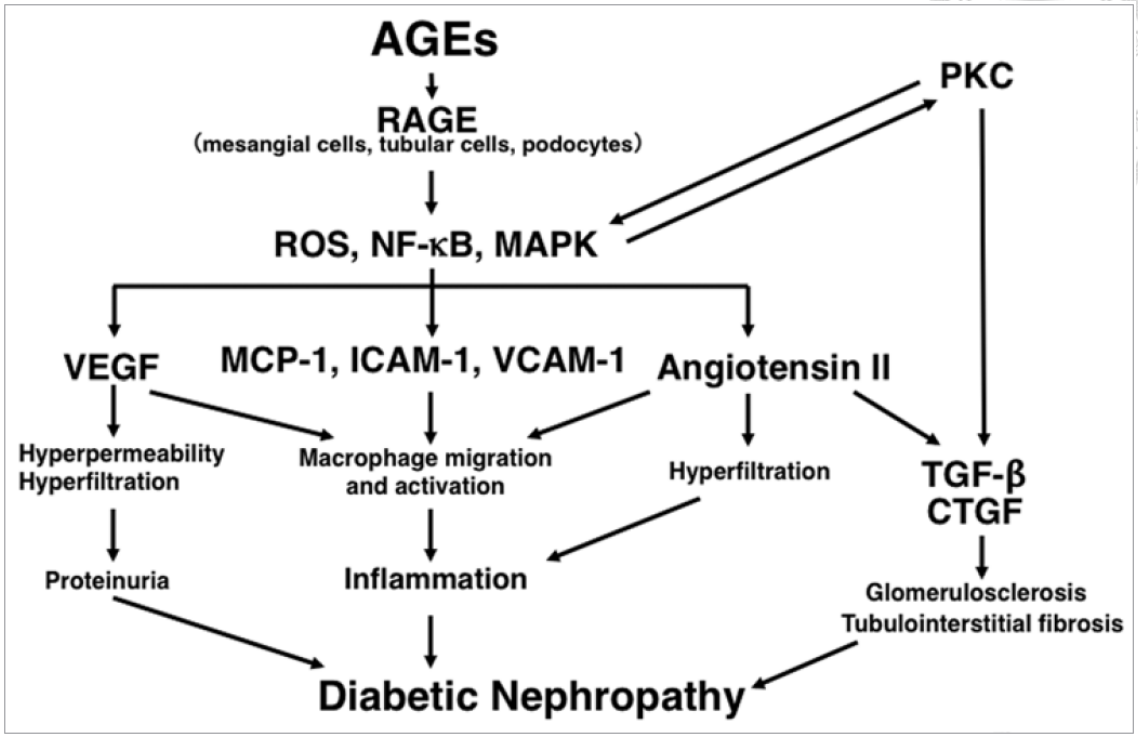


Figure 2. Pathophysiological role of the AGE-RAGE pathway in diabetic nephropathy

1.4. Endoplasmic reticulum (ER) stress

The endoplasmic reticulum (ER) is an organelle that has essential roles in multiple cellular processes, and it has the ability to regulate synthetic, metabolic as well as adaptive responses to both intra- and extracellular stress and plays a pivotal role in maintaining cell homeostasis [18-19]. ER stress occurs in response to a variety of stimuli, various physiological and pathological conditions that can cause the accumulation of unfolded and misfolded proteins in the ER lumina (Figure 3). Consequently, an adaptive response called the unfolded protein response (UPR) is triggered to resolve the ensuing stress by activating intracellular signal transduction

pathways. In eukaryotic cells, UPR is mediated by three ER membrane-associated proteins: inositol requiring enzyme 1 (IRE1), protein kinase r-like endoplasmic reticulum kinase (PERK), and activating transcription factor (ATF) 6. These ER membrane-associated proteins are inhibited under basal or stress-free conditions by their association with the chaperone protein glucose-regulated protein 78 (GRP78)/Bip, but are activated when released from GRP78 during ER stress. These UPR sensors detach from GRP78, causing oligomerization and activation of PERK and IRE1 and leading to the activation of downstream signaling pathways. The phosphorylation of eukaryotic initiation factor 2α (eIF2α) by PERK during ER stress down-regulates efficient translation of most mRNAs, thereby inhibiting protein synthesis. ATF6 is translocated to the Golgi in response to ER stress, where it is cleaved by golgi-resident serine proteases site-1 protease (S1P) and site-2 protease (S2P). The cleaved ATF6 N-terminal fragment migrates to the nucleus to activate the transcription of UPR target genes. The UPR can alleviate the accumulation of misfolded proteins in the ER by producing chaperones to assist with protein folding, inhibiting new protein synthesis, or accelerating the degradation of proteins. However, if the function of ER cannot be re-established, extensive or prolonged ER stress will eventually lead to cell death through activating apoptosis [18, 19, 21].

ER stress-induced apoptosis is mainly mediated by CCAAT/enhancer-binding protein homologous protein (CHOP) [22]. CHOP is a transcription factor, which induces several proapoptotic factors, and is downstream of the PERK (PERK-eIF2α signal followed by ATF4 expression) and ATF6 UPR pathways [22]. CHOP also downregulates anti-apoptotic Bcl-2, which leads to enhanced oxidant injury and apoptosis [22]. A previous observation indicated that overexpression of Bcl-2 specifically in the ER protected renal tubular cells against ER stress-induced apoptosis. Further, AGEs-induced ER stress in murine podocytes resulted in increasing expression of GRP78 and apoptosis in a dose- and time-dependent manner [21, 23]. Also, collagen type I modified by 3-deoxyglucosone induces reactive oxygen species (ROS) and apoptosis mediated through ER stress pathway via CHOP activation and not through the RAGE signaling, providing a link between AGEs and ER stress in the pathogenesis of diabetic wounds [20].

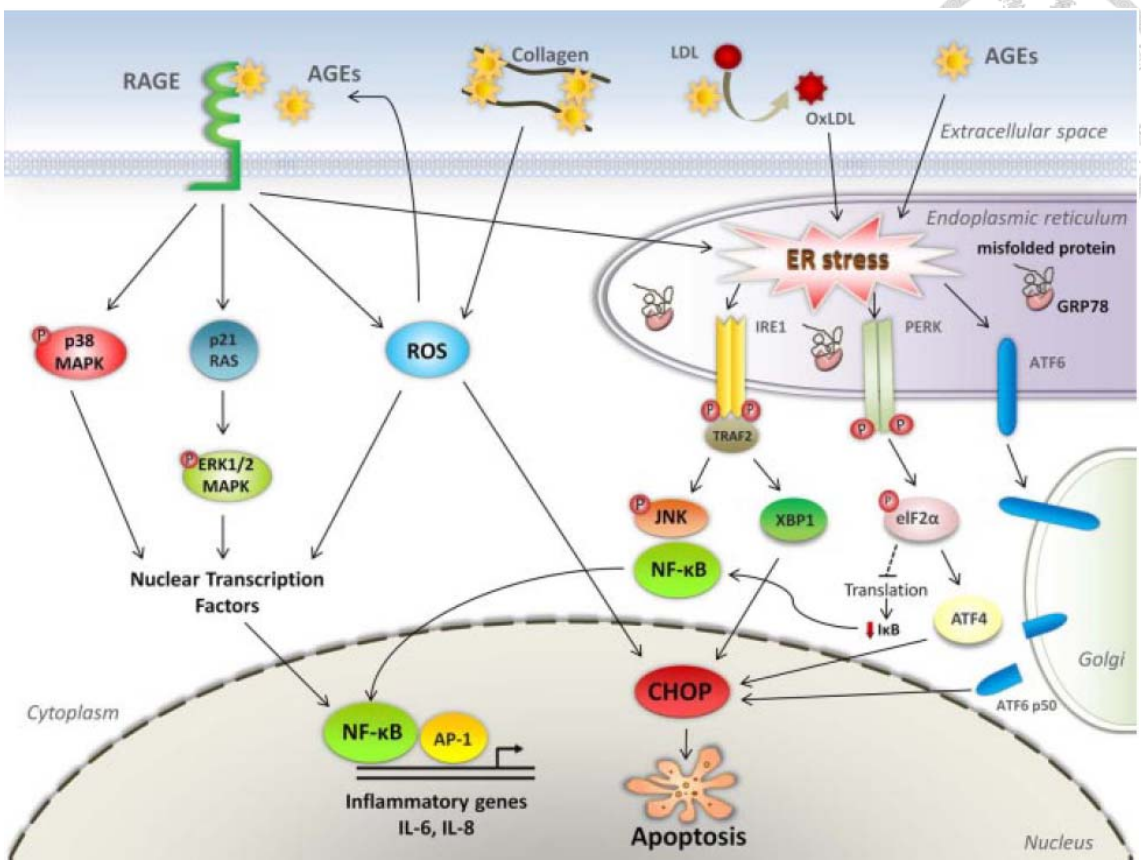
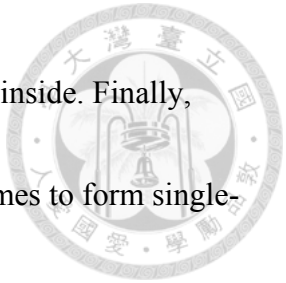


Figure 3. Crosstalk of AGEs and UPR [20]

1.5. Autophagy

Autophagy is a tightly regulated system in which the endogenous cellular protein aggregates and damaged organelles are degraded via the lysosomal pathway [24]. There are three types of autophagy, namely macroautophagy, microautophagy and chaperone-mediated autophagy. Macroautophagy, generally referred simply as autophagy, is the most studied and the major type of autophagy [24]. The first step of autophagy is initiating with the formation of autophagophores around substances which will be sequestered (Figure 4). Secondly, autophagophores elongate and expand, and finally



form double-membrane vacuoles, called autophagosomes with cargo inside. Finally, outer membranes of autophagosomes fuse with lysosomes or endosomes to form single-membrane autolysosomes, which contain lysosomal enzymes to digest the enclosed substrates [24-26]. Autophagy is a multiple-step process (Figure 5), which involves many proteins complexes and autophagy-related genes (Atg) [24, 25]. In the beginning, autophagy is initiated by the unc-51-like kinase (Ulk) 1 complex [25-28]. Next, phagophore nucleation is dependent on Beclin 1, a mammalian vacuolar protein sorting 34 homologue (hVps34) or class III phosphatidylinositol 3-kinase (PI3K) complex [25-28]. During autophagosome elongation/closure, two dependent ubiquitin-like conjugation systems are involved: Atg12 and microtubule-associated protein 1A/1B-light chain 3 (LC3). The Atg12-Atg5 conjugate, which forms the Atg12-Atg5-Atg16 complex, contributes to the stimulation and localization of the LC3 conjugation reaction [25-28]. The cytosolic isoform of LC3 (LC3-I) is conjugated to phosphatidylethanolamine through two consecutive ubiquitin-like reactions catalyzed by E1-like enzyme Atg7 and the E2-like enzyme Atg3 to form LC3-II [25-28]. Therefore, LC3-II formation is recognized as a marker of the existence of autophagosomes in cell or animal experiments. After formation, autophagosomes merge with the lysosomal compartment to form autolysosomes [25-28]. The protein p62, also

known as sequestosome 1, is known to recognize cargo and localize to autophagosomes via LC3 interaction and to be constantly degraded by the autophagy-lysosome system [25-29].

