The Hong Kong Polytechnic University

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Effects of Ghrelin on Doxorubicin-induced Toxicity in Skeletal Muscle

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Certificate of originality

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Abstract

Doxorubicin is a potent anti-cancer drug that has been used for treating a wide range of cancers. However, doxorubicin causes multi-organ toxicity which has largely limited the clinical usage of it. There are more than 30% of cancer patients suffer from cancer cachexia which is characterized by significant weight loss and muscle wasting resulting in weakness and fatigue. Notably, application of doxorubicin may further compromise muscle function through the induction of muscle impairment in patients with cancer cachexia. Ghrelin is a peptide that has been proposed as a potential candidate for treating cancer cachexia due to its potent ability in stimulating growth hormone release and increasing food intake. Administration of ghrelin has been shown to preserve muscle mass during fasting-induced muscle atrophy. This study aimed to investigate whether ghrelin protects skeletal muscle from doxorubicin-induced damage. Wild type adult C57BL/6 mice were randomly assigned to saline control group (CON; n = 7), doxorubicin group (DOX; n = 7), doxorubicin with treatment of ghrelin group (DOX+Ghrelin; n = 7) and doxorubicin with treatment of ghrelin plus GHSR-1a antagonist [D-Lys3]-GHRP-6 group (DOX+Ghrelin+[D-Lys3]-GHRP-6; n = 5). Mice in all groups were injected with doxorubicin (15 mg/kg, i.p.) at the beginning except the mice in the CON group received saline as a placebo. Mice assigned to the DOX+Ghrelin and DOX+Ghrelin+[D-Lys3]-GHRP-6 groups then received ghrelin treatment (100 μg/kg, i.p.) every 12 hours while mice in both the CON and DOX groups received same volume of saline as a placebo. Mice in the DOX+Ghrelin+[D-Lys3]-GHRP-6 group had GHSR-1a antagonist – [D-Lys3]-GHRP-6, (3.75mg/kg, i.p.) injected just before the administration of

ghrelin. All mice were sacrificed 5 days after the first injection. Histological analysis revealed that abnormal high percentage of centronucleated fibers was only observed in doxorubicin-treated muscle. The increased abundance of cleaved-actin in doxorubicin-treated muscle was accompanied by the upregulation of Bax, increase in number of TUNEL positive nuclei and elevation of apoptotic DNA fragmentation whereas these changes were not found in mice receiving ghrelin treatment. Protein abundances of autophagic markers, including LC3 II/I ratio, Atg12-5 complex, Atg5 and Beclin-1 were not changed after doxorubicin administration but upregulated by ghrelin administration. Application of GHSR-1a antagonist did not blunt the anti-apoptotic effects of ghrelin in doxorubicin-treated muscle but further augmented the phosphorylation of Akt and protein abundances of autophagic markers. It is noted that the basal levels of apoptosis and autophagy were not affected by administration of ghrelin alone under normal physiological condition. It is also observed that autophagy was upregulated 24 hours after doxorubicin administration and return to basal level after 5 days. Collectively, our data suggest that ghrelin upregulates autophagy in the skeletal muscle following doxorubicin treatment in concomitant with the suppression of apoptosis and muscle protein degradation. It is worth noting that the modulating effects of ghrelin on apoptosis and autophagy in doxorubicin-treated skeletal muscle are possibly not mediated through the GHSR-1a signaling. These findings are consistent with the hypothesis that ghrelin protects skeletal muscle against the doxorubicin-induced injury.

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List of Abbreviations

AIDS	Acquired immunodeficiency syndrome
Akt	Thymoma viral proto-oncogene
Apaf-1	Apoptotic protease activating factor 1
Atg	Autophagy related gene
Bad	BCL-2-associated death promotor
Bak	BCL2-antagonist.killer 1
Bax	BCL-2 associated X
Bcl-2	B cell leukemia.lymphoma 2
Bcl-X	BCL-2 like-1
Bid	BH3 interacting domain death agonist
tBid	Truncated BH3 interacting domain death agonist
Bik	BCL-2 interacting killer
Bim	BCL-2 like 11
Bmf	BCL2 modifying factor
Bok	BCL2-related ovarian killer
$Ca^{2}+$	Calcium ion
Calpain	Calcium-activated neutral proteinase
Caspase	Cysteine-dependent aspartateidiected proteases
Smac/Diablo	Inhibitor of apoptotic protein -binding mitochondrial protein
DNA	Deoxyribonucleic acid
eIF2α	Eukaryotic translation initiation factor 2 alpha
ELISA	Enzyme-linked immunosorbent assay
ERK	Mitogen-activated protein kinase 1
FoxO3	Forhead box O3
GAPDH	Glyceradehyde-3-phosphate dehydrogenase
Hrk	Harakiri, BCL2 interacting protein
Htr/Omi	HtrA serine peptidase 2
IGF-1	Insulin-like growth factor 1
i.p.	Intraperitoneal injection
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
LC3	Microtubule-associated protein 1 light chain 3 alpha
MAFbx	F-box protein 32

mRNA	Messenger ribonucleic acid
MurF1	Muscle-specific RING finger protein 1
NF-kB	Nuclear factor kappa B
NIP	BCL2/adenovirus E1B 19kDa interacting protein 2
NOD2	Nucleotide-binding oligomerization domain containing 2
Noxa	Thymoma viral proto-oncogene
p-Akt	Phosphorylated thymoma viral proto-oncogene
Par-4	Protease-activated receptor 4
PGC1α	Peroxisome proliferator activated receptor gamma coactivator 1 alpha
PI3	Peptidase inhibitor 3
Puma	BCL2 binding component 3
TNFα	Tumor necrosis factor alpha
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick labeling

CHAPTER 1

Introduction

According to the Global Cancer Figure reported by the American Cancer Society, there were 12.7 million new cases of cancer and 7.6 million deaths from cancer in 2008. It is predicted that there will be 21.4 million new cases of cancer and 13.2 million of related deaths by 2030 (Argiles *et al.*, 2008). The increasing number of cancer patients generates a huge financial burden due to the associated medical costs (Argiles *et al.*, 2008). The effective anti-cancer drugs are in huge demand.

Doxorubicin is one of the effective anti-cancer drugs and is widely used nowadays since its introduction in 1960s (Bonadonna *et al*, 1969). This can be attributed to its high potency in treating various types of cancer, including breast cancer, bladder cancer and leukemia (American Cancer Society, 2011). At the same time, the clinical use of doxorubicin is somehow restricted due to its induction of multi-organ toxicity. Doxorubicin induces damage to many important organs, such as the heart (Hayward & Hydock, 2007), liver (Abbas, 2011) and kidney (Peng *et al.*, 2012a).

Toxicological research in doxorubicin revealed the underestimation of myotoxicity

when compared to the well documented cardiotoxicity and renal toxicity (Hayward *et al.*, 2012). Hayward and colleagues have demonstrated that the maximal twitch force and rate of force generation in skeletal muscle were reduced after doxorubicin exposure, thereby impair the proper function of muscles (Hayward *et al.*, 2012). A significant reduction in muscle mass has also been reported after doxorubicin intervention (Smuder *et al.*, 2011b).

Doxorubicin can increase the production of mitochondrial reactive oxygen species (ROS) (Subashini *et al.*, 2006). The increased oxidative stress results in cell death (i.e., apoptosis) in muscles. Extensive apoptosis due to cellular damage in skeletal muscle may cause excessive loss of muscle cells and subsequent functional decline (Dupont-Versteegden, 2005). Acute inflammation and fibrosis in the skeletal muscle following doxorubicin treatment have been reported (Cullu *et al.*, 2003). Recently, autophagy was found to be upregulated in skeletal muscle after doxorubicin intervention (Smuder *et al.*, 2011a), suggesting that autophagy might contribute, at least partly, to doxorubicin-induced myotoxicity.

It is well known that cancer cachexia, the major cause of actual mortality of cancer, occurs in 30% to 80% of cancer patients (Jurdana, 2009). Patients with cancer cachexia have symptoms of fatigue and body weakness (Lee & Glass, 2011) that affect patients' quality of life (Gilliam & St Clair, 2011). More importantly, cancer cachexia is associated with progressive muscle wasting and leads to metabolic dysfunction (Lee & Glass, 2011). Collectively, doxorubicin-induced muscle injury impairs the muscle function of cancer patients and may aggravate the muscle wasting in cachetic cancer patients, thus deteriorates the quality of life of cancer patients and even causes death.

Ghrelin is a 28-amino acid long multifunctional peptide and has been proposed as a potential therapeutic candidate for treating cancer cachexia due to its modulating roles in regulating appetite and stimulating gastric acid secretion and gastric motility (Inui *et al.*, 2004). This peptide is produced mainly in the stomach and can be found in various tissues, such as the bowel, kidney, pituitary and pancreas (van der Lely *et al.*, 2004). Ghrelin is a well known inducer of growth hormone release by activating the inositol 1,4,5-triphosphate (PI3) pathway

(Ueno *et al.*, 2005). In addition, ghrelin was found to prevent inflammation, fibrosis and oxidative stress by inhibiting the release of proinflammatory cytokines and suppressing neutrophils infiltration (Iseri *et al.*, 2008), decreasing the collagen content (Cetin *et al.*, 2011) and increasing the activities of the anti-oxidative enzymes (El *et al.*, 2007;Zwirska-Korczala *et al.*, 2007;Iseri *et al.*, 2008), while these effects are all related to the pathological mechanisms of the doxorubicin-induced myotoxicity.

Besides inflammation, oxidative stress and fibrosis, doxorubicin has also been shown to induce apoptosis (Liu *et al.*, 2008;Hosseinzadeh *et al.*, 2011) and autophagy (Smuder *et al.*, 2011a) in skeletal muscle. In this MPhil study, we hypothesized that ghrelin could protect the skeletal muscle from doxorubicin-induced damages by suppressing the upregulation of apoptosis and enhancing the activation of autophagy.

CHAPTER 2

Background and Significance

2.1 Global Cancer Figure

Cancer is a major health issue worldwide as the number of cancer patients is rapidly increasing (Jemal *et al.*, 2008). According to the Global Cancer Facts & Figures in 2008 from the American Cancer Society, there were 12.7 million new cancer cases and 7.6 million deaths in 2008. It is predicted that by 2030, the number of new cases and cancer deaths will reach to 24.1 million and 13.2 million, respectively (Jemal *et al.*, 2008). The increasing number of cancer cases will generate a financial burden due to the escalating patient numbers and medical costs. Hence, effective cancer therapies are important for relieving the financial burden as well as for saving human lives.

2.2 Cancer Cachexia - The Major Cause of Death to Cancer Patients

Approximately 30% to 80% of cancer patients develop cancer cachexia depending on the types of cancer (Tisdale, 2009). It has been reported that cancer cachexia accounts for approximately 22% of actual mortality (Argiles *et al.*, 2008) and is cited as the major cause of death among cancer patients (Lee & Glass, 2011). Lee and Glass suggested that cancer cachexia should be a concern during cancer treatments (Lee & Glass, 2011).

Cancer cachexia is a multifactorial syndrome characterized by a significant loss of body weight (Bosaeus *et al.*, 2001). Weight loss is considered as an important prognostic factor for cancer patients. It has been reported that 25% to 30% of total weight loss can be fatal to a cancer patient (Wigmore *et al.*, 1997). Muscle wasting due to the increased catabolism of proteins, in conjunction with decreased protein synthesis in skeletal muscle mainly contributes to the excessive weight loss in patients with cancer cachexia (Jurdana, 2009). It has been reported that this cancer cachexia-associated weight loss cannot be reverted by increasing food intake (Lee & Glass, 2011). Adipose and muscle tissue wasting and

inflammation usually occur with cachexia (Argiles *et al*, 2008).

The metabolism of patients with cancer cachexia is different from that of the normal population (Dahele & Fearon, 2004). For example, despite both the patients with cancer cachexia and normal people under long-term starvation demonstrating weight loss, however, patients with cancer cachexia exhibit a highly differential profile of metabolism compared to normal people (Dahele & Fearon, 2004). The basal metabolic rate in people under long-term starvation is decreased to reduce the energy expenditure, accompanied by the shift of energy source from gluconeogenic amino acid generated by muscle catabolism to liver-stored lipids to preserve functional muscle mass. Protein breakdown and glucose turnover are both decreased to conserve energy and materials for life maintenance (Tisdale, 1997). In contrast, the opposite applies in patients with cancer cachexia. The cachectic cancer patients exhibit a normal or even increased basal metabolic rate and glucose turnover. Their energy source remains as gluconeogenic amino acid generated by muscle catabolism. Protein synthesis in skeletal muscle is also slowed down due to the insufficiency of the substrate as

most of the amino acids have been consumed for the increase of acute-phase protein synthesis in liver (Tisdale, 1997). The increased degradation rate and decreased synthesis rate of muscle proteins result in a rapid loss of muscle mass (Moley *et al.*, 1987). Muscle fiber shifting also occurs in the wasting muscle of cachectic cancer patients. It was observed that muscle tended to shift from slow twitch fiber to fast twitch fiber during muscle wasting (Mitch, 1996). Thus, the patients with cancer cachexia often experience loss of muscle mass, decline in muscle function and increase of fatigubility.

The skeletal muscle is an important organ that contributes approximately 40% of the body mass in humans (Henriksen, 2002). Skeletal muscle is vital to postural support, locomotion and breathing, through force production during contraction and confers cold tolerance. The skeletal muscle carries out fatty acid uptake and oxidation and is the venue that glucose disposal takes place upon insulin or exercise stimulation (Henriksen, 2002). In addition, skeletal muscle is essential for the proper functioning of the circulatory and respiratory systems by returning venous blood to the heart and enlarging the chest cavity (Kaneko & Harey, 1997).

Kaneko and Harey have suggested that it is essential to consider skeletal muscle function when assessing both the healthy and diseased states of other organ systems (Kaneko & Harey, 1997). In other words, excessive loss of muscle can cause many health problems and may result in functional decline in other important organ systems of cancer patients.

Fatigue is a common consequence secondary to skeletal muscle wasting due to cancer cachexia (Bing *et al.*, 2000). The reduction of muscle function impairs the quality of life of cancer patients and may even be fatal (Gilliam & St Clair, 2011). The decrease in muscle mass results in reduced endurance, asthenia, and weakness (Bing *et al.*, 2000). Cachectic cancer patients may also exhibit reduced cold tolerance due to the decrease in core body temperature (Bing *et al.*, 2000). Functional decline of the respiratory muscle could be lethal to patients (Winsor & Hill, 1988). It has been reported that decline in respiratory muscle function could lead to hypostatic pneumonia which is responsible for 48% of cancer patients' death (Tisdale, 2009).

To conclude, cancer cachexia is considered as the major cause of death in cancer patients while more than 30% cancer patients suffer from cachexia. Cancer cachexia causes severe weight loss due to muscle wasting. The reduced muscle mass greatly affects the patients' quality of life and may be fatal.

2.3 Doxorubicin is a Widely Used Potent Anti-cancer Drug

There are several types of cancer therapies including radiotherapy, chemotherapy and surgery. Each of them has their own advantages. However, among them, chemotherapy has an unique advantage that the drug is introduced to the entire body so that the treatment is also effective in treating metastatic tumors (National Cancer Institute, 2007). This renders chemotherapy irreplaceable, and doxorubicin is one of the effective and widely used chemotherapeutics.

Doxorubicin is a potent anti-cancer drug that was introduced in the 1960s (Bonadonna *et al.*, 1969). It is still widely used to treat a broad range of cancers including Hodgkin's disease, leukemia, non-Hodgkin's lymphoma, neuroblastoma, sarcoma, Wilms' tumor, bladder cancer, breast cancer, lung cancer, ovarian cancer, stomach cancer, germline cancer, and thyroid cancer (American Cancer Society, 2011).

2.3.1 Working Mechanism of Doxorubicin as an Anti-cancer Drug

Topoisomerase II is an important enzyme that takes part in Deoxyribonucleic acid (DNA) replication or transcription. It is responsible for unwinding the DNA supercoil by decatenation, relaxation or unknocking to provide access for the transcription factors or polymerases to bind on the DNA during DNA replication and transcription so that the target gene can be transcribed. During the process of decatenating, relaxing and unknocking of the DNA supercoil, topoisomerase II creates a temporarily double-strand break to allow other DNA strands to pass through the junction. It then ligates the junction once the process is completed. Doxorubicin, as a topoisomerase II inhibitor, inhibits the topoisomerase-mediated ligation of the broken strands. If the topoisomerase II fails to ligate back the broken DNA strands, the strand breaks will trigger apoptosis, hence eliminating the treated cancer cells (Deweese & Osheroff, 2009).

The doxorubicin-induced elevation of oxidative stress has also been implicated in the elimination of cancer cells (Sharma *et al.*, 2007). Reactive oxygen species

(ROS) are generated when doxorubicin undergoes redox cycling in mitochondrial complex I (Subashini *et al.*, 2006;Berthiaume & Wallace, 2007). The accumulation of oxidative stress results in damages of DNA and proteins, hence subjects the cancer cells to undergo cell death through apoptosis or necrosis (Ozben, 2007).

Although new drugs have been discovered and developed after doxorubicin, the usage of doxorubicin is still popular as indicated by the sales figure showing that doxorubicin is still generating a large amount of profit due to the large consumption in the market. According to EvaluatedPharma, a profit of more than 600 million US dollars was made from the sales of doxorubicin between the year 2009 and 2010. It is predicted that the usage of doxorubicin will be continuously increased in the coming years (EvaluatedPharma, 2011).

2.3.2 Limited Usage of Doxorubicin Due to Multi-organ Damage

Despite doxorubicin has a potent anti-cancer capability, its clinical use is limited by its multi-organ toxicity that causes damages to vital organs including heart,

lung, kidney, and liver (Agapito *et al.*, 2001;Saad *et al.*, 2001).

2.3.2.1 Doxorubicin-induced Cardiac Toxicity

The heart has been suggested as a highly susceptible target to doxorubicin induced-damages. Previous report showed that around 10% of patients with doxorubicin treatment developed cardiomyopathy (Octavia *et al.*, 2012). Administration of doxorubicin has been demonstrated to impair cardiac function, as indicated by the decrease in cardiac fractional shortening (Hydock *et al.*, 2009).

Research efforts have been spent to investigate the mechanisms of doxorubicin-induced cardiomyopathy. Oxidative stress generated by doxorubicin has been postulated to be the major cause of cardiac injury (Xu *et al.*, 2001), although other mechanisms such as intracellular calcium dysregulation, apoptosis, and changes of the high-energy phosphate pool were possibly involved (Octavia *et al.*, 2012). Extracellular matrix remodeling and fibrosis were observed in doxorubicin-treated hearts (Goetzenich *et al.*, 2009). In particular, the increase in collagen content in the left ventricle was demonstrated to be

causative for the reduction of heart function (Spinale, 2007).

2.3.2.2 Doxorubicin-induced Liver Toxicity

Doxorubicin can induce hepatotoxicity. Serum glutamic pyruvic transaminase, which is an indicative marker of liver damage, was increased by more than 50-fold after doxorubicin treatment. Malonaldehyde, a marker of oxidative stress, was also elevated in the doxorubicin-treated liver (Patel *et al.*, 2010), accompanied with acute inflammation and neutrophils infiltration (Abbas, 2011). Necrosis and vacuolation also occurred in the liver after prolonged exposure to doxorubicin (Abbas, 2011).

2.3.2.3 Doxorubicin-induced Renal Toxicity

The kidney-to-body weight ratio was found to decrease after doxorubicin administration (Rashikh *et al.*, 2013). Doxorubicin treatment resulted in a reduction of renal function, as indicated by concomitant reductions of serum protein, creatinine clearance, urea clearance (Rashikh *et al.*, 2013), increased serum blood urea nitrogen (Mohan *et al.*, 2010) and urinary protein (Pal & Sil, 2012).

Histological analysis revealed that degeneration of glomeruli and tubules occurred after doxorubicin intervention. This finding is paralleled by extended mesangial matrix and dilated urinary spaces and capillaries (Ayla *et al.*, 2011). Ultrastructural analysis demonstrated that the widths of the basement membrane and podocyte foot process base were both increased in doxorubicin-treated kidney (Rashikh *et al.*, 2013) due to the effacement of podocyte foot process after the administration of doxorubicin (Zhou *et al.*, 2011). Recently, Peng and colleagues reported that glomerulosclerosis was induced by doxorubicin treatment in concomitant with tubulointerstitial inflammation and fibrosis (Peng *et al.*, 2012a).

2.3.2.4 Doxorubicin-induced Lung Toxicity

Relatively fewer studies have been conducted in studying the doxorubicin-induced damages in the lung. However, damages caused by doxorubicin in the lung can be fatal and should not be depreciated. The elevation of collagen content in the alveolar walls followed by an increase in stiffness of the lung have been reported after doxorubicin treatment (Take *et al.*, 2008). This leads to the loss of pulmonary function in response to the reduction of lung volume. The increase in collagen

deposition was also known to be associated with inflammation of the lungs (Laurent, 1986). Furthermore, doxorubicin was found to induce mitochondrial degeneration in the lung, thereby synergistically deteriorating the lung function (Take *et al.*, 2008).

2.3.2.5 Doxorubicin-induced Skeletal Muscle Toxicity

Following an array of studies in doxorubicin-induced damages of the important organs mentioned earlier, the attention of researchers has recently been extended to doxorubicin-induced skeletal muscle injury.

Body weight, muscle mass and muscle performance were decreased by doxorubicin treatment. It was reported that the maximal twitch force and maximal rate of force production were decreased after doxorubicin treatment (Hydock *et al.*, 2011;Hayward *et al.*, 2012), indicating that skeletal muscle function was impaired in terms of force production and endurance capacity. Furthermore, relaxation time required for the muscle to relax from 90% return to 10% of maximal force was observed to be increased, meaning that the process of muscle

relaxation was also impaired (van *et al.*, 2009). Interestingly, doxorubicin was found to retain in the skeletal muscle several days following administration, suggesting damage induced by doxorubicin in the skeletal muscle could be accumulative (Hayward *et al.*, 2012).

A dose-dependent acute inflammatory reaction was observed in the skeletal muscle within one week after doxorubicin injection while considerable fibrosis occurred six weeks later (Cullu *et al.*, 2003). Direct injection of doxorubicin to the skeletal muscle altered myofilament structure and induced necrosis (Gilliam & St Clair, 2011).

Reactive oxygen species (ROS) were generated by doxorubicin in the skeletal muscle (Gilliam & St Clair, 2011). The increase in oxidative stress-induced DNA damage triggered apoptosis by activating the cysteine-dependent aspartate-directed proteases (caspase) cascade, resulting in a loss of muscle mass (Gilliam & St Clair, 2011).

The loss of muscle mass can also be ascribed to the proteasome pathway. Gillian and St Clair reported that the protein abundance of E3 ubiquitin-ligase F-box protein 32 (MAFbx) was increased in doxorubicin treated skeletal muscle (Gilliam & St Clair, 2011). Activation of cellular proteases, such as Calcium-activated neutral proteinase (calpain) and caspase-3, were also suggested to be involved in the increased proteolysis of the skeletal muscle after doxorubicin intervention (Smuder *et al.*, 2011b).

Recently, autophagy was found to be activated in response to doxorubicin intervention. The protein and Messenger ribonucleic acid (mRNA) abundances of autophagy markers, including Beclin-1, autophagy related gene (Atg) 5, Atg 7, Atg 12 and the microtubule-associated protein 1 light chain 3 alpha (LC3) II/I ratio were elevated in doxorubicin-treated muscle, suggesting the pathogenic role of autophagy in doxorubicin-induced muscle injury (Smuder *et al.*, 2011a).

To conclude, doxorubicin is a potent anti-cancer drug that is widely used for cancer treatment. However, its multi-organ toxicity largely limits its clinical use.

Toxicological studies of doxorubicin are now extended to skeletal muscle. It has been demonstrated that exposure to doxorubicin can induce functional loss of skeletal muscle by promoting muscle wasting, inflammation, autophagy, cell death and muscle fibrosis.

2.4 Apoptosis

In a multicellular organism, an individual cell may commit suicide for the benefit of the whole. This cellular behavior, termed programmed cell death or apoptosis, is important for the development and maintenance of the biological systems of multicellular organisms (Gewies, 2003). For example, morphogenesis and the immune system process to eliminate damaged and harmful cells which are not necessary for cell functions.

Regulation of apoptosis is sophisticated. Apoptosis can be triggered by a wide range of stimuli from diverse origins including DNA damages, cytotoxic drugs, irradiation and activation of the death receptors on cell surface, survival signal deficiency, contradictory cell cycle signaling and developmental death signals. After receiving the stimulus, the cell will either undergo intrinsic or extrinsic pathway depending on the type of stimulus received (Gewies, 2003). Depending on the type of stimulus, execution of apoptosis would be mediated differentially by either the intrinsic or extrinsic pathway.

2.4.1 Intrinsic Pathway of Apoptosis

In the intrinsic pathway, apoptotic stimuli stimulates the release of cytochrome c from the mitochondria via the regulation of the Bcl-2 family proteins (Salvesen & Renatus, 2002). Following its release to cytosol, cytochrome c binds to apoptotic protease activating factor 1 (Apaf-1) to form a complex called apoptosome, leading to the activation of procaspase-9 by dimerization (Denault & Salvesen, 2002). The effector caspases are then activated to induce cleavages of other proteins resulting in the execution of apoptosis (Earnshaw *et al.*, 1999).

2.4.2 Extrinsic Pathway of Apoptosis

The extrinsic pathway is initiated by the activation of specific death receptors on the cell surface. Ligand-receptor binding causes the activation of cytoplasmic death domain of the death receptors to recruit caspase-8 through adaptor molecules. This process results in the formation of the death-inducing signaling complex and increases the local concentration of procaspase-8. Auto-activation of procaspase-8 then occurs due to the high local concentration of itself. The activation of caspase-8 leads to activation of downstream effector caspases

through the caspase cascade and ultimately results in the cleavage of specific substrates and execution of cell death (Scaffidi *et al.*, 1998). When the cell death signal generated by the activated receptors is insufficient to execute cell death, caspase-8 will cleave BH3 interacting domain death agonist (Bid), a Bcl-2 family member, into its truncated form (tBid) to induce the release of cytochrome c with coordination of the proapoptotic proteins BCL-2 associated X (Bax) and BCL2-antagonist.killer 1 (Bak) (Luo *et al.*, 1998). The released cytochrome c will then bind to Apaf-1 to form apoptosome, leading to the downstream signaling which converges with the intrinsic pathway (Gewies, 2003).

2.4.3 Bcl-2 Family Proteins as Regulators of Apoptosis

The Bcl-2 family proteins play an important role in apoptotic regulation. Members of the Bcl-2 family are characterized by the presence of Bcl-2 homology domains (BH1 to BH4) and can be divided generally into the prosurvival group and the proapoptotic group (Borner, 2003). Bcl-2 and BCL-2 like-1 (Bcl-X) are members of the prosurvival group and contain all the homology domains (BH1 to BH4). The proapoptotic group can be further divided into two groups as the Bax subfamily

(contains BH1 to BH3) including Bax, Bak and BCL2-related ovarian killer (Bok) and BH3-only subfamily (contains BH3 motif only) comprising Bid, BCL-2 like 11 (Bim), BCL-2 interacting killer (Bik), BCL-2-associated death promotor (Bad), BCL2 modifying factor (Bmf), Harakiri, BCL2 interacting protein (Hrk), Thymoma viral proto-oncogene (Noxa), BCL2 binding component 3 (Puma), BCL2/adenovirus E1B 19kDa interacting protein 2 (NIP) and Spike (Cory & Adams, 2002;Mund *et al.*, 2003). Members of the prosurvival group inhibit the members of the proapoptotic group to inhibit the release of apoptotic factors like cytochrome c to cytosol. Hence the balance between the members of the prosurvival group and the proapoptotic group determines the execution of apoptosis (Gewies, 2003).

Each BH3 only-protein responds differentially to different stimuli. In response to stimulation, interaction between the BH3-only proteins and the proteins of the prosurvival group occurs, resulting in the activation of the proapoptotic proteins (Bouillet & Strasser, 2002). Bax and Bak undergo conformational change after activation and form channels at the mitochondrial outer membrane, thus trigger

the release of apoptotic factors, such as cytochrome c, apoptosis-inducing factor (AIF) (Susin *et al.*, 1999), endonuclease G (EndoG) (Li *et al.*, 2001), inhibitor of apoptotic protein-binding mitochondrial protein (Smac/Diablo) (Verhagen *et al.*, 2000) and HtrA serine peptidase 2 (Htr/Omi) to cytosol (Verhagen *et al.*, 2002).

It has been reported that Bax and Bak act as gatekeepers of apoptotic signaling. This notion is supported by the fact that mitochondrial permeabilization pore cannot be formed in the absent of Bax and Bak, thus the release of apoptotic factors from the mitochondrial intermembrane space and execution of mitochondria-driven apoptosis is hindered. It has been demonstrated in vitro that deficiency in Bax and Bak confer resistance to multiple apoptosis stimuli, such as ultraviolet radiation, growth factor deprivation, and endoplasmic reticulum stress (Wei *et al.*, 2001).

