Investigation of mechanisms responsible for myocyte cell death in metabolic syndrome

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ABSTRACT

The aim of this study is to investigate the mechanism involved in cardiomyocyte and skeletal muscle cell death in metabolic syndrome. Specific metabolic syndrome conditions focused on in this research are iron overload and hyperglycemia. Both in skeletal muscle cells and cardiomyocytes, cell death occurred via intrinsic pathway of apoptosis in response to these stimuli. Apoptosis in these cells can lead to further problems such as sarcopenia and heart failure. We tested the hypothesis that the mechanism responsible for apoptosis was through inhibition of autophagy. Under both iron overload and hyperglycemia conditions, I noted that autophagosome formation was significantly increased. Furthermore, when autophagy was defective, activation of apoptosis was more sensitive to the applied stimuli, or defect in autophagy maximized the event under basal conditions. Extending out from this, in cardiomyocytes hyperglycemia-induced cell death was alleviated by adiponectin, and less autophagosome formation occurred. Thus adiponectin, which is considered to be anti-apoptotic adipokine, acted to alleviate hyperglycemia-induced apoptosis. Detailed understanding of the mechanisms involved in cell death can uncover new potential therapeutic targets for treating complications of metabolic syndrome.

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TABLE OF CONTENTS

ABSTRACT……ii

ACKNOWLEDGEMENTS……iii

TABLE OF CONTENTS ……iv

LIST OF TABLE ……vii

LIST OF FIGURES ……viii

LIST OF ABBREVIATION……ix

Chapter One: Introduction ……1

1.1 Diabetes and associated complication……1

1.1.1 Diabetes prevalence……1

1.1.2 Metabolic syndrome as predictor of cardiovascular disease (CVD).3

1.1.3 Heart failure in diabetes……3

1.2 Apoptosis……4

1.3 Autophagy……7

1.4 Iron metabolism/Iron overload……9

1.4.1 Iron metabolism……9

1.4.2 Iron overload and diabetes……10

1.4.3 Effect of iron overload on cellular mechanisms……11

1.4.3.1 Iron and oxidative stress. ……11

1.4.3.2 Iron and autophagy……11

1.4.3.3 Iron and apoptosis……12

1.4.4 The role of oxidative stress on apoptosis.....13

1.5 Adiponectin.....13

1.5.1 Adiponectin structure.....13

1.5.2 Associations between adiponectin and heart failure.....14

1.5.3 Cardioprotective effects of adiponectin.....14

1.6 Effect of hyperglycemia on cellular mechanisms.....15

1.6.1 Hyperglycemia and autophagy.....15

1.6.2 Hyperglycemia and apoptosis.....15

1.7 Hypothesis and research aims.....16

Chapter Two: Iron-overload inducing cell death via inhibition of autophagic flux and/or by promotion of oxidative stress in L6 skeletal muscle cells

2.1 Preface.....17

2.2 Introduction.....18

2.3 Materials and Methods.....20

2.4 Results.....24

2.5 Discussion and Future Plan.....32

Chapter Three: Adiponectin alleviating hyperglycemia-induced cell death via improving autophagic pathway in cardiomyocyte

3.1 Preface.....35

3.2 Introduction.....36

3.3 Materials and Methods.....37

3.4 Results.....41

3.5 Discussion and Future Plan.....47

Chapter Four: General Conclusions and Future Direction 49

4.1 Summary.....49

4.2 Future Direction.....52

Chapter Five: Reference.....54

Appendix A: Copyright Permission.....60

Appendix B: List of Contributions.....65

LIST OF TABLE

Chapter One: Introduction

Table 1.1 Diabetes statistics in Canada……..……..2

LIST OF FIGURES

Chapter One: Introduction

Figure 1.1: Estimated diabetes prevalence in 2015 and predicted number in 2040 worldwide ……2

Figure 1.2: Different modes of activating apoptosis- extrinsic and intrinsic pathways ……6

Figure 1.3: Autophagy pathway……8

Figure 1.4: Autophagic Flux……9

Chapter Two: Iron-overload inducing cell death via inhibition of autophagic flux and/or by promotion of oxidative stress in L6 skeletal muscle cells

Figure 2.1: Iron overload inducing apoptosis in L6 cells……25

Figure 2.2: Iron overload inducing autophagosome accumulation……27

Figure 2.3: Reduction of iron overload-induced apoptosis with autophagy inhibitor……28

Figure 2.4: Reduced apoptosis activity in autophagy deficient cell line……30

Figure 2.5: Iron overload inducing ROS production in L6 cells……31

Chapter Three: Adiponectin alleviating hyperglycemia-induced cell death via improving autophagic pathway in cardiomyocyte

Figure 3.1: Adiponectin treatment attenuated high glucose-induced apoptosis in H9c2 cells……42

Figure 3.2: High glucose inducing intrinsic apoptotic pathway……43

Figure 3.3: High glucose disturbing autophagy flux……44

Figure 3.4: Increased apoptosis in autophagy deficient cells……45

LIST OF ABBREVIATION

AMEM	Alpha modification of Eagle's Medium
AMP	Adenosine Monophosphate
AMPK	Adenosine Monophosphate –activated protein kinase
Apaf-1	Apoptotic protease activating factor 1
ASP	Acylation-stimulating protein
Atg	Autophagy related genes
ATP	Adenosine triphosphate
BAK	BCL-2 antagonist or killer
BAX	BCL-2 associated X protein
BCL-2	B cell lymphoma 2
BH3	BCL-2 homology 3
BMI	Body Mass Index
BSA	Bovine Serum Albumin
CAT	Cationic amphiphilic tracer
CHD	Coronary heart disease
CVD	Cardiovascular disease
Cyt c	Cytochrome C
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
EV	Empty vector
fAd	Full length adiponectin
FADD	Fas-associated protein with death domain
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HDL	High-density lipoprotein
HF	Heart failure
HG	High glucose
HMW	High molecular weight
HRP	Horseradish peroxidase
Hsc70	Heat shock protein 70
IRE	Iron responsive element

IRP	Iron regulatory protein
KO	Knockout
LC3	Light chain 3
LMW	Low molecular weight
MMW	Middle molecular weight
MnTBAP	Manganese (III) tetrakis (4-benzoic acid) porphyrin
MOMP	Mitochondrial outer membrane permeabilization
mTORC	Mammalian target of rapamycin complex
NAC	N-acetyl cysteine
NCOA4	Nuclear receptor co-activator 4
NG	Normal glucose
PBS	Phosphate-buffered saline
PI	Propidium Iodide
PI3K	Phosphatidylinositol-3-kinase
PS	Phophotidyl serine
PVDF	Polyvinylidene fluoride
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
Ser	Serine
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

Chapter One: Introduction

1.1 Diabetes and associated complications

1.1.1 Diabetes Prevalence

Nowadays people are aware that the incidence of diabetes is increasing. Diabetes mellitus is a global metabolic disorder characterized by high blood glucose levels, insensitivity to insulin, and/or lack of insulin production [1]. Two major types of diabetes are Type 1 (T1D) and Type 2 (T2D). T1D occurs due to the destruction of pancreatic beta cells caused by malfunctioning of the immune system. As a result, insulin secretion is significantly reduced or absent. On the other hand, T2D is commonly developed later in life, and beta cells continue to produce insulin but the production is either not enough or insufficient to compensate for the body's insensitivity to insulin. This condition is referred to as insulin resistance. In the United States, about 9% of population had diabetes in 1994, and this number increased to 14% in 2012. [2]. Diabetes is the major cause of illness and death in the United States, and it causes a burden on the health care system [2]. However, this is not only restricted to the USA. Increasing prevalence is also observed in Canada, according to Diabetes Canada (**Table 1.1**), and even worldwide (**Figure 1.1**).