Autophagy activity is regulated by both nutrient state and intracellular stresses [25]. The role of autophagy in the kidney is complex, since both the up- and downregulation of autophagy have been shown to be protective against different kidney diseases [30]. There are studies indicated that activation of autophagy might exacerbate kidney lesions [31, 32]. On the other hand, recent studies suggested that hyperglycemia reduces autophagy activity in podocytes which may lead to diabetes-related podocyte injury [25]. Furthermore, previous studies indicated that autophagy has a renal protective role in proximal tubular cells under acute and chronic kidney injury, such as diabetic nephropathy [25].

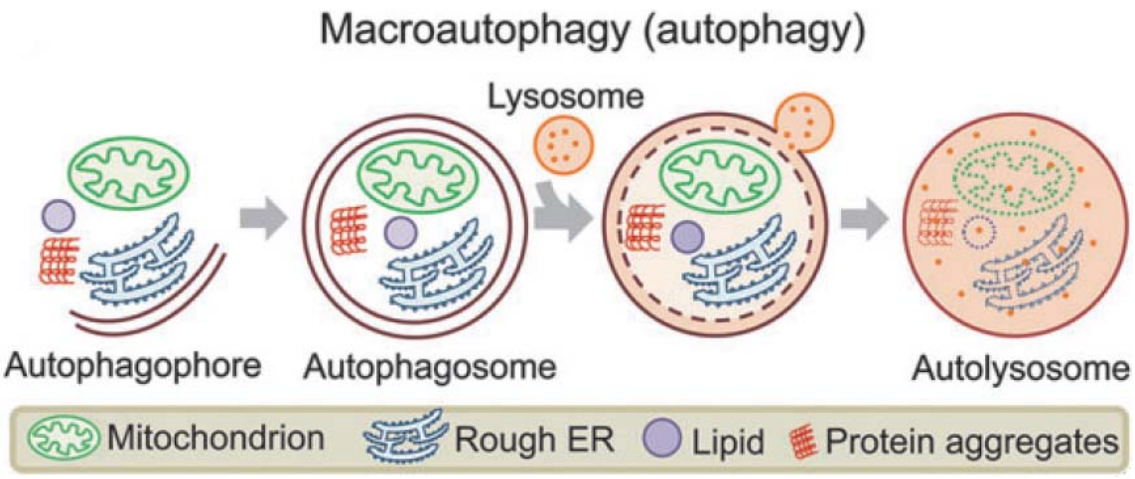


Figure 4. Schematic illustration of the process of autophagy [21]

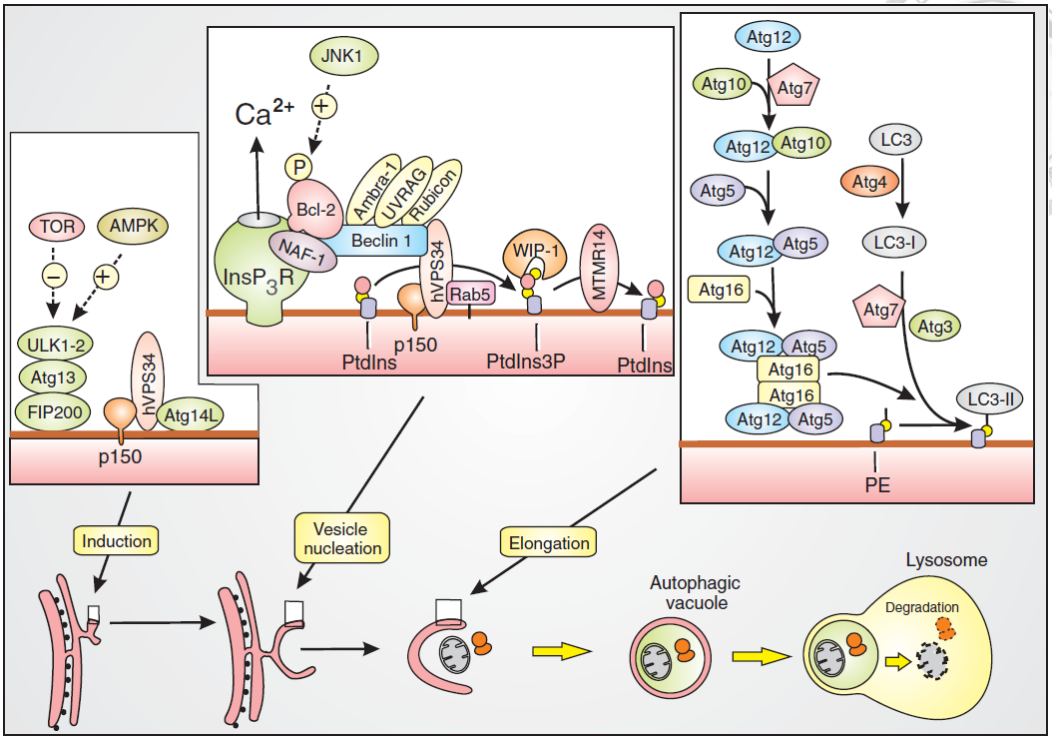


Figure 5. Autophagy signaling mechanisms [26]

1.6. The interaction between autophagy and ER stress

Increasing evidence has indicated that in addition to starvation, ER stress is also a potent trigger of autophagy. Autophagy can act as an ER-associated degradation system in mammalian cells, and it plays a fundamental role in preventing toxic accumulation of disease-associated mutant proteins in the ER [19]. Studies suggested that PERK, IRE1 and increased [Ca^{2+}] have been implicated as mediators of ER stress-induced autophagy in mammalian cells [19], as depicted in Figure 6. Autophagy deficiency in kidneys of diabetic animals can lead to tubule cells being sensitive to hypoxia and ER stress and can result in progression of diabetic nephropathy [18]. Therefore, activation of

autophagy may be a therapeutic option for end-stage diabetic nephropathy. On the other hand, autophagy may have opposite effects in determining cell fate in response to ER stress in apoptosis-competent cells in which autophagy serves as a survival mechanism, and in apoptosis-deficient cells that utilize autophagy as a means to promote non-apoptotic cell death [19]. Thus, even though autophagy is known to be associated with ER stress, the molecular mechanisms of autophagy induction by ER stress is not fully elucidated and its cellular effects appear to vary with cell types and the stimuli [19].

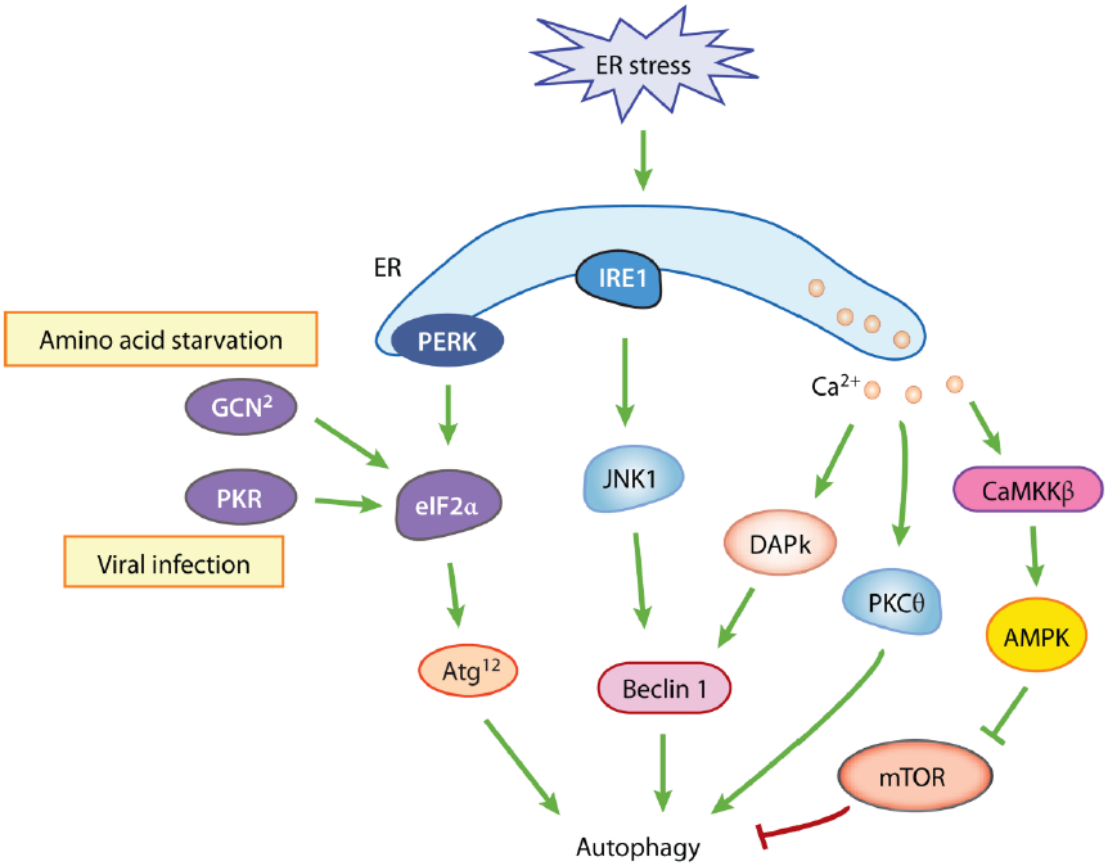


Figure 6. Schematic of molecular mechanisms of ER stress-induced autophagy [26]

1.7. Hypothesis and aims

Despite the emerging evidences suggested that AGEs-induced ER stress, apoptosis and autophagy participate in podocyte and tubule cells injury in diabetic nephropathy, the involvement of ER stress, apoptosis and autophagy in AGEs-induced mesangial cells injury in diabetic nephropathy remains unclear. Therefore, our study wants to clarify the interaction between ER stress, apoptosis and autophagy in mesangial cells accumulating with AGEs in diabetic nephropathy. Furthermore, the activation of autophagy could be a protective or an injury role in kidney. We want to investigate the role of autophagy in mesangial cells in diabetic nephropathy.