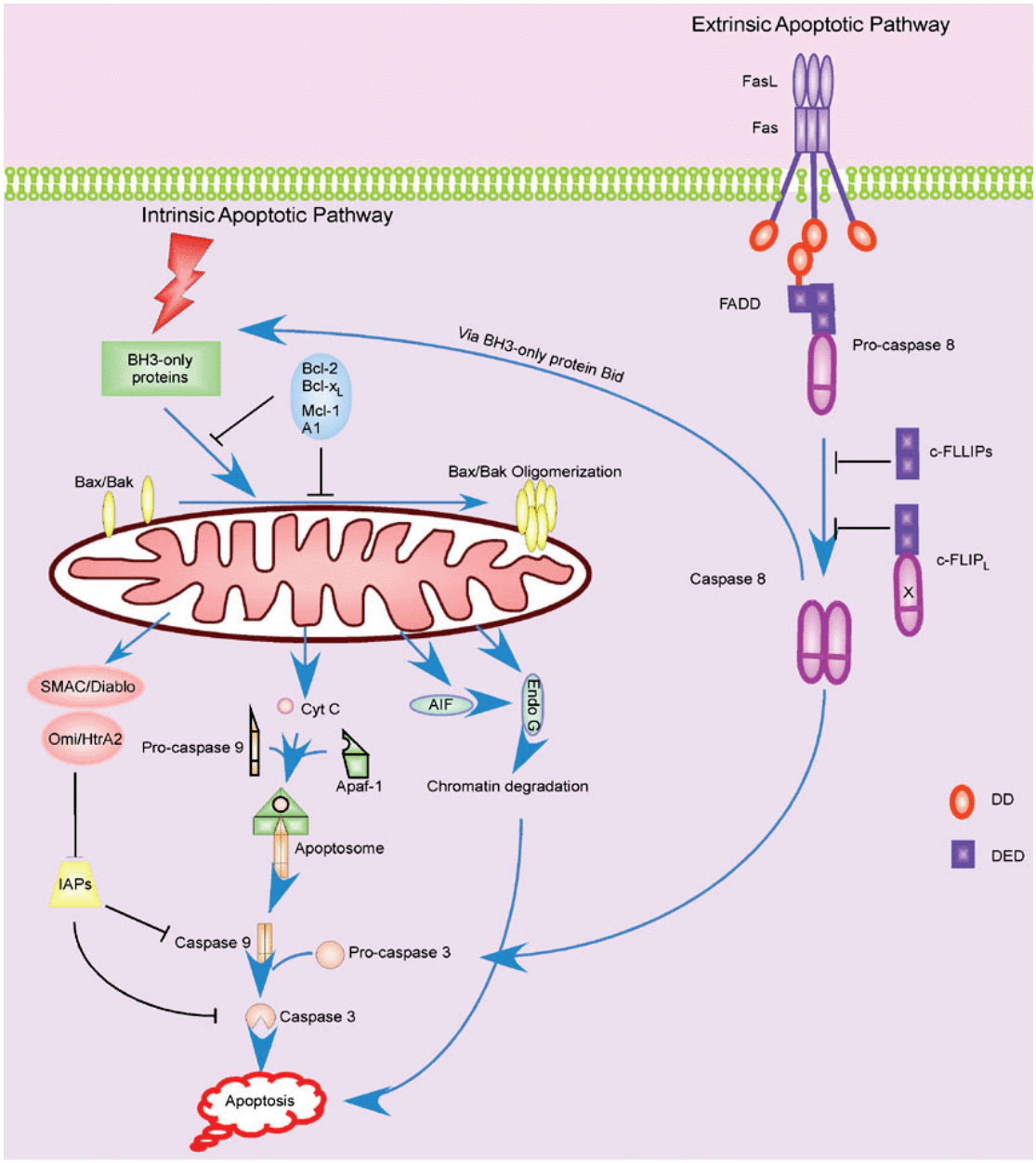

Figure 1. Intrinsic and Extrinsic Pathways of Apoptosis.

(Adopted from Pope (Pope, 2002)).

2.4.4 Association between Apoptosis and Skeletal Muscle

Apoptosis impacts development and adaptation of skeletal muscle and causes disorders if its homeostasis is disturbed. During the developmental stage, apoptosis eliminates excessive immature cells under the government of the developmental program. In the skeletal muscle, apoptosis eliminates the undesired muscle cells during the myotubular stage (Webb, 1972). Some muscle fibers are also removed during gestation via apoptosis such that the final number of muscle fibers can be sculptured (Fidzianska & Goebel, 1991). In contract, apoptotic nuclei were found in muscles undergoing atrophy following unloading (Allen *et al.*, 1997) or denervation (Tews *et al.*, 1997), indicating that apoptosis may mediate the elimination of myonuclei during muscle atrophy. Similar observation was also found in spinal muscular atrophy, a genetic degenerative disease of motor neurons (Roy *et al.*, 1995).

Emerging evidence suggests the role of apoptosis in muscle pathology. Sandri and co-workers identified the role of apoptosis in muscle degeneration in muscle dystrophy (Sandri *et al.*, 1998). It has also been reported that pressure-induced

deep-tissue injury triggers apoptosis, evidenced by the increased expression of proapoptotic markers including Bax, caspase-3, caspase-8 and caspase-9, paralleled by the elevation of DNA fragmentation (Siu *et al.*, 2009).

Apoptosis is also involved in the elimination of damaged muscle fibers caused by excessive exercise (Carraro & Franceschi, 1997), as well as the pathogenesis of other muscle diseases such as thyroid-associated ophthalmopathy (Koga *et al.*, 1998). The implications of apoptosis in regulating muscle function during development, adaptations, and disorders shall receive attention.

2.5 Autophagy

Autophagy was discovered in 1950 by examining the presence of specific vesicles, named autophagosomes, in cells under electron microscope (Mizushima *et al.*, 2010). Autophagy, as its name in Greek describes, is a self-engulfing process which double-membrane vesicles in cytoplasm enwrap the functionally impaired organelles and damaged proteins in the cytoplasm and directs a lysosomal transfer for degradation (Mizushima *et al.*, 2010). Different forms of autophagy such as macroautophagy, microautophagy and mitophagy are characterized by its distinct mechanism or its specific removal targets (Klionsky, 2005;Massey *et al.*, 2006). Among them, macroautophagy is the focus in this MPhil study.

2.5.1 General Functions of Autophagy

Autophagy is regarded as the quality control machinery for proteins in the cells through the removal of damaged proteins and dysfunctional organelles (Yorimitsu & Klionsky, 2007) and is considered primarily as a pro-survival pathway (Mizushima *et al.*, 2008;Maiuri *et al.*, 2007). Downregulation of autophagy leads to an increase in cell death (Levine & Kroemer, 2008). It has been reported that

autophagy responds to different cellular stresses and generally confers cytoprotective effects. Accumulation of abnormal proteins and damaged organelles have been observed in several types of autophagy-deficient cells (Komatsu *et al.*, 2006;Hara *et al.*, 2006;Nakai *et al.*, 2007).

Autophagy operates at different activity levels among different tissues to meet their specific demands. Autophagy is particularly important in tissues consisting of post-mitotic differentiated cells (Komatsu *et al.*, 2006;Hara *et al.*, 2006;Nakai *et al.*, 2007;Mizushima *et al.*, 2008). This removal process is tightly regulated since some organelles, like mitochondria, are necessary for cell survival. Excessive removal of these essential components in response to uncontrolled autophagy can lead to cell death. While autophagy modulates various physiological events to confer cytoprotection, altered level of autophagy has been identified in various diseases (Mizushima *et al.*, 2008).

2.5.2 Autophagic Regulation and Mechanism

Autophagy is important for maintaining homeostasis in cells. Hence, this process must be tightly regulated, or otherwise over-activation of autophagy may lead to extensive loss of cytoplasmic contents and results in cell death. A set of genes known as autophagy-related genes (Atg) have been identified to be involved in autophagic regulation (Tsukada & Ohsumi, 1993;Thumm *et al.*, 1994;Harding *et al.*, 1995).

2.5.2.1 Formation of Autophagosome

Autophagy begins with the formation of autophagosomes, the vesicles which internalize proteins and organelles for removal. The elongation of a small membrane structure, known as the autophagosome precursor, is a prerequisite for the formation of autophagosomes in mammalian cells. Knowledge on this membrane structure is limited but it has been demonstrated in Atg 5-dificient mouse embryonic cells that this membrane structure is not divided from any pre-existing large membrane structure organelles such as the endoplasmic reticulum (Mizushima *et al.*, 2001). The mechanism of autophagosome formation

has been characterized in more detail in yeast than in mammalian cells.

Similar to mammalian cells, the autophagosome formation in yeast begins with a pre-autophagosomal structure (PAS), which is generated by assembling several Atg proteins as a site (Kim *et al.*, 2001;Suzuki & Ohsumi, 2007). Although the actual structure of PAS remains unknown, 18 out of the 31 characterized Atg proteins have been shown to be essential for PAS formation in yeast (Klionsky *et al.*, 2003;Kabeya *et al.*, 2007;Suzuki *et al.*, 2007b). The recruitment of these Atg proteins are inter-dependent which requires Atg17 as the scaffold and the assistance of Atg29 and Atg31 (Kawamata *et al.*, 2005;Suzuki *et al.*, 2007b;Kabeya *et al.*, 2007).

2.5.2.2 Membrane Elongation

Upon the autophagosome precursor, the structure starts to elongate and becomes an isolation membrane under the co-regulation of the Atg 12 system and LC3 system. The Atg 12 system is an ubiquitin-like conjugation system that was first identified in yeasts and was found highly conserved in mammalian cells

(Mizushima *et al.*, 1998;Ohsumi, 2001;Mizushima *et al.*, 2002a;Mizushima *et al.*, 2003). In this system, the conjugation process is initiated by Atg 7 so that Atg 12 and Atg 5 can be assembled to form a conjugate, known as Atg 12-5 Complex (Mizushima *et al.*, 2001;Tanida *et al.*, 2001). Atg 7 first activates the carboxy-terminal glycine residue of Atg 12 to form an Atg 12-Atg 7 thioester intermediate (Tanida *et al.*, 2001). It is subsequently transferred to Atg10 and this process is essential for the formation of a Atg 12-Atg10 thioester intermediate (Mizushima *et al.*, 2002b) prior to the covalent attachment of the carboxy-terminal glycine of Atg 12 to lysine 130 of Atg 5 by which requires isopeptide bonds (Mizushima *et al.*, 1998).

Atg16, a β-transducin repeat protein, also participates in the Atg12 system (Mizushima *et al.*, 2002a). The structure of Atg16 in mammalian cells is different from yeast cells. The Atg16 protein in yeast is in form of a small coiled-coil protein whereas its mammalian counterpart is a relatively big protein containing seven repeated β-transducin repeats in the C-terminal domain (Mizushima *et al.*, 1999;Mizushima *et al.*, 2002a;Kuma *et al.*, 2002). It is thus expected that the

structure of protein complex formed by these two Atg16 proteins are also different. The integration of Atg 12-Atg 5 conjugate with mammalian Atg16 yields a protein complex of about 800 kDa whereas the corresponding complex in yeasts is only approximately 350 kDa in size (Mizushima *et al.*, 1999;Mizushima *et al.*, 2002a;Kuma *et al.*, 2002). As the β-transducin repeat protein generally provides a platform for protein-protein interaction, it is believed that mammalian Atg16 can interact further with other proteins (Mizushima *et al.*, 2002a). These complexes localize on the isolation membrane during the elongation process, although most of them reside in the cytosol. The localization of the Atg12-Atg5.Atg16 complex also changes with the elongation of the isolation membrane. They first distribute evenly on both sides of the membrane but then distribute asymmetrically with most of them locate on the outer side. These complexes are then released to cytosol following completion of the elongation process (Mizushima *et al.*, 2002a).

To further understand the role of Atg12-Atg5.Atg16 complex in the elongation process, Muzushima and co-workers conducted a study in embryonic stem cells that expressed mutated Atg5. They mutated the Atg12 attachment site in Atg5 by

substituting lysine 130 with arginine (Mizushima *et al*., 2001). It has been observed that the mutants were defective in forming the Atg12-Atg5.Atg16 complex since the binding of Atg12 to the mutated Atg5 was abolished (Mizushima *et al*., 2001). The mutated Atg5 together with Atg16 was still able to localize on the autophagosome precursors despite the membrane elongation was unsuccessful in the absence of Atg12 (Mizushima *et al*., 2001). This result implied that the Atg12-Atg5.Atg16 complex is indispensable for the isolation membrane to elongate but not for the formation of autophagosome precursor (Mizushima *et al*., 2001).

The other system that regulates membrane elongation is an ubiquitin-like system named LC3 system. LC3 was the first Atg protein discovered in autophagosome and named as Atg8 in yeast (Nakatogawa *et al*., 2007). There are two isoforms of LC3, which are the cytosol residue form, LC3 I and the truncated form, LC3 II. The Atg4 catalyzed a post-translational cleavage at the C-terminal of the LC3 to produce the LC3 I immediately after the translation of LC3 mRNA (Tanida *et al*., 2001). During the elongation process of isolation membrane, LC3 I targets to the

isolation membrane (Tanida *et al.*, 2001). Atg7 then activates the second cleavage of LC3 (Tanida *et al.*, 2001). LC3 I is transferred to Atg3, a specific E2 enzyme, where protein lipidation takes place and generates LC3 II (Tanida *et al.*, 2002). It has been reported that the abundance of LC3 II and LC3II/I ratio are the standard markers used to assess the level of autophagic activation due to their correlation with the number of autophagosomes formed (Kabeya *et al.*, 2000). The targeting of isolation membrane and cleavage of LC3 I is Atg5-dependent. It has been demonstrated in Atg5-deficient cells that LC3 I failed to target to the isolation membrane, although it was produced normally and remained in cytosol. There was no LC3 II detected in the Atg5-deficient cells (Mizushima *et al.*, 2001). Nevertheless, further investigation is needed to elucidate these two regulatory mechanisms. It was proposed that the Atg12-Atg5 conjugate might serve as E3-like enzyme in the LC3 system or stabilizer of LC3 II. Unlike the Atg12–Atg5 conjugate, LC3 was found to remain on the membrane even when the autophagosome had been completely formed (Kabeya *et al.*, 2000;Mizushima *et al.*, 2001).

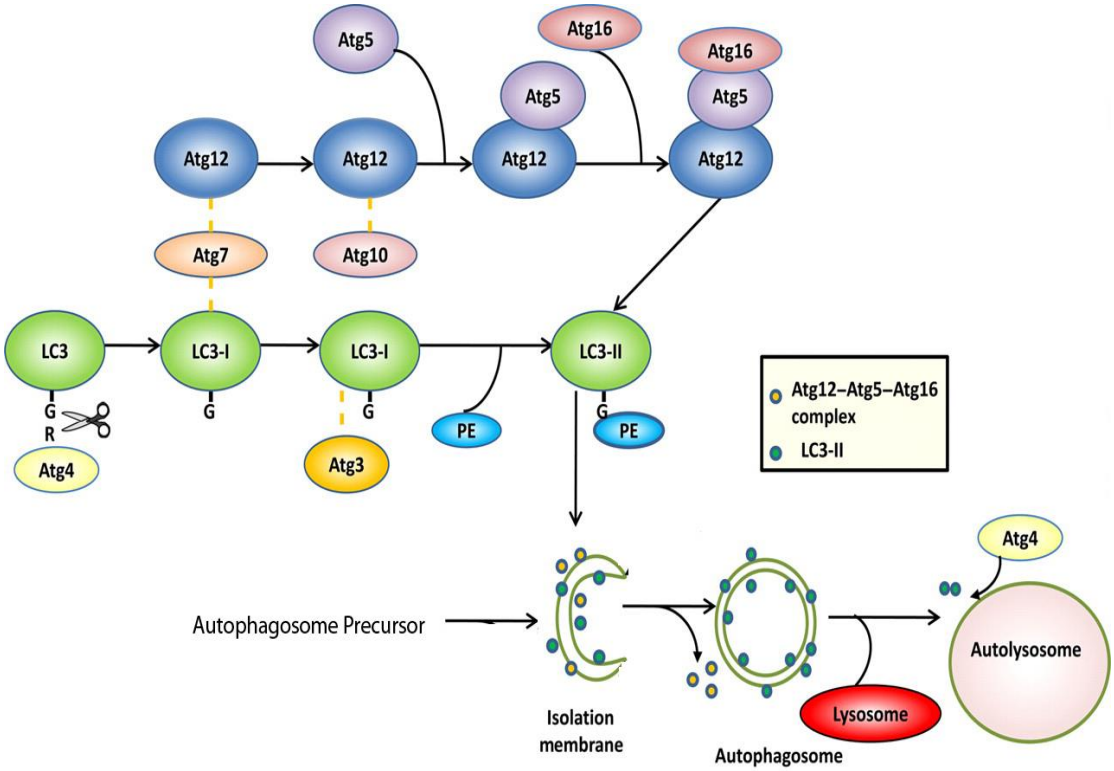

Figure 2. Autophagy Is Regulated by Autophagy-related Gene Proteins.

(Adopted and modified from Ding et al. (Ding *et al.*, 2011)).

2.5.2.3 Specificity of Autophagic Removal for Proteins and Organelles

Autophagosome non-specifically enwraps proteins and organelles during the elongation process of isolation membrane. However, the specificity of isolation membrane in the recognition and removal of certain proteins and organelles has been reported (Mizushima, 2007). The composition of the outer side and the inner side of the autophagosomal membrane is different while LC3 is the only protein that has been identified on the inner side of the isolation membrane and is proposed as a mediator of selective autophagy (Kabeya *et al.*, 2000). It has been demonstrated that LC3 serves as a receptor for the binding of targeted proteins to the autophagosomal membrane for selective autophagic degradation (Bjorkoy *et al.*, 2005).

In 2006, Wang and co-workers demonstrated that p62/SQSTM1 accumulated in autophagy-deficient cells and brought about the selective nature of autophagic degradation (Wang *et al.*, 2006). The ubiquitin-binding domain of p62/SQSTM1 may explain the selective process further as ubiquitinated p62/SQSTM1 was found in the autophagy-deficient cells. These results suggested that ubiquitination

of proteins may be involved in the coordination of autophagy (Bjorkoy *et al.*, 2005;Pankiv *et al.*, 2007). Besides p62/SQSTM1, peroxisomes, catalase, mitochondria, and invading bacteria have also been reported as the targets of autophagy-mediated selective degradation (Luiken *et al.*, 1992;Nakagawa *et al.*, 2004;Ogawa *et al.*, 2005;Iwata *et al.*, 2006;Yu *et al.*, 2006;Kim *et al.*, 2007).

2.5.2.4 Degradation

The maturation of autophagosome and its fusion with lysosome precedes the enwrapped proteins and organelles to degradation. The inner membrane of autophagosome and its contents are degraded by hydrolase in the lysosome (Epple *et al.*, 2001;Teter *et al.*, 2001). The structure that brings about the degradation process is named autolysosome or autophagolysosome. The formation of amphisome by the fusion of autophagosomes and endosomes (Tooze *et al.*, 1990) was proposed to be a machinery of endosome to facilitate subsequent fusion with lysosome (Mizushima, 2007). Provided that lysosomes undergo continual endocytosis and amphisomes are fused with lysosome for degradation, autolysosomes may contain heterophagic materials in addition to its self-materials

(Gordon & Seglen, 1988). It is difficult to distinguish these membrane structures under electron microscope since the definition of autolysosome, amphisome and autophagosome are characterized by their functions instead of their morphologies. In autolysosomes, proteins or organelles are degraded into monomeric units and exported to the cytosol for reuse purpose (Mizushima, 2007).

2.5.2.5 Reutilization of Autophagy Degraded Material

Autophagy is critical for cell survival during long-term starvation (Kuma *et al.*, 2004;Onodera & Ohsumi, 2005). Upregulation of autophagy is an adaptive response to recycle essential amino acids by degrading damaged cytoplasmic proteins and dysfunctional organelles (Mizushima, 2007). Regulation of autophagy in response to starvation is complicated. Although autophagy was reported to be induced in response to the depletion of total amino acid, downregulation of autophagy has been observed in ex vivo liver following the depletion of a particular kind of amino acid (Mortimore & Poso, 1987). The alteration of autophagy level due to the depletion of amino acid is cell-type specific

since amino acid metabolism varies among tissues (Mortimore & Poso, 1987). The mechanisms of reusing autophagy-recycled amino acids are still unknown, although three possible mechanisms have been proposed. The autophagy-generated amino acid can be 1) consumed during hepatic gluconeogenesis; 2) served as fuel in tricarboxylic cycle; or 3) consumed for synthesizing new proteins as a starvation-induced adaptive response (Mizushima, 2007). In addition to the depletion of nutrients, it is reported that autophagy was induced during hypoxia for temporary promotion of cell survival (Degenhardt *et al.*, 2006).

2.5.3 Physiological Roles of Autophagy

2.5.3.1 Removal of Pathogen

Autophagy is an antimicrobial mechanism. This process is considered to be mediated by a special class of autophagy called xenophagy (Levine, 2005). For the removal of harmful materials and pathogens including bacteria, parasites, phagosomes, pathogen-containing vacuoles, and newly synthesized virons (Huang & Klionsky, 2007;Levine & Deretic, 2007). The autophagosomes

generated by this special type of autophagy is larger than that in the macroautophagy (Levine & Deretic, 2007). It is believed that the increased size of the autophagosome in xenophagy is an adaptive response to remove bulky microbes or pathogens (Levine & Deretic, 2007). Apart from direct removal via lysosomes, xenophagy also participates in innate and adaptive immunity by serving as a trafficking system. For example, xenophagy mediates the delivery process of viral nucleic acids to the endosomal toll-like receptor TLR7 to activate the type 1 interferon signaling (Levine & Deretic, 2007).

While autophagy is an anti-infection machinery, development of strategies to counteract autophagic removal was reported in various pathogens (Kirkegaard *et al.*, 2004;Huang & Klionsky, 2007), including the inhibition of autophagic regulatory signaling and blockage in autophagy trafficking (Levine & Deretic, 2007). One example is the herpes simplex virus. This virus can cause fatal encephalitis by downregulating Beclin-1 with a viral neurovirulent protein (Orvedahl *et al.*, 2007). Thus, specific modulation of the level of autophagic proteins may be able to prevent infection-induced pathology.

2.5.3.2 Relieve of Endoplasmic Reticulum Stress

Autophagy was reported as a potential mechanism for relieving endoplasmic reticulum stress to avoid cell death in both yeast and mammal (Ogata *et al.*, 2006;Yorimitsu *et al.*, 2006;Kouroku *et al.*, 2007;Ding *et al.*, 2007). Autophagosomes containing endoplasmic reticulum materials were found in the cytoplasm of the endoplasmic reticulum stress inducer-treated yeast (Bernales *et al.*, 2006). Interestingly, the endoplasmic reticulum materials-containing autophagosomes did not fuse with vacuoles for degradation (Bernales *et al.*, 2006). It has also been reported that vacuolar proteases are not essential to decrease the endoplasmic reticulum stress relieving effect (Mizushima, 2007). Collectively, it is believed that autophagy depends mainly on endoplasmic reticulum sequestration to protect cells against endoplasmic reticulum stress.

2.5.3.3 Suppression of Tumor Formation

Tumorigenesis is a multifactorial process that involves autophagy as one of the factors (Mizushima *et al.*, 2008). Cancer is regarded as a disease secondary to the disturbed regulation of autophagy, and the genetic linkage between cancer and

defective autophagy has been established (Mathew *et al.*, 2007;Levine & Kroemer, 2008). Botti and co-workers demonstrated that activation of oncogenes inhibited autophagy whereas autophagy-activating tumor suppressor genes were found to be silenced through mutation or epigenetic mechanisms (Botti *et al.*, 2006). It has been reported that monoallelic deletion of Beclin-1 accounts for 40-75% of human breast cancer, ovarian cancer, and prostate cancer (Qu *et al.*, 2003). The implication of Beclin-1 in tumorigenesis was supported by the findings obtained in animals with monoallelic deletion of Beclin-1. These animals developed lymphomas, lung carcinomas, hepatocellular carcinomas spontaneously together with mammary precancerous lesions (Yue *et al.*, 2003). Conversely, tumor suppression can be induced by the introduction of extra copies of Atg genes (Marino *et al.*, 2007). It was proposed that autophagy served as a tumor-suppressor pathway although the mechanism has not been fully established.

The dual role of autophagy in delivering pro-survival signal and tumor suppression seems contradictory (Mathew *et al.*, 2007). There are several

possible explanations for the paradox. First, necrotic cell death predominates when apoptosis fails to eliminate cancer cells. However, this may promote tumor growth by exacerbating local inflammation (Mizushima *et al.*, 2008). Autophagy can inhibit necrosis and subsequently suppresses tumor growth (Mizushima *et al.*, 2008). Second, autophagy may prevent cell growth under metabolic stress. The dominant effect of tumor suppression often disrupts the balance between the pro-survival effects and the tumor suppressing effects of autophagy (Mizushima *et al.*, 2008). It was reported that the cytotoxicity induced by chemotherapeutic agents was enhanced upon genetic or pharmacological inhibition of autophagy (Abedin *et al.*, 2007;Carew *et al.*, 2007). Clinical trials of augmenting the anti-tumor effects of cancer cytotoxic agents by autophagic inhibition are now in progress (Amaravadi *et al.*, 2007). It can be concluded that upregulation of autophagy may prevent tumor formation and progression while suppressing autophagy may promote tumor regression.

2.5.3.4 Alteration of Autophagy with Aging

The level of autophagy alters with age (Del *et al.*, 2003;Cuervo *et al.*, 2005).

Accumulation of damaged proteins and organelles was commonly observed in aged cells (Del *et al.*, 2003;Cuervo *et al.*, 2005). It has been demonstrated in liver that the level of autophagy was decreased progressively with age, thereby possibly accounting for the loss of function and accumulation of damaged proteins and organelles particularly in the postmitotic differentiated cell types (Del *et al.*, 2003;Mizushima *et al.*, 2008).

Mitochondrial DNA mutations were found to be accumulated with age (Kujoth *et al.*, 2007). Increased frequency of mitochondrial DNA mutations was observed in autophagy-deficient yeast (Mathew *et al.*, 2007;Levine & Kroemer, 2008). The roles of Beclin-1 and Atg 5 in genome protection have been proposed. Increased DNA damages, gene amplifications, and aneuploidy were observed in immortalized epithelial cells upon the removal of either or both copies of Beclin-1 or Atg 5 (Mathew *et al.*, 2007). These results demonstrated the effect of abolishing basal autophagy on genotoxic stress and DNA damages.

To conclude, autophagy is a pro-survival mechanism under careful regulations of

Atg proteins. It is responsible for protein quality control and removal of malfunctioned organelles. Autophagy was found to recycle amino acids by degradation of damaged proteins and organelles. It also takes part in removal of pathogens, alleviation of endoplasmic reticulum stress, and prevention of cancer development. The aging-induced reduction in autophagy may associate with the pathogenesis of age-related diseases.

2.5.4 Association Between Autophagy and Skeletal Muscle Maintenance

Autophagy is an important catabolic mechanism in the skeletal muscle. Proteins and organelles may be damaged following muscle contraction (Sandri, 2010). In this context, the autophagy-mediated removal of useless substances is necessary to maintain the healthy cellular environment of muscle cells.

2.5.4.1 Role of Autophagy in Skeletal Muscle Catabolism

Many studies have implicated the role of autophagy in muscle atrophy. Transgenic animal models were employed to unmask the roles of autophagy in Forhead box O3 (FoxO3) and oxidative stress-mediated loss of the skeletal muscle (Mammucari

et al., 2007). Catabolic conditions including fasting, denervation and muscle wasting were shown to activate FoxO3 to enhance the ubiquitin-proteasome protein degradation pathway by upregulating MAFbx and Muscle-specific RING finger protein 1 (MuRF-1) (Sandri *et al.*, 2004;Stitt *et al.*, 2004). It was observed that deletion of a critical autophagic gene, LC3, could preserve muscle mass partially in the presence of constitutively active FoxO3 (Mammucari *et al.*, 2007). Furthermore, reductions of specific force generation and tetanic force generation in the atrophying soleus muscle and the atrophying extensor digitorum longus muscle were observed following the elevation of oxidative stress (Dobrowolny *et al.*, 2011). In addition, blunting the expression of LC3 was found to be protective against reactive oxygen species (ROS)-induced muscle atrophy and weakness (Dobrowolny *et al.*, 2011).

The effects of aging on autophagy were found to be varies in different tissues. The finding of aging-induced reduction of autophagy cannot be reproduced in the skeletal muscle and some studies reported that the level of autophagy in skeletal muscle was even increased with age (Del *et al.*, 2003;Wenz *et al.*, 2009). In

addition, autophagy has been proposed to mediate age-related sarcopenia. It has been demonstrated that upregulation of peroxisome proliferator activated receptor gamma coactivator 1 alpha (PGC1α) preserved muscle mass and concomitantly abrogated the induction of autophagy with aging (Wenz *et al.*, 2009).

It can be summarized from previous studies that an increase in autophagic flux leads to muscle loss while alleviation of autophagy can potentially preserve muscle mass under several catabolic conditions or diseased states such as fasting, denervation, and aging. Future research may need to target the autophagic pathway for exploring therapeutic targets to treat muscle diseases.

2.5.4.2 Important Roles of the Basal Level of Autophagy in Skeletal Muscle

Abnormally high level of autophagy in catabolic condition contributes to muscle loss whereas downregulation of autophagy was found to prevent muscle loss (Mammucari *et al.*, 2007;Masiero & Sandri, 2010;Dobrowolny *et al.*, 2011). Interestingly, autophagy is constitutively active in skeletal muscle. Both

conventional and inducible Atg7 knockout mouse models have been generated by Masiero and co-workers to elucidate the roles of the basal autophagy in the skeletal muscle (Masiero & Sandri, 2010). As the cleavage of the C-terminal of LC3 is a Atg7-dependent process critical to autophagy, the formation of autophagosome is hampered in the absence of Atg7 and subsequent autophagic machinery is abolished (Tanida *et al.*, 2001).

Muscle mass was expected to be preserved in Atg7-deficient mice in congruent with the maintenance of contractile proteins and higher muscle strength. Surprisingly, muscle loss and fiber degeneration were not abolished in Atg7-deficient mice, suggesting that abrogation of basal autophagy neither preserved muscle mass nor retained muscle strength (Masiero & Sandri, 2010). The Atg7-deficient mice indeed exhibited lower body weight due to the reduced muscle mass. Histological analysis showed that the size of muscle fibers in Atg7-deficient mice was 40% smaller than that of wild type mice and an increased percentage of centronucleated muscle fibers was observed (Masiero & Sandri, 2010). The elevated abundances of two E3 ligases, MAFbx and MurF1, following

the deletion of Atg7 might account for the reduction in muscle fiber size (Masiero & Sandri, 2010). Notably, Atg7-deficient mice showed no deterioration in proteasome function. However, ubiquitinated proteins which is supposed to be removed via proteasome were found to be accumulated in the muscle, suggesting that the removal of certain ubiquitinated proteins may specifically depend on lysosomes and autophagy (Masiero & Sandri, 2010).