It is well known that diabetes is a major risk factor for cardiovascular diseases (CVD). Recent studies showed that adults with diabetes have up to 4 times greater risk of dying from heart disease compared to those without diabetes [3]. Diabetic individuals may exhibit cardiac dysfunction in the absence of hypertension or coronary artery disease, a condition known as diabetic cardiomyopathy [4]. Diabetic patients or animals have hyperglycemia (elevated blood glucose levels), due to impaired insulin activity and glucose catabolism. Progression of diabetes can cause numerous complications not only including CVD, but also damage to different organs such as the kidneys, eyes, and nerves.

Table 1.1: Diabetes statistics in Canada Estimation of prevalence in 2015 and prediction for 2025. Copied from Diabetes Canada (http://www.diabetes.ca/how-you-can-help/advocate/why-federal-leadership-is-essential/diabetes-statistics-in-canada). Copyright permission attached on page 60.

Key Statistics[1]	2015	2025
Estimated diabetes prevalence (n/%)	3.4 million/9.3%	5 million/12.1%
Estimated prediabetes prevalence in Canada (n/%) (age 20+)	5.7 million/22.1%	6.4 million/23.2%
Estimated diabetes prevalence increase (%)	44% from 2015-2025	
Estimated diabetes cost increase (%)	25% from 2015-2025	

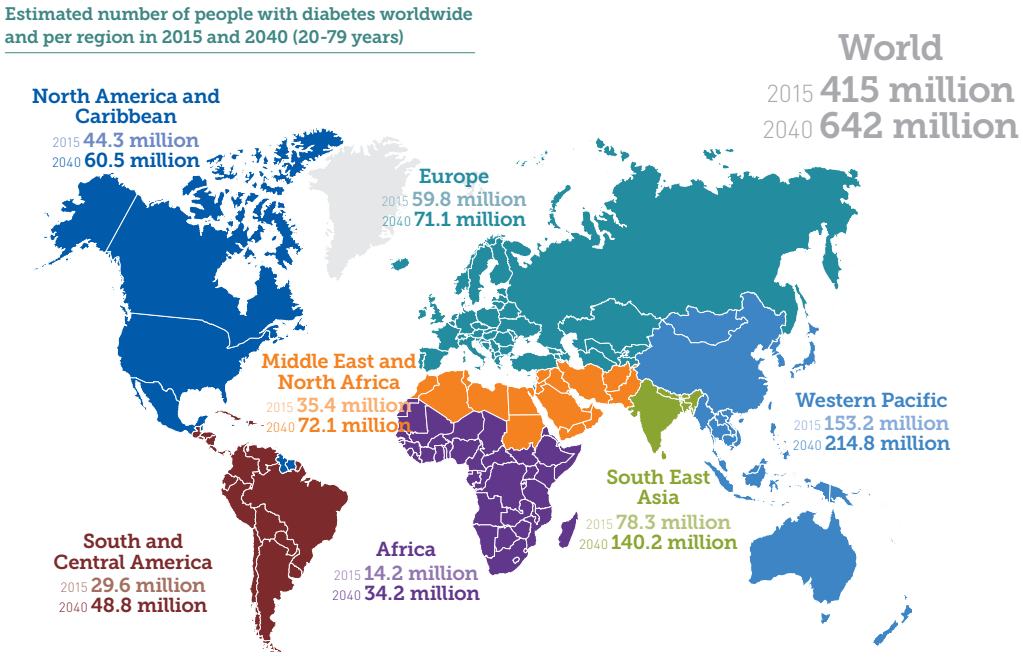


Figure 1.1: Estimated diabetes prevalence in 2015 and predicted number in 2040 worldwide. Source from International Diabetes Federation. (http://www.diabetesatlas.org/resources/2015-atlas.html) Copyright permission attached on page 61.

1.1.2 Metabolic Syndrome as predictor of cardiovascular disease (CVD)

There are many risk factors associated with the development of CVD, and some of them can be grouped together under the term metabolic syndrome. Dyslipidemia, hypertension, hyperglycemia, low HDL cholesterol level are examples that fall into this category [5]. Metabolic syndrome can be a predictor of diabetes, and also a predictor of CVD. People who have metabolic syndrome have a high occurrence of new-onset diabetes. Furthermore, diabetes itself can predict CVD development. The Framingham Heart Study showed that people with diabetes had an almost 20% chance of developing Coronary Heart Disease (CHD) within ten years of its onset. Metabolic syndrome can be treated by modification of lifestyle, such as increased physical activity and change in diet [6]; however, this might not be the case for everyone. Therefore, understanding metabolic syndrome at the molecular level is also required.

1.1.3 Heart Failure in diabetes

There are number of events that can be defined as CVD events, such as cardiovascular death, myocardial infarction, ischemic stroke, revascularization, and acute coronary syndromes [7]. Heart failure (HF), a condition associated with a damaged or weakened heart, is caused by these CVDs, which will further be able to cause losing significant number of myocardial cells [8]. Functionally, HF occurs when there is not enough pressure in the heart to pump blood effectively through the circulation, or the heart muscle cannot relax properly to accommodate blood flow in and out of heart [8]. Impaired contraction and relaxation of cardiomyocyte potentially result in heart failure, which is closely related with Ca^{2+} homeostasis in cardiac muscle cells. When cardiomyocytes lose function to properly contract and relax, it would hinder

blood to be filled in ventricle, which is known as ventricle diastole [9]. And HF is resulted to compensate for abnormal cardiac output, such as increased heart rate, where the cardiac output is determined by the product of heart rate (HR) and stroke volume (SV) [8]. Studies have shown that heart failure is partly, if not all, due to loss of cardiomyocytes. Unlike other cells, cardiac muscle cells have limited ability to be self-renewed once damaged [10]. Recently, there is increasing evidence showing that apoptosis is the key modulator in heart failure [11]. As mentioned previously, it was known that people with diabetes have higher risk to develop CVD. The exact mechanisms T2D causing HF is multifaceted and need to be further investigated [12]. HF is a very serious disease, and at present there are not many therapeutic options available, highlighting the need to prevent HF in advance.

1.2 Apoptosis

Apoptosis, also known as programmed cell death, is a highly conserved process with roles in development, maintenance of homeostasis, and disease. Apoptosis is involved in cell turnover in adult tissues as well as in embryonic development. Kerr and colleagues first used the terminology in 1972. They discovered that there was a previously undescribed process that reverses mitosis. They observed a two-stage process during which nuclear and cytoplasmic condensation occurred, followed by breakage into fragments. Then these fragments were to taken up by phagosomes [13].

Apoptosis can be broadly grouped into two pathways depending on the triggering signals– intrinsic and extrinsic (**Figure 1.2**). A third pathway was also discovered which involves T-cell mediated cytotoxicity. While the signalling cascades these pathways use are different, they merge at the same endpoint, activation of caspase-3 by cleavage [14].

As the name indicates, the intrinsic pathway is normally initiated by internal signals, such as damaged DNA, chromosome rearrangement, and hypoxic conditions. These signals cause damage to the mitochondria. When this occurs, their membrane potential is disturbed, a event called mitochondrial outer membrane permeabilization (MOMP), through activation of B cell lymphoma (BCL-2) homology 3 (BH3) -only protein [15]. Cytochrome c is then released from the mitochondria into cytosol and binds to Apaf-1 (apoptotic protease activating factor-1) proteins. This binding changes its conformation and recruits procaspase-9 to form the apoptosome complex in the presence of ATP. Procaspase-9 will cleave itself and further it will cleave procaspase-3 to potentiate activation [16].