2. Materials and Methods

2.1. Antibodies

Anti-caspase-3, anti-CHOP, anti-phospho-eIF2α, anti-LC3B, anti-Atg5, anti-beclin-1, anti-SQSTM1/p62 antibodies as well as secondary goat anti-mouse and anti-rabbit horseradish peroxidase (HRP)-conjugated antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-β-actin and anti-mouse HRP-conjugated secondary antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cell culture

Mouse mesangial cells were obtained from Food Industry Research and Development Institute and cultured in growth medium consisting of 3:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium, 95%; fetal bovine serum,5%; supplemented with 14 mM HEPES and incubated in 5% carbon dioxide (CO_2) at 37°C.

2.3. Preparation of AGEs

Bovine serum albumin (BSA) (1mg/ml) was incubated under sterile conditions with D-glucose (1mg/ml) in 0.2 M phosphate buffer (pH 7.4) at 37℃ for 8 weeks. After incubation, AGEs were dialyzed against PBS for 24 hours to remove unbound

sugars and filter-sterilized using a 0.22 μm Millipore filter (Millipore, Billerica, MA, USA).

2.4. Cell viability assay

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) assay. Mesangial cells were cultured in 24-well plates. After treatment with or without AGEs or with BSA alone for 24 hours, culture medium was replaced with equal volume of MTT (0.5 mg/ml) and incubated for 2 hours at 37℃. The yellow tetrazolium MTT is reduced by mitochondrial succinate dehydrogenase in metabolically active cells. The resulting intracellular purple formazan crystals were dissolved with DMSO and shaken at room temperature for 30 minutes. After transfer the mixture in each well to a 96-well plate, the absorbance was measured at 570 nm.

2.5. Apoptosis analysis

Cell apoptosis were analyzed using fluoresceine isothiocyanate (FITC) Annexin V Apoptosis Detection Kit (BD Pharmingen^{TM}, Franklin Lakes, NJ, USA) by flow cytometry. After cells treated with AGEs for 24 hours, old mediums were transferred to flow tubes, and cells were washed with PBS twice and incubated with 0.5% trypsin-EDTA. Cells were neutralized with old mediums and transferred to flow tubes. Samples

were then centrifuged at 4℃, 1000 rpm for 5 minutes. After centrifuged, supernatants were disposed and cells were washed with PBS twice. Cells were stained with FITC Annexin V and Propidium Iodide (PI), and then percentages of cells with different death patterns were determined by flow cytometry. Positive control (PC) was mesangial cells incubated with 3% formaldehyde for 30 minutes.

2.6. Preparation of total cell lysates

Cells were washed with PBS and lysed with RIPA buffer (20 mM Tris-base (pH7.4), 150mM NaCl, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid (EGTA), 0.1% Nonidet P-40, 0.2% protein inhibitor). The lysates were left on ice for 30 minutes and centrifuged at 13,000 rpm for 30 minutes at 4℃. The supernatants were transferred to new eppendorfs. The protein concentration of supernatants was normalized by BCA™ Protein Assay Kit (Pierce, IL, USA).

2.7. Western blot analysis

The cell lysates were prepared. Equal proteins were resolved on 6-15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinyl difluoride (PVDF) membranes. The membranes were blocked with 5% fat-free milk in Tris-buffered saline/Tween-20 (TBST) buffer (20 mM Tris, 150 mM NaCl, 0.02% Tween-20, pH 7.5) for 1 hour and followed by immunoblotting with primary

antibodies overnight at 4℃. Membranes were washed with TBST for 10 minutes for three times. After washing, membranes were incubated with secondary goat anti-rabbit or anti-mouse HRP-conjugated antibodies for 1 hour. After membranes were washed three times for 15 minutes each, the signals were visualized by enhanced chemiluminescence using Fujifilm Super RX (Fujifilm, Tokyo).

2.8. RNA interference

For autophagy-blocking experiments, we used small interfering RNA (siRNA) molecules targeted to Atg5 mRNA. Atg5 siRNA was purchased from Invitrogen, USA. The siRNA was a mixture of three different siRNA against Atg5 (sense1, 5'-GCGAGCAUCUGAGCUACCCAGAUAA-3'; antisense1, 5'-UUAUCUGGGUAGCUCAGAUGCUCGC-3'; sense2, 5'-GCCAUCAACCGGAAACUCAUGGAAU-3'; antisense2, 5'-AUUCCAUGAGUUUCCGGUUGAUGGC-3'; sense3, 5'-GACAGCUGCACACACUUGGAGAUCU-3'; antisense3, 5'-AGAUCUCCAAGUGUGUGCAGCUGUC-3'). The negative control siRNA was Stealth^{TM} RNAi Negative Control Duplexes Medium GC Duplex (Invitrogen, USA). The final concentration of siRNA was 80nM. Cells were transfected with siRNA using siLentFect^{TM} Lipid (Bio-Rad, USA) for 6 hours and recovered with culture medium

overnight. The efficacy of RNA interference was determined by western blot.

2.9. Statistics

The results are presented as mean ± SEM (standard error of mean). Each experiment was performed three times or more to ensure the reproducibility. Statistical differences between control and treated group were determined by Student's *t*-test. The difference is significant if the p-value is less than 0.05. Software used：GraphPad Prism 5.

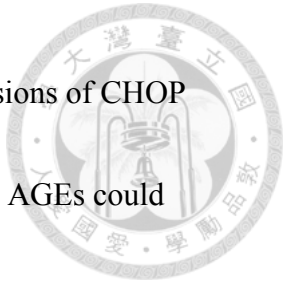
3. Results

3.1. AGEs induced apoptosis response in mesangial cells

Mesangial cells were treated with various concentrations of AGEs (10, 20, 40, 80, 160 μg/ml) and BSA (160 μg/ml) for 24 hours to evaluate the influence of AGEs on mesangial cells. The cell viability significantly reduced at concentrations 20, 40, 80 and 160 μg/ml in a dose-dependent manner as compared with normal control or BSA control (Figure 5.1), and it indicated that AGEs had toxicity on mesangial cells. Further, AGEs upregulated cleavage form of caspase-3, which is an apoptosis-related protein, at concentrations 80 and 160 μg/ml in a dose-dependent manner (Figure 5.2), and it indicated that accumulation of AGEs might induce apoptosis in mesangial cells. Moreover, the results of flow cytometry showed that AGEs significantly induced apoptosis at concentration 40, 80 and 160 μg/ml in a dose-dependent manner (Figure 5.3). These results indicated that AGEs are capable of inducing apoptosis responses in mesangial cells, and the cell viability of mesangial cells was reduced by AGEs-induced apoptosis responses.

3.2. Administered with AGEs induced ER stress in mesangial cells

Mesangial cells were cultured with various concentrations of AGEs (20, 40, 80 and 160 μg/ml) and BSA (160 μg/ml) for 24 hours. Whole cell lysates were prepared for



western blot, and AGEs significantly up-regulated the protein expressions of CHOP (Figure 5.4A) and p-eIF2α (Figure 5.4B). These results indicated that AGEs could induce ER stress in mesangial cells. Furthermore, AGEs could induce ER stress through regulate the activity of p-eIF2α/CHOP pathway in mesangial cells.

3.3. Accumulation of AGEs induced autophagy in mesangial cells

Mesangial cells were cultured with various concentrations of AGEs (20, 40, 80 and 160 μg/ml) and BSA (160 μg/ml) for 24 hours. Whole cell lysates were prepared for western blot, and AGEs significantly induced the protein expressions of LC3-II (Figure 5.5 A), Atg5 (Figure 5.5 B) and becline-1 (Figure 5.5 C) in a dose-dependent manner. These results suggested that AGEs accumulation in mesangial cells might induce autophagy activity through beclin-1 related pathway.

3.4. 4-phenylbutyric acid (4PBA) reversed AGEs-induced ER stress and apoptosis response in mesangial cells

Mesangial cells were treated with AGEs (160 μg/ml) and with or without chemical chaperone, 4PBA (1 mM) for 24 hours. BSA (160 μg/ml) were treated with or without 4PBA (1 mM) as control groups. Whole cell extracts were prepared for western blot, and co-treatment with 4PBA significantly reversed AGEs-induced CHOP (Figure 5.6 A) and p-eIF2α (Figure 5.6 B) protein expressions. These results indicated that 4PBA

was capable of inhibiting ER stress at the concentration of 1 mM, therefore, we chose the dose for further experiments. Co-treatment with 4PBA significantly reversed AGEs-induced protein expression of cleaved caspase-3 (Figure 5.6 C), and it indicated that AGEs could regulate cleaved caspase-3 through p-eIF2α/CHOP pathway. Also, co-treatment with 4PBA significantly reversed AGEs-induced protein expression of LC3-II (Figure 5.6 D), and this indicated that AGEs might regulate LC3-II through p-eIF2α/CHOP pathway. Furthermore, cells with same treatments were stained with PI and Annexin V, and percentages of cells with different death patterns were determined by flow cytometry. Co-treatment with 4PBA significantly mitigated AGEs-induced apoptosis response (Figure 5.7), and this indicated that inhibition of ER stress could further inhibit apoptosis. Among these results indicated that AGEs could induce mesangial cells apoptosis through the induction of p-eIF2α/CHOP pathway related ER stress. Moreover, AGEs could also activate autophagy through the induction of p-eIF2α/CHOP pathway related ER stress in mesangial cells.