It was demonstrated in inducible Atg7 knockout mice that a three-week inactivation of autophagic machinery induced severe loss of muscle mass and strength (Masiero & Sandri, 2010). The upregulation of endoplasmic reticulum stress marker accompanied with the accumulation of protein aggregates and abnormal mitochondria were found in the skeletal muscle with depressed autophagy (Masiero & Sandri, 2010). The presence of abnormal mitochondria and increase in Eukaryotic translation initiation factor 2 (eIF2α) phosphorylation in autophagy-deficient muscle indicated that basal autophagy is responsible for preventing accumulation of oxidative stress and activation of unfolded protein response (Masiero & Sandri, 2010). The increase in oxidative stress and unfolded

protein response due to the inactivation of autophagic machinery have triggered the activation of ubiquitin-proteasome pathway and apoptotic pathway to induce muscle loss (Masiero & Sandri, 2010). A consensus was reached regarding the indispensable role of basal autophagy in the maintenance of muscle mass and function through the elimination of damaged proteins and non-functional organelles.

To conclude, the importance of autophagy is supported by the notion that severe loss of muscle mass and function were resulted from attempts to inhibit or abrogate basal autophagy in skeletal muscle.

2.6 Satellite Cells in Skeletal Muscle and Centronuclear Myopathy

2.6.1 The Structure of the Skeletal Muscle Cell

The skeletal muscle is consisted of muscle fibers enclosed by a layer of connective tissue. A single cylinder-shaped muscle cell defines a muscle fiber. Multinucleation is a distinguished feature of muscle cells from cells of other systems and is achieved by the merging of myoblasts during muscle formation in the fetus (Minguetti & Mair, 1986). One single muscle cell can be 3-centimeter long, hence multinucleation is needed for controlling the whole bulky cell (Cochran, 2003). Muscle nuclei are located and distributed evenly along the peripheral muscle cells. Multinucleation is beneficial to the skeletal muscle fibers in allowing collective accumulation of genetic materials to support massive production of enzymes and structural proteins needed for muscle contraction (Martini & Bartholomew, 2009).

2.6.2 Satellite Cell and Centralized Nucleation of Muscle Fiber

Skeletal muscle fibers are terminally differentiated cells. As muscle cells do not divide, other mechanisms are required for muscle regeneration or repair after injury. Satellite cells were discovered in 1961 and named in accordance with their

unique locations within the basal lamina surrounding individual myofibers and juxtaposing between the plasma membrane of muscle fibers and basement membrane (Mauro, 1961;Charge & Rudnicki, 2004). These mononucleated myogenic cells are quiescent and do not actively proliferate in the absence of stimuli such as muscle injury (Hawke & Garry, 2001;Cabral *et al.*, 2008). Upon stimulation, satellite cells proliferate and express myogenic markers. The satellite cells are then differentiated into myoblasts and fused to the existing muscle fibers for repair and regeneration (Hawke & Garry, 2001).

Satellite cells fuse to the damaged fibers during fiber regeneration. The nuclei of the fused satellite cells are firstly located in the center of the fiber and progressively migrate to the peripheral position (Hawke & Garry, 2001). Hence, histological identification of nuclei present at the center of muscle fibers is indicative of muscle regeneration (Charge & Rudnicki, 2004). Under normal physiology, centralized nuclei are present in approximately 3% of muscle fibers (Schochet, 1986).

Centronucleation in skeletal muscle fibers serves as a robust histological evidence

of skeletal muscle injury. Centralized nuclei in skeletal fibers are a sign of muscle regeneration after damage as they indicate the fusion of satellite cells to existing muscle fibers. Thus, abnormal increase of centronucleated fiber portion can serve as a sign of muscle damage (Chiquet & Fluck, 2003). The percentage of centralized nuclei containing fibers reflects the degree of damage (Baker, 2007). For example, the centronucleation of muscle fibers is a good indicator of a genetic disease called Duchenne Muscular Dystrophy which is a condition with substantially high frequency of muscle degeneration and regeneration (Briguet *et al.*, 2004).

2.7 The Growth-hormone Secretagogues Receptor and Its Natural Ligand - Ghrelin

2.7.1 Growth-hormone Secretagogues Receptor

The growth-hormone secretagogues receptor (GHSR) was identified in 1966 as a regulatory machinery for growth hormone secretion (Howard *et al.*, 1996). This receptor is a G-protein coupled receptor encoded in chromosome 3 at location 3q26.2 (Mckee *et al.*, 1997a;Mckee *et al.*, 1997b). There are two isoforms of this receptor including the full-length isoform, GHSR-1a, and the truncated isoform, GHSR-1b (Mckee *et al.*, 1997b). These two isoforms are produced by alternative splicing (Howard *et al.*, 1996;Mckee *et al.*, 1997a;Smith *et al.*, 1997). The translational product of the mRNA of GHSR-1a is a protein of approximately 41 kDa with seven transmembrane regions encoded by 366 amino acids whereas the translational product of the mRNA of GHSR-1b is constituted by only five transmembrane regions of the GHSR-1a protein and encoded by only 289 amino acids (Mckee *et al.*, 1997b). It was reported that protein products of GHSR-1a of human and rat share more than 90% identity with its homologs in rat and pig (Palyha *et al.*, 2000).

The existence of GHSR-1b isoform is due to a read through of intron in which an in-frame stop codon is located. This stop codon terminates transcription before the entire gene is transcribed. Incomplete transcription due to the stop codon results in the generation of a shortened form of GHSR mRNA. Subsequent translation of the shortened form of GHSR mRNA produces the GHSR-1b isoform that lacks the transmembrane regions 6 and 7 of GHSR-1a. The function of this truncated isoform 1b remains elusive (Mckee *et al.*, 1997a;Smith *et al.*, 2001;Gnanapavan *et al.*, 2002).

2.7.2 Discovery of Ghrelin

The natural endogenous ligand of GHSR was not known until 1999. Kojima and co-workers identified the ligand by using an orphan-receptor strategy (Kojima *et al.*, 1999). This strategy monitored the increase in calcium ion (Ca^{2+}) influx in a cultured cell line transfected with GHSR. With reference to the high-expression level of GHSR in the hypothalamus, pituitary and hippocampus, a considerable amount of ligand was hypothesized to be present in the brain tissue. However, the brain tissue extract only induced a weak activation of GHSR (Guan *et al.*, 1997).

Attempts were then made using extracts of other tissues to identify the ligand. Stomach-tissue extract, surprisingly, was found to generate a strong response in the experiment. The active peptide was then identified after several steps of purification. Kojima and co-workers named this peptide “ghrelin” where the word root “ghre” meaned “growth” in Proto-Indo-European languages (Kojima *et al.*, 1999).

Ghrelin is a 28-amino acid long multifunctional peptide with octanoylation on serine 3 (Kojima *et al.*, 1999). The gene of this natural ligand peptide is located at chromosome 3p25-26 in both the human and rat genome (Kojima *et al.*, 2001). The ghrelin gene of human and rat consists of three introns and four exons (Kojima *et al.*, 2001). The mouse ghrelin gene, however, consists of five exons and four introns (Kojima *et al.*, 2001). Genomic structural analysis has shown that the mature peptide of ghrelin is encoded in exon 1 and exon 2 (Kojima *et al.*, 1999). Two isoforms of the ghrelin precursor mRNA exist in rats due to alternative splicing (Hosoda *et al.*, 2000a). Apart from the one encode ghrelin precursor, the other mRNA encodes des-Gln14-ghrelin, a 27-amino acid peptide that also has an

octanoylation at serine 3. This 27-amino acid ghrelin derivative contributes 10% to the total immunoreactive ghrelin and is potent to stimulate growth hormone secretion and increase intracellular Ca^{2+} as the 28-amino acid ghrelin (Hosoda *et al.*, 2000a). Both the 27-amino acid long des-Gln14-ghrelin and the 28-amino acid long ghrelin are able to bind to GHSR-1a and are regarded as the bioactive forms, although they are not the dominant forms of ghrelin in vivo. The des-n-octanoyl ghrelin is the dominant form of ghrelin and structurally identical to the 28-amino acid ghrelin except the absence of octanoylation at its serine 3. Besides, the des-n-octanoyl ghrelin is incapable to affect the level of intracellular Ca^{2+} (Matsumoto *et al.*, 2001).

Ghrelin is mainly produced in the stomach (Kojima *et al.*, 1999;Date *et al.*, 2000;Dornonville de la *et al.*, 2001). In addition to its expression in the bowel, ghrelin is also found in the pituitary glands, kidney, lung, placenta, testis, pancreas, leukocyte, and hypothalamus with low expression level. The adrenal gland, adipocytes, gall bladder, skeletal muscle, myocardium, skin, spleen, liver, ovary, and prostate also express trace amounts of ghrelin. Ghrelin can also be detected in the

circulation (Gnanapavan *et al.*, 2002).

2.7.3 Physiological Roles of Ghrelin

2.7.3.1 Regulatory Effects of Ghrelin on Growth Hormone Release

As a multifunctional peptide, ghrelin impacts a variety of physiological events. It is expected that ghrelin, as a natural ligand of GHSR, is able to induce growth hormone secretion. Ghrelin was found to induce growth hormone release from the anterior pituitary cells in a dose-dependent manner (Kojima *et al.*, 1999) with the inducing effect more pronounced through intracerebroventricular administration than intravenous administration (Date *et al.*, 2000). Notably, ghrelin induced a stronger release of growth hormone than the endogenous growth hormone-releasing hormone (Kojima *et al.*, 1999). It was also observed that co-administration of ghrelin and the growth hormone-releasing hormone exerted a synergetic effect on the secretion of growth hormone (Hataya *et al.*, 2001).

The remarkable increase in the transcription of ghrelin and GHSR-1a in response to the growth hormone-release hormone administration demonstrated the regulatory role of growth hormone-release hormone in ghrelin (Kamegai *et al.*, 2001). In contrast, pharmacological inhibition of the growth hormone-releasing hormone by the use of its anti-sera or antagonist abolished the effect of ghrelin in inducing growth hormone release (Kamegai *et al.*, 2001).

Growth hormone secretion in the pituitary gland is mediated by the vagus nerve which is a cranial nerve consisting of afferent and efferent fibers (Schwartz *et al.*, 2000). Afferent and unmyelinated fibers account approximately 90% of the total vagus nerve fibers in subdiaphragma (Date *et al.*, 2002). Some of the afferent vagus nerve fiber endings were located at the gastrointestinal mucosa and submucosa, where are the optimal position for detecting substances in the lumen. The mediatory role of the vagus nerve in the ghrelin-induced release of the growth hormone was demonstrated further by the attenuated secretion of growth hormone in response to selective de-afferentation of the gastric vagus nerve (Date *et al.*, 2002).

2.7.3.2 Ghrelin Increases Food Intake and Appetite

In addition to the inducing effect of ghrelin on growth hormone secretion, ghrelin also regulates food intake and energy homeostasis without affecting the locomotion activity and stress-relating behaviors (Nakazato *et al.*, 2001). GHSR-1a is expressed at high level in the hypothalamus, a regulatory center for energy homeostasis (Spiegelman & Flier, 2001). It has been demonstrated that reduced expression of GHSR-1a in the hypothalamus led to a reduction of food intake, body fat mass and growth hormone secretion, thus suggesting GHSR-1a involves in the mediatory machinery of ghrelin for growth hormone secretion and energy homeostasis regulation (Shuto *et al.*, 2002).

Chronic administration of ghrelin can lead to weight gain and adiposity (Wren *et al.*, 2000;Tschop *et al.*, 2000;Nakazato *et al.*, 2001). Nakazato and co-workers have demonstrated that the food intake of free-feeding rats can be increased by intracerebroventricular administration of ghrelin in both the light and dark phase (Nakazato *et al.*, 2001). They have also demonstrated that the administration of anti-ghrelin immunoglobulin G abolished the starvation-induced feeding in a

dose-dependent manner. Moreover, food intake was found to be increased significantly in growth hormone-deficient rats after ghrelin administration (Nakazato *et al.*, 2001), suggesting that ghrelin is highly orexigenic and its inducing effect on food intake is growth hormone-independent.

Ghrelin regulates not only energy uptake but also appetite. Administration of ghrelin to healthy subjects induced hunger sensation (Arvat *et al.*, 2000;Muller *et al.*, 2001). Studies on healthy humans showed an approximately 30% increase in energy uptake after ghrelin administration (Wren *et al.*, 2001). The level of plasma ghrelin and plasma insulin demonstrated regulatory profile with the ghrelin content increased about two-fold before each meal followed by its return to the basal level an hour after the meal (Cummings *et al.*, 2001;Tschop *et al.*, 2001;Yildiz *et al.*, 2004). Ghrelin concentration in plasma was not affected by water intake (Asakawa *et al.*, 2001;Shiiya *et al.*, 2002) whereas high-fat diet and low-protein diet were found to reduce and elevate, respectively, the level of ghrelin (Broglio *et al.*, 2002). The level of ghrelin in plasma can be decreased by 80% after gastric bypass surgery (Dornonville de la *et al.*, 2001). Hence, it is

believed that ghrelin secretion from the stomach and ingested nutrients are related, although the underlying mechanism for the regulation of ghrelin secretion is still unclear.

Ghrelin may serve as a signaling molecule to regulate gastric secretion and gastric motility (Date *et al.*, 2001). It has been demonstrated that gastric secretion and motility for food digestion were both increased by ghrelin via intravenous administration in a dose-dependent manner under the mediation of vagal nerve (Kamegai *et al.*, 2001;Date *et al.*, 2002). While ghrelin was suggested to possess the capability to pass through the blood-brain barrier, intraperitoneal injection of ghrelin failed to induce food intake in vagotomized mice (Asakawa *et al.*, 2001). It was demonstrated that the starvation signal of ghrelin was diminished when the gastric vagal afferent fibers were blocked by surgical or chemical approaches (Date *et al.*, 2002).

Synthesis of GHSR-1a also occurs at vagal afferent neurons in nodosa ganglion (Date *et al.*, 2002). The synthesized GHSR-1a are then transferred to the afferent

end of the neurons that located in the stomach (Date *et al.*, 2002). Hence, it can be concluded that the vagal nerve relays the signal of ghrelin bi-directionally between the central nervous system and the stomach (Ueno *et al.*, 2005).

2.7.3.3 Ghrelin as an Anti-inflammatory, Anti-oxidative and Anti-fibrotic Agent

Further to the effects on growth hormone release, food intake, and energy homeostasis mentioned above, ghrelin was also suggested to possess anti-inflammatory, anti-oxidative and anti-fibrotic capacity.

Results of studies investigating the anti-oxidative effect of ghrelin are encouraging. Zwirska-Korczala and co-workers reported that ghrelin treatment increased the activities of glutathione peroxidase, superoxide dismutase and catalase in the cultured preadipocyte cells (Zwirska-Korczala *et al.*, 2007). Consistently, injections of ghrelin enhanced the activity of superoxide dismutase in ovarian tissue (Kheradmand *et al.*, 2010). Increased superoxide dismutase mRNA in trout phagocytic leukocytes after ghrelin administration has been reported (Kheradmand *et al.*, 2010). There is also evidence suggesting the inhibitory effect

of ghrelin on ROS formation (El *et al.*, 2007). It has been demonstrated that ghrelin administration suppressed oxidative stress in hypertensive rats (Kawczynska-Drozdz *et al.*, 2006).

Administration of ghrelin confers protection against fibrosis. It has been demonstrated that exogenous ghrelin protected the liver from oxidative stress-induced fibrosis (Iseri *et al.*, 2008). Anti-fibrotic effect of ghrelin in the liver was replicated consistently in rodents and humans during acute and chronic injury (Moreno *et al.*, 2010). Hence, ghrelin has been proposed as an anti-fibrotic drug for treating liver fibrosis (Moreno *et al.*, 2010). On the other hand, Interleukin 1 beta (IL-1β) was demonstrated as a pro-fibrotic agent in the lung (Kolb *et al.*, 2001) whereas ghrelin was found to prevent fibrosis of IL-1β treated lungs, left ventricle and cardiomyocytes by downregulating IL-1β (Kolb *et al.*, 2001;Moreno *et al.*, 2010;Imazu *et al.*, 2011).

The abrogating effect of ghrelin on infiltration of immune cells following ischemia/reperfusion and exposure to toxic substances suggested the

anti-inflammatory potency of ghrelin. (Jaeschke & Hasegawa, 2006;Wu *et al.*, 2007;Imazu *et al.*, 2011). Administration of ghrelin has been shown to reduce the level of proinflammatory cytokines such as tumor necrosis factor alpha (TNFα), IL-1β and Interleukin 6 (IL-6). Similar results were obtained in the liver (Cetin *et al.*, 2011;Iseri *et al.*, 2008), lung (Peng *et al.*, 2012b;Imazu *et al.*, 2011) and gastric intestines (El *et al.*, 2007) with a notable reduction of proinflammatory cytokines concentration in plasma (Wu *et al.*, 2007).

2.7.3.4 Ghrelin Exerts Protective Effects in Many Organs

Protective effects of ghrelin have been demonstrated in many tissues including the liver, lung, neuron, kidney, heart and skeletal muscle. It has been demonstrated that administration of ghrelin protected the liver from CCL_4-induced damage. Ghrelin increased the activities of anti-oxidative enzymes to antagonize CCL_4-induced oxidative stress (Jaeschke & Hasegawa *et al.*, 2006). Inflammation and fibrosis were observed in chronic hepatic damage (Moreno *et al.*, 2010) and biliary obstruction-induced acute hepatic damage (Cetin *et al.*, 2011). Interestingly, these damages were abolished by ghrelin administration by

reducing neutrophils infiltration and collagen content and by downregulating cytokines including IL-6, IL-8 and TNF-α (Moreno *et al*., 2010; Cetin *et al*., 2011). It has been demonstrated that administration of ghrelin blunted the phenotypical changes in the kidney induced by ischemia/reperfusion (Takeda *et al*., 2006). It has been observed in ghrelin-treated rats that the renal function and survival rate were improved following ischemia/reperfusion-induced injury (Takeda *et al*., 2006).

Ghrelin has also been demonstrated to confer resistance against apoptosis induced by ischemia/reperfusion in neurons (Hwang *et al*., 2009) and tubular cells (Takeda *et al*., 2006). It was observed that ghrelin treatment reduced caspase-3 activity level and the Bax/Bcl-2 ratio in the neuron challenged by ischemia/reperfusion (Hwang *et al*., 2009), exposure to pilocarpine (Xu *et al*., 2009) and lipopolysaccharide-treated gastric tissue (Slomiany & Slomiany, 2010).

The potency of ghrelin in the inhibition of inflammation has been demonstrated in the lung. Ghrelin treatment reduced the pro-inflammatory status through the

downregulation of IL-6, IL-8 and TNF-α following the induction of inflammation in the lung by various strategies such as cegal ligation and puncture (Peng *et al.*, 2012b), exposure to bleomycin (Imazu *et al.*, 2011) and pancreatitis (Zhou & Xue et al., 2010). It has also been demonstrated that the administration of ghrelin can ameliorate fibrosis in the lung induced by cegal ligation and puncture (Peng *et al.*, 2012b) and pulmonary hypertension induced by hypoxia due to its anti-fibrotic effects (Zhou & Xue, 2010).

Since skeletal muscle is the focus of this study and the cardiac muscle data can be employed as a reference due to the similarities between skeletal muscle and cardiac muscle, the protective effects of ghrelin on these two muscle systems are discussed in details as below.

2.7.3.4.1 The Protective Effects of Ghrelin in the Cardiac System

Researchers have reported that ghrelin protected various organs against a wide range of stress among which the protective effects of ghrelin against cardiac dysfunction are well-documented. Intravenous infusion of ghrelin reduced the

mean arterial pressure but did not affect heart rate (Nagaya *et al.*, 2001a). Cardiac output was found to be increased in both healthy volunteers and chronic heart failure patients following ghrelin administration (Nagaya *et al.*, 2001a;Nagaya *et al.*, 2001b). Larger amounts of ghrelin were detected in the plasma of patients suffered with cachectic chronic heart failure when compared with those without cachexia (Nagaya *et al.*, 2001c). Chronic administration of ghrelin was beneficial to the cardiac system. The left-ventricle function was improved after a three-week treatment of ghrelin concomitantly with increases in growth hormone and insulin-like growth factor 1 (IGF-I) (Nagaya *et al.*, 2001d). The same intervention has also been shown to protect rats with chronic heart failure against left ventricle remodeling and cardiac cachexia (Nagaya *et al.*, 2001d). Ghrelin was shown to confer apoptotic resistance in H9C2 cardiomyocytes under oxidative stress by activating the peptidase inhibitor 3 (PI3) kinase/thymoma viral proto-oncogene (Akt) and mitogen-activated protein kinase 1 (ERK) 1/2 pathway (Baldanzi *et al.*, 2002).

In 2010, Zhang and co-workers demonstrated that ghrelin suppressed the

phosphorylation of nuclear factor kappa B (NF-kB), expression of Bax, activities of caspase-3 and caspase-9 and Bcl-2. These findings suggested that the anti-apoptotic effect of ghrelin is related to the intrinsic apoptotic pathway (Zhang *et al.*, 2010;Zhang *et al.*, 2011). In addition to the government of cell death, the cardioprotective effect of ghrelin can also be attributed to the induction of growth hormone release. This notion is supported by the indispensible role of growth hormone in the maintenance of cardiac structure and function (Zheng *et al.*, 2005).

2.7.3.4.2 Protective Effects of Ghrelin in Skeletal Muscle

Compared with the cardiac muscle, the effects of ghrelin in skeletal muscle are less understood. Extension of ghrelin research to the skeletal muscle revealed that the skeletal muscle is also a protective target of ghrelin. Balasubramaniam and co-workers reported that ghrelin prevented degradation of skeletal muscle proteins after a burn injury through the inhibition of thermal damage-induced upregulation of the two E3 ligases, MAFbx and MurF1 (Balasubramaniam *et al.*, 2009). The reduced expression of TNFα and IL-6 following ghrelin treatment

suggested the anti-inflammatory effect of ghrelin on the skeletal muscle (Balasubramaniam *et al.*, 2009). It has been suggested that ghrelin promotes growth hormone release, increases food intake and exerts protective effects such as preserving muscle mass and preventing proteins degradation (Fujitsuka *et al.*, 2011). The beneficial effects of ghrelin on patients with cachexia induced by diseases like cancer and acquired immunodeficiency syndrome (AIDS) were reported (Tisdale, 1997). Ghrelin intervention has been demonstrated to preserve muscle mass and average muscle fiber area and suppress the upregulation of E3 ubiquitin ligases MuRF1 and MAFbx in response to fasting-induced atrophy (Porporato *et al.*, 2013). Angiotensin is known to mediate loss of body weight by enhancing catabolism of the skeletal muscle (Sugiyama *et al.*, 2012). It was reported that ghrelin hampered the angiotensin-induced upregulations of MuRF1 and MAFbx to preserve the lean mass and fat mass (Sugiyama *et al.*, 2012). Collectively, these findings suggest the therapeutic potential of ghrelin in cancer cachexia. Table 1 summarizes the protective effects of ghrelin on different organs.

To conclude, ghrelin is a natural ligand of GHSR-1a that promotes growth

hormone release, food intake, and preserves of both lean mass and fat mass. Ghrelin is suggested to serve as a potent therapeutic agent for treating cachexia. In parallel, ghrelin has also been demonstrated to antagonize inflammation, fibrosis and oxidative stress.

In contrast, the detrimental effects of doxorubicin on skeletal muscle are reviewed in details in Chapter 2.3. These detrimental effects are probably mediated through inflammation, oxidative stress, and fibrosis and the upregulation of apoptosis and autophagy. As emerging evidence suggested that ghrelin conferred protection to multiple organs by abrogating cell death, inflammation, fibrosis, and oxidative stress-induced damage, ghrelin might be a potential therapeutic agent against doxorubicin-induced damage in skeletal muscle. These provide a strong rationale for the present MPhil study to test the hypothesis that ghrelin protects against the skeletal muscle injury induced by doxorubicin.

Table 1. Summary of the Ghrelin Protective Effects in Various Organs

Organ / Tissue	Protective effects of ghrelin	Species	Reference
Liver	Protect liver from biliary obstruction induced hepatic fibrosis, inflammation and oxidative stress. Collagen content in liver and neutrophils infiltration were reduced with downregulated IL-6, IL-1β and TNFα.	Rat	(Cetin *et al*, 2011)
	Recombinant ghrelin in rat protected liver from fibrosis caused by chronic injury by preventing the change of expression pattern during fibrogeneis. Mice with ghrelin knockout were more subject to CCl_4-induced acute liver injury. Administration of ghrelin suppressed inflammation by preventing neutrophils infiltration and reduced apoptosis in hepatic cells by activating Akt and ERK 1/2 signaling pathway. Patients suffering from liver fibrosis due to chronic hepatitis and alcoholic hepatitis had a lower concentration of ghrelin in their serum.	Rat and human	(Moreno *et al*, 2010)
	Protect CCl_4 induced acute liver damage by tackling oxidative stress. Retain antioxidant enzyme activities from being reduced by CCl_4.	Rat	(Jaeschke & Hasegawa, 2006)
	Protect acetaminophen induced liver injury by downregulating TNFα.	Rat	(Iseri *et al*, 2008)
Lung	Protect acute lung injury due to cecal ligation and puncture by inhibiting NF-kB activity, suppressing Nucleotide-binding oligomerization domain containing 2 (NOD2) expression and downregulating TNFα and IL-6.	Rat	(Peng *et al*, 2012b)
	Protect bleomycin-induced actue lung injury from fibrosis, inflammation and apoptosis by reducing collagen content, preventing neutrophils infiltration and downregulating IL-6 and IL-1β.	Mice	(Imazu *et al*, 2011)
	Ameliorate hypoxia-induced pulmonary hypertension by exerting anti-fibrotic effects in lung, preventing medial thickening of pulmonary arteriole and pulmonary microvascular endothelial cell death by activating Akt and GSK3β pathway.	Rat	(Xu *et al*, 2011)
	Protect lung from pancreatitis-induced injury by reducing inflammation by preventing neutrophils infiltration, downregulating serum IL-6, IL-1β and TNFα and inhibiting substance P. Pulmonary microvascular permeability and water content were also decreased, indicating that lung function is preserved.	Rat	(Zhou & Xue, 2010)
Gastric	Ghrelin intervention suppressed apoptosis and increased caspase-3 activity induced by lipopolysaccharide in a dose-dependent manner. Ghrelin administration also bunt the effect of lipopolysaccharide on constitutive synthase and inducible nitric oxide synthase expression.	Rat Mucosa cell	(Slomiany & Slomiany, 2010)
	Pretreatment of ghrelin can prevent upregulation of TNFα caused by ischemia/ reperfusion. Ghrelin pretreatment suppressed the increase of vascular permeability, serum lactic acid dehydrogenase level and markers of oxidative stress. Histologically analysis showed that ghrelin pretreatment prevented inflammatory cell infiltration.	Rat	(El *et al*, 2007)7)
Neuron	Protect hippocampal neurons from apoptosis induced by pilocarpine by inhibiting caspase-3 activation increasing Bcl-2/Bax ratio with activation of Akt/PI3k Signaling pathway.	Rat	(Xu *et al*, 2009)
	Prevent substantia nigra pars compacta dopamine cell loss by uncoupling protein 2 mediation of respiration, ROS production and biogenesis of mitochondria. Mice with ghrelin or GHSR-1a knockout showed increased substantia nigra pars compacta dopamine cell loss.	Rat and Mice	(Andrews *et al*, 2009)
	Protected hippocampus from ischemia/ reperfusion together with an increase of uncoupling protein-2 expression.	Rat	(Liu *et al*, 2009)
	Reduced infarct volume against brain ischemia in dose dependent manner with suppressing the upregulation of Protease-activated receptor 4 (Par-4) due to brain ischemia. Ghrelin treatment suppressed apoptosis by increasing Bcl-2/Bax ratio, preventing cytochrome c release and caspase 3 activation.	Rat	(Hwang *et al*, 2009)

Kidney	Blunt the phenotypic changes caused by ischemia and reperfusion, such as increase of tubular casts, interstitial edema and epithelial detachment. The intervention also suppressed apoptosis in tubular cells. Kidney function and survival rate of mice with ghrelin administration were also higher than those without.	Mice	(Takeda *et al*, 2006)
Heart	Decreased mean arterial pressure without changing heart rate while increase cardiac output in health volunteers.	Human	(Nagaya *et al*, 2001a)
	Increased cardiac output in chronic heart failure patients.	Human	(Nagaya *et al*, 2001b)
	Chronic intervention of ghrelin can increase left ventricle function with plasma GH and IGF-I increase. Same intervention can protect rat suffering from chronic heart failure from left ventricle remodeling and cardiac cachexia.	Rat	(Nagaya *et al*, 2001d)
	Protected H9c2 cardiomyocyte from oxidative stress-induced apoptosis by activating Akt Signaling and ERK1/2 Signaling.	H9c2 cardiomyocyte	(Baldanzi *et al*, 2002)
	Protected H9c2 cardiomyocyte from oxidative stress induced apoptosis by suppressing NF-kB Signaling pathway. Ghrelin intervention was able to downregulate Bax, upregulate Bcl-2 and reduce the activity of caspase-3 and caspase-9.	H9c2 cardiomyocyte	(Zhang *et al*, 2010)
Skeletal muscle	Prevented skeletal muscle protein breakdown after burn injury by suppressing the upregulation of MAFbx and MuRF1 caused by thermal damage and downregulating TNFα and IL-6.	Rat	(Balasubramaniam *et al*, 2009)
	Reduced muscle loss and muscle area loss caused by fasting induced muscle atrophy by activating Akt Signaling pathway and downregulating MAFbx	mice	(Porporato *et al*, 2013)
	Prevent skeletal muscle from angiotensin induced muscle weight loss and body weight loss by suppressing MuRF1 and MAFbx	mice	(Sugiyama *et al*, 2012)2)

2.8 Objectives

1. To investigate whether ghrelin can protect skeletal muscle from doxorubicin-induced damage by modulating the signaling of apoptosis and autophagy and to determine whether the protective effects of ghrelin against doxorubicin-induced damage are mediated through the GHSR-1a pathway.
2. To investigate whether ghrelin exerts any effect on basal levels of apoptosis and autophagy under normal physiological condition.
3. To study the acute effect of doxorubicin on autophagy in skeletal muscle.

