INVOLVEMENT OF AUTOPHAGY IN OCHRATOXIN-A (OTA)-MEDIATED TOXICITY IN HK-2 CELL LINE

by

Hilal Kahraman

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To my beloved family...

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ABSTRACT

INVOLVEMENT OF AUTOPHAGY IN OCHRATOXIN-A (OTA)-MEDIATED TOXICITY IN HK-2 CELL LINE

Ochratoxin A (OTA) is a secondary metabolite produced by fungi and found in a variety of food and feed. It is classified as a possible human carcinogen and immense amount of work has revealed to its nephrotoxic and carcinogenic properties. Autophagy is a cellular degradation process in which long-lived or defective organelles and proteins are cleared in order to maintain cellular homeostasis. In this study, we investigated the involvement of autophagy and related signaling pathways MAPK/ERK and PI3K/AKT during progression of OTA-induced toxicity in human proximal tubule epithelial HK-2 cell lines. Time- and dose-dependent autophagic activity in response to OTA exposure was tested by examining the alterations of the amount of autophagy-related proteins, the conversion of microtubule-associated protein 1 light chain 3 (LC3)-1 to LC3-II, and by visualization of autophagasomes and acidic compartments. The results showed that OTA at 10 μM concentration induces autophagy at very early hours of treatment. After 6 hours, autophagasome formation decreases while AKT phosphorylation is up-regulated. Concordantly, inhibition of PI3K/AKT pathway increases the autophagasome formation while inhibition of MAPK/ERK had no effect on the regulation of autophagy in response to OTA. OTA has also decreased global steady-state levels of protein ubiquitinylation. In addition, the decrease in p62/SQSTM and increase in acidic vacuoles at later time points when the autophagic activity decreased could be the consequence of OTA-induced fast degradation by ubiquitin proteasome system (UPS) rather than autophagy. Taken together, this study suggest that OTA up-regulates autophagy at early hours of treatment and then down-regulates by the OTA-mediated PI3K/AKT pathway activation at later time points in HK-2 cell lines.

ÖZET

HK-2 HÜCRE HATTINDA OKRATOKSİN-A GÜDÜMLÜ TOKSİSİTEDE OTOFAJİNİN KATILIMI

Okratoksin-A (OTA) küf mantarı tarafından üretilen, birçok çeşitli besin ve yemde bulunan ikincil metabolittir. OTA'nın nefrotoksik ve karsinojenik etkileri önemli sayıdaki çalışmada gösterilmiş ve OTA olası insan karsinojeni olarak sınıflandırılmıştır. Otofaji, hücre homeostazının sürdürülebilmesi için uzun ömürlü ya da kusurlu organel ve proteinlerin yıkılması işlemidir. Bu çalışmada, insan böbrek proksimal tübül epitel hücreleri olan HK-2 hücreleri kullanılarak otofaji ve ona bağlı olan MAPK/ERK ve PI3K/AKT yolaklarının OTA-güdümlü toksisiteye olan katılımı incelenmiştir. OTA-teşvikli zamana ve doza bağlı otofajik aktivite, otofaji ile bağlantılı proteinlerden mikrotübül bağlayıcı LC3-I proteininin LC3-II'ye dönüşmesine, otofagozom ve asidik kesecikli yapıların oluşumuna bağlı olarak test edilmiştir. 10 μM dozlu OTA muamelesinin çok erken saatlerinde otofaji aktive olmuştur. Bunun yanında 6 saatlik OTA muamelesiyle AKT fosforilasyonu artış gösterirken otofagozom oluşumu da azalmaya başlamıştır. Bu sonuçlarla paralel olarak, PI3K/AKT yolağı wortmannin ile inhibe edildiğinde otofagozom oluşumunda artış gözlemlenirken, MAPK/ERK yolağı inhibasyonunun OTA-güdümlü otofajinin d üzenlenmesi üzerinde bir rolü görülmedi. OTA aynı zamanda denge durumundaki ubikitinlenmenin seviyesini azalttı. Bu sebeple otofajinin azaldığı ilerleyen saatlerdeki p62/SQSTM1 azalımı ve asidik yapılardaki artışın yine OTA tarafından tetiklenen übikitin-proteozom yolağından kaynaklı olabileceğini gösterdi. Sonuçlar birlikte ele alındığında, bu çalışma OTA'nın HK-2 hücrelerinde erken saatlerde otofajiyi tetiklerken, ilerleyen saatlerde PI3K/AKT yolağının aktive olmasıyla otofajinin azaltıldığını göstermektedir.

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LIST OF SYMBOLS

°C	Centigrade degree
g	Gram
hrs	Hours
kDa	Kilodalton
L	Liter
mg	Milligram
min	Minute
ml	Mililiter
mM	Milimolar
ng	Nanogram
rpm	Revolutions per minute
V	Volt
μg	Microgram
μl	Microliter
μM	Micromolar
κ	Kappa

LIST OF ABBREVIATIONS

AMPK	Adenosine mono-phosphate-activated protein kinase
AO	Acridine orange
APS	Ammonium persulphate
ATG	Autophagy related proteins
AVO	Acidic vesicular organelles
Baf	Bafilomycin A
BEN	Balkan Endemic Nephropathy
BSA	Bovine serum albumin
CMA	Chaperone-mediated autophagy
CO_2	Carbon dioxide
CQ	Chloroquine
DAPI	Diamidinophenylindoleamine
ddH_2O	Double distilled water
dH_2O	Distilled water
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetate
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinase 1/2
EtOH	Ethanol
FBS	Fetal bovine serum
GFP	Green flourescent protein
HBSS	Hank's Balanced Salt Solution
HK-2	Human kidney proximal cell line
IARC	International Agency for Research on Cancer
JNK	C-jun amino terminal kinase
LB	Luria-Bertani broth

LC3B	Microtubule-associated protein 1 light chain 3
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MDC	Monodansylcadaverine
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2
NAC	*N*-acetyl-L-cysteine
NaCl	Sodium Chloride
NF-κB	Nuclear factor kappa B
Nrf2	Nuclear factor erythroid 2-related factor 2
OTA	Ochratoxin A
pAKT	Phospho-AKT
PAS	Pre-autophagasomal structures
PBS	Phosphate buffered saline
PE	Phosphatidylethanolamine
pERK1/2	Phospho-ERK1/2
PFA	Paraformaldehyde
PI3K	Phosphoinositide-3-OH kinase
PVDF	Polyvinylidene difluoride
ROS	Reactive oxygen species
rpm	Rotations per minute
RT	Room temperature
SAPK	Stress-activated protein kinases
Ser	Serine
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
siRNA	Small interfering RNA
TBS-T	Tris Buffered Saline Tween
TEMED	Tetramethylethylenediamine
TORC1	TOR complex 1

TORC2	TOR complex 2
UPS	Ubiquitin Proteosome Pathway System
UTT	Urinary tract tumour
Wort	Wortmannin
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

1. INTRODUCTION

1.1. Ochratoxin-A (OTA)

Environmental factors are important in triggering diseases as much as genetic factors. Food contaminants such as the secondary metabolites of fungal species, namely mycotoxins are examples of these factors whose toxic effects result in developing of certain diseases. This thesis has focused on one of these mycotoxins; Ochratoxin A (OTA). In humans, OTA has been linked to the kidney disease Balkan Endemic Nephropathy (BEN) and urinary tract tumors (UTT) [1–4]. In 1993, the International Agency for Research on Cancer (IARC) stated that Ochratoxin A is a possible carcinogen to humans [5]. Its wide occurrence in food stuff and its potential carcinogenic effects attracted the attention of food safety organizations and scientists over the last few decades. Even though there is a growing body of evidence suggesting the genotoxic and nephrotoxic effects of OTA, its mode of action in carcinogenicity is still uncertain.

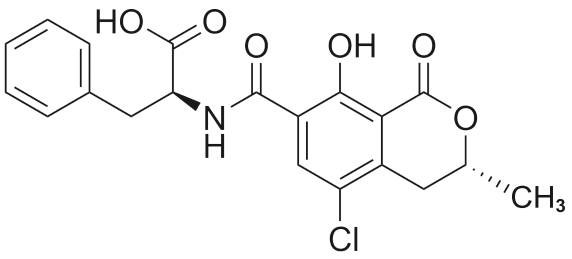

Figure 1.1. Structure of OTA.

OTA was first characterized as a toxic metabolite of *Aspergillus ochreceus* by van der Merwe and coworkers in 1965 [6]. After 24 years, OTA was reported to be produced by several other species of *Aspergillus* including *A. fresenii*, *A. mellus*, *A. ostianus*, *A. petrakii*, and *A. sclerotiorum* [7]. Since it contaminates a wide variety of food stuff such as chocolate, cereal, wine, beer, coffee, potato, spices etc., the rate of OTA exposure in our daily lives is very high.

OTA is a weak organic acid containing L-phenylalanine in its structure (Figure 1.1). Because of its lipophilic structure, it is well membrane-permeable and can accumulate in the cells. Animal studies have revealed that OTA is absorbed from the gastrointestinal tract and it is distributed via blood [8]. Its high affinity to plasma proteins (especially to albumin) and its slow elimination in urine and feces maintain long half-life in organisms [9].

1.2. Toxic and Carcinogenic Properties of OTA

Several animal studies and epidemiological studies in human populations have revealed a diverse range of OTA-induced responses including genotoxicity, nephrotoxicity, immunotoxicity, neurotoxicity, teratogenicity, and hepatotoxicity [7, 8, 10].

1.2.1. Nephrotoxicity

Kidney is the main target organ for many toxic materials due to the high renal blood flow per tissue weight and presence of high number of transporters. Lipophilic structure and toxicokinetic features of OTA result in accumulation of it in renal tissues, mostly in renal tubule epithelial cells [11]. *In vivo* experiments in a range of animal species have shown higher distribution of OTA in kidneys compared to the liver and other tissues. In a study, OTA was found to be mostly accumulated in kidneys after 24 hours of treatment in rats [12]. OTA was also transferred from the blood to the milk of lactating rabbit and the highest concentration of OTA was found in kidney [13]. Alternatively, another study on pigs has revealed that OTA reduced the protein synthesis and enzyme activities in kidney cells and caused detoriation of kidney structure [14]. For these reasons, OTA is considered as highly nephrotoxic agent causing a range of renal diseases.

Progressive renal diseases are generally characterized by cell death, inflammation, fibrosis and epithelial-to-mesenchymal transition. Similarly, exposure to OTA causes development of renal diseases accompanied by proximal tubular atrophy, cortical interstitial fibrosis and inflammation in animal studies [15]. Epidemiological studies

associate OTA with BEN which is characterized by tubular degeneration, interstitial fibrosis, and impaired renal function without degenerative nephrotoxicity and BEN is a common disease in Balkan region where foods are commonly contaminated by OTA. [2]. Moreover, the people live in certain Balkan regions were shown to have higher blood concentrations of OTA than the healthy individuals in countries throughout the world [3, 7].

1.2.2. Carcinogenicity

OTA-related carcinogenicity in humans has been investigated since OTA was implicated as a possible risk factor in the etiology of BEN and UTT. The studies have indicated that BEN patients with higher amounts of OTA in their serum were more likely to have tumour formation especially in their kidneys [1]. More evidence on possible carcinogenic effects of OTA has arisen with *in vivo* studies utilizing different model organisms. When F344 rats were exposed to OTA by gavage, they have shown benign and malignant tumours in their renal tubular epithelial cells with cytoplasmic alteration, karyomegaly, degeneration and cyst formations [16]. Since being exposed to OTA by gavage is not common in humans, another group designed an experiment with rats being fed daily with low-dose OTA. Even though, the results were less severe than the rats exposed to OTA by gavage, tumour formations were detected later than the previous studies. The authors have also detected renal carcinomas and metastases in the rats [17]. Furthermore, high incidence of renal lesions were also observed in kidney and gastrointestinal tract of pigs treated with daily oral doses of OTA [18].

In the light of these evidence, the International Agency for Research on Cancer (IARC) issued that Ochratoxin-A is possible carcinogen to humans in 1993 [5]. Up to now, many *in vivo* and *in vitro* studies have been performed to elucidate how OTA initiates tumour formation. Although some possible mechanisms were proposed to explain the carcinogenicity of OTA , the exact mechanism is still unknown.

1.3. Mode of Action

Altough OTA-induced DNA damaging has been proposed in several studies, the exact mechanism of OTA-derived carcionegenicity has not been elucidated yet. Whether direct genotoxic effect of OTA or indirect mechanisms such as oxidative stress, protein synthesis inhibition, alterations of cell signaling pathways, apoptosis playing role in OTA-derived carcinogenicity are still under debate [1, 7, 19].

1.3.1. Oxidative Stress

Oxidative stress arises when antioxidant defenses against reactive oxygen species (ROS) (such as free radicals and peroxides) decreases in a cell. If generation of ROS exceeds the capacity of endogenous defense agents, ROS start to damage cellular macromolecules including proteins and DNA. These damages may trigger cell death or tumorigenesis. The fate of the cell in response to oxidative stress is influenced by the type and duration of the stress, stress levels and the ability of the cells to cope with the source of stress and its destructive outcomes. If stress goes beyond a certain threshold, activation of stress signaling cascades may trigger death pathways such as apoptosis, necrosis or autophagy.

OTA-mediated toxicity studies have shown that oxidative stress has an important role in tumor formation primarily in kidney and liver [20, 21]. Therefore, oxidative damage has been proposed as a possible factor in carcinogenic effect of OTA. *In vitro* experiments in which ROS levels were measured by flow cytometry indicated an increase in oxidative stress after 24 hours exposure to OTA in kidney tubule cells. Moreover, down-regulation of glutathione S-transferase involved in cellular protection against oxidative stress was also observed after OTA treatment [22]. When ROS steals an electron from lipids, lipid peroxidation occurs and it can be monitored by analyzing malondialdehyde (MDA) formation. In many studies, OTA has been shown as an inducer of lipid peroxidation. For example, when the chicks were fed with OTA in their diet with two antioxidant vitamins, vitamins E and C, their MDA values in the liver were less than the chicks fed with only OTA [23]. In another study, there were

increases in oxidative stress response markers in plasma, kidney and liver of rats after treatment of OTA by gavage [21].

As an anti-oxidant defense mechanism, the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway is activated in response to elevated ROS in many cell systems, including proximal tubule cells [24]. However, OTA was shown to impaire this Nrf2 mechanism in kidney cells of rats and also human proximal tubule epithelial HK-2 cells [25]. OTA inhibited Nrf2 activity and, decreased Nrf2-dependent gene expressions in kidney of rats fed with OTA and also in porcine renal epithelial proximal tubule cells [26, 27]. Therefore, impairment in antioxidant defense mechanism may be critical in OTA-mediated cytotoxicity and carcinogenicity.

1.3.2. Genotoxicity

In some studies, OTA was shown to be a genotoxic substance. Genotoxic effect is considered as a source of tumor formations. Covalent binding of chemicals to DNA forms DNA adducts which have a strong relation with increasing mutation rates and subsequently tumour initiation. *In vivo* experiments have revealed that the DNA adduct formation depends on species, cell type, gender, exposure time and OTA concentration [28].

Genotoxic effect of OTA is a very controversial issue as there are two different claims to explain OTA-mediated genotoxicity: direct and indirect effect. As for the former, Pfohl-Leszkowicz *et. al.* have postulated a genotoxic mechanism of action for OTA based on ^{32}P-postlabelling experiments showing OTA-DNA adducts in kidney, liver and spleen of rats 24 hours after oral administration of OTA [29]. On the contrary, another group has claimed that when they repeated the same experiment, DNA-OTA adducts were not detectable with the same method and also with a more sensitive LC-MS/MS method [30].

In the literature, there are more studies indicating the presence of indirect genotoxic effect of OTA. In these studies, ROS-mediated oxidative stress is considered as

the main reason of DNA adducts, DNA strand breaks, unscheduled DNA replication and micronuclei. It was demonstrated that antioxidant supplementation before OTA treatment decreased DNA adduct formations in liver and kidneys of the rats [31]. Furthermore, oxidative DNA damage was also shown in HK-2 cell line, and pre-treatment with ROS scavenger *N*-acetyl-L-cysteine (NAC) protected the cells from DNA damage in response to OTA treatment [32].

1.3.3. OTA-mediated Cell Death or Survival

A number of study have clearly indicated that OTA caused to cell loss in time- and concentration-dependent manner. *In vivo* experiments with rats have shown alterations in the expression of key regulators of mitosis and genes linked to chromosomal instability [33]. Specific assays have revealed that OTA induces apoptotic or necrotic cell death in a variety of cell lines [19]. As a consequence of cell death, surviving cells lose their cell-cell adhesions activating signals for proliferation pathways to restore the original number of cells and size of the organ.

Long term administration of OTA increased cell loss and correspondingly proliferation of proximal tubule epithelium cells in outer stripe of outer medulla where OTA-induced tumours arise [34]. When rodents were subjected to different concentrations of OTA for long-time (up to 13 weeks), histopathological results have shown that OTA gave rise to renal alterations, single-cell death and prominent nuclear enlargement in the proximal tubules. Furthermore, sustained administration of OTA up to 90 days resulted in time- and dose-dependent decrease in relative kidney weight [35]. *In vitro* studies of the same group have revealed that OTA blocks metaphase/anaphase transition and causes formation of aberrant mitotic figures and giant cells with enlarged or multiple nuclei. Moreover, immunostaining with tubulin antibody have shown that there were defects in spindle formation [36].

The remarkable results supporting OTA-mediated mitotic disruptions and cell cycle abnormalities have led to proposition of another mode of action for OTA carcinogenicity. OTA most probably disrupts the cell signaling cascades leading to activation

of cell death mechanisms such as apoptosis, and necrosis. As an proposed mechanism, some of the genetically disrupted cells survive by activating the cell survival and proliferation pathways resulting in tumor formation [35].

1.4. Signaling Pathways Activated in Response to OTA

Several studies on OTA-induced carcinogenicity, nephrotoxicity and genotoxicity have associated these effects with perturbations of specific cell signaling cascades.

Mitogen activated protein kinases (MAPKs) have role in transducing signals from the cellular membrane to the nucleus. Extracellular signal-regulated kinases (ERKs), stress-activated protein kinases (SAPK)/Jun amino-terminal kinases (JNK) and mitogen activated protein kinase p38 are essential members of MAPKs. These signaling cascades are activated in response to mitogenic stimuli and associated with both survival and apoptosis [37]. OTA-mediated MAPK/ERK pathway was believed to activated in response to oxidative stress [38]. OTA exposure was shown to activate MAPK/ERK pathway in time- and dose-dependent manner in Madin-Darby canine kidney (MDCK) cells [39]. In the same study, as the activity of ERK increased, alkali-induced dedifferentiation of the cells also increased. It was concluded that the change in epithelial phenotype after OTA exposure could be mediated by the activation of ERK1/2 in renal epithelia. Another study in proximal tubular cell line NRK-52E, ERK, JNK and p38 were activated by OTA in a dose dependent manner [15].

It was also shown that chronic dietary administration of a carcinogenic dose of OTA to male rats over 7 days, 21 days and 12 months, activated PKC (protein kinase C) and downstream MAPK/ERK effectors [40]. Constitutive activation of MAPK in human renal tumors implies a strong association between sustained MAPK activation and carcinogenic transformation of human renal cells [41].