3.5. Transfection of siAtg5 aggravated AGEs-induced injury in mesangial cells

Mesangial cells were transfected with siScrumble (80 nM) or siAtg5 (80 nM) for 6 hours and recovered with culture medium. After cells were treated with BSA (160

μg/ml) or AGEs (160 μg/ml) for 24 hours, whole cell extracts were prepared for western blot. Transfection with siAtg5 significantly reduced Atg5 (Figure 5.8 A) and LC3-II (Figure 5.8 B) protein expressions, and these indicated that transfection with concentration of 80 nM could significantly inhibit AGEs-induced autophagy. Also, transfection with siAtg5 significantly reversed AGEs-reduced p62 protein expression (Figure 5.8 C), and this indicated that the degradation of p62 was inhibited. Moreover, siAtg5-inhibited degradation of p62 could further supporte that autophagy was inhibited by siAtg5. On the other hand, the protein expression of cleaved caspase-3 was significantly up-regulated by siAtg5 (Figure 5.8 D), and this indicated that inhibition of Atg5-related autophagy could further provoke AGEs-induced cleaved caspase-3. Similarly, transfection of siAtg5 significantly aggraveated apoptosis response (Figure 5.9), suggesting that inhibition of Atg5-related autophagy could exaggerate AGEs-induced apoptosis in mesangial cells. Further, transfection of siAtg5 made no difference in the protein expressions of CHOP (Figure 5.10 A) and p-eIF2α (Figure 5.10 B), this indicated that inhibition of Atg5-related autophagy could not further inhibit ER stress. These results indicated that inhibition of autophagy could exaggerate AGEs-induced apoptosis response, suggesting that autophagy play a protection role in AGEs-induced injury in mesangial cells. Furthermore, AGEs-induced ER stress might not be regulated

by Atg5-related autophagy.

4. Discussion

Diabetic nephropathy is a serious complication of diabetic mellitus, and it can lead to end-stage renal disease, which will cause inconvenient and disability in patients' life. Studies indicated that the amount of AGEs in tissue in diabetic patients with renal disease is twice as much as diabetic patients without renal disease. Also, growing evidences suggested that AGEs-induced mesangial cells apoptosis is involved in the pathogenesis of diabetic nephropathy [13, 16]. And, AGEs could induce apoptosis response through ER stress in podocytes. Furthermore, both up and down-regulation of autophagy is implicated in protection of kidney in renal disease. Therefore, in this study, we aimed to investigate the links between ER stress, apoptosis and autophagy in AGEs-induced mesangial cells injuries.

AGEs' toxicity may occur through at least three mechanisms: interaction with RAGE, tissue deposition, and in situ glycation [17]. AGEs were found to decrease the cell viability and to induce apoptosis in both human embryonic kidney cells (HEK293) and human mesangial cells (HMCs) [33]. Also, AGEs induced the protein expression of caspase-3/7, an indicator of apoptosis, in both mouse mesangial cells and mouse podocytes [34]. Consistent with previous reports, our data revealed that AGEs decreased the cell viability of mesangial cells in a dose-dependent manner. Furthermore,

AGEs induced cleavage form of caspase-3 in mesangial cells in a dose-dependent manner. However, the interactions between ER stress, autophagy and AGEs-induced apoptosis in mesangial cells remain unclear. Therefore, we next investigated the involvement of ER stress and autophagy in AGEs-induced mesangial cells apoptosis.

Previous studies demonstrated that AGEs induce ER stress in both human aortic endothelial cells and chondrocytes, which plays an important role in cell apoptosis [35, 36]. Further, ER stress can induce apoptosis through different signaling pathways, such as CHOP-mediated pathway, IRE1-mediated pathway and caspase-mediated pathway [22]. In CHOP-mediated pathway, CHOP is downstream of PERK (PERK- eIF2α-ATF4 signaling pathway) and ATF6 UPR pathways. In IRE1-mediated pathway, IRE1interacs with tumor necrosis factor receptor-associated factor-2 (TRAF2), leading to apoptosis. In caspase-mediated pathway, activated caspase-12 cleaves caspase-9 to activate procaspase-3. Our data revealed that AGEs-induced ER stress could lead to mesangial cells apoptosis. Moreover, p-eIF2α/CHOP pathway and caspase-3 were significantly induced, suggesting that AGEs induced apoptosis through both CHOP-mediated pathway and caspase-mediated pathway.

Autophagy is a complex response regulated by nutrients and stress, whether it plays a protective role or a harmful role in cells is not fully understood. AGEs induced

cardiomyocyte autophagy by inhibiting PI3K/Akt/mammalian target of rapamycin (mTOR) pathway via RAGE and impaired the cell viability in dose-dependent manner [37]. Further, previous study indicated that heavy metal cadmium induced autophagy by Ca^{2+}-extracellular signal-regulated kinase (ERK) signaling pathway, which impaired the cell viability of mesangial cells [38]. On the other hand, studies suggested that autophagy played a cytoprotective role in osteoblastic cells and podocytes under hyperglycemia circumstance [25, 39]. Our data revealed that autophagy was downstream of AGEs-induced ER stress. Furthermore, inhibition of autophagy by transfection of siAtg5 could significantly induce cleaved caspase-3 and exaggerate apoptosis, suggesting that autophagy played a protective role in AGEs-induced mesangial cells injury.

In conclusion, the present study provides the evidence that AGEs induce mesangial cell apoptosis through ER stress via p-eIF2α/CHOP signaling pathway. Moreover, autophagy is activated by AGEs-induced ER stress and it protects mesangial cells from AGEs-induced apoptosis.

However, our data did not fully elucidate the molecular mechanisms involved in AGEs induced ER stress, apoptosis and autophagy, thus, further studies should investigate in the molecular mechanisms more detailed. Moreover, none of our data

demonstrates the interaction between ER stress, apoptosis and autophagy on AGEs-induced mesangial cells injury *in vivo.* Future studies needs to clarify the links between ER stress, apoptosis and autophagy *in vivo.*

5. Figures and Figure Legends

Figure 5.1

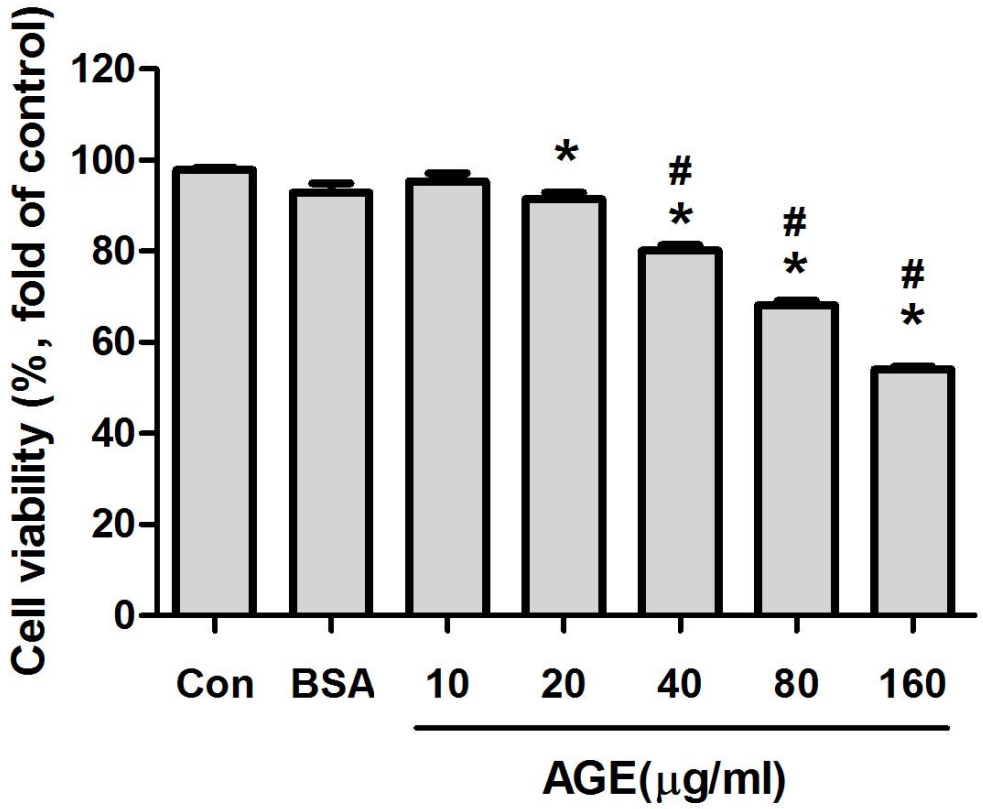


Figure 5.1. AGEs decreased the cell viability of mesangial cells.

Mesangial cells were treated with BSA (160 μg/ml) and different concentrations of AGEs (10, 20, 40, 80, 160 μg/ml) for 24 hours. The cytotoxic effect of AGEs was examined by MTT assay. Statistical results are presented as mean ± SEM of three separate experiments. *, significant different ($p<0.05$) compared to control group (culture medium-treated group). #, significant different ($p<0.05$) compared to BSA-treated group.

Figure 5.2

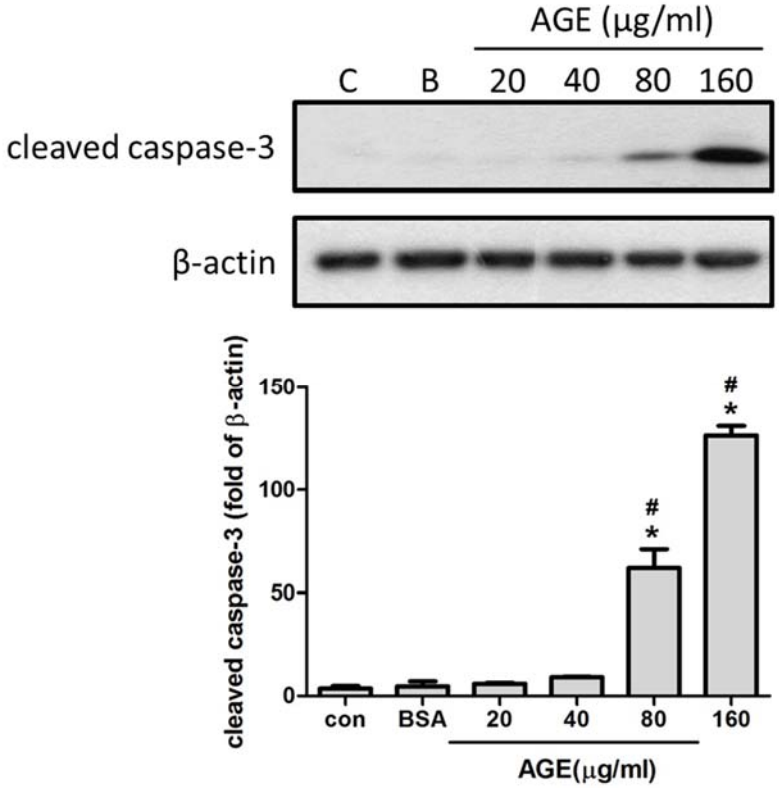


Figure 5.2. AGEs induced the protein expression of cleaved caspase-3 in mesangial cells. Cells were treated with BSA (160μg/ml) and different concentrations of AGEs (20, 40, 80, 160 μg/ml) for 24 hours. Whole cell extracts were prepared, and protein expression level was evaluated by western blot using antibodies against caspase-3 and β-actin . β-actin was used as internal control. Statistical results are presented as mean ± SEM of three separate experiments. *, significant different ($p<0.05$) compared to control group (culture medium-treated group). #, significant different ($p<0.05$) compared to BSA-treated group.