2.9 Project Significance

Many cancer patients suffer from cancer cachexia and experience severe loss of muscle mass and function (Bing *et al.*, 2000). Chemotherapy, such as doxorubicin, may predispose the muscle to exercise intolerance, fatigue, and muscle weakness by impairing muscle function (Gilliam & St Clair, 2011). In fact, extensive loss of muscle is fatal as skeletal muscle contributes to the function of many vital organ systems such as circulatory system (Hilton, 1982;Laughlin, 1987) and respiratory system (Tolep & Kelsen, 1993;Serres *et al.*, 1998). The study of doxorubicin-induced myotoxicity is essentially important because the doxorubicin-induced damages to the skeletal muscle may considerably compromise the quality of life and even be life-threatening in cancer patients (Gilliam & St Clair, 2011). As ghrelin has been proposed as a potential candidate to treat cancer cachexia, ghrelin might also have a therapeutic role in treating the anti-cancer drug doxorubicin-induced myotoxicity in addition to cancer cachexia. Therefore, this MPhil project aimed to examine the protective role of ghrelin in doxorubicin-induced toxicity in skeletal muscle.

CHAPTER 3

Ghrelin Protects Skeletal Muscle from Doxorubicin-induced Injury by Supressing Apoptosis and Enhancing Autophagy

3.1 Introduction

Doxorubicin administration can lead to muscle loss and functional decline. Upregulation of apoptosis (Hilder *et al.*, 2005;Liu *et al.*, 2008) and autophagy (Smuder *et al.*, 2011a), inflammation (Cullu *et al.*, 2003), elevation of oxidative stress (Smuder *et al.*, 2011b;Gilliam *et al.*, 2012), degradation of skeletal muscle proteins (Smuder *et al.*, 2011b), and fibrosis (Cullu *et al.*, 2003) were reported to be induced by doxorubicin. The present experiment aimed to examine whether ghrelin, a potent anti-oxidative (El *et al.*, 2007;Zwirska-Korczala *et al.*, 2007), anti-inflammatory (Iseri *et al.*, 2008;Cetin *et al.*, 2011) and anti-fibrotic agent (Iseri *et al.*, 2008;Cetin *et al.*, 2011) can protect skeletal muscle from doxorubicin-induced damage by inhibiting the doxorubicin-induced upregulations of apoptosis and modulating autophagy.

Ghrelin is known as the natural ligand of the GHSR-1a (Kojima *et al.*, 1999). Many cellular effects of ghrelin including the induction of growth hormone release (Sun *et al.*, 2004), proliferation of endothelial cells (Rossi *et al.*, 2008) and inhibition of apoptosis in osteoblastic cells (Kim *et al.*, 2005) have been demonstrated to be

mediated through the GHSR signaling. Although the corresponding effects in skeletal muscle remained to be elucidated, several studies reported that the effects of ghrelin were blunted by applying GHSR-1a antagonist in various cell types and tissues (Kim *et al.*, 2005;Rak *et al.*, 2009;Rossi *et al.*, 2008;Ma *et al.*, 2012). Therefore, this current experiment was conducted based on the hypothesis that the effects of ghrelin on doxorubicin treated muscle were mediated through GHSR-1a pathway.

3.2 Methods

3.2.1 Animals

Eight to twelve-week old C57/B6 wild type mice were employed in this study. Animals were maintained on a 12:12-h light-dark cycle. Food and water were provided *ad libitum* throughout the experimental period. Animal ethics approval was obtained from the Animal Ethics Sub-committee of The Hong Kong Polytechnic University.

3.2.2 Experimental Design

The effects of ghrelin on the doxorubicin-induced skeletal muscle damage were examined by assigning mice randomly to one of the following groups: saline control group (CON; n = 7); doxorubicin group (DOX; n = 7); doxorubicin with treatment of ghrelin group (DOX+Ghrelin; n = 7); and doxorubicin with treatment of ghrelin plus GHSR-1a antagonist [D-Lys3]-GHRP-6 group (DOX+Ghrelin+[D-Lys3]-GHRP-6; n = 5). At the beginning of the experimental period, mice of the DOX, DOX+Ghrelin and DOX+Ghrelin+[D-Lys3]-GHRP-6 groups were injected intraperitoneally with doxorubicin (15mg/kg body weight) (Hydock *et al.*, 2011) while mice assigned to the CON group received intraperitoneal injection of the corresponding volume of saline as placebo.

Twelve hours after the doxorubicin injection, ghrelin was administered to mice in the DOX+Ghrelin group and DOX+Ghrelin+[D-Lys3]-GHRP-6 groups by intraperitoneal injection (100 μg/kg body weight) every 12 hours (Li *et al.*, 2006), while counterparts in the CON group and DOX groups were injected with saline as a placebo at the same time. Mice in the DOX+Ghrelin+[D-Lys3]-GHRP-6 group

were injected with the GHSR-1a antagonist [D-Lys3]-GHRP-6 (Tocris Bioscience, USA) just before the ghrelin administration. This practice allowed the abrogation of GHSR-1a signaling so that the contribution of the GHSR-1a signaling mechanism to the effects of ghrelin could be revealed.

The dosage of [D-Lys3]-GHRP-6 adopted was 3.75 mg/kg body weight (37.5-fold dose of ghrelin; adopted from the published protocol of Kobashi and co-workers) (Kobashi *et al.*, 2009), which has been demonstrated to effectively abolish the ghrelin-GHSR signaling. Mice were sacrificed 5 days after the first injection by overdose of ketamine and xylazine. Gastrocnemius muscles were harvested and washed with cold phosphate buffered saline. Tissues were frozen quickly in liquid nitrogen-cooled isopentane and stored at -80^{o}C until further use.

3.2.3 Histological Analysis

Gastrocnemius muscles were divided into two halves by cutting down the middle transversely. Twenty micrometers thick frozen muscle cross-sections were prepared in a cryostat at −20 °C.

3.2.3.1 Hematoxylin and Eosin Staining

Sections were air dried at room temperature for 20 min and fixed with 10% formalin (HT-5011, Sigma-Aldrich) at room temperature for 10 min before counter-staining in Mayer's haematoxylin (MHS-1, Sigma-Aldrich) and 1% eosin in $CaCl_2$ (318906, Sigma-Aldrich). Sections were then dehydrated and mounted.

3.2.3.2 Centralized Nuclei

Images of sections with hematoxylin and eosin staining were examined and captured by confocal microscopy (Confocal Microscope C1, Nikon) with a 20X objective. The number of fibers and number of fibers containing centralized nuclei were counted in five random non-overlapping views. More than 800 fibers were counted for each tissue sample.

3.2.4 Cytoplasmic and Nuclear Fraction Protein Preparation

Muscle proteins were prepared in accordance with the published protocol of Teng and co-workers (Teng *et al.*, 2011). Tissues were minced and homogenized on ice in the ice-cooled Rothermal's lysis buffer (10mM NaCl, 1.5mM $MgCl_2$, 20mM

HEPES, 1mM DTT, 20% glycerol, 0.1% Triton X-100, pH7.4). Homogenate was centrifuged at 3000 rpm (805 x g) to pellet the nuclei and cell debris. Cytoplasmic proteins were prepared by subjecting the supernatants to centrifugation at 6000 rpm (3220 x g) to remove nuclei residues and were stored regarded as a cytoplasmic protein fraction. A portion of the cytoplasmic fraction was stored at -80^{o}C directly for activity assays while protease inhibitor cocktail (P8340; Sigma Aldrich) was added to the remaining portion to prevent protein degradation, thereby allowing the conduction of western blot and Cell Death Enzyme-linked Immunosorbent Assay (ELISA).

Pellets were washed with Rothermal's lysis buffer and centrifuged at 3000 rpm (805 x g) three times to remove any remaining cytoplasmic proteins. Pellets were rotated at 4^{o}C for 1 hour after adding 300 µl Rothermal's lysis buffer, 41.5 µl NaCl and 5 µl protease inhibitor for releasing the nuclear proteins. The mixture was then centrifuged at 15000 rpm (17530 x g). Supernatants were collected as the nuclear fraction of muscle proteins.

3.2.5 Analysis of Apoptotic Signaling

3.2.5.1 TUNEL and Dystrophin Staining Analysis

Apoptotic nuclei were detected by double immunofluorescent labeling of terminal dUTP nick-end labeling (TUNEL) (TUNEL in Situ Cell Death Detection Kit, Roche Applied Science) as described (Teng *et al.*, 2011). Sections were also counterstained with anti-dystrophin mouse monoclonal antibody at 1:400 dilution (D8168, Sigma-Aldrich) to confirm the TUNEL positive nuclei were located in the peripheral position and mounted with DAPI mounting medium (Vectashield Mounting Medium, Vector Laboratories, Burlingame, CA, USA) to visualize the nuclei.

TUNEL-stained nuclei, DAPI stained nuclei and dystrophin staining was examined by a confocal microscope (Confocal Microscope C1, Nikon). Images of five random and non-overlapping fields were captured at an objective magnification of 20x for analysis. Data was expressed as apoptotic index by through dividing the number of TUNEL-positive nuclei by the number of total nuclei multiplied by 100.

3.2.5.2 Apoptotic Cell Death ELISA

The Cell Death Detection ELISA kit was purchased from Roche Diagnostics. The experiment was conducted according to the manufacturer's protocol. Briefly, the wells of the microplate were first coated with the primary mouse monoclonal histone antibody in a coating solution at 4^{o}C overnight. Coating solution was then removed by suction. Wells were then incubated with incubation buffer for 30 min at room temperature. Sample solutions were prepared during the 30 min incubation by diluting the cytoplasmic protein fraction with incubation buffer with the ratio of 1:9. Two hundred µl of sample solutions were added accordingly and left at room temperature for 90 min. After three washes, the microplate was incubated with the conjugate solution for 90 min except for the blank position, which was employed as a negative control. Suction removal and three washes were performed prior to the incubation with substrate solution for 20 min at room temperature. Photometric analysis was at an absorbance of 405 nm conducted by a spectrophotometer (Benchmark Plus, BIORAD). The resulting optical density was normalized to the milligrams of the proteins used in the assay (OD 405/mg protein used) and presented as the apoptotic DNA

fragmentation index.

3.2.5.3 Fluorometric Caspase-3 Activity Assay

The caspase-3 protease activity in skeletal muscle was measured the by a fluorometric assay using a published protocol adopted from Teng and co-worker (Teng *et al.*, 2011). Briefly, 50 µl of the cytoplasmic protein fraction without protease inhibitor cocktail were added to a black microplate together with 50 µl assay reaction buffer and 7-amino-4-trifluoromethl coumarin (AFC)-conjugates substrates. Fluorescence intensity was measured immediately after the mixture was prepared and after 2 hours of incubation on a microplate reader (infinite F200; Tecan) with an excitation wavelength of 405 ± 10 nm and emission wavelength of 500 ± 25 nm. Caspase-3 enzymatic activity was presented as the change in fluorescence intensity normalized to amount of protein loaded.

3.2.6 Western Blotting

The protein expression levels of the markers of Akt Signaling (Akt and phosphorylated thymoma viral proto-oncogene (p-Akt)), apoptosis (Bax and Bcl-2), autophagy (Beclin-1, Atg 5, Atg 12-5 Complex, LC3) and muscle protein

degradation (cleaved-actin) were measured by using proteins of cytoplasmic fraction. Protein extracts were boiled at 95^{o}C with 5% Beta-mercaptoethanol for 15 min. Equal amounts of protein were loaded on 10% (Akt, p-Akt, Bax, Bcl-2, Beclin-1, Atg 5, Atg 12-5 Complex) or 12% (LC3 and cleaved-actin) polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose membranes (Akt, p-Akt, Bax, Bcl-2, Beclin-1, Atg 5, Atg 12-5 Complex) or polyvinylidene difluoride membranes (LC3 and cleaved-actin) by electroblotting. After the transfer, the membranes were blocked with 10 ml of 5% non-fat milk for 1 hour at room temperature, followed by overnight incubation with the corresponding primary antibodies at 4^{o}C (Table 2). Membranes were then washed in TBST for three times and incubated with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 1 hour (1: 4000 dilution, 7076 for antimouse IgG antibody, 7074 for anti-rabbit IgG antibody; Cell Signaling Technology). Membranes were washed again for three times with TBST. Luminol reagent (NEL103001EA; Perkin Elmer, Waltham, MA, USA) was then applied to the membrane for chemiluminescent detection of horseradish peroxidase activity. The chemiluminescent signal was detected using a Chemidoc^{tm}

MP imaging system, BIORAD. The resulting bands were quantified by Imagej as an optical density X band area. Glyceradehyde-3-phosphate dehydrogenase (GAPDH) was employed as an internal control for all measurements. Data were expressed by normalizing to the corresponding internal control and expressed as percentage change relative to CON group.

3.2.7 Statistical Analysis

Statistical analysis was performed by using the SPSS 20.0 software package (IBM). The normality of data distribution was assessed by the Kolmogorov-Smirnov test. One-way ANOVA with tukey's HSD post hoc or Kruskal-Wallis test followed by post hoc test were employed to detect the significant difference among groups depended on the results of the Kolmogorov-Smirnov test. P value < 0.05 was considered as statistical significantly different. All data were expressed as mean ± standard error of mean.

Table 2. Primary Antibodies Employed in Western Blot Analysis of Chapter 3

Antibody	**Dilution Factor**	**Source**
Anti-Akt rabbit polyclonal	1:1000	9272, Cell Signaling Technology
Anti-phospho-Akt polyclonal antibody	1:1000	9271, Cell Signaling Technology
Anti-Bax rabbit polyclonal	1:1000	ab7977, Abcam
Anti-Bcl-2 rabbit polyclonal	1:1000	2870, Cell Signaling Technology
Anti-Beclin-1 rabbit polyclonal	1:1000	3738, Cell Signaling Technology
Anti-Atg5 rabbit polyclonal	1:1000	8540, Cell Signaling Technology
Anti-Atg12 rabbit polyclonal	1:1000	2011, Cell Signaling Technology
Anti-LC3 rabbit monoclonal	1:1000	3868, Cell Signaling Technology
Anti-α actin mouse polyclonal	1:1000	A3853, Sigma-Aldrich

3.3 Results

3.3.1 Muscle Histology

Normal skeletal myofiber histology was generally observed in the muscles of all groups except that of the DOX group in which an abnormally high percentage of centronucleated myofibers was found (Figure 3). Quantification of the degree of damage in skeletal muscle was done by counting the percentage of fibers with centralized nuclei. About 3.8%, 2.6% and 4.2% of fibers were centronucleated in the muscle sections of the CON, Dox+Ghrelin and DOX+Ghrelin+[D-Lys3]-GHRP-6 groups, respectively. The percentage of centronucleated fiber increased to around 9% in the muscle sections of the DOX group (Figure 4). The number of myofibers with centralized nuclei was significantly increased by 155% in the muscle sections of DOX group compared to that in the CON group (P=0.003) (Figure 4). The number of myofibers with centronuclei was found to be significantly reduced by 61% and 54% in doxorubicin-treated muscles following ghrelin administration no matter with or without GHSR-1a antagonist in the DOX+Ghrelin (P=0.001) and DOX+Ghrelin+[D-Lys3]-GHRP-6 (P=0.007) groups, respectively (Figure 4).

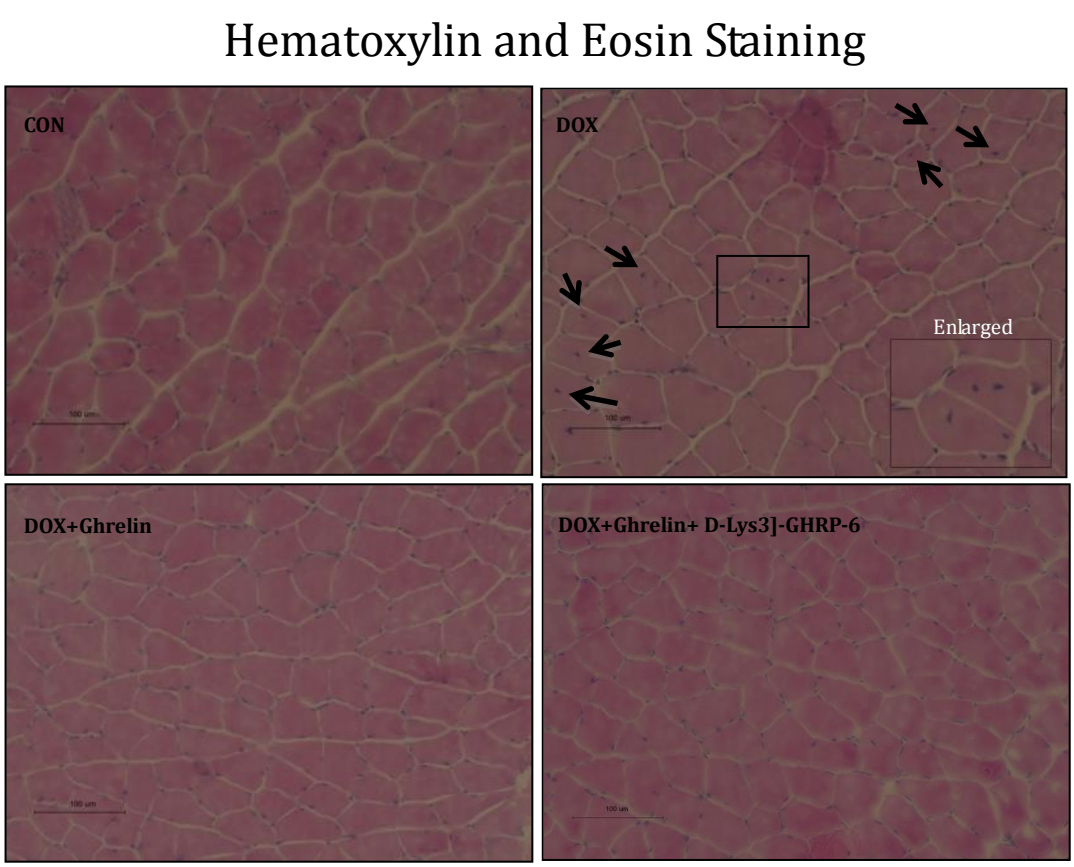

Figure 3. Histological Analysis of Effects of Ghrelin on Doxorubicin-induced Muscle Injury. Hematoxylin and eosin staining were performed on transverse cross-section of gastrocnemius muscle. Muscle sections of CON, DOX+Ghrelin and DOX+Ghrelin+[D-Lys3]-GHSR-6 groups exhibited normal skeletal muscle morphology whereas increased number of myofibers with centrolised nuclei was observed in DOX group. Arrows indicate the myofibers with centralized nuclei. Images were taken using an objective of 20x.

(CON, saline control; DOX, doxorubicin; DOX+Ghrelin, doxorubicin with treatment of ghrelin; DOX+Ghrelin+[D-Lys3]-GHRP-6, doxorubicin with treatment of ghrelin plus GHSR-1a antagonist [D-Lys3]-GHRP-6).

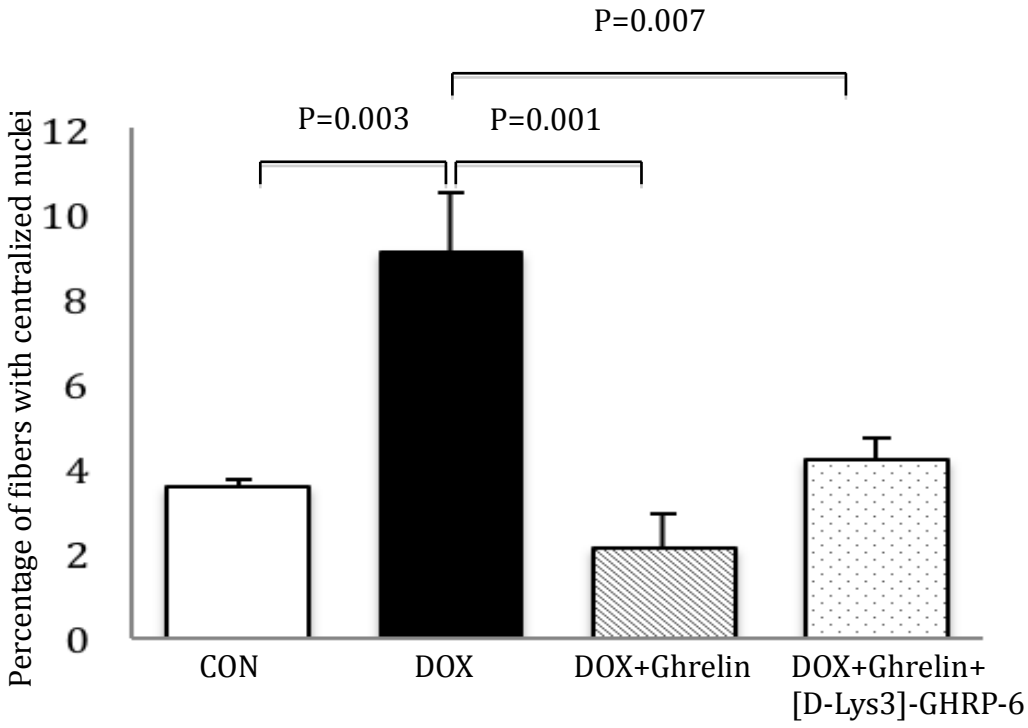

Figure 4. Quantification of the Degree of Damage in Skeletal Muscle by Histological Analysis.

Muscle damage was quantified by counting the percentage of fibers with centralized nuclei to total number of fibers. Data are expressed as means ± SEM with significant level set at $P<0.05$. The data were analysed by one-way ANOVA with Turkey's HSD post hoc test.

(CON, saline control; DOX, doxorubicin; DOX+Ghrelin, doxorubicin with treatment of ghrelin; DOX+Ghrelin+[D-Lys3]-GHRP-6, doxorubicin with treatment of ghrelin plus GHSR-1a antagonist [D-Lys3]-GHRP-6).

3.3.2 Analysis of Skeletal Muscle Protein Degradation

The cleaved-actin is determined as the indicator of muscle protein degradation (Zhou *et al.*, 2007;Plant *et al.*, 2009). The abundance of cleaved-actin in the muscles of the DOX group was 150% greater than that of the CON group (Ratio of protein abundance relative to internal control, CON V.S. DOX: 0.13±0.03 V.S. 0.31±0.03) (P=0.001) (Figure 5). The cleaved-actin level significantly decreased after ghrelin treatment and was reduced by 60% in muscles of the DOX+Ghrelin group relative to the DOX group (Ratio of protein abundance relative to internal control, DOX V.S. DOX+Ghrelin: 0.31±0.03 V.S. 0.13±0.01) (Figure 5). However, there was no significant difference in the level of cleaved-actin in the muscles of the DOX+Ghrelin+[D-Lys3]-GHRP-6 group when compared to the DOX group (Ratio of protein abundance relative to internal control, DOX V.S. DOX+Ghrelin+[D-Lys3]-GHRP-6: 0.31±0.03 V.S. 0.29±0.04) (Figure 5) .

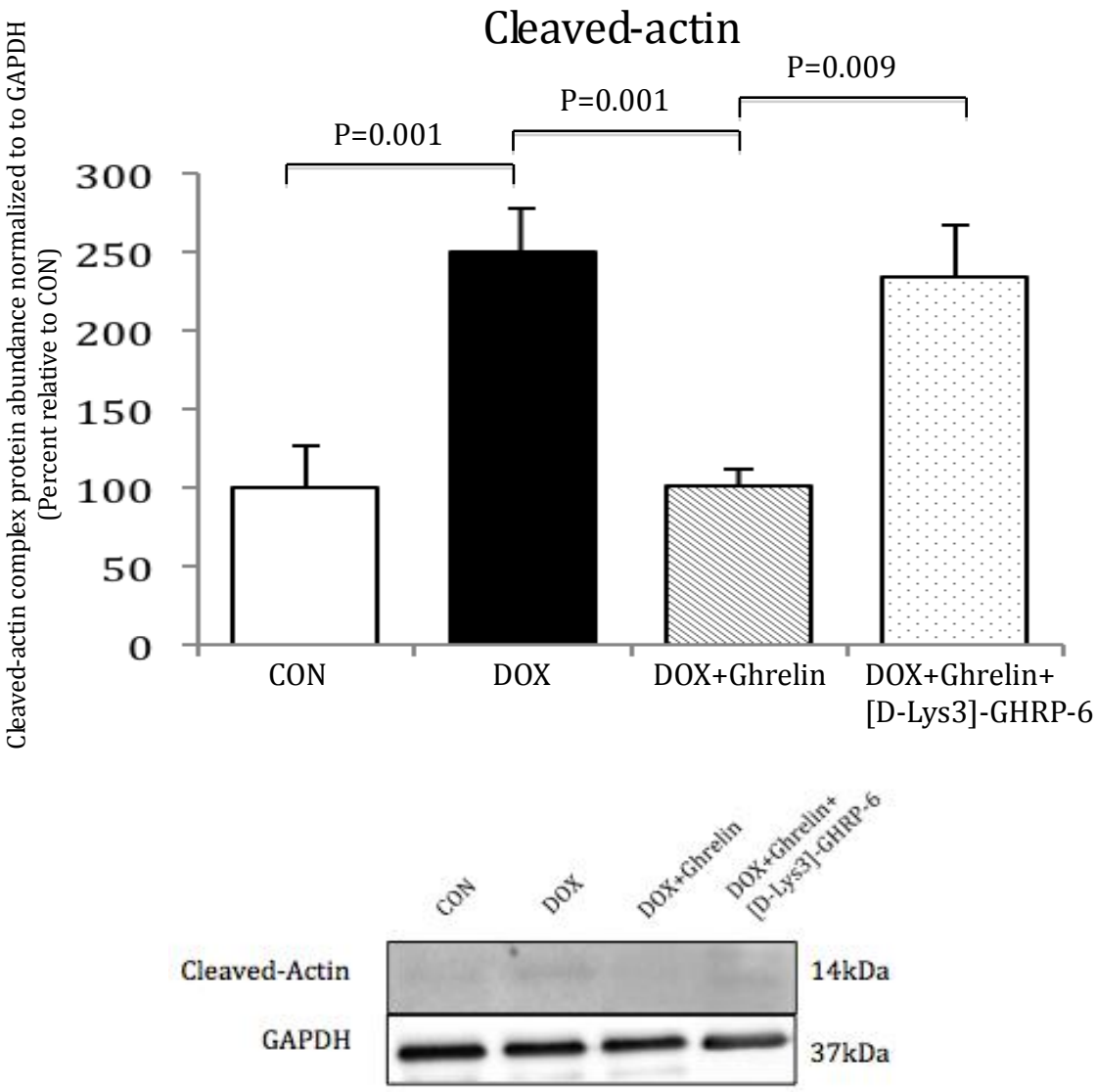

Figure 5. Protein Degradation Analysis of Effects of Ghrelin on Doxorubicin-induced Muscle Injury.

Protein abundances of cleaved-actin were determined by Western blotting. Data are analysed as net intensity x resulting band area and expressed in percent relative to CON. Results of cleaved-actin were normalized to corresponding GAPDH signal. Data are expressed as means ± SEM with significant level set at $P<0.05$. The data were analysed by one-way ANOVA with Turkey's HSD post hoc test.

(CON, saline control; DOX, doxorubicin; DOX+Ghrelin, doxorubicin with treatment of ghrelin; DOX+Ghrelin+[D-Lys3]-GHRP-6, doxorubicin with treatment of ghrelin plus GHSR-1a antagonist [D-Lys3]-GHRP-6).

3.3.3 Analysis of Apoptotic Signaling

Apoptotic index were obtained from TUNEL staining (Figure 6). The apoptotic index of the muscles of the DOX group was 583% significantly higher than that of the CON group (P=0.038) (Figure 7) whereas ghrelin treatment significantly reduced the apoptotic index by 86% in the muscles of the DOX+Ghrelin group relative to the DOX group (P=0.038) (Figure 7). Consistently, the extent of apoptotic DNA fragmentation, as measured by Cell Death ELISA, in the muscles of the DOX group was significantly increased by 115%, compared with that of the CON group (P=0.001) but significantly reduced by 45% in the muscles of DOX+Ghrelin group (P=0.006) and 60% in the muscles of DOX+Ghrelin+[D-Lys3]-GHRP-6 group respectively relative to that of the DOX group (P=0.001) (Figure 8). There was no significant difference in apoptotic DNA fragmentation in muscles between the DOX+Ghrelin+[D-Lys3]-GHRP-6 and DOX+Ghrelin groups (Figure 8). A consistent trend of the changes was noted in the caspase-3 activity but the differences among groups did not reach to the significant level (Figure 9). The protein abundance of Bax was significantly increased by 111% in the muscles of the DOX group when compared with the

CON group (Ratio of protein abundance relative to internal control, CON V.S. DOX: 0.54±0.08 V.S. 1.14±0.25) (P=0.037), whereas it was decreased by approximately 50% in the DOX+Ghrelin (Ratio of protein abundance relative to internal control, DOX V.S. DOX+Ghrelin: 1.14±0.25 V.S. 0.56±0.08) (P=0.047) and the DOX+Ghrelin+[D-Lys3]-GHRP-6 groups relative to the DOX group (Ratio of protein abundance relative to internal control, DOX V.S. DOX+Ghrelin+[D-Lys3]-GHRP-6: 1.14±0.25 V.S. 0.69±0.03) (Figure 10). The protein abundance of Bcl-2 tended to be increased approximately 2-fold in the muscles of the DOX group and the DOX+Ghrelin group when compared with the CON group (Ratio of protein abundance relative to internal control, CON V.S. DOX V.S. DOX+Ghrelin: 0.53±0.08 V.S. 1.12±0.24 V.S. 1.08±0.13), however it did not reach to a significant level (Figure 11). The Bcl-2 protein level was found to be significantly upregulated by 127% in the muscles of the DOX+Ghrelin+[D-Lys3]-GHRP-6 group (Ratio of protein abundance relative to internal control, DOX V.S. DOX+Ghrelin V.S. DOX+Ghrelin+[D-Lys3]-GHRP-6: 1.12±0.24 V.S. 1.08±0.13 V.S. 2.45±0.19) relative to the DOX (P=0.001) and DOX+Ghrelin groups (P=0.001) (Figure 11). The protein abundance of p-Akt

relative to total Akt in the muscles of DOX group was significantly reduced by 72%, compared with the CON group (Ratio of protein abundance relative to internal control, CON V.S. DOX: 0.72±0.13 V.S. 0.20±0.06) (P=0.005) (Figure 12). With treatment of ghrelin, the reduction in p-Akt abundance relative to total Akt abundance induced by doxorubicin exposure was alleviated in the muscles of the DOX-Ghrelin group (Figure 12). The p-Akt abundance relative to total Akt abundance in the muscles of the DOX+Ghrelin+[D-Lys3]-GHRP-6 group was significantly higher than that in the DOX+Ghrelin (p=0.006) and DOX groups (P=0.001), and was similar compared with the CON group (Ratio of protein abundance relative to internal control, DOX V.S. DOX+Ghrelin V.S. DOX+Ghrelin+[D-Lys3]-GHRP-6: 0.20±0.06 V.S. 0.40±0.08 V.S. 0.96±0.14).