Phosphoinositide-3-OH kinase/AKT (PI3K/AKT) pathway is another cascade that is critical for essential processes in the cell such as protein synthesis, cell cycle, cell survival and growth. In a study, it was shown that the topical application of OTA

enhanced short term markers of skin tumor promotion such as DNA synthesis and hyperplasia [42]. Furthermore, exposure of primary murine keratinocytes with non-cytotoxic dose of OTA enhanced phosphorylation and activation of epidermal growth factor receptor (EGFR) and its downstream signaling pathways AKT, ERK1/2, p38, and JNK [42]. The authors also observed an increase in binding of nuclear-factor-kappaB (NF-κB) and AP-1 transcription factors to the promoter regions of cyclin-D1 and COX-2 genes which are important in cell cycle, apoptosis and proliferation . In a recent study, OTA was shown to induce sustained activation of PI3K/AKT and MAPK/ERK1/2 pathways in HK-2 cell line [43]. Moreover inhibition of MAPK/ERK pathway led to increased cell viability and decreased apoptosis while inhibition of PI3K/AKT pathway decreased cell viability and increased apoptosis in the same study.

It is well known that these OTA-induced signaling pathways are associated with the regulation of apoptosis, necrosis and autophagy. There are several studies showing the apoptotic and necrotic affects of OTA; however, there is no study in the literature exploring the possible autophagy inducing effects of OTA to the best of our knowledge.

1.5. Autophagy

Autophagy is a bulk degradation process that is essential for cell homeostasis. "Auto" means self and "phagy" means "to eat". As its etymology indicates, the cell's cytoplasmic content is degraded by its own degradation machinery through this process.

Autophagy is recognized as a cellular response to nutrient starvation in which the cell recycle its own content and provides its basic elements and energy to survive [44]. However, studies have shown that the cells use this mechanism to renew their own macromolecules and organelles at a basal level in most tissue types and also they up-regulate this mechanism in response to stressful conditions, such as amino acid deprivation, hypoxia, oxidative stress, and toxic insults [45]. Defects in autophagy have been implicated in the pathogenesis of diverse diseases including cancer, diabetes, Alzheimer's, Parkinson's and Huntington's diseases [46, 47]. Three types of autophagy have been described so far; macroautophagy, microautophagy and chaperone-mediated

autophagy (CMA) .

1.5.1. Types of Autophagy

In macroautophagy (hereafter will be referred to as autophagy), the cellular content is sequestered into formed vesicles which are called autophagasomes. This process takes different name with respect to cellular content of the cargo such as mitophagy for mitochondria, ribophagy for ribosome or pexophagy for peroxisomes, etc. These processes end up with fusion of vesicles and acidic lysosomes to be degraded [48].

Microautophagy is the least understood type of autophagy by which the cellular content is directly engulfed by lysosomal membranes [44]. In CMA, the proteins are selectively translocated from cytosol to lysosome by chaporones recognizing a specific polypeptide motif KFERQ [44, 49].

1.5.2. Molecular Machinery of Autophagy

Autophagy is initiated by the formation of autophagasome in which the cargo will be enclosed. The initiation of autophagasome formation is still controversial issue whether it is generated by assembly of pre-autophagasomal structures (PAS) or generated from pre-existing organelle such as endoplasmic reticulum, golgi apparatus or mitochondria. Close proximity of the organelle-specific proteins and autophagy proteins have provided evidence for pre-existing membrane source of autophagasome [49, 50]. In fact, PAS contain highly regulated coordination of components containing several proteins expressed by the autophagy-related (*Atg*) genes [51].

Autophagasome formation and degradation processes are analyzed in three main stages; initiation, elongation and fusion with maturation (Figure 1.2). The studies have shown that nucleation of initial phagaphore membrane requires two complexes: ULK1 complex (Atg1 complex in yeast) and class III phosphatidylinositol 3-kinase (PI3K III). As the target of autophagy inhibitor, 3-methyladenine (3-MA), Vps34 is the catalytic subunit of PI3K complex. This complex contains Beclin-1 (Atg6 in

yeast), Atg14 and Vps15. In a recent study, starvation-induced ULK kinase has been shown to be recruited to Vps34 and Beclin-1 complexes and initiate the autophagasome formation by phosphorylating Beclin-1 and so activating the Vps34 [52].

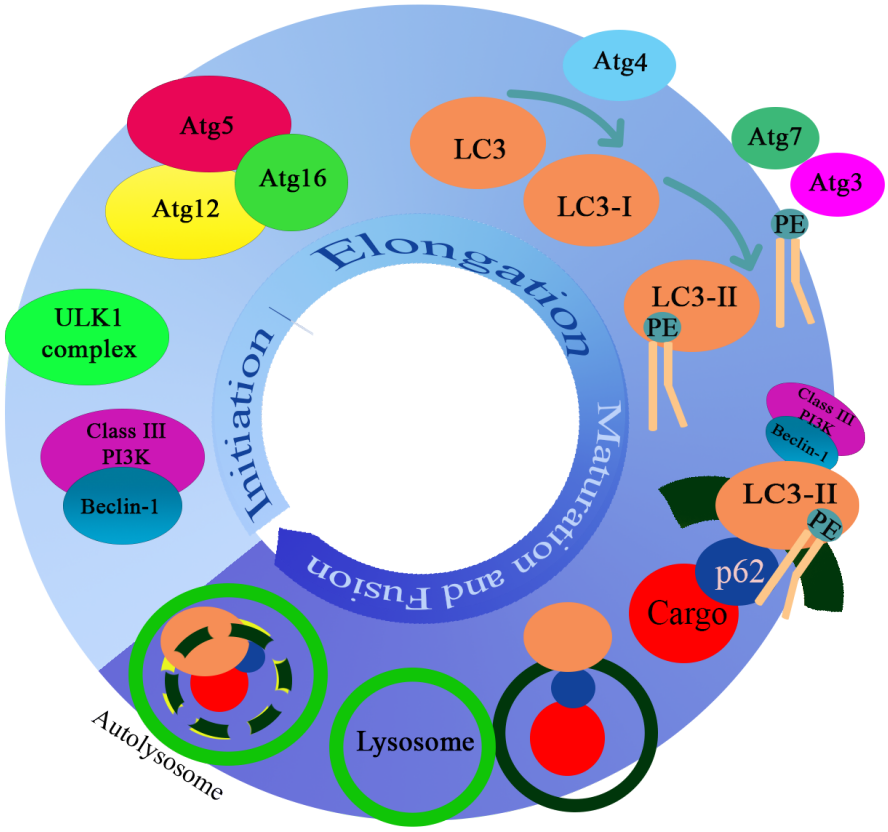

Figure 1.2. The stages of autophagasome formation.

Autophagasome elongation requires important ubiquitin-like reactions in pre-autophagasomal structures. Firstly, the ubiquitin-like protein Atg12 is activated by E1 ubiquitin activating enzyme-like Atg7 and then transferred to the Atg10 which is E2 ubiquitin conjugating enzyme-like. This process ensures covalent linkage of Atg12 to Atg5. Then Atg12-Atg5 complex associates with Atg16L1 protein. This large tetramer complex is very important for the elongation of autophagasomal membrane and also responsible for the activation of E2-like protein Atg3 required in the other ubiquitin-like reaction containing microtubule-associated protein 1 light chain 3 (LC3; Atg8 in yeast). LC3 protein firstly cleaved at its COOH terminus by Atg4B to form LC3-I.

Then, LC3-I is conjugated to phosphatidylethanolamine (PE) by a reaction involving E1-like protein Atg7 and Atg3 to form LC3-II [53]. This reaction takes place after the induction of autophagy, therefore, conversion of LC3-I to LC3-II is widely used as a marker for autophagy detection.

Mature autophagasome fuse with late endosome or lysosome which is called as "autolysosome". The acidic conditions of lysosome degrade the cargo with autophagy machinery. The high rate of autophagic flux results in fast degradation process which has an effect on the detectable amount of autophagy related proteins. Therefore, specific inhibitors toward degradation process are used to monitor autophagic flux by LC3 conversion [48].

1.5.3. Selective Autophagy and the Ubiquitin-Proteasome System

Autophagy has long been thought as nonselective bulk degradation process. However, recent studies have revealed that specific adaptor proteins such as p62/SQSTM1 and NBR1 facilitate selective uptake and targeting of the cargo for degradation [54].

p62/SQSTM1 is a scaffold protein involved in various signaling pathways and mainly found in the inclusion bodies containing polyubiquitinated protein aggregates. It binds polyubiquitin via its ubiquitin binding site, and then it is shuttled to the proteasome for degradation [55]. However, in recent years p62/SQSTM1 has been shown to interact with LC3 via its N-terminal LC3 binding domain. Hence, p62/SQSTM1 is thought to be a central player connecting the ubiquitin-proteasome system (UPS) and autophagy [56]. When the interaction of p62/SQSTM1 with LC3 is blocked by mutuation on p62/SQSTM1, ubiquitin-positive inclusion bodies were formed as in autophagy deficient cells [57]. p62/SQSTM1 is required for only selective turnover of polyubiquitinated macromolecules but not for LC3 lipidation or autophagasome formation and also basal level of autophagy was not affected by p62/SQSTM1 knockdown [58]. Since p62/SQSTM1 is incorporated into the autophagasomes and degraded in lysosomes, the decrease in p62/SQSTM1 has been considered as an autophagy indicator. Nonetheless, it has multiple domains interacting with various signaling molecules [48]. For instance,

it plays a role in the activation of pro-survival-antioxidant cell responses involving Nrf2 and KEAP1 through proteasomal degradation pathway [59].

Degradation and recycling of organelles, proteins and other components are important for the maintenance of cellular homeostasis. Beside autophagy, UPS is another route for cell to recycle excess, dysfunctional or damaged cellular contents. These two systems are essential together to maintain cellular homeostasis, therefore, they need to be properly coordinated. Both systems have common protein targets, and impairment of the UPS have been shown to induce autophagy [54].

1.5.4. Signaling Pathways Regulating Mammalian Autophagy

In nutrient and energy deprivation conditions autophagy is activated in order to recycle its own content and provide essential basic elements to survive. It is also activated as a cellular response against stressful conditions to destroy damaged organelles and other macromolecules. Therefore, it has direct interactions with several essential cellular signaling cascades.

The most studied pathway that regulates autophagy is the mTOR (mammalian target of rapamycin) pathway, also known as nutrient cascade, having two functional complexes; mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is a key cellular nutrient sensor and its activated form phosphorylates ULK1 and subsequently inhibits formation of autophagasome initiation complex [60]. It is inactivated in amino-acid deprivation conditions. The energy sensor adenosine mono-phosphate-activated protein kinase (AMPK) or rapamycin blocks its activity. Inactive mTORC1 cannot phosphorylate ULK1 which enables formation of autophagasome initiation complex. Class I PI3K/AKT is the upstream regulator of mTOR and its activation suppresses autophagy by blocking mTOR while class III PI3K/Beclin complex activates autophagasome formation [45, 61]. (Figure 1.3)

As an upstream regulator of mTORC1, class I PI3K/AKT, plays roles in protein synthesis, cell cycle, cell survival and cell growth. In nutrient rich conditions,

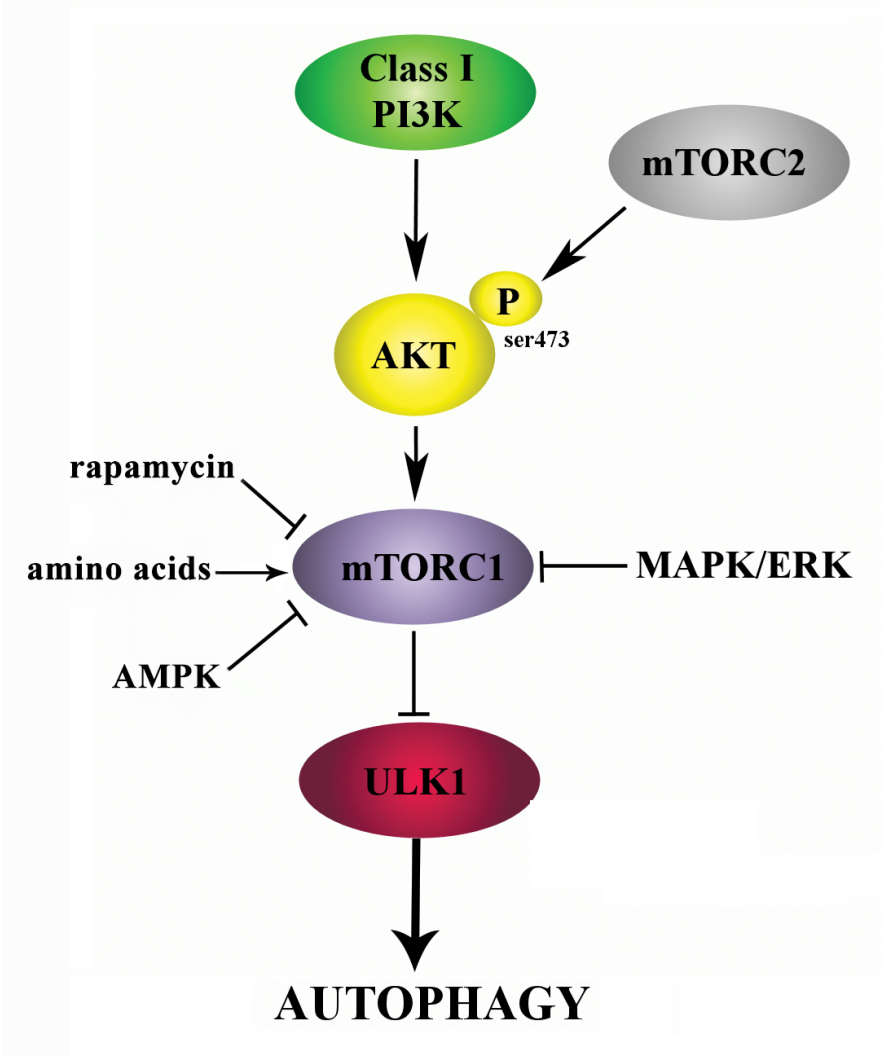

Figure 1.3. Signaling pathways regulating autophagy.

AKT is phosphorylated by mTORC2 on ser-473, and active AKT represses autophagy through mTORC1. In addition, inhibition of PI3K/AKT pathway is known to induce autophagy [45].

The studies have shown that nutrient starvation and oxidative stress activate ERK signaling together with autophagy, and amino acids suppress ERK1/2 and autophagy [45,62]. Furthermore, inhibition of MAPK/ERK pathway by specific inhibitors attenuated autophagy in various cell types [63, 64].

1.5.5. Autophagy in Chemotoxicity

Exposure to a toxin under specific conditions triggers a cascade of events in the cell and the cell elicits dose- and time-dependent responses. Toxic chemicals or their metabolites can interact and damage important macromolecules such as proteins and DNA and may trigger specific signaling pathways.

In many cases, autophagic response has been observed after toxic stimuli with different outcomes. Firstly, toxins may cause cellular stress leading to impairment of organelles such as mitochondria and ER which are cleared by autophagic process. Secondly, the cells can cope with the toxins by activating their defense mechanisms up to a certain threshold. If the cells cannot overcome the stress, they activate cell death mechanisms such as autophagy [65]. Autophagic cell death, also known as type II programmed cell death, is defined by the observations of increased autophagic markers in dying cells [66]. The general idea is that a moderate level of autophagic activity serves as a cell survival mechanism while high levels of autophagy results in death due to excess degradation of essential molecules or organelles [67]. Most of the toxicologic studies suggest the autophagy as a cell death mechanism since the inhibition of toxin-mediated autophagy usually increases cell viability [65, 68, 69].

Toxins mostly harm cells via generation of ROS which is another triggering effector of autophagy as damaged macromolecules and organelles are removed by this process. Mitochondria-derived intracellular ROS have been shown as activators of mitophagy to remove damaged mitochondria [70]. Oxidative stress-mediated nephropathic cystinosis has been implicated in induction of mitophagy [71]. Endoplasmic reticulum (ER), another target of toxins in the cells, senses accumulation of proteins that are not properly folded and this ER stress triggers autophagy [72, 73]. Autophagy induction in response to ROS encompasses multiple signaling cascades including MAPK/ERK pathway. Furthermore, p62/SQSTM1, which assigns cargo for autophagic degradation, also plays a role in activation of pro-survival antioxidant cell responses including the Nrf2 and the nuclear factor kappa B (NF-κB) [59]. Deficiency of p62/SQSTM1 or aggregation by accumulation are found to increase ROS levels and

subsequent tumorogenesis [74].

The role of autophagy in tumorogenic processes has been studied in certain extent. Autophagy is thought to take place in the tumorogenic process at several levels. First, any defect in autophagic system prevents clearance of damaged proteins/organelles and DNA which may lead to mutagenesis and tumorogenesis. Secondly, tumor cells suffer from nutrient deprivation and hypoxia due to rapid growth and proliferation, therefore they show higher levels of basal autophagic activity [46]. Based on this knowledge, the chemicals that inhibit autophagy are widely used as cancer therapeutics [75, 76].

1.6. OTA and Autophagy

As described earlier, OTA is one of the mycotoxins that induce cytotoxicity in different types of cells and tissues. There exist some evidence that corroborate the involvement of autophagy in OTA-mediated cytotoxicity.

In the first place, the signaling pathways MAPK/ERK and PI3K/AKT, also regulating autophagy, are activated by OTA exposure in proximal tubule epithelial cell line [43]. Even though the contributions of these pathways to OTA-mediated autophagy are not known, OTA-induced activation of MAPK/ERK pathway could activate autophagy whereas OTA-induced activation of PI3K/AKT pathway could suppress autophagy.

In the second place, oxidative stress a known inducer of autophagy has been observed in OTA-treated animals and cells in culture. In a number of study, it has been shown that OTA-induced ROS generation causes DNA damage, protein oxidation and lipid peroxidation [20, 21, 23]. Furthermore, nephropathic cystinosis has been implicated as an activator of autophagy through oxidative stress [71]. OTA, as a strong nephrotoxin, may trigger autophagy through oxidative stress in renal cells.

Moreover, a number of *in vivo* and *in vitro* studies have revealed that OTA induces cellular death mechanisms [19, 33]. These studies have focused on necrosis and

apoptosis; however autophagic cell death may also be involved in OTA-induced cytotoxicity. Furthermore, increased DNA adduct and giant cell formations with enlarged and multiple nuclei are also observed in OTA-mediated toxicity [28, 29]. At this step, autophagy may serve as a pro-survival mechanism leading to survival of genetically disrupted cells and subsequently to carcinogenesis.

2. PURPOSE

Ochratoxin-A (OTA) is a mycotoxin with nephrotoxic and carcinogenic potential. Toxicity of OTA has been associated with oxidative stress induction which is one of the triggers of autophagy. Autophagy is a bulk degradation process in which abundant or defective organelles and cytosolic, long-lived and aggregated proteins are destroyed in order to maintain cellular homeostasis. OTA-induced ROS production may result in disruption of proteins and organelles resulting in the activation of autophagy for clearence. Furthermore, OTA has been shown to induce apoptotic and necrotic cell death mechanisms. However, autophagic cell death in response to OTA exposure has not been studied yet. Last but not the least, the opposing autophagy regulatory MAPK/ERK and PI3K/AKT pathways are activated in response to OTA exposure.

Based on these circumstantial evidence, the aim of the study is to examine the involvement of autophagy in OTA-mediated toxicity in human renal proximal tubule epithelial cell line HK-2 in relation to MAPK/ERK and PI3K/AKT pathways.

3. MATERIALS

3.1. Cell Line

Human kidney proximal tubular cell line HK-2 (ATCC, Manassas) was used for this study.

3.2. Plasmid Construct

pBABEpuroGFP-LC3 (Addgene plasmid 22405, Cambridge, MA) was used in this study.

3.3. Chemicals, Plastic and Glassware

All solid and liquid chemicals used in this study were purchased from Sigma (USA), AppliChem (Germany) and Merck (Germany) unless it is declared in tables. Plasticware were purchased from TPP (Switzerland) and Sarstedt (Germany) while microcentrifuge tubes and tips were from Axygen (USA). All glassware were sterilized by autoclaving at 121°C for 20 minutes.