Figure 5.3

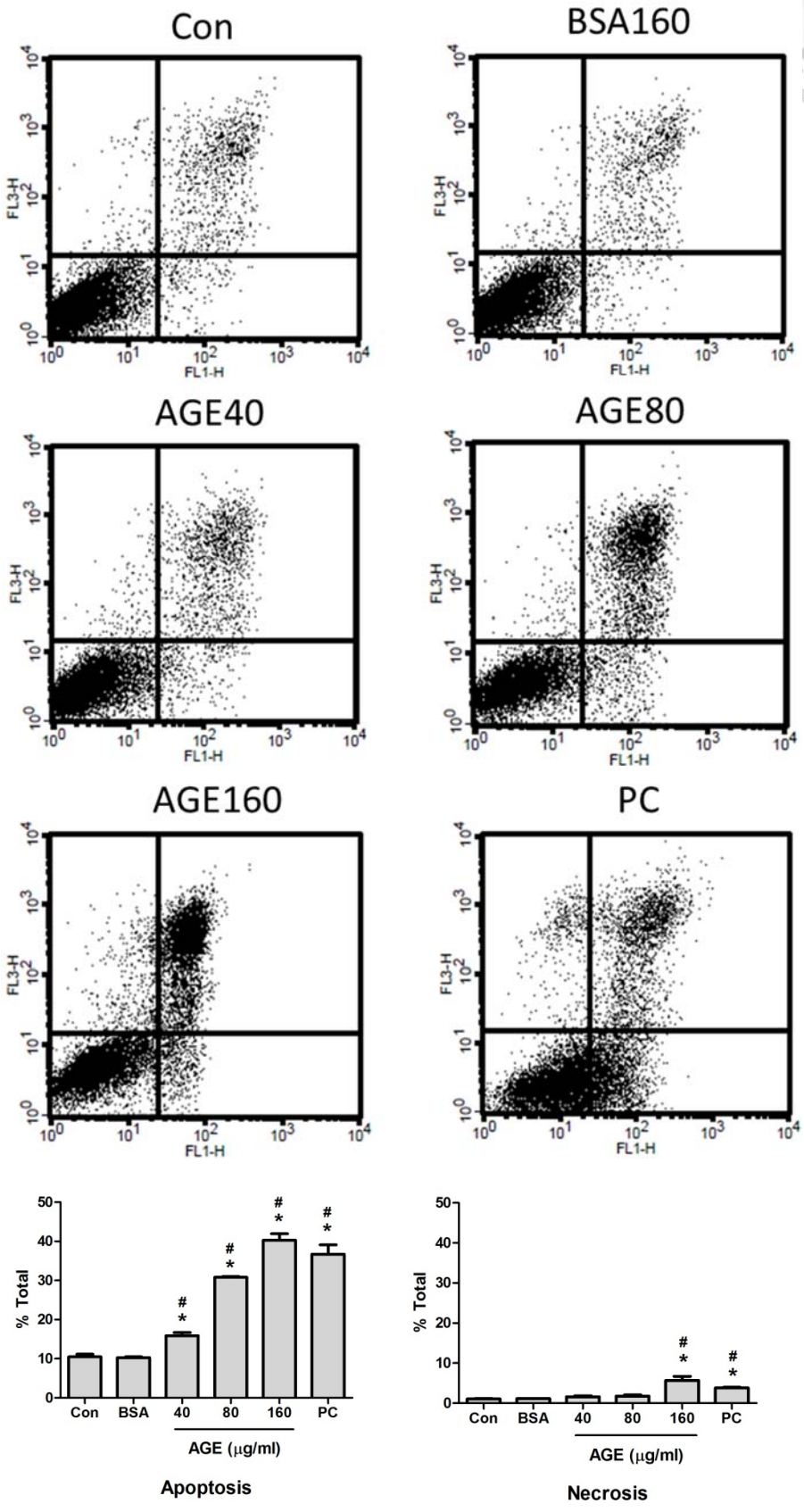
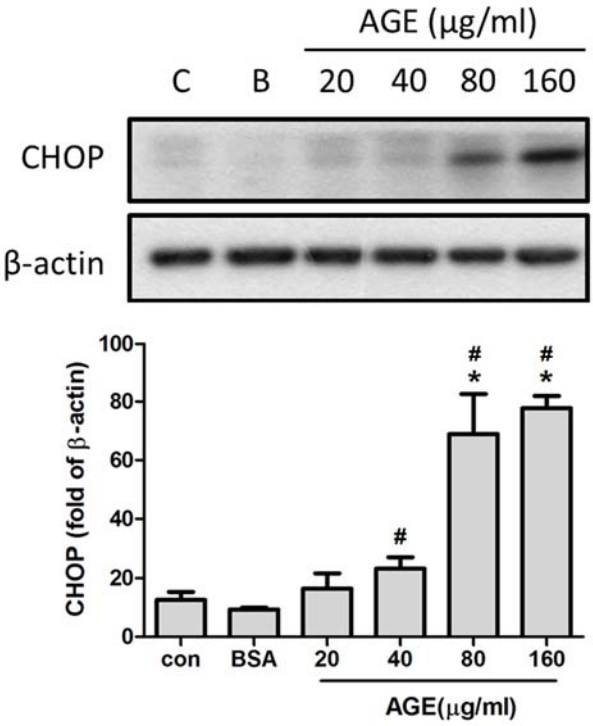

Con
BSA160
AGE40
AGE80
AGE160
PC
FL3-H
FL1-H
% Total
Con
BSA
40
80
160
PC
AGE (μg/ml)
Apoptosis
Necrosis

Figure 5.3. AGEs induced apoptosis responses in mesangial cells. Cells were incubated with BSA (160μg/ml) and different concentrations of AGEs (40, 80, 160 μg/ml) for 24 hours. Then cells were stained with Annexin V and PI. Percentages of cells with different death patterns were determined by flow cytometry. Apoptosis positive control cells were treated with 3% formaldehyde for 30 minutes. Upper left panel means necrosis cells; upper right panel means apoptosis cells; lower left panel means normal cells; lower right panel means early apoptosis cells. The apoptosis statistical result was the sum of apoptosis and early apoptosis. Statistical results are presented as mean ± SEM of three separate experiments. *, significant different ($p<0.05$) compared to control group (culture medium-treated group). #, significant different ($p<0.05$) compared to BSA-treated group.

Figure 5.4

(A)


AGE (μg/ml)
C
B
20
40
80
160
CHOP
β-actin
CHOP (fold of β-actin)
100
80
60
40
20
0
con
BSA
20
40
80
160
AGE(μg/ml)

(B)

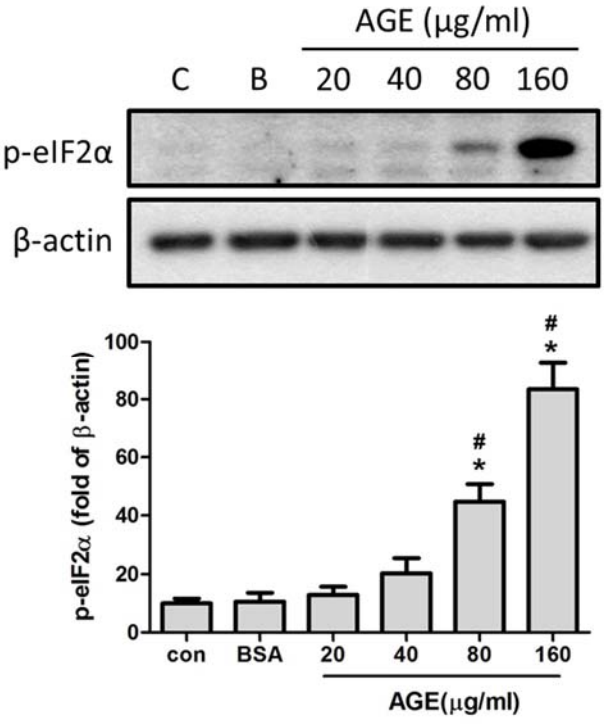
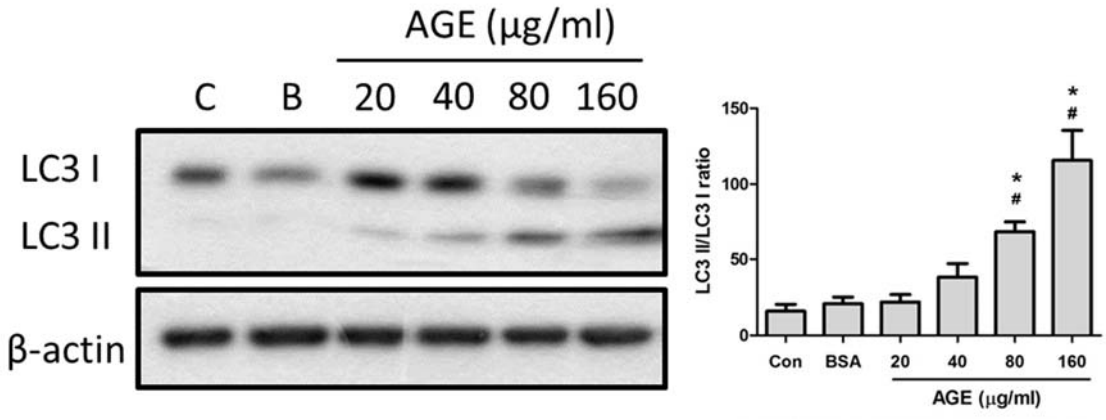

AGE (μg/ml)
C
B
20
40
80
160
p-eIF2α
β-actin
p-eIF2α (fold of β-actin)
100
80
60
40
20
0
con
BSA
20
40
80
160
AGE(μg/ml)

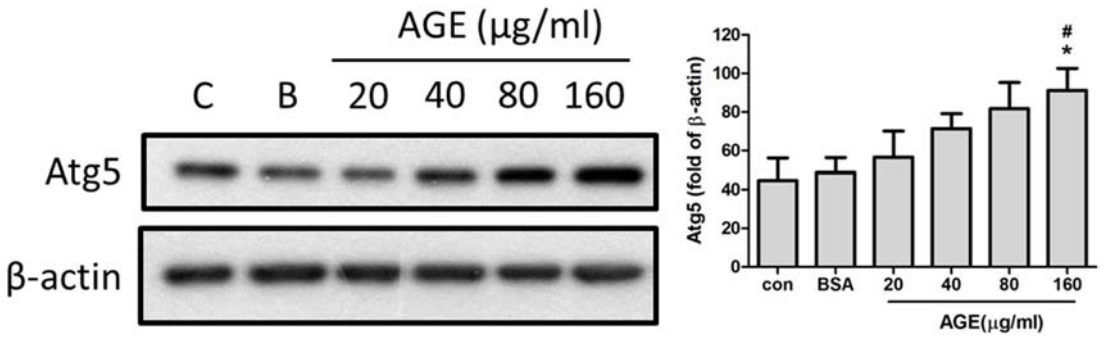
Figure 5.4. AGEs induced CHOP and p-eIF2α protein expressions in mesangial cells. Cells were treated with BSA (160 μg/ml) and different concentrations of AGEs (20, 40, 80, 160 μg/ml) for 24 hours. Whole cell extracts were prepared, and protein expression levels were evaluated by western blot using antibodies against CHOP (A), p-eIF2α (B) and β-actin. β-actin was used as internal control. The relative levels of CHOP, p-eIF2α of three separate experiments were analyzed and expressed as mean ± SEM. *, significant different ($p<0.05$) compared to control group (culture medium-treated group). #, significant different ($p<0.05$) compared to BSA-treated group.

