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PROTECTIVE EFFECTS OF METFORMIN IN AN IN VITRO MODEL OF PROTEINURIC KIDNEY DISEASE

BY

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ABSTRACT

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Title: PROTECTIVE EFFECTS OF METFORMIN IN AN IN VITRO MODEL OF PROTEINURIC KIDNEY DISEASE

Supervisor of Thesis: Shankar Munusamy

Albuminuria, a hallmark of chronic kidney disease (CKD), plays a crucial role in the etiology of CKD. High albumin levels are thought to induce endoplasmic reticulum (ER) stress and activate the AKT pathway, and lead to inactivation of AMP-activated kinase (AMPK), an energy-sensing molecule within cells. This in turn, activates mTOR (mammalian target of rapamycin), which inhibits autophagy and induces epithelial-to-mesenchymal transition (EMT), and ultimately results in accelerated renal cell apoptosis. Thus, the objectives of this study are: 1) to investigate the effects of restoration of AMPK signaling in renal cells using metformin on ER stress, AKT, EMT, autophagy and apoptosis that are thought to mediate renal cell injury during proteinuria, and 2) to dissect the AMPK- and non-AMPK mediated effects of metformin using an *in vitro* model of albumin-induced renal cell injury. Normal rat kidney proximal tubular (NRK-52E) cells grown to 60% confluency were exposed to 10 and 15 mg/ml of albumin for 72 hours in the presence or absence of 1 mM Metformin and/or 0.5 μM compound C, (an AMPK inhibitor). Cells were assessed for alterations in AMPK, AKT and mTOR pathways, and the markers of ER stress, EMT, autophagy and apoptosis. Metformin treatment significantly induced phosphorylation of AMPK, and attenuated the phosphorylation of AKT and mTOR markers in NRK-52E cells exposed to albumin. More importantly,

metformin treatment prevented albumin-mediated induction of α-SMA (an EMT marker) and the expressions of pro-apoptotic ER stress marker CHOP and apoptotic caspases -12 and -3 but augmented the levels of autophagy markers (P-ULK-1 and LC3-II) in renal cells. Blockade of metformin-induced AMPK activation with compound C blunted the ER defense response and autophagy but had no effect on the markers of EMT (α-SMA) and apoptosis (caspase-12 and BAX) in renal cells exposed to albumin. Our studies suggest that metformin protects renal cells against proteinuric cytotoxicity via suppression of AKT and mTOR activation, EMT and apoptosis, and augmentation of autophagy and ER defense response in renal tubular cells. Studies with Compound C reveal that metformin's effects on autophagy and ER chaperone expression were AMPK-mediated whereas its effects on EMT and apoptosis were AMPK-independent.

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Dedication

To my Exquisite Family

Eng. Najib Allouch

Dr. Oussama Allouch

Dr. Hind Ben Tkhayat

Dr. Sonia Allouch

Asma Allouch

Israa Allouch

Eng. Atef Jrad

And

My loving Mother

Mrs. Saida Jrad (1962-2004)

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Abbreviations

ACE, angiotensin-converting-enzyme

AICAR, N(1)-(β-d-Ribofuranosyl)-5-aminoimidazole-4-carboxamide

AMPK, AMP-activated protein kinase

AMPKK, AMP-activated protein kinase kinase

ARBs, angiotensin-receptor blockers

ATF-6, activating transcription factor-6

ATG13, autophagy-related gene 13

BAX, Bcl-2-associated death promoter

CaMKK, Ca^{2+}/calmodulin-dependent protein kinase kinase

CHOP, CCAAT-enhancer-binding protein homologous protein

CKD, chronic kidney disease

EBP1, eukaryotic translation initiation factor 4E-binding protein 1

eIF2α, translation initiation factor 2α

eIF4E, eukaryotic initiation factor 4E

EMT, epithelial-to-mesenchymal transition

EndoMT, endothelial-to-mesenchymal transition

ER, endoplasmic reticulum

ERK, extracellular signal–regulated kinases

ESRD, end stage renal disease

FSP1, fibroblast-specific protein 1

GFR, glomerular filtration rate

GRP78, 78 kDa glucose-regulated protein

GTPases, rag guanosine triphosphatases

HIF-1α, hypoxia-inducible factor-1 alpha

IRE, inositol-requiring protein 1

JNK, c-Jun N-terminal kinase

LDH, lactate dehydrogenase

LKB1, liver kinase B1

MAPK, mitogen activated protein kinase

mSIN1, mammalian stress-activated protein kinase

mTORC1, mammalian target of rapamycin complex 1

mTORC2, mammalian target of rapamycin complex 2

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

NADPH, nicotinamide adenine dinucleotide phosphate

NRK-52E, normal rat kidney epithelial cells

P70S6K, ribosomal protein S6 kinase

PERK, double-stranded RNA-activated (PKR)-like endoplasmic reticulum kinase

PKB, protein kinase B

PKC, protein kinase C

ROS, reactive oxygen species

Sgk1, serine/threonine-protein kinase

STN, subtotal nephrectomy

TGF-β1, transforming growth factor beta 1

TSC-2, tuberous sclerosis complex 2

ULK1, UNC-51-like kinase 1

UPR, unfolded protein response

UUO, unilateral ureteral obstruction

v-ATPase, vacuolar H^+-adenosine triphosphatase

α-SMA, alpha smooth muscle actin

Chapter 1: Introduction

1.1. CHRONIC KIDNEY DISEASE: DEFINITION, STAGING, EPIDEMIOLOGY AND CURRENT THERAPY

Kidneys play a pivotal role in the homeostasis of the human body. Under physiological conditions, the renal system, through controlling water, hydrogen ions and essential electrolytes such as sodium, potassium, and bicarbonate, maintains the intravascular volume, acid-base equilibrium, electrolyte and water balance. Chronic kidney disease (CKD) is a broad term that refers to diverse disease conditions affecting the structure and function of the renal system. CKD is characterized by either the existence of renal impairment or the diminution of renal function (1). Glomerular filtration rate (GFR), a cornerstone indicator to estimate renal function, is used to determine the prognosis and progression of CKD. Based on the GFR, CKD is classified into five stages as shown in **Table 1.1** (2).

Table 1.1.

Stages of chronic kidney disease based on the glomerular filtration rate (GFR)

Stages	Description	Renal Function (ml/min/1.73 m^2)
1	Kidney damage but normal GFR	≥ 90
2	Mild decreases in GFR	60 to 89
3	Moderate decrease in GFR	30 to 59
4	Severe decrease in GFR	15 to 29
5	End stage renal disease (ESRD)	< 15

Various factors such as diabetes, hypertension, obesity, aging, and other cardiovascular diseases could lead to CKD (**Figure 1.1**). Irrespective of its etiology, CKD is associated with multiple complications ranging from increased hospitalization to aggravated cardiovascular events and mortality (3). Worldwide, a trend of a sharp increase in the incidence and prevalence of CKD is noted. In the United States, more than 20 million adults are estimated to have CKD in 2015 (4). In Qatar, CKD affects about 13% of the population and the prevalence of end stage renal disease (the advanced phase of CKD) was found to be 212 per million population (5). The increased risk of complications associated with CKD combined with the high prevalence of CKD warrants its prevention and management as a high national and international priority.

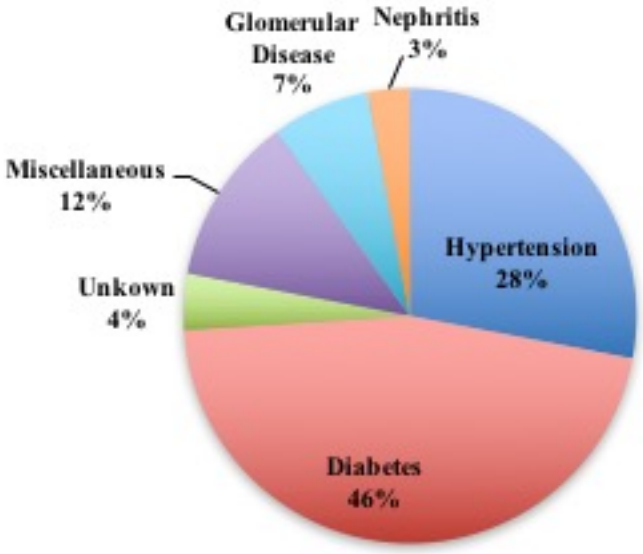

Figure 1.1. The different risk factors responsible for the development of CKD in the USA.

The current management of CKD is mainly aimed at slowing the progression of renal impairment and albuminuria through the use of angiotensin-converting-enzyme (ACE) inhibitors and angiotensin-receptor blockers (ARBs) (1). Nonetheless, these drugs were only shown to be beneficial in patients with macroalbuminuria (i.e., the urinary albumin to creatinine ratio between 30 to 300 mg/g) and advanced renal impairment (6). Furthermore, the existing therapies neither reverse established kidney damage nor restore normal renal function (1, 6). Thus, there is an urgent need for therapeutic agents that can prevent and/or treat CKD.

1.2. METFORMIN

Metformin, a chemically derived compound from biguanides (**Figure 1.2**), is considered a cornerstone in the management of type 2 diabetes (T2DM). Metformin's exact mechanism of action is yet to be fully elucidated. Nonetheless, its ability to normalize blood glucose levels without the incidence of hypoglycemia in patients is shown to be exerted through an increase in insulin sensitivity in the tissues and a decrease in hepatic gluconeogenesis and intestinal absorption of glucose (7). Additionally, several reports have documented metformin's ability to improve lipid profile (8), and promote weight loss (9) in patients with T2DM and obesity. Numerous studies also reveal the potential therapeutic benefits that metformin confers in the management of CKD (7).

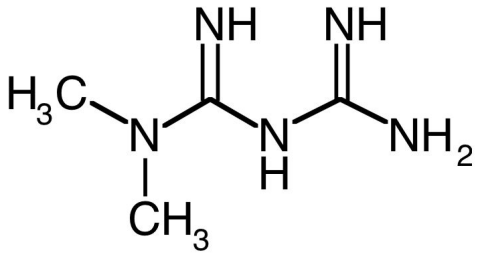

Figure 1.2. Structure of Metformin (3-(diaminomethylidene)-1,1-dimethylguanidine).

The renal elimination of metformin in its unchanged form, in conjunction with metformin's tendency to cause lactic acidosis led to its cautious use in patients with CKD. Despite their initial development in 1950, the cautious use of biguanides (phenformin and metformin) started only in mid-1970s when phenformin was implicated

in a number of fatal cases of lactic acidosis (10). Phenformin was ultimately withdrawn from the market due to its lethal metabolic side effects. In spite of its structural similarity with phenformin, the incidence of lactic acidosis was 10 to 20 times less frequent with metformin (11). Pharmacovigilance reports from France, United Kingdom and Sweden showed that the risk for lactic acidosis with metformin is as low as was 2.4 cases per 100,000 patient-years (12). This is further supported by the very few cases of lactic acidosis reported with metformin despite the exponential increase in its use worldwide. Eventually in 1995, the U.S. Food and Drug Administration (FDA) approved the use of metformin to treat type II diabetes and the risk of lactic acidosis was deemed minimal. Nonetheless, the use of metformin in patients with CKD remained contraindicated until recently. In April 2016, the US FDA revised the labeling of metformin and approved its use in patients with mild-moderate renal impairment (13) based on the eGFR status. Currently, metformin is contraindicated only in patients with an eGFR below 30 mL/minute/1.73 m^2. This expands the patient population (i.e., patients with stages 1 to 3 of CKD) who could benefit from metformin (13).

Although the exact mechanisms through which metformin exerts its beneficial action are yet to be fully understood, a majority of reports link metformin's therapeutic effects to its ability to activate AMP-activated protein kinase (AMPK) signaling (14-17). Nonetheless, emerging evidences also suggest for AMPK-independent actions of metformin. For instance, Ben Sahra et al. (2011) showed that the anti-proliferative effects of metformin in prostate cancer is AMPK-independent, and was mediated through p53 and REDD1 (regulated in development and DNA damage responses 1) axis (18). Similarly, Kelly et al. (2015) reported that metformin inhibited reactive oxygen species

(ROS) production in macrophages in an AMPK-independent manner (19). The multiple beneficial effects of metformin therapy paired with its poorly characterized mechanism of action necessitates investigation of its therapeutic potential and the mechanistic pathways involved also in non-diabetic states such as CKD.

1.3. FACTORS CONTRIBUTING TO ALBUMINURIA AND CKD

1.3.1. Diabetes Mellitus

Diabetes mellitus is a metabolic condition characterized by an insufficient insulin production or signaling resulting in continuously elevated glucose levels. Proteinuria was first identified in diabetes mellitus patients in the late 18th century, but an association between the two was proven only later (20). In the 1950s, kidney disease was identified as one of the most prominent complications of diabetes. Furthermore, about 50% of patients suffering from diabetes for more than 20 years developed CKD (20). Presently, diabetes is considered as a major risk factor for the development of CKD worldwide (20). Moreover, in terms of morbidity and mortality rates, diabetic nephropathy is classified as one of the most serious long-term complications of diabetes. The most prominent histologic changes observed in the renal glomeruli of patients with diabetes are: thickening of glomerular basement membrane (GBM), expansion of the mesangium, and glomerular sclerosis (21). The sum of these three morphological changes leads to extracellular matrix accumulation and proteinuria (21).

Different signaling pathways contribute to the histologic changes and the onset of nephropathy in patients with diabetes (7, 22). For instance, high glucose exposure in

glomerular mesangial cells was reported to increase the levels of angiotensin II (Ang II), a vasoactive peptide implicated in the pathogenesis of diabetic nephropathy (22). Studies suggest that hyperglycemia added to the increase in Ang II expression in the kidney triggers a multitude of intracellular signaling cascades, such as the polyol (23) and the reactive oxygen species pathways (24) in diabetes patients. The sum of the aforementioned signaling processes triggers the Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway in glomerular mesangial cells, eventually intensifying cell proliferation and growth, thus, leading to proteinuria and nephropathy (22).

1.3.2. Hypertension

The association between hypertension and renal injury is complex and multifactorial (25). For instance, different studies explained the causative relation between elevated blood pressure and CKD through the observed increase in the intraglomerular pressure, which leads to increased glomerular filtration and glomeruli injury. Consequently, the ultrafiltration increases, which in conjunction with altered glomerular membrane permeability causes an abnormal leakage of protein into the urine, i.e., proteinuria (25).

Apart from its effects on the modulation of blood pressure, a direct association between the renin-angiotensin system (RAS) and the development and progression of renal injury is well documented (26). In particular, in cases where RAS was inappropriately triggered, a significant elevation in the intrarenal Ang II levels was noted (27). Ang II is then sequestered into the renal interstitial fluid and the proximal tubular compartments (27), resulting in the activation of the tubular angiotensin II type 1 (AT1)

receptors in the zona glomerulosa of the adrenal cortex. Stimulation of AT1 receptors triggers the production of mineralocorticoids and reactive oxygen species (ROS) (28). Increased ROS production was reported to activate mitogen-activated protein kinases (MAPK) and extracellular signal–regulated kinases (ERK), which together stimulate transforming growth factor-beta (TGF-β)/Smad pathway. This eventually leads to epithelial-to-mesenchymal transition (EMT) in proximal tubular epithelial cells and contributes to the development and progression of CKD (29).

1.4. CELLULAR TRANSPORTATION AND FATE OF ALBUMIN

Albumin, the most prominent plasma protein in mammals, is constituted of three homologous domains (I, II and III). Each homologous domain is composed of two sub-domains: sub-domain A - comprised of six helices and sub-domain B - comprised of 4 helices (30). The domain-based structure of albumin facilitates its binding to a wide range of molecules, thereby allowing albumin to function as a plasma carrier and protein transporter. Additionally, albumin plays a major role in the regulation osmotic pressure by controlling the distribution of body fluids' within the vasculature and across tissues (31).

On a cellular level, albumin is transported into the cells by endocytosis via clathrin-dependent or independent pathways, where small vesicles are formed to facilitate albumin uptake (30). The exact mechanism responsible for albumin internalization is highly dependent on the cell type. For instance, in human skin fibroblasts and endothelial cells, albumin is taken up via caveolae-mediated endocytosis (30). On the other hand, in kidney cells, internalization of albumin is triggered by its binding to the low-density lipoprotein membrane receptor megalin, which functions through clathrin-dependent

process (32). Following its uptake, the intermediary pathways and the final target of albumin in many cells are yet to be determined. In kidney, albumin uptake and its fate are well-characterized in Madin-Darby kidney cell line (MDCK). Further to megalin-mediated endocytosis, the clathrin-coated pits deliver albumin to early and late endosomes. The degradation process is then initiated by the transport of albumin containing endosomes to lysosomes in which albumin gets degraded to amino acids and recycled for various cellular processes such as energy metabolism and protein synthesis (30).

1.5. AMP-ACTIVATED PROTEIN KINASE (AMPK): STRUCTURE, FUNCTION AND REGULATION

AMP-activated protein kinase (AMPK) is an intrinsic energy sensor that coordinates the intricate balance between energy production and expenditure in all eukaryotic cells. The structure of AMPK was characterized as a heterotrimeric complex with one catalytic (α) and two regulatory (β and γ) subunits (33). Owing to its vital role in the maintenance of cellular homeostasis, it is phylogenetically conserved across eukaryotes. In mammalian cells, seven genes - two isoforms each of the α and the β subunit, and three isoforms of the γ subunit - with 12 distinct heterotrimeric combinations were found to play a major role in the control of AMPK (34). Typically, the N-terminal of the α subunit is comprised of a serine/threonine kinase domain that is only activated subsequent to phosphorylation with a variety of upstream kinases (34). The regulatory adenine nucleotide-binding sites, which serve as cellular energy sensor, are located on the γ subunits. The catalytic binding site (CBS) motif, which is composed of four tandems repeats, is the main composition of the adenine-binding site. Different combinations of

these repeats form four adenine nucleotide-binding clefts, namely, site 1 to 4 (35). Site 1 and 3 are thought to be the most essential as they are the binding clefts for AMP, ADP, and ATP, and act as cellular energy sensors (**Figure 1.3**).

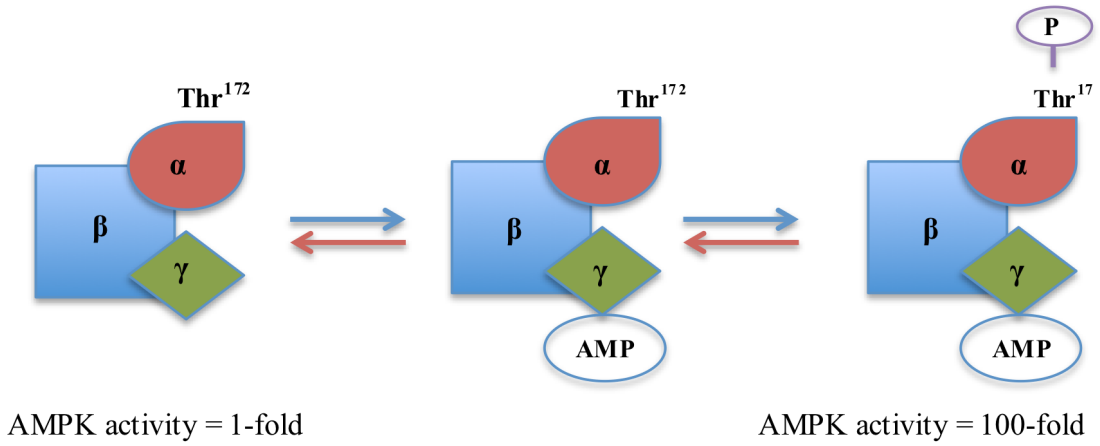

Figure 1.3. AMPK Structure and Its Regulation Via Phosphorylation**.** In the presence of AMP threonine 172 gets phosphorylated, increasing the AMPK activity by 100 Folds. AMPK, AMP activated Kinase.

The core function of AMPK is dependent on cellular energy levels, which is determined by the concentration of AMP over ATP and the resultant AMP/ATP ratio (36). An increase in the AMP concentration coupled with a decrease in the ATP level leads to binding of AMP to the catalytic subunit of AMPK and its subsequent phosphorylation at Thr172. Further to the AMP/ATP ratio increase, AMPK activation

takes place through three main mechanisms (37). The first pathway takes place via a direct allosteric activation. The second mechanism is initiated by the binding of AMP to the catalytic loop allowing its phosphorylation via one of the upstream AMPK kinases (38). Following its phosphorylation, a 100-fold increase in the AMPK activity is noted (39). Furthermore, at least two upstream kinases are known to increase the AMPK activity by up to a 1,000-fold (40). The final pathway functions mainly through the activation of a protein phosphatase that inhibits the dephosphorylation of Thr172 (35). The concerted actions of the three pathways make the system exquisitely sensitive to even minimal changes in AMP levels. Conversely, these three mechanisms are inhibited in cases where the AMP/ATP ratio is decreased.

1.6. EXPRESSION OF AMPK IN THE RENAL SYSTEM AND ITS ROLE IN RENAL HOMEOSTASIS

AMPK expression in the renal system was best characterized in rat models (41). The predominantly expressed AMPK subunits in the kidneys were found to be α1, β2, γ1, and γ2 (41). Immunostaining of kidneys for AMPK by Fraser et al. (2005) revealed that the strongest expression of AMPK occurs at the cortical thick ascending limbs, the macula densa and the basolateral surface of collecting ducts (42). The localization of AMPK within the renal system served as an initial step in determining its role in renal homeostasis.

Disproportionate to their size, kidneys receive 20 to 25% of cardiac output and accounts for about 7% of total oxygen consumption. This high perfusion rate and energy consumption by the kidneys in comparison to their tissue mass could be justified by their role in active tubular reabsorption of considerably large quantities of sodium and other

ions. Initial studies showed that tubular sodium transport and tubular respiration are closely related; nonetheless, the pathways by which the coupling takes place were yet to be defined. Recent studies reveal that AMPK plays a crucial role in the regulation of several ion transport proteins (41, 43). However, the exact pathway by which AMPK maintains a firm coupling between energy metabolism and tubular transport is yet to be known.

1.7. AMPK AND CKD: SPECULATED PATHWAYS

1.7.1 AMP-activated protein kinase (AMPK)

Several studies have attempted to determine the link between these upstream kinases and the phosphorylation of AMPK (44, 45). Hitherto, two kinases - the tumor suppressor gene (LKB1) and Ca^{2+}/calmodulin-dependent protein kinase (CaMKKβ) - were identified as the major upstream signal for activation of AMPK (44, 45). Both of which were shown to cause an AMP-independent phosphorylation of threonine-172 residue in AMPK-α subunit (46).

A study by Woods et al. (2003) in HeLa S3 and G361 cell lines revealed a strong correlation (Pearson correlation coefficient of 0.945, $p< 0.005$) between LKB1 levels and AMPK activity (46). Contrary to these findings, a study by Hurley et al. (2005) demonstrated that the phosphorylation of AMPK further to cellular stimulation with ionomycin and 2-DG was not impaired in $LKB1^{-/-}$ cells as compared with $LKB1^{+/+}$ cells (46). In fact, the LKB1 deficient cells showed a paradoxical increase in the phosphorylation and activation of AMPK, which can be further increased in the presence of calcium ionophores. Pharmacologic inhibition of CaMKKβ and silencing with

isoform-specific siRNAs proved that CaMKKβ is essential for AMPK phosphorylation (47). A variety of studies were conducted to identify other protein kinases responsible for AMPK phosphorylation. Three kinases were recently identified in yeast, and evidence of a third AMPK kinase (Tak1) in mammalian cells was reported. Nonetheless, the physiological significance of these kinases is yet to be determined (48).