3.4. Cell Culture Chemicals and Reagents

Table 3.1. Chemicals and reagents used in cell culture.

Ochratoxin-A	O1877, Sigma-Aldrich, USA
Bafilomycin A1	B1793, Sigma-Aldrich, USA
Chloroquine	A2143, Applichem, Germany
U0126	9903, Cell Signaling, USA)
Wortmannin	9951, Sigma-Aldrich, USA
MG132	Sigma-Aldrich, USA
Dulbecco's Modified Eagle Medium (DMEM)	GibcoBRL, USA

Table 3.1. Chemicals and reagents used in cell culture (cont.).

Fetal Bovine Serum (FBS)	GibcoBRL, USA
0.25% Trypsin/0.913 mM EDTA (1X), Phenol Red	GibcoBRL, USA
Penicillin-Streptomycin Solution	GibcoBRL, USA
Hank's Balanced Salt Solution (HBSS)	Sigma-Aldrich, USA
Freezing Medium	10% DMSO FBS
PBS	GibcoBRL, USA
Lipofectamine2000	Invitrogen, USA
siRNA Transfection Reagent	Santa Cruz, USA
BECN1 (Beclin) and Control siRNA	Santa Cruz, USA
siRNA Transfection Medium	Santa Cruz, USA
Paraformaldehyde (4% in PBS)	GibcoBRL, USA
Acridine Orange	Santa Cruz, USA
Monodansylcadaverine	Sigma-Aldrich, USA

3.5. Protein Extraction and Quantification

Table 3.2. Chemicals used for protein extraction and quantification.

RIPA	150 mM NaCl 1% NP40 0.5% Sodiumdeoxycolate 0.1% SDS 50 mM Tris pH 7.4
Protease Inhibitor Cocktail	Roche, Germany
Phosphatase Inhibitor Cocktail	Roche, Germany
Bovine Serum Albumin (BSA) (2 mg/ml)	Thermo Scientific, USA

3.6. Western Blotting

Table 3.3. Buffers and solutions used for western blot analysis.

4X Protein Loading Dye	200mM TrisHCl pH 6.8 8% (w/v) SDS 40% (w/v) 100% Glycerol 4% (w/v) β-mercaptoethanol 50 mM EDTA 0.08% (w/v) Bromophenol Blue
12% Resolving gel	375 mM TrisHCl pH 8.8 0.1% (w/v) SDS Acrylamide:Bisacrylamide (12%/0.32% w/v) 0.05% (w/v) APS 0.005% (w/v) TEMED
Stacking gel	0.125 mM TrisHCl pH 6.8 0.1% (w/v) SDS Acrylamide:Bisacrylamide (4%/0.1% w/v) 0.05% (w/v) APS 0.0075% (w/v) TEMED
10X SDS Buffer	1% (w/v) SDS 1% (w/v) Tris Base 14.4% (w/v) Glycine
Transfer Buffer	1% (w/v) Tris Base 14.4% (w/v) Glycine
TBS-T	50 mM TrisHCl pH 7.4 150 mM NaCl %0.05 Tween-20
Blocking Solution	5% (w/v) skim milk powder TBS-T
Primary Antibody Solution	5% (w/v)BSA

Table 3.3. Buffers and solutions used for western blot (cont.).

	0.02% (w/v) Sodium Azide TBS-T
Secondary Antibody Solution	5% (w/v) skim milk TBS-T

3.7. Antibodies

Table 3.4. Antibodies used for western blot analysis.

Antibody	**Company**	**Dilution**	**Host**
Actin	Cell Signaling	1:3000	Mouse
LC3B	Sigma-Aldrich	1:5000	Rabbit
p62	Sigma-Aldrich	1:5000	Rabbit
Atg3	Cell Signaling	1:1000	Rabbit
Beclin	Cell Signaling	1:1000	Rabbit
Akt	Cell Signaling	1:1000	Rabbit
Phospho-Akt(S473)	Cell Signaling	1:1000	Rabbit
Erk1/2	Cell Signaling	1:1000	Rabbit
Phospho-Erk1/2(T202/Y204)	Cell Signaling	1:1000	Rabbit
Ubiquitin	Santa Cruz	1:4000	Mouse
Rabbit IgG, HRP	Cell Signaling	1:5000	Horse
Mouse IgG, HRP	Cell Signaling	1:5000	Goat

3.8. Kits

Table 3.5. Kits used for this study.

BCA Protein Assay Kit	Pierce, USA
XTT	Roche, Germany

Table 3.5. Kits used for this study (cont.).

CytoTox-Glo™ Cytotoxicity Assay	Promega, USA
NucleoBond Xtra Midi Kit	Macherey-Nagel, Germany

3.9. Equipments

Table 3.6. Equipments used for this study.

Autoclaves	Model MAC-601, Eyela, Japan Model ASB260T, Astell, UK
Balances	AY123, Satorius, Germany
Camera	Nikon CoolPix 5400, Japan
Centrifuges	Allegra X-22, Beckman, USA J2-21 Centrifuge, Beckman, USA
Cold room	Birikim Elektrik Soğutma, Turkey
Confocal Microscope	Leica SP5-AOBS, USA
CO_2 Incubator	MCO-18AC, Sanyo, Japan
CO_2 Tank	Genç Karbon, Turkey
Deep Freezers (-20°C)	Ugur, UFR 370 SD, Turkey
Deep Freezers (-80°C)	Sanyo Ultra Low, UK,Thermo Scientific, USA
Deep Freezers (-150°C)	Sanyo MDF-1156, UK,Thermo Scientific, USA
Dish Washer	Mielabor G7783, Miele, Germany
Flow Cytometer	FACSCalibur, Becton Dickinson, USA
Heat blocks	DRI-Block DB-2A, Techne, UK
Ice Machine	Scotsman Inc. AF20, Italy
Inverted Microscope	Z1 Axio Observer, Zeiss, USA
Laminar Flow Cabinet	Class II B, Tezsan, Turkey
Luminometer	Fluoroskan Ascent™ FL, Thermo Scientific, USA
Magnetic Stirrer	MS-H-S, Dragonlab, China
Micro-centrifuge	VWR CT15RE, Japan

Table 3.6. Equipments used for this study (cont.).

Microplate Reader	680, Bio-Rad, USA
Micro-Spin	VWR Galaxy Ministar, USA
Oven	Gallenkamp 300, UK
pH Meter	WTW, Germany
Pipettes	Axygen, USA
Pipettor	Brandtech Accu-jet, USA
Power Supply	Power Pac Universal, Bio-Rad, USA
Refrigerator (4°C)	Ugur, USS 300 DTK, Turkey
SDS-PAGE Transfer System	Bio-Rad, USA
Spectrophometer	NanoDrop1000, Thermo Scientific, USA
Stella	Raytest, Germany
Vortex	VWR, USA
Water Bath	Memmert, Germany
Water purification	WA-TECH UP Water Purification Sys. Germany

4. METHODS

4.1. Plasmids

4.1.1. Transformation

Competent DH5α strain of *Escherichia coli* was transformed by 100 ng of purchased plasmid DNA in a microcentrifuge tube. The cells were incubated on ice for 10 min, then they were heat shocked at 42°C for 90 seconds. After another incubation on ice for 5 min, 900 μl of Luria-Bertani broth (LB) were added onto sample and the bacteria were grown for 1 hour at 37°C as they would express antibiotic resistance. The bacteria were plated on ampicillin-containing LB agar plate and incubated overnight at 37°C. On the next day, a single colony was selected from plate by using a sterile pipette tip, and transferred into 80 ml of LB containing 100ng/ml of ampicillin.

4.1.2. Plasmid Isolation

Overnight inoculated 80 ml bacterial cultures were used for plasmid DNA isolation process. It was performed by using Roche Plasmid Midi Kit according to manufacturer's instructions. In the final step, concentration of eluted plasmids was measured by using NanoDrop Spectrophotometer (Thermo Scientific, USA). The plasmid DNA was stored in -20 °C freezer.

4.2. Cell Culture Techniques

A human-derived renal proximal tubule epithelial cell line HK-2 was purchased from American Type Culture Collection (Manassas, USA).

4.2.1. Thawing

The vials containing HK-2 cells were frozen in -150°C freezer . After complete melting, the cells were immediately transferred into 15 ml falcon tubes, and centrifuged at 300 g for 5 min. Then the supernatants were discarded, and the pellets were re-suspended in growth medium. Finally, the cell suspensions were transferred into cell culture plates.

4.2.2. Maintanence of Cells

HK-2 cells were cultured in Dulbecco's Modified Eagle Medium F-12 (DMEM/F-12) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were incubated in CO_2 incubator at 37°C in 5% CO_2 .

When the cells reached 70% confluency, the cells were washed with PBS and treated with 0.05% trypsin for 5 min at 37 °C. The cells were transferred into 15 ml falcon tubes, and centrifuged at 300 g for 5 min. After supernatant was discarded, the pellet was resuspended with growth medium. If a new experiment would be set, the cells were counted by using hemocytometer, and appropriate number of cells in growth medium was seeded into new plates.

4.2.3. Storage

The cells were washed with PBS once. After trypsinization with 0.25% Trypsin/0.913 mM EDTA for 5 min at 37 °C, the cells were collected in a 15 ml falcon tube with growth medium. Then the cells were centrifuged at 300 g for 5 min. Supernatant was discarded and the pellet was resuspended with FBS. Then appropriate amount of dimethyl sulfoxide (DMSO) was added to the final volume of 10%. Finally, $0.5x10^6$ cells were transferred into a cryovial and stored in -150 °C freezer.

4.2.4. Treatments

Before the treatments, the cells were transferred into assay medium (DMEM/F-12 containing 5% FBS, 100U/ml penicillin and 100 μg/ml streptomycin) and treatments were done in assay medium. OTA was dissolved in absolute EtOH at a concentration of 10 mM and stored at -80°C. Wortmannin (wort), U0126, Bafilomycin A (baf), Chloroquine (CQ) and MG132 were dissolved in DMSO at concentrations of 10 mM, 10 mM, 50 μM, 10 mM and 12,5 mM respectively, and they were stored at -20 °C. If the cells would be treated with an inhibitor, the cells were pretreated with inhibitor for 1 hour then exposed to appropriate amount of OTA.

4.2.5. Cell Viability Assay

The viability of the cells was measured using XTT Cell Proliferation Kit II (Roche) according the protocol provided by the company. Briefly the cells were seeded in 96-well cell culture plate as each well would have 7.5x10^3 HK-2 cells in complete medium. The cells were treated in triplicates with different concentrations of chemicals or vehicle as control. After appropriate time period, freshly prepared XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] solution was added onto cells, and the cells were incubated for an additional 4 hrs at 37 °C. The amount of colorimetric change was measured at 495 nm using a microplate reader (Bio-Rad, Model 680). Background absorbance values were measured at 655 nm, and substracted from the corresponding values at 495 nm. The results were expressed as the percentage of viability relative to the control, according to the following formula:

$$\%Viability = \frac{Absorbance\ of\ the\ sample\text{-}absorbance\ of\ the\ blank}{Absorbance\ of control - absorbance of the blank} \times 100$$

4.2.6. Cell Cytotoxicity Assay

OTA-induced necrotic cell death in HK-2 cells was examined using CytoTox-Glo™ Cytotoxicity Assay (Promega, USA) which measures the activity of proteases released from cells as a result of cell membrane damage. The cells were seeded in white-walled 96-well cell culture plate as each well would have 7.5x10^3 HK-2 cells in complete medium. On the next day, the cells were treated with different concentrations of OTA (0.1, 1 and 10 μM) in assay medium for 24 hrs. The luminescence signal was measured according to the manufacturer's recommendation using Fluoroskan Ascent™ FL (Thermo Scientific, USA). The total cell protease acitivity, representing 100% cell death, was measured by cell lysis using digitonin containing lysis reagent as provided in the assay kit. The percentage of necrotic cell death relative to the control cells was calculated according to the following formula;

$$\%Necrosis = \frac{Luminescence\ of\ sample\ -\ Luminescence\ of\ control}{Luminescence\ of whole cell - Luminescence\ of control} \times 100$$

4.2.7. Transient Transfection

HK-2 cells were plated on small coverslips at 30x10^3 cells/well in 24-well plate as each well contained 500 μl growth medium. On the next day, equal volume of plasmid and transfection reagent containing medium were mixed in a microcentrifuge tube. The summary of transfection mix is shown in Table 4.1.

Table 4.1. Transfection mix prepared for transient transfection.

Plasmid DNA	0.5 μg
Lipofectamine2000	1,25 μl
DMEM/F-12	100 μl

Transfection mix was incubated at room temperature for 30 min. The cells were washed with PBS for 3 times and DMEM/F-12 medium was added to the transfection mix as the final volume would be 200 μl/well in 24-well plate. The transfection mix was added onto the cells thoroughly. After 6 hrs of incubation in the incubator, the transfection mix was discarded, and fresh growth medium was added onto the transfected cells.

4.2.8. siRNA Transfection

For transfections in 96-well plate, 7.5x10^3 HK-2 cells per well were plated in 100 μl of growth medium without antibiotics . For transfection in 60 mm plates, 3.5x10^5 HK-2 cells were seeded in 3 ml of growth medium without antibiotic and incubated overnight. On the next, siRNA transfection mix was prepared according to manufacturer's instructors. Transfection mix was incubated for 30 min at RT and then, added onto the cells so that the final concentration of the siRNAs was 3 μM. After 6 hrs, equal volume of fresh growth medium containing twice the amount of FBS and antibiotics was added into each well and plates in the same amount of transfection medium. On the next day, the cells were washed with PBS and fresh medium was replaced. After 18 hours, the cells were transferred to assay medium containing 5% FBS and then, the treatments were carried out after 6 hours.

4.2.9. Acridine Orange (AO) and Monodansylcadaverine (MDC) Staining

Cells were seeded in 60 mm plates in 3 ml growth medium. Next day, the cells were treated with either ethanol as vehicle control or OTA in 5% FBS containing assay medium. After 12 hours, 3 μl of 1 mg/ml acridine orange solution or 50 mM MDC was added onto medium and the plate was shaken thoroughly. Then the cells were incubated in the incubator for 15 min. The cells were visualized under fluorescent microscope (Zeiss Z1 Axio Observer), confocal microscope (Leica SP5-AOBS) or the development of AVO was quantified by flow cytometric analysis by using flow cytometer (FACSCalibur).

4.3. SDS-PAGE and Western Blotting

4.3.1. Cell Lysis and Protein Extraction

The cells in 6 cm or 10 cm plates were washed once with PBS and then lysed with 150 μl or 400 μl RIPA buffer, respectively, supplemented with Phos-STOP phosphatase inhibitor cocktail and complete EDTA-free protease inhibitor cocktail (Roche). After incubating them for 5 min on ice, they were scrapped by using rubber policeman. The lysates were incubated on ice for 30 min and they were homogenized by passing through 25-gauge syringes for 5-6 times. After centrifugation at 14000 rpm for 15 min at 4°C, the supernatants were collected into new 1.5 ml eppendorf.

4.3.2. Quantification of Protein Lysates

Protein concentrations were measured by using BCA Protein Assay Kit (Pierce). Stock BSA /2 mg/ml) (Thermo Scientific 23210) solution was serially diluted in PBS to final concentrations of 0.125, 0.25, 0.5, 1 mg/ml to serve as BSA standards. Protein samples were also diluted in PBS (1:4). BCA working solution was prepared by mixing Reagent A and Reagent B in 50:1 ratio. Firstly 1X PBS (blank standard), 10 μl of diluted proteins samples and BSA standards were added into 96-well plate and then 200 μl of BCA working solution was added. After plate was incubated in 37°C for 30

min, the absorbance was measured at 562 nm on the plate reader.

Finally, blank measurement was subtracted from the measurements of BSA standards and the protein samples. Standard curve was obtained by using subtracted BSA standard measurements vs. concentrations. According to the standard curve the concentrations of protein samples were calculated.

4.3.3. Preparation of Protein Lysates

In order to prepare the protein lysates for gel loading, appropriate volumes of PBS and 4X protein loading buffer were added to the 20-25 μg of protein samples. Then the samples were boiled at 95°C for 5 min.

4.3.4. SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Western Blotting

12% resolving and 5% stacking polyacrylamide gel solutions were prepared and poured between the glass plates. After polymerization, 4 μl of Fermentas pre-stained marker and the samples were loaded on gel and run in 1X SDS buffer at 100 V until the samples were reached to the resolving gel and then the voltage was increased to 120 V. This process was continued until the front bromophenol dye reached to the end of the gels.

The separated proteins were transferred onto Polyvinylidene difluoride (PVDF) membrane (Millipore) with Bio-Rad wet transfer system. The membrane and the Watmann papers were cut according to the size of the gel. The membrane was immersed firstly in absolute methanol and then in distilled water. The gel and membrane were sandwiched between Watmann papers and sponges. Transfer were done in 1X transfer buffer and the duration of the transfer was arranged according to protein sizes at 4°C.

Membranes were washed in TBS-T once for 5 min and they were incubated in blocking solution (5% skimmilk in TBS-T) for 1 hour. After blocking the membranes

were washed thrice in TBS-T each for 5 min. Then the membranes were incubated in primary antibody solution at 4°C overnight. The next day, the membranes were washed in TBS-T three times and they were incubated in secondary antibody solution for 1 hour at RT. After three more washing steps, the membranes were incubated in LumiGLO solution (Cell Signaling Technologies) for 1 min at RT and then the chemiluminescent signals were captured using Stella digital bioimaging system (Raytest). Later, the band intensities were analyzed using ImageJ software (NIH, Bethesda).

4.4. Flow Cytometry Analysis

HK-2 cells ($3.5x10^5$) were seeded in 60 mm cell culture plates in growth medium, and then treated with the chemicals as described above. After 12 hrs, 3 μl of 1 mg/ml acridine orange solution was added onto the medium for 15 min in 37°C. Then the cells were trypsinized, and they were collected by centrifugation at 1200 rpm. The cell pellets resuspended in 1 ml of PBS on ice. The samples were analyzed twice on a BD FACSCalibur^{TM} system (Becton Dickinson Biosciences) using the CellQuest-Pro software.

4.5. Sample Preparation/Fluorescent Imaging

Transfected and OTA-treated cells on coverslips were washed twice with PBS and then fixed in 4% PFA in PBS for 10 min. After washing the slides twice with PBS again, the cells were treated with Diamidinophenylindoleamine (DAPI) for 5 min. Afterward, the cells were washed again with PBS twice, and the coverslips were mounted with a tiny droplet of mounting medium and inverted onto glass slide. The cells were visualized under Zeiss Axio Observer inverted fluorescent microscope. The cells were visualized at 40x magnification.

4.6. Confocal Imaging

Treated cells grown in 24-well plates containing glass coverslips were exposed to acridine orange solution at 1 μg/ml final concentration for 15 min. The medium was

discarded and the sample was washed with PBS for 1 time. Then immediately the coverslips were placed on glass slides to be analyzed under Leica TCS SP5 II upright confocal microscope. The cells were visualized at 200x magnification.

4.7. Statistical Analysis

All experiments were performed in at least three independent replicates, and quantitive data were analyzed using GraphPad Prism v5.04. The data are presented as mean ± SEM, and the statistical significance of the differences among the groups was calculated by one-way analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparison Test. "*" denotes statistical significance (*, $p<0.05$, **, $p<0.01$ and ***, $p<0.001$.)