Figure 5.5

(A)



(B)



(C)

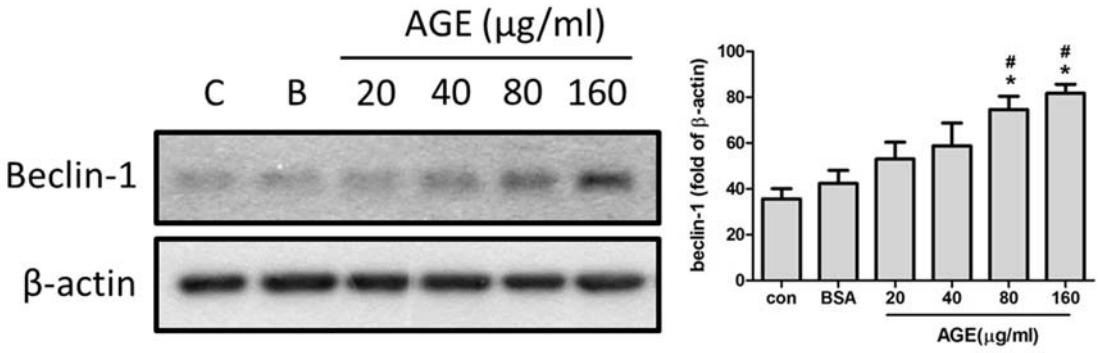


Figure 5.5. AGEs induced autophagy activity in mesangial cells. Cells were treated with BSA (160 μg/ml) and different concentrations of AGEs (20, 40, 80, 160 μg/ml) for 24 hours. Whole cell extracts were prepared, and protein expression levels were evaluated by western blot using antibodies against LC3 (A), Atg5 (B), beclin-1 (C) and β-actin. β-actin was used as internal control. The relative levels of LC3-II, beclin-1 and Atg5 of three separate experiments were analyzed and expressed as mean ± SEM. *, significant different ($p<0.05$) compared to control group (culture medium-treated group). #, significant different ($p<0.05$) compared to BSA-treated group.

Figure 5.6

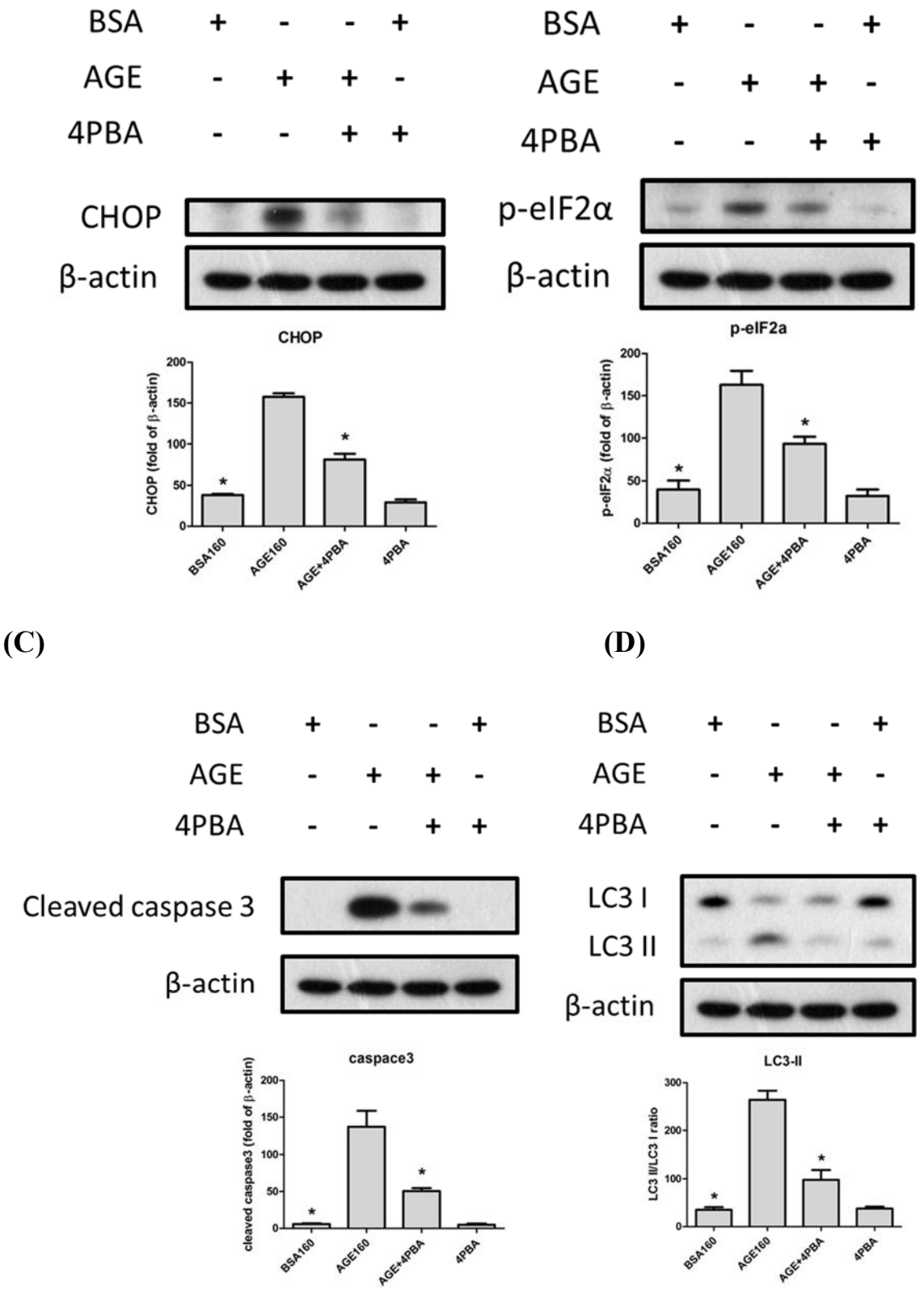

(A)
BSA + - - +
AGE - + + -
4PBA - - + +
CHOP
β-actin
CHOP
CHOP (fold of β-actin)
BSA160
AGE160
AGE+4PBA
4PBA
(B)
BSA + - - +
AGE - + + -
4PBA - - + +
p-eIF2α
β-actin
p-eIF2a
p-eIF2α (fold of β-actin)
BSA160
AGE160
AGE+4PBA
4PBA
(C)
BSA + - - +
AGE - + + -
4PBA - - + +
Cleaved caspase 3
β-actin
caspace3
cleaved caspase3 (fold of β-actin)
BSA160
AGE160
AGE+4PBA
4PBA
(D)
BSA + - - +
AGE - + + -
4PBA - - + +
LC3 I
LC3 II
β-actin
LC3-II
LC3 II/LC3 I ratio
BSA160
AGE160
AGE+4PBA
4PBA

Figure 5.6. 4PBA reversed AGEs-induced CHOP, p-eIF2α, cleaved caspase-3, and LC3-II protein expressions in mesangial cells. Cells were treated with BSA (160 μg/ml) or AGEs (160 μg/ml) and with or without 4PBA (1 mM) for 24 hours. Whole cell extracts were prepared, and protein expression levels were evaluated by western blot using antibodies against CHOP (A), p-eIF2α (B), cleaved caspase-3 (C), LC3-II (D) and β-actin. β-actin was used as internal control. The relative levels of CHOP, p-eIF2α, cleaved caspase-3, LC3-II and β-actin of three separate experiments were analyzed and expressed as mean ± SEM. *, significant different ($p<0.05$) compared to AGEs-alone group.