Metformin, a commonly used medication for the management of type II diabetes, is known to exert its action through activation of AMPK. A study by Hawley et al. (2002) showed that exposure of cells to metformin leads to phosphorylation of the Thr-172 residue on the catalytic (α) subunit of AMPK. In AMPK-α1 knockout animal models, p-AMPK levels were found to be blunted despite metformin treatment (49). Conversely, administration of metformin to mice for three days showed an elevation in p-AMPK levels in the whole-body (49).

1.7.2. Mammalian target of rapamycin (mTOR)

The mammalian target of rapamycin (mTOR) pathway regulates an array of cellular processes ranging from transcription and protein synthesis to cell growth and proliferation (50). So far, two distinct protein complexes of mTOR were identified: mTOR complex 1 (mtorc1) and mTOR complex 2 (mTORC 2). Both protein complexes are thought to have the subunits mTOR, mlst8, and deptor in common. Nonetheless, the rapamycin-insensitive companion of tor (rictor), mammalian stress-activated protein kinase (msin1) and proline-rich protein 5 (protor) were only identified in mTORC 2 (50). In regards to their function, both mtor complexes phosphorylate a variety of proteins that are involved in regulation of cellular function. For example, mtorc1 stimulates the initiation of cap-dependent translation through its two downstream targets: eif4e-binding

proteins (4e-bps) and s6 kinases (s6k1 and s6k2), leading to cell growth and proliferation. Whereas mtorc2 phosphorylates akt, sgk1, and pkc, and is responsible for cell survival and cytoskeletal organization (51).

Multiple signaling pathways control the activity of mTORC in cells (**Figure 1.4**). For instance, a study by Inoki et al. (2003) in HEK293 cells demonstrated that ATP depletion activates AMPK and increases the activity of tuberous sclerosis complex 2 (TSC2) (52). The study also revealed that overexpression of TSC2 reduces the phosphorylation of mTORC1 targets: 4E-BPs and S6Ks, and thereby decreasing the activity of mTOR (52).

Similarly, a study by Takiar et al. (2011) in canine kidney cells revealed that activation of AMPK via metformin led to time-dependent inhibition of the downstream targets of mTOR - ribosomal S6 kinase (S6K) and p70 subunit (p70S6K). Furthermore, a dose-dependent decrease in cell proliferation was observed with metformin treatment in the wild-type cells but not in the AMPK-deficient cells, indicating that the inhibitory effects of metformin on mTOR are mediated via AMPK activation (53).

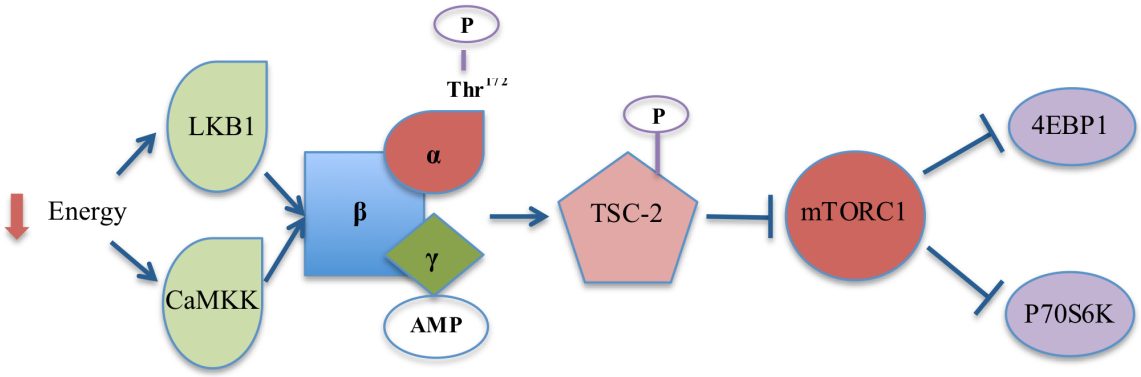

Figure 1.4. Signaling Pathways Linking AMPK and mTOR. Energy starvation triggers the phosphorylation of LKB1 and increases the expression of CaMKK. This stimulates the phosphorylation of AMPK and increases the expression of mTORC1 regulatory protein TSC-2. TSC-2 inhibits mTORC activation, thereby inhibiting 4EBP-1 and P70S6K. LKB1, Liver kinase B1; CaMKK, Ca^{2+}/calmodulin-dependent protein kinase kinase; TSC-2, Tuberous Sclerosis Complex 2; mTORC1, Mammalian target of rapamycin complex 1; 4EBP1, Eukaryotic translation initiation factor 4E-binding protein 1; P70S6K, Ribosomal protein S6 kinase.

1.7.3. Endoplasmic Reticulum (ER) Stress

The endoplasmic reticulum (ER) is the chief organelle responsible for protein folding and lipid synthesis. Furthermore, the ER functions as a sensor and a regulator of cellular homeostasis. In cases of cellular stress, unfolded proteins accumulate in the ER and activate three main cellular sensors: inositol-requiring enzyme-1α (IRE-1α), RNA-dependent protein kinase-like ER kinase (PERK), and transcription factor 6 (ATF6).

Activation of these sensors is known as the unfolded protein response (UPR), which is an adaptive mechanism aimed at restoration of cellular homeostasis. ER stress ensues when UPR fails to restore homeostasis (54).

AMPK plays a major role in sensing cellular stress and restoring cellular homeostasis in response to stress (33). A study by Dong et al. (2010) revealed that AMPK activation confers cytoprotection through stimulation of cell survival and inhibition of inflammatory and oxidative response in cells (55). Xi et al. (2013) demonstrated that ER stress induced with 2-deoxyglucose and tunicamycin activates AMPK via Ca^{2+}- calmodulin-dependent protein kinase kinase-β (CaMKKβ) and consequently stimulates autophagy. This study highlights the function of AMPK as an ER stress sensor (56). Alternatively, Jia et al. (2015) showed that activation of AMPK by N(1)-(β-d-Ribofuranosyl)-5-aminoimidazole-4-carboxamide (AICAR) attenuates ER stress via inhibition of the phosphorylation of IRE-1α and PERK (54). Furthermore, the knockdown of AMPK in the above model diminished the protective effects of AICAR against ER stress (54). Similarly, a study by Kim et al. (2015) showed that AMPK activation by metformin suppressed tunicamycin and thapsigargin-induced ER stress (57). Moreover, when AMPK was pharmacologically inhibited using Compound C, the effect of metformin was abolished; further proving that the reduction of ER stress observed with metformin is mediated via AMPK activation (57).

1.7.4. Autophagy

Autophagy, a highly conserved process across eukaryotes, encompasses all pathways leading to the delivery of cytoplasmic material to the lysosome for recycling or degradation (58). In general, three different classes of autophagy (macro-, micro- and

chaperone-mediated) were identified in eukaryotes. The most studied class is the macroautophagy, in which sequestering of a minute portion of the cytoplasm takes place through an isolation membrane known as phagophore, which sequentially form autophagosomes. Further fusion of autophagosomes with lysosome leads to the formation of autolysosome and results in cargo degradation and recycling. In microautophagy, an inward invagination of the lysosome membrane takes place to engulf small components of the cytoplasm. The third category of autophagy, which is mediated by chaperones, is the only type of autophagy that does not involve membrane restructuring. Nonetheless, a direct translocation of proteins across lysosomal membrane occurs during this process (58).

As mentioned earlier, AMPK plays a central role as a cellular energy sensor, while mTOR, particularly mTORC1 regulates nutrient signaling and cellular proliferation (52). The link between mTOR and autophagy is thought to be mediated through the mTORC1-aminoacid signal (59). The presence of a significant concentration of amino acids leads to the activation of the Rag guanosine triphosphatases (GTPases), which favors mTORC1 translocation to its activation site on the lysosomal surface. Studies indicate that the vacuolar H^+-adenosine triphosphatase ATPase (v-ATPase) is necessary for amino acids to activate mTORC1 (59). The translocation of mTORC1 to the lysosomes plays a crucial role in the regulation of autophagy. In non-starving conditions, the phagophore formation is suppressed through the attenuation of a serine/threonine-protein kinase UNC-51-like kinase 1 (ULK1) and autophagy-related gene 13 (ATG13) (60). Conversely, during cellular starvation or AMPK activation, an inhibition of mTORC1 takes place, which allows phagophore formation and the subsequent fusion of

autophagosomes with lysosomes to enable protein digestion (61).

Emerging evidence supports a linear relationship between AMPK and ULK1. Using bioinformatics, Egan et al. (2011) identified four potential AMPK-sensitive phosphorylation sites on ULK1 (62). Furthermore, treating cells with phenformin confirmed the phosphorylation of the described site (62). Consistent with these findings, Kim et al. 2011 showed that glucose starvation (a strong stimulus for AMPK activation) activates ULK1 in cells. Whereas cellular exposure to an AMPK inhibitor prevented ULK1 activation and attenuated autophagy (**Figure 1.5**) (61).

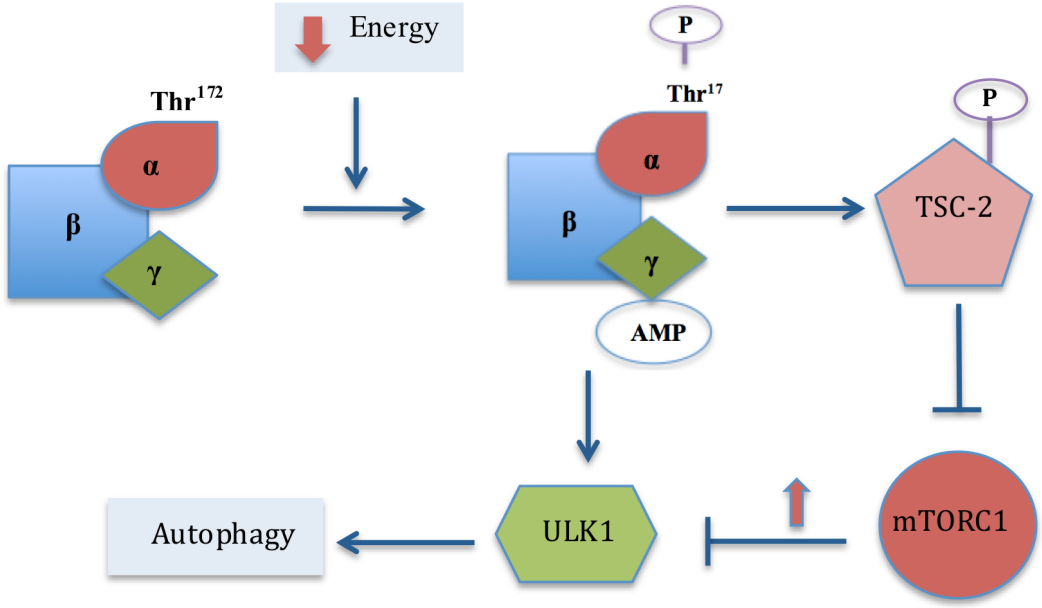

Figure 1.5. AMPK mediated autophagy. Energy starvation triggers the phosphorylation of AMPK. The increase in phosphorylated AMPK levels stimulates the activation of mTOR regulatory protein TSC-2 and increases the phosphorylation of ULK1. Increased ULK1 activity in conjunction with the inhibition of mTORC1 stimulates autophagy. ULK1: UNC-51-like kinase 1.

To further explore the cytoprotective effects of autophagy, Declèves et al. (2014) and Wei et al. (2015) investigated the mechanism by which autophagy promotes cell survival in kidney ischemia-reperfusion model (63) and cisplatin-induced acute kidney injury model (64) respectively. Intriguingly, in both models, AMPK activation inhibited mTOR pathway and induced autophagy, while chemical inhibition or knockdown of

AMPK blocked autophagy and resulted in DNA damage and cellular apoptosis (63, 64).

1.7.5. Epithelial-to-Mesenchymal Transition (EMT)

Epithelial-to-mesenchymal transition (EMT) is characterized by a defined sequence of three major events in which epithelial cells lose cell-cell adhesion (1), undergo cytoskeletal reorganization (2), and transcriptional reprogramming (3) to acquire a mesenchymal phenotype. The loss of cell-cell adhesion during EMT allows the epithelial cells to get released from the surrounding cells. Secondly, the restructuring of the cytoskeleton enables the cells to move across the extracellular matrix (ECM). These two processes in conjunction with induction of transcriptional reprogramming enables the epithelial cells to transform into mesenchymal phenotype (65). EMT is essential during embryonic period as it allows organ differentiation and development (66). EMT is systematized through cell-cell and cell-ECM interactions (66). Disturbances to the coordination between the aforementioned processes lead to the initiation of spontaneous and cell-autonomous EMT, thereby increasing the production of mesenchymal cells and disrupting the epithelial tissue, eventually perpetuating alterations in the cellular architecture (65, 66).

A prominent pathological feature of CKD is the replacement of normal renal structures by fibrotic tissues in both the glomeruli and the tubulo-interstitial space (67). Studies by Iwano et al (2002) using transgenic mice with a reporter gene on proximal tubular epithelial cells revealed that the origin of more than 35% of fibroblast-specific protein-1 (FSP1) positive fibroblast is renal proximal tubules. This study highlighted the significant impact EMT has on the development and progression of CKD (68). Using a similar cell lineage tracking technique, studies conducted by Zeisberg et al. (2008) and Li

J et al. (2009) showed that a significant proportion of interstitial fibroblasts are originated from capillary endothelia, i.e., endothelial to mesenchymal transition (EndoMT). Taking into account that endothelial cells represent a specialized type of epithelial cells, EndoMT is therefore, a form of EMT occurring in injured renal tissues (69, 70). Consistent with these findings, studies in different animal models of diabetic (71) and obstructive nephropathy (72) showed similar findings. Additionally, studies in human renal biopsies have shown that the expression of vimentin (a mesenchymal protein) and FSP1 within tubular cells correlates with the progression of renal injury in various types of kidney diseases (73, 74). Together, these studies provide substantial evidence for the involvement of EMT in the pathogenesis of CKD.

A strong correlation between AMPK inactivation and induction of EMT was reported in multiple organs including hepatic (75), cardiac (76) and renal systems (77). For instance, Lee et al. (2013) showed that activation of AMPK using metformin in human proximal tubular cells blocked ROS generation through induction of heme oxygenase-1 and an endogenous antioxidant thioredoxin, thereby suppressing EMT. Furthermore, ablation of AMPK signaling by chemical inhibition and small interference RNA-mediated silencing hindered the effects of metformin, thereby proving the beneficial effects AMPK activation has in attenuating tubulointerstitial fibrosis (78). Alternatively, knockdown of AMPKα2 in a murine model of unilateral ureteral obstruction (UUO) led to the up-regulation of pro-fibrotic factors such as β-catenin and Smad3 and concomitant induction of EMT and renal fibrosis. On the other hand, promoting AMPKα Thr172 phosphorylation in the kidney attenuated EMT in renal tubular epithelia, and mitigated renal injury induced by UUO (77). Similar findings were

also reported by Wang et al. (2016) in a rat model of acute renal ischemia-reperfusion (79). Altogether, these studies support the fact that AMPK activation could serve as a therapeutic strategy to attenuate tubulointerstitial fibrosis and EMT in the kidney.

1.7.6. Apoptosis

Apoptosis (also known as programmed cell death) is defined as a rigorously controlled form of cell death essential for healthy proliferation and development of multicellular organisms. Defective apoptosis can result in abnormal development and pathogenesis (80).

AMPK is thought to be an anti-proliferation kinase; this is due to its association with tumor suppressor genes (LKB1 and TSC2). The activation of AMPK takes place through its upstream stimulating kinase LKB1, which links the regulation of cellular metabolism to cell proliferation and growth (81). Several studies reported that LKB1 stimulates AMPK and thus serves as an AMPK kinase (AMPKK) (82, 83). In addition, studies from two independent groups Hawley et al. (2005) and Hurley et al. (2005) revealed that Ca^{2+}/ calmodulin-dependent protein kinase kinase (CaMKK) also acts as an upstream activator of AMPK (45, 46). In this regard, a variety of studies over the last decade demonstrated the anti-apoptotic effects of activated AMPK in mammalian cells (57, 82, 84).

Studies by Stefanelli et al. (1998) and Durante et al. (1999) revealed that activation of AMPK with AICAR protects rat thymocytes and fibroblasts from apoptosis induced by dexamethasone (84) and serum starvation (85) respectively. Furthermore, Kim et al. (2011) showed that specific activation of AMPK using A769662 ameliorates

palmitate-induced ER stress, tau hyperphosphorylation and apoptosis in neuroblastoma cells (86).

In consistence with the aforementioned findings, studies conducted in the renal system also support the inhibitory effects AMPK activation has on apoptosis. In a recent study by Han et al., specific deletion of the upstream kinase LKB1 in the renal distal tubules and the resultant diminution in AMPK signaling in mice induced energy deficiency marked by reduced fatty acid oxidation and glycolysis, and increased cellular apoptosis. Pharmacological activation of AMPK with A769662 in LKB1-deficient cells improved fatty acid oxidation and lessened apoptosis (87). Additionally, exposure of podocytes to high glucose levels led to programed cell death through blockade of AMPK phosphorylation, and increase in the expression of NADPH oxidase (a pro-oxidant enzyme) and phosphorylation of its downstream target p53. Restoration of AMPK phosphorylation reversed the detrimental effects of high glucose and promoted cell survival (88). Furthermore, glomeruli isolated from diabetic mice revealed a major decrease in phosphorylated AMPK levels and pharmacological activation of AMPK exerted a renoprotective effect via decreasing NADPH oxidase levels (88). Similarly, Li et al. (2015) reported reduced levels of AMPK and hypoxia-inducible factor-1 (HIF-1α) and accelerated apoptosis in rat kidneys subjected to subtotal nephrectomy (STN), a model of CKD (89). Intriguingly, activation of HIF-1α induced AMPK in the kidney and inhibited apoptosis (89). In vitro studies demonstrated that the anti-apoptotic effects of HIF-1α were lost when AMPK is inhibited in cells. Thus, it is clear that AMPK activation exerts cytoprotective effects on various organs including the kidney.

Thesis Objectives

Increased albumin excretion, commonly referred to as proteinuria, is strongly associated with the development and progression of chronic kidney disease (CKD). Proteinuria is thought to induce endoplasmic reticulum (ER) stress, consequently triggering AKT pathway and inhibiting AMP-activated kinase (AMPK). The inactivation of AMPK was found to trigger mTOR (mammalian target of Rapamycin) pathway, subsequently inhibiting autophagy and inducing epithelial-to-mesenchymal transition (EMT), eventually accelerating renal cell apoptosis and contributing to CKD. AMPK activation may potentially reverse the aforementioned pathways, and therefore, may protect against CKD.

The objectives of this study are:

1) To standardize and characterize an *in vitro* model of albumin-induced ER stress and renal cell injury,
2) To investigate the effects of metformin (an AMPK activator) on ER stress, AKT, mTOR, EMT, autophagy and apoptosis in the established *in vitro* model of albumin-induced renal cell injury, and
3) To dissect the AMPK- and non-AMPK mediated effects of metformin in the established *in vitro* model using an AMPK inhibitor Compound C.

Chapter 2: Methods

2.1. CELL CULTURE

Normal Rat kidney cells (NRK-52E; Health Protection Agency, UK) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies, UK) complemented with 5% fetal bovine serum (FBS), 1% L-Glutamine and 1% Penicillin/Streptomycin and incubated at 37°C in 5% CO_2 atmosphere. Trypan blue exclusion assay was performed regularly to monitor the viability of cells.

2.2. STANDARDIZATION OF AN I*N VITRO* MODEL OF ALBUMIN-INDUCED RENAL CELL INJURY

NRK-52E cells (a rat renal proximal tubule cell line) grown to 60% confluency were divided into two main groups based on the FBS concentration (1% FBS vs. 5% FBS); each of the groups were further divided into six sub-groups based on the albumin treatment concentration (as shown in **Figure 2.1**):

1. Control (Vehicle-treated)
2. 1 mg/ml Albumin treated
3. 5 mg/ml Albumin treated
4. 10 mg/ml Albumin treated
5. 15 mg/ml Albumin treated
6. 30 mg/ml Albumin treated

After exposure of cells to albumin for 48, 72 and 120 hours, the following parameters were assessed as described in the **Table 2.1**.

Table 2.1.

Parameters assessed in NRK-52E cells following 48 and 72 h albumin treatment

Parameter assessed	**Protein measured**	**Time point**
AMPK pathway	Phospho-AMPK and total-AMPK	
mTOR pathway	Phospho-4EBP-1 and Phospho-P70S6K	48 hours
AKT pathway	Phospho-AKT and total-AKT	
ER Stress	GRP78 and CHOP	
Epithelial-to-Mesenchymal	α-SMA and E-cadherin	
Transition (EMT)		72 hours
Autophagy	Phospho-ULK1 and LC3-II	
Apoptosis	Caspase 12 and Caspase 3	

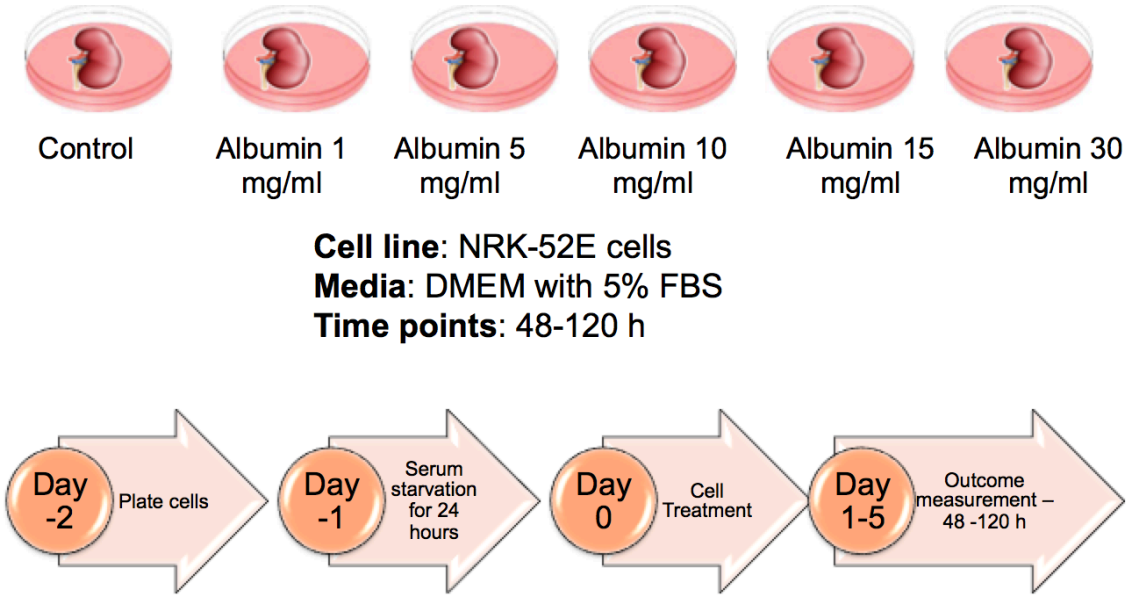

Figure 2.1. Experimental design for standardization of an *in vitro* model of albumin-induced renal cell injury using rat renal proximal tubular (NRK-52E) cells.