5. RESULTS

5.1. Induction of Cytotoxicity in HK-2 Cell Line

Cytotoxic effect of OTA in time- and dose-dependent manner is shown in various *in vivo* and *in vitro* studies [19]. The kidney is considered as the target organ of OTA according to animal studies and epidemiologic studies in human populations. Therefore, HK-2, a human-derived renal proximal tubule epithelial cell line, was used in this study. In order to confirm OTA's cytotoxicity on HK-2 cells and to determine the cytotoxic dosage of OTA, color-based cell viability and luminescence-based cell cytotoxicity assays were performed.

5.1.1. Effect of OTA on Cell Viability

The effect of OTA on cell viability was examined by XTT assays. The cells were treated with either vehicle or OTA in appropriate concentration. After 24 and 48 hrs XTT assays were performed according to the manufacturer's instructions. In these assays, metabolically active cells convert the yellow tetrazolium salt XTT to an orange formazan dye whose absorbance is then measured with a plate reader. The results show that the cell viability decreases as the concentration of OTA and exposure time increases (Figure 5.1). The cell viability was significantly decreased to 65,3 $\pm$ 1,97 percent of control cells by the treatment of 10 μM OTA for 24 hrs while there was no significant effect up to 1 μM OTA treatment. OTA further decreased the cell viability to 39,6 $\pm$ 2,78 percent of control cells at 48^{th} hour.

5.1.2. Effect of OTA on Necrotic Cell Death

OTA-induced apoptotic cell death was shown in HK-2 cell line by the increased caspase activity after 10 μM OTA exposure [43]. In this study, OTA-induced necrotic cell death was detected by CytoTox-Glo$^{\text{TM}}$ assay. It measures the activity of proteases which are released from cell as a result of cell membrane damage. In this assay, the

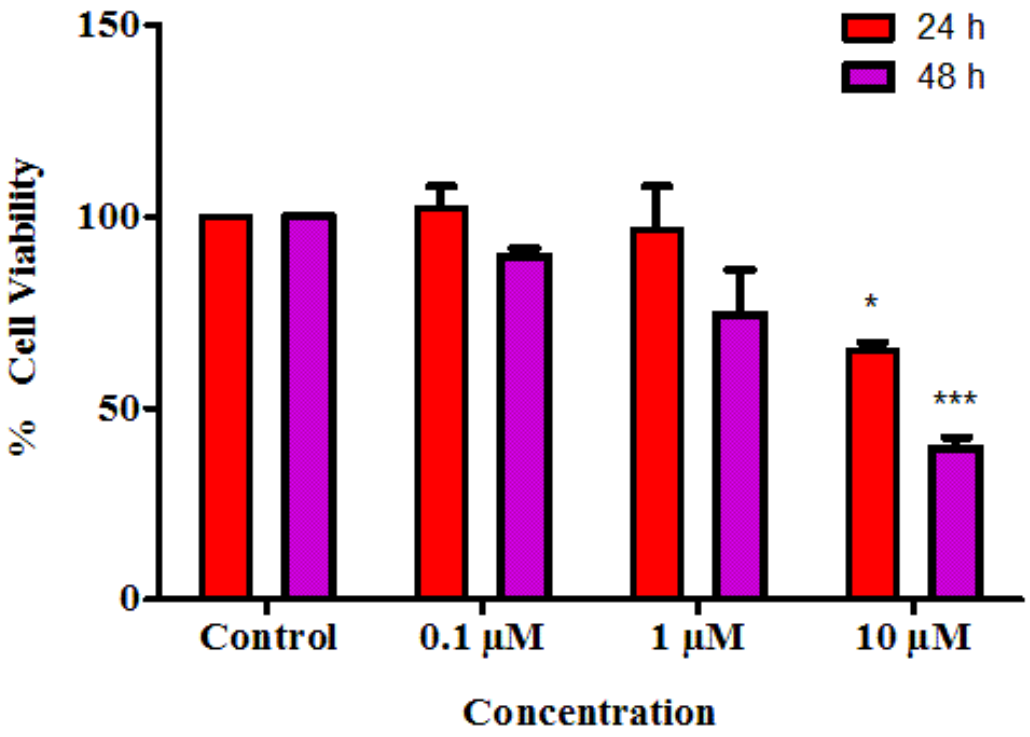

Figure 5.1. Effect of OTA on cell viability in HK-2 cells. Percentage of viability was calculated with the formula as described in the methods. The represented results plotted as means ± SEMs based on three experiments (*, $p<0.05$; and ***, $p<0.001$).

cells were treated with either vehicle or 0.1 to 10 μM OTA for 24 hrs. In CytoTox-Glo assay, each well provides its own control with the measurement of whole cell's proteases activity after addition of digitonin containing lysis buffer. The results show that cytotoxic effect of OTA is dose dependent (Figure 5.2). Necrotic cell death was significantly increased to 13,11 ± 3,86 percent after 24 hrs of 10 μM OTA treatment while the lower concentrations of OTA did not increase necrotic cell death significantly.

5.2. Dose- and Time-Dependent Effect of OTA on Autophagic Process in HK-2 Cell Line

Autophagy is known to be activated in response to multiple forms of cellular stress such as oxidative stress, nutrient deprivation, hypoxia damaged organelles [45,

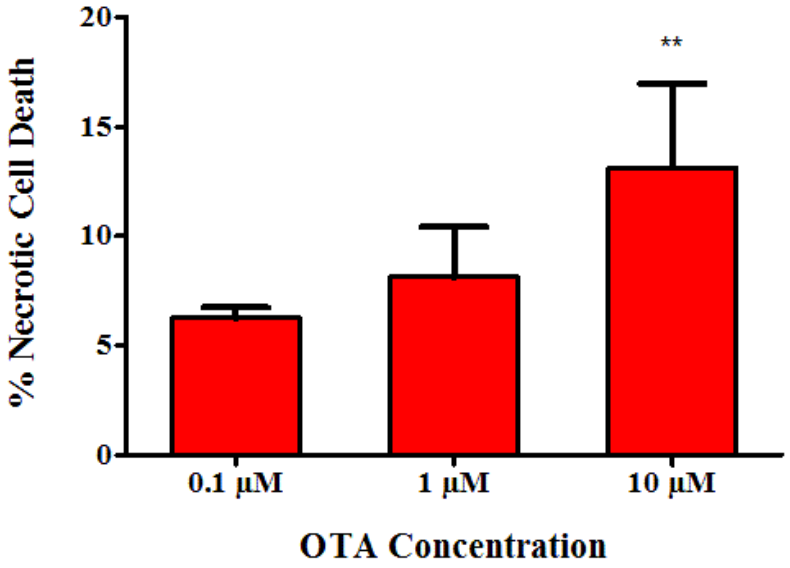

Figure 5.2. Effect of OTA on necrotic cell death in HK-2 cells. Percentage of necrotic cell death was calculated by using the formula as described in methods. The represented results plotted as means ± SEMs based on three experiments (**, $p<0.01$).

70, 77–79]. Excess autophagy result in autophagic cell death which is defined as type II programmed autophagic cell death [45, 67]. A dose- and time-dependent induction of autophagy was examined by the expression of autophagic proteins and formation of autophagasomes during progression of OTA-induced injury to HK-2 cells.

5.2.1. Analysis of Dose Dependent Autophagy Induction in Response to OTA

An important step for elongation of autophagasomal membrane is the conjugation of cytosolic LC3-I (18 kDa) with phosphatidylethanolamine (PE) producing LC3-II (16 kDa) [53]. The conversion of LC3-1 to LC3-II (faster migration on SDS gels) is considered as a reliable marker of autophagy, and this conversion could be observed by Western blot analysis [48]. In addition to LC3, p62/SQSTM1 is another autophagy marker, since it has been reported to directly bind to LC3, incorporate into autophagosome, and finally degraded by autophagy [48, 56].

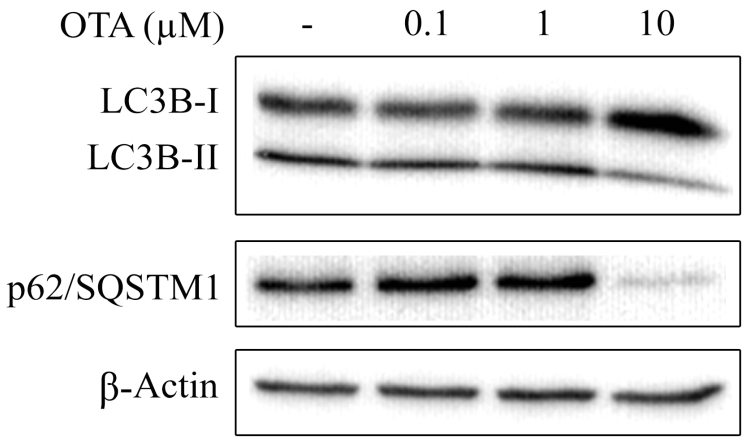

Figure 5.3. Dose-dependent decrease in autophagasome formation after OTA treatment in HK-2 cells. Western blot analysis of extracts from HK-2 cells which were treated with appropriate concentration of OTA or vehicle (EtOH) for 24 hrs.

In order to examine if OTA induces autophagy in HK-2 cells, the cells were treated with different doses of OTA for 24 hrs and the expression of levels of LC3B and p62/SQSTM1 were analyzed by Western blot analysis. The results demonstrate that conversion of LC3-I to LC3-II protein was decreased in response to 10 μM OTA after 24 hrs (Figure 5.3). On the other hand, there is a great reduction in p62/SQSTM1 amount after 10 μM OTA exposure indicating high autophagic vesicle turnover. As p62/SQSTM1 and LC3B-II are degraded in lysosomes after autophagasome fusion, the decrease in p62/SQSTM1 and LC3-II amount could be the consequence of increased autophagic flux and faster degradation.

In order to examine if the decline in LC3B-II amount is due to fast degradation, specific inhibitors were used to block autophagic flux. Bafilomycin A1 (Baf) inhibits fusion of autophagasome with lysosome, and chloroquine (CQ) inhibits acidification in vesicles and degradation, respectively [48]. First, the cells were pre-treated with 50 nM Baf or 10 μM CQ 1 hour before OTA treatment. Then they were subjected to 10 μM OTA or vehicle (EtOH) for 12 hrs. The results support the previous observations

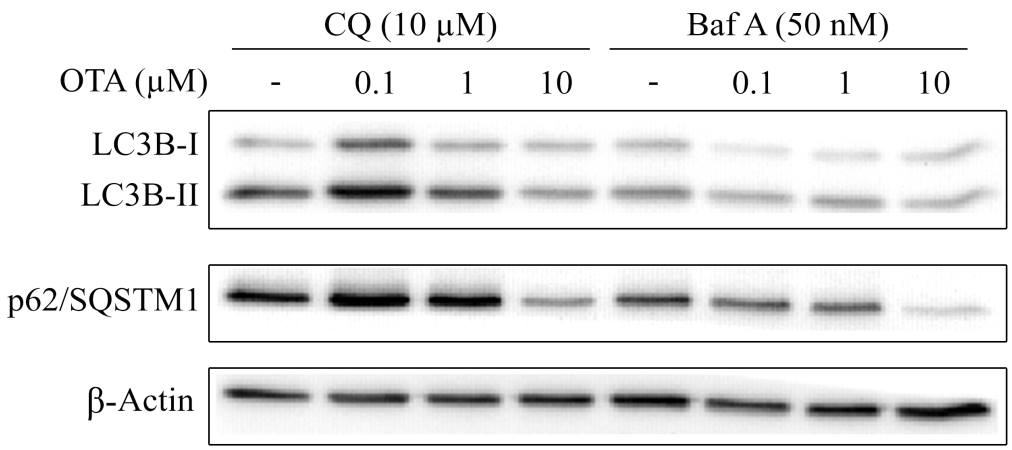

Figure 5.4. OTA-induced suppression of basal autophagy in HK-2 cell line. Western blot analysis of extracts from HK-2 cells pre-treated with either 10 μM CQ or 20 nM Baf for 1 hour, then they were subjected to 10 μM OTA for 12 hrs.

since LC3-II remains significantly lower in the cells co-treated with Baf or CQ than the control cells treated with only CQ or Baf (Figure 5.4). LC3B-II amount was also higher in the cells exposed to lower dosage of OTA. These results suggest that the decrease in LC3B-II amount after exposure to 10 μM OTA for 24 hrs is due to autophagy suppression rather than increased autophagic flux.

5.2.2. Analysis of Time-Dependent Autophagy Induction in Response to OTA

Higher doses of OTA are known to be cytotoxic for the cells and activate cell death mechanisms. In our work, a decrease in cell viability and an increase in necrotic cell death were observed. Moreover, after 24 hrs of OTA exposure, higher numbers of floating cells were detectable under the light microscope. A number of study indicates that autophagy first acts as a pro-survival mechanism, while excess amount of autophagy results in autophagic cell death. Based on these information and observations, autophagy could be activated in early hours of OTA treatment and the viable cells could suppress autophagic process to avoid autophagic cell death at later time

points. In order to test this hypothesis, a time course analysis of the changes in the expression of autophagy-related proteins by Western blotting in response to 10 μM OTA was performed.

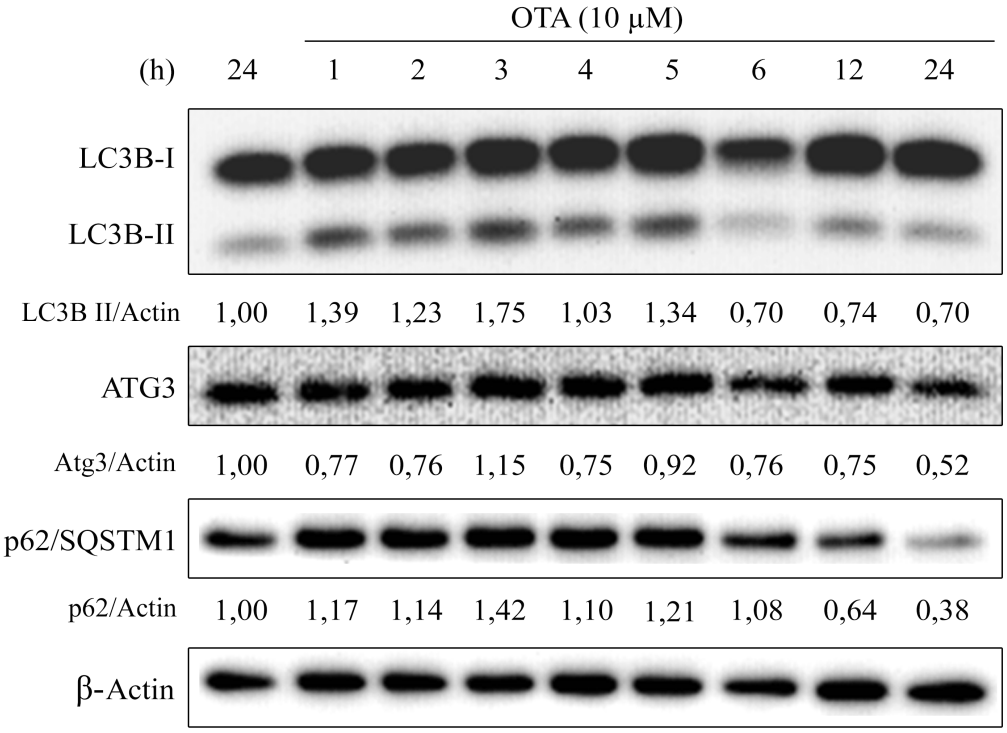

Figure 5.5. Transient activation of autophagy by OTA in HK-2 cell line. Western blot analysis of extracts from HK-2 cells treated with 10 μM OTA for different time periods or vehicle for 24 hrs.

The cells were treated with 10 μM of OTA or vehicle, and they were lysed at different time intervals from 1 to 24 hrs. The Western blot results indicate that LC3-II conversion began very early at 1 hour of OTA treatment, continues up to 5 hrs and gradually decreased after 6 hrs (Figure 5.5). Moreover, Atg3 catalyzing the conjugation of LC3B-I and PE, was used as another autophagic marker [51]. It also begin to decrease along with p62/SQSTM1 after 6 hrs. These results suggest that autophagy is triggered in early hours of treatment and then suppressed after 6 hrs. Early up-regulation of p62/SQSTM1 could be the result of oxidative stress since it also regulates antioxidant cell responses, and subsequent decline of p62/SQSTM1 could be the consequence of

overactivated UPS. However further experiments are required to verify this hypothesis.

5.2.3. Examination of GFP-LC3 Puncta Formation After OTA Treatment

The recruitment of LC3-II to autophagic vesicles was further tested by expression of GFP-LC3 in HK-2 cells in response to 10 μM tretament at early time points. When autophagy is not induced, GFP-LC3 proteins are found as diffused throughout the cell. On the contrary, if autophagy is induced, LC3 proteins come together to form autophagasome which lead to visualization of dot formations under fluorescence microscope. The cells were transfected with plasmids expressing GFP tagged LC3 protein and then, exposed to 10 μM OTA for different time intervals or to vehicle for 6 hrs. The cells were incubated in HBSS for 2 hrs to mimic starvation conditions serving as a positive control. According to the observations under fluorescence microscope, there is an increase in dot formations in HK-2 cells after exposure to OTA at early time points (Figure 5.6). The highest number of dot formation was observed in the cells that were exposed to OTA for 1 hour similiar to the cells incubated in HBSS. The least amount of dots were detected in the cells treated with OTA for 6 hrs. The results support the Western blot analyses with early activation of autophagy probably as a pro-survival mechanism.