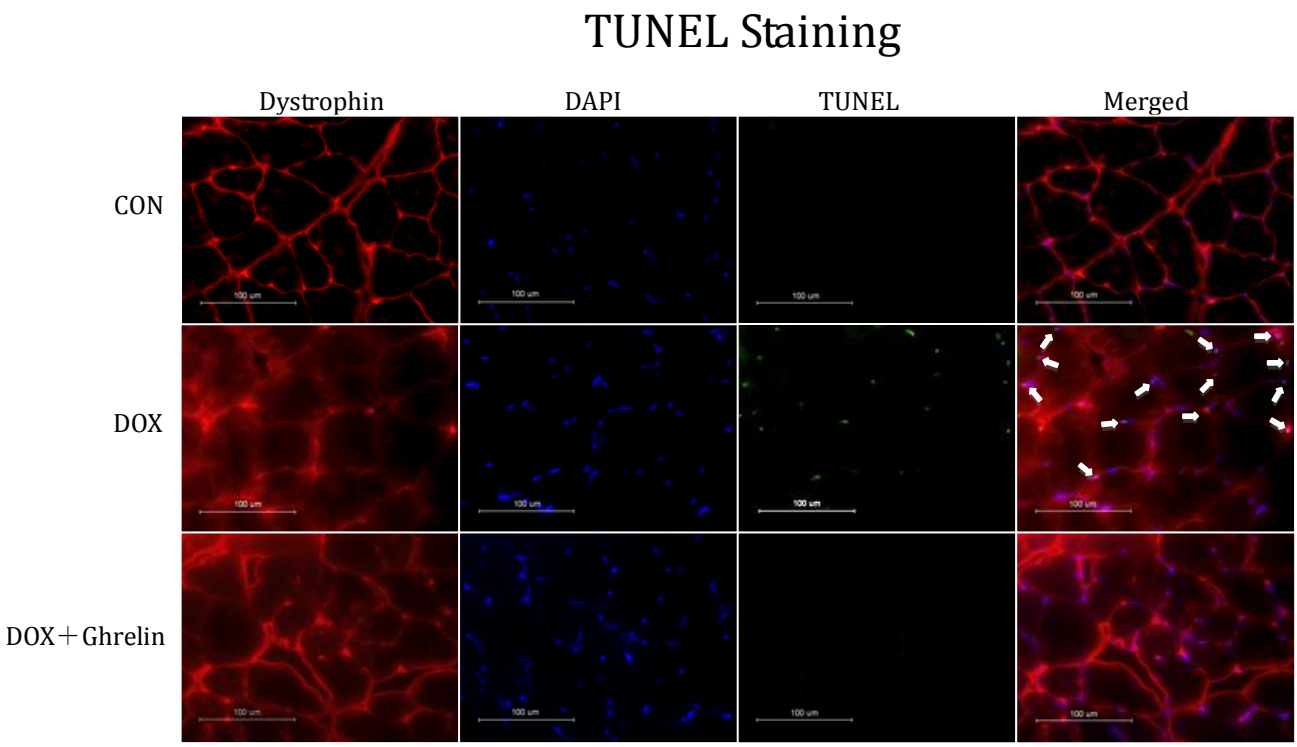

Figure 6. Apoptotic Analysis of Effects of Ghrelin on Doxorubicin-induced Muscle Injury-TUNEL staining.

Apoptotic DNA breaks were determined by TUNEL staining. Immunofluorescent labelling of dystrophin (red) was performed to identify the localization of the TUNEL-positive nuclei (green) with regard to muscle sarcolemmal membrane. Total nuclei were labelled by 4',6-diamidino-2-phenyl-lindole (DAPI) (blue). Images of dystrophin, TUNEL, and total nuclei staining were taken using an objective of 20x.

(CON, saline control; DOX, doxorubicin; DOX+Ghrelin, doxorubicin with treatment of ghrelin).

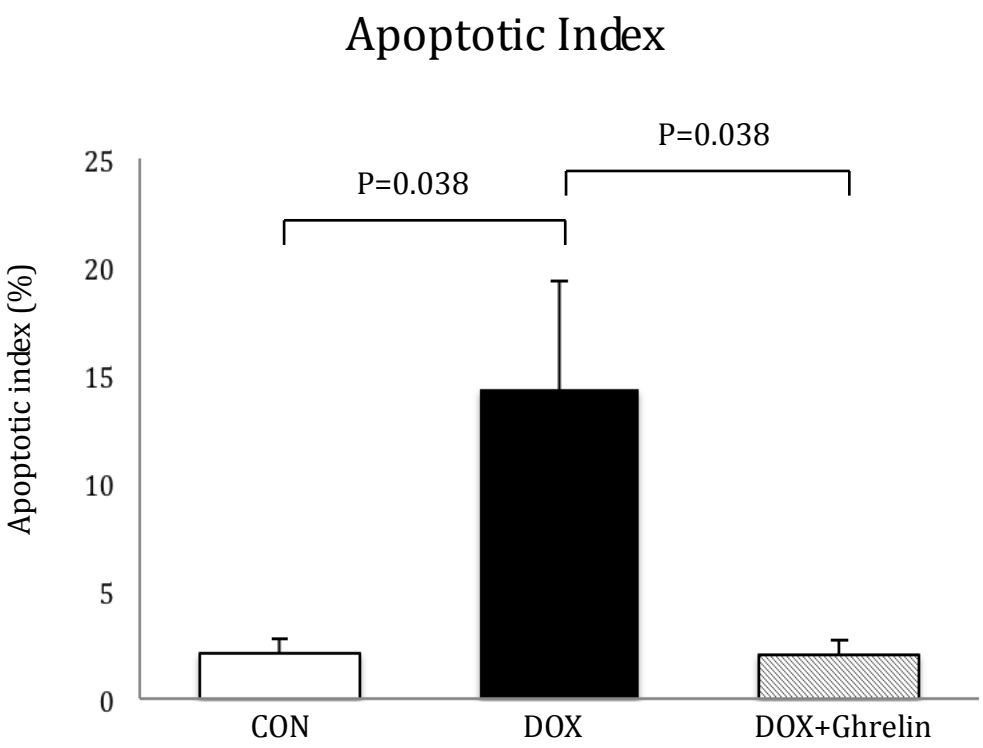

Figure 7. Quantification of Apoptotic Level by TUNEL Staining.

Apoptotic DNA breaks were determined by TUNEL staining and were expressed as TUNEL apoptotic index (i.e., numbers of TUNEL-positive muscle-relative nuclei relative to the total number of nuclei). Data are expressed as means ± SEM with significant level set at $P<0.05$. The data of TUNEL apoptotic index were analysed by Kruskal-Wallis test followed by Mann-Whitney U test.

(CON, saline control; DOX, doxorubicin; DOX+Ghrelin, doxorubicin with treatment of ghrelin; DOX+Ghrelin+[D-Lys3]-GHRP-6, doxorubicin with treatment of ghrelin plus GHSR-1a antagonist [D-Lys3]-GHRP-6).

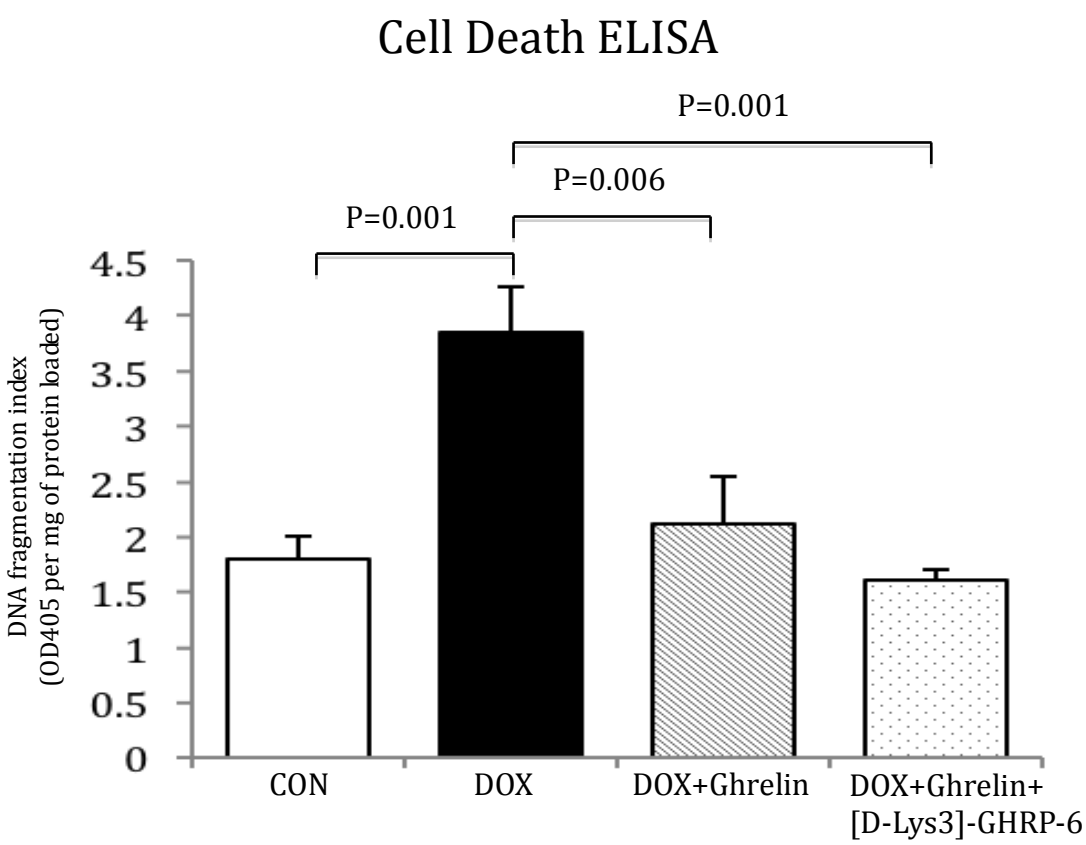

Figure 8. Apoptotic Analysis of Effects of Ghrelin on Doxorubicin-induced Muscle Injury-Cell Death ELISA.

The extent of apoptotic DNA fragmentation was estimated by measuring the cytosolic mono- and oligonucleosomes with Cell Death ELISA. The optical density at 405nm (OD405) was normalized to total milligrams of protein loaded in the assay. The data of Cell Death ELISA were analysed by one-way ANOVA with Turkey's HSD post hoc test.

(CON, saline control; DOX, doxorubicin; DOX+Ghrelin, doxorubicin with treatment of ghrelin; DOX+Ghrelin+[D-Lys3]-GHRP-6, doxorubicin with treatment of ghrelin plus GHSR-1a antagonist [D-Lys3]-GHRP-6).

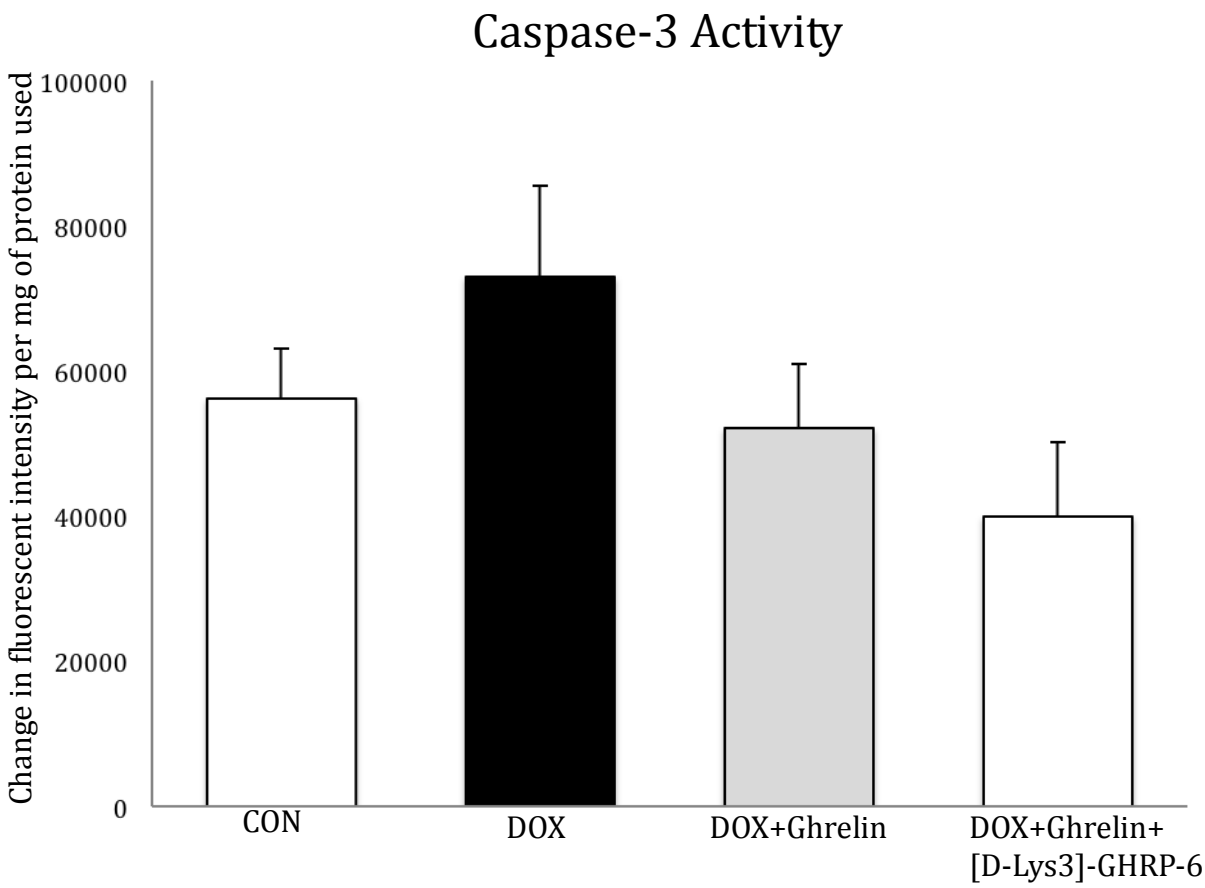

Figure 9. Apoptotic Signaling Analysis of Effects of Ghrelin on Doxorubicin-induced Muscle Injury-Caspase-3 protease activity.

Caspase-3 protease activities are determined as the change of fluorescence intensity normalized to total milligram protein used in the assay. Data are expressed as means ± SEM with significant level set at $P<0.05$. The data were analysed by one-way ANOVA with Turkey's HSD post hoc test.

(CON, saline control; DOX, doxorubicin; DOX+Ghrelin, doxorubicin with treatment of ghrelin; DOX+Ghrelin+[D-Lys3]-GHRP-6, doxorubicin with treatment of ghrelin plus GHSR-1a antagonist [D-Lys3]-GHRP-6).

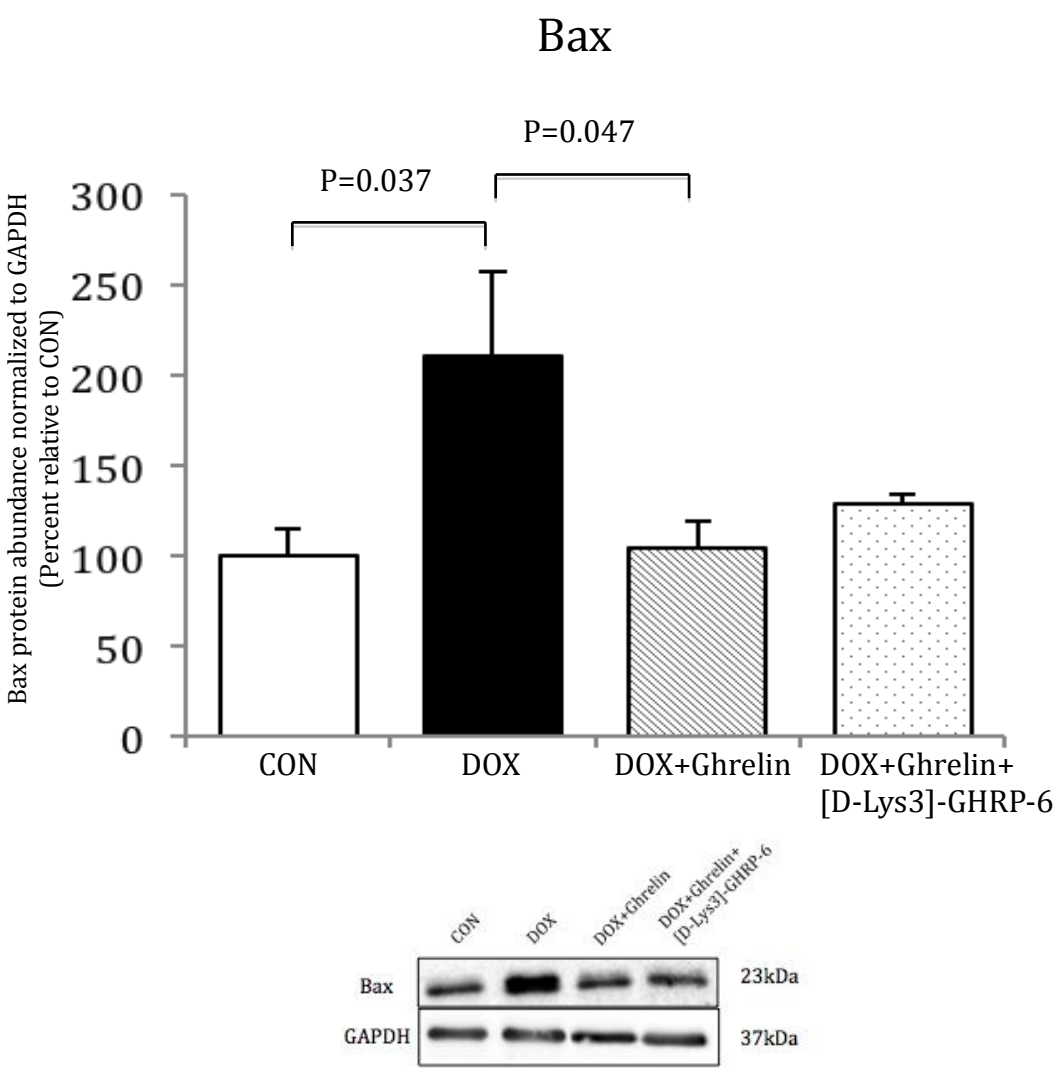

Figure 10. Apoptotic Signaling Analysis of Effects of Ghrelin on Doxorubicin-induced Muscle Injury-Protein Abundance of Bax.

Protein abundances of Bax were determined by Western blotting. Data are presented as net intensity x resulting band area and expressed in percent relative to CON. Results of Bax were normalized to corresponding GAPDH signal. Data are expressed as means ± SEM with significant level set at $P<0.05$. The data were analysed by one-way ANOVA with Turkey's HSD post hoc test.

(CON, saline control; DOX, doxorubicin; DOX+Ghrelin, doxorubicin with treatment of ghrelin; DOX+Ghrelin+[D-Lys3]-GHRP-6, doxorubicin with treatment of ghrelin plus GHSR-1a antagonist [D-Lys3]-GHRP-6).

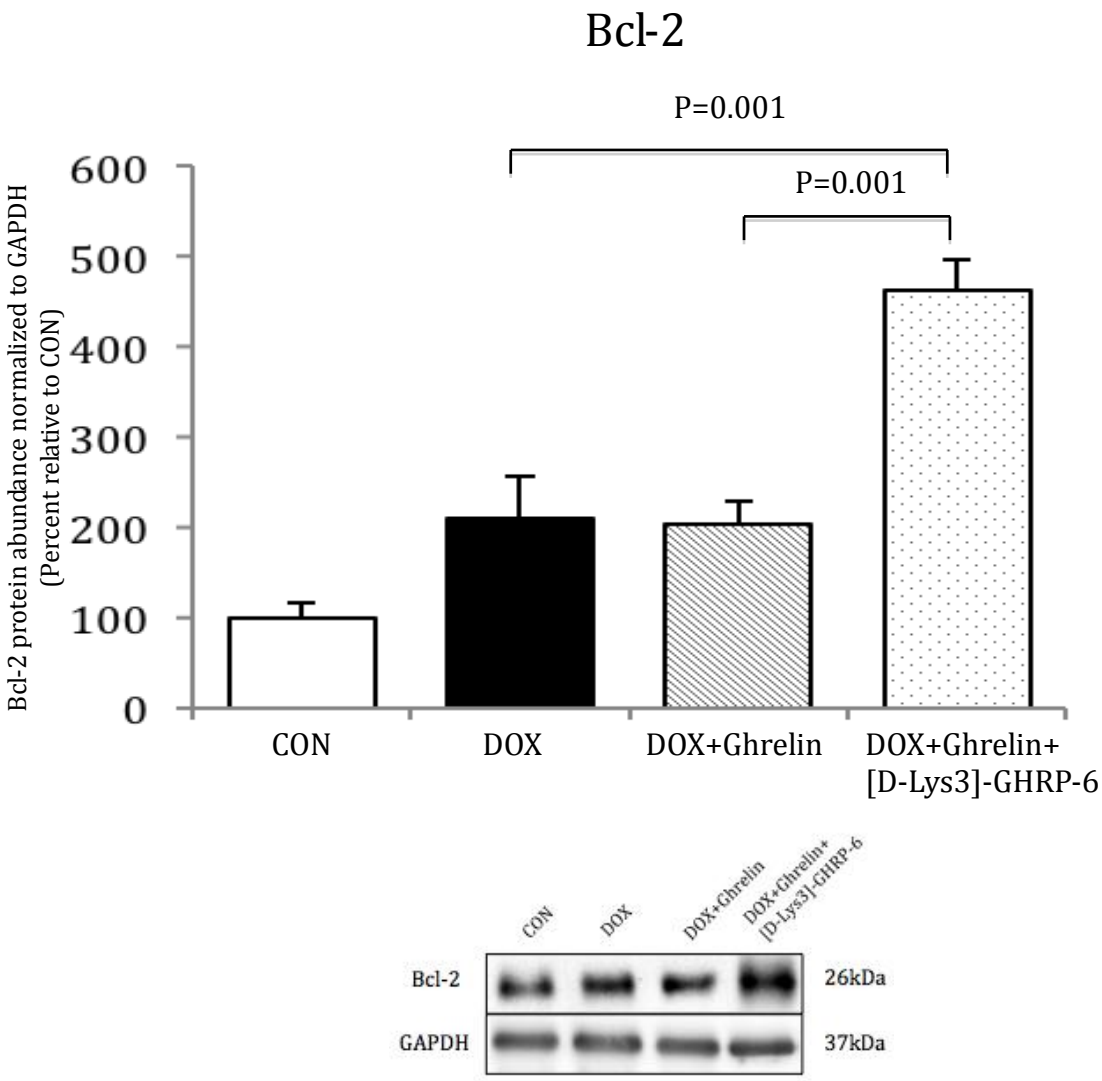

Figure 11. Apoptotic Signaling Analysis of Effects of Ghrelin on Doxorubicin-induced Muscle Injury-Protein Abundance of Bcl-2.

Protein abundances of Bcl-2 were determined by Western blotting. Data are presented as net intensity x resulting band area and expressed in percent relative to CON. Results of Bcl-2 were normalized to corresponding GAPDH signal. Data are expressed as means ± SEM with significant level set at $P<0.05$. The data were analysed by one-way ANOVA with Turkey's HSD post hoc test.

(CON, saline control; DOX, doxorubicin; DOX+Ghrelin, doxorubicin with treatment of ghrelin; DOX+Ghrelin+[D-Lys3]-GHRP-6, doxorubicin with treatment of ghrelin plus GHSR-1a antagonist [D-Lys3]-GHRP-6).

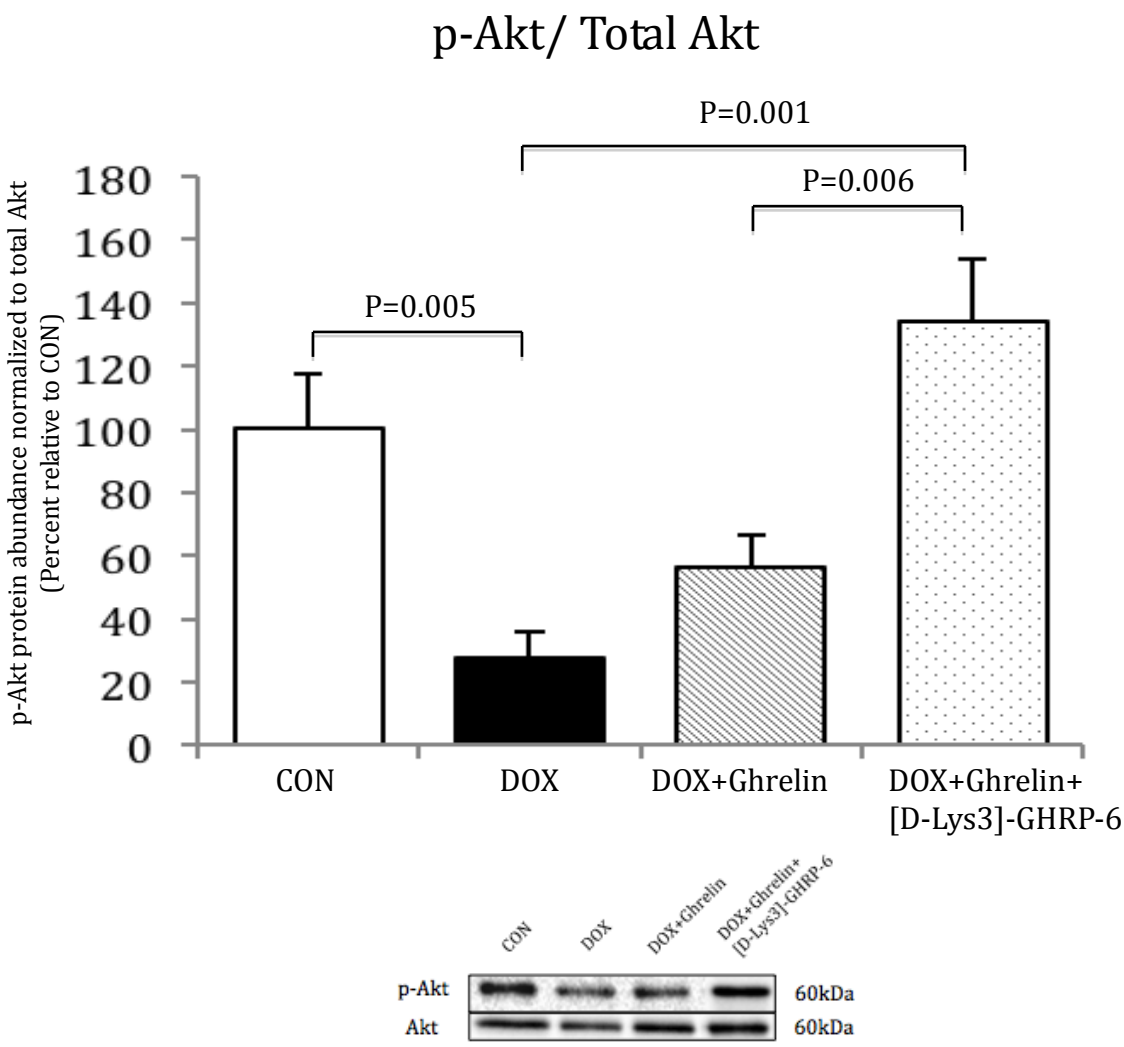

Figure 12. Apoptotic Signaling Analysis of Effects of Ghrelin on Doxorubicin-induced Muscle Injury-Akt Signaling.

Protein abundances of p-Akt and Akt were determined by Western blotting. Data are presented as net intensity x resulting band area and expressed in percent relative to CON. Results of p-Akt were normalized to corresponding total Akt signal. Data are expressed as means ± SEM with significant level set at $P<0.05$. The data were analysed by one-way ANOVA with Turkey's HSD post hoc test.

(CON, saline control; DOX, doxorubicin; DOX+Ghrelin, doxorubicin with treatment of ghrelin; DOX+Ghrelin+[D-Lys3]-GHRP-6, doxorubicin with treatment of ghrelin plus GHSR-1a antagonist [D-Lys3]-GHRP-6).