2.3. EVALUATION OF THE EFFECTS OF METFORMIN AGAINST ALBUMIN-INDUCED RENAL INJURY

NRK-52E cells were grown to 60% confluency and divided into six treatment groups:

1. Control (Vehicle-treated)
2. 1 mM Metformin treated
3. 10 mg/ml Albumin treated
4. 10 mg/ml Albumin +1 mM Metformin treated
5. 15 mg/ml Albumin treated
6. 15 mg/ml Albumin +1 mM Metformin treated

After exposing the cells to metformin for 48 and 72 hours, the following parameters were assessed as described in the **Table 2.2**.

Table 2.2.

Parameters assessed in NRK-52E cells following 48 and 72 h albumin and metformin co-treatment

Parameter assessed	**Protein measured**	**Time point**
AMPK pathway	Phospho-AMPK and total-AMPK	48 hours
mTOR pathway	Phospho-4EBP-1 and Phospho-P70S6K	
AKT pathway	Phospho-AKT and total-AKT	
ER Stress	GRP78 and CHOP	72 hours
Epithelial-to-Mesenchymal Transition (EMT)	α-SMA and E-cadherin	
Autophagy	Phospho-ULK1 and LC3-II	
Apoptosis	Phospho-SAPK/JNK, Caspase 12 and Caspase 3	

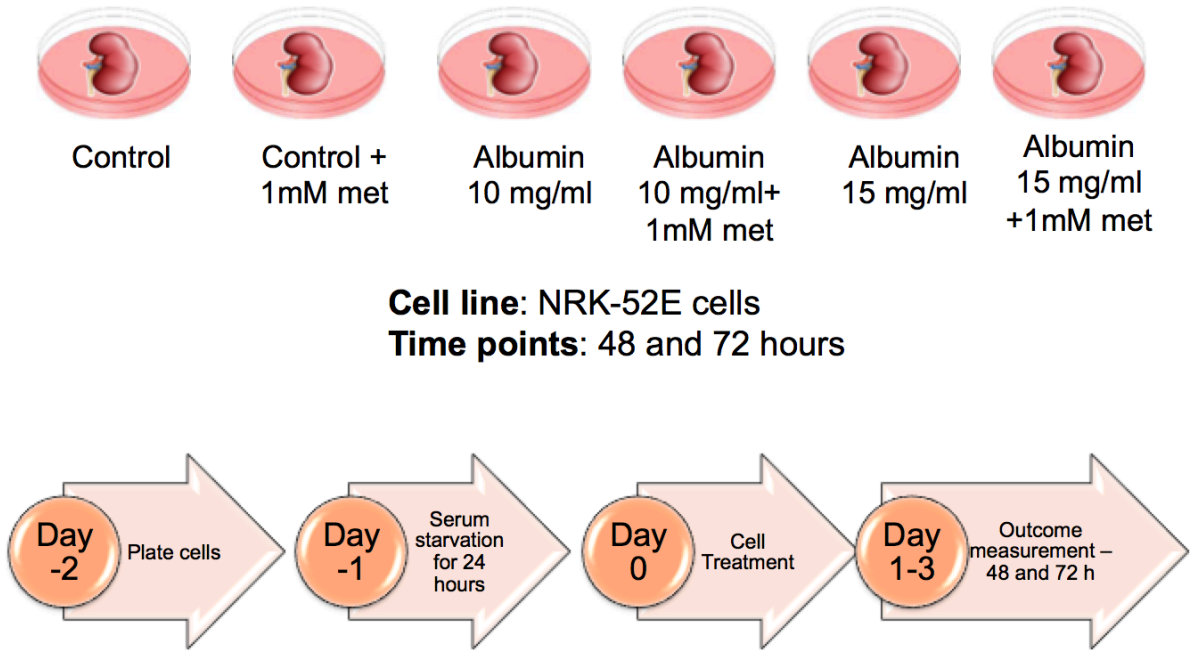

Figure 2.2. Experimental design for Evaluation of the effects of Metformin against albumin-induced renal injury.

2.4. IDENTIFICATION OF THE AMPK-MEDIATED AND NON-AMPK MEDIATED EFFECTS OF METFORMIN AGAINST ALBUMIN-INDUCED RENAL INJURY

2.4.1. Concentration-Response Studies for Compound C

NRK-52E cells were grown to 60% confluency and divided into treatment groups as follows:

1. Control
2. Metformin (1 mM) treated

3. Compound C (0.05, 0.1, 0.5, 1, 3, 5, 10, 20 or 40 μM) treated
4. Metformin (1 mM) + Compound C (0.05, 0.1, 0.5, 1, 3, 5, 10, 20 or 40 μM) treated

After exposing the cells to metformin for 48 and 72 hours, the following parameters were assessed as described in the **Table 2.3**.

Table 2.3.

Parameters assessed in NRK-52E cells following 48 h of compound C treatment

Parameter assessed	Protein measured	Time point
AMPK pathway	Phospho-AMPK and total-AMPK	48 hours
mTOR pathway	Phospho-4EBP-1	

2.4.2. Effects of Compound C-mediated AMPK inhibition on metformin-mediated outcomes

NRK-52E cells were grown to 60% confluency and divided into eight treatment groups as follows.

1. Control
2. Albumin (10 mg/ml)
3. Metformin (1 mM)
4. Albumin (10 mg/ml) + Metformin (1 mM)
5. Compound C (0.5 μM)

6. Albumin (10 mg/ml) + Compound C (0.5 μM)
7. Metformin (1 mM) + Compound C (0.5 μM)
8. Albumin (10 mg/ml) + Metformin (1 mM) + Compound C (0.5 μM)

After exposing the cells to metformin for 48 and 72 hours, the following parameters were assessed as described in the **Table 2.4**.

Table 2.4.

Parameters assessed in NRK-52E cells following 48 and 72 h albumin, metformin and compound C treatment

Parameter assessed	**Protein measured**	**Time point**
AMPK pathway	Phospho-AMPK and total-AMPK	48 hours
mTOR pathway	Phospho-4EBP-1	
ER Stress	GRP78 and CHOP	
Epithelial-to-Mesenchymal Transition (EMT)	α-SMA	72 hours
Autophagy	LC3-II	
Apoptosis	Caspase 12 and Caspase 3	

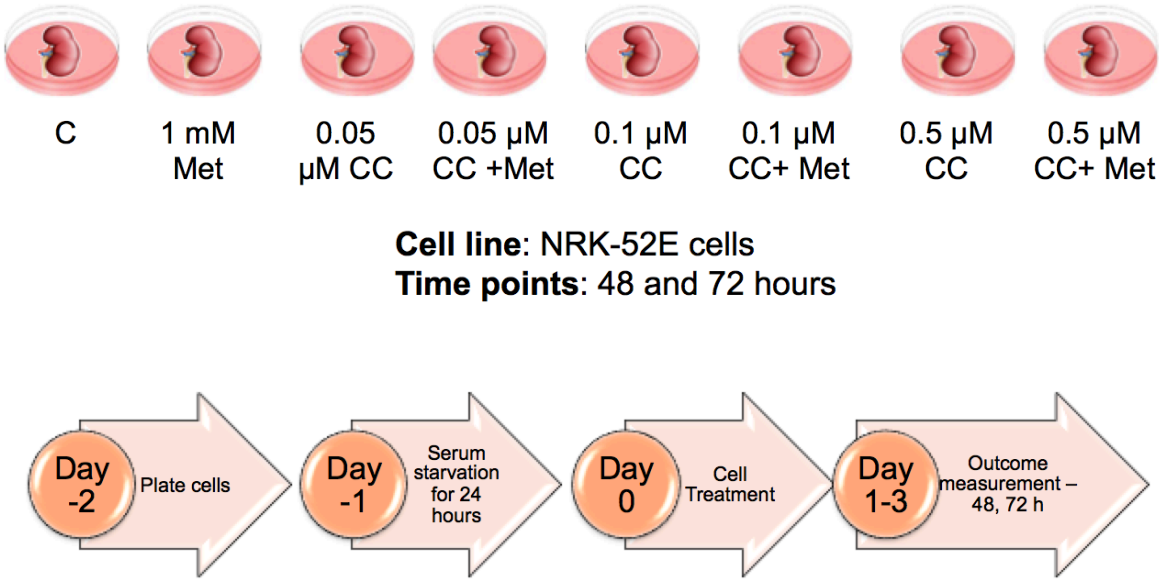

Figure 2.3. Experimental design to identify the AMPK mediated effects of metformin using Compound C.

2.5. GENERAL METHODS

2.5.1. Western Blotting

Western blotting was used to determine the expression of proteins of interest following drug treatments. Cells were seeded onto 6-well plates, and treated as per treatment protocol. At the endpoint, cells were lysed in 60 mM Tris pH 6.8 and 2% SDS lysis buffer and collected. Samples were sonicated for 15 seconds at 5 second intervals and centrifuged at 16,000xg for 15 minutes at 4°C. Protein concentrations were quantified using Bicinchonic acid (BCA) Protein Assay Kit (Pierce, USA) as per manufacturer's protocol. 12% and 15% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) were loaded with 40-50 µg of proteins. Electrophoresis conditions were set

at 60 volts for 20 minutes followed by 150 volts for 90 minutes. Thereafter, proteins were transferred from the gel to polyvinylidene fluoride (PVDF) membranes at 100 volts for 90 minutes via wet transfer technique.

Blockade of non-specific binding was ensured by incubating PVDF membranes with either 5% skimmed milk or bovine serum albumin (BSA) diluted in Tris-buffered saline (TBS) - depending on the protein of interest - for 1 hour at room temperature. Primary antibodies diluted in TBS containing 3% skimmed milk or BSA at specific concentrations listed in the **Table 2.5** were added to the membrane and incubated overnight at 4°C. The next day, membranes were washed using 0.1% Tween-20 containing TBS (TBS-T) for 3 times at 10 minute intervals. Secondary antibodies of either horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:10,000; Abcam, UK) or HRP-conjugated goat anti-rabbit IgG (1: 20,000; Abcam, UK) (diluted in TBS) were added to the membrane for 1 hour at room temperature. To visualize the protein bands, Optiblot ECL detection kit (Abcam, UK) was added to the membranes, and the images were captured using FluroChem M imaging system (Protein Simple, USA). The protein bands were analyzed using NIH ImageJ 1.48V software, and the densitometry values were normalized to beta-actin (a loading control) and expressed as folds of control (90).

Table 2.5.

Primary and secondary antibodies used in the study

Protein	**Primary Antibody**	**Secondary Antibody**	**Source**
Phospho-AMPKα	Rabbit Monoclonal Antibody (Thr172) (D79.5E), 1:1000	Goat anti-rabbit (GAR): HRP conjugated	Cell Signaling Technology, Netherlands
AMPKα	Rabbit Monoclonal Antibody, 1:1000	Goat anti-rabbit (GAR): HRP conjugated	Cell Signaling Technology, Netherlands
AKT1	Rabbit Monoclonal antibody (phospho S473) [EP2109Y], 1:1000	Goat anti-rabbit (GAR): HRP conjugated	Abcam, UK
Anti-AKT1	Rabbit Monoclonal antibody [Y89], 1:1000	Goat anti-rabbit (GAR): HRP conjugated	Abcam, UK
Phospho-p70 S6 Kinase	Rabbit Monoclonal antibody, (Thr389) (108D2), 1:1000	Goat anti-rabbit (GAR): HRP conjugated	Cell Signaling Technology, Netherlands
Phospho-4E-BP1	Rabbit Monoclonal antibody,	Goat anti-rabbit (GAR): HRP	Cell Signaling Technology,

	(Thr37/46) (236B4), 1:1000	conjugated	Netherlands
Phospho-eIF2α	Rabbit Monoclonal antibody, (Ser51) (D9G8) XP®, 1:1000	Goat anti-rabbit (GAR): HRP conjugated	Cell Signaling Technology, Netherlands
GADD153 (CHOP)	Rabbit Monoclonal antibody (R-20), 1:1000	Goat anti-rabbit (GAR): HRP conjugated	Santa Cruz, Germany
GRP78/BiP	Rabbit Polyclonal antibody, 1:1000	Goat anti-rabbit (GAR): HRP conjugated	Abcam, UK
Alpha-Smooth Muscle Actin	Mouse monoclonal antibody, 1:500	Goat anti-mouse (GAM): HRP conjugated	Abcam, UK
Phospho-ULK1	Rabbit Monoclonal antibody (Ser555) (D1H4), 1:1000	Goat anti-rabbit (GAR): HRP conjugated	Cell Signaling Technology, Netherlands
ULK1	Rabbit Monoclonal antibody, (D8H5), 1:1000	Goat anti-rabbit (GAR): HRP conjugated	Cell Signaling Technology, Netherlands
LC3B	Rabbit Monoclonal antibody, 1:1000	Goat anti-rabbit (GAR): HRP	Cell Signaling Technology,

		conjugated	Netherlands
Caspase-12	Rabbit Monoclonal antibody, 1:1000	Goat anti-rabbit (GAR): HRP conjugated	Abcam, UK
Caspase-3	Rabbit Monoclonal antibody, 1:1000	Goat anti-rabbit (GAR): HRP conjugated	Abcam, UK
β-Actin	Rabbit Monoclonal antibody (13E5), 1:1000	Goat anti-rabbit (GAR): HRP conjugated	Cell Signaling Technology, Netherlands

2.5.2. Cell Viability Assay (MTT Assay)

MTT assay was used to quantify cell viability following albumin treatment. The assay is based on the ability of mitochondrial dehydrogenases to reduce the yellow colored MTT reagent (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to insoluble purple formazan crystals. The formed crystals were then solubilized in inorganic solvent and the intensity of the produced color was quantified using spectrophotometry. Following 72 hours of treatment, the old media was removed and 100 µl of serum free media containing 0.5 mg/ml MTT reagent (Sigma Aldrich, M2128) was added to the plate and incubated at 37°C for 4 hours. Thereafter, the media was carefully aspirated and 50 µl of dimethylsulfoxide (DMSO) was added to solubilize the formazan crystals. Using a microplate reader, absorbance was then measured at 570 nm; the control

readings were set to 100% and treatment readings were expressed as a percentage of the control (91).

2.5.3. Statistical Analysis

A minimum of three independent experimental samples were obtained and tested for statistical significance. Values were expressed as Mean ± SEM. The data sets were tested for Gaussian distribution using normality and equal variance test. One-way ANOVA followed by post-hoc Tukey test was used for comparison of multiple groups. A P value of less than 0.05 was considered to be statistically significant.

Chapter 3: Results

3.1. STANDARDIZATION OF AN *IN VITRO* MODEL OF ALBUMIN-INDUCED RENAL CELL INJURY

NRK-52E cells were exposed to various concentrations of albumin ranging from 1 to 30 mg/ml in culture media with or without 5% fetal bovine serum (FBS) for 24 hours to 120 hours. Untreated cells were used as control. Cell injury was quantified using MTT assay.

3.1.1. Effect of Albumin on Renal Cell Injury Following 24 hours of Treatment

No significant differences in cell injury were observed in albumin-treated groups following 24 hours of exposure in serum-free media or the 5% FBS containing media (**Figure 3.1**).

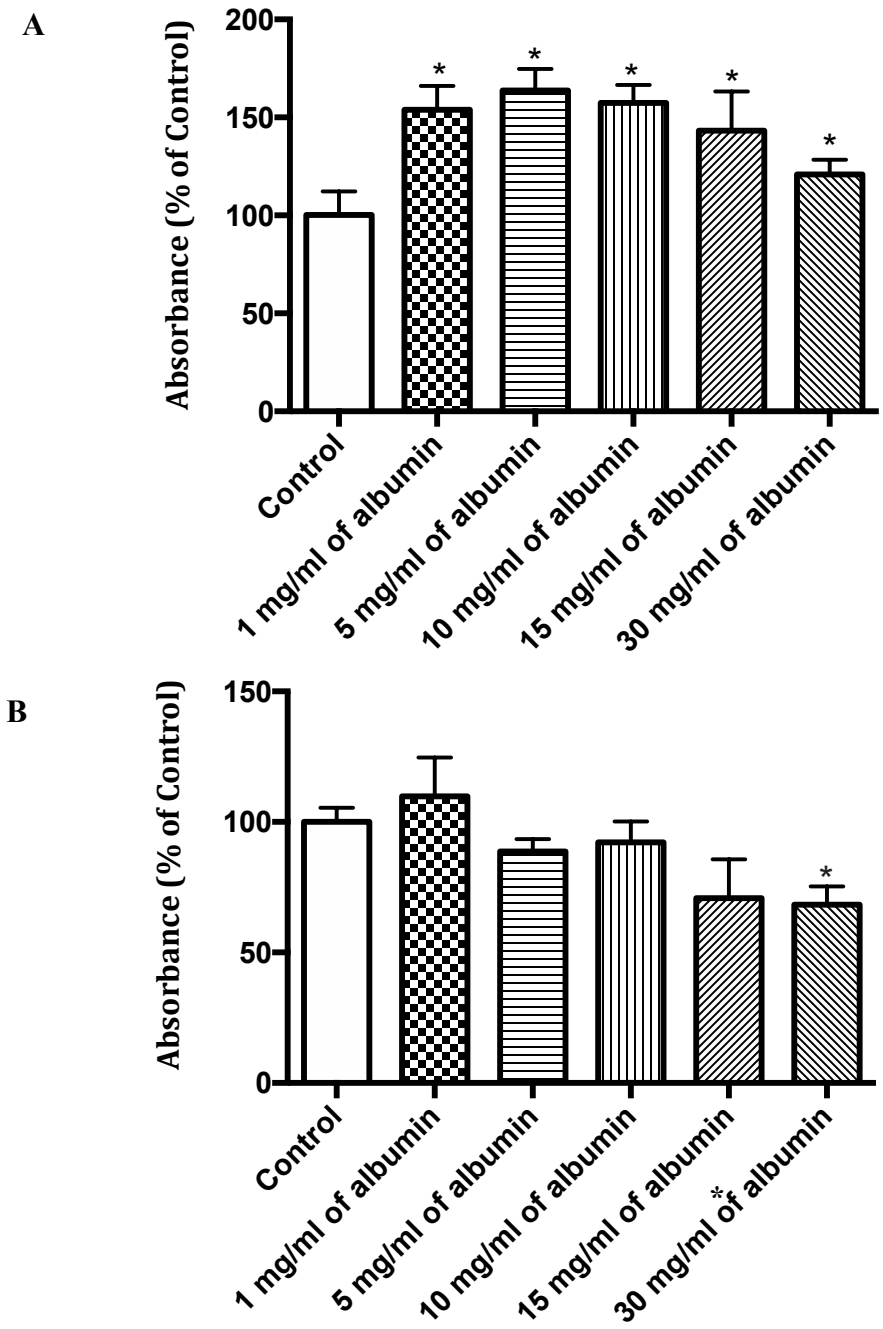

Figure 3.1. Effect of various concentrations of albumin on the viability of NRK-52E cells following 24 hours of treatment in serum-free media (A) and in media containing 5% FBS (B). Values were expressed as Mean ± SEM; n = 6. * $p < 0.05$ vs. Control.

3.1.2. Effect of Albumin on Renal Cell Injury Following 48 hours of Treatment

Under serum-free conditions, treatment with albumin had no prominent effect on the viability of NRK-52E cells (**Figure 3.2A**). Whereas, cells exposed to albumin in media containing 5% FBS showed a progressive decline in cell viability (**Figure 3.2B**). Furthermore, a statistically significant ($P < 0.05$) decrease in cell viability was noted in cells treated with 30 mg/ml of albumin as compared to the control.

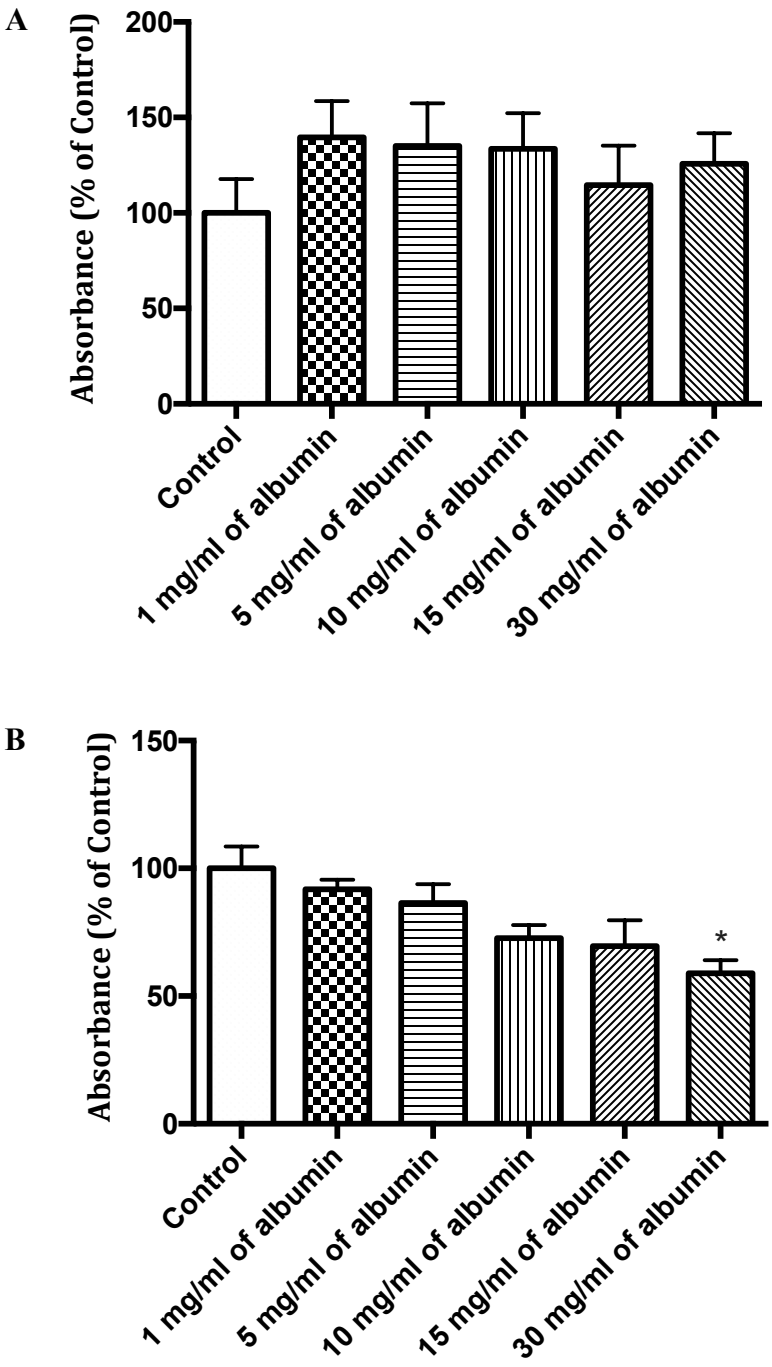

Figure 3.2. Effect of various concentrations of albumin on the viability of NRK-52E cells following 48 hours of treatment in serum-free media (A) and in media containing 5% FBS (B). Values were expressed as Mean ± SEM; n = 6. *P < 0.05 vs. Control.

3.1.3. Effect of Albumin on Cell Injury Following 72 hours of Treatment

Intriguingly, NRK-52E cells exposed to albumin in serum-free or 5% FBS containing media for 72 hours showed no differences in cell viability (**Figure 3.3**).