5.2.4. Examination of AVO Formation After OTA Treatment

Autophagy induction at early hours was also revealed by using specific dyes to detect acidic vesicular organelles (AVO) such as autophagasomes and lysosomes. Acridine orange (AO) and monodansylcadaverine (MDC) were used to monitor autophagasomes; however, they label later stages of degradation process together with lysosomes [48]. At neutral pHs, AO is a green fluorescent molecule. When the dye is accumulated in acidic organelles, it is protonated and forms aggregates which emit red fluorescence. MDC is also an autofluorescent marker which preferentally accumulates in acidic vesicular organelles. AO and MDC staining of the live cells were employed to visualize AVO in HK-2 cells exposed to vehicle or 10 μM OTA for 2 hrs (Figure 5.7). As the positive control, one set of HK-2 cells were incubated in HBSS for 2 hrs. After incubating the

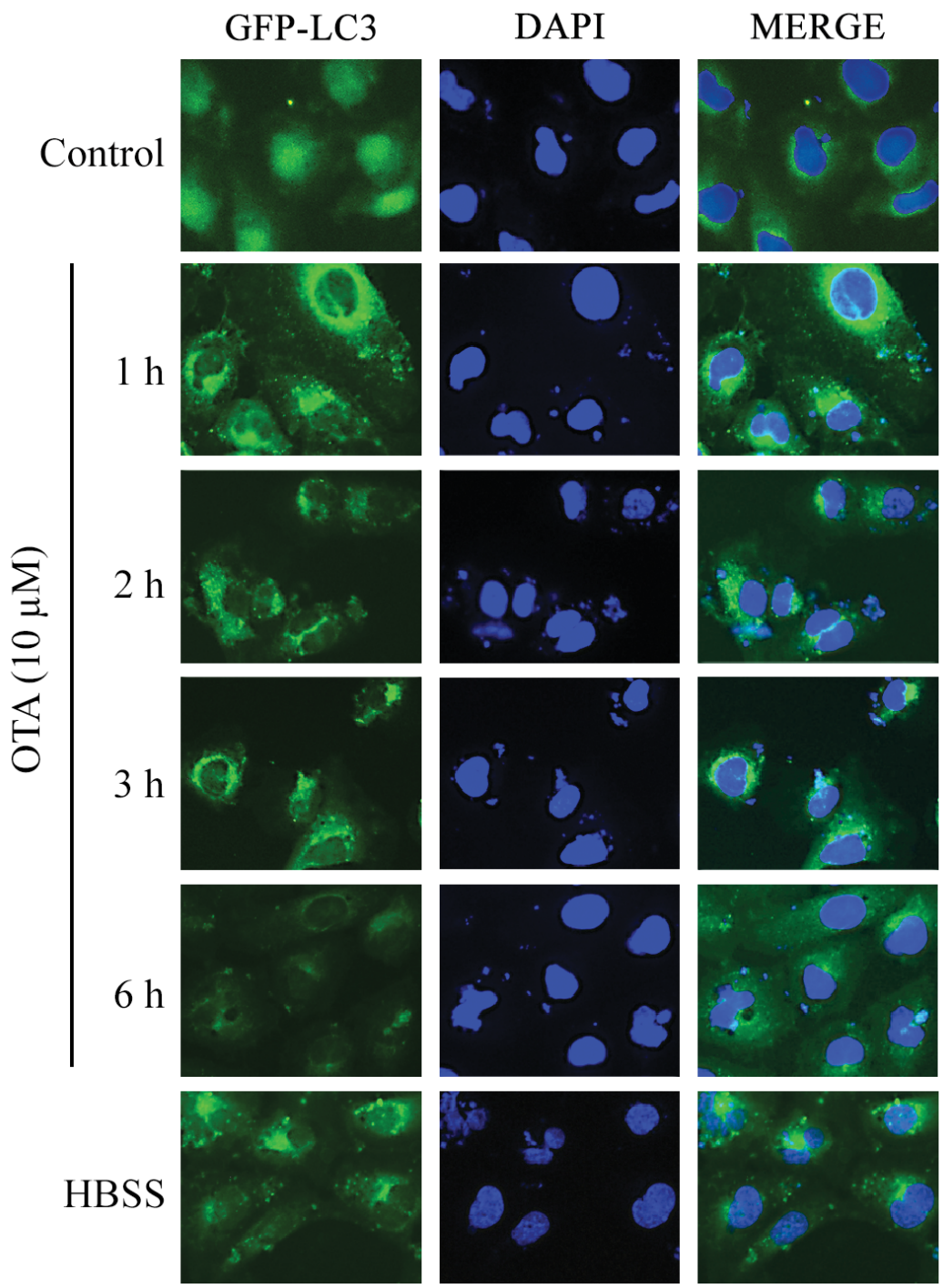

Figure 5.6. Early activation of autophagasome formation upon treatment with OTA in HK-2 cell line. The cells expressing GFP-LC3 were subjected to 10 μM OTA for different time periods. Punctate formation was observed under fluorescence microscopy.

cells with 1 μg/ml AO or 50 μM MDC for 15 min, the cells were immediately analyzed under fluorescence microscope. The results show that there were augmentation in red compartments in OTA-treated cells with respect to control cells but not as much as seen in the positive control cells. In MDC staining, control cells show more diffuse staining while the cells treated with OTA exhibit punctuate vesicular staining. The cells incubated in HBSS showed much more punctuate vesicular staining.

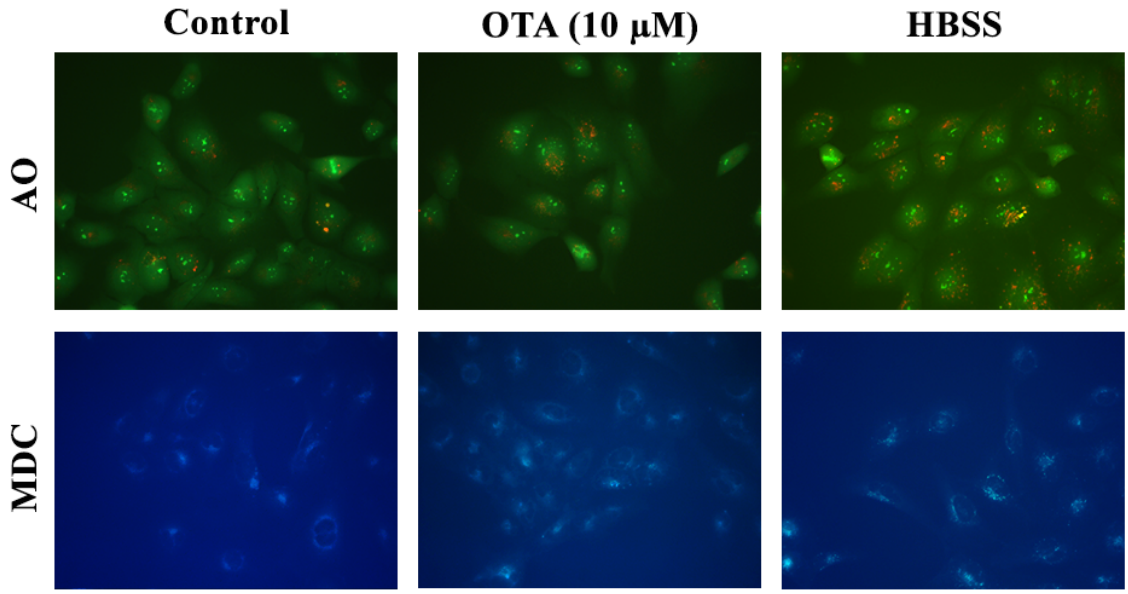

Figure 5.7. Increase in AVO formation in early hours of OTA treatment in HK-2 cell line. The cells were incubated in medium containing 10 μM OTA or in HBSS for 2 hours. AVO formation was observed after incubation in AO and MDC for 15 min under fluorescence microscopy.

5.3. Analysis of Involvement of MAPK/ERK and PI3K/AKT Pathways in OTA-mediated Autophagy Suppression

5.3.1. Effect of OTA on ERK1/2 and AKT Activation

The previous experiments demonstrated that autophagy is triggered by OTA in early hours of treatment while it is suppressed at later hours. According to the literature, OTA activates cellular signaling cascades including MAPK/ERK pathway [15,39].

Furthermore, OTA was shown to increase phosphorylation level of AKT protein in HK-2 cells [43]. PI3K/AKT pathway is known as a negative regulator of autophagy through mTOR activation [42]. Since the signaling pathways that regulates autophagy have roles in OTA-mediated toxicity, differential induction of autophagy could be the consequence of activationf of these signaling pathways. In order to observe the association between the differential activation of autophagy and these signaling pathways in OTA-induced toxicity, time-course analysis of ERK1/2 and AKT phosphorylation was performed by Western blotting (Figure 5.8). According to the results, OTA induced phosphorylation of ERK1/2 in earlier hours than phosphorylation of AKT at ser473. Furthermore, the activation of PI3K/AKT pathway sustained up to 24 hrs that could be the reason of autophagy suppression.

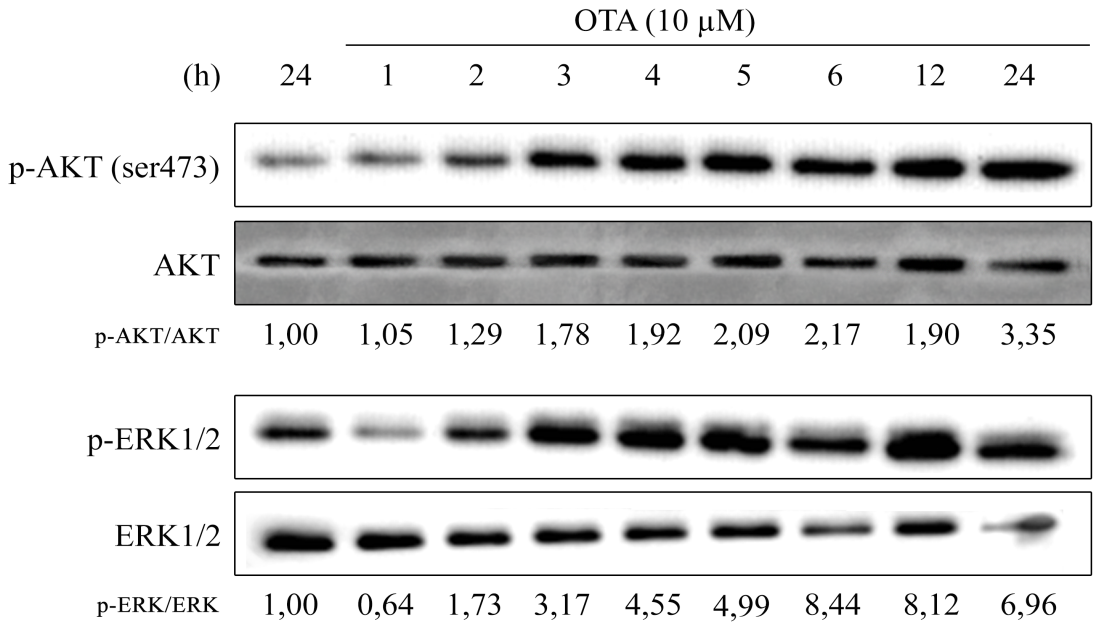

Figure 5.8. OTA-mediated activation of MAPK/ERK and PI3K/AKT pathways in HK-2 cell line. Western blot analysis of extracts from HK-2 cells treated with 10 μM OTA for different time periods or vehicle for 24 hours.

5.3.2. Effect of the MAPK/ERK and PI3K/AKT Pathways on OTA-mediated Autophagy Suppression

Both MAPK/ERK and PI3K/AKT pathways have been shown to regulate autophagy. The MAPK/ERK pathway regulates autophagy positively, while the PI3K/AKT pathway is a negative regulator of autophagy [45]. In order to further examine the relation of autophagy with MAPK/ERK and PI3K/AKT pathways in OTA treated cells, activation of autophagy was examined using selective chemical inhibitors by Western blot analysis. In this set of experiments, Wortmannin (Wort), PI3K inhibitor and U0126, MAPK/ERK inhibitor were used.

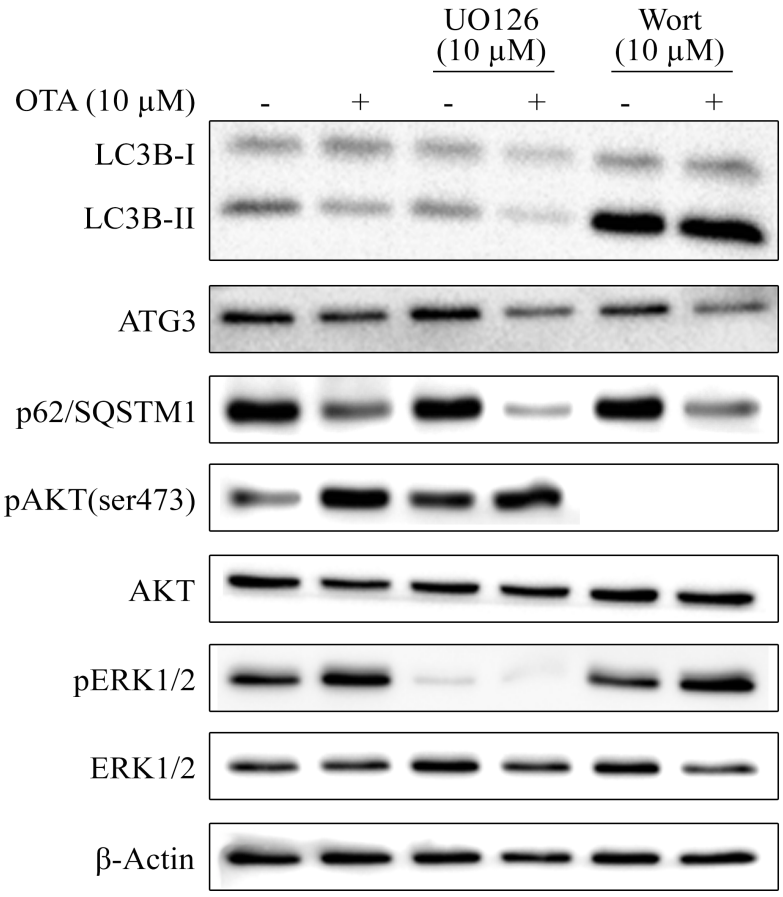

Figure 5.9. Involvement of PI3K/AKT and MAPK/ERK pathways in OTA-mediated autophagy in HK-2 cell line. Western blot analysis of of extracts from HK-2 cells pre-treated with Wort or U0126 1 hour before 10 μM OTA treatment.

The cells were pre-treated with 10 μM Wort or 10 μM U0126 1 hour before OTA treatment (Figure 5.9). Then, the cells were subjected to 10 μM OTA exposure for 12 hrs. As demonstrated in the Western blot results, inhibitors successfully blocked phosphorylation of ERK1/2 and AKT. Wortmannin not only inhibits class I PI3K which is positive regulator of AKT and negative regulator of autophagy, but also class III PI3K which is required for autophagasome formation. The western blot results demonstrate that it successfully inhibited class III PI3K since it blocked the phosphorylation of AKT [80]. When PI3K/AKT pathway is inhibited, autophagy is up-regulated in response to OTA after 12 hrs. Morphological examination of the cells under light microscope also indicates an increase in vesicular formations when PI3K/AKT pathway is inhibited (See Figure A.1 in Appendix A). Moreover, the presence of OTA seems to be ineffective in suppression of autophagy when AKT phosphorylation is blocked. This result provide strong evidence that autophagy is suppressed by activation of PI3K/AKT pathway at later times under the OTA exposure. However, OTA further decreased LC3-II amount in the presence of U0126 which implies that OTA-induced autophagy suppression is ERK1/2 independent. Surprisingly, p62/SQSTM1 levels were decreased no matter what inhibitor was used as long as OTA was present.

5.3.3. Effect of the PI3K/AKT Pathway on OTA-mediated AVO Formation

Inhibitory effect of PI3K/AKT pathway on autophagy was further confirmed in OTA-treated cells by the AO staining (Figure 5.10). Moreover, CQ, a flux blocker, was also used as a negative control since it inhibits acidification of the autophagolysosomal vacuoles [48]. In this experiment, the cells were pre-treated with 10 μM Wort or 10 μM CQ for 1 hour, then exposed to 10 μM OTA . After 12 hrs, the cells were immediately prepared for fluorescent confocal microscopy and analyzed (Figure 5.10). Moreover, the development of AVO was quantified by flow cytometric analysis (Figure 5.11). Untreated control cells showed predominantly green fluorescence with lower red fluorescence whereas cells treated with 10 μM OTA displayed considerable red fluorescence as it was also verified in flow cytometric analysis with significant increase in red:green intensity ratio. Furthermore, inhibition of PI3K/AKT pathway increased red compartments significantly and, OTA increased AVO formation further when PI3K/AKT

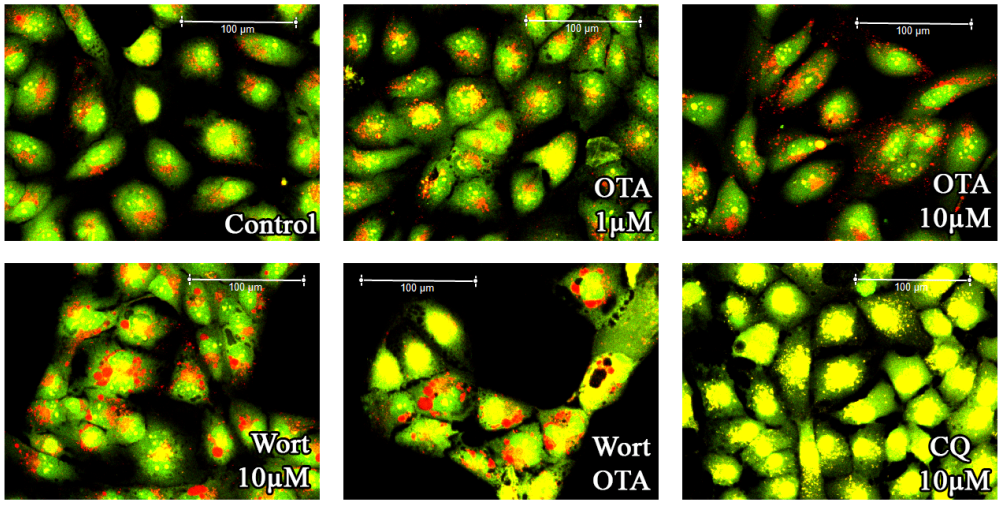

Figure 5.10. Effect of OTA on AVO formation in the presence of PI3K inhibitor Wortmannin. The cells pre-treated with inhibitors and then treated with 10 μM OTA for 12 hours. Fluorescent confocal microscopy analysis of the OTA-treated cells was performed after incubation in AO for 15 min.

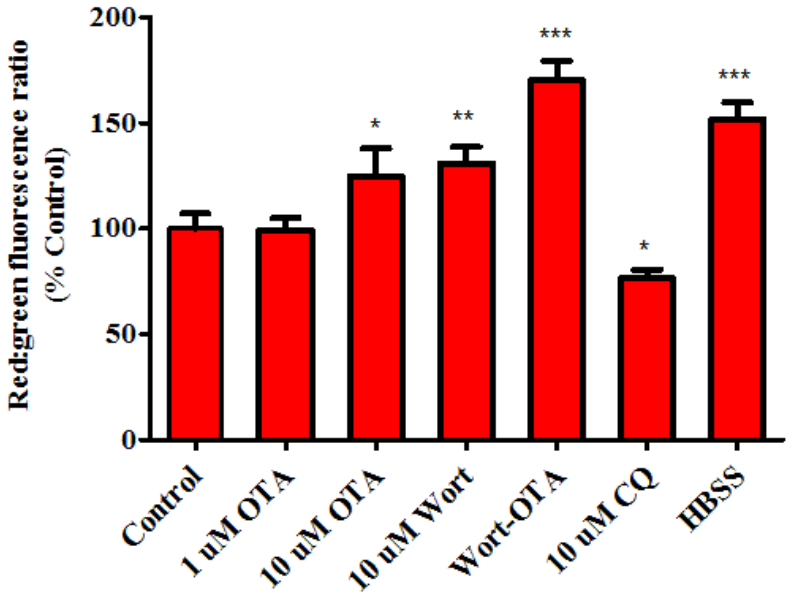

Figure 5.11. Flow cytometric analysis of OTA-induced AVO formation. The red:green fluorescence ratio was calculated using FlowJo software. The represented results plotted as means ± SEMs based on three experiments (*, $p<0.05$; **, $p<0.01$ and ***, $p<0.001$).

pathway is blocked. Positive control cells incubated in HBSS for 2 hrs showed high red intensity as expected, while negative control cells treated with CQ had lower red intensity with respect to untreated cells.