3.3.4 Analysis of Autophagic Signaling

No significant difference in the ratio of the protein abundance of LC3 II to the protein abundance of LC3 I (Ratio of LC3 II protein abundance relative to LC3 I, CON V.S. DOX: 0.65±0.16 V.S. 0.70±0.17) (Figure 13), protein abundance of Atg 12-5 Complex (Ratio of protein abundance relative to internal control, CON V.S. DOX: 1.40±0.12 V.S. 1.11±0.08) (Figure 14), Atg 5 (Ratio of protein abundance relative to internal control, CON V.S. DOX: 1.04±0.10 V.S. 0.85±0.06) (Figure 15) and Beclin-1 (Ratio of protein abundance relative to internal control, CON V.S. DOX: 1.35±0.24 V.S. 0.91±0.14) (Figure 16) was found in the muscles between the CON and DOX groups. The LC3 II/I ratio tended to be increased approximately 2-fold in the muscles of the DOX-Ghrelin group (Ratio of LC3 II protein abundance relative to LC3 I, DOX V.S. DOX+Ghrelin: 0.70±0.17 V.S. 1.47±0.24) compared with the DOX group, while it was significantly increased with 3.5-fold change in the muscles of the DOX+Ghrelin+[D-Lys3]-GHRP-6 group (Ratio of LC3 II protein abundance relative to LC3 I, DOX+Ghrelin V.S. DOX+Ghrelin+[D-Lys3]-GHRP-6: 1.47±0.24 V.S. 5.14±1.35) relative to the DOX+Ghrelin group (Figure 13). The Atg 12-5 Complex protein level was significantly increased by 48% and 92% in the

muscles of the DOX+Ghrelin and the DOX+Ghrelin+[D-Lys3]-GHRP-6 groups, respectively, when compared with the DOX group (Ratio of protein abundance relative to internal control, DOX V.S. DOX+Ghrelin V.S. DOX+Ghrelin+[D-Lys3]-GHRP-6: 1.11±0.08 V.S. 1.64±0.19 V.S. 2.12±0.14) (Figure 14). The Atg 5 protein level was significantly increased by 59% and 235% in the muscles of the DOX+Ghrelin and DOX+Ghrelin+[D-Lys3]-GHRP-6 groups respectively, relative to the DOX group (Ratio of protein abundance relative to internal control, DOX V.S. DOX+Ghrelin V.S. DOX+Ghrelin+[D-Lys3]-GHRP-6: 0.85±0.06 V.S. 1.35±0.17 V.S. 2.84±0.18) (Figure 15). No significant differences were found in the protein abundance of Beclin-1 in the muscles of the DOX+Ghrelin group relative to that of the DOX group. In the presence of the GHSR-1a antagonist, the Beclin-1 protein level was significantly upregulated with 2-fold increase in the muscles of the DOX+Ghrelin+[D-Lys3]-GHRP-6 group relative to the DOX+Ghrelin group (P=0.001) and 4-fold increase relative to the DOX group (P=0.001), respectively (Ratio of protein abundance relative to internal control, DOX V.S. DOX+Ghrelin V.S. DOX+Ghrelin+[D-Lys3]-GHRP-6: 0.91±0.14 V.S. 1.71±0.23 V.S. 5.16±0.41) (Figure 16).

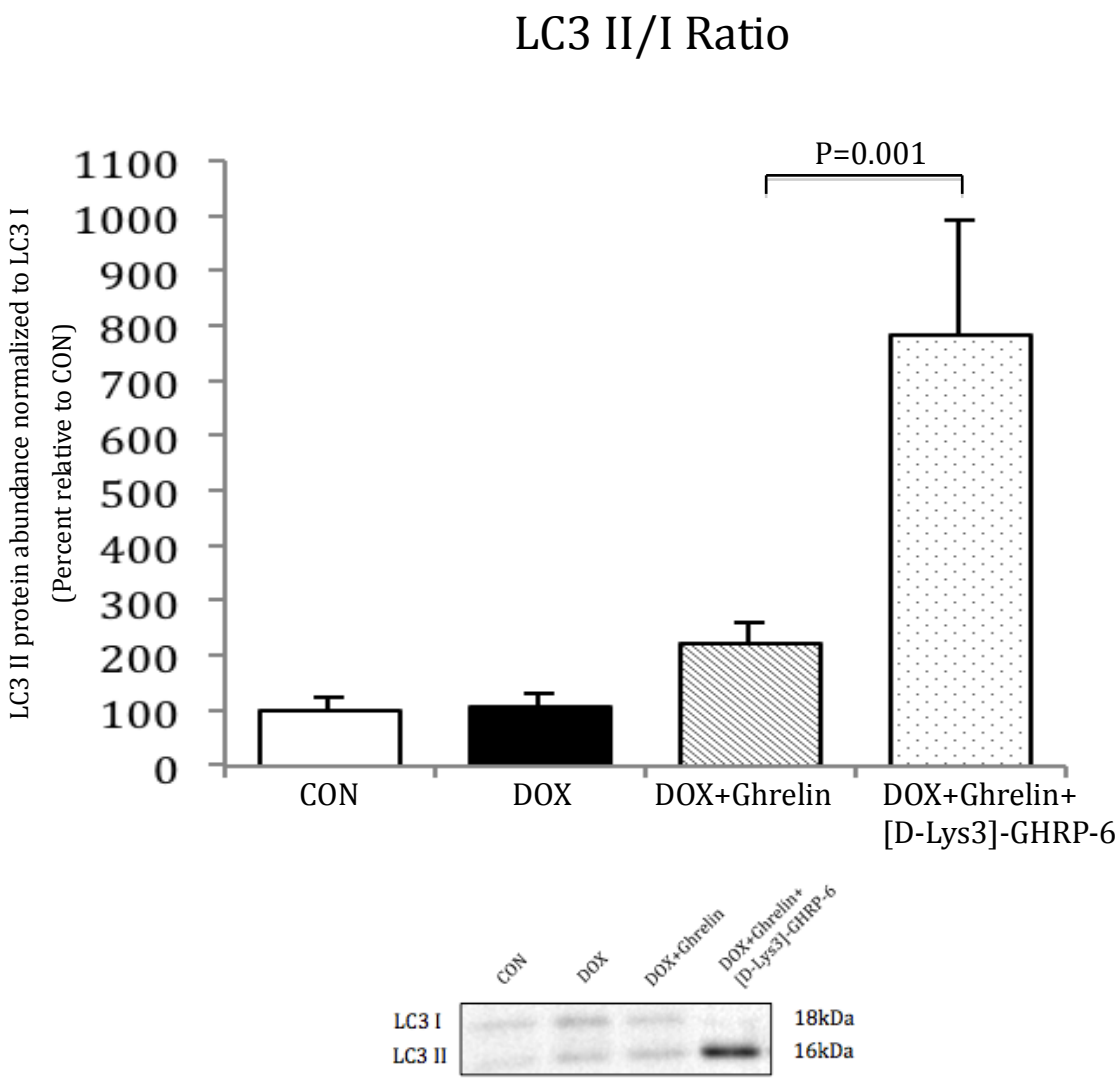

Figure 13. Autophagic Analysis of Effects of Ghrelin on Doxorubicin-induced Muscle Injury-LC3II/I Ratio.

Protein abundances of LC3 II and LC3 I were determined by Western blotting. Data are presented as net intensity x resulting band area and expressed in percent relative to CON. Results of LC3 II was normalized to corresponding LC3 I to generate LC3 II/I ratio. Data are expressed as means ± SEM with significant level set at $P<0.05$. The data were analysed by one-way ANOVA with Turkey's HSD post hoc test.

(CON, saline control; DOX, doxorubicin; DOX+Ghrelin, doxorubicin with treatment of ghrelin; DOX+Ghrelin+[D-Lys3]-GHRP-6, doxorubicin with treatment of ghrelin plus GHSR-1a antagonist [D-Lys3]-GHRP-6).

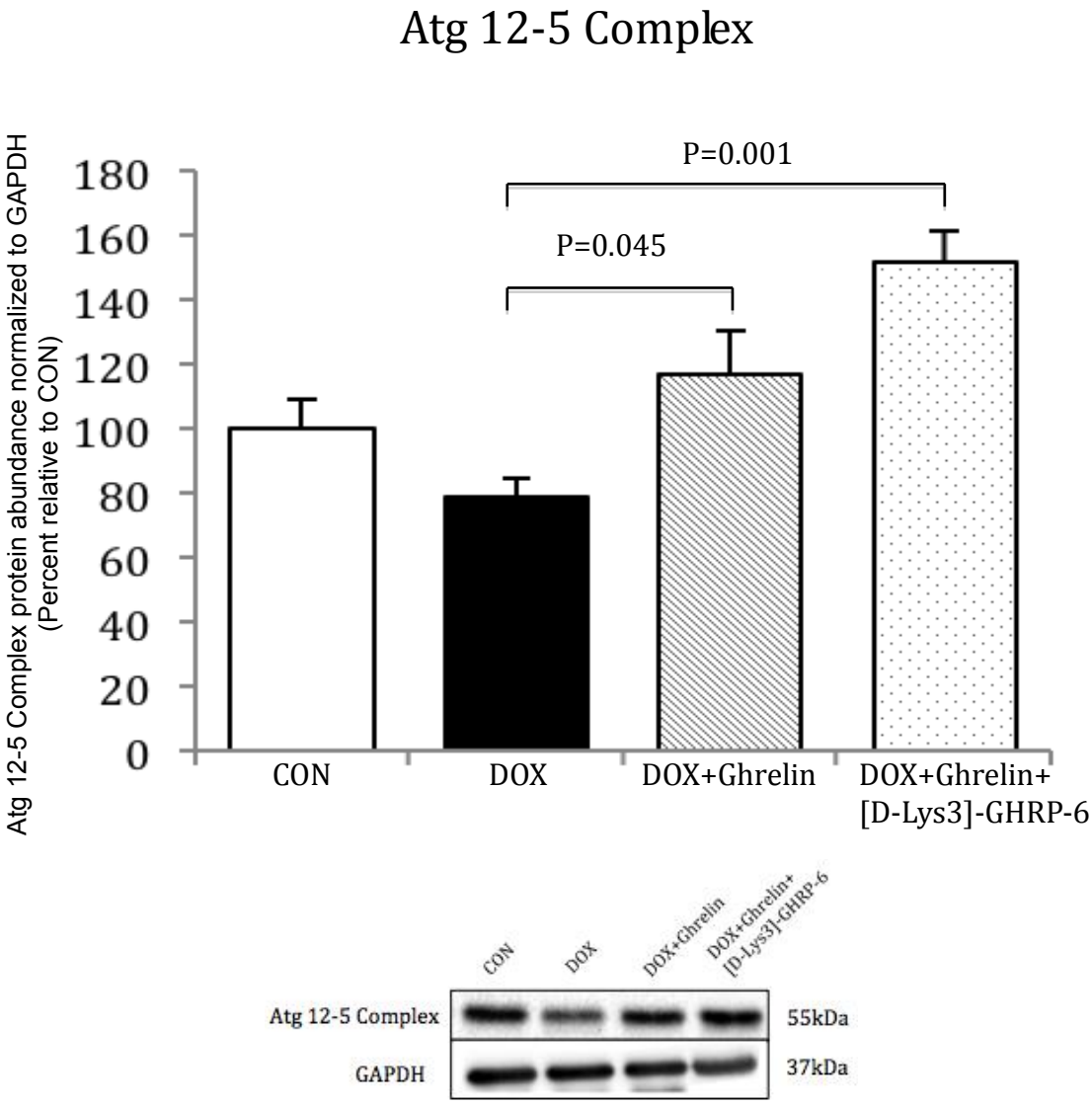

Figure 14. Autophagic Analysis of Effects of Ghrelin on Doxorubicin-induced Muscle Injury-Protein Abundance of Atg 12-5 Complex.

Protein abundances Atg 12-5 Complex were determined by Western blotting. Data are presented as net intensity x resulting band area and expressed in percent relative to CON. Results of Atg 12-5 Complex were normalized to corresponding GAPDH signal. Data are expressed as means ± SEM with significant level set at $P<0.05$. The data were analysed by one-way ANOVA with Turkey's HSD post hoc test.

(CON, saline control; DOX, doxorubicin; DOX+Ghrelin, doxorubicin with treatment of ghrelin; DOX+Ghrelin+[D-Lys3]-GHRP-6, doxorubicin with treatment of ghrelin plus GHSR-1a antagonist [D-Lys3]-GHRP-6).

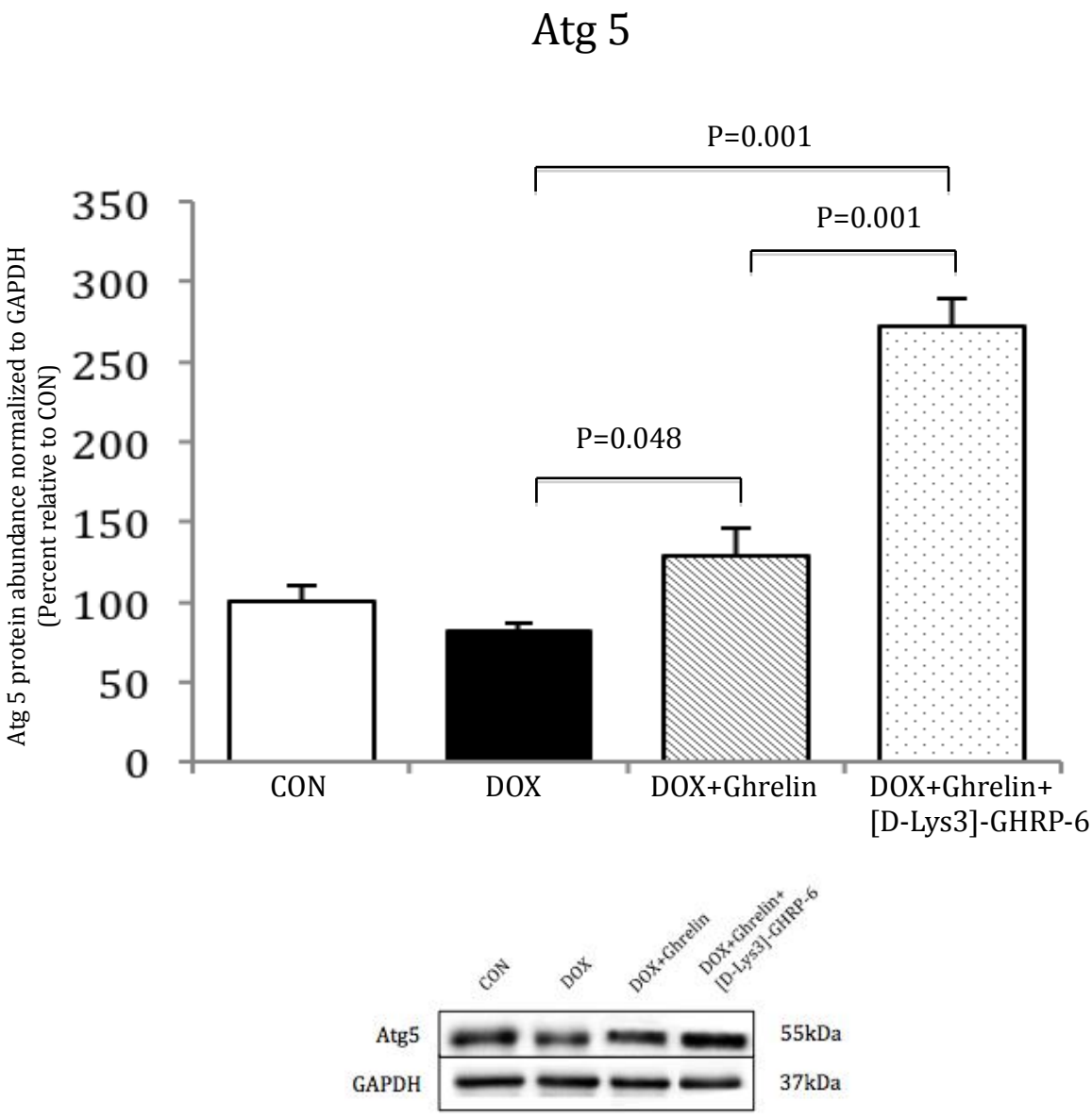

Figure 15. Autophagic Analysis of Effects of Ghrelin on Doxorubicin-induced Muscle Injury-Protein Abundance of Atg 5.

Protein abundances of Atg 5 were determined by Western blotting. Data are presented as net intensity x resulting band area and expressed in percent relative to CON. Results of Atg 5 were normalized to corresponding GAPDH signal. Data are expressed as means ± SEM with significant level set at $P<0.05$. The data were analysed by one-way ANOVA with Turkey's HSD post hoc test.

(CON, saline control; DOX, doxorubicin; DOX+Ghrelin, doxorubicin with treatment of ghrelin; DOX+Ghrelin+[D-Lys3]-GHRP-6, doxorubicin with treatment of ghrelin plus GHSR-1a antagonist [D-Lys3]-GHRP-6).

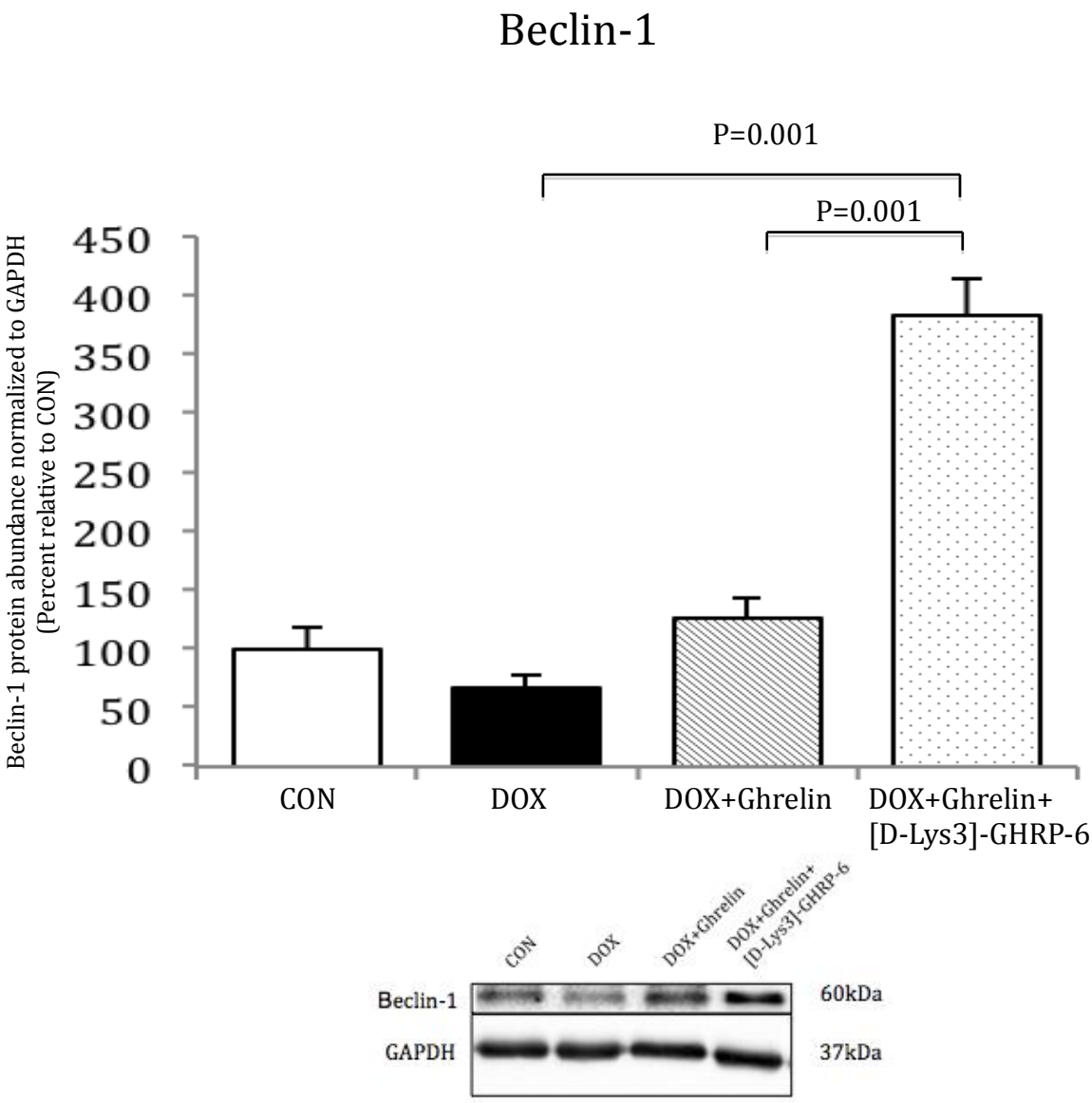

Figure 16. Autophagic Analysis of Effects of Ghrelin on Doxorubicin-induced Muscle Injury-Protein Abundance of Beclin-1.

Protein abundances of Beclin-1 were determined by Western blotting. Data are presented as net intensity x resulting band area and expressed in percent relative to CON. Results of Beclin1 were normalized to corresponding GAPDH signal. Data are expressed as means ± SEM with significant level set at $P<0.05$. The data were analysed by one-way ANOVA with Turkey's HSD post hoc test.

(CON, saline control; DOX, doxorubicin; DOX+Ghrelin, doxorubicin with treatment of ghrelin; DOX+Ghrelin+[D-Lys3]-GHRP-6, doxorubicin with treatment of ghrelin plus GHSR-1a antagonist [D-Lys3]-GHRP-6).

3.4 Discussion

In the present study, exposure to doxorubicin revealed an increase in muscle fibers containing centralized nuclei, suggesting the processes of degeneration and regeneration were activated in muscles following the exposure of doxorubicin (Chiquet & Fluck, 2003). The reduction in the percentage of centronucleated fibers in muscles co-administered with doxorubicin and ghrelin suggests the protection of ghrelin against doxorubicin-induced injury in the skeletal muscle. Under normal physiology, centronucleated fibers account for around 3% of the total fibers in the skeletal muscle (Schochet, 1986). In this experiment, muscle sections of the CON, the DOX+Ghrelin and the DOX+Ghrelin+[D-Lys3]-GHRP-6 groups had around 3 % of the total fibers that were centronucleated, indicating that muscles from both groups were healthy.

Cleaved-actin, a 14 kDa actin fragment, is a widely adopted cellular marker of protein degradation in skeletal myofibers (Zhou *et al*., 2007;Plant *et al*., 2009). In addition to the histological findings of centralized myonuclei, our immuoblot analysis demonstrated that doxorubicin significantly induced the cleavage of actin in the muscles whereas this induction was not observed in the

doxorubicin-treated muscles with ghrelin intervention. These results are in agreement with the previous reports demonstrating the induction of cellular damage and proteolysis in skeletal muscle following doxorubicin-administration to the skeletal muscle (Smuder *et al.*, 2011a;Smuder *et al.*, 2011b). These results are also in line with the previous findings showing that the inhibitory effects of ghrelin on proteins catabolism in skeletal muscle following thermal injury (Balasubramaniam *et al.*, 2009) and fasting-induced atrophy (Porporato *et al.*, 2013).

3.4.1 Ghrelin Suppresses Muscle Apoptosis and Enhances Autophagy after Doxorubicin Exposure

Doxorubicin has been demonstrated previously as an inducer of apoptosis in cardiomyocytes (Takemura & Fujiwara, 2007) and C2C12 myotubes (Hilder *et al.*, 2005). The results of TUNEL and Cell Death ELISA in this experiment supported the notion of doxorubicin-induced activation of apoptosis in the skeletal muscle. More importantly, our results demonstrated that ghrelin inhibited the doxorubicin-induced apoptosis in the skeletal muscle. These findings are in agreement with the previous reports demonstrating the anti-apoptotic properties

of ghrelin in response to different stimuli, such as H_2O_2 (Zhang *et al.*, 2011), carbon tetrachloride (Cetin *et al.*, 2011), and doxorubicin (Baldanzi *et al.*, 2002) in various tissues including the heart (Vedam *et al.*, 2010), liver (Cetin *et al.*, 2011), and gastric tissue (El *et al.*, 2007).

In this study, the protein contents of pro-apoptotic Bax and anti-apoptotic Bcl-2 were elevated concurrently in doxorubicin-treated muscles. While the doxorubicin-induced upregulation of Bax was well documented (Wang *et al.*, 1998;Tsang *et al.*, 2003), the corresponding modulating effects of Bcl-2 expression varied with tissues (Tu *et al.*, 1996;Wang *et al.*, 1998;Jang *et al.*, 2004;Romano *et al.*, 2004;Zhao *et al.*, 2009). Our results are in agreement with the *in vivo* study conducted by Jang and co-workers (Jang *et al.*, 2004) and the *in vitro* study conducted by Wang and colleagues (Wang *et al.*, 1998), reporting the upregulation of Bcl-2 after the exposure to doxorubicin in cardiac muscles. We speculated the observed increase in Bcl-2 protein content as a self-protective mechanism of muscle cells in response to doxorubicin challenge. Furthermore, ghrelin intervention was shown to reduce the protein content of Bax but did not exert

any modulating effects on the expression of Bcl-2 in doxorubicin-treated muscle. This finding is consistent with previous report demonstrating that the administration of ghrelin suppressed apoptosis by reducing the expression of proapoptotic Bax in the absence of the modulation of the expression of antiapoptotic Bcl-2 (Kheradmand *et al.*, 2012). The decrease in Bax protein abundance explained partly the reduction in the doxorubicin-induced activation of apoptosis by the intervention of ghrelin.

The present findings of the effects of doxorubicin and ghrelin on the Akt cellular signaling in skeletal muscle are consistent with the previous studies demonstrating the inhibitory effect of doxorubicin on the phosphorylation status of Akt in cardiac muscle (Xiang *et al.*, 2009) and the modulating role of ghrelin in activating the Akt cellular pro-survival pathway (Baldanzi *et al.*, 2002). It has been demonstrated in this experiment that the doxorubicin-induced elevation of apoptosis was completely suppressed while the Akt signaling was only partially restored by the treatment of ghrelin in the muscle, suggesting that there might be other signaling pathways involved in the ghrelin-mediated inhibition of apoptosis.

In addition to the activation of Akt signaling pathway, the intervention of ghrelin has been shown to activate ERK1/2 signaling pathway (Baldanzi *et al.*, 2002). It has been demonstrated in cardiomyocytes (Kui et al., 2009), endothelial cells (Favaro et al., 2012) and neuronal cells (Chung et al., 2008) that administration of ghrelin can activate the ERK1/2 signaling pathway by upregulating the phosphorylation of ERK. Both activation of Akt signaling and activation of ERK1/2 signaling pathways are known to suppress apoptosis (Favaro *et al.*, 2012), implying that activation of ERK1/2 signaling pathway could be one of the possible mechanisms that contribute to the inhibition of apoptosis following ghrelin treatment. The Akt signaling pathway was proposed to inhibit both apoptosis and autophagy in the skeletal muscle (Sandri, 2010) while activation of ERK1/2 pathway was known to promote the execution of autophagy (Ellington *et al.*, 2006;Sridharan *et al.*, 2011). Indeed, the cross-talk between Akt signaling and ERK1/2 signaling may serve as the "switch" of the activation of autophagy (Gozuacik & Kimchi, 2004). It is postulated that administration of ghrelin may activate the ERK1/2 signaling pathway and promote the upregulation of autophagy in doxorubicin-treated muscle.

3.4.2 Inhibition of GHSR-Signaling Did Not Blunt the Effects of Ghrelin on Suppressing Apoptosis and Activating Autophagy

Ghrelin is known as the natural ligand of the GHSR-1a (Kojima *et al.*, 1999). Many cellular effects of ghrelin including induction of growth hormone release (Ma *et al.*, 2012), and suppression of apoptosis in osteoblastic cells (Kim *et al.*, 2005) were demonstrated to be mediated through the GHSR-signaling. Hence, GHSR-1a was hypothesized as the mediating machinery for the effects of ghrelin against doxorubicin-induced toxicity.

The doxorubicin-induced pathological damages were not observed in doxorubicin-treated muscles with ghrelin treatment, regardless of the co-administration of GHSR-1a antagonist. The inhibitory effects of ghrelin on the activation of apoptotic signaling induced by doxorubicin were not observed to be blunted in the muscles treated with the GHSR-1a antagonist based on the results that the apoptotic DNA fragmentation, caspase-3 activity level and protein abundance of pro-apoptotic Bax were not different in ghrelin treatment no matter with or without application of GHSR-1a antagonist. The protein abundance of pro-survival Bcl-2 was even higher in the ghrelin-treated muscles in the presence

of the GHSR-1a antagonist. These results agreed with previous studies showing that the inhibitory effects of ghrelin on apoptosis (Baldanzi *et al.*, 2002;Zhang *et al.*, 2011) and caspase-3 activity (Rak *et al.*, 2009) were not dependent on GHSR-1a signaling. Notably, GHSR-1a antagonist was shown to further augment elevation in autophagic markers, including the LC3 II/I ratio, Atg 5 and Beclin-1 in the muscles co-administrated with ghrelin and GHSR-1a antagonist when compared to the muscle treated with ghrelin alone, suggesting that the ghrelin-induced upregulation of autophagy was not blunted by blockage of GHSR-1a. Recently, Tong and co-workers reported that administration of ghrelin induced autophagy in H9C2 cardiomyocytes, a cell type which do not express GHSR-1a (Tong *et al.*, 2012). Results of this experiment and Tong's study support the notion that ghrelin induces autophagy through a GHSR-independent mechanism. These findings collectively supported that the effects of ghrelin on both the suppression of apoptosis and the enhancement of autophagy were not blunted by blocking GHSR-1a Signaling, indicated that the myoprotective effects of ghrelin on the doxorubicin-induced alteration of apoptosis and autophagy were mediated through the cellular signaling pathways that were independent of GHSR.