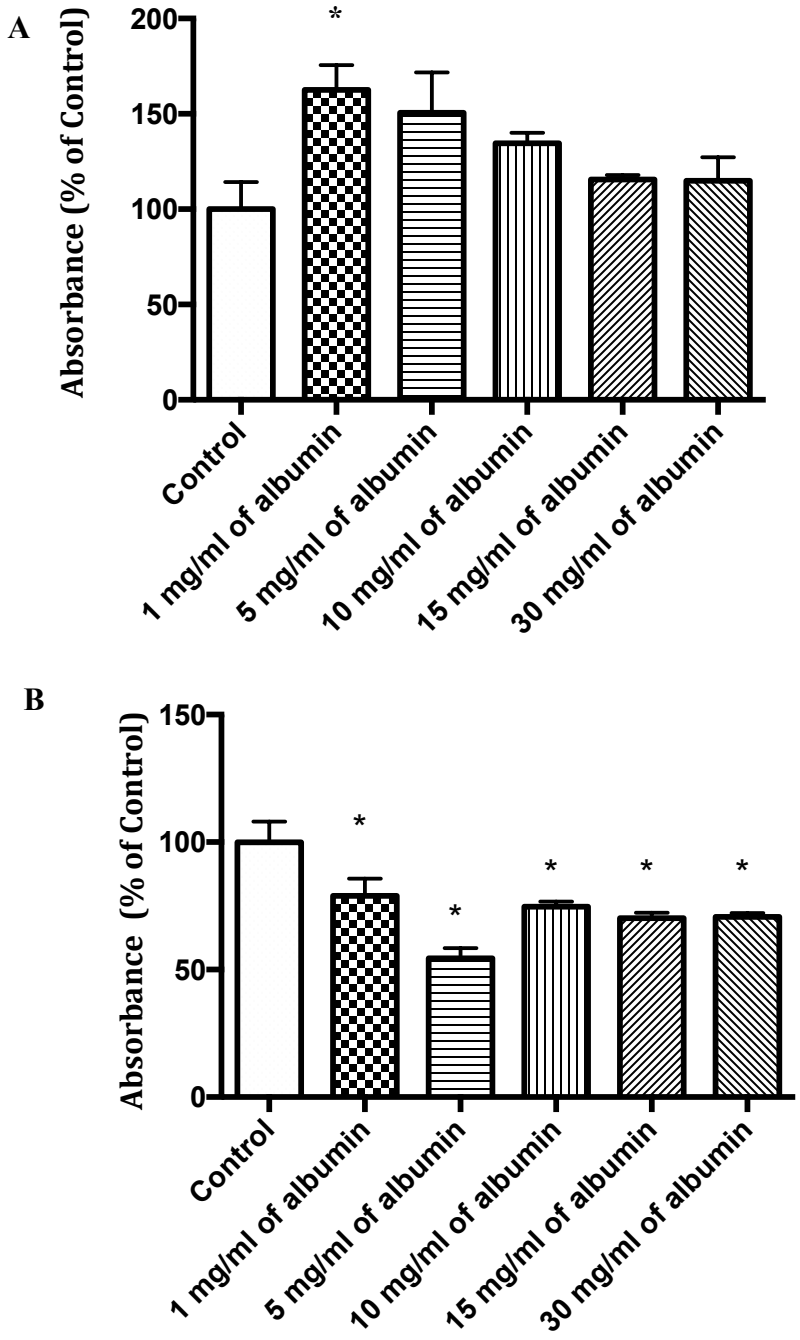

Figure 3.3. Effect of various concentrations of albumin on the viability of NRK-52E cells following 72 hours of treatment in serum-free media (A) and in media containing 5% FBS (B). Values were expressed as Mean ± SEM; n = 6. *P < 0.05 vs. Control.

3.1.4. Effect of Albumin on Cell Injury Following 120 hours of Treatment

Consistent with 24, 48 and 72 hours' albumin treatment groups under serum-free conditions, cells exposed to albumin for 120 hours in serum-free media showed no differences in cell viability compared to control (**Figure 3.4A**). In contrast, cells exposed to albumin in 5% FBS containing media showed statistically significant decreases in cell viability in all albumin-treated groups as compared to the control (**Figure 3.4B**).

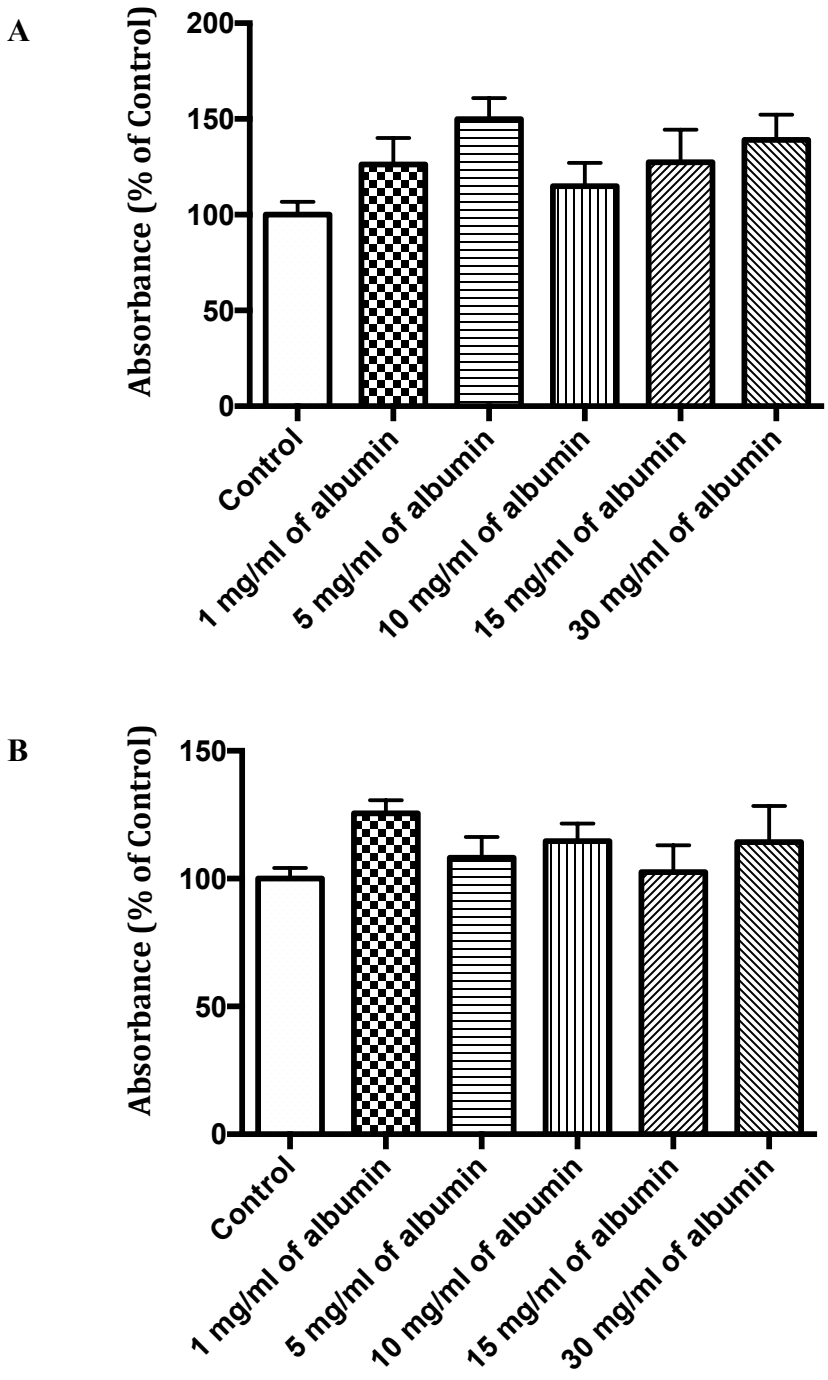

Figure 3.4. Effect of various concentrations of albumin on the viability of NRK-52E cells following 120 hours of treatment in serum-free media (A) and in media containing 5% FBS (B). Values were expressed as Mean ± SEM; n = 6. *P < 0.05 vs. Control.

3.1.5. Effect of Albumin on ER Stress Following 72 hours of Treatment

Based on the findings from MTT assay, albumin treatments in media containing 5% FBS was found to yield more consistent results as compared to those conducted in serum-free conditions. Therefore, all cell treatments were performed in media containing 5% FBS.

Western blotting followed by densitometry was used to quantitate the expression of ER stress response proteins GRP78 and CHOP in NRK-52E cells subjected to 1 to 30 mg/ml albumin in 5% FBS containing media for 72 hours. Our results revealed that the expression of GRP78 and CHOP was significantly increased ($p < 0.05$) in all albumin-treated groups as compared to the control (**Figure 3.5**).

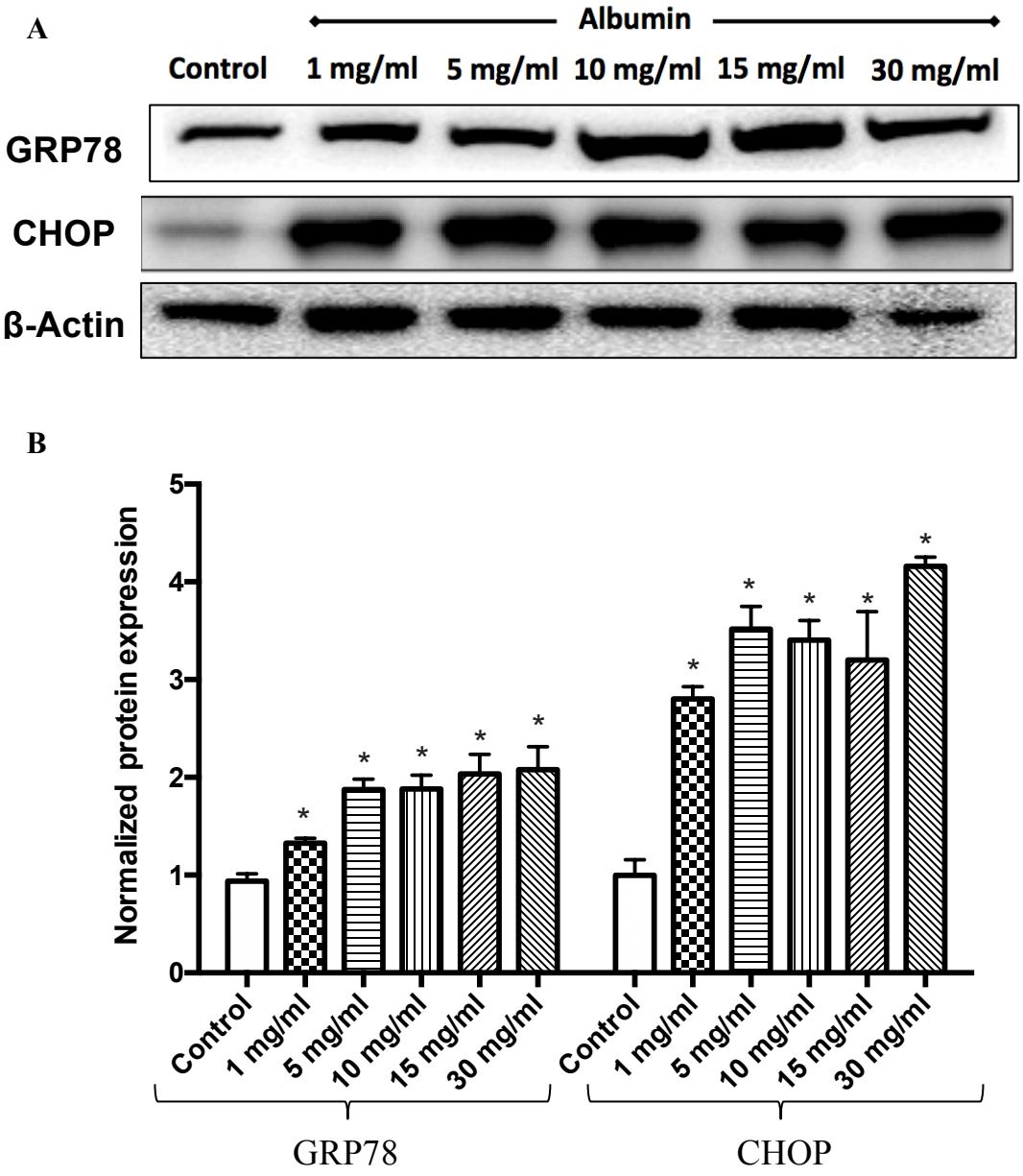

Figure 3.5. Effect of albumin on the expression of GRP78 and CHOP in NRK-52E cells following 72 hours of treatment. Upper panel (A) showing a representative western blot and the lower panel (B) showing the densitometry measurements normalized to β-actin. Values were expressed as Mean ± SEM; n = 3; *P < 0.05 vs. Control.

3.1.6. Effect of Albumin on EMT and Autophagy Following 72 hours of Treatment

To examine the effect of albumin on EMT and autophagy, the expression of α-SMA and LC3-II expression was measured in NRK-52E cells subjected to various concentrations of albumin (1 to 30 mg/ml) in 5% FBS containing media for 72 hours. α-SMA expression increased in a concentration-dependent manner with more than 2-fold increase in cells treated with 1 mg/ml of albumin and 6-fold increase in cells treated with 30 mg/ml of albumin (**Figure 3.6**). Intriguingly, the levels of LC3-II were unchanged in renal cells exposed to low concentrations of albumin (1, 5 and 10 mg/ml). Nonetheless, exposure of cells to 15 mg/ml and 30 mg/ml of albumin caused a significant increase in LC3-II expression as compared to the control.

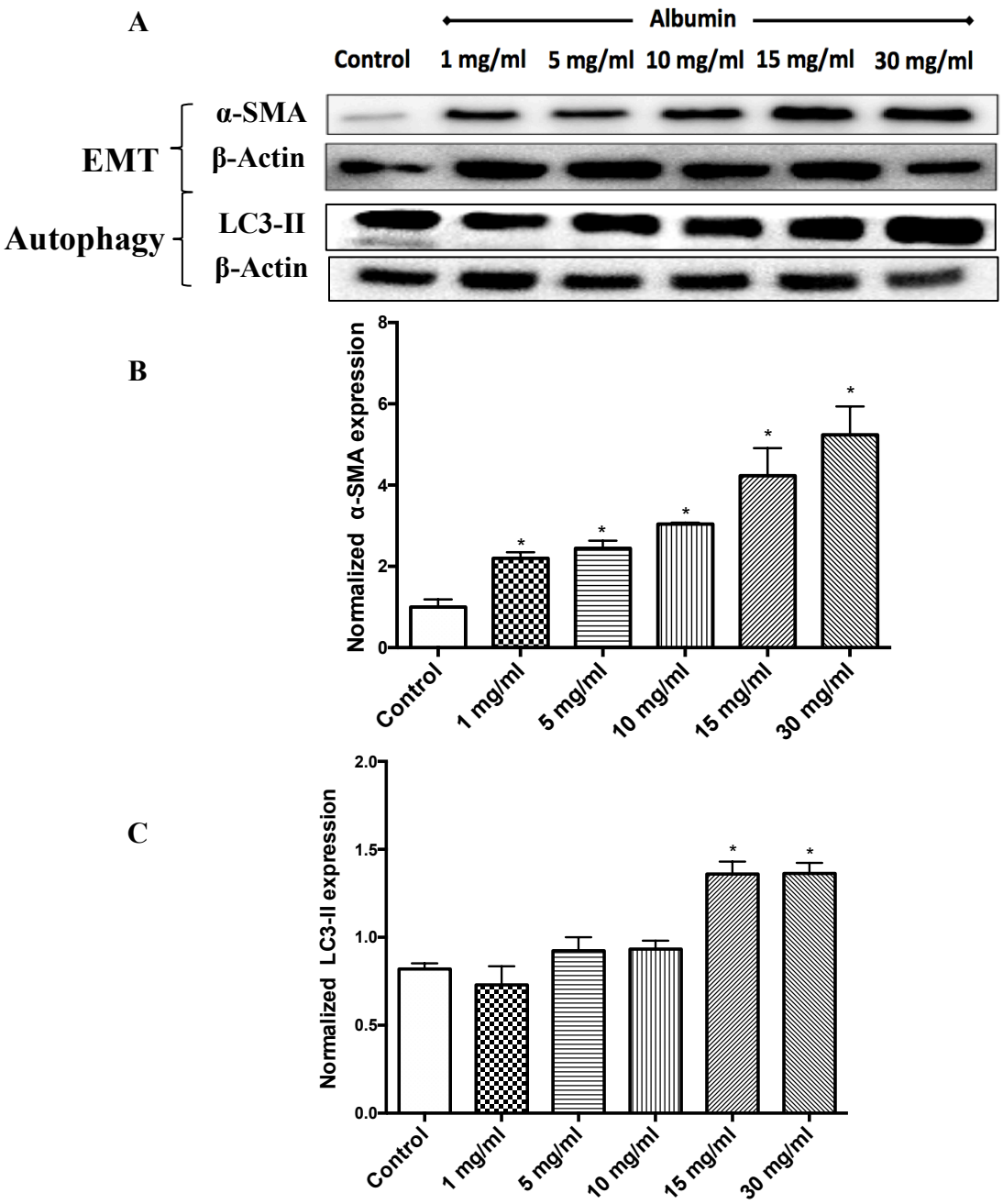

Figure 3.6. Effect of albumin on EMT and autophagy in NRK-52E cells following 72 hours of treatment. Upper panel (A) showing a representative western blot, the middle (B) and the lower (C) panels showing the densitometry measurements normalized to β-actin. Values were expressed as Mean ± SEM; n = 3; *P < 0.05 vs. Control.

3.2. EFFECT OF METFORMIN ON SIGNALING PATHWAYS ALTERED BY ALBUMIN IN RENAL CELLS

3.2.1. Effect of Metformin on AMPK Signaling Pathway in Albumin-Induced Renal Cell Injury

Western blotting followed by densitometry was used to quantify the effect of metformin treatment on AMPK phosphorylation in NRK-52E cells exposed to albumin (10 mg/ml and 15 mg/ml) in 5% FBS containing media for 48 hours. We noted a significant decrease by about 0.5-fold in phospho-AMPK levels with 10 and 15 mg/ml of albumin treatment as compared to the control. Metformin treatment was able to prevent the reduction in phospho-AMPK expression caused by albumin (**Figure 3.7**).

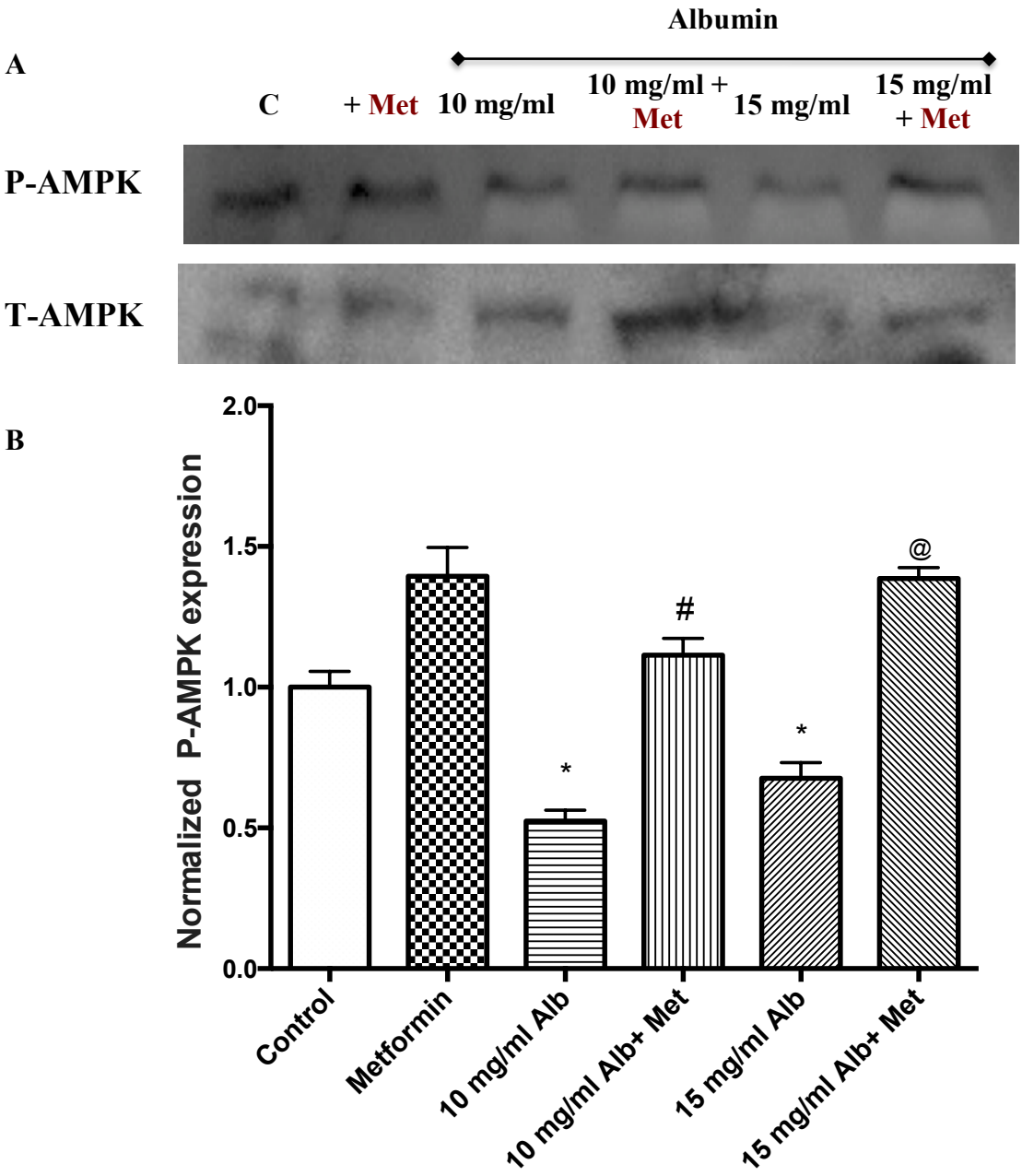

Figure 3.7. Effect of metformin on phospho-AMPK (Thr172) expression in NRK-52E cells following 48 hours of albumin treatment. Upper panel (A) showing a representative western blot and the lower panel (B) showing the densitometry measurements normalized to β-actin. Values were expressed as Mean ± SEM; n = 3; *P<0.05 vs. untreated control (C), # and @ P<0.05 vs. 10 and 15 mg/ml Albumin treated groups respectively.

3.2.2. Effect of Metformin on AKT Signaling Pathway in Albumin-Induced Renal Cell Injury

To determine the effect 1 mM of metformin has on phospho-AKT expression in our model, western blotting followed by densitometry was used. Subsequent to 48 hours of albumin treatment phospho-AKT levels significantly increased by 1.5-fold as compared to the control. On the other hand, when cells were co-treated with metformin and albumin, the phospho-AKT expression was reduced to 0.5-fold as compared to albumin treated groups (**Figure 3.8**).