5.4. Effect of OTA on Ubiquitin Proteasome System

5.4.1. Effect of OTA on Steady-State Ubiquitinylated Protein Levels

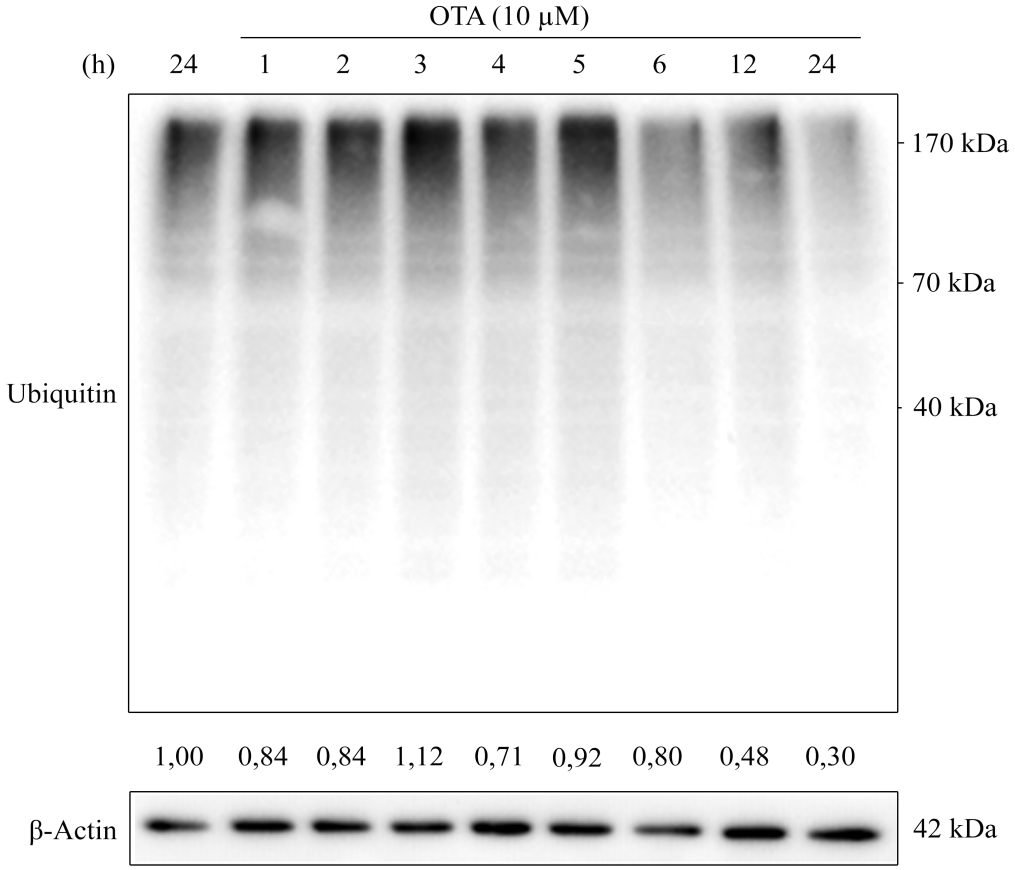

Figure 5.12. Time-dependent effects of OTA on steady-state levels of ubiquitinylated proteins. Western blot analysis of extracts from HK-2 cells treated with 10 μM OTA by using antibody against ubiquitin.

p62/SQSTM1 is a protein used frequently as a marker of induced autophagy. Previous Western blot analyses demonstrated that p62/SQSTM1 was decreased by 10 μM OTA treatment after 6 hrs when the autophagy is suppressed in HK-2 cells which

is generally correlated with autophagy induction. p62/SQSTM1 is also a well known substrate of ubiquitin-proteasome system (UPS).

In order to test if this decrease could be due to UPS, steady state levels of ubiquitinated proteins were analyzed by Western blot using antibody against ubiquitin after treating the cells with 10 μM of OTA for different time points (Figure 5.12). The results showed that OTA significantly decreased ubiquitinylated protein amount over time especially after 6 hrs of OTA exposure. The results suggest that OTA may cause sequential activation of autophagy and UPS and that the decrease in p62/SQSTM1 levels after 6 hrs of OTA exposure may be because of the UPS activation.

5.4.2. Effect of UPS Inhibition on Autophagy in Response to OTA Exposure

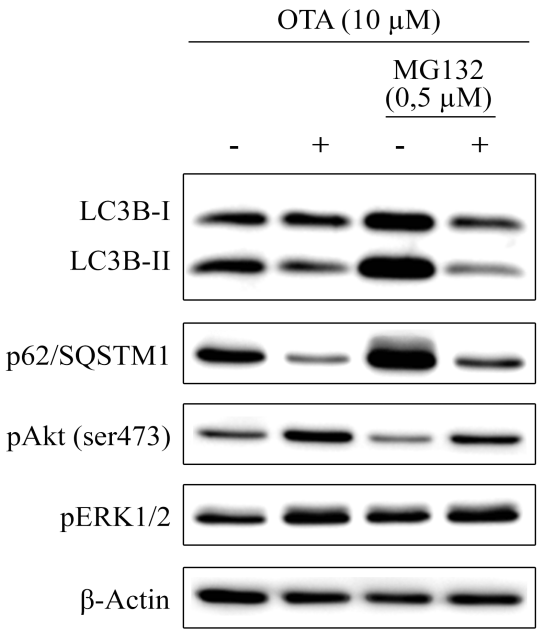

Figure 5.13. The effect of the UPS inhibitor MG132 on OTA-induced autophagy. Western blot analysis of extracts from HK-2 cells pre-treated with 0.5 μM MG132 for 1 hour and then, subjected to 10 μM OTA for 24 hrs.

It has been shown that inhibition of UPS triggers autophagy in several studies [54]. In order to probe the possible interplay between the autophagy and UPS under OTA exposure, the expression levels of autophagic markers and the activation

status of the ERK and AKT were studied in the presence of UPS inhibitor MG132 (Figure 5.13). It blocks the chymotrypsin-like activity of the 26S proteasome complex and also, lysosomal protease activity [81]. Thus, MG132 can inhibit degradation of autophagasomes in lysosomes too. The results indicate that inhibition of proteasome system and lysosomal proteases enhanced accumulation of LC3-II and p62/SQSTM1 under normal conditions as expected. However, the presence of OTA blocked this accumulation suggesting that OTA suppresses autophagy upstream of autophagosome formation at later time points. Even though p62/SQSTM1 was again diminished in OTA-treated cells in the presence of MG132, the levels were higher than that of cells treated with only OTA. Thus, p62/SQSTM1 is most probably degraded faster by UPS under OTA exposure. However, this postulation should be verified by further experimentation.

6. DISCUSSION

OTA is a widespread secondary metabolite of fungus and food contaminant having various toxicological effects on tissues especially on the kidney [12, 14, 16–18]. Despite much of evidence pointing towards genotoxic, carcinogenic, and nephrotoxic effects of OTA, the mechanism of its toxicity has not been elucidated yet. The present study provides evidence for the involvement of autophagy and the relating signaling pathways in OTA toxicity in human renal proximal tubule epithelial HK-2 cell line. The autophagic response to OTA was examined by specific hallmarks including conversion of LC3-I to LC3-II, the levels of other critical autophagic proteins p62/SQSTM1 and Atg3, formation of punctated autophagasome-associated LC3-II and, acidic vesicular organelles including autophagolysosomes. These studies demonstrated that autophagy is an immediate response to OTA toxicity in HK-2 cells probably to adapt OTA-induced stress conditions. However, it is suppressed by the activation of PI3K/AKT pathway at later time points.

Activation of autophagy has essential role in protecting renal proximal tubular cells from many stresses including cystinosis and ischemia [71, 82]. The cells could utilize this mechanism at different basal levels to renew their organelles and activate this mechanism in response to stress conditions such as nutrient depletion, hypoxia, oxidative stress or toxic stimuli [45, 70, 77–79]. Based on these information, autophagy may be activated as a cell protection mechanism toward OTA-induced injury in proximal tubule epithelial cells. The cells may utilize autophagy as pro-survival mechanism under oxidative stress while excess amount of autophagy may result in autophagic cell death caused by excessive degradation of essential organelles necessary for cellular homeostasis [67]. In this study, it was shown that OTA decreases cell viability and increases necrotic cell death in dose- and time-dependent manner in HK-2 cells. Previous studies from our laboratory have also shown the apoptotic effects of OTA [43]. Nevertheless, there was no study investigating the autophagic cell death in response to OTA exposure.

In the present study, autophagic response under OTA exposure was examined by the visualization of specific protein markers that participate in the development of autophagy. One of the marker of autophagy induction is the conversion of LC3-I to its lipid-conjugated form LC3-II which is required for double-membraned autophagasome formation [48]. This conjugation enables idetification of the conversion by an electrophoretic mobility shift in SDS-PAGE gels, and by a fluorescent staining pattern which changes from diffuse cytosolic to punctate form with the help of GFP tagged LC3 protein. Using both experimental approaches, it was demonstrated that LC3B-II conversion is initiated at the early hours of OTA exposure and continues up to 5 hrs of treatment. However this increase diminishes in the subsequent time points conciding with the AKT activation. Moreover, Atg3 and p62/SQSTM1 also displayed a similiar patterns of expression in response to OTA. When autophagy is induced, p62/SQSTM1 levels are supposed to attenuate due to degradation by autophagy machinery. On the contrary, we observed that the levels of p62 were somehow increased when the autophagic activity peaked. Moreover, it was significantly down-regulated when autophagic activity was reduced upon OTA treatment.

The decrease in LC3B-II amount at later hours of treatment was also shown to be independent from fast autophagic flux by co-treating the cells with specific inhibitors; Baf and CQ. Baf is a specific inhibitor of vacuolar H^+ ATPase blocking autophagasome and lysosome fusion by raising the lysosomal pH [83]. CQ attenuates lysosomal acidity and block degradation of autophagasomes [48]. In our studies, we found that even the autophagasome degradation was inhibited by these inhibitors, there was lower amount of LC3B-II in the cells treated with higher dosage of OTA than the untreated cells. This result suggests that OTA possibly activates an upstream autophagy inhibitory pathway at later time points.

OTA was shown to increase ROS level and suppress antioxidant defense mechanism in a number of study [21, 22, 24, 26, 27]. During starvation, ROS was shown to modulate cysteine protease Atg4 which cleaves C-terminus of LC3 to form LC3-I [84]. Another role of Atg4 in autophagy regulation is deconjugation of PE from LC3 [85]. If Atg4 is upregulated by ROS, it may also act as converting LC3-II proteins to LC3-I

resulting decrease in LC3-II as in our results.

In a bountiful study, OTA was found to activate PI3K/AKT and MAPK/ERK pathways which are known to regulate autophagy [15, 38, 39, 43]. The most studied signaling cascade regulating autophagy is the mTOR pathway which is inactivated in starvation conditions [45,61]. mTOR is regulated by the PI3K/AKT pathway essential for cell growth and survival [60, 61]. mTOR has two complexes which are mTORC1 and mTORC2. mTORC1 is a key cellular nutrient sensor activated by aminoacids [60, 82]. Moreover, energy sensor AMPK, rapamycin, and starvation conditions blocks its activity. Inactivation of mTORC1 results in dephosphorylation of ULK1 required for initiation of autophagasome formation, and subsequently stimulation of autophagy [51, 86]. mTORC2 also regulates autophagy by activating AKT via ser473 phosphorylation [87].

Another autophagy regulatory pathway is the MAPK/ERK pathway shown to be activated in response to mitogenic stimuli and stress [37]. MAPKs are serine/threonine kinases that mediate responses to various extracellular stimuli which lead to a sequential phosphorylation cascades. ERK is associated with cell proliferation, migration, differentiation and cell death [88]. It has also been shown to play role in regulating cell death through apoptosis and autophagy [62]. Contrary to PI3K/AKT, the MAPK/ERK pathway is mostly implicated as positive regulator of autophagy, and inhibiton of MAPK/ERK pathway has been shown to suppress autophagic process in a number of study [62, 89–91].

The present study demonstrates that the phosphorylation of ERK1/2 and AKT increases in a time- and dose-dependent manner in response to OTA. The involvement of the MAPK/ERK and PI3K/AKT pathways on OTA-mediated autophagy was analyzed by using specific inhibitors. PI3K inhibitor Wort causes accumulation of LC3B-II even in the presence of OTA. In line with these findings, inhibition of PI3K/AKT pathway increased AVO formation. In contrast to PI3K/AKT inhibition, MAPK/ERK inhibition pathway decreased basal autophagy levels. Further decrease was observed in response to OTA which suggests that OTA-induced autophagy regula-

tion is MAPK/ERK independent at later hours of treatment. These findings taken together supports that OTA-induced autophagy at early hours of exposure is suppressed by the trailing activation of PI3K/AKT pathway.

Being essential for cell survival, growth and proliferation, PI3K/AKT has been shown to be deregulated in several human cancers and its constitutive activity correlated with tumor progression in different cell types [92]. In this study, it is demonstrated that inhibition of PI3K/AKT pathway increases HK-2 cell death indicating its pro-survival activity (See Figure A.2 in appendix A). Based on these findings, OTA-mediated autophagy most probably serves as a pro-survival mechanism, and then it is inactivated by PI3K/AKT to prevent autophagic cell death. However this suppression may activate apoptotic pathway if stress conditions persist. In a study, cisplatin, another nephrotoxic chemical, was shown to activate autophagy during the initial period of treatment [93]. Moreover, later inhibition of autophagy took place when the caspases and subsequent apoptotic pathway were activated.

In order to test if autophagy serves as a pro-survival mechanism or pro-death mechanism in response to OTA, class III PI3K inhibitor 3-MA, and siRNA against Beclin1 were used. However, 3-MA failed to block autophagy in our experimental paradigm, as it also decreased phosphorylation of AKT (See Figure A.3 in Appendix A). Similarly, siRNA against Beclin1, required in the initial step of autophagasome formation, was also used to inhibit autophagy. Western blot was performed to confirm its knock-down and, cell viability was measured to examine the role of autophagy after OTA exposure (See Figure A.4A and A.4B in Appendix A). However Beclin1 siRNA failed to decrease Beclin1 protein, and therefore autophagy as it can be seen in Western blot analyses. Thus, the role of autophagy in cell viability under OTA exposure could not be determined using Beclin1 siRNA and 3-MA in the present study.

We have noticed that the levels of p62/SQSTM1 are decreased upon OTA exposure at later time points when the autophagy is suppressed. This is paradoxical that reduction in p62/SQSTM1 amount has been associated with higher autophagy flux in several studies [56–58]. Impairment in LC3B-II and p62/SQSTM1 accumula-

tion is thought to be the consequence of overactive UPS since p62/SQSTM1 is also involved in UPS activities [94]. Furthermore, p62/SQSTM1 has multiple domains interacting with various signaling molecules [48]. It has a role in the activation of pro-survival-antioxidant cell responses involving the nuclear factor erythroid 2-related factor 2 (Nrf2) and KEAP1 through proteasomal pathway [59, 95]. OTA was also shown to impair antioxidant defense mechanism as it decreases Nrf2 acitivity and also Nrf2-dependent gene expressions in the kidney of rats and also in porcine renal epithelial proximal tubulus cells [25–27]. Therefore, expression of p62/SQSTM1 can be regulated at transcriptional and translational level in response to OTA. In the present study it is shown that OTA reduces the steady-state levels of ubiquitinylated proteins. OTA-induced reduction in ubiquitinylated proteins may be due to decreases in ubiquitination or increase in degradation of ubiquitinylated proteins. If the latter is the case, then the polyubiquitinylated proteins may rapidly be degraded with the help of p62/SQSTM1.

When the proteasome system was inhibited by MG132 (carbobenzoxyl-L-leucyl-L-leucyl-L-leucine), LC3B-II and p62/SQSTM1 were accumulated in the cells suggesting that the impairment of UPS activates autophagy. MG132 is a proteasome inhibitor that inhibits 20S proteasome activity reversibly by covalently binding to catalytic site, and subsequently blocks the proteolytic activity of the 26S proteasome complex [81, 96]. Inhibition of proteasome system by MG132 was shown to lead upregulation of p62/SQSTM1 [97]. Moreover, it was shown that MG132 also decreases lysosomal protease activity resulting in blockage of autophagasome degradation and accumulation of LC3-II [81]. In this study, treating the HK-2 cells with MG132 increased p62/SQSTM1 levels as well as LC3B-II accumulation as expected. However, OTA decreased this accumulation which supports the idea that OTA suppresses autophagy upstream of autophagasome formation as MG132 inhibits autophagasome degradation. The results show that p62/SQSTM1 amount is higher in the cells co-treated with OTA than the cells treated with only MG132. Since the inhibitory effect of MG132 is reversible and does not inhibit all the proteolytic activities, OTA-induced p62/SQSTM1 reduction may be the consequence of UPS activities other than the chemotrypsin-like. Moreover, the presence of MG132 does not effect AKT and ERK1/2 phosphorylations.

Therefore, phosphorylation of AKT inhibits autophagy even in the presence of UPS inhibitor.

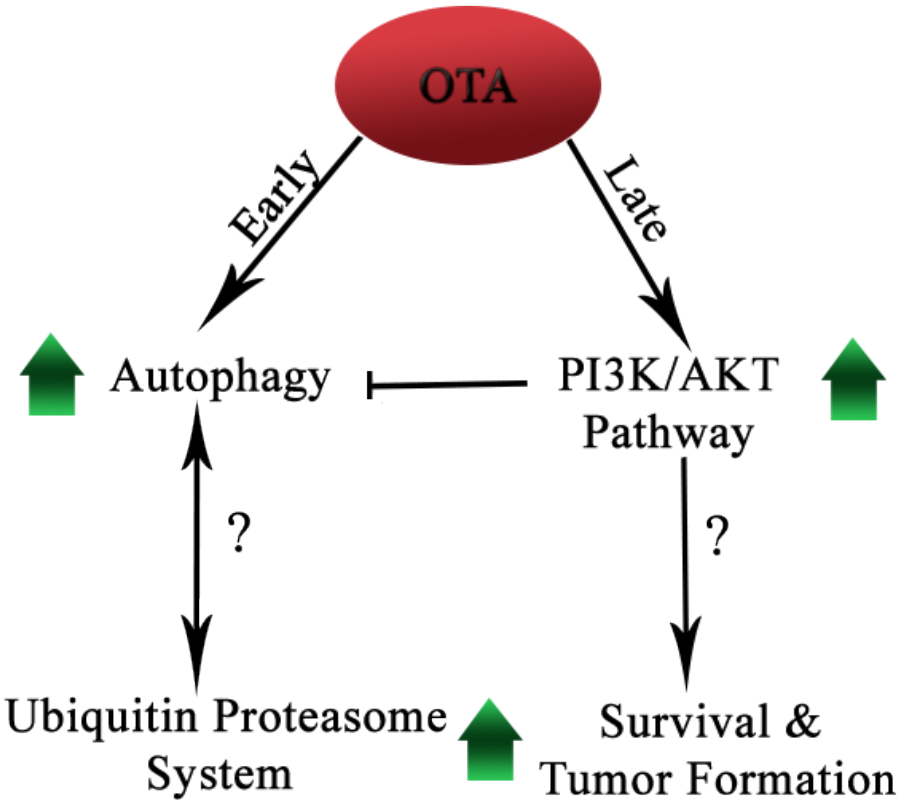

Figure 6.1. Proposed mechanism of OTA-mediated toxicity.

In conclusion, our results demonstrate that, autophay is up-regulated as an early response to OTA, and the trailing activation of PI3K/AKT pathway down-regulates autophagy Therefore, we hypothesize that OTA induces autophagy in order to cope with the oxidative stress generated by OTA exposure in the earlier time points. Subsequently, the cells utilize constitutively active PI3K/AKT pathway to escape autophagic or apoptotic cell death with the disadvantage of transformation and initiating tumors (Figure 6.1). Nonetheless, this hypothesis should be further tested with more appropriate experimentation in the future.