On the other hand, extrinsic pathway of apoptosis is activated by signals come through death receptors, tumor necrosis factor (TNF) receptor superfamily. Stimulating these receptors such as TNF-related apoptosis-inducing ligand (TRAIL) and FAS, will recruit Fas-associated death domain (FADD) and caspase-8 and this will further cleave procaspase-3, an effector caspase protein [17, 18].

However, intrinsic and extrinsic pathways are not mutually exclusive. Even though the signal stimulated by binding to death receptors, it can also cross to intrinsic pathway by activating Bcl-2 associated X protein (BAX) and Bcl-2 antagonist or killer (BAK) to cause MOMP [17].

Apoptosis in myocyte can potentially result in serious diseases such as HF and sarcopenia. HF is characterized by an increased apoptotic rate of cardiomyocytes, a change which is known to play an important role in the progression of the disease [19]. Apoptosis has emerged as a potential therapeutic target for treating HF, because unlike necrosis, apoptosis can be regulated and by intervening earlier step, it can be prevented

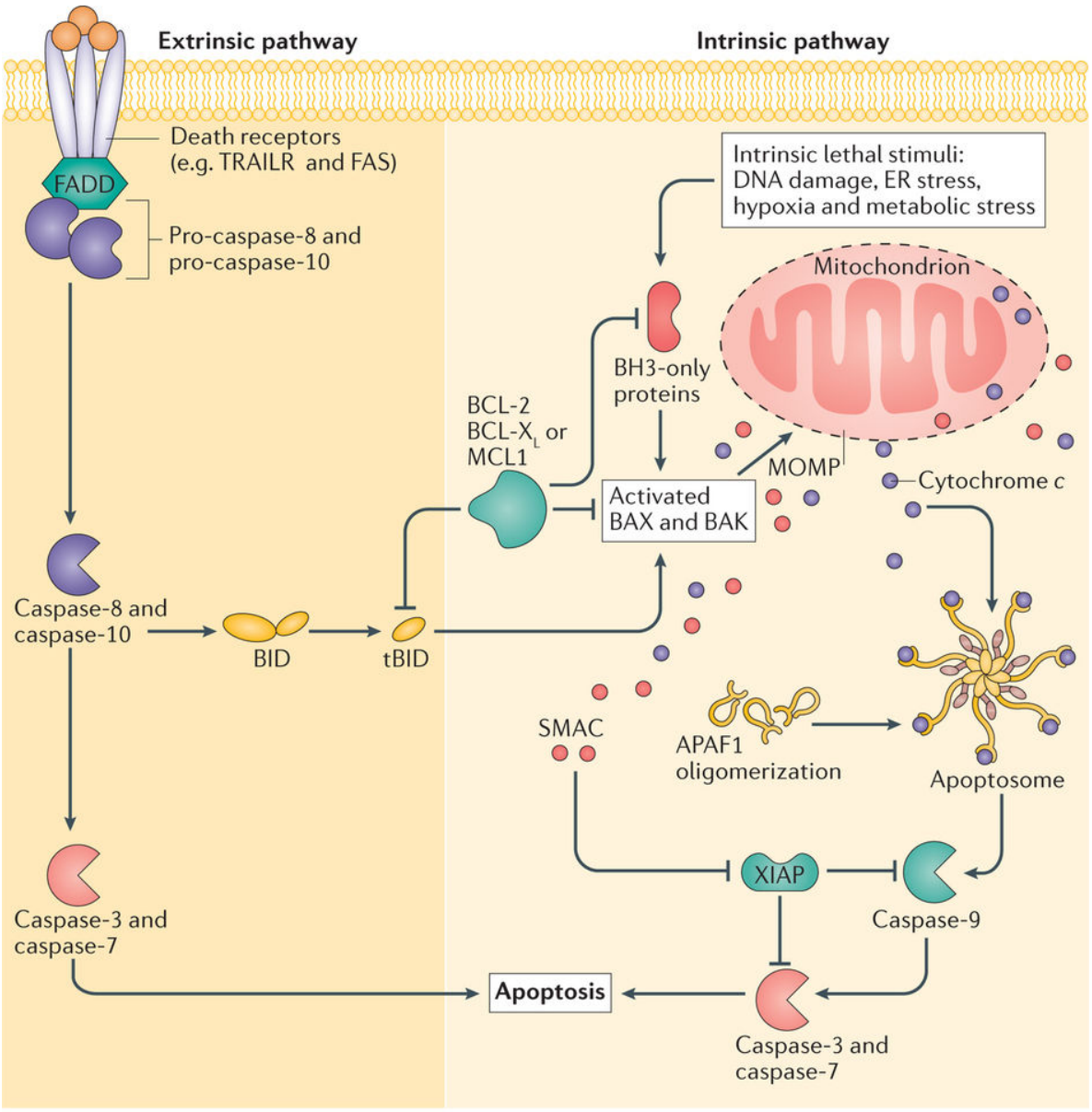


Figure 1.2: Different modes of activating apoptosis- extrinsic and intrinsic pathways. Extrinsic pathway begins with signals that activate through death receptors (TRAIL and FAS). FADD and procaspase-8 would be recruited and further activate effector proteins, casapse-3 and caspase-7. Intrinsic pathway is activated by intrinsic lethal signals, which will activate BH-3-only proteins to activate Bax and Bak and cause MOMP. Disturbance in mitochondrial membrane will release cytochrome c to cytosol, which can bind to Apaf1 to form apoptosome. Apoptosome will cause self-cleavage of caspase-9 and will activate caspase-3 and casapse-7. Source from "A fate worse than death: apoptosis as an oncogenic process" [20]. Copyright permission attached on page 62.

[11]. Since cardiomyocytes do not proliferate, and even a small reduction in the number of cardiomyocytes is detrimental to cardiac function, prevention is very crucial. In terms of skeletal muscle cells, sarcopenia can occur possibly due to loss of muscle nuclei through apoptosis. This will affect the quality of muscle, which can further change muscle metabolism as well [21].

1.3 Autophagy

Autophagy is the process that eukaryotes have adapted in order to maintain homeostasis at the cellular level [22, 23]. This process is involved in the turnover of cellular components, especially under the stress. This stress can be defined as changes in nutrient availability, oxygen levels, or energy status [24]. Autophagy can be grouped into 3 categories: microautophagy, macroautophagy, and chaperone-mediated autophagy. Microautophagy, studied mainly in yeast, is the process in which lysosomes or vacuoles engulf cytosol or organelles by invagination. Chaperone-mediated autophagy occurs only in mammalian cells and requires the presence of a specific sequence on the substrate, and a heat shock chaperone (HSC70) to carry out the process. Macroautophagy is the most well-known pathway, which can be further sub-classified into 2 categories: non-selective autophagy, a process to adapt to starvation, and selective autophagy, which maintains homeostasis of cytosolic proteins and organelles. In macroautophagy (hereafter referred to as autophagy), a double membrane autophagosome is formed and fuses with lysosomes to be degraded [25].