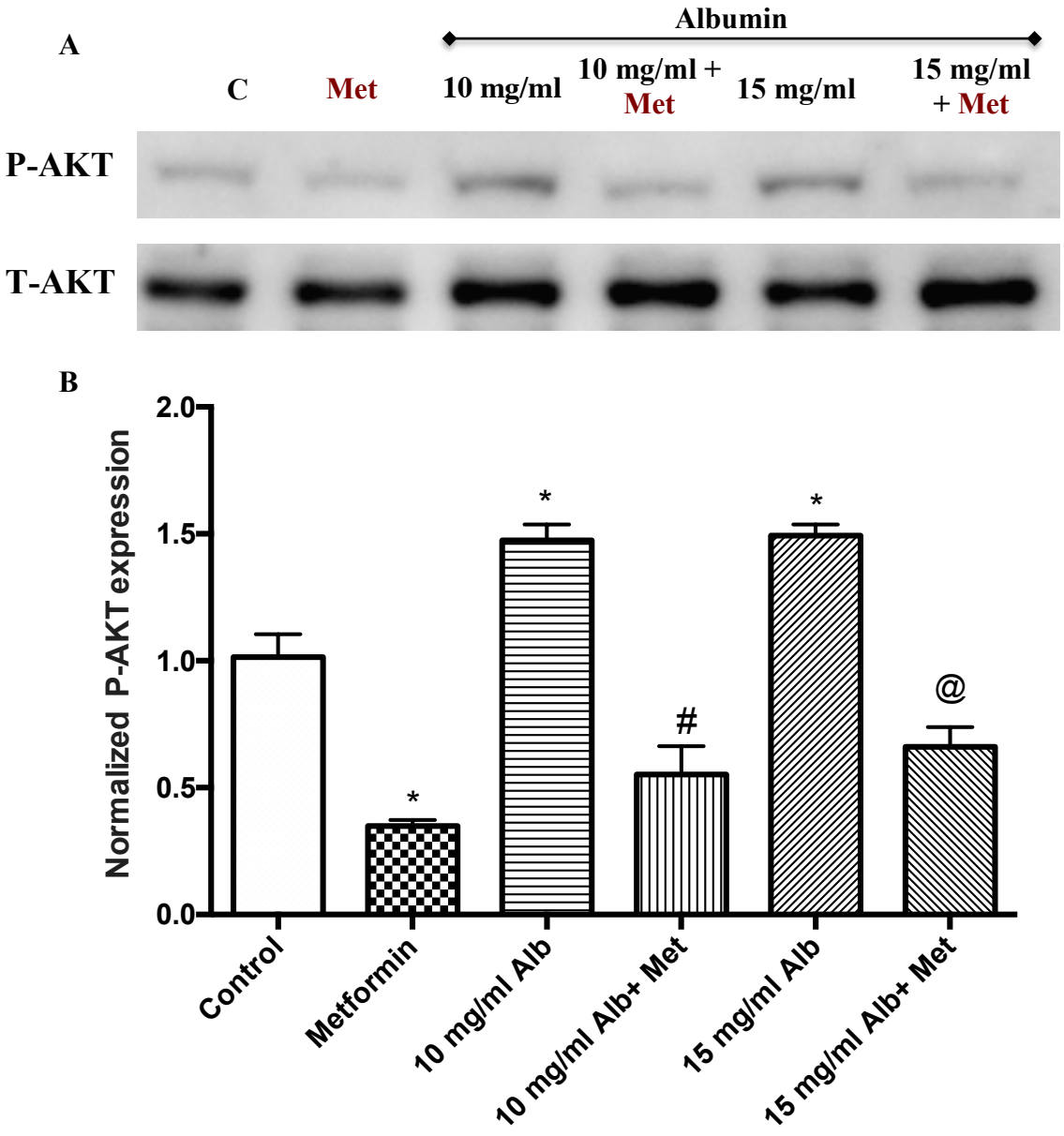

Figure 3.8. Effect of metformin on phospho-AKT (Ser129) expression in NRK-52E cells following 48 hours of albumin treatment. Upper panel (A) showing a representative western blot and the lower panel (B) showing the densitometry measurements normalized to β-actin. Values were expressed as Mean ± SEM; n = 3; *P<0.05 vs. untreated control (C), # and @ P<0.05 Vs. 10 and 15 mg/ml Albumin treated groups respectively.

3.2.3. Effect of Metformin on mTOR Signaling Pathway in Albumin-Induced Renal Cell Injury

Western blotting followed by densitometry was employed to measure changes in the expression of downstream targets mTOR pathway - phospho-4EBP-1 and phospho-p70S6K - following the addition of 1 mM of metformin in the *in vitro* model. Both phospho-4EBP-1 and phospho-p70S6K were found to significantly increase subsequent to cellular treatment with 10 mg/ml of albumin. Co-treatment with 10 mg/ml of albumin and 1mM of metformin significantly decreased phospho-4EBP-1 (**Figure 3.9**) and phospho-p70S6K (**Figure 3.10**) to 0.8-fold and 1.2-fold respectively as compared to albumin treated groups.

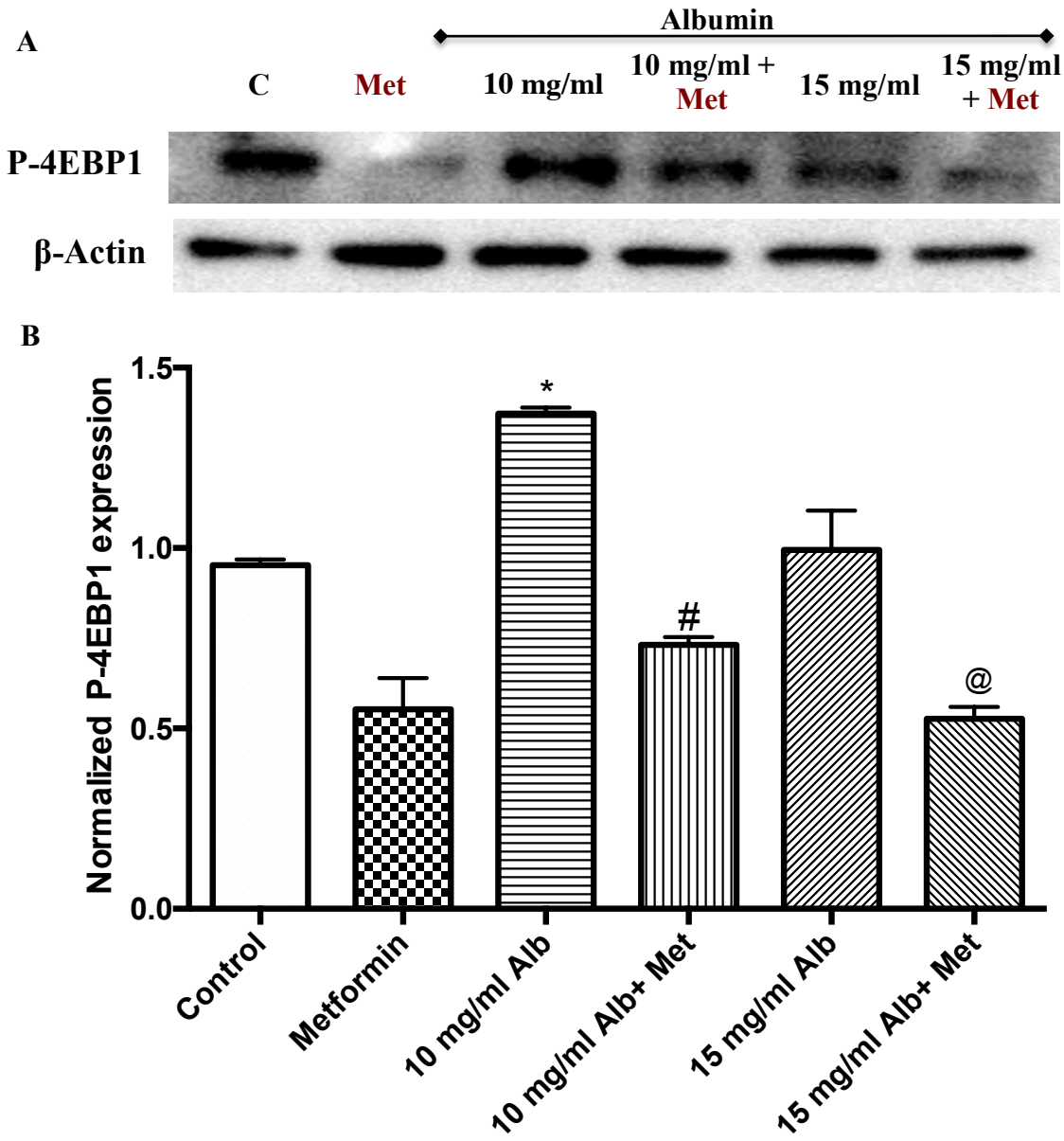

Figure 3.9. Effect of metformin on phospho-4EBP1 (Thr37/46) expression in NRK-52E cells following 48 hours of albumin treatment. Upper panel (A) showing a representative western blot and the lower panel (B) showing the densitometry measurements normalized to β-actin. Values were expressed as Mean ± SEM; n = 3; *P<0.05 vs. untreated control (C), # and @ P<0.05 vs. 10 and 15 mg/ml Albumin treated groups respectively.

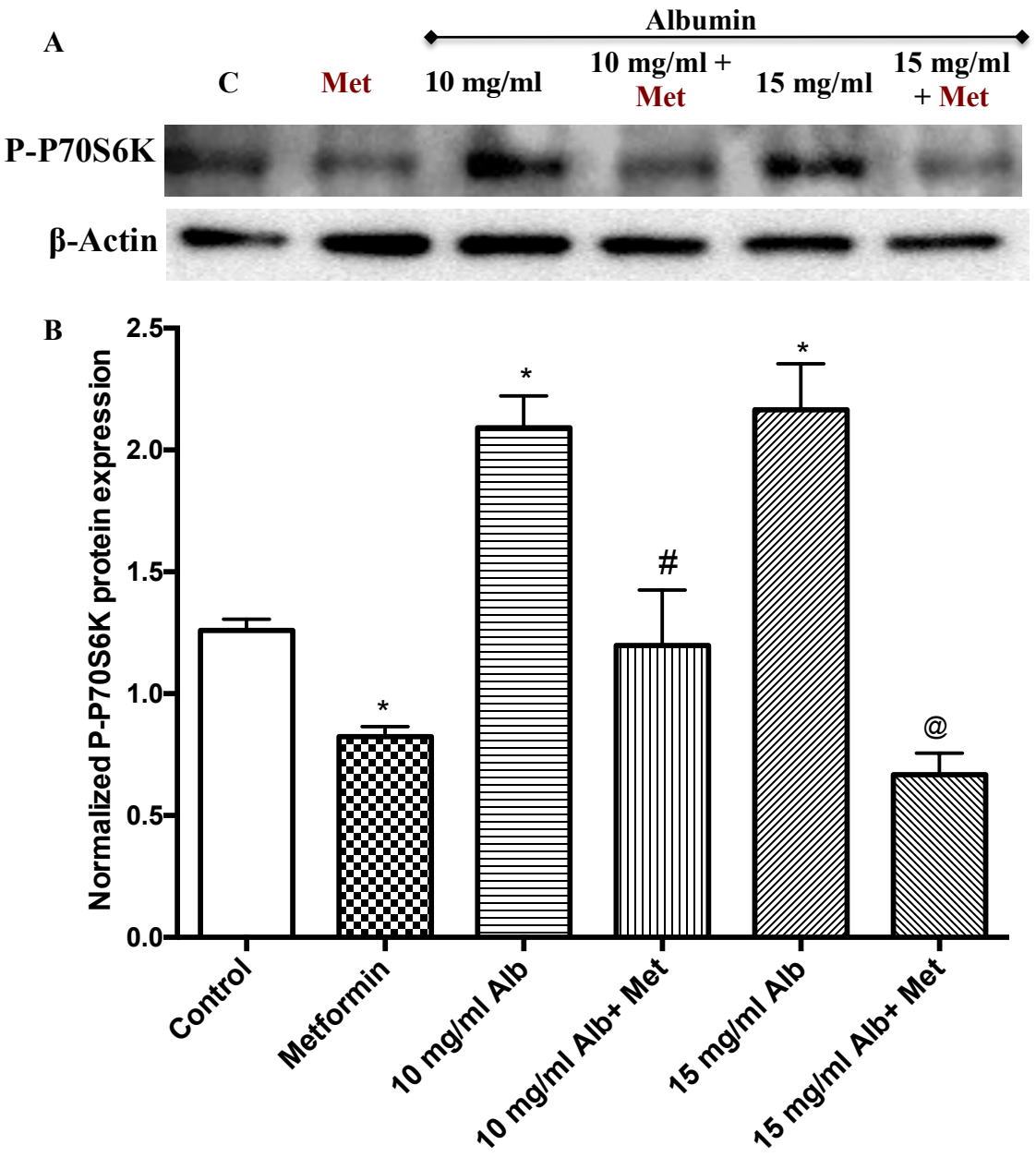

Figure 3.10. Effect of metformin on phospho-P70S6K (Thr389) expression in NRK-52E cells following 48 hours of albumin treatment. Upper panel (A) showing a representative western blot and the lower panel (B) showing the densitometry measurements normalized to β-actin. Values were expressed as Mean ± SEM; n = 3; *P<0.05 vs. untreated control (C), # and @ P<0.05 vs. 10 and 15 mg/ml Albumin treated groups respectively.

3.2.4. Effect of Metformin on Albumin-Induced ER Stress in Renal Cells

To measure metformin-mediated alterations on albumin-induced ER stress in renal cells, western blotting was employed. The expression of ER stress markers GRP78, phospho-eIF2α and CHOP was quantified in NRK-52E cells following treatment with 10 mg/ml and 15 mg/ml of albumin in the presence or absence of metformin for 72 hours. Treatments with either 10 mg/ml of albumin or 1 mM of metformin caused a significant induction in GRP78 expression. Nonetheless, cells subjected to albumin and metformin co-treatment showed profound increase in GRP78 levels as compared to albumin only treatment. On the other hand, the induction of other two ER stress markers - phospho-eIF2α and CHOP - by 10 mg/ml albumin treatment was normalized following co-treatment with metformin to 0.8-fold and 1.2-fold respectively as compared to control (**Figure 3.11**). Intriguingly, metformin treatment was unable to further increase GRP78 expression and attenuate the induction of phospho-eIF2α and CHOP in 15 mg/ml albumin treated group.

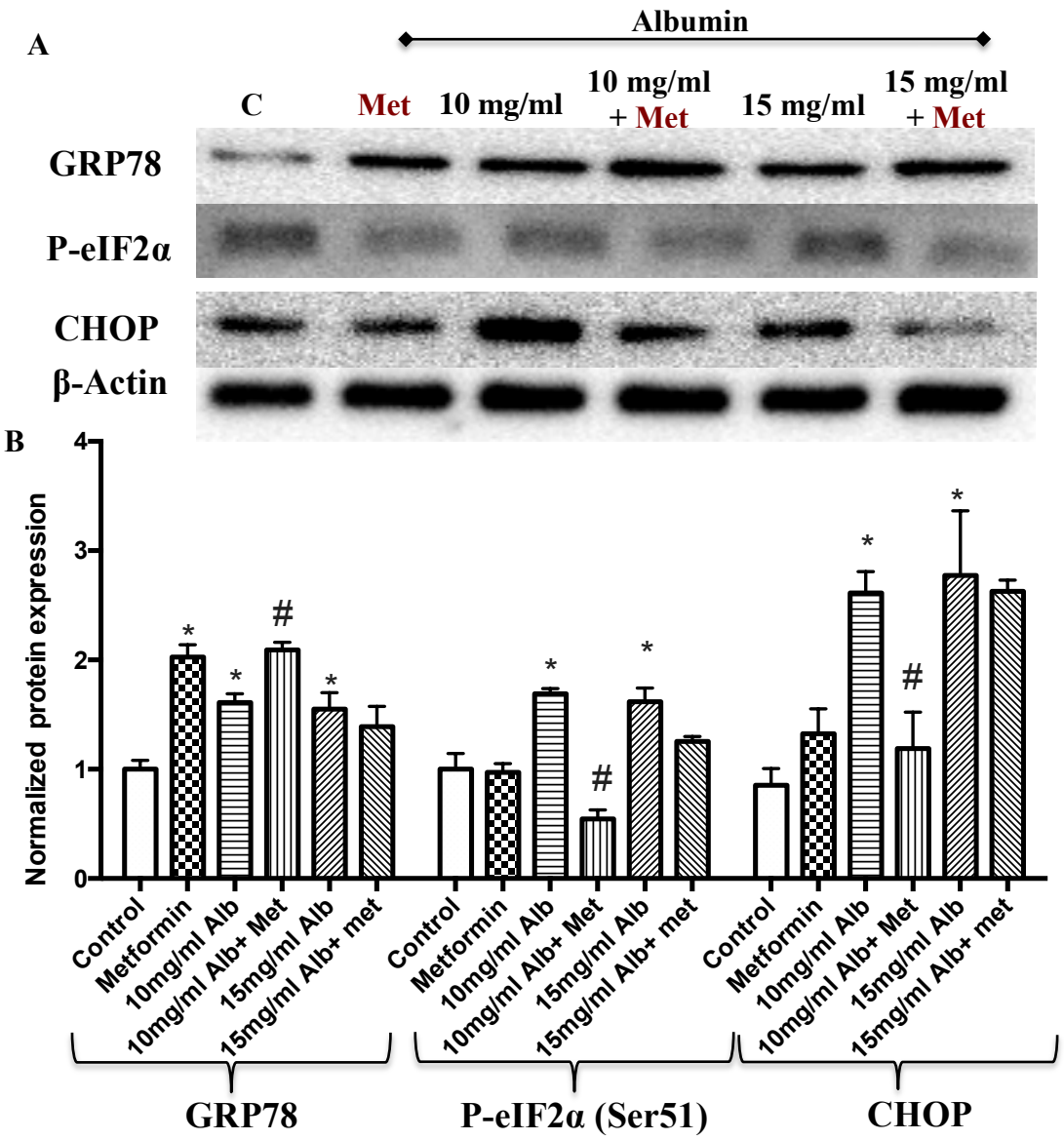

Figure 3.11. Effect of metformin on the expression of ER stress markers (GRP78, P-eIF2α (Ser51) and CHOP) in NRK-52E cells following 72 hours of albumin treatment. Upper panel (A) showing a representative western blot and the lower panel (B) showing the densitometry measurements normalized to β-actin. Values were expressed as Mean ± SEM; n = 3; *P<0.05 vs. untreated control (C), # and @ P<0.05 vs. 10 and 15 mg/ml Albumin treated groups respectively.

3.2.5. Effect of Metformin on EMT in Albumin-Induced Renal Cell Injury

Metformin's effect on α-SMA (a biomarker of EMT) in albumin-induced cell injury model was examined following 72 hours of treatment. As shown previously in **Figure 3.6**, albumin treatments (10 mg/ml and 15 mg/ml) induced the expression of α-SMA in NRK-52E cells. Cellular exposure to metformin for 72 hours in the presence of 10 mg/ml and 15 mg/ml of albumin caused a significant decrease in the expression of α-SMA to 0.4-fold and 0.6-fold respectively (**Figure 3.12**).

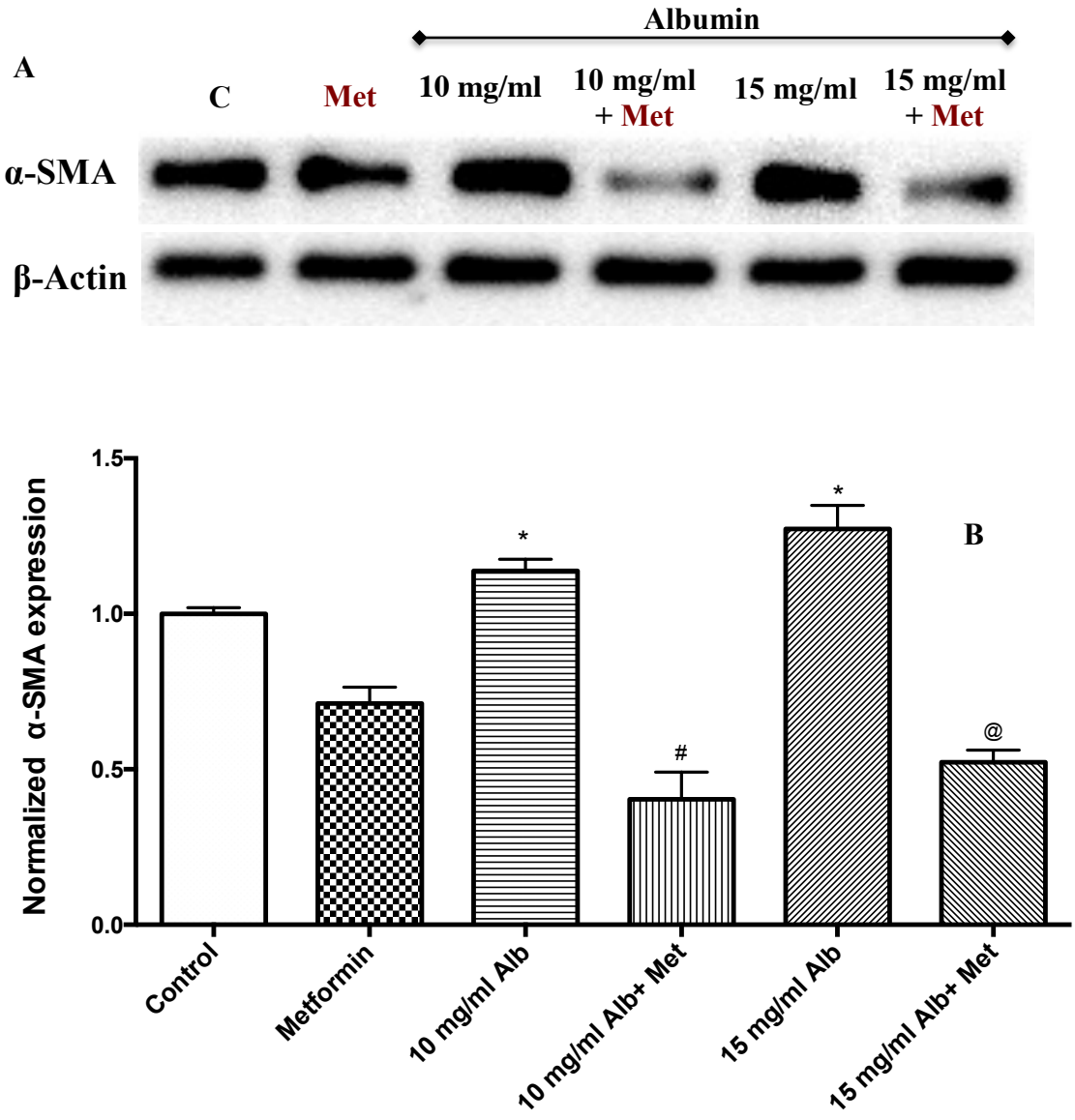

Figure 3.12. Effect of metformin on the expression of α-SMA (an EMT marker) in NRK-52E cells following 72 hours of albumin treatment. Upper panel (A) showing a representative western blot and the lower panel (B) showing the densitometry measurements normalized to β-actin. Values were expressed as Mean ± SEM; n = 3; *P<0.05 vs. untreated control (C), # and @ P<0.05 Vs. 10 and 15 mg/ml Albumin treated groups respectively.