APPENDIX A: SUPPLEMENTAL RESULTS

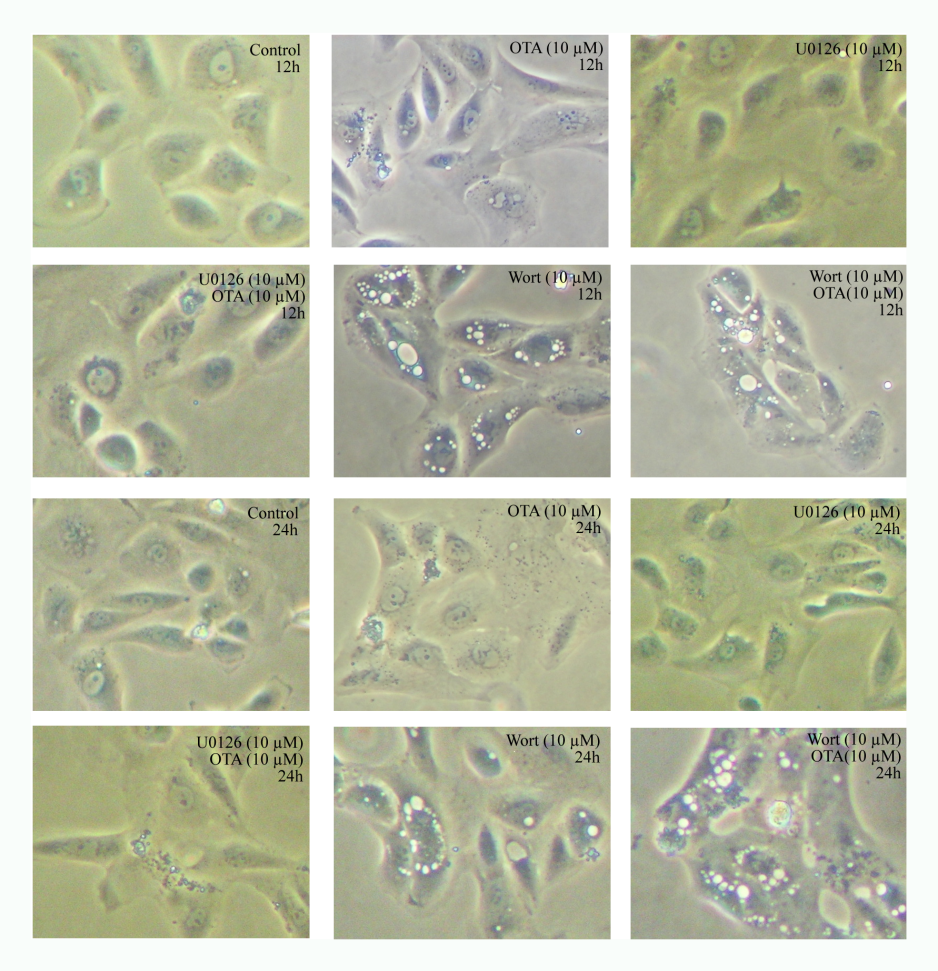

Figure A.1. The effects of OTA alone or in combination with Wort, U0126 on the moorphology of HK-2 cells. The cells were visualized under light microscope after treatments. These cells were also used in Western blot experiments whose results can be seen in Figure 5.9

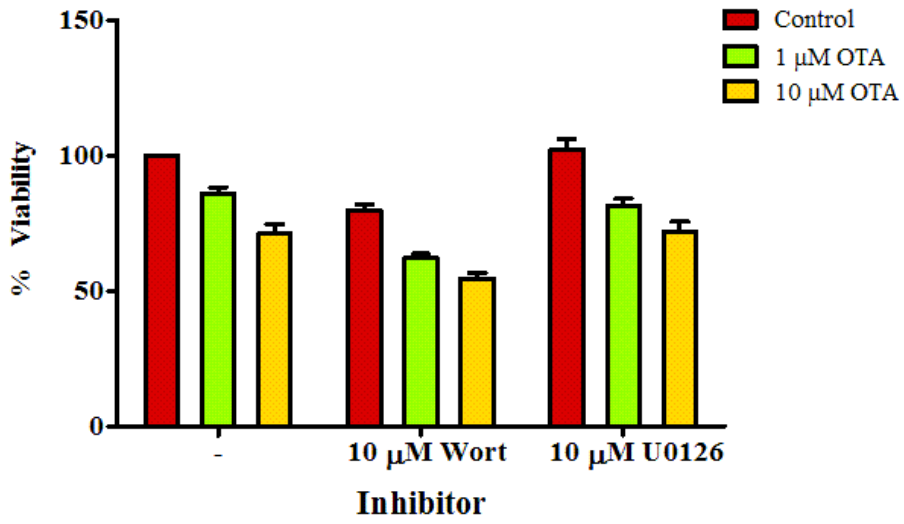

Figure A.2. The effects of PI3K/AKT and MAPK/ERK inhibitors on the viability of HK-2 cells exposed to OTA. The cells were pre-treated with inhibitors and then exposed to OTA for 24 hrs.

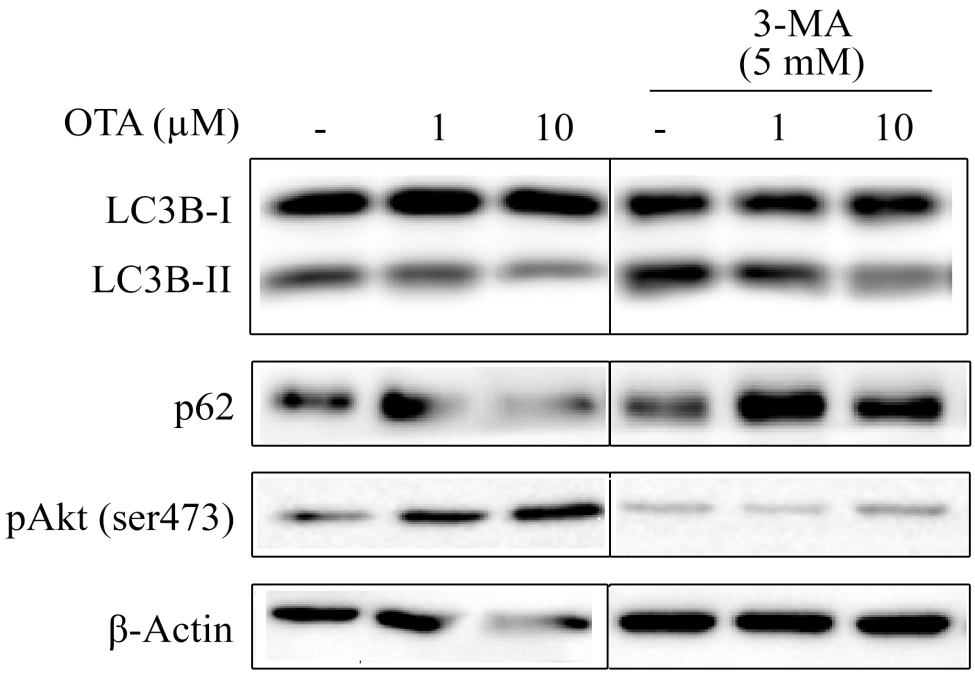

Figure A.3. The effect of 3-MA alone or in combination with OTA on AKT phosphorylation and autophagy induction.

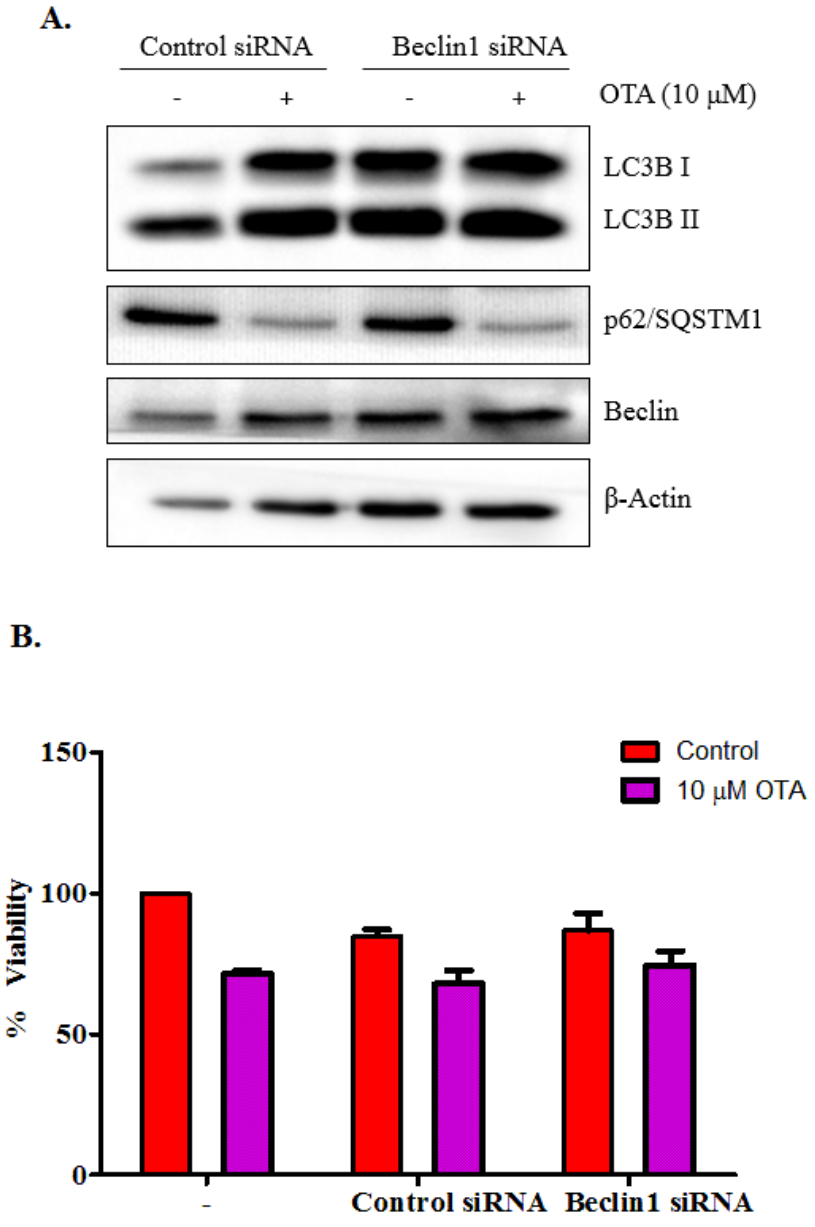

Figure A.4. Knockdown of autophagy-related gene Beclin1 by siRNA. A. Western blot analysis of extracts from HK-2 cells transfected with control or beclin1 siRNA and then exposed to 10 μM OTA for 24 hrs. B. Cell viability analysis after siRNA-mediate knockdown and following treatment with 10 μM OTA for 24 hrs.

REFERENCES

1. Pfohl-Leszkowicz, A. and R. A. Manderville, "Ochratoxin A: An Overview on Toxicity and Carcinogenicity in Animals and Humans.", *Molecular Nutrition and Food Research*, Vol. 51, No. 1, pp. 61–99, 2007.

2. Vrabcheva, T., T. Petkova-Bocharova, F. Grosso, I. Nikolov, I. N. Chernozemsky, M. Castegnaro, and S. Dragacci, "Analysis of Ochratoxin A in Foods Consumed by Inhabitants from an Area with Balkan Endemic Nephropathy: a 1 Month Follow-up Study.", *Journal of Agricultural and Food Chemistry*, Vol. 52, No. 8, pp. 2404–2410, 2004.

3. Castegnaro, M., D. Canadas, T. Vrabcheva, T. Petkova-Bocharova, I. N. Chernozemsky, and A. Pfohl-Leszkowicz, "Balkan Endemic Nephropathy: Role of Ochratoxins A Through Biomarkers.", *Molecular Nutrition and Food Research*, Vol. 50, No. 6, pp. 519–529, 2006.

4. Petkova-Bocharova, T. and M. Castegnaro, "Ochratoxin A in Human Blood in Relation to Balkan Endemic Nephropathy and Urinary Tract Tumours in Bulgaria.", *IARC Scientific Publications*, , No. 115, pp. 135–137, 1991.

5. "Ochratoxin A.", *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, Vol. 56, pp. 489–521, 1993.

6. van der Merwe, K. J., P. S. Steyn, L. Fourie, D. B. Scott, and J. J. Theron, "Ochratoxin A, a Toxic Metabolite Produced by Aspergillus Ochraceus Wilh.", *Nature*, Vol. 205, No. 976, pp. 1112–1113, 1965.

7. Clark, H. A. and S. M. Snedeker, "Ochratoxin A: Its Cancer Risk and Potential for Exposure.", *Journal of Toxicology and Environmental Health. Part B, Critical Reviews*, Vol. 9, No. 3, pp. 265–296, 2006.

8. Ringot, D., A. Chango, Y.-J. Schneider, and Y. Larondelle, "Toxicokinetics and Toxicodynamics of Ochratoxin A, an Update.", *Chemico-Biological Interactions*, Vol. 159, No. 1, pp. 18–46, 2006.

9. Kamp, H. G., G. Eisenbrand, C. Janzowski, J. Kiossev, J. R. Latendresse, J. Schlatter, and R. J. Turesky, "Ochratoxin A Induces Oxidative DNA Damage in Liver and Kidney After Oral Dosing to Rats.", *Molecular Nutrition and Food Research*, Vol. 49, No. 12, pp. 1160–1167, 2005.

10. Malir, F., V. Ostry, A. Pfohl-Leszkowicz, and E. Novotna, "Ochratoxin A: Developmental and Reproductive Toxicity-an Overview.", *Birth Defects Research. Part B, Developmental and Reproductive Toxicology*, Vol. 98, No. 6, pp. 493–502, 2013.

11. Schwerdt, G., K. Bauer, M. Gekle, and S. Silbernagl, "Accumulation of Ochratoxin A in Rat Kidney In Vivo and in Cultivated Renal Epithelial Cells In Vitro.", *Toxicology*, Vol. 114, No. 3, pp. 177–185, 1996.

12. Zepnik, H., W. Völkel, and W. Dekant, "Toxicokinetics of the Mycotoxin Ochratoxin A in F 344 Rats After Oral Administration.", *Toxicology and Applied Pharmacology*, Vol. 192, No. 1, pp. 36–44, 2003.

13. Ferrufino-Guardia, E. V., E. K. Tangni, Y. Larondelle, and S. Ponchaut, "Transfer of Ochratoxin A During Lactation: Exposure of Suckling via the Milk of Rabbit does Fed a Naturally-Contaminated Feed.", *Food Additives and Contaminants*, Vol. 17, No. 2, pp. 167–175, 2000.

14. Elling, F., J. P. Nielsen, E. B. Lillehøj, M. S. Thomassen, and F. C. Størmer, "Ochratoxin A-induced Porcine Nephropathy: Enzyme and Ultrastructure Changes After Short-Term Exposure.", *Toxicon*, Vol. 23, No. 2, pp. 247–254, 1985.

15. Sauvant, C., H. Holzinger, and M. Gekle, "The Nephrotoxin Ochratoxin A Induces Key Parameters of Chronic Interstitial Nephropathy in Renal Proximal Tubular

Cells.", *Cellular Physiology and Biochemistry*, Vol. 15, No. 1-4, pp. 125–134, 2005.

16. Boorman, G. A., M. R. McDonald, S. Imoto, and R. Persing, "Renal Lesions Induced by Ochratoxin A Exposure in the F344 Rat.", *Toxicologic Pathology*, Vol. 20, No. 2, pp. 236–245, 1992.

17. Mantle, P. and E. Kulinskaya, "Lifetime, Low-dose Ochratoxin A Dietary Study on Renal Carcinogenesis in Male Fischer Rats.", *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*, Vol. 27, No. 11, pp. 1566–1573, 2010.

18. Szczech, G. M., W. W. Carlton, J. Tuite, and R. Caldwell, "Ochratoxin A Toxicosis in Swine.", *Veterinary Pathology*, Vol. 10, No. 4, pp. 347–364, 1973.

19. Schilter, B., M. Marin-Kuan, T. Delatour, S. Nestler, P. Mantle, and C. Cavin, "Ochratoxin A: Potential Epigenetic Mechanisms of Toxicity and Carcinogenicity.", *Food Additives and Contaminants*, Vol. 22 Suppl 1, pp. 88–93, 2005.

20. Palma, N., S. Cinelli, O. Sapora, S. H. Wilson, and E. Dogliotti, "Ochratoxin A-Induced Mutagenesis in Mammalian Cells is Consistent with the Production of Oxidative Stress.", *Chemical Research in Toxicology*, Vol. 20, No. 7, pp. 1031–1037, 2007.

21. Gautier, J. C., D. Holzhaeuser, J. Markovic, E. Gremaud, B. Schilter, and R. J. Turesky, "Oxidative Damage and Stress Response from Ochratoxin A Exposure in Rats.", *Free Radical Biology and Medicine*, Vol. 30, No. 10, pp. 1089–1098, 2001.

22. Boesch-Saadatmandi, C., A. Loboda, A. Jozkowicz, P. Huebbe, R. Blank, S. Wolffram, J. Dulak, and G. Rimbach, "Effect of Ochratoxin A on Redox-Regulated Transcription Factors, Antioxidant Enzymes and Glutathione-S-transferase in Cultured Kidney Tubulus Cells.", *Food and Chemical Toxicology*, Vol. 46, No. 8, pp. 2665–2671, 2008.

23. Hoehler, D. and R. R. Marquardt, "Influence of Vitamins E and C on the Toxic

Effects of Ochratoxin A and T-2 Toxin in Chicks.", *Poultry Science*, Vol. 75, No. 12, pp. 1508–1515, 1996.

24. Wilmes, A., D. Crean, S. Aydin, W. Pfaller, P. Jennings, and M. O. Leonard, "Identification and Dissection of the Nrf2 Mediated Oxidative Stress Pathway in Human Renal Proximal Tubule Toxicity.", *Toxicology in Vitro*, Vol. 25, No. 3, pp. 613–622, 2011.

25. Jennings, P., C. Weiland, A. Limonciel, K. M. Bloch, R. Radford, L. Aschauer, T. McMorrow, A. Wilmes, W. Pfaller, H. J. Ahr, C. Slattery, E. A. Lock, M. P. Ryan, and H. Ellinger-Ziegelbauer, "Transcriptomic Alterations Induced by Ochratoxin A in Rat and Human Renal Proximal Tubular In Vitro Models and Comparison to a Rat In Vivo Model.", *Archives of Toxicology*, Vol. 86, No. 4, pp. 571–589, 2012.

26. Cavin, C., T. Delatour, M. Marin-Kuan, D. Holzhäuser, L. Higgins, C. Bezençon, G. Guignard, S. Junod, J. Richoz-Payot, E. Gremaud, J. D. Hayes, S. Nestler, P. Mantle, and B. Schilter, "Reduction in Antioxidant Defenses may Contribute to Ochratoxin A Toxicity and Carcinogenicity.", *Toxicological Sciences*, Vol. 96, No. 1, pp. 30–39, 2007.

27. Boesch-Saadatmandi, C., A. E. Wagner, A. C. Graeser, C. Hundhausen, S. Wolffram, and G. Rimbach, "Ochratoxin A Impairs Nrf2-Dependent Gene Expression in Porcine Kidney Tubulus Cells.", *Journal of Animal Physiology and Animal Nutrition*, Vol. 93, No. 5, pp. 547–554, 2009.

28. Mally, A., "Ochratoxin A and Mitotic Disruption: Mode of Action Analysis of Renal Tumor Formation by Ochratoxin A.", *Toxicological Sciences*, Vol. 127, No. 2, pp. 315–330, 2012.

29. Pfohl-Leszkowicz, A., K. Chakor, E. E. Creppy, and G. Dirheimer, "DNA Adduct Formation in Mice Treated with Ochratoxin A.", *IARC Scientific Publications*, , No. 115, pp. 245–253, 1991.

30. Mally, A., H. Zepnik, P. Wanek, E. Eder, K. Dingley, H. Ihmels, W. Völkel, and W. Dekant, "Ochratoxin A: Lack of Formation of Covalent DNA Adducts.", *Chemical Research in Toxicology*, Vol. 17, No. 2, pp. 234–242, 2004.

31. Grosse, Y., L. Chekir-Ghedira, A. Huc, S. Obrecht-Pflumio, G. Dirheimer, H. Bacha, and A. Pfohl-Leszkowicz, "Retinol, Ascorbic Acid and Alpha-tocopherol Prevent DNA Adduct Formation in Mice Treated with the Mycotoxins Ochratoxin A and Zearalenone.", *Cancer Letters*, Vol. 114, No. 1-2, pp. 225–229, 1997.