Indeed, ghrelin was shown to modulate the activation of Akt signaling, inhibition of cell death and protein degradation through GHSR-independent pathways based on the results obtained from studies using GHSR-deficient cells (Baldanzi *et al.*, 2002) and GHSR-deficient mice (Porporato *et al.*, 2013). These studies consistently demonstrated that ghrelin inhibited the oxidative stress-induced cell death in the GHSR-1a absent H9C2 cardiomyocytes (Baldanzi *et al.*, 2002;Zhang *et al.*, 2011), suggesting that the protective effects of ghrelin could be possibly mediated through other cellular signaling pathways that do not involve GHSR-1a.

Porporato and colleagues have tested the hypothesis that GHSR-1a was not required to mediate the protective effects of ghrelin against fasting-induced muscle atrophy by employing the GHSR deficient mice (Porporato *et al.*, 2013). Their data clearly demonstrated that ghrelin exerted the protective effects by activating the Akt signaling and suppressing the upregulation of ubiquitin E3 ligase even in muscle cells with GHSR-1a deficiency (Porporato *et al.*, 2013). In this experiment, application of GHSR-1a antagonist did not affect the activation of Akt signaling after ghrelin treatment which is in agreement with their findings

(Porporato *et al.*, 2013). Indeed, the involvement of GHSR-1a has been shown as unnecessary for exerting the effects of ghrelin in the inhibition of apoptosis and activation of Akt signaling, suggesting that GHSR-1a is not the only receptor that ghrelin binds with (Chang *et al.*, 2004;Rak *et al.*, 2009) In addition, unknown receptors that are responsible for coordinating the cellular effects of ghrelin may exist. Therefore, the unknown receptors and GHSR-1a might possibly compete with each other for ghrelin binding. Reasonably, it was speculated that the blockage of GHSR-1a with the introduction of an antagonist would restrict the binding of ghrelin to GHSR-1a and so increase the cellular amount of free ghrelin. This would result in increased ghrelin binding to the unknown receptor, which seems to explain partly our observation that the activation of Akt signaling and autophagy were enhanced further in the muscle with ghrelin treatment in the presence of the GHSR-1a antagonist. Further investigation is needed to unravel the unknown receptors of ghrelin and to unmask the exact mechanisms of the myoprotective effects of ghrelin fully.

3.4.3 Inhibition of GHSR-Signaling Abolished the Effect of Ghrelin in Preventing Degradation of Skeletal Muscle Protein

It was noted that the abundance of cleaved-actin in the muscles co-administrated with ghrelin and GHSR-1a antagonist was similar to that in the DOX group, implying that the inhibitory effect of ghrelin on degradation of muscle proteins was GHSR-1a dependent. Ghrelin has been shown to exert anti-inflammatory effects attributing to the activation of GHSR signaling mechanism (Li *et al.*, 2004;Waseem *et al.*, 2008;Deboer, 2011). Administration of both ghrelin or des-n-octanoyl ghrelin has been shown to suppress apoptosis in a dose-dependent manner whereas only ghrelin was able to inhibit the release of proinflammatory cytokines and this was probably due to the binding ability of ghrelin to GHSR-1a (Li *et al.*, 2004;Deboer, 2011). The importance of GHSR signaling was further substantiated by the evidence showing that the administration of GHSR-1a antagonist [D-Lys3]-GHRP-6 reduce the anti-inflammatory effect of ghrelin on the TNF-α-treated endothelial cells (Waseem *et al.*, 2008). As inflammation is proposed to have an important role in the doxorubicin-induced muscle injury (Gilliam *et al.*, 2009), the prohibition of engagement of ghrelin and GHSR-1a by adopting GHSR-1a antagonist [D-Lys3]-GHRP-6 in this experiment reasonably

resulted in diminishing the anti-inflammatory effect of ghrelin (Li *et al.*, 2004;Waseem *et al.*, 2008) in the doxorubicin-exposed muscle, and therefore failed to suppress the protein degradation induced by doxorubicin.

Cleaved-actin was reported to be generated by caspase-3 cleavage action during muscle protein degradation (Plant *et al.*, 2009). The trend of caspase-3 activity levels in the muscles of the CON, DOX and DOX+Ghrelin groups were consistent with the protein level of cleaved-actin. Both protein levels of cleaved actin and caspase-3 activities were upregulated after doxorubicin intervention and suppressed by ghrelin treatment. However, the protein level of cleaved-actin in muscle of DOX+Ghrelin+[D-Lys3]-GHRP-6 group was significantly higher than that in DOX+Ghrelin group while the caspase-3 activity levels in the muscle of these two groups were similar. These results suggest that other proteases may mediate the cleavage of actin during protein degradation. Calpain is regarded as a potential candidate as the actin-cleavage during proteolysis was inhibited by the administration of calpain inhibitors but not caspase inhibitors (Villa *et al.*, 1998).

CHAPTER 4

Effects of Ghrelin on Basal Apoptosis and Autophagy under Normal Physiological Condition

4.1 Introduction

It has been demonstrated that ghrelin administration can suppress the elevation of apoptosis following thermal-induced injury or exposure to toxic substances (Rodriguez *et al*., 2012). The cytoprotective effects of ghrelin via the mediation of autophagy has also been reported (Tong *et al*., 2012). However, the effects of ghrelin on basal apoptosis and autophagy in the skeletal muscle remain unknown. This sub-experiment aimed to investigate the regulatory effects of ghrelin on the basal levels of apoptosis and autophagy in the skeletal muscle.

4.2 Methods

4.2.1 Animals

Eight to twelve-week old C57BL/6 wild type mice were employed in this study. Animals were maintained on a 12:12-h light-dark cycle. Food and water were provided *ad libitum* throughout the experimental period. Animal ethics approval was obtained from the Animal Ethics Sub-committee of The Hong Kong Polytechnic University.

4.2.2 Experimental Design

Mice were randomly assigned to two groups (n = 5), namely the Saline control group (CON) and the Saline control with treatment of ghrelin group (CON+Ghrelin). Both groups were first intraperitoneally injected with saline (the same volume of doxorubicin administration as described in the previous Chapter). Ghrelin intervention was begun 12 hours after the saline injection. Mice in the CON+Ghrelin group were subsequently injected with 100 μg/kg body weight ghrelin intraperitoneally (acyl ghrelin, Tocris Bioscience, USA) (Li *et al.*, 2006) whereas the CON group received saline injection (the same volume of ghrelin injected in CON+Ghrelin group) as placebo twice daily for four consecutive days. After the experimental period, all mice were sacrificed and the gastrocnemius muscles were collected. Tissues were frozen rapidly in liquid nitrogen and stored at -80^{o}C until further use.

4.2.3 Cytoplasmic and Nuclear Fraction Protein Preparation

Muscle proteins were prepared in accordance with the published protocol of Teng and co-workers (Teng *et al.*, 2011). Tissues were minced and homogenized on ice

in the ice-cooled Rothermal's lysis buffer (10mM NaCl, 1.5mM $MgCl_2$, 20mM HEPES, 1mM DTT, 20% glycerol, 0.1% Triton X-100, pH7.4). Homogenate was centrifuged at 3000 rpm (805 x g) to pellet the nuclei and cell debris. Cytoplasmic proteins were prepared by subjecting the supernatants to centrifugation at 6000 rpm (3220 x g) to remove nuclei residues and were stored regarded as a cytoplasmic protein fraction. A portion of the cytoplasmic fraction was stored at -80^{o}C directly for activity assays while protease inhibitor cocktail (P8340; Sigma Aldrich) was added to the remaining portion to prevent protein degradation, thereby allowing the conduction of western blot and Cell Death ELISA.

Pellets were washed with Rothermal's lysis buffer and centrifuged at 3000 rpm (805 x g) three times to remove any remaining cytoplasmic proteins. Pellets were rotated at 4^{o}C for 1 hour after adding 300 µl Rothermal's lysis buffer, 41.5 µl NaCl and 5 µl protease inhibitor for releasing the nuclear proteins. The mixture was then centrifuged at 15000 rpm (17530 x g). Supernatants were collected as the nuclear fraction of muscle proteins.

4.2.4 Apoptotic Cell Death ELISA

The Cell Death Detection ELISA kit was purchased from Roche Diagnostics. The experiment was conducted according to the manufacturer's protocol. Briefly, the wells of the microplate were first coated with the primary mouse monoclonal histone antibody in a coating solution at 4^{o}C overnight. Coating solution was then removed by suction. Wells were then incubated with incubation buffer for 30 min at room temperature. Sample solutions were prepared during the 30 min incubation by diluting the cytoplasmic protein fraction with incubation buffer with the ratio of 1:9. 200 µl of sample solutions were added accordingly and left at room temperature for 90 min. After three washes, the microplate was incubated with the conjugate solution for 90 min except for the blank position, which was employed as a negative control. Suction removal and three washes were performed prior to the incubation with substrate solution for 20 min at room temperature. Photometric analysis was at an absorbance of 405 nm conducted by a spectrophotometer (Benchmark Plus, BIORAD). The resulting optical density was normalized to the milligrams of the proteins used in the assay (OD 405/mg protein used) and present as the apoptotic DNA fragmentation index.

4.2.5 Western Bloting

The protein expression levels of the markers of autophagy (Beclin-1, Atg 5, Atg 12-5 Complex, LC3) were measured by using proteins of cytoplasmic fraction. Protein extracts were boiled at 95^{o}C with 5% Beta-mercaptoethanol for 15 min. Equal amounts of protein were loaded on 10% (Beclin-1, Atg 5, Atg 12-5 Complex) or 12% (LC3) polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose membranes (Beclin-1, Atg 5, Atg 12-5 Complex) or polyvinylidene difluoride membranes (LC3) by electroblotting. After the transfer, the membranes were blocked with 10 ml of 5% non-fat milk for 1 hour at room temperature, followed by overnight incubation with the corresponding primary antibodies at 4^{o}C (Table 3). Membranes were then washed in TBST for three times and incubated with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 1 hour (1: 4000 dilution, 7076 for antimouse IgG antibody, 7074 for anti-rabbit IgG antibody; Cell Signaling Technology). Membranes were washed again for three times with TBST. Luminol reagent (NEL103001EA; Perkin Elmer, Waltham, MA, USA) was then applied to the membrane for chemiluminescent detection of horseradish

peroxidase activity. The chemiluminescent signal was detected using a Chemidoc^{tm} MP imaging system, BIORAD. The resulting bands were quantified by Imagej as an optical density X band area. GAPDH was employed as an internal control for all measurements. Data were expressed by normalizing to the corresponding internal control and expressed as percentage change relative to CON group.

4.2.6 Statistical Analysis

Statistical analysis was performed by using the SPSS 20.0 software package (IBM). The normality of data distribution was assessed by using the Kolmogorov-Smirnov test. Independent t-test was employed to detect the significant difference between the CON group and CON+Ghrelin group. P-value < 0.05 was considered as statistical significant different. All data were expressed as mean ± standard error of mean (SEM).

Table 3. Primary Antibodies Employed in Western Blot Analysis of Chapter 4

Antibody	**Dilution Factor**	**Source**
Anti-Beclin-1 rabbit polyclonal	1:1000	3738, Cell Signaling Technology
Anti-Atg5 rabbit polyclonal	1:1000	8540, Cell Signaling Technology
Anti-Atg12 rabbit polyclonal	1:1000	2011, Cell Signaling Technology
Anti-LC3 rabbit monoclonal	1:1000	3868, Cell Signaling Technology

4.3 Results

4.3.1 Analysis of Apoptotic Signaling

Cell Death ELISA was conducted to determine the level of apoptosis in the skeletal muscle. There was no significant difference in the apoptotic DNA fragmentation index (OD405/mg protein used) observed between the muscles of CON and CON+Ghrelin groups (Figure 17), indicating that there was no significant difference in the apoptotic level in the skeletal muscle between these two groups.

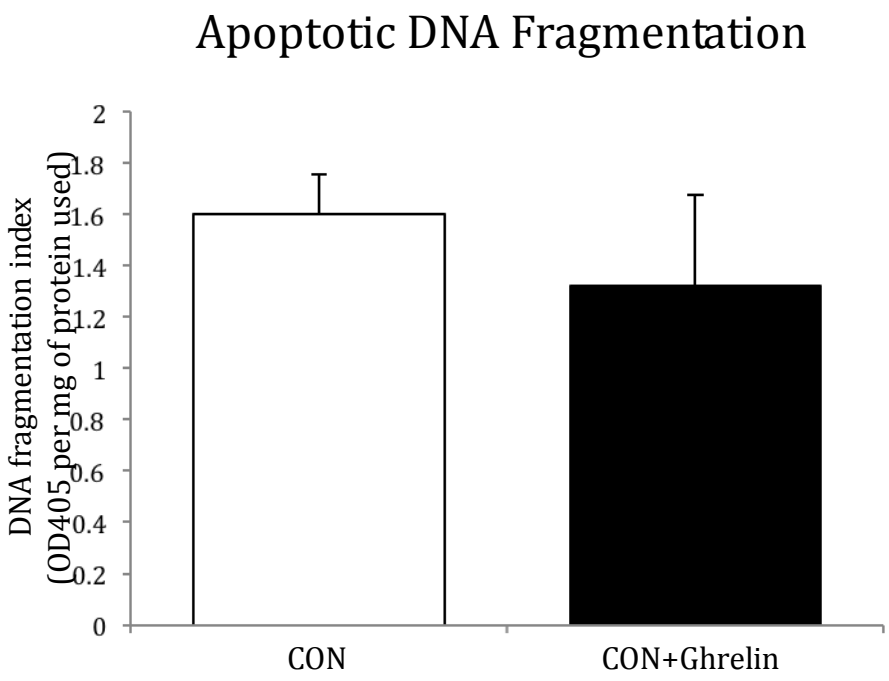

Figure 17. Apoptotic Analysis of Effects of Ghrelin in Normal Physiological Condition.

The effects of ghrelin on apoptosis in normal condition were determined. The extent of apoptotic DNA fragmentation was estimated by measuring the cytosolic mono- and oligonucleosomes with Cell Death ELISA. The optical density at 405 nm (OD405) was normalized to total milligrams of protein loaded in the assay. Data were presented as means ± SEM

4.3.2 Analysis of Autophagic Signaling

According to our Western blot analysis, the autophagic markers including the protein abundances of LC3 II/I ratio (Ratio of LC3 II protein abundance relative to LC3 I, CON V.S. CON+Ghrelin: 0.50±0.11 V.S. 0.69±0.23) (Figure 18), Atg 12-5 Complex (Ratio of protein abundance relative to internal control, CON V.S. CON+Ghrelin: 1.57±0.07 V.S. 1.53±0.11) (Figure 19), Atg 5 (Ratio of protein abundance relative to internal control, CON V.S. CON+Ghrelin: 0.85±0.06 V.S. 1.30±0.18) (Figure 20) and Becin-1 (Ratio of protein abundance relative to internal control, CON V.S. CON+Ghrelin: 1.47±0.32 V.S. 1.86±0.12) (Figure 21) in the skeletal muscle were not significantly different between the CON and CON+Ghrelin groups . This indicated that the levels of autophagy in skeletal muscle were not significantly different between these two groups.

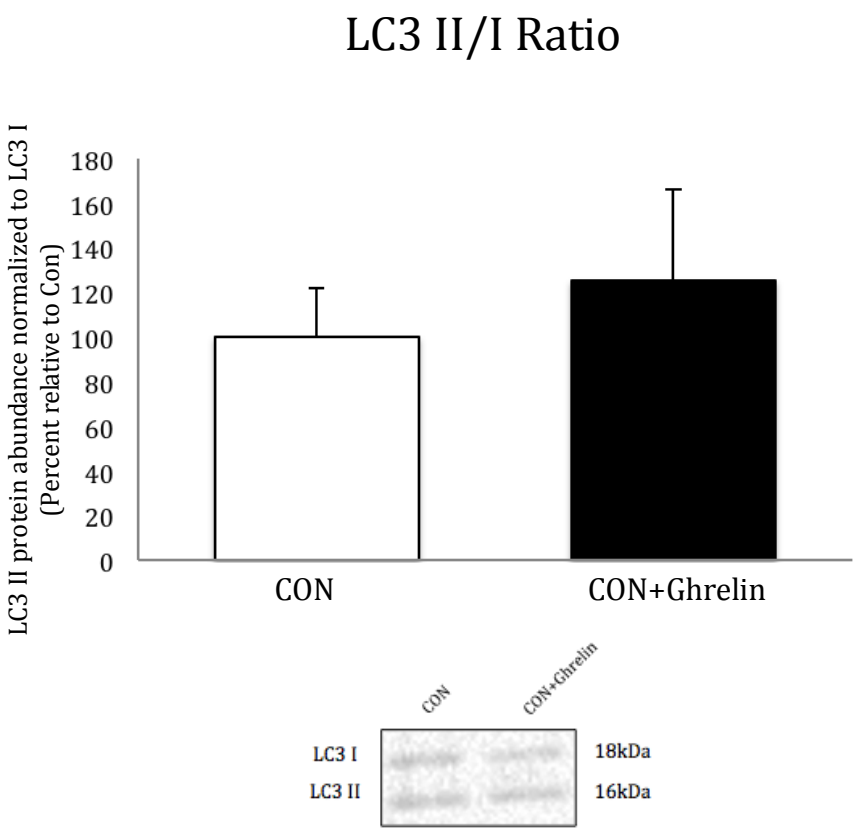

Figure 18. Autophagic Analysis of Effects of Ghrelin in Normal Condition - LC3 II/I ratio.

Autophagic marker - LC3 II/I ratio was determined by Western blot analysis. Data are presented as net intensity x resulting band area and expressed in percent relative to CON. Results of LC3 II were normalized to corresponding LC3 I signal. Data are expressed as means ± SEM with significant level set at $P<0.05$. The data were analyzed by independent t-test. (CON, saline control; CON+Ghrelin, saline control with treatment of ghrelin).

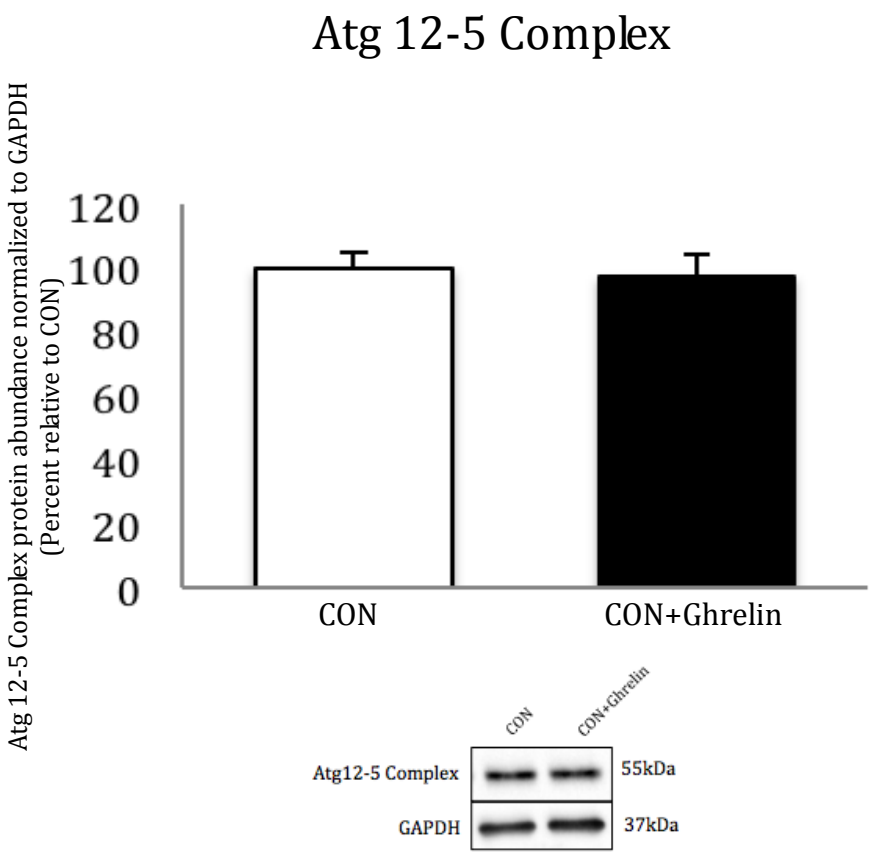

Figure 19. Autophagic Analysis of Effects of Ghrelin in Normal Condition - Protein abundance of Atg 12-5 Complex.

Autophagic marker - protein abundances of Atg 12-5 Complex were determined by Western blot analysis. Data are presented as net intensity x resulting band area and expressed in percent relative to CON. Results of Atg 12-5 Complex were normalized to corresponding GAPDH signal. Data are expressed as means ± SEM with significant level set at $P<0.05$. The data were analyzed by independent t-test. (CON, saline control; CON+Ghrelin, saline control with treatment of ghrelin).

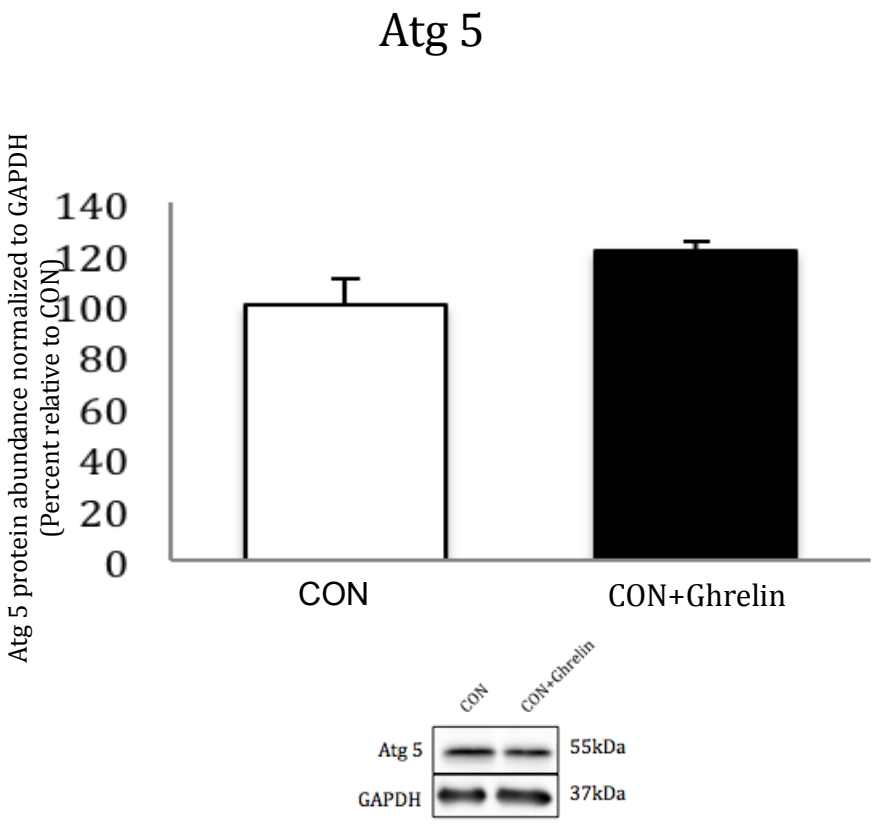

Figure 20. Autophagic Analysis of Effects of Ghrelin in Normal Condition - Protein abundance of Atg 5.

Autophagic marker - protein abundances of Atg 5 were determined by Western blot analysis. Data are presented as net intensity x resulting band area and expressed in percent relative to CON. Results of Atg 5 were normalized to corresponding GAPDH signal. Data are expressed as means ± SEM with significant level set at $P<0.05$. The data were analyzed by independent t-test. (CON, saline control; CON+Ghrelin, saline control with treatment of ghrelin).

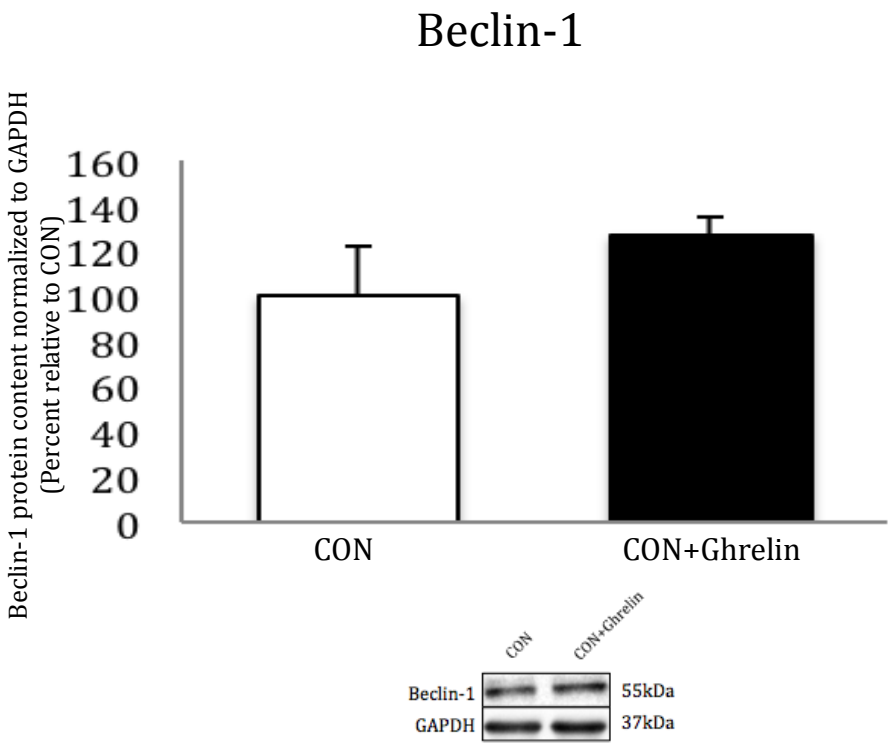

Figure 21. Autophagic Analysis of Effects of Ghrelin in Normal Condition - Protein abundance of Beclin-1.

Autophagic marker - protein abundances Beclin-1 were determined by Western blot analysis. Data are presented as net intensity x resulting band area and expressed in percent relative to CON. Results of Beclin-1 were normalized to corresponding GAPDH signal. Data are expressed as means ± SEM with significant level set at $P<0.05$. The data were analyzed by independent t-test. (CON, saline control; CON+Ghrelin, saline control with treatment of ghrelin).

4.4 Discussion

Chapter 4 aimed to examine the effects of ghrelin on autophagy and apoptosis in the skeletal muscle under normal physiological condition.

The Cell Death ELISA analysis indicated that the level of DNA fragmentation in the skeletall muscle was not affected upon ghrelin administration. These results are thus in congruence with a similar study performed in cardiomyocytes (Lear *et al.*, 2010). In 2010, Lear and colleagues have demonstrated that ghrelin treatment did not affect the basal level of apoptosis in cultured cardiomyocytes (Lear *et al.*, 2010). Results obtained in the cardiac muscle are often translatable to the skeletal muscle due to the similarities between cardiac and skeletal muscles (Richardson *et al.*, 1998).

Western blot analysis revealed that the levels of all autophagic markers including Atg 5, Atg 12-5 Complex, Beclin-1 and LC3 II/I ratio did not significantly change in response to ghrelin treatment in skeletal muscle. These results suggested that ghrelin did not affect the basal autophagic signaling under normal physiological condition.

It is known that the autophagic machinery is still activated at basal level in the skeletal muscle in order to maintain muscle health (Fanzani *et al.*, 2012). However, the modulating effects of ghrelin on autophagy in the skeletal muscle were rarely studied. In a recent study, ghrelin was shown to suppress apoptosis and autophagy in adipocytes, suggesting the inhibitory role of ghrelin in apoptosis and autophagy (Rodriguez *et al.*, 2012). However, we did not observe any modulating effects of ghrelin in apoptosis and autophagy in the skeletal muscle in our current experimental setting. This can be, at least in part, attributed to the difference in the tissue type used for analysis (i.e., adipocytes vs. myocytes). With reference to the extensive degeneration of myofibers and the subsequent loss of muscle mass in autophagy-deficient mice, it is revealed that a certain level of autophagy in the skeletal muscle is necessary to prevent muscle damages caused by daily contractile activities (Sandri, 2010;Masiero & Sandri, 2010).

CHAPTER 5

Acute Response of Autophagy in Skeletal Muscle Following Doxorubicin Administration

5.1 Introduction

According to the results of Chapter 3 in this MPhil thesis, the level of autophagy in the skeletal muscle was not affected 5 days after doxorubicin. It is noted that these findings are not quite consistent with the data reported by Smuder and co-workers in 2011, in which it has been demonstrated that autophagy is upregulated 24 hours following doxorubicin administration as indicated by the increase in protein abundances of the beclin-1, Atg 7, Atg 12, Atg 12-5 Complex and LC3II/I ratio (Smuder *et al.*, 2011a). We suspected that these contradictory results may be due to the difference of dosage of doxorubicin (20 mg/kg vs. 15 mg/kg) and experimental period (5 days vs. 24 hours) between studies. Therefore, this sub-experiment was conducted with the aim to confirm the findings reported by Smuder and co-workers in our present experimental setting. We would like to replicate the findings of Smuder et al. that autophagy is activated acutely (i.e., 24 hours) after the administration of doxorubicin (Smuder *et al.*, 2011a) in our experimental setting.

5.2 Methods

5.2.1 Animals

Eight to twelve-week old C57BL/6 wild type mice were employed in this study. Animals were maintained on a 12:12-h light-dark cycle. Food and water were provided *ad libitum* throughout the experimental period. Animal ethics approval was obtained from the Animal Ethics Sub-committee of The Hong Kong Polytechnic University.

5.2.2 Experimental Design

This experiment aimed to examine the autophagic response in a shorter timeframe after the administration of doxorubicin (i.e., 24 hours instead of 5 days). Mice were randomly assigned to Saline control group (CON; n=4) and Doxorubicin group (DOX24h; n=4). Mice in the DOX24h group received the administration of doxorubicin (15 mg/kg body weight by intraperitoneal injection) (Hydock *et al.*, 2011) whereas their counterpart in the CON group received the same volume of saline as a placebo. All mice were sacrificed 24 hours after injection. Gastrocnemius muscles were collected and frozen quickly in liquid nitrogen. Tissues were stored at -80^{o}C until further use.