Figure 5.7

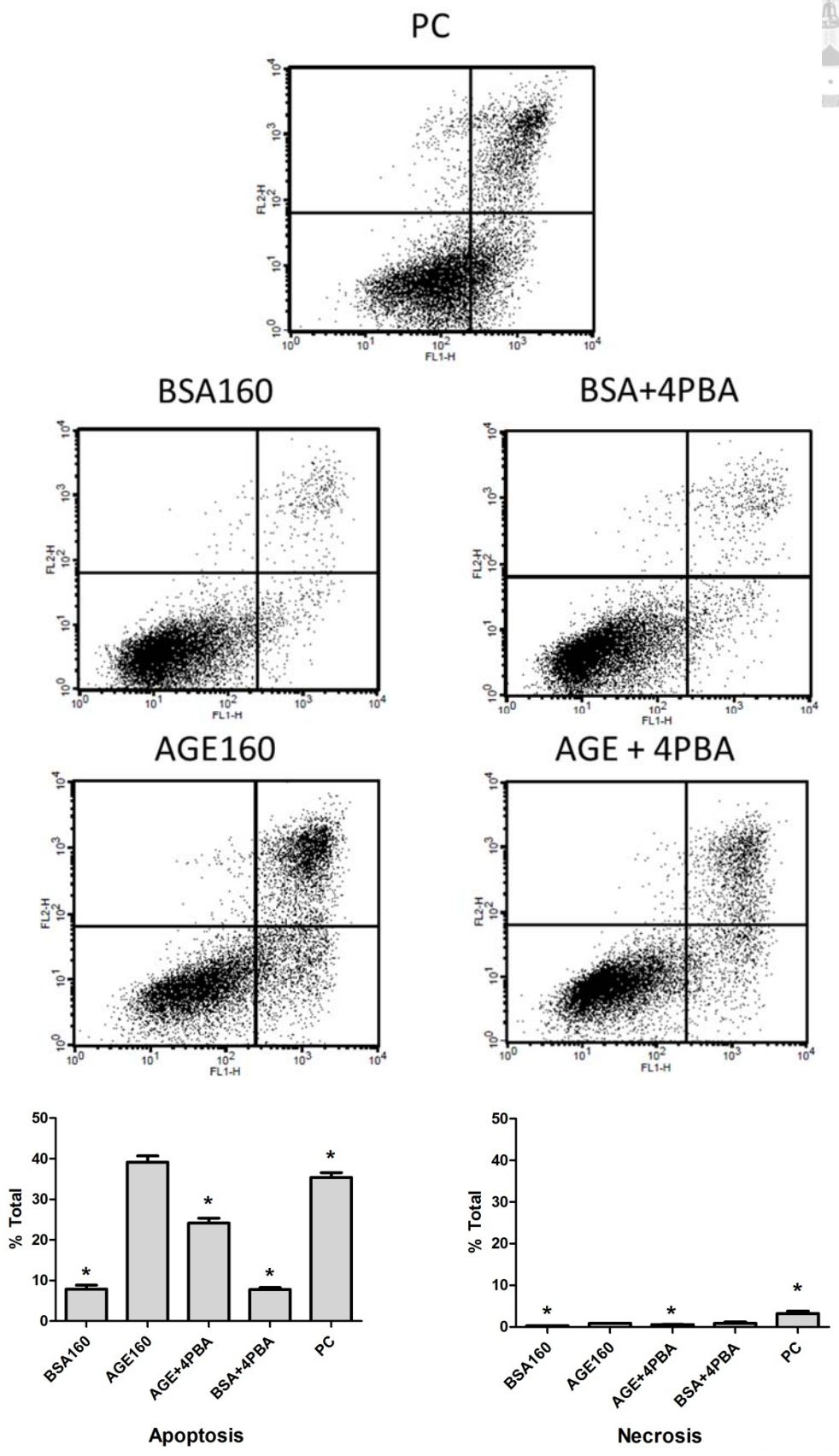

PC
BSA160
BSA+4PBA
AGE160
AGE + 4PBA
FL2-H
FL1-H
% Total
BSA160
AGE160
AGE+4PBA
BSA+4PBA
PC
Apoptosis
Necrosis

Figure 5.7. 4PBA decreased AGEs-induced apoptosis response in mesangial cells. Cells were treated with BSA (160 μg/ml) or AGEs (160 μg/ml), and with or without 4PBA (1 mM) for 24 hours. Then cells were stained with Annexin V and PI. Percentages of cells with different death patterns were determined by flow cytometry. Apoptosis positive control cells were treated with 3% formaldehyde for 30 minutes. Upper left panel means necrosis cells; upper right panel means apoptosis cells; lower left panel means normal cells; lower right panel means early apoptosis cells. The apoptosis statistical result was the sum of apoptosis and early apoptosis. Statistical results are presented as mean ± SEM of three separate experiments. *, significant different ($p<0.05$) compared to AGEs-alone group.

Figure 5.8

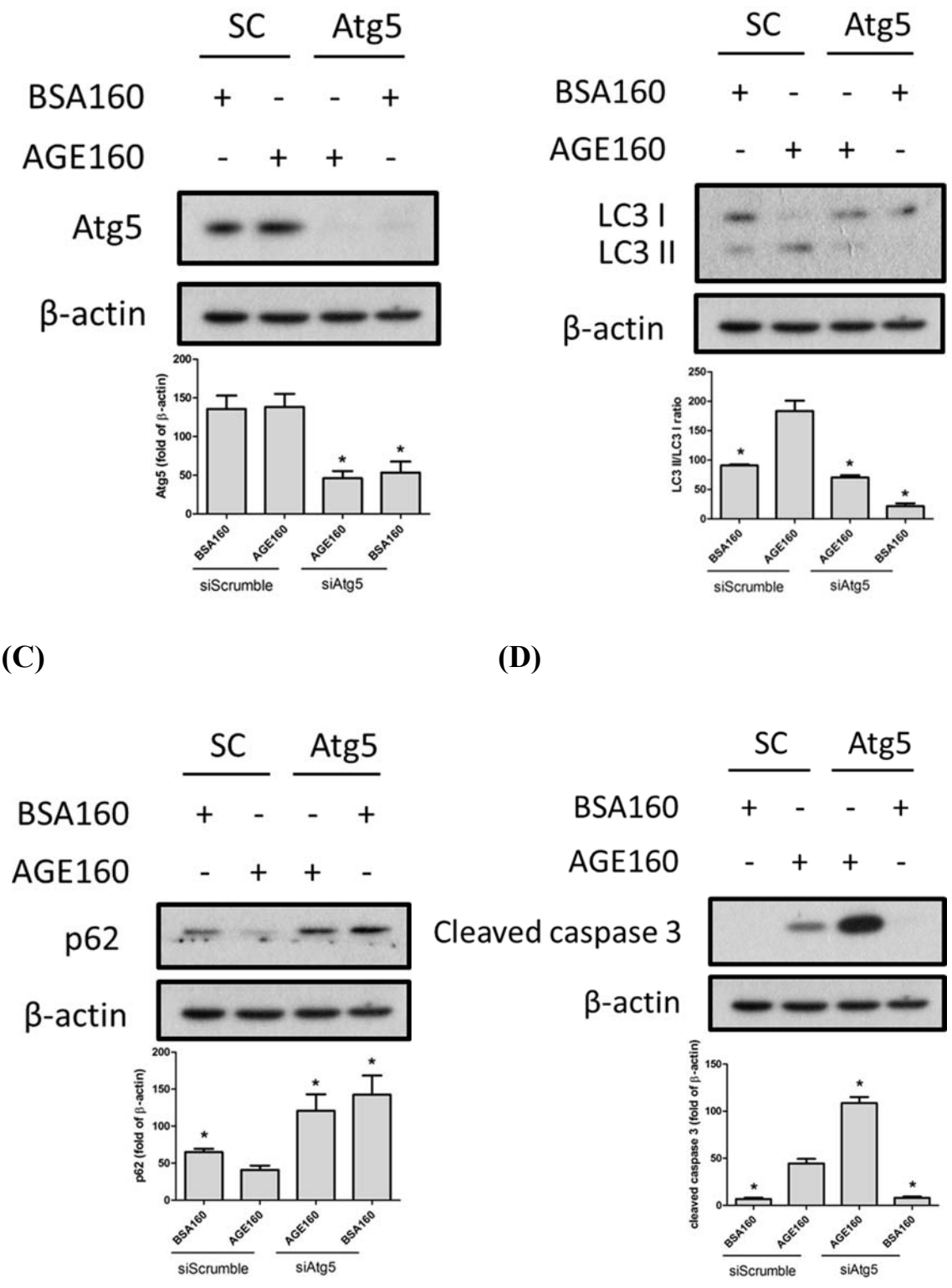

(A)
SC
Atg5
BSA160
AGE160
Atg5
β-actin
Atg5 (fold of β-actin)
siScrumble
siAtg5
(B)
LC3 I
LC3 II
LC3 II/LC3 I ratio
(C)
p62
p62 (fold of β-actin)
(D)
Cleaved caspase 3
cleaved caspase 3 (fold of β-actin)

Figure 5.8. Transfection of siAtg5 downregulated AGEs-induced LC3-II protein expression, and upregulated p62 and cleaved caspase-3 protein expressions in mesangial cells. Cells were transfected with siScrumble (80nM) or siAtg5 (80nM) for 6 hours. After recovered with culture medium overnight, cells were treated with BSA (160 μg/ml) or AGEs (160 μg/ml) for 24 hours. Whole cell extracts were prepared, and protein expression levels were evaluated by western blot using antibodies against Atg5 (A), LC3 (B), p62 (C), cleaved caspase-3 (D) and β-actin. β-actin was used as internal control. The relative levels of Atg5, LC3-II, p62 and cleaved caspase-3 of three separate experiments were analyzed and expressed as mean ± SEM. *, significant different ($p<0.05$) compared to AGEs-alone group.