32. Arbillaga, L., A. Azqueta, O. Ezpeleta, and A. L. de Cerain, "Oxidative DNA Damage Induced by Ochratoxin A in the HK-2 Human Kidney Cell Line: Evidence of the Relationship with Cytotoxicity.", *Mutagenesis*, Vol. 22, No. 1, pp. 35–42, 2007.

33. Adler, M., K. Müller, E. Rached, W. Dekant, and A. Mally, "Modulation of Key Regulators of Mitosis Linked to Chromosomal Instability is an Early Event in Ochratoxin A Carcinogenicity.", *Carcinogenesis*, Vol. 30, No. 4, pp. 711–719, 2009.

34. Rásonyi, T., J. Schlatter, and D. R. Dietrich, "The Role of Alpha2u-globulin in Ochratoxin A Induced Renal Toxicity and Tumors in F344 Rats.", *Toxicology Letters*, Vol. 104, No. 1-2, pp. 83–92, 1999.

35. Rached, E., G. C. Hard, K. Blumbach, K. Weber, R. Draheim, W. K. Lutz, S. Ozden, U. Steger, W. Dekant, and A. Mally, "Ochratoxin A: 13-week Oral Toxicity and Cell Proliferation in Male F344/n Rats.", *Toxicological Sciences*, Vol. 97, No. 2, pp. 288–298, 2007.

36. Rached, E., E. Pfeiffer, W. Dekant, and A. Mally, "Ochratoxin A: Apoptosis and Aberrant Exit from Mitosis due to Perturbation of Microtubule Dynamics?", *Toxicological Sciences*, Vol. 92, No. 1, pp. 78–86, 2006.

37. Tibbles, L. A. and J. R. Woodgett, "The Stress-Activated Protein Kinase Pathways.", *Cellular and Molecular Life Sciences*, Vol. 55, No. 10, pp. 1230–1254, 1999.

38. Rumora, L. and T. Z. Grubisić, "A Journey Through Mitogen-Activated Protein Kinase and Ochratoxin A Interactions.", *Arhiv za Higijenu Rada i Toksikologiju*, Vol. 60, No. 4, pp. 449–456, 2009.

39. Schramek, H., D. Wilflingseder, V. Pollack, R. Freudinger, S. Mildenberger, and M. Gekle, "Ochratoxin A-Induced Stimulation of Extracellular Signal-Regulated Kinases 1/2 is Associated with Madin-Darby Canine Kidney-C7 Cell Dedifferentiation.", *Journal of Pharmacology and Experimental Therapeutics*, Vol. 283, No. 3, pp. 1460–1468, 1997.

40. Marin-Kuan, M., S. Nestler, C. Verguet, C. Bezencon, D. Piguet, T. Delatour, P. Mantle, C. Cavin, and B. Schilter, "MAPK-ERK Activation in Kidney of Male Rats Chronically Fed Ochratoxin A at a Dose Causing a Significant Incidence of Renal Carcinoma.", *Toxicology and Applied Pharmacology*, Vol. 224, No. 2, pp. 174–181, 2007.

41. Oka, H., Y. Chatani, R. Hoshino, O. Ogawa, Y. Kakehi, T. Terachi, Y. Okada, M. Kawaichi, M. Kohno, and O. Yoshida, "Constitutive Activation of Mitogen-Activated Protein (MAP) Kinases in Human Renal Cell Carcinoma.", *Cancer Research*, Vol. 55, No. 18, pp. 4182–4187, 1995.

42. Kumar, R., S. Alam, B. P. Chaudhari, P. D. Dwivedi, S. K. Jain, K. M. Ansari, and M. Das, "Ochratoxin A-Induced Cell Proliferation and Tumor Promotion in Mouse Skin by Activating the Expression of Cyclin-D1 and Cyclooxygenase-2 Through Nuclear Factor-Kappa B and Activator Protein-1.", *Carcinogenesis*, Vol. 34, No. 3, pp. 647–657, 2013.

43. Özcan, Z., G. Gül, and İ. Yaman, "Ochratoxin A Activates Opposing c-MET/PI3K/Akt and MAPK/Erk 1-2 Pathways in Human Proximal Tubule HK-2 Cells", *Archives of Toxicology*, 2014, [Press release].

44. Mizushima, N., "Autophagy: Process and Function.", *Genes and Development*, Vol. 21, No. 22, pp. 2861–2873, 2007.

45. Ravikumar, B., S. Sarkar, J. E. Davies, M. Futter, M. Garcia-Arencibia, Z. W. Green-Thompson, M. Jimenez-Sanchez, V. I. Korolchuk, M. Lichtenberg, S. Luo, D. C. O. Massey, F. M. Menzies, K. Moreau, U. Narayanan, M. Renna, F. H. Siddiqi, B. R. Underwood, A. R. Winslow, and D. C. Rubinsztein, "Regulation of Mammalian Autophagy in Physiology and Pathophysiology.", *Physiological Reviews*, Vol. 90, No. 4, pp. 1383–1435, 2010.

46. Chen, N. and V. Karantza-Wadsworth, "Role and Regulation of Autophagy in Cancer.", *Biochimica et Biophysica Acta*, Vol. 1793, No. 9, pp. 1516–1523, 2009.

47. Nixon, R. A., "The Role of Autophagy in Neurodegenerative Disease.", *Nature Medicine*, Vol. 19, No. 8, pp. 983–997, 2013.

48. Klionsky, D. J., F. C. Abdalla, H. Abeliovich, R. T. Abraham, A. Acevedo-Arozena, K. Adeli, L. Agholme, M. Agnello, P. Agostinis, J. A. Aguirre-Ghiso, *et al.*, "Guidelines For the Use and Interpretation of Assays for Monitoring Autophagy", *Autophagy*, Vol. 8, No. 4, pp. 445–544, 2012.

49. Boya, P., F. Reggiori, and P. Codogno, "Emerging Regulation and Functions of Autophagy.", *Nature Cell Biology*, Vol. 15, No. 7, pp. 713–720, 2013.

50. Hamasaki, M., S. T. Shibutani, and T. Yoshimori, "Up-to-date Membrane Biogenesis in the Autophagosome Formation.", *Current Opinion in Cell Biology*, Vol. 25, No. 4, pp. 455–460, 2013.

51. Hurley, J. H. and B. A. Schulman, "Atomistic Autophagy: The Structures of Cellular Self-Digestion.", *Cell*, Vol. 157, No. 2, pp. 300–311, 2014.

52. Russell, R. C., Y. Tian, H. Yuan, H. W. Park, Y.-Y. Chang, J. Kim, H. Kim, T. P. Neufeld, A. Dillin, and K.-L. Guan, "ULK1 Induces Autophagy by Phosphorylating Beclin-1 and Activating VPS34 Lipid Kinase.", *Nature Cell Biology*, Vol. 15, No. 7, pp. 741–750, 2013.

53. Tanida, I., "Autophagy Basics.", *Microbiology and Immunology*, Vol. 55, No. 1, pp. 1–11, 2011.

54. Shaid, S., C. H. Brandts, H. Serve, and I. Dikic, "Ubiquitination and Selective Autophagy.", *Cell Death and Differentiation*, Vol. 20, No. 1, pp. 21–30, 2013.

55. Seibenhener, M. L., T. Geetha, and M. W. Wooten, "Sequestosome 1/p62–More Than Just a Scaffold.", *FEBS Letters*, Vol. 581, No. 2, pp. 175–179, 2007.

56. Pankiv, S., T. H. Clausen, T. Lamark, A. Brech, J.-A. Bruun, H. Outzen, A. Øvervatn, G. Bjørkøy, and T. Johansen, "p62/SQSTM1 Binds Directly to Atg8/LC3 to Facilitate Degradation of Ubiquitinated Protein Aggregates by Autophagy.", *Journal of Biological Chemistry*, Vol. 282, No. 33, pp. 24131–24145, 2007.

57. Ichimura, Y., T. Kumanomidou, Y. shin Sou, T. Mizushima, J. Ezaki, T. Ueno, E. Kominami, T. Yamane, K. Tanaka, and M. Komatsu, "Structural Basis for Sorting Mechanism of p62 in Selective Autophagy.", *Journal of Biological Chemistry*, Vol. 283, No. 33, pp. 22847–22857, 2008.

58. Shvets, E., E. Fass, R. Scherz-Shouval, and Z. Elazar, "The N-terminus and Phe52 Residue of LC3 Recruit p62/SQSTM1 into Autophagosomes.", *Journal of Cell Science*, Vol. 121, No. Pt 16, pp. 2685–2695, 2008.

59. Moscat, J., M. T. Diaz-Meco, and M. W. Wooten, "Signal Integration and Diversification Through the p62 Scaffold Protein.", *Trends in Biochemical Sciences*, Vol. 32, No. 2, pp. 95–100, 2007.

60. Kim, J., M. Kundu, B. Viollet, and K.-L. Guan, "AMPK and mTOR Regulate Autophagy Through Direct Phosphorylation of Ulk1.", *Nature Cell Biology*, Vol. 13, No. 2, pp. 132–141, 2011.

61. He, C. and D. J. Klionsky, "Regulation Mechanisms and Signaling Pathways of Autophagy.", *Annual Review of Genetics*, Vol. 43, pp. 67–93, 2009.

62. Cagnol, S. and J.-C. Chambard, "ERK and Cell Death: Mechanisms of ERK-induced Cell Death–Apoptosis, Autophagy and Senescence.", *FEBS Journal*, Vol. 277, No. 1, pp. 2–21, 2010.

63. Sivaprasad, U. and A. Basu, "Inhibition of ERK Attenuates Autophagy and Potentiates Tumour Necrosis Factor-Alpha-Induced Cell Death in MCF-7 Cells.", *Journal of Cellular and Molecular Medicine*, Vol. 12, No. 4, pp. 1265–1271, 2008.

64. Zeng, Y., X. Yang, J. Wang, J. Fan, Q. Kong, and X. Yu, "Aristolochic acid I Induced Autophagy Extenuates Cell Apoptosis via ERK 1/2 Pathway in Renal Tubular Epithelial Cells.", *PLoS One*, Vol. 7, No. 1, p. e30312, 2012.

65. Orrenius, S., V. O. Kaminskyy, and B. Zhivotovsky, "Autophagy in Toxicology: Cause or Consequence?", *Annual Review of Pharmacology and Toxicology*, Vol. 53, pp. 275–297, 2013.

66. Clarke, P. G., "Developmental Cell Death: Morphological Diversity and Multiple Mechanisms.", *Anatomy and Embryology*, Vol. 181, No. 3, pp. 195–213, 1990.

67. Gozuacik, D. and A. Kimchi, "Autophagy and Cell Death.", *Current Topics in Developmental Biology*, Vol. 78, pp. 217–245, 2007.

68. Singh, B. N., D. Kumar, S. Shankar, and R. K. Srivastava, "Rottlerin Induces Autophagy which Leads to Apoptotic Cell Death Through Inhibition of PI3K/Akt/mTOR Pathway in Human Pancreatic Cancer Stem Cells.", *Biochemical Pharmacology*, Vol. 84, No. 9, pp. 1154–1163, 2012.

69. Thévenod, F. and W.-K. Lee, "Cadmium and Cellular Signaling Cascades: Interactions Between Cell Death and Survival Pathways.", *Archives of Toxicology*, Vol. 87, No. 10, pp. 1743–1786, 2013.

70. Frank, M., S. Duvezin-Caubet, S. Koob, A. Occhipinti, R. Jagasia, A. Petcherski, M. O. Ruonala, M. Priault, B. Salin, and A. S. Reichert, "Mitophagy is Trig-

gered by Mild Oxidative Stress in a Mitochondrial Fission Dependent Manner.", *Biochimica et Biophysica Acta*, Vol. 1823, No. 12, pp. 2297–2310, 2012.

71. Sansanwal, P., B. Yen, W. A. Gahl, Y. Ma, L. Ying, L.-J. C. Wong, and M. M. Sarwal, "Mitochondrial Autophagy Promotes Cellular Injury in Nephropathic Cystinosis.", *Journal of the American Society of Nephrology*, Vol. 21, No. 2, pp. 272–283, 2010.

72. Gozuacik, D., S. Bialik, T. Raveh, G. Mitou, G. Shohat, H. Sabanay, N. Mizushima, T. Yoshimori, and A. Kimchi, "DAP-Kinase is a Mediator of Endoplasmic Reticulum Stress-Induced Caspase Activation and Autophagic Cell Death.", *Cell Death and Differentiation*, Vol. 15, No. 12, pp. 1875–1886, 2008.

73. Qin, L., Z. Wang, L. Tao, and Y. Wang, "ER Stress Negatively Regulates AKT/TSC/mTOR Pathway to Enhance Autophagy.", *Autophagy*, Vol. 6, No. 2, pp. 239–247, 2010.

74. Mathew, R., C. M. Karp, B. Beaudoin, N. Vuong, G. Chen, H.-Y. Chen, K. Bray, A. Reddy, G. Bhanot, C. Gelinas, R. S. Dipaola, V. Karantza-Wadsworth, and E. White, "Autophagy Suppresses Tumorigenesis Through Elimination of p62.", *Cell*, Vol. 137, No. 6, pp. 1062–1075, 2009.

75. Yang, Z. J., C. E. Chee, S. Huang, and F. A. Sinicrope, "The Role of Autophagy in Cancer: Therapeutic Implications.", *Molecular Cancer Therapeutics*, Vol. 10, No. 9, pp. 1533–1541, 2011.

76. Cheong, H., C. Lu, T. Lindsten, and C. B. Thompson, "Therapeutic Targets in Cancer Cell Metabolism and Autophagy.", *Nature Biotechnology*, Vol. 30, No. 7, pp. 671–678, 2012.

77. Kongara, S. and V. Karantza, "The Interplay Between Autophagy and ROS in Tumorigenesis.", *Front Oncol*, Vol. 2, p. 171, 2012.

78. Lee, J., S. Giordano, and J. Zhang, "Autophagy, Mitochondria and Oxidative Stress: Cross-talk and Redox Signalling.", *Biochemical Journal*, Vol. 441, No. 2, pp. 523–540, 2012.

79. Lee, Y. J., N.-Y. Kim, Y.-A. Suh, and C. Lee, "Involvement of ROS in Curcumin-induced Autophagic Cell Death.", *Korean Journal of Physiology and Pharmacology*, Vol. 15, No. 1, pp. 1–7, 2011.

80. Foster, F. M., C. J. Traer, S. M. Abraham, and M. J. Fry, "The Phosphoinositide (PI) 3-Kinase Family.", *Journal of Cell Science*, Vol. 116, No. Pt 15, pp. 3037–3040, 2003.

81. Guo, N. and Z. Peng, "MG132, a Proteasome Inhibitor, Induces Apoptosis in Tumor Cells.", *Asia-Pacific Journal of Clinical Oncology*, Vol. 9, No. 1, pp. 6–11, 2013.

82. Inoki, K., "mTOR Signaling in Autophagy Regulation in the Kidney.", *Seminars in Nephrology*, Vol. 34, No. 1, pp. 2–8, 2014.

83. Yamamoto, A., Y. Tagawa, T. Yoshimori, Y. Moriyama, R. Masaki, and Y. Tashiro, "Bafilomycin A1 Prevents Maturation of Autophagic Vacuoles by Inhibiting Fusion Between Autophagosomes and Lysosomes in Rat Hepatoma Cell Line, H-4-II-E Cells.", *Cell Structure and Function*, Vol. 23, No. 1, pp. 33–42, 1998.

84. Li, Z.-y., Y. Yang, M. Ming, and B. Liu, "Mitochondrial ROS Generation for Regulation of Autophagic Pathways in Cancer.", *Biochemical and Biophysical Research Communications*, Vol. 414, No. 1, pp. 5–8, 2011.

85. Yu, Z.-Q., T. Ni, B. Hong, H.-Y. Wang, F.-J. Jiang, S. Zou, Y. Chen, X.-L. Zheng, D. J. Klionsky, Y. Liang, and Z. Xie, "Dual Roles of Atg8-PE Deconjugation by Atg4 in Autophagy.", *Autophagy*, Vol. 8, No. 6, pp. 883–892, 2012.

86. Nazarko, V. Y. and Q. Zhong, "ULK1 Targets Beclin-1 in Autophagy.", *Nature*

Cell Biology, Vol. 15, No. 7, pp. 727–728, 2013.

87. Tato, I., R. Bartrons, F. Ventura, and J. L. Rosa, "Amino Acids Activate Mammalian Target of Rapamycin Complex 2 (mTORC2) via PI3K/Akt Signaling.", *Journal of Biological Chemistry*, Vol. 286, No. 8, pp. 6128–6142, 2011.

88. Murphy, L. O. and J. Blenis, "MAPK Signal Specificity: the Right Place at the Right Time.", *Trends in Biochemical Sciences*, Vol. 31, No. 5, pp. 268–275, 2006.

89. Dagda, R. K., J. Zhu, S. M. Kulich, and C. T. Chu, "Mitochondrially Localized ERK2 Regulates Mitophagy and Autophagic Cell Stress: Implications for Parkinson's Disease.", *Autophagy*, Vol. 4, No. 6, pp. 770–782, 2008.

90. Sridharan, S., K. Jain, and A. Basu, "Regulation of Autophagy by Kinases.", *Cancers (Basel)*, Vol. 3, No. 2, pp. 2630–2654, 2011.

91. Pedro, J. M. B.-S., M. Niso-Santano, R. Gómez-Sánchez, E. Pizarro-Estrella, A. Aiastui-Pujana, A. Gorostidi, V. Climent, R. L. de Maturana, R. Sanchez-Pernaute, A. L. de Munain, J. M. Fuentes, and R. A. González-Polo, "The LRRK2 G2019S Mutant Exacerbates Basal Autophagy Through Activation of the MEK/ERK Pathway.", *Cellular and Molecular Life Sciences*, Vol. 70, No. 1, pp. 121–136, 2013.

92. Nicholson, K. M. and N. G. Anderson, "The Protein Kinase B/Akt Signalling Pathway in Human Malignancy.", *Cellular Signalling*, Vol. 14, No. 5, pp. 381–395, 2002.

93. Yang, C., V. Kaushal, S. V. Shah, and G. P. Kaushal, "Autophagy is Associated with Apoptosis in Cisplatin Injury to Renal Tubular Epithelial Cells.", *American Journal of Physiology. Renal Physiology*, Vol. 294, No. 4, pp. F777–F787, 2008.

94. Seibenhener, M. L., J. R. Babu, T. Geetha, H. C. Wong, N. R. Krishna, and M. W. Wooten, "Sequestosome 1/p62 is a Polyubiquitin Chain Binding Protein Involved

in Ubiquitin Proteasome Degradation.", *Molecular and Cellular Biology*, Vol. 24, No. 18, pp. 8055–8068, 2004.

95. Nezis, I. P. and H. Stenmark, "p62 at the Interface of Autophagy, Oxidative Stress Signaling, and Cancer.", *Antioxidants and Redox Signalling*, Vol. 17, No. 5, pp. 786–793, 2012.

96. Lee, D. H. and A. L. Goldberg, "Proteasome Inhibitors Cause Induction of Heat Shock Proteins and Trehalose, which Together Confer Thermotolerance in Saccharomyces Cerevisiae.", *Molecular and Cellular Biology*, Vol. 18, No. 1, pp. 30–38, 1998.

97. Nakaso, K., Y. Yoshimoto, T. Nakano, T. Takeshima, Y. Fukuhara, K. Yasui, S. Araga, T. Yanagawa, T. Ishii, and K. Nakashima, "Transcriptional Activation of p62/A170/ZIP During the Formation of the Aggregates: Possible Mechanisms and the Role in Lewy Body Formation in Parkinson's Disease.", *Brain Research*, Vol. 1012, No. 1-2, pp. 42–51, 2004.