3.2.6. Effect of Metformin on Autophagy in Albumin-Induced Renal Cell Injury

To determine metformin's effects on autophagy, the expression of P-ULK and LC3-II was measured in NRK-52E cells following metformin and albumin co-treatment for 72 hours. Cells that were exposed to 10 mg/ml of albumin showed caused minimal or no difference in the expression of both autophagy biomarkers compared to control. Co-treatment with metformin increased the expression of the autophagy markers in a significant manner ($P < 0.05$) as compared to 10 mg/ml albumin-treated group (**Figure 3.13**). Intriguingly, not only the albumin treatment at 15 mg/ml per se increased the expression of P-ULK and LC3-II by about 2-folds ($P < 0.05$) as compared to control, but the induction also remained unaltered despite metformin co-treatment.

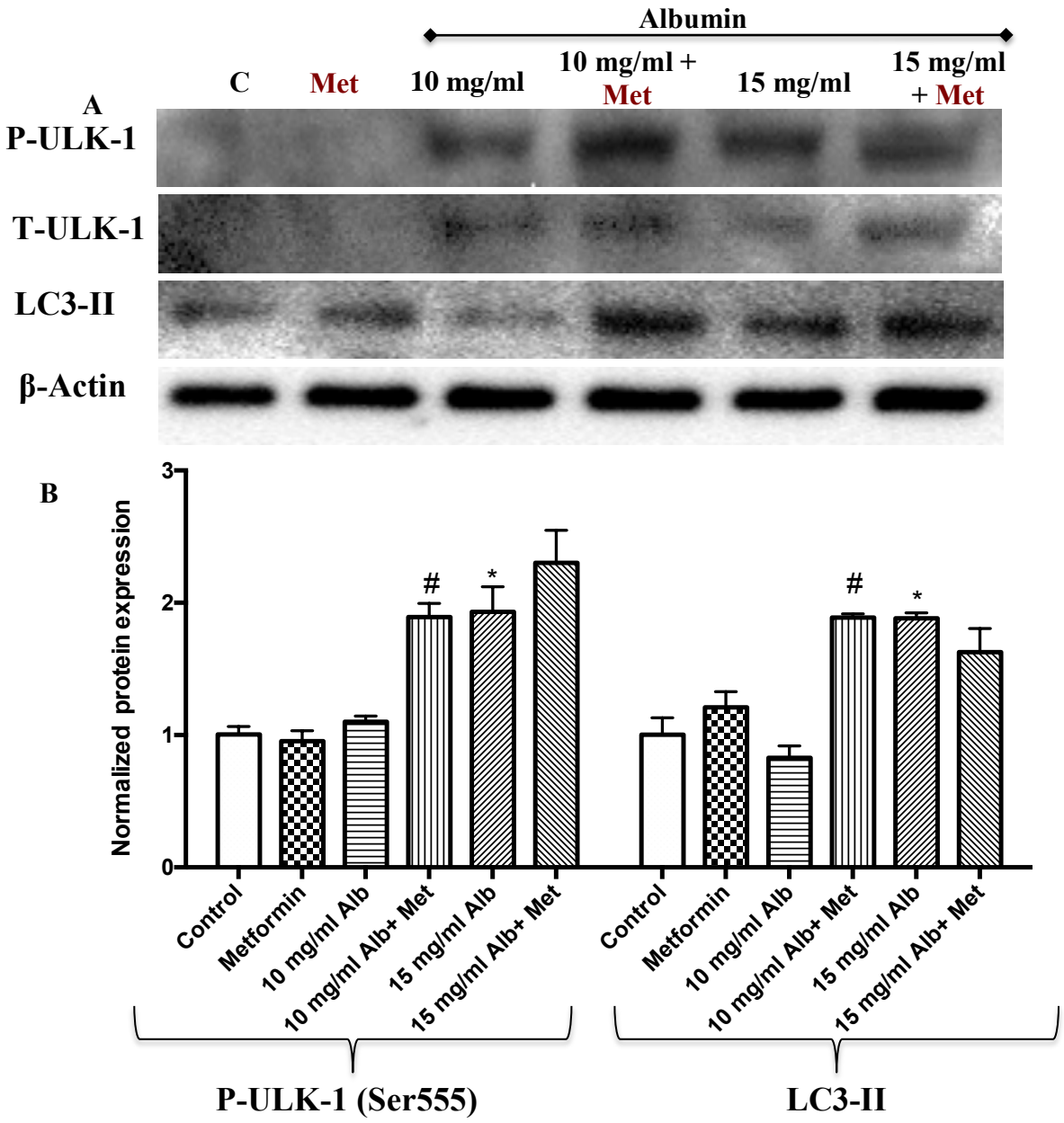

Figure 3.13. Effect of metformin on the expression of autophagy markers P-ULK-1 (Ser555) and LC3-II in NRK-52E cells following 72 hours of albumin treatment. Upper panel (A) showing a representative western blot and the lower panel (B) showing the densitometry measurements normalized to β-actin. Values were expressed as Mean ± SEM; n = 3; *P<0.05 vs. untreated control (C), # and @ P<0.05 vs. 10 and 15 mg/ml Albumin treated groups respectively.

3.2.7. Effect of Metformin on Apoptosis in Albumin-Induced Renal Cell Injury

The levels of caspase-12 and caspase-3 expression were measured as indices of apoptosis using western blotting. Cellular exposure to 10 and 15 mg/ml of albumin for 72 hours showed a 2-fold increase in both apoptotic markers as compared to the control. Metformin treatment caused a significant decrease in caspase-12 expression in 10 and 15 mg/ml of albumin groups to around 0.5-fold as compared to albumin-treated cells. Concurrently, a similar pattern was noted with caspase-3 expression in cells co-treated with albumin and metformin in which the caspases-3 levels were normalized to control (**Figure 3.14**).

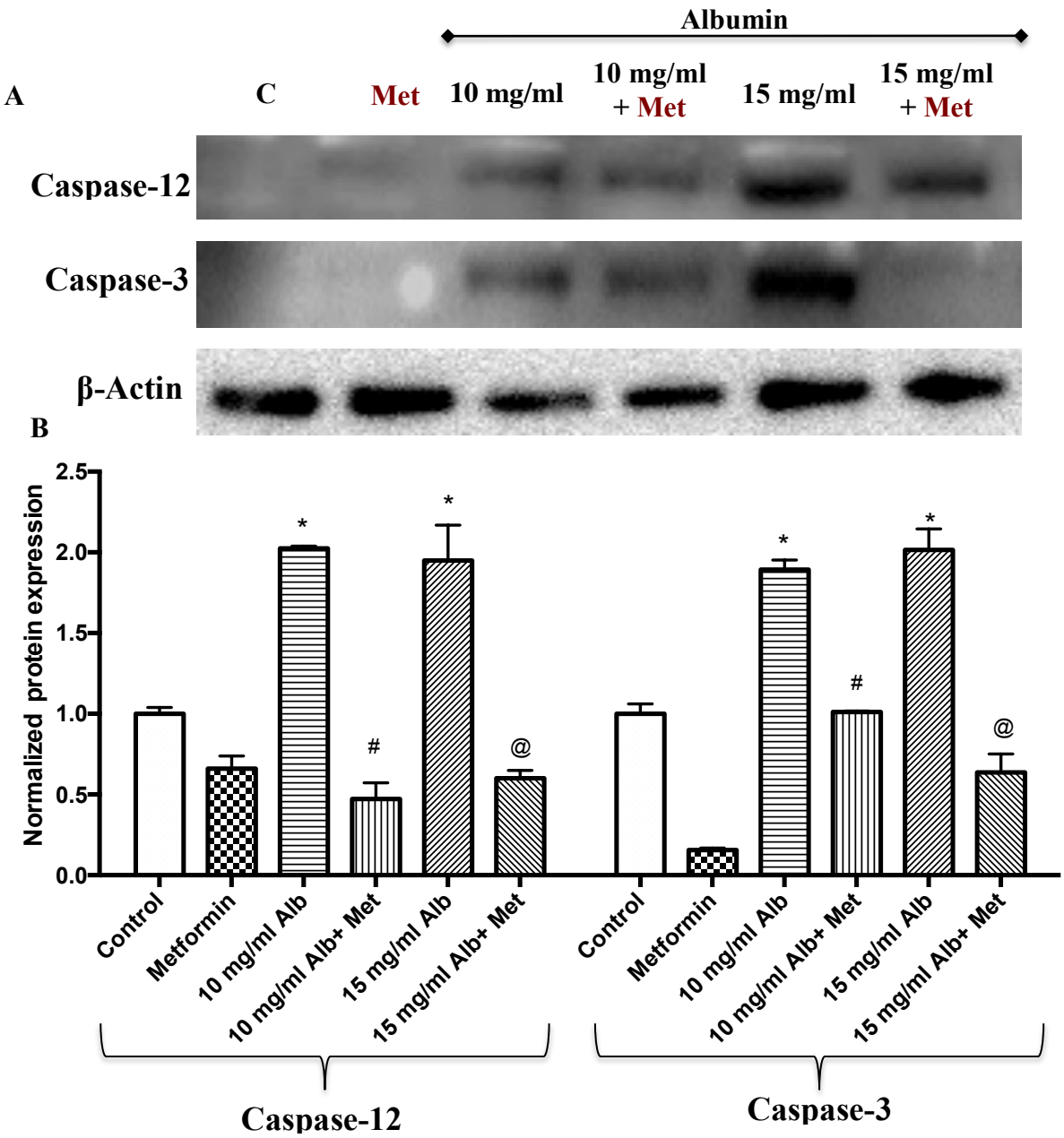

Figure 3.14. Effect of metformin on the expression of apoptotic caspases – 12 and -3 in NRK-52E cells following 72 hours of albumin treatment. Upper panel (A) showing a representative western blot and the lower panel (B) showing the densitometry measurements normalized to β-actin. Values were expressed as Mean ± SEM; n = 3; *P<0.05 vs. untreated control (C), # and @ P<0.05 Vs. 10 and 15 mg/ml Albumin treated groups respectively.

3.3. EFFECTS OF AMPK INHIBITION (USING COMPOUND C) ON EMT, AUTOPHAGY, AND APOPTOSIS IN ALBUMIN-INDUCED RENAL CELL INJURY

3.3.1. Concentration-Response Study of Compound C in NRK-52E Cells

To identify an optimal concentration of compound C (CC) that would cause a significant AMPK inhibition without substantial cytotoxicity, a concentration-response study was conducted. The screened concentrations of compound C ranged from 0.05 to 50 μM. Compound C was found to be cytotoxic even at concentration as low as 1 μM. Hence, the optimal concentration for compound C was concluded to exist between 0.05 and 0.5 μM (**Figure 3.15**).

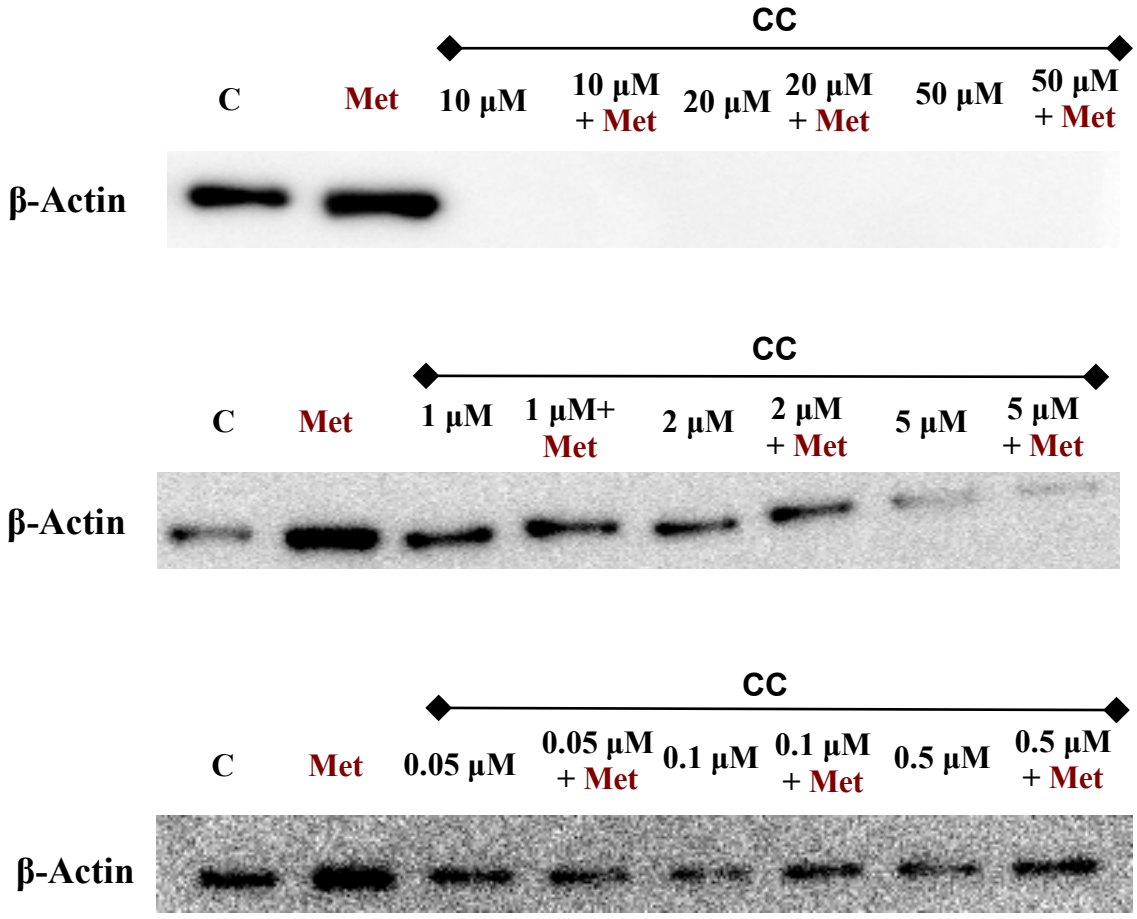

Figure 3.15. Effect of different concentrations of compound C (CC) on β-Actin expression in NRK-52E cells following 48 hours of treatment.

3.3.2. Effect of Compound C on AMPK Signaling Pathway in Albumin-Induced Renal Cell Injury

Further to determining the tolerable range of concentrations of compound C in our model, we determined the ability of these concentrations to cause a significant AMPK inhibition. We found that treatments with compound C at lower concentrations - 0.05 μM and 0.1 μM - failed to inhibit metformin-induced phosphorylation of AMPK. Nevertheless, co-treatment with 0.5 μM of compound C significantly reduced metformin-induced AMPK phosphorylation ($p<0.05$).

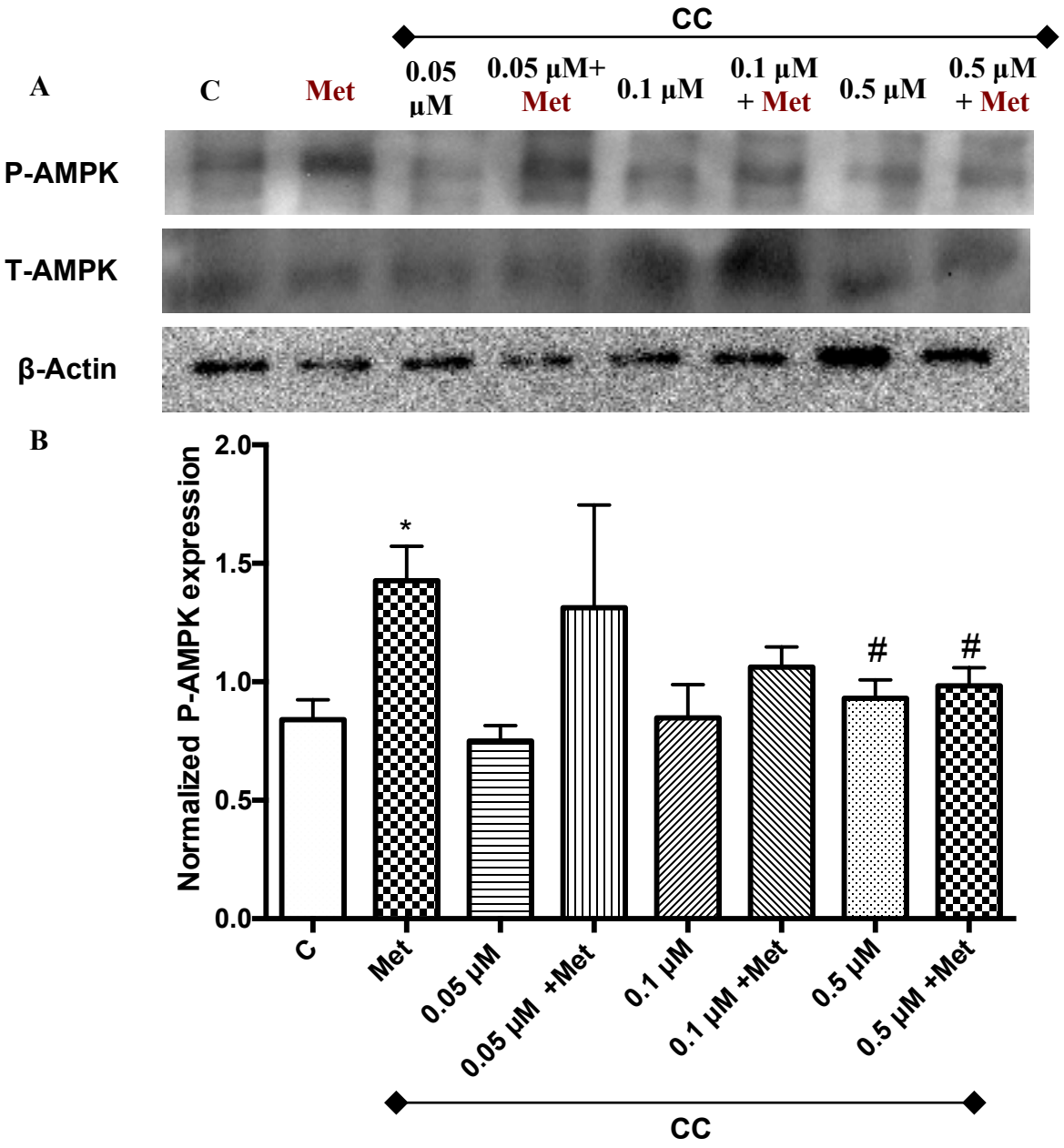

Figure 3.16. Effect of different concentrations of compound C (0.05 to 0.5 µM) on AMPK (Thr172) signaling in NRK-52E cells in the presence and absence of metformin following 48 hours of treatment. Upper panel (A) showing a representative western blot and the lower panel (B) showing the densitometry measurements normalized to β-actin. Values were expressed as Mean ± SEM; n = 3; * $p<0.05$ vs. untreated control and # $p<0.05$ vs. Metformin treated group.

3.4. EFFECT OF AMPK INHIBITION (USING COMPOUND C) ON ALBUMIN-INDUCED RENAL CELL INJURY

To determine whether the observed effects of metformin are mediated by AMPK, we conducted co-incubation studies with compound C (an inhibitor of AMPK) in our model. Western blotting was performed to quantify the effects of compound C on the markers of ER stress, EMT, autophagy and apoptosis.

3.4.1. Effects of Compound C on AMPK Phosphorylation in Albumin-induced Renal Cell Injury

Western blotting followed by densitometry was performed to quantify the effects 0.5 μM of compound C has on the AMPK phosphorylation in the presence and absence of albumin and/or metformin. Consistent with our previous findings, treatment with 1 mM metformin for 48 h caused a 2-fold induction in phospho-AMPK levels in our model. Co-incubation of cells with compound C significantly attenuated metformin-mediated AMPK phosphorylation in our model (**Figure 3.17**).

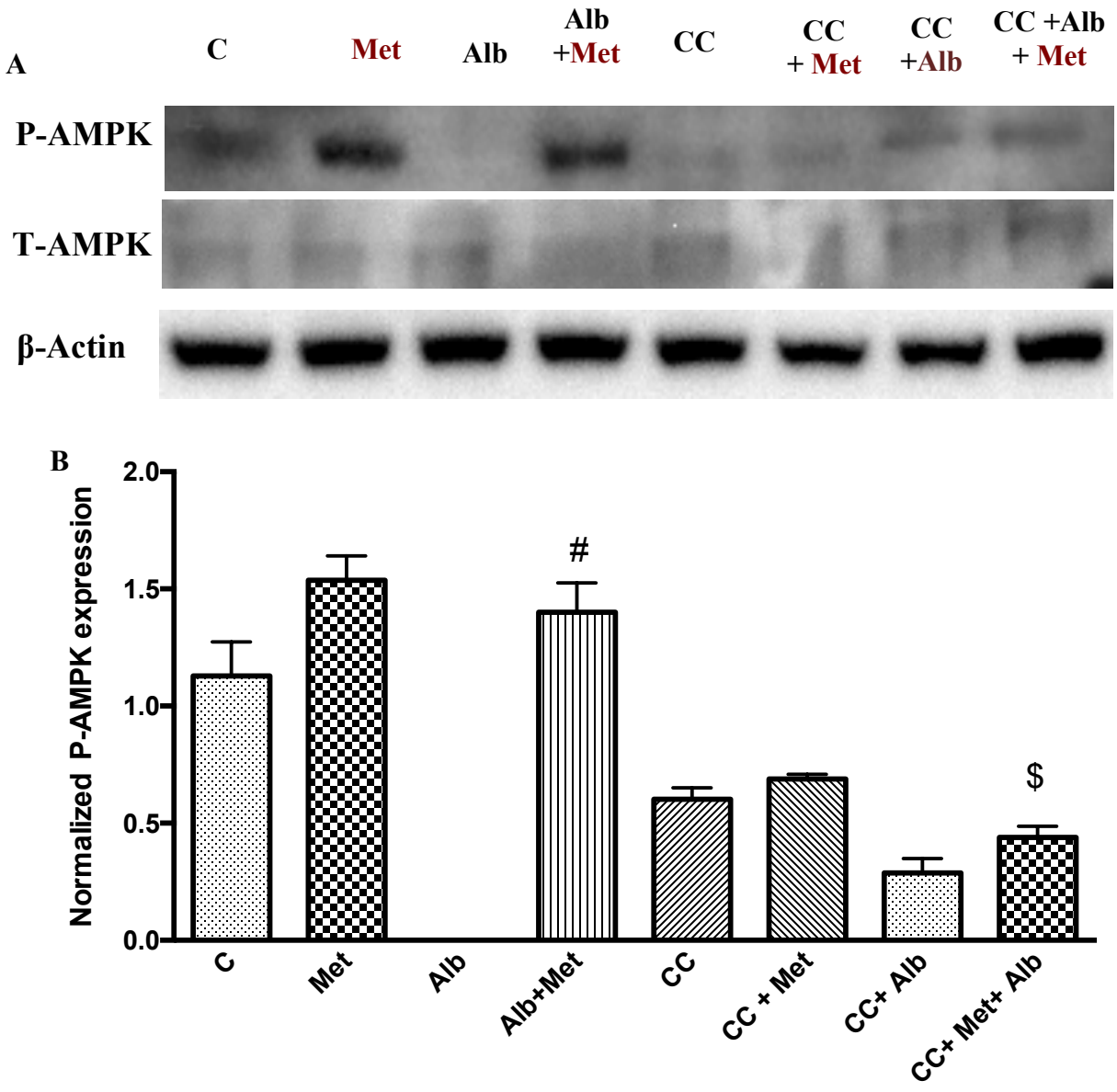

Figure 3.17. Effect of compound C on phospho-AMPK (Thr172) expression in NRK-52E cells following 48 hours of treatment. Upper panel (A) showing a representative western blot and the lower panel (B) showing the densitometry measurements normalized to β-actin. Values were expressed as Mean ± SEM; n = 3; * P<0.05 Vs. untreated control (C), # P<0.05 Vs. Albumin treated groups, and $ P<0.05 Vs. Albumin and Metformin co-treated group.