Figure 5.9

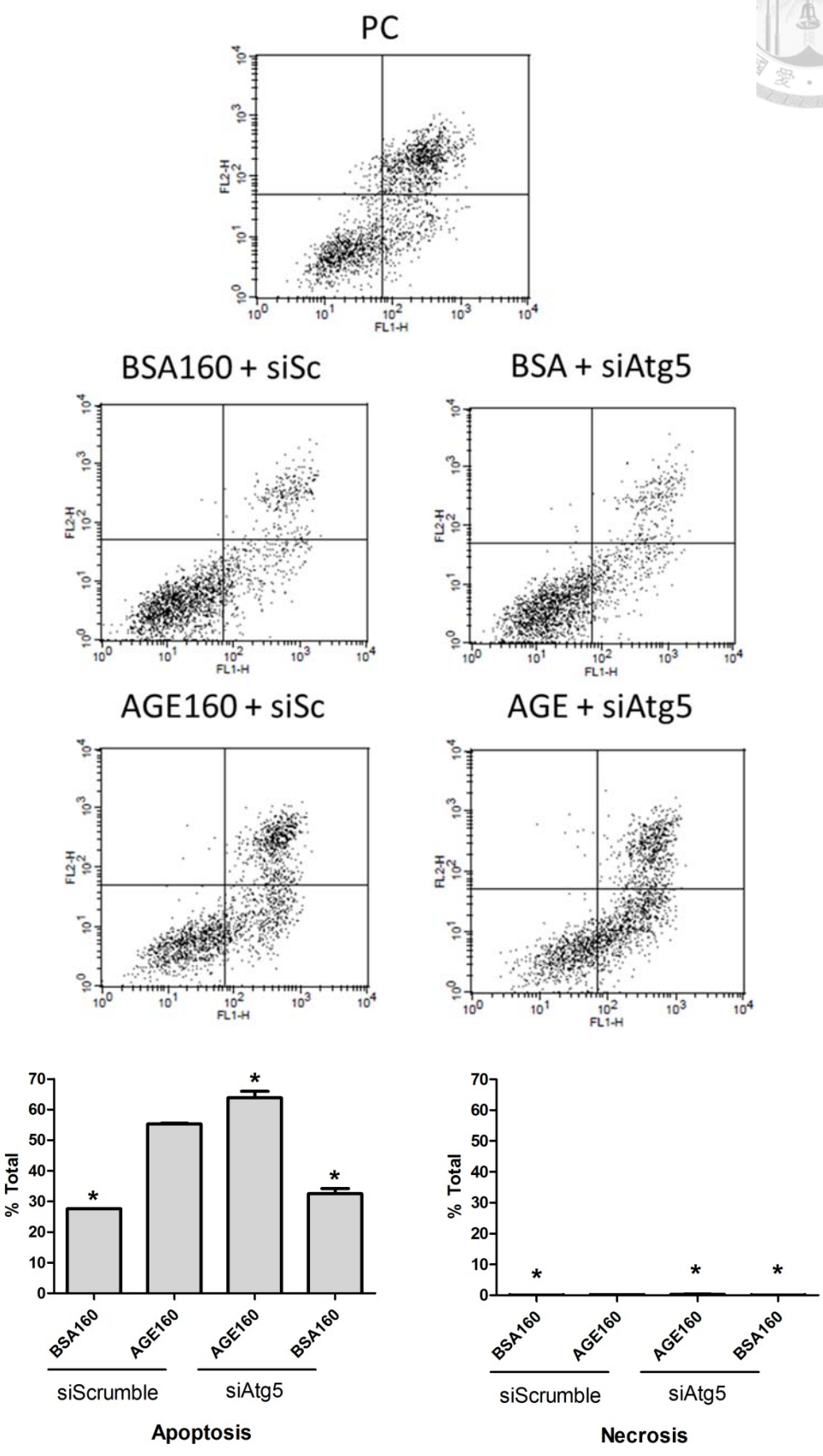
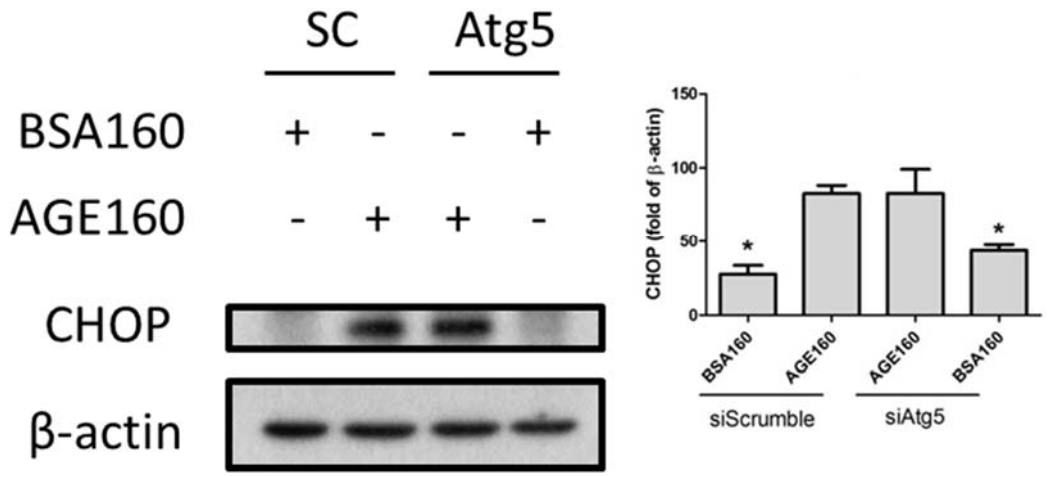

PC
BSA160 + siSc
BSA + siAtg5
AGE160 + siSc
AGE + siAtg5
FL2-H
FL1-H
% Total
BSA160
AGE160
AGE160
BSA160
siScrumble
siAtg5
Apoptosis
Necrosis

Figure 5.9. Transfection of siAtg5 induced apoptosis response in mesangial cells. Cells were transfected with siScrumble (80nM) or siAtg5 (80nM) for 6 hours. After recovered with culture medium overnight, cells were treated with BSA (160 μg/ml) or AGEs (160 μg/ml) for 24 hours. Then cells were stained with Annexin V and PI. Percentages of cells with different death patterns were determined by flow cytometry. Apoptosis positive control cells were treated with 3% formaldehyde for 30 minutes. Upper left panel means necrosis cells; upper right panel means apoptosis cells; lower left panel means normal cells; lower right panel means early apoptosis cells. The apoptosis statistical result was the sum of apoptosis and early apoptosis. Statistical results are presented as mean ± SEM of three separate experiments. *, significant different ($p<0.05$) compared to AGEs-alone group.

Figure 5.10

(A)



(B)

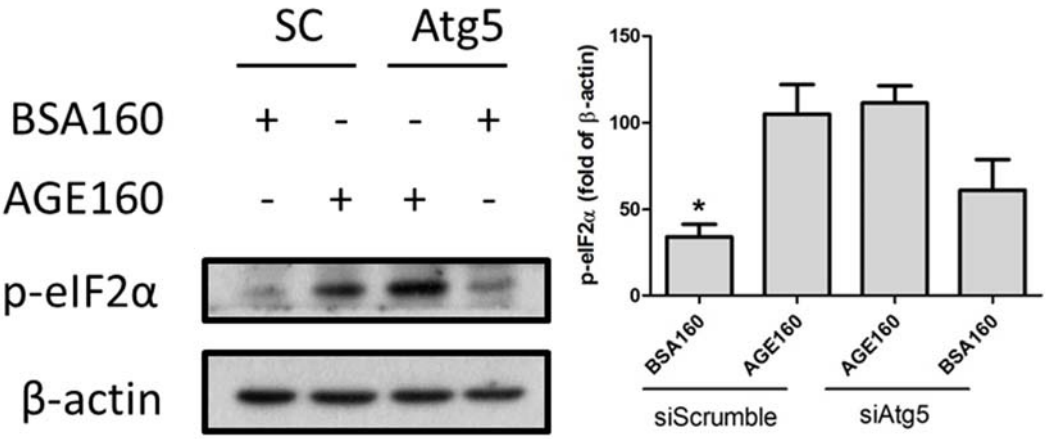
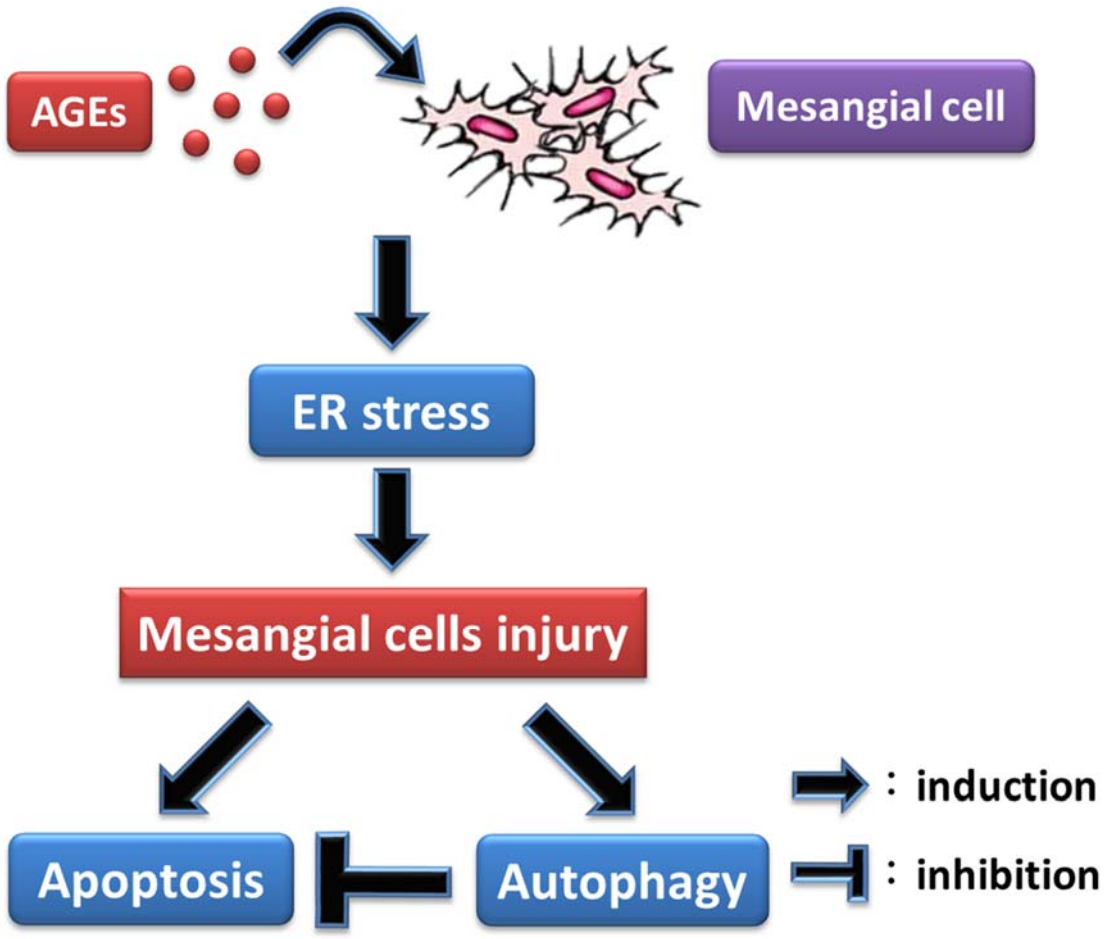


Figure 5.10. Transfection of siAtg5 had no effect on CHOP and p-eIF2α protein expressions in mesangial cells. Cells were transfected with siScrumble (80nM) or siAtg5 (80nM) for 6 hours. After recovered with culture medium overnight, cells were treated with BSA (160 μg/ml) or

AGEs (160 μg/ml) for 24 hours. Whole cell extracts were prepared, and protein expression levels were evaluated by western blot using antibodies against CHOP (A), p-eIF2α (B) and β-actin. β-actin was used as internal control. The relative levels of CHOP (A), p-eIF2α of three separate experiments were analyzed and expressed as mean ± SEM. *, significant different ($p<0.05$) compared to AGEs-alone group.

6. Conclusion

Taken together, these findings suggested that AGEs induced mesangial cells apoptosis through ER stress via p-eIF2α/CHOP sinaling pathway. Also, AGEs-induced ER stress might activate autophagy which plays a protective role in AGEs-induced mesangial cells apoptosis.



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