There are many proteins involved in autophagy (**Figure 1.3**), called Atg (autophagy related genes) proteins. The induction of autophagy is controlled by a multi-protein complex- Atg1/ULK1 (unc-51-like kinase-1), FIP200 (FAK family kinase-interacting protein of 200kDa), Atg13, and Atg101. This complex is activated when mammalian target of

rapamycin (mTOR) is inactivated [26, 27]. There are two phosphorylation sites that are closely related to autophagy, Ser757 and Ser555. The first is known to be phosphorylated by mammalian target of rapamycin complex (mTORC), and the latter is phosphorylated by AMP-activated protein kinase (AMPK). The process progresses with the formation of a phagophore by forming a complex with Beclin1/Atg6 and vascular protein sorting (VPS) 34 and VPS 15, which will further activate the class III PI3K complex. Then, elongation of the phagophore occurs, involving Atg12-Atg5 and Atg8/LC3 (microtubule-associated protein 1 light chain 3) [28] [29]. Lastly, the autophagosme will fuse with the lysosome to form an autophagolysosome or autolysosome, to be further degraded by lysosomal enzymes [30].

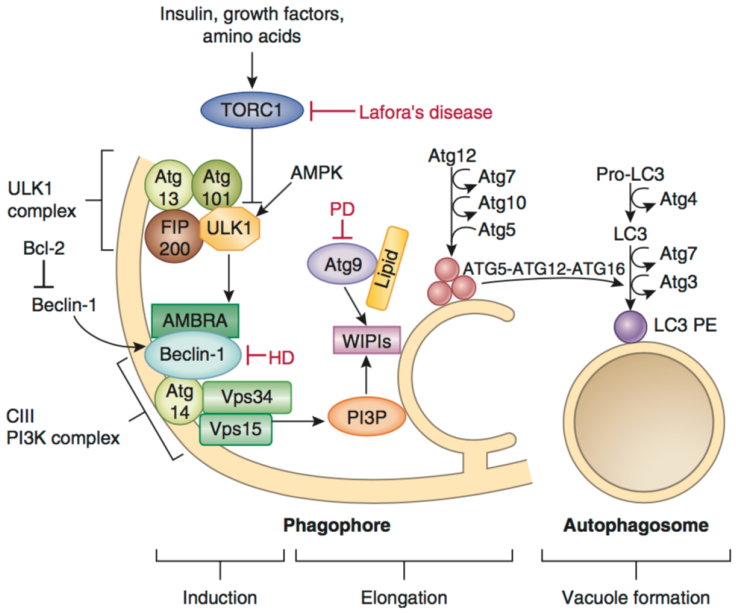


Figure 1.3: Autophagy pathway. Overview of the autophagy pathway. Autophagy begins with inactivation of mTOR to activate the ULK1 and P13K-III complexes. Induction of autophagy is followed by phagophore formation, or also recognized as elongation, and subsequently autophagosome, the vacuole, formation. Source from “The role of autophagy in neurodegenerative disease” [31]. Copyright permission attached on page 63.

Autophagic flux (**Figure 1.4**) refers to the whole process from synthesis of the

autophagosome to autophagosome degradation by the lysosome. Since an increase in autophagosomes can mean either increase in induction of autophagy or decrease in degradation, careful observation is needed [29].

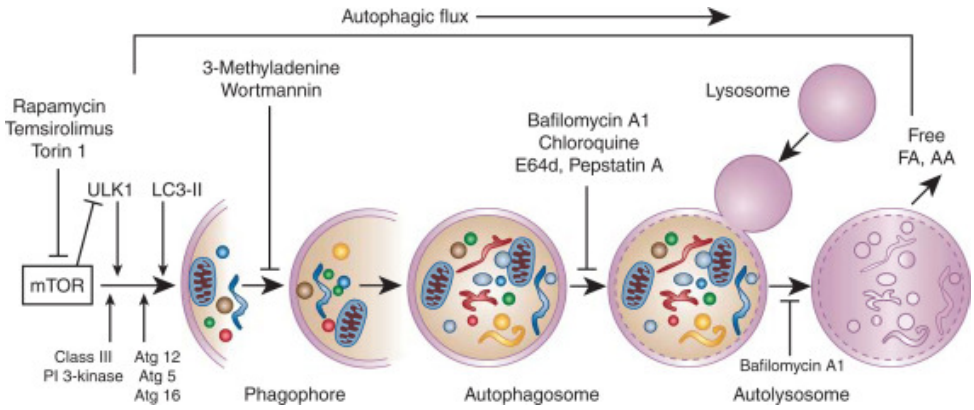


Figure 1.4: Autophagic Flux. A description of the dynamic process of autophagic flux, from formation of autophagosomes to degradation by lysosomes. The diagram features inhibitors to block normal autophagic flux. Rapamycin, Temsirolimus, and Torin acts as autophagy inducer to inhibit mTOR. 3-Methyladenine and Wortmannin **inhibit** phagophore formation in earlier stage. BafilomycinA1, Chloroquine, E64d, and PepstatinA blocks autophgosome binding to lysosome. Source from "Autophagy protects proximal tubular cells from injury and apoptosis" [32]. Copyright permission attached on page 64.

1.4 Iron metabolism/Iron overload

1.4.1 Iron Metabolism

The most common states of iron in biological and physiological settings are the ferrous (Fe^{2+}) and ferric (Fe^{3+}) forms. Different forms of iron can be found depending on the site in the body. The majority of iron in the body is stored in the ferric form. Iron is first taken up by the cells in the ferrous form, primarily by the small intestine, but is quickly oxidized to the ferric form by enzymes [33]. Maintenance of internal iron homeostasis is important because iron can participate in ROS formation [34]. Iron participates in the redox reaction due to its unpaired electrons. Fenton discovered that the oxidation of tartaric acid

was catalyzed by the presence of iron and hydrogen peroxide (Fenton, 1894). Later, Haber and Weiss demonstrated that in the presence of iron and hydrogen peroxide, the hydroxyl radical produced is very reactive, and this will initiate the chain reaction of lipid peroxidation [35].

$$Fe^{2+} + H2O2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$$

Cells maintain their iron balance by two mechanisms. First through ferritin, which is known as an iron storage protein. Ferritin deposits excess iron and allows iron to be transported. The second means of regulation is through iron regulatory proteins (IRPs). Basically, this protein regulates production and degradation of iron responsive elements (IREs) that control iron metabolism [34].

1.4.2 Iron overload and diabetes

People are aware that a deficiency in the daily iron supply can cause many health problems such as anemia, fatigue, and impaired immune function [36]. Iron deficiency is reportedly the most common micro-nutritional deficiency in the world [37]. Conversely, there are also a rising number of studies showing that excessive iron in the body can impair cellular activities. Iron utilization must be tightly regulated, since there is no physiological process that mediates excretion of large amounts of iron, except for losing a large amount of blood [38]. The disorder characterized by an overabundance of iron is called hemochromatosis, and this can be caused by genetic mutation or overdosing on iron supplements. Iron overload can result in cardiac toxicity and dysfunction of the endocrine system by damaging tissue with an excessive amount of free radical production [39]. In particular, previous research has found that the concentration iron stores in the body are correlated with the development of T2D [40]. This raises the possibility that

excessive iron can lead to diabetes [41]. This is supported by studies showing that iron plays a role in the regulation of glucose metabolism and insulin resistance.