3.4.2. Effects of Compound C on ER Stress in Albumin-induced Renal Cell Injury

To determine whether the observed beneficial effects of metformin on ER stress (shown in Figure 3.11) were mediated through AMPK activation, the levels of ER stress markers GRP78 and phosphorylated eIF2α were assessed following the addition of 0.5 μM of compound C in our model. Our studies demonstrate that compound C per se significantly decreases the levels of GRP78. On the other hand, cellular co-treatment of compound C with metformin in the presence or absence of albumin showed a significant decrease in GRP78 expression as compared to albumin and metformin treated group.

Intriguingly, the phosphorylation of eIF2α in the presence of compound C showed an opposite trend to that of GRP78. For instance, compound C per se significantly increased the phospho-eIF2α levels as compared to the control. However, addition of compound C to cells co-treated with albumin and metformin had no significant effect on eIF2α phosphorylation as compared to albumin and metformin treated group (**Figure 3.18**).

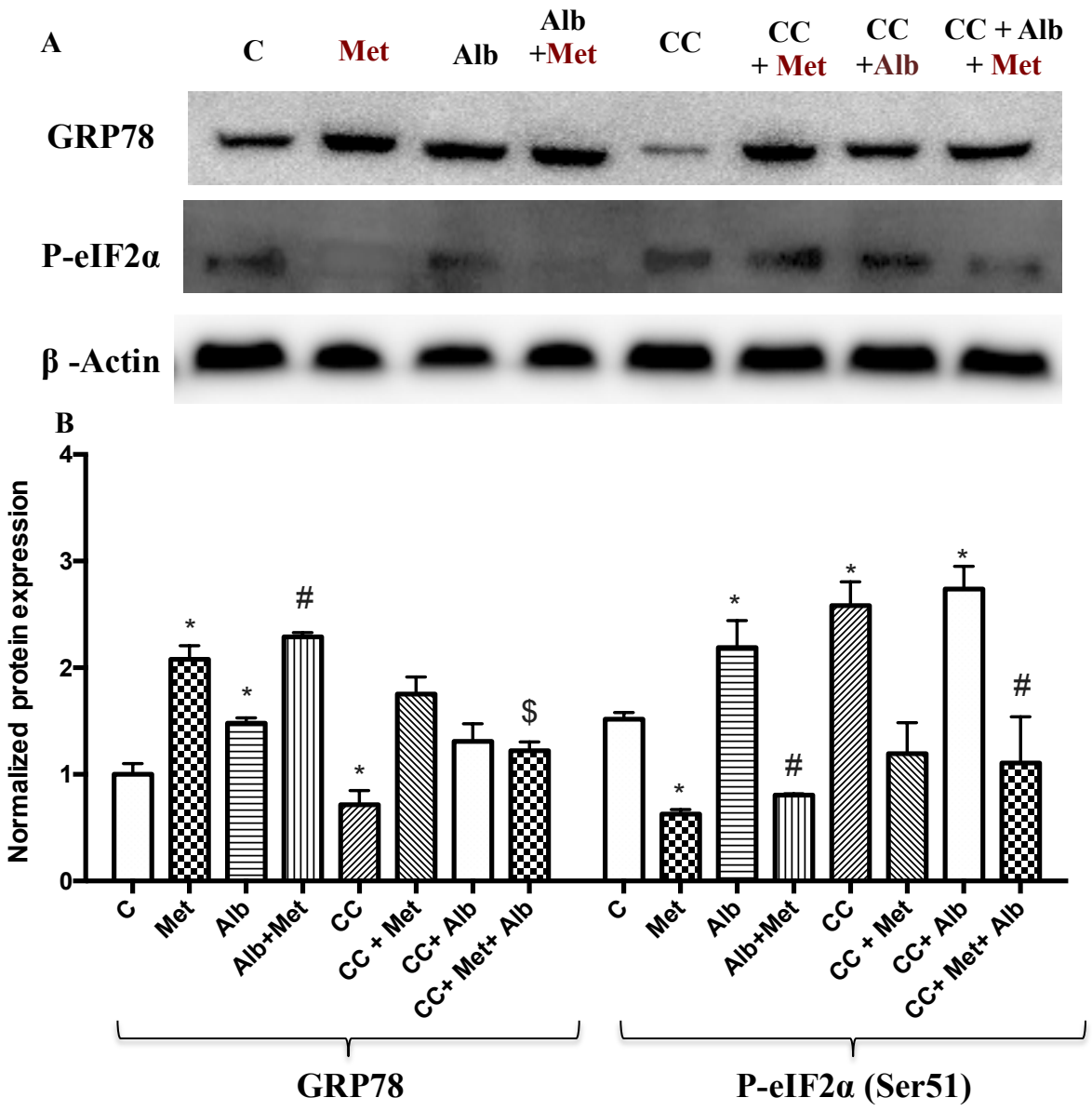

Figure 3.18. Effect of compound C on ER stress markers (GRP78 and P-eIF-2α (Ser51)) in NRK-52E cells following 72 hours of treatment. Upper panel (A) showing a representative western blot and the lower panel (B) showing the densitometry measurements normalized to β-actin. Values were expressed as Mean ± SEM; n = 3; * P<0.05 vs. untreated control (C), # P<0.05 vs. Albumin treated group, and $ P<0.05 vs. Albumin and Metformin co-treated group.

3.4.3. Effects of Compound C on EMT in Albumin-induced Renal Cell Injury

To identify the mechanism by which metformin exerts its protective effects against EMT, compound C was used to inhibit AMPK mediated effects of metformin. α-SMA expression was used as a biomarker for EMT. Consistent with our previous findings (as shown in **Figure 3.12**), exposure to albumin treatment caused a significant induction of α-SMA expression, which was attenuated in metformin co-treated group. However, no difference was detected between compound C, metformin and albumin treated group as compared to albumin and metformin co-treated arm (**Figure 3.19**).

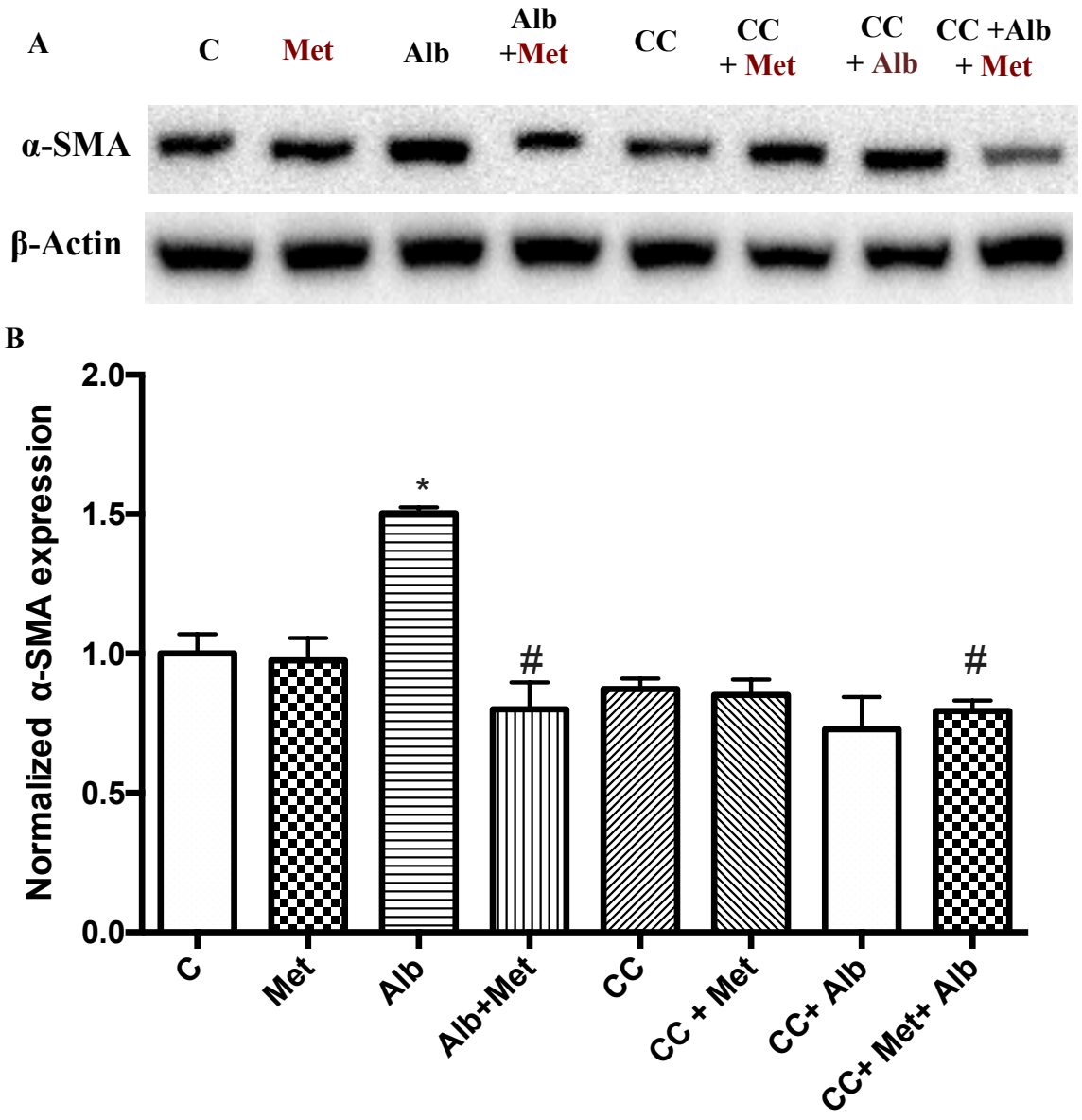

Figure 3.19. Effect of compound C on α-SMA (an EMT marker) expression in NRK-52E cells following 72 hours of treatment. Upper panel (A) showing a representative western blot and the lower panel (B) showing the densitometry measurements normalized to β-actin. Values were expressed as Mean ± SEM; n = 3; *P<0.05 Vs. untreated control (C) and # P<0.05 Vs. Albumin treated group.

3.4.4. Effects of Compound C on Autophagy in Albumin-induced Renal Cell Injury

To assess the effects of AMPK inhibition on cellular autophagy, the expression of LC3-II was measured in the presence of compound C in our model. Our data revealed a significant attenuation of LC3-II expression in cells treated with compound C, metformin and albumin as compared to cells that were only co-treated with albumin and metformin (**Figure 3.20**).

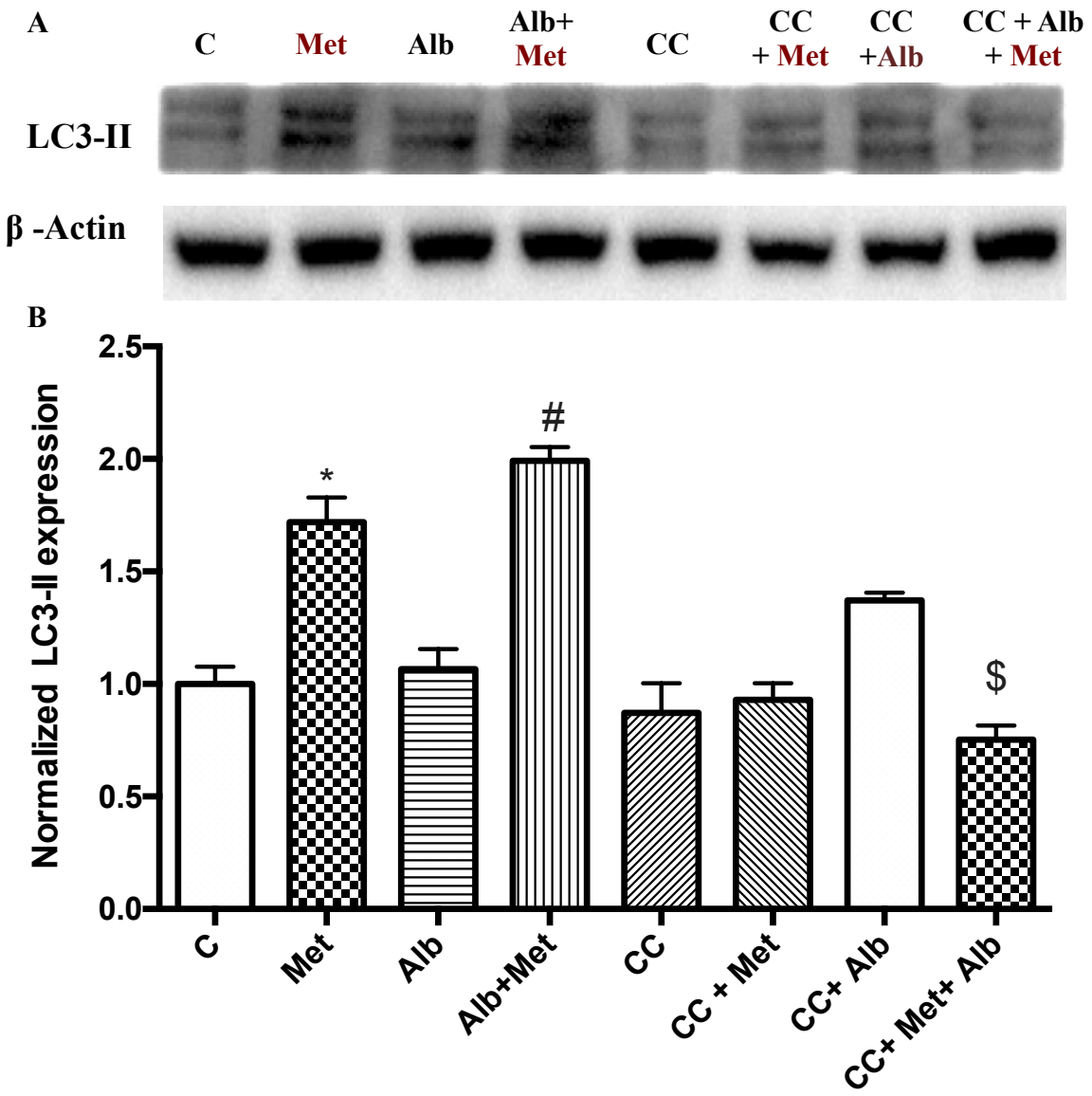

Figure 3.20. Effect of compound C on autophagy marker LC3-II in NRK-52E cells following 72 hours of treatment. Upper panel (A) showing a representative western blot and the lower panel (B) showing the densitometry measurements normalized to β-actin. Values were expressed as Mean ± SEM; n = 3; *P<0.05 Vs. untreated control (C), # P<0.05 Vs. Albumin treated group, and $ P<0.05 Vs. Albumin and Metformin co-treated group.

3.4.5. Effects of Compound C on Apoptosis in Albumin-induced Renal Cell Injury

To study whether the anti-apoptotic effects of metformin were AMPK mediated or not, caspase-12 and BAX expressions were measured in presence of compound C in our in vitro model. The expression of both caspase-12 and BAX was found to significantly increase in the presence of compound C alone and the degree of induction was similar to that of the albumin-treated group. Interestingly, metformin retained its anti-apoptotic effects - evidenced by the normalization of caspase-12 and BAX levels - in albumin treated NRK-52E cells despite AMPK inhibition through compound C (**Figure 3.21**).

A summary of the effects of albumin, metformin and compound C treatment in NRK-52E cells on AMPK, AKT and mTOR signaling pathways following 48 hours of treatment and on ER stress, EMT, autophagy and apoptosis makers were represented in the **Table 3.1**.

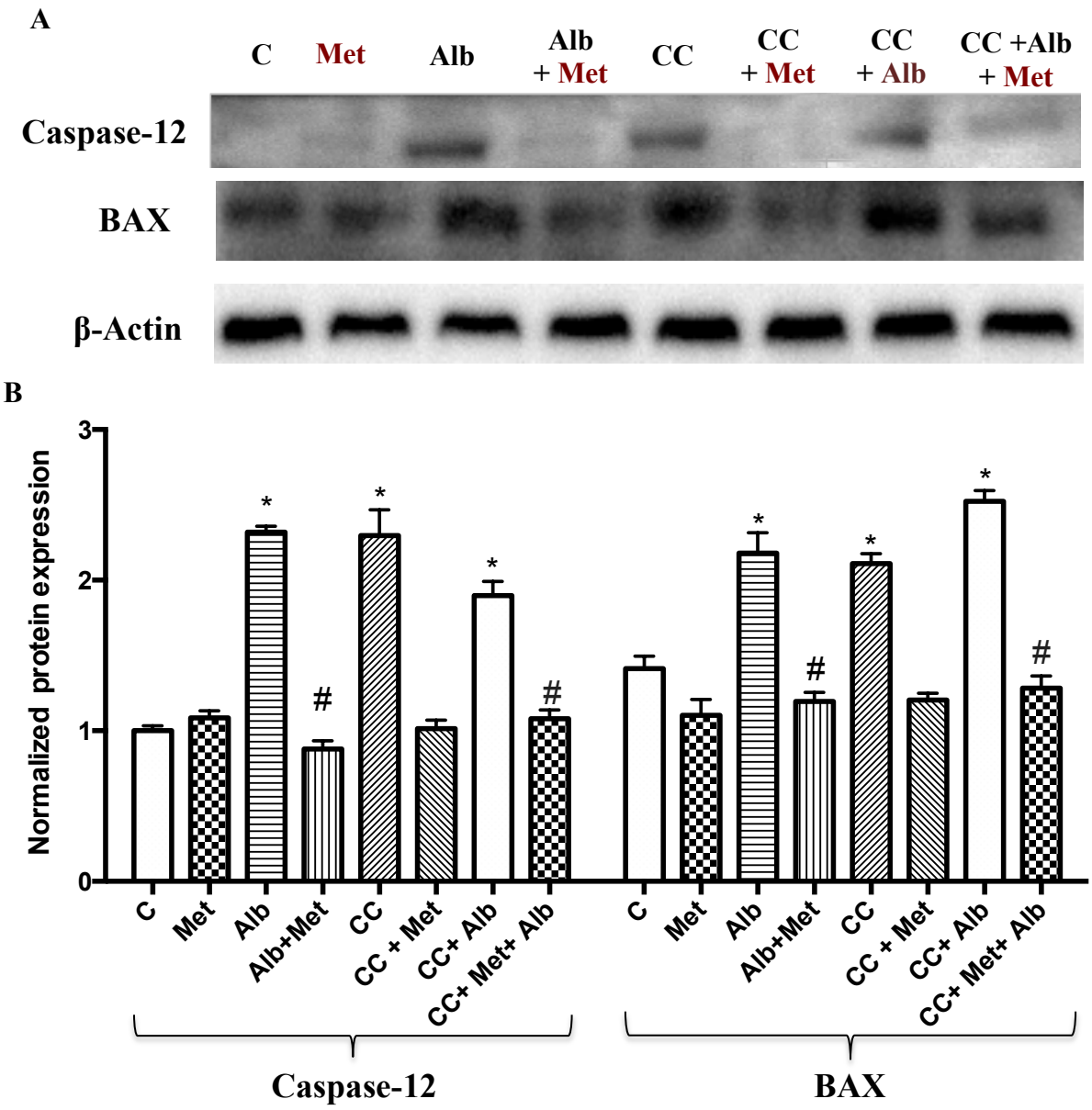

Figure 3.21. Effect of compound C on pro-apoptotic proteins – Caspase-12 and BAX - in NRK-52E cells following 72 hours of treatment. Upper panel (A) showing a representative western blot and the lower panel (B) showing the densitometry measurements normalized to β-actin. Values were expressed as Mean ± SEM; n = 3; *P<0.05 vs. untreated control (C) and # P<0.05 vs. Albumin treated group.

Table 3.1.

Summary of the effects of albumin, metformin and compound C in the established in vitro model of proteinuric kidney disease. The upward (↑) and downward (↓) arrows represent upregulation and downregulation of the protein of interest respectively. Double upward arrows indicate profound induction of protein expression as compared to albumin-treated group.

Protein of interest	Albumin	Metformin	Compound C
AMPK Signaling			
P-AMPK	↓	↑	↓
AKT Signaling			
P-AKT	↑	↓	-
mTOR Signaling			
P-4EBP-1	↑	↓	-
P-P70S6K	↑	↓	-
ER Stress			
GRP78	↑	↑↑	↓
P-eIF2α	↑	↓	**No change**
CHOP	↑	↓	-

Protein of interest	Albumin	Metformin	Compound C
EMT			
α-SMA	↑	↓	**No change**
Autophagy			
P-ULK-1	↑	↑↑	↓
LC3-II	↑	↑↑	↓
Apoptosis			
Caspase-12	↑	↓	**No change**
Caspase-3	↑	↓	**-**
BAX	↑	↓	**No change**

Chapter 4: Discussion

In the current study, immortalized rat proximal tubular epithelial cells (NRK-52E cells) were exposed to different concentrations of albumin that mimics conditions observed in CKD. Following standardization of a cellular model of CKD, the effects of metformin, a widely used anti-diabetic drug, was tested for its ability to modulate AMPK, AKT, and mTOR pathways, ER stress, EMT, autophagy and apoptosis. Furthermore, compound C, an AMPK inhibitor, was used to differentiate between the AMPK-dependent and AMPK-independent effects of metformin.

4.1. AMPK PHOSPHORYLATION

Our study for the first time indicates that exposure to albumin (at concentrations 10 and 15 mg/ml) causes a significant decrease in the phosphorylation of AMPK in renal cells, and metformin treatment reverses albumin's effect on AMPK phosphorylation. A previous study in human proximal tubular cell line (HK-2 cells) by Lee et al. (2012) used 5 mg/ml concentration of albumin but failed to showed changes in phospho-AMPK levels as compared to the control (92). This discrepancy in comparison to our findings could be attributed to the suboptimal concentration of albumin (i.e., 5 mg/ml) used in their study. Although we noted an increase in the expression of phospho-AMPK in metformin treated cells as compared to the control, the increase was not statistically significant. Using a similar cell line, Li et al. (2016) showed a similar pattern of a non-significant increase in AMPK phosphorylation in metformin treated cells as compared to the control (93). Thus, our findings with metformin in albumin-treated cells are in close alignment with the above study (92) and other models of kidney injury such as hypoxia-induced renal injury

(94), gentamicin-induced acute renal failure (95) and contrast-induced acute kidney injury (96).

To determine an optimal concentration of compound C in our cellular model, we tested different concentrations of compound C for their activity against AMPK phosphorylation with little or no cytotoxicity. This is because compound C was shown to be cytotoxic in various *in vivo* and *in vitro* models of astroglioma (97, 98). For instance, compound C (at 10 μM concentration) was shown to effectively diminish astrocytic tumors proliferation and growth (97). Subsequently, as study by Liu et al. (2014) revealed that concentrations as low as 1 μM for 72 hours causes significant cytotoxicity in three different glioma cell lines (99). The aforementioned cytotoxic effects of compound C could be explained by its ability to bind to a range of kinases other than AMPK (100, 101). For instance, studies conducted in zebra fish embryos revealed compound C's potent inhibitory action on bone morphogenetic protein (BMP) signaling (102); in particular, Yu et al. (2008) showed a significant decrease in BMP type I receptors and BMP-mediated phosphorylation of SMAD1/5/8 following treatment with compound C at concentrations ranging from 1 to 4 μM (102). Furthermore, a full-scale SAR study performed in vertebrates to screen for the effects of compound C and its analogs on cell signaling showed that compound C is a potent inhibitor of vascular endothelial growth factor (VEGF) type-II receptor at concentrations ranging from 5 to 10 μM (103).