5.2.3 Cytoplasmic and Nuclear Fraction Protein Preparation

Muscle proteins were prepared in accordance with the published protocol of Teng and co-workers (Teng *et al.*, 2011). Tissues were minced and homogenized on ice in the ice-cooled Rothermal's lysis buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 20 mM HEPES, 1 mM DTT, 20% glycerol, 0.1% Triton X-100, pH7.4). Homogenate was centrifuged at 3000 rpm (805 x g) to pellet the nuclei and cell debris. Cytoplasmic proteins were prepared by subjecting the supernatants to centrifugation at 6000 rpm (3220 x g) to remove nuclei residues and were stored regarded as a cytoplasmic protein fraction. A portion of the cytoplasmic fraction was stored at -80^{o}C directly for activity assays while protease inhibitor cocktail (P8340; Sigma Aldrich) was added to the remaining portion to prevent protein degradation, thereby allowing the conduction of western blot and Cell Death ELISA.

Pellets were washed with Rothermal's lysis buffer and centrifuged at 3000 rpm (805 x g) three times to remove any remaining cytoplasmic proteins. Pellets were rotated at 4^{o}C for 1 hour after adding 300 µl Rothermal's lysis buffer, 41.5 µl NaCl and 5 µl protease inhibitor for releasing the nuclear proteins. The mixture was

then centrifuged at 15000 rpm (17530 x g). Supernatants were collected as the nuclear fraction of muscle proteins.

5.2.4 Western Blotting

The protein expression levels of the markers of autophagy (Beclin-1, Atg 7,Atg 5, Atg 12-5 Complex, LC3) were measured by using proteins of cytoplasmic fraction. Protein extracts were boiled at 95^{o}C with 5% beta-mercaptoethanol for 15 min. Equal amounts of protein were loaded on 10% (Beclin-1, Atg 7, Atg 5, Atg 12-5 Complex) or 12% (LC3) polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose membranes (Beclin-1, Atg 5, Atg 12-5 Complex) or polyvinylidene difluoride membranes (LC3) by electroblotting. After the transfer, the membranes were blocked with 10 ml of 5% non-fat milk for 1 hour at room temperature, followed by overnight incubation with the corresponding primary antibodies at 4^{o}C (Table 4). Membranes were then washed in TBST for three times and incubated with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 1 hour (1:4000 dilution, 7076 for antimouse IgG antibody, 7074 for anti-rabbit IgG antibody; Cell

Signaling Technology). Membranes were washed again for three times with TBST. Luminol reagent (NEL103001EA; Perkin Elmer, Waltham, MA, USA) was then applied to the membrane for chemiluminescent detection of horseradish peroxidase activity. The chemiluminescent signal was detected using a Chemidoc^{tm} MP imaging system, BIORAD. The resulting bands were quantified by Imagej as an optical density X band area. GAPDH was employed as an internal control for all measurements. Data were expressed by normalizing to the corresponding internal control and expressed as percentage change relative to CON group.

5.2.5 Statistical Analysis

Statistical analysis was performed by using the SPSS 20.0 software package (IBM). The normality of data distribution was assessed by using the Kolmogorov-Smirnov test. Independent t-test was employed to detect the significant difference between the CON group and DOX24h group. A P-value of < 0.05 was considered as statistical significantly different. All data were expressed as mean ± standard error of mean.

Table 4. Primary Antibodies Employed in Western Blot Analysis of Chapter 5

Antibody	**Dilution Factor**	**Source**
Anti-Beclin-1 rabbit polyclonal	1:1000	3738, Cell Signaling Technology
Anti-Atg5 rabbit polyclonal	1:1000	8540, Cell Signaling Technology
Anti-Atg12 rabbit polyclonal	1:1000	2011, Cell Signaling Technology
Anti-Atg7 rabbit polyclonal	1:1000	2631, Cell Signaling Technology
Anti-LC3 rabbit monoclonal	1:1000	3868, Cell Signaling Technology

5.3 Results

5.3.1 Analysis of Autophagic Signaling

The LC3 II/I ratio in the skeletal muscle was found to be significantly increased by 243% in the DOX24h group when compared to CON group (P=0.03) (Ratio of LC3 II protein abundance relative to LC3 I, CON V.S. DOX24h: 0.47±0.12 V.S. 1.62±0.40) (Figure 22). No significant changes were observed in the protein abundances of Atg7 (Ratio of protein abundance relative to internal control, CON V.S. DOX24h: 0.35±0.03 V.S. 0.43±0.05) (Figure 23), Atg12-5 Complex (Ratio of protein abundance relative to internal control, CON V.S. DOX24h: 0.83±0.04 V.S. 0.91±0.07) (Figure 24), Atg5 (Ratio of protein abundance relative to internal control, CON V.S. DOX24h: 1.01±0.10 V.S. 0.93±0.07) (Figure 25) and Beclin-1 (Ratio of protein abundance relative to internal control, CON V.S. DOX24h: 0.89±0.13 V.S. 0.83±0.05) (Figure 26) in the skeletal muscles between CON and DOX24h groups .

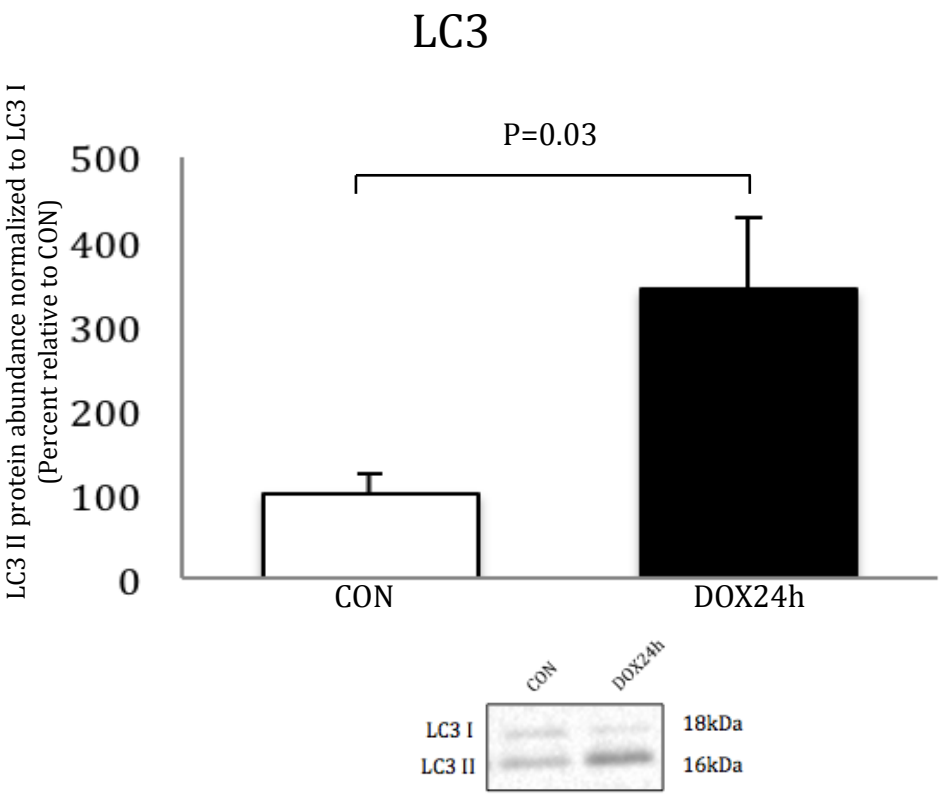

Figure 22. Acute Autophagic Response to Doxorubicin Administration-LC3 II/I Ratio.

The autophagic response to doxorubicin was examined 24 hours after the doxorubicin administration. Protein abundances of LC3 II and LC3 I were examined by Western blotting. Data are presented as net intensity x resulting band area and expressed in percent relative to CON. Results of LC3 II was normalized to corresponding LC3 I. Data are expressed as means ± SEM with significant level set at $P<0.05$. The data were analyzed by independent t-test.
(CON, saline control; DOX24h, 24 hours after the doxorubicin administration).

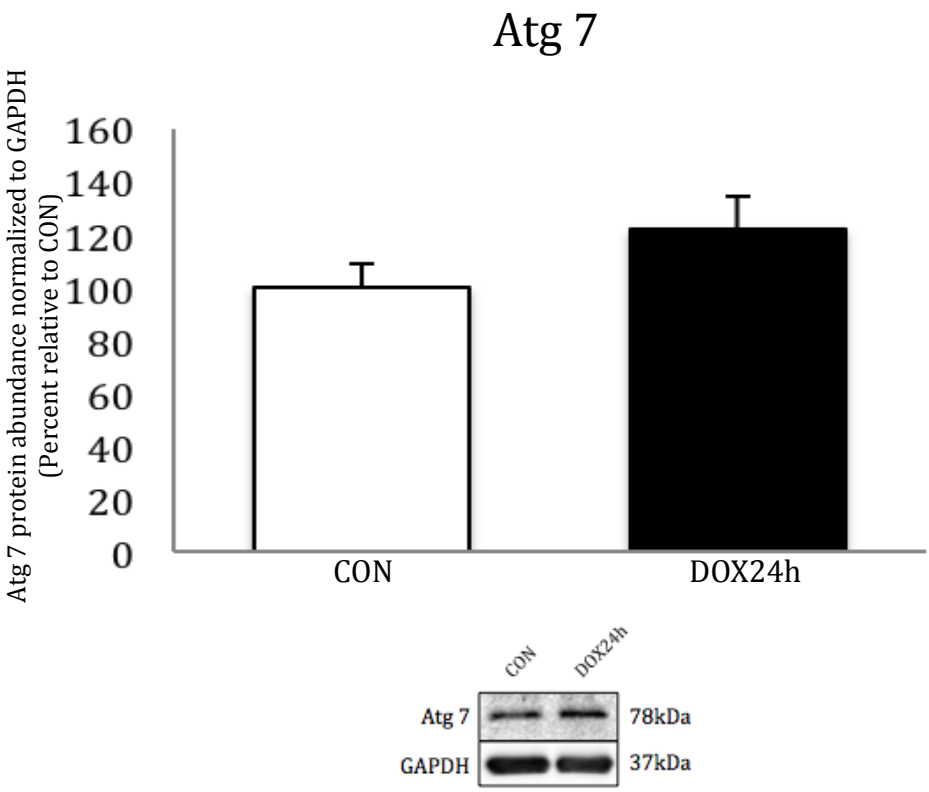

Figure 23. Acute Autophagic Response to Doxorubicin Administration-Protein Abundance of Atg7.

The autophagic response to doxorubicin was examined 24 hours after the doxorubicin administration. Autophagic marker-protein abundances of Atg7 were examined by Western blotting. Data are presented as net intensity x resulting band area and expressed in percent relative to CON. Results of Atg7 were normalized to corresponding GAPDH signal. Data are expressed as means ± SEM with significant level set at $P<0.05$. The data were analyzed by independent t-test. (CON, saline control; DOX24h, 24 hours after the doxorubicin administration).

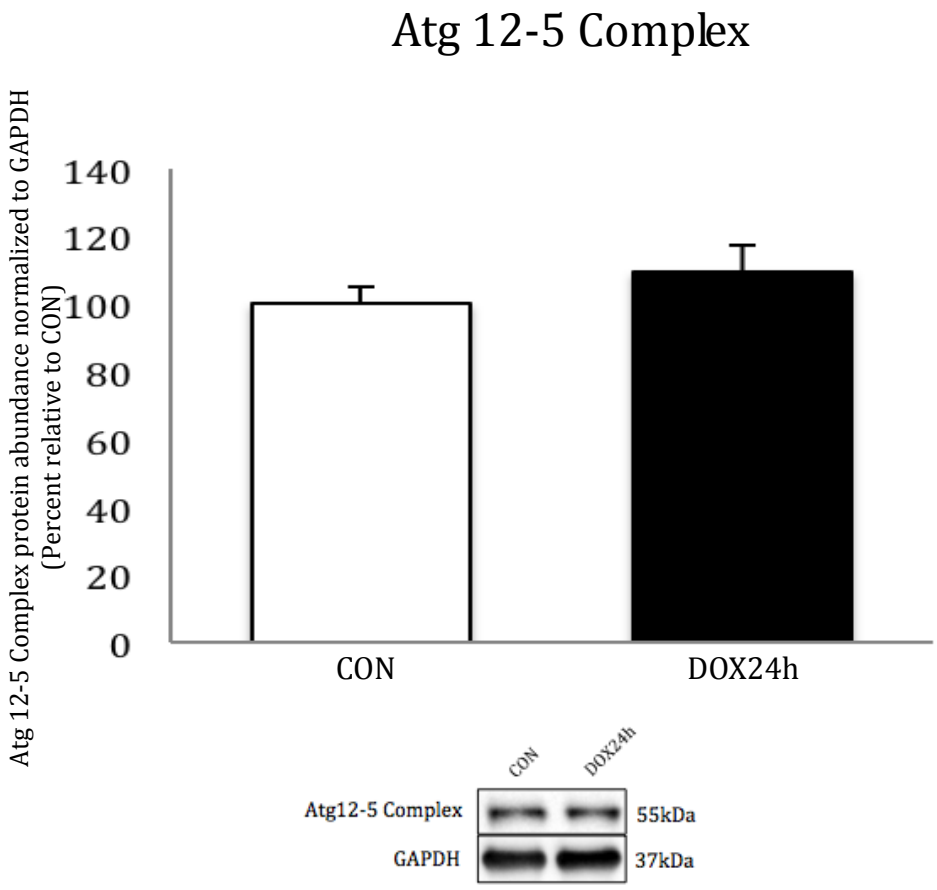

Figure 24. Acute Autophagic Response to Doxorubicin Administration-Protein Abundance of Atg12-5 Complex.

The autophagic response to doxorubicin was examined 24 hours after the doxorubicin administration. Autophagic marker-protein abundances of Atg12-5 Complex were examined by Western blotting. Data are presented as net intensity x resulting band area and expressed in percent relative to CON. Results of Atg12-5 Complex were normalized to corresponding GAPDH signal. Data are expressed as means ± SEM with significant level set at $P<0.05$. The data were analyzed by independent t-test.

(CON, saline control; DOX24h, 24 hours after the doxorubicin administration).

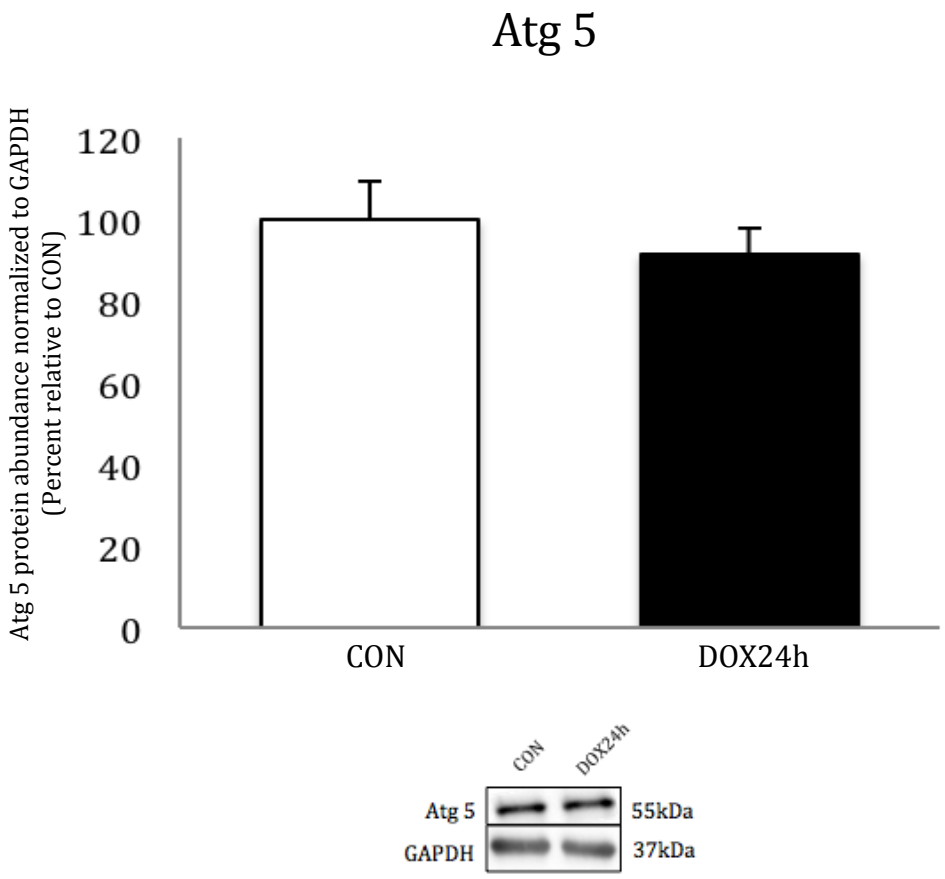

Figure 25. Acute Autophagic Response to Doxorubicin Administration-Protein Abundance of Atg5.

The autophagic response to doxorubicin was examined 24 hours after the doxorubicin administration. Autophagic marker-protein abundances of Atg5 were examined by Western blotting. Data are presented as net intensity x resulting band area and expressed in percent relative to CON. Results of Atg5 were normalized to corresponding GAPDH signal. Data are expressed as means ± SEM with significant level set at $P<0.05$. The data were analyzed by independent t-test. (CON, saline control; DOX24h, 24 hours after the doxorubicin administration).

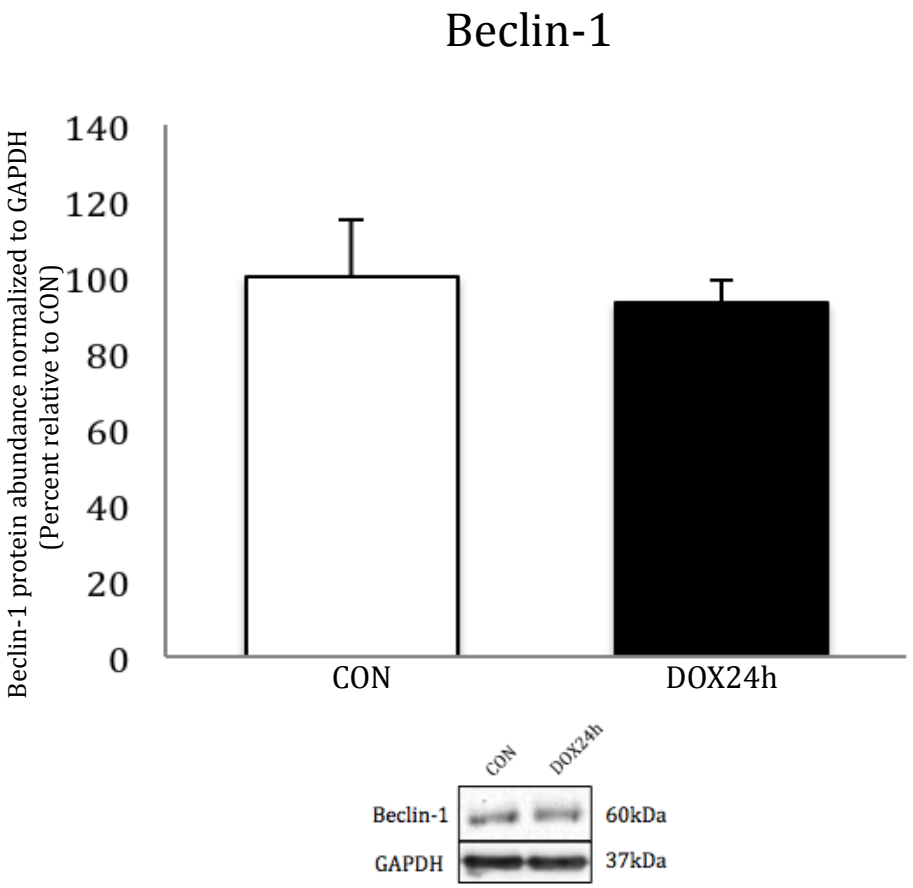

Figure 26. Acute Autophagic Response to Doxorubicin Administration-Protein Abundance of Beclin-1.

The autophagic response to doxorubicin was examined 24 hours after the doxorubicin administration. Autophagic marker-protein abundances of Beclin-1 were examined by Western blotting. Data are presented as net intensity x resulting band area and expressed in percent relative to CON. Results of Beclin-1 were normalized to corresponding GAPDH signal. Data are expressed as means ± SEM with significant level set at $P<0.05$. The data were analyzed by independent t-test.

(CON, saline control; DOX24h, 24 hours after the doxorubicin administration).

5.4 Discussion

In constrast to the results of Smuder and colleagues who demonstrated the upregulation of autophagy in doxorubicin-treated muscle, we observed, in Chapter 3, that the levels of all autophagic markers measured in the skeletal muscle were unaffected at 5-day after doxorubicin administration (Smuder *et al.*, 2011a). It was suspected that the inconsistent results were attributed to the variation of the experimental period (i.e., 5 days vs. 24 hours after the administration of doxorubicin). Therefore, a sub-experiment was conducted to address the autophagic response in the skeletal muscle 24 hours after doxorubicin administration (i.e., the experimental period adopted by Smuder and co-workers) in our experimental setting (Smuder *et al.*, 2011a). The results of this sub-experiment have demonstrated that the crucial autophagic signaling indicated by LC3 II/I ratio was elevated 24 hours after the exposure to doxorubicin. LC3 II/I ratio is a robust indicator of the formation of autophagosome which is directly proportional to the number of autophagosome formed and is commonly used as a marker of autophagy (Kabeya *et al.*, 2000). We interpreted that our results are generally in agreement with the data obtained by Smuder and colleagues (Smuder *et al.*, 2011a),

There were no changes observed in the protein abundances of Beclin-1, Atg5, Atg12-5 Complex, and Atg7 in the doxorubicin-treated muscle in our setting. These may be due to the following reasons: 1) the dosage of doxorubicin used in the present study was different from that of Smuder and co-workers. The higher dosage of doxorubicin (20mg/kg) employed in the study conducted by Smuder and co-workers might reasonably lead to a more intensive response when compared to the moderate dose (15mg/kg) used in this MPhil study. 2) The type of animal employed in smuder's study (rats) and this mphil study (mice) was different, hence different expression patterns of these autophagic markers were observed.

According to the findings reported in Chapter 3 and the present chapter (Chapter 5), we interpreted that the autophagic signaling was upregulated at 24 hours after the exposure of doxorubicin and it would return to the basal level 5 days after the doxorubicin exposure. We suspected that the acute activation of autophagy following doxorubicin administration might serve as the protective mechanism of muscle cells against doxorubicin-induced myotoxicity. The elevation

of autophagic signaling possibly enhanced the removal of damaged proteins and organelles produced by the doxorubicin-induced oxidative stress, and thereby alleviated the cellular damages and facilitated the process of cellular repairing. Our findings in Chapter 3 demonstrated that ghrelin protected the skeletal muscle from the doxorubicin-induced injury by upregulating autophagy. These results are supported by the previous findings demonstrating that the activation of autophagy was a possible mechanism to prevent the toxic chemical-induced cellular injury in H9C2 cardiac muscle cells (Tong *et al.*, 2012).

CHAPTER 6

Overall Discussions, Limitations and Future Prospectives

6.1 Overall Discussion

In this MPhil project, we have provided novel data demonstrating that ghrelin protects the skeletal muscle against doxorubicin-induced injury by suppressing protein degradation and apoptosis and enhancing autophagy. It was observed that the effects of ghrelin on the inhibition of apoptosis and activation of Akt signaling and autophagy were not abolished by the treatment of the GHSR-1a antagonist. These observations suggest that the protective effects of ghrelin are largely mediated through mechanisms other than the GHSR-1a pathway.

Interestingly, the suppressing effect of ghrelin on doxorubicin-induced catabolism of muscle proteins was abolished upon the application of GHSR-1a antagonist, suggesting that the inhibitory effect of ghrelin on protein degradation might be mediated through GHSR-1a signaling. It has been demonstrated that skeletal muscle protein degradation can be induced by inflammatory cytokines including TNFα (Bicer *et al.*, 2009). The anti-inflammatory effect of ghrelin was shown to be mediated through GHSR-1a (Waseem *et al.*, 2008). In our study, we speculated that the anti-inflammatory effects of ghrelin might have been diminished in the

presence of GHSR-1a antagonist, which made ghrelin failed to prevent specifically the doxorubicin-induced degradation of skeletal muscle proteins.

A growing number of studies demonstrated that des-n-octanoyl ghrelin also exerts similar protective effects to ghrelin in various cell types including cardiomyocytes, endothelial cells and skeletal muscle (Baldanzi *et al.*, 2002;Sheriff *et al.*, 2012). The application of ghrelin in GHSR-deficient cells (Baldanzi *et al.*, 2002) and mutant mice (Porporato *et al.*, 2013) has also been demonstrated to activate Akt signaling and to suppress ubiquitin-proteasome protein degradation pathway. These findings suggested that there might be unidentified common receptors for ghrelin and des-n-octanoyl ghrelin that are responsible for the effects of ghrelin in inhibiting apoptosis and muscle atrophy, activating Akt signaling and upregulating autophagy (Baldanzi *et al.*, 2002;Sheriff *et al.*, 2012;Porporato *et al.*, 2013;Tong *et al.*, 2012). Further investigation is needed to elucidate the precise molecular mechanisms of the protective effects of ghrelin.

The modulating effects of ghrelin on the basal levels of apoptosis and autophagy

varied among tissues. It has been demonstrated in adipose tissue that ghrelin intervention reduced the basal levels of apoptosis and autophagy whereas this finding was not reproducible in cardiomyocytes (Rodriguez *et al.*, 2012) and skeletal muscle. This may be due to the specific need for autophagy to remove harmful proteins in certain cell types, especially in those non-dividing cell types such as skeletal muscle (Fanzani *et al.*, 2012). In the present study, autophagy was found to be upregulated 24 hours after the administration of doxorubicin (as demonstrated by Smuder and colleagues) followed by its restoration to basal level at 5-day after the doxorubicin administration (Smuder *et al.*, 2011a). It was proposed by Smuder and co-workers that the doxorubicin-induced upregulation of autophagy might have contributed to the doxorubicin-induced damages in the skeletal muscle (Smuder *et al.*, 2011a). On the contrary, the findings of the present study appear to support that the upregulation of autophagy indeed protects the skeletal muscle from doxorubicin-induced toxicity rather than to exacerbate the damages in doxorubicin-treated muscles. Tong and co-workers have demonstrated that ghrelin protected cobalt chloride-induced injury in H9C2 cardiomyocytes by upregulating autophagy (Tong *et al.*, 2012). The upregulation of autophagy was

proposed as an acute mechanism to counteract the doxorubicin-induced toxicity. Further investigation is required to fully reveal the exact role of autophagy in doxorubicin-induced damages in the skeletal muscle.

6.2 Limitations and Future Prospectives

Cancer patients have different metabolic profiles from healthy individuals, particularly those with cachexia (Argiles *et al.*, 2008). Inflammation, wasting of muscle and adipose tissue, and failure in metabolic adaptation all occur in cancer cachexia patients (Argiles *et al.*, 2008). This study employed normal mice instead of mice with cancer for the investigation, which might not mimic exactly the situation of applying ghrelin to cancer patients due to the differences in metabolism and environment between normal mice and cancer mice. As muscle wasting and inflammation were reported in cancer patients with cachexia (Tisdale, 2009), exposure to doxorubicin might further aggravate the situation. Nonetheless, our study was the first to demonstrate the myoprotective effects of ghrelin against doxorubicin-induced myotoxicity in normal mice. This can be taken as the first step for exploring ghrelin as the potential myoprotective agent

against doxorubicin-induced myotoxicity and this merits the extension of ghrelin research to tumor-bearing animals or animals with cancer cachexia.

Apart from the protective effects of ghrelin against doxorubicin myotoxicity, it is not known whether ghrelin would affect the therapeutic effects of doxorubicin against cancer, although this is not likely to happen. In 2004, Neary and co-workers conducted clinical trials using ghrelin for treating impaired appetite in cancer patients caused by chemotherapy (Neary *et al.*, 2004). Indeed, no side effects were reported in the cancer patients in this clinical study. Nevertheless, further study is needed to determine whether long term treatment of ghrelin along with doxorubicin chemotherapy would affect the chemotherapeutic performance of doxorubicin.

CHAPTER 7

Conclusions

In the present study, the doxorubicin-induced damage in skeletal muscle was exhibited by the observed increase in the percentage of centronucleated fibers and muscle protein degradation in the doxorubicin-treated murine muscle. This damage is accompanied with the inhibition of Akt signaling pathway and the activation of apoptosis in skeletal muscle. Our data further demonstrated that ghrelin treatment protected the doxorubicin-treated muscle from apoptosis and muscle protein degradation. Ghrelin also enhanced the autophagic signaling in the doxorubicin-treated muscle. The application of GHSR-1a antagonist was not found to affect the activation of Akt signaling, inhibition of apoptosis, and induction of autophagy. These findings suggested that the cellular mechanisms that mediated the protective effects of ghrelin were independent of GHSR-1a signaling pathway. Additionally, we did not observe any modulating effects of ghrelin on apoptosis and autophagy under normal physiological condition. Our data also demonstrated that the upregulation of autophagy at 24 hours after the administration of doxorubicin would return to basal level at 5 days after the doxorubicin administration.

The results from this MPhil project demonstrated that ghrelin intervention could protect skeletal muscle from doxorubicin-induced muscle damage. Ghrelin has been suggested as a potential therapeutic agent for treating cancer cachexia by inducing food intake and reducing muscle loss. Chemotherapeutics, such as doxorubicin, may predispose the muscle to exercise intolerance, fatigue, and muscle weakness by exacerbating the loss of muscle mass and function especially in cancer patients with cachexia, hence affecting their quality of life and limiting their cancer treatment. In addition to the beneficial effects of ghrelin on cancer cachexia, our present findings suggested that ghrelin also protects skeletal muscle against doxorubicin-induced damages by inducing autophagy and inhibiting apoptosis. These findings imply that ghrelin might be able to preserve the muscle mass and function of cancer patients during chemotherapy. These novel findings are valuable for exploring the novel effective therapies to treat the toxicity induced by anti-cancer drug doxorubicin.

CHAPTER 8

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