1.4.3 Effect of iron overload on cellular mechanisms

1.4.3.1 Iron and oxidative stress

At the cellular level, iron plays an important role in the formation and destruction of reactive oxygen species (ROS) through a process known as the Fenton reaction [42]. When there is massive ROS production in the cells, cells experience oxidative stress. Oxidative stress is a condition in which the balance of ROS and antioxidant defense is unstable [43]. Progression of oxidative stress will further cause cell death [44]. Mitochondria are organelles that are very closely related to iron metabolism. The electron transport chain, one of the processes in which iron participates, occurs in mitochondria. Also, there are mitochondrial proteins that contain iron. MitoNEET, which is named based on the amino acid sequence Asn-Glu-Glu-Thr (NEET), is one of the proteins that resides in the outer mitochondrial membrane and regulates oxidative capacity [45].

1.4.3.2 Iron and autophagy

As mentioned previously, autophagy is a well-known for survival mechanism under conditions of disturbance in cellular homeostasis, during which catabolic processes are initiated to conserve energy and nutrients [46, 47].

As more studies on autophagy are done, researchers have found more specific autophagic pathways, as known as selective autophagy. In these cases, they name the selective autophagy based on the substrate it degrades, i.e., mitophagy for mitochondria and ferritinophagy for ferritin. In terms of iron, there are no direct studies showing effect of

iron overload on autophagy. When there is an increase in iron, ferritin is stabilized and NCOA4, which is a cargo protein for ferritinophagy, will be degraded, which is observed as a decrease in NCOA4. Such an observation would indicate a decrease in ferrtinophagy [48]. The study of autophagy with iron metabolism is a relatively new area, which has potential to be a great therapeutic target to treat many diseases.

1.4.3.3 Iron and apoptosis

Apoptosis, also known as programmed cell death, is a conserved process which aids in development, homeostasis, and disease. Similar to autophagy, apoptosis can be considered as either a beneficial or detrimental process to cells. Previous studies have shown iron overload can cause apoptosis in different cell types such as cardiomyocytes [49], hepatocytes [50], and osteocytes [49]. However, the mechanism(s) involved in iron-overload cell death are not fully understood.

There is special area that study iron induced cell death, called ferroptosis. This is found to be distinct from apoptosis, where features of event are slightly different. In apoptosis, the main biochemical feature is the activation of caspases as well as PS exposure. However, in ferroptosis, neither events are predominant, rather glutathione (GSH) depletion and increasing Nicotinamide adenine dinucleotide phosphate (NAPDH) oxidation occur [51]. Ferroptosis is normally resulted from lipid peroxide accumulation from iron-dependent pathway [52]. Discovering role of iron in apoptosis and ferroptosis will be very helpful to understand cell death in iron-overload associated diseases.

1.4.4 The role of oxidative stress in appoptosis

Both oxidative stress and apoptosis are essential for normal function of

cells and for survival. However, over-activation of these processes can cause diseases. Recently, studies illustrated that oxidative stress plays crucial role in apoptosis. For example, experiments showed that excess oxidative stress kills cells by apoptosis [53, 54] and antioxidants can delay, or even block apoptosis [55]. Mitochondria are the place where apoptosis and oxidative stress share in common. They are the sites where majority of oxygen radicals generated, which can cause oxidative stress. Also, cytochrome C release from mitochondria mediates apoptosis.

1.5 Adiponectin

1.5.1 Adiponectin structure

Adipokines are cytokines that are produced from adipose tissue [56]. There are several examples of adipokines including leptin, tumor necrosis factor-a (TNF-a), Visfatin, lipocalin-2, and acylation-stimulating protein (ASP) [57, 58]. Lipocalin-2, for example, is a pro-inflammatory hormone that is positively correlated with various metabolic parameters such as body mass index (BMI) and body fat percentage [58]. Adiponectin (also known as GBP-28, apM1, adipoQ, and Acrp30) is another example of an adipokine. Adiponectin can be found in either a 17kDa globular form (gAd) or the full-length form (fAd), and fAd can further be divided into 3 categories according to its polymerization and molecular weight: low (LMW), middle (MMW), and high molecular weight (HMW). These forms are a 90kDa trimer, a 180kDa hexamer, and a higher order oligomer, respectively [59]. Among these different sizes of adiponectin, it is the HMW one that is thought to have the most biological activity in the body [60].

1.5.2 Associations between adiponectin and heart failure

Unlike Lipocalin-2, described above, there is a growing body of evidence that suggests a negative relationship between adiponectin levels and HF. One study showed that in adiponectin knockout (KO) mice, cardiac hypertrophy and mortality rates increased [61]. Another study demonstrated that acute myocardial infarction (MI) was observed to correlate with a decline in adiponectin levels [62]. In one study, it was shown that hypoadiponectinemia, where there is a low plasma adiponectin level, was closely related to diseases related to obesity such T2D, CHD, and hypertension [63].

1.5.3 Cardioprotective effects of adiponectin

There is evidence that adiponectin has many physiological functions such as insulin-sensitization, anti-diabetic, anti-inflammatory, and anti-atherosclerotic effects [58, 64]. Of note, many studies have shown that adiponectin has a cardioprotective function in the heart under pathological conditions [63]. Adiponectin knockout mice showed a greater extent of stress response in cardiac tissue compared to wild-type mice. For instance, after myocardial ischemia-reperfusion injury, adiponectin knockout mice showed a larger infarct area, enhanced concentric cardiac hypertrophy, and increased mortality [63]. However, the function was rescued when exogenous adiponectin was supplied [60]. One of the ways that adiponectin is believed to protect against cardiomyopathy is through enhancing the AMP-activated protein kinase (AMPK)- dependent signalling pathway. There is evidence proves that adiponectin helps from apoptosis through AMPK pathway from high glucose toxicity in tubular cells [65] and in endothelia cells [66]. Therefore, adiponectin is thought to have an anti-apoptotic function through activation of the AMPK signaling pathway to inhibit apoptosis in cardiac cells [63].

1.6 Effect of hyperglycemia on cellular mechanisms

1.6.1 Hyperglycemia and autophagy

In animal models, diabetic rats have been shown to have suppressed autophagy [67]. When autophagy was up-regulated using a pharmacological inducer of autophagy, metformin, a cardiac protective role was demonstrated in the diabetic heart [68]. Further, induction of autophagy prevented hyperglycemia-induced cardiomyocyte cell death in H9c2 cells[69]. In other model, glucose infused- hyperglycemia inhibited autophagy in rat skeletal muscle [70].

1.6.2 Hyperglycemia and apoptosis

In cardiomyocytes, hyperglycemia induces apoptosis [71]. HF is characterized by an increased apoptotic rate of cardiomyocytes, which is known to play an important role in the progression of the disease. Apoptosis has emerged as a potential therapeutic target for treating heart failure, since cardiomyocytes do not proliferate and even a small reduction in the number of cardiomyocytes is detrimental to cardiac function [19]. Hyperglycemia increases cardiomyocyte apoptosis, which leads to diabetic cardiomyopathy and HF [72]. In the study by Cai and his colleague, they showed that STZ-induced diabetic mice had more apoptosis occurrence in the heart compared to control or insulin-supplemented diabetic mice. And this was supplemented with *in vitro* experiment, which showed that increased caspase-3 activation upon high levels of glucose treatment.

1.7 Hypothesis and research aims

Studies had proven that iron-overload and hyperglycemia are observed in metabolic syndrome patients and animal models of diabetes, and furthermore, that these conditions can induce apoptosis. However, the mechanism(s) behind how these stimuli cause

apoptosis are not yet fully understood. Thus, the aim of my study is to investigate the mechanism(s) through which conditions commonly observed in metabolic syndrome induce cell death in relevant target cell types.

For my first project, the hypothesis was that iron overload induces intrinsic apoptosis, and that this was mediated by reduced autophagic flux and/or increased oxidative stress, and possibly by crosstalk between them. The relationship between autophagy and iron-overload induced apoptosis is not well elucidated. To investigate their interactions, I used *in vitro* models of both wild type and autophagy-deficient L6 myoblast cells treated with iron for 24 hours, and tested ROS production, autophagic flux, and apoptosis.

For my second project, I hypothesized that adiponectin would have a beneficial effect on apoptosis by activating autophagy under hyperglycemic conditions in cardiomyocytes. In hyperglycemia, reduced levels of autophagy and increased levels of apoptosis are expected. However, when treated with adiponectin, the autophagy level would increase to prevent cells from undergoing apoptosis. In this project, I used H9c2 cardiomyocyte cells treated with adiponectin in both normal glucose and high glucose conditions and examined autophagy and apoptosis.

Chapter 2 Iron-overload induces cell death via inhibition of autophagic flux and/or by promotion of oxidative stress in L6 skeletal muscle cells

2.1 Preface

Iron overload can result in cardiac toxicity and dysfunction of the endocrine system by damaging tissue through an excessive amount of free radical production. When there is massive ROS production in cells they experience oxidative stress, and this will cause cell death. Muscle is one of the largest reservoirs for iron in the body, along with liver, spleen, and bone marrow. Though the concentration of iron in muscle is lower than other organs, muscle mass is much larger, thus the total amount of iron is very similar to other tissue types. Based on three different apoptosis assays, caspase-3 western blot and immunofluorescence, and a cytochrome C/mitotracker co-localization assay, I concluded that iron overload conditions increase apoptosis in L6 skeletal muscle cells. Also, iron overload caused accumulation of autophagosomes, as demonstrated by cytoID immunofluorescence. In autophagy deficient cells ($Atg5^{K130R}$), there was a significant increase in apoptotic activity. Lastly, in order to link autophagy, oxidative stress, and apoptosis, I measured ROS production with CellROX reagents, and showed an elevation in ROS production with iron treatment, and reduction upon co-treatment with the antioxidant reagent, MnTBAP.

In conclusion, my study confirmed that iron overload induced the intrinsic pathway of apoptosis. Additionally, iron overload also provoked autophagosome formation. Based on results thus far, inhibition of autophagy appears to exacerbate apoptosis.

2.2 Introduction

People are aware that a deficit in the daily iron supply can cause many health problems such as anemia, fatigue, and impaired immune function [36]. Iron deficiency is reportedly the most common micro-nutritional deficiency in the world [37]. However, an increasing number of studies have now shown that excessive iron in the body can impair cellular activities. Iron overload can result in cardiac toxicity and dysfunction of the endocrine system by damaging tissue through an excessive amount of free radical production [39]. In particular, previous research has found that the concentration of iron stores in the body are correlated with development of T2D [40] raising the possibility that iron overload can lead to diabetes [41]. Muscle is one of the largest reservoirs for iron in the body along with the liver, spleen, and bone marrow. Even though the concentration of iron in the muscle is lower than other organs, muscle mass is much larger, thus the total amount of iron contained in the tissue is very similar to others [73].

At the cellular level, iron plays an important role in the formation and destruction of reactive oxygen species (ROS), through a process known as the Fenton reaction [42]. When there is massive ROS production in cells they experience oxidative stress, and this will cause cell death [44]. Therefore, the level of ROS has to be tightly controlled and autophagy plays a role in this process.

Autophagy is a well-known survival mechanism utilized by cells in response to disturbance in their homeostasis, during which catabolic processes are initiated to conserve energy and nutrients [46, 47]. It can be divided into two main steps- formation of autophagosomes by engulfing organelles, and breakdown of these autophagosomes by lysosomes [47]. An increasing number of discoveries about the autophagic process have been published, generating controversy over whether it is beneficial or detrimental to cells.

Arguments can be different depending on their context; however, it has been observed that autophagy can be a defense mechanism against oxidative stress [74].

Apoptosis, also known as programmed cell death, is a conserved process, which aids in development, homeostasis, and disease. It is a natural process that is involved in cell turnover in adult tissues, as well as during embryonic development. Similar to autophagy, apoptosis can be considered to be either a beneficial or detrimental process to cells depending on the context. There have been studies showing that inhibition of autophagy elevates apoptosis in various cell types [75-77]

The relationship between autophagy and iron-overload induced apoptosis is not well elucidated. Based on previous research, I hypothesized that iron overload induces intrinsic apoptosis, and this is mediated by reduced autophagic flux and/or increased oxidative stress, and potentially crosstalk between them. To investigate interactions between these pathways, I used both wild type and autophagy deficient L6 myoblast cells treated with iron for 24 hours and tested ROS production, autophagic flux, and apoptosis activity.

2.3 Materials and Methods

2.3.1 L6 myoblast cell culture and treatment

L6 skeletal muscle cells were maintained in Alpha modification of Eagle's Medium (AMEM) from Wisent Bioproduct supplemented with 10% fetal bovine serum (FBS) from Gibco and 1% Antibiotic-antimycotic (vol/vol) from Gibco. Cells were maintained at 37 °C with 5% CO_2 in a 75cm^2 flask. During cell growth, when cells reached 80% confluence, they were trypsinized and seeded for experiments. Prior to treatments, cells were starved for 2 hours in FBS-free AMEM. According to treatment conditions specified in the results, cells were treated with 0.5% FBS AMEM and $FeCl_3$ 250uM, Chloroquine 30uM, and MnTBAP 10uM.

2.3.2 Generation of L6-ATG5K130R mutant cell line

Autophagy-deficient L6 cell stably overexpressing dominant negative Atg5 with lysine(K) mutated to arginine(R) at position 130 was generated. The virus produced from ecotropic packaging cell line, Ecopack2-293, was infected to L6 myoblast cells in sterile condition. Using retroviral expression vector, pQCXIP, containing puromycin resistance, virus was produced. Cells were incubated with virus for 24hours in the incubator. Cells were maintained in growth media containing puromycin (2ug/mL; Sigma-Aldrich).

2.3.3 Western blot Analysis

After treatment, L6 cells were washed in phosphate buffered saline (PBS). 120uL of lysis buffer [0.5 M tris-hydrochloride (Tris-HCL) pH 6.8, 2% (vol/vol) sodium dodecyl sulphate (SDS), 15% (vol/vol) glycerol, 10% (vol/vol) 2-mercaptoethanol, and protease inhibitor cocktail] was added to each well and cells were scraped off. The lysates were then incubated at 95°C for 10 minutes. Approximately 35ug of protein was loaded to each

well. Samples were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidine fluoride (PVDF) membrane. The membranes were blocked in 3% BSA for 1 hour and were left in primary antibodies overnight. Western blot analysis was performed using specific primary antibodies to Caspase-3, LC3B, and GAPDH (1:1000, Cell Signaling). HRP-linked secondary anti-rabbit (1:5000, Cell Signaling, MA) was used as secondary antibody and proteins were detected by enhanced chemiluminescence reagent (BioRad). The membranes for caspase-3 were stripped each time and re-probed with antibodies against GAPDH (1:1000, Cell Signaling, MA) as loading controls. Band intensity was quantified using ImageJ software.

2.3.4 Detection of mitochondrial cytochrome c release

Mitotracker Red CMXRos (ThermoFisher Scientific) at 250nM was loaded into wells for 30 minutes at 37°C. Cells were fixed in 4% Paraformaldehyde for 20 minutes at room temperature and permeabilized using 0.1% Triton X-100. Overnight incubation with Cytochrome C (clone 6H2.B4, BD Pharmingen™, 1:400) primary antibody was followed by 1 hour incubation with Goat anti-Mouse IgG, Alexa Fluor 488 (ThermoFisher, 1:800). Coverslips were mounted on microscope glass slides using DAPI mounting medium (Vector Laboratories Inc., USA) and analyzed using fluorescence microscopy. The images were taken using 40x objective of LSM 700 confocal microscope with DAPI, FITC, and TRITC channels for nucleus, cytochrome c, and mitotracker, respectively. Experiments were done 3 separate experiments and each experiment consisted of 3 fields of view that were selected randomly. Pearson Coefficients were calculated using the JACoP plug-in in ImageJ software to calculate co-localization.

2.3.5 CYTO-ID® Assay (Product from Enzo)

L6 cells were seeded onto 18mm coverslips and treated according to their respective treatment plan. Cells were washed twice with 1x Assay Buffer, then 2uL of CYTO-ID® Green Detection Reagent, and 1 uL of Hoechst 33342 Nuclear Stain was mixed into 1mL of 1x Assay Buffer. 100uL of this mixture was added on top of the coverslip. Samples were incubated for 30 minutes at 37°C in the dark. Then cells were washed with 100uL of 1X Assay Buffer and fixed with formalin for 20 minutes. Cells were washed with 1X Assay Buffer three times and mounted on the glass slide with Prolong mounting medium (Prolong). Using the FITC filter for autophagic signal and DAPI filter for nuclear signal by confocal microscope (Zeiss LSM700), images were taken at 40x objectives. 3 different fields of view were selected randomly per experiments for 3 separate experiments and analyzed using Image J software.

2.3.6 CellROX Green Assay

5uM of CellROX Green Oxidative Stress Reagent (Life Technologies) was added to each well and incubated for 30 minutes at 37°C in the dark. The cells were fixed with 4% PFA for 15 minutes, and mounted on a glass slide with DAPI mounting medium (Vector Laboratories Inc., USA). Using FITC and DAPI filters in confocal microscopy, images were taken using the 20x objective (Zeiss LSM700).

2.3.7 CellROX Deep Red Assay

5uM of CellROX Deep Red Oxidative Stress Reagent (Life Technologies) was added to each well and incubated for 30 minutes at 37°C in the dark. The cells were fixed with 4% PFA for 15 minutes, and mounted on a glass slide with DAPI mounting medium (Vector

Laboratories Inc., USA). Using TRITC and DAPI filters in confocal microscopy, images were taken using the 20x objective (Zeiss LSM700).

2.3.8 Statistical analysis

All data are presented as mean ± SEM. Statistical analysis was performed using Student's t-test when comparing 2 groups. To compare experiment with more than 2 groups, one way ANOVA followed by Dunnett's post-test was performed. The differences among groups were considered statistically significant when $p<0.05$ and are indicated by symbols as described in figure legends.

2.4 Results

2.4.1 Iron overload causing apoptosis in L6 skeletal muscle cells.

To confirm iron overload causes apoptosis in L6 skeletal muscle cells, cells were first treated with 250uM of $FeCl_3$ for 24 hours. Two different assays, western blot and immunofluorescence, were done to see treatment induced activation of caspase-3, which is a known effector protein of apoptosis. A significant increase in cleaved caspase-3 was observed with 24 hours of iron treatment by western blot (**Figure 2.1 A, B**). Additionally, Cellevent™ Caspase3/7 Green Detection Reagent, a fluorescent indicator activated by active caspase-3, demonstrated localization of caspase-3 within nucleus (**Figure 2.1 C**). This was quantified as co-localization of FITC to DAPI, and there was significant increase of co-localization upon iron treatment (**Figure 2.1 D**).

To further investigate the activation of intrinsic apoptosis, a cytochrome C/mitotracker™ co-localization assay was performed. This assay allows us to determine whether cytochrome C has been released out of mitochondria to the cytoplasm to further activate the cascade reaction of apoptosis. Merged images of the two different conditions showed that in controls, cytochrome C primarily resided inside mitochondria (represented by yellow punctae). There was a significant decrease in cytochrome C and mitotracker co-localization upon iron treatment. Images were then quantified and represented as a Pearson's coefficient ratio (**Figure 2.1 E, F**).

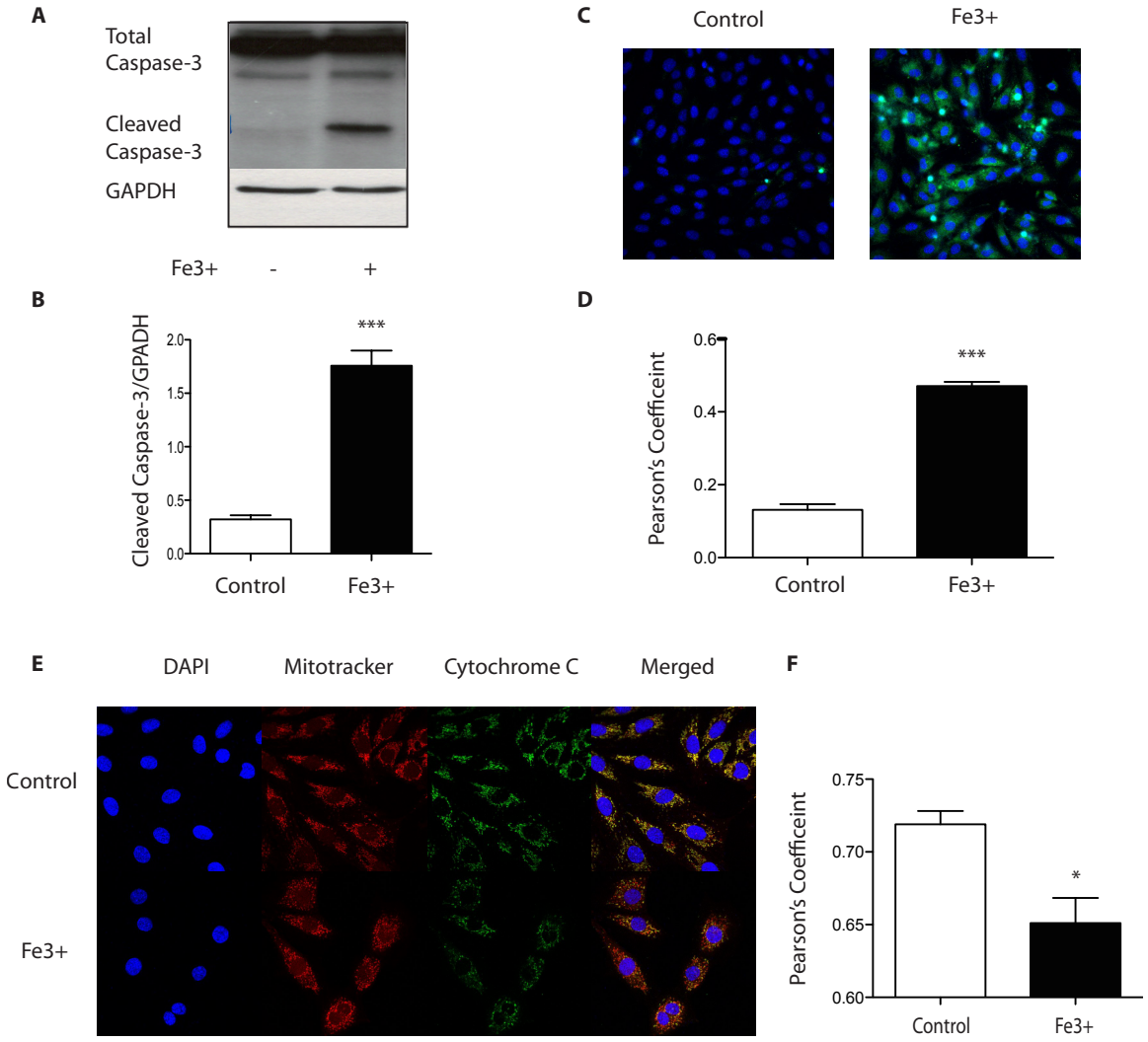


Figure 2.1 Iron overload inducing apoptosis in L6 cells. L6 cells were treated with $FeCl_3$ (250uM) for 24 hours. Western blots showing total and cleaved forms of caspase-3 and the loading protein, GAPDH (A) and quantification for cleaved caspase-3 form over GAPDH (B). Representative images to show increased activation of caspase-3/7 (green) within nucleus (C) and quantification of its co-localization using Pearson's coefficient (D). Representative images to show increase in activation of intrinsic apoptosis was visualized using cytochrome C (green) and mitotracker (red) co-localization (E) and quantification using Pearson's coefficient (F). * indicates significant difference from control $p<0.05$. *** indicates significant difference from control $p<0.001$ (n=3).

2.4.2 Iron overload inducing autophagosome formation.

To establish the relationship between autophagy and apoptosis upon iron overload conditions, I focused on autophagic flux. To visualize autophagosome formation, cytoID reagent was used. This novel reagent allows visualization of the autophagosome by cationic amphiphilic tracer (CAT) dye that goes into cells in a similar way as phospholipidosis, and labels autophagic vacuoles. Result showed that upon treatment with iron, there was a significant increase in total green fluorescence (**Figure 2.2 A, B**). Western blot supported IF images, in which the lipidated form of LC3, LC3II, a marker for autophagosomes, increased upon iron treatment (**Figure 2.2 C, D**).

2.4.3 Autophagy deficiency did not accelerate apoptosis

Chloroquine, which is the inhibitor of autophagic degradation, was co-treated with iron to see whether blocking autophagy can further activate apoptosis. Consistent with previous data, iron overload increased cleaved caspase-3 activation. However, when chloroquine was treated along with iron, there was a decrease in not only cleaved caspase-3, but also total caspase-3 levels (**Figure 2.3 A, B**).

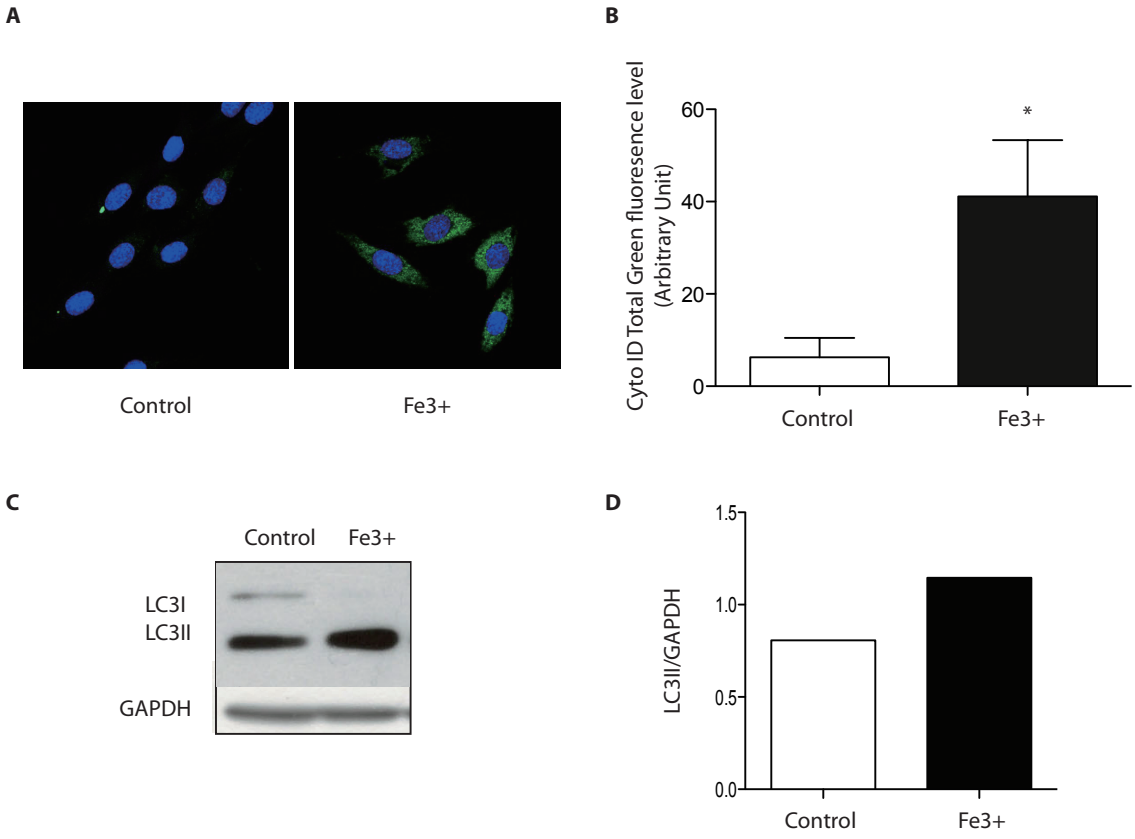


Figure 2.2 Iron overload inducing autophagosome accumulation. L6 cells were treated with $FeCl_3$ (250uM) for 24 hours. Representative images to show increase in autophagosome formation by CytoID (A) and its quantification (B). * indicates significant difference from control $p<0.05$ (n=3). Increase in autophagosome formation also supported by western blot of LC3 (C) and its quantification (n=1) (D).

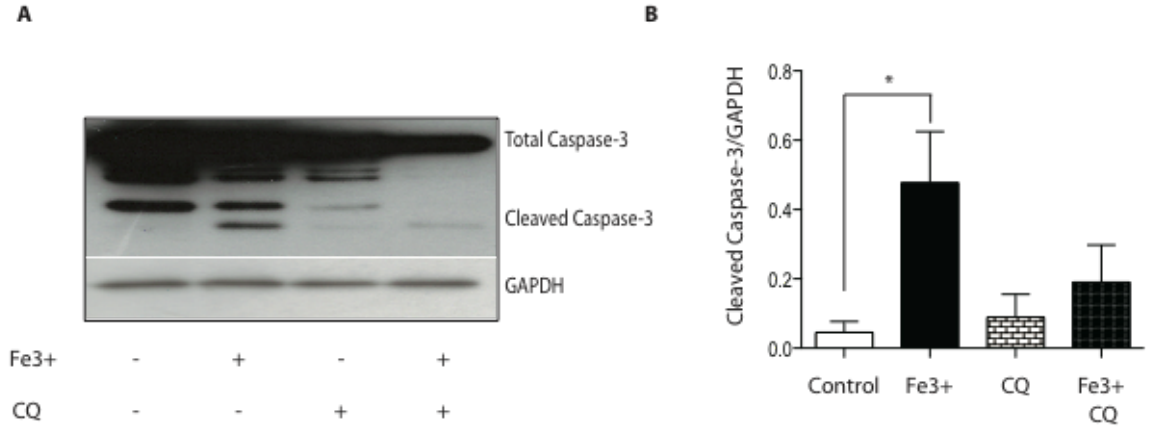