It is remarkable to note that studies using non-cancerous cell lines to understand the effects of compound C on the phosphorylation of AMPK are scarce. Studies in cancer cell lines such as glioma (99) and human fibrosarcoma (104) indicate that concentrations ranging from 5 μM to 10 μM of compound C are required to cause a significant inhibition

of AMPK phosphorylation. In our studies, we found that the use of compound C at 0.5 μM concentration caused significant inhibition of metformin-induced AMPK phosphorylation without substantial cytotoxicity.

4.2. MTOR PATHWAY

Our study showed that cellular exposure to albumin leads to a significant increase in mTOR activation portrayed by an increase in 4EBP-1 and P70S6K phosphorylation. mTOR pathway modulation was extensively studied in different renal disease states (105, 106). Intriguingly, the effects of mTOR activation vary significantly depending on the type of renal injury. For instance, inhibition of mTOR activation with rapamycin (an mTOR inhibitor) in renal ischemia-reperfusion injury, delayed the rate of renal recovery and repair as compared to cells in which mTOR was not inhibited (106). These effects could be explained by the proliferative and regenerative properties mediated through mTOR, which are essential in the restoration of glomerular filtration following AKI (106). In contrast, the proliferative and regenerative effects of mTOR were found to exacerbate CKD and diabetic nephropathy through stimulation of the extracellular matrix protein synthesis, leading to basement membrane thickening and mesangial matrix enlargement, and ultimately into glomerular hypertrophy (105). Additionally, data from animal studies showed that rapamycin-mediated inhibition of mTOR significantly ameliorated interstitial inflammation, fibrosis, and loss of renal function associated with CKD (105, 106). In our study, albumin was used to evoke cellular responses that mimic those observed in cases of CKD. Therefore, an increase in mTOR activity caused by albumin in our model was anticipated. In corroboration to our findings, a study by Lee et al. (2010) in which renal proximal tubular cells were exposed to 5 mg/ml of albumin for

5 days also showed a significant increase in mTOR activity as compared to the control (107).

Our findings also demonstrated that 1 mM of metformin was able to significantly reduce mTOR activity induced by albumin. A similar inverse association between AMPK and mTOR pathway is also shown in many cell types such as hepatocytes (108), airway smooth muscle cells (98) and cellular models of cancer (109, 110). For example, studies by Dowling et al. (2007) in MCF-7 breast cancer cells demonstrated that metformin treatment blocks the phosphorylation of P70S6 kinase and eIF4E-1, the makers of mTOR activation in cells. The study also indicated that the effect metformin had on mTOR pathway was AMPK dependent, as these effects were diminished in presence of compound C (111). Comparably, data from renal injury models further indicate the inhibitory effects of metformin on mTOR pathway. Consensus with our findings, Liu et al. (2013) and Declèves et al. (2014) demonstrated that metformin treatment stimulates AMPK phosphorylation and blocks mTOR pathway *in vitro* and *in vivo* in renal cells (63, 112).

4.3. P-AKT PATHWAY

We found that exposure to albumin per se for 48 hours induces significant AKT phosphorylation in our model. Similar to our findings, Jones et al. (2003) reported a significant increase in AKT phosphorylation in B-chronic lymphocytic leukemia (CLL) cells following treatment with 25 mg/ml of human albumin for 24 hours (113). Additionally, the AKT activation was reported to play a key role in the progression of renal carcinoma (114). The direct evidence for the role of AKT in CKD was demonstrated in a study by Canaud et al. (2013), which measured AKT activity in

podocytes extracted from patients with different forms of CKD. In line with our findings, the study showed that AKT phosphorylation was significantly increased in all forms of CKD (115).

Further, we studied the effects of metformin treatment for 48 hours on AKT pathway in our cellular model. Similar to our findings with the mTOR pathway, metformin was able to restore phospho-AKT activity in renal cells exposed to albumin. Congruent with our findings, metformin was shown to inhibit AKT activation in breast cancer cells (111). In addition, metformin's effects on AKT were reported to be AMPK-dependent as silencing of AMPK activity using siRNA blocked metformin's effect on AKT pathway in these cells (111).

4.4. ER STRESS

As previously described, the accumulation of misfolded proteins within the endoplasmic reticulum is the principal stimulus for ER stress. To protect cells from transient ER stress, unfolded protein response is triggered, through which, the cellular structure and function is preserved. One of the mechanisms by which transient UPR maintains ER homeostasis is by increasing the levels of GRP78 (the ER chaperone) and via phosphorylation of eIF2α, which inhibits the translation of new proteins (92). In contrast, prolonged ER stress induces CHOP, which promotes ER stress mediated apoptosis (116). To characterize the nature of albumin-induced ER stress, we measured the effects of albumin on GRP78, phospho-eIF2α and CHOP. We found that albumin significantly increased the expression of CHOP in addition to induction of GRP78 and phosphorylation of eIF2α. In consensus with our findings, Lee et al. (2012) reported a significant increase in the levels of GRP78 and P-eIF2α in human proximal tubular cells

(HK-2) following 3 days' exposure to 5 mg/ml of albumin (92). Similarly, the expression of CHOP increased significantly following albumin treatment in proximal tubular cells (117). In support to *in vitro* studies, findings by Ohse et al. (2006) in proteinuric rat kidneys reveal a prominent induction of GRP78 in proximal and distal tubules, which is otherwise restricted to distal tubules in normal rat kidneys (118).

Further, we studied metformin-mediated alterations on albumin-induced ER stress in our model. Intriguingly, a paradoxical increase in GRP78 levels was noted following metformin treatment as compared to albumin only treated cells. These findings could be explained by the unique functions of transient UPR activation and its anti-apoptotic effects mediated through the ER chaperone GRP78 (119). As a multi-functional protein GRP78 assists in several process such as protein folding and assembly, translocation of newly synthesized proteins across the ER and initiation of ER-associated degradation (ERAD) to degrade misfolded proteins. The sum of the described functionalities mediated through GRP78 decreases ER stress and thereby, promotes cellular survival (119). The observed increase in GRP78 expression in renal cells subjected to metformin treatment might be an early compensatory mechanism through which ER machinery attempts to minimize ER stress. Paradoxical to our findings, a similar study by Lee et al. (2012) reported a 2-fold reduction in the levels of GRP78 following 48 h metformin treatment in HK-2 cells that were pretreated with albumin for 72 h (92). However, the discrepancy could be attributed to the differences in treatment protocol, i.e. albumin pre-treatment and limited duration of metformin treatment. Our studies also demonstrated that metformin causes a significant decrease in the phosphorylation of eIF2α, indicative

of attenuation of new protein translation, which is consistent with the findings from Lee et al. (2012) (92).

In our model, CHOP expression significantly decreased following metformin treatment signifying a potential inhibition to its pro-apoptotic properties. Intriguingly, metformin's effect on CHOP seems to be controversial in the literature and is highly dependent on the cellular model and the concentration used. For instance, a study by Leclerc et al. (2013), which investigated the effects of metformin (5 mM) in acute lymphoblastic leukemia (ALL), reported a significant increase in IRE1α and CHOP, and a resultant increase in UPR-mediated cell death (120). In contrast, Kim et al. (2010) demonstrated that 0.5 mM of metformin was able to lower CHOP levels in palmitate-induced ER stress in HepG2 cells (121). The differential alterations in the expression of CHOP by metformin can be attributed to the differences in the concentrations used (i.e. 0.5 mM vs. 5mM). Our findings in conjunction with reported studies suggest a concentration-dependent modulation of ER stress by metformin, i.e., anti-ER stress properties at lower concentrations and pro-ER stress and apoptosis at higher concentrations.

To identify whether metformin's effects on ER stress were mediated through AMPK pathway, we used compound C to inhibit metformin-mediated AMPK phosphorylation in cells exposed to albumin. Intriguingly, we found that metformin's induction of GRP78 was AMPK-dependent whereas eIF2α inhibition was AMPK-independent. It is important to note GRP78 is involved in all UPR pathways including IRE1α, ATF6α and PERK, while the eIF2α is a downstream target for PERK pathway. Therefore, our finding denotes that PERK inhibition is AMPK-independent while the

metformin's effects on IRE1α and/or ATF6α may or may not be AMPK-dependent. Congruently, Gomez et al. (2008) demonstrated that the use of AICAR (a specific AMPK activator) in a model of glucose-stimulated ER stress in pancreatic cells had no effect on the phosphorylation of eIF2α. Taken together, it is clear that PERK modulation is independent of AMPK status in cells (122). Moreover, studies conducted in mouse embryonic fibroblast and Phoenix cells to investigate the effects of AMPK on IRE1α pathway revealed that phenformin (an analog of metformin) activates IRE1α signaling pathway in an AMPK-dependent manner (123). However, metformin-mediated modulation of ATF6α and its dependency on AMPK status are yet to be evaluated.

4.5. EMT

To determine the effects of albumin on EMT, we examined for alterations in the expression of α-SMA following cellular exposure to albumin. As expected, cellular treatment with albumin for 72 hours increased α-SMA significantly, indicating the involvement of EMT in albumin-induced renal injury. Consistently, studies by Hu et al. (2015) and Ibrini et al. (2012) in rat renal tubular cells revealed a clear induction in EMT - marked by a decrease in the levels of epithelial marker E-cadherin and an increase in the expression of mesenchymal markers - fibroblast-specific protein 1, α-smooth muscle actin and collagen I - following exposure to albumin (124, 125).

To determine the anti-fibrotic effects of metformin, i.e. to inhibit epithelial-mesenchymal transformation, we examined the metformin-induced changes to α-SMA levels in our cell model. Our studies demonstrate that metformin prevents albumin-induced EMT - evident from a significant decrease in α-SMA expression with metformin treatment. Consistent with our findings, Lee et al. (2013) showed a significant suppression in albumin-induced EMT in human proximal tubular cell line following metformin treatment (78). Furthermore, the study postulated that metformin mediated EMT inhibition maybe due to its inhibitory action on ROS (78).

Intriguingly, we found that metformin's effects on albumin-induced EMT are not AMPK-dependent. This was evident from metformin's persistent inhibitory effect on α-SMA expression despite AMPK inhibition using compound C. In consensus with our findings, Liu et al. (2016) also reported that the addition of compound C to metformin in KLE cells did not change metformin's ability to prevent EMT (126). Thus, our study indicates that metformin's inhibitory effects on EMT are AMPK-independent.

4.6. AUTOPHAGY

We found that NRK-52E cells subjected to lower concentrations of albumin showed minimal or no changes in the expression of autophagy marker LC3II as compared to the control. Nonetheless, exposure to higher concentrations of albumin caused a significant increase in LC3-II levels in NRK-52E cells. Our findings indicate a concentration-dependent activation of autophagy by albumin in renal cells. This is in close correlation with the findings from a study by Liu et al. (2014), which reported only a slight increase in the number of LC3-II positive immunofluorescence staining puncta in HK-2 cells when treated with lower concentration of urinary proteins (0.5 to 1 mg/ml) as compared to the control (127). Increasing the concentration of urinary proteins to 8 mg/ml was shown to cause a significant elevation in LC3-II expression detected by western blotting and immunofluorescence staining (127). Remarkably, immunostaining of kidneys obtained from rats injected with cationic bovine serum albumin (C-BSA) revealed a significant elevation in the amount of LC3-II puncta as compared to healthy rat kidneys (127).

Following standardization of the albumin-induced renal injury model, we studied the effects of metformin on autophagy. This was done via determining the phosphorylation of ULK-1 and the expression of LC3-II in cells subjected to metformin co-treatment as compared to the albumin-treated group. Induction of P-ULK-1 and LC3-II expression in renal cells revealed that metformin significantly induced autophagy. In conjunction with our findings, Li et al. (2016) also reported a prominent induction of autophagy following metformin treatment in cisplatin-induced renal injury model (93).

Furthermore, when cells were subjected to compound C in the presence of metformin in our albumin-induced renal injury model, metformin's activation of autophagy was abolished indicating that metformin's effects on autophagy are AMPK-dependent. In agreement with our findings, Li et al. (2016) and Wang et al. (2013) revealed through chemical inhibition and small hairpin shRNA silencing of AMPK that metformin induces autophagy through AMPK activation in cisplatin and ischemia/reperfusion-induced renal tubular cell injury models respectively (93, 128).

4.7. APOPTOSIS

The effects of 10 mg/ml and 15 mg/ml of albumin on apoptosis in NRK-52E cells were quantified through determining the differences in caspase-12 and caspase-3 expression in cells subjected to albumin for 72 hours. We showed a statistically significant 2-fold induction in the expression of both caspase-12 and caspase-3 following albumin treatment as compared to the control. Our findings suggest that albumin induces apoptosis in renal tubular cells. In further support to our findings, a study by Ohse et al. (2006) in immortalized rat proximal tubule cells (IRPTC) also revealed apoptotic cellular alterations such as nuclear shrinkage and condensation following albumin treatment at concentrations ranging from 10 mg/ml to 40 mg/ml. In addition, the extent of apoptosis was then confirmed using Annexin V assay (118). Similarly, Wu et al. (2010) and Fontana et al. (2015) also reported albumin-induced apoptosis in proximal tubule cells (117, 129).

To study metformin's protective effects against albumin-induced apoptosis, changes in the expression of apoptotic markers caspase-12 and caspase-3 were measured following metformin treatment in our model. Our findings indicate that metformin

treatment attenuates albumin-mediated induction of caspases-12 and caspase-3 and protects renal cells from apoptosis. Similarly, multiple studies revealed metformin's protective effects on renal cell apoptosis. For instance, in a model similar to this study, Lee et al. (2012) showed that albumin-induced apoptosis was prevented by metformin, which was marked by a reduction in the caspase-3 expression (92). It is interesting to note that metformin's renoprotective effects were not limited to albumin-mediated apoptosis and extends to other models of renal injury. For example, metformin's ability to inhibit apoptosis in renal cells was also demonstrated in *in vivo* models of gentamycin-induced (95) and renal ischemia/reperfusion-induced (130) renal cell apoptosis.

Using compound C mediated chemical inhibition of AMPK, we determined that metformin's anti-apoptotic effects are not AMPK mediated. This was evident through no alterations to the metformin-mediated reduction in caspase-12 and BAX expression following compound C treatment in our model. Intriguingly, the majority of studies that investigated the link between AMPK activation and the anti-apoptotic effects of metformin are conducted in cancerous cell lines. In consensus with our findings, Janjetovic et al. (2010) demonstrated that metformin prevents cisplatin-induced apoptosis in U251 glioma cells through blockade of oxidative stress and caspase activation (131). Furthermore, metformin's cytoprotective action was shown to be AMPK-independent as the use of other AMPK activators such as AICAR did not prevent cisplatin-mediated apoptosis (131).

Chapter 5: Conclusion

The current study indicates that *in vitro* exposure to albumin inhibits AMPK but activates AKT and mTOR pathways, and induces ER stress, EMT, autophagy and cellular apoptosis. Furthermore, metformin's reno-protective effects against proteinuric cytotoxicity are exerted via the inhibition of AKT and mTOR signaling pathways and the attenuation of EMT and apoptosis. Additionally, treatment with metformin significantly augments cytoprotective mechanisms such as autophagy and ER defense response in renal tubular cells. Studies with Compound C reveal that metformin's effects on autophagy and ER chaperone expression are AMPK-mediated whereas its effect on EMT and apoptosis are independent of AMPK activation (**Figure 5.1**).

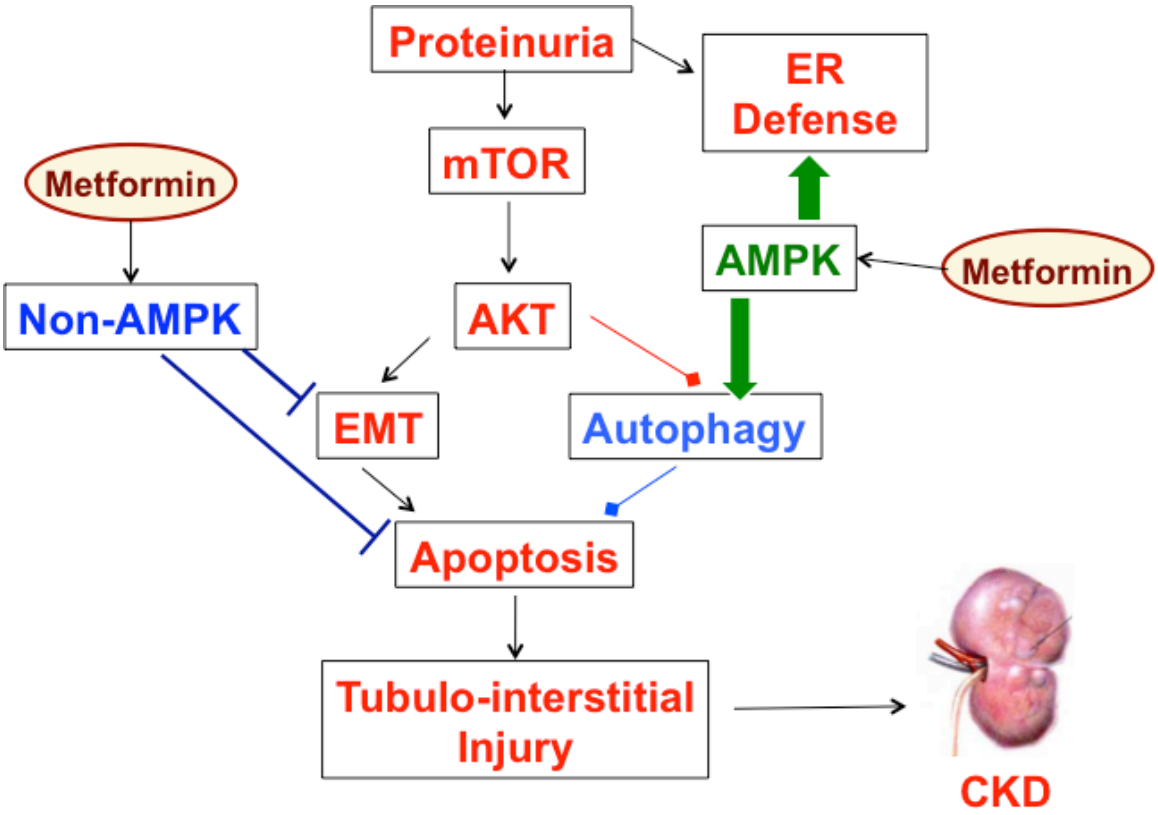

Figure 5.1. Signaling pathways through which metformin exerts its AMPK-dependent and AMPK-independent effects on albumin-induced renal cell injury.

5.1. CLINICAL IMPLICATIONS

Our study unveiled the potential therapeutic effects of metformin in preventing the development and progression of CKD (**Figure 5.2**). In alignment with the revised guidelines from the US FDA, metformin could be used in patients with stages 1 and 2 of CKD without any dose-reduction and in patients with stage 3A of CKD with 50% dose-reduction (7). In support to our findings, multiple studies *in vivo* revealed metformin's ability to ameliorate tubular injury associated with hyperglycemia (93, 94, 132). Nonetheless, clinical trials in patients with established renal injury are yet to be performed to ascertain the therapeutic potential of metformin in the management of CKD.

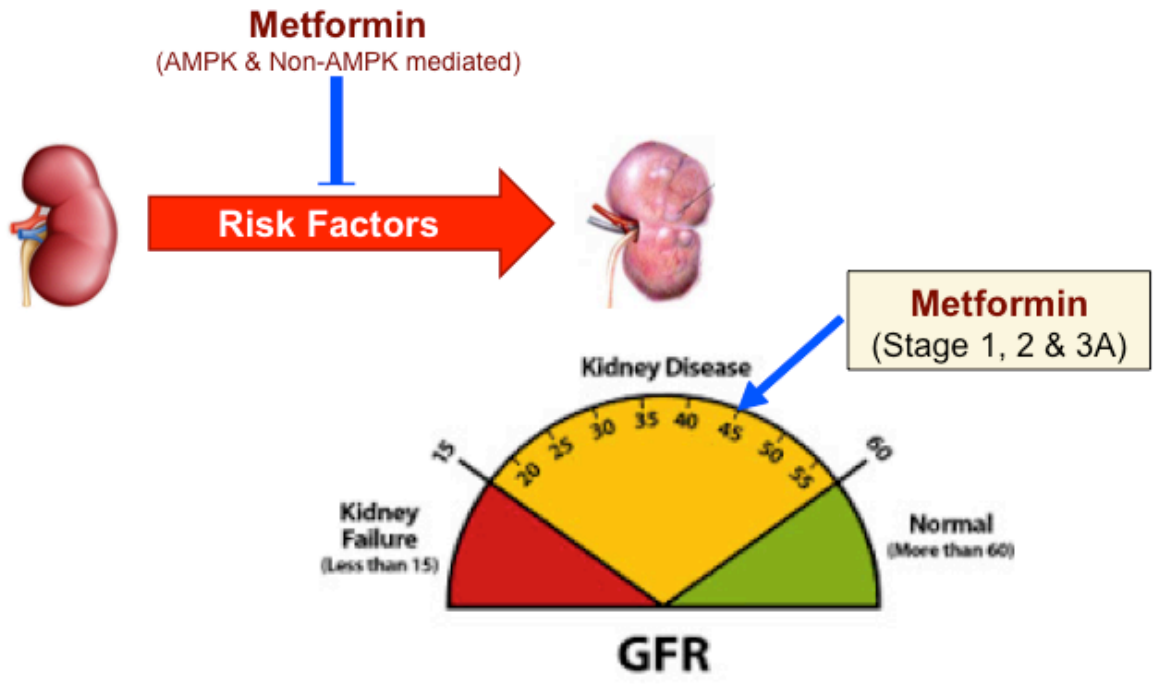

Figure 5.2. Potential therapeutic uses of metformin therapy in patients with CKD.

5.2. FUTURE DIRECTIONS

- One major limitation of our study is the use of a chemical inhibitor for AMPK, i.e., compound C, which is known for its non-specific effects and potential cytotoxicity. Thus, to validate the findings from compound C studies, an siRNA-mediated AMPK gene silencing in NRK-52E cells could be considered.
- To validate our findings *in vivo*, an animal model with renal-specific deletion of both AMPKα1 and α2 catalytic subunits (133) could be used to dissect AMPK-dependent and independent effects of metformin.
- Studies to delineate the AMPK-independent pathways such as blockade of HIF-1 activation (94), TGF-β expression (134) and ROS production (95) through which metformin could inhibit EMT and apoptosis would unveil novel drug targets for the treatment of CKD.
- One of the main phenomenon that was discussed in our study is autophagy nonetheless, its effects on metformin-mediated cell survival were not explored. Therefore, studying the consequences of attenuation of autophagy on metformin's protective effects would be an intriguing area to investigate.
- To study the effects of metformin on UPR, we investigated its effects on proteins regulated by all 3 pathways (i.e. GRP78 and CHOP) in addition to its effects on PERK pathway (P-eIF2α). Nonetheless, metformin's effects on IRE1 and ATF6 pathways are yet to be explored. Hence, investigating the effects of metformin on XBP-1 and P50, the downstream targets of IRE1 and ATF6, would be an interesting area of research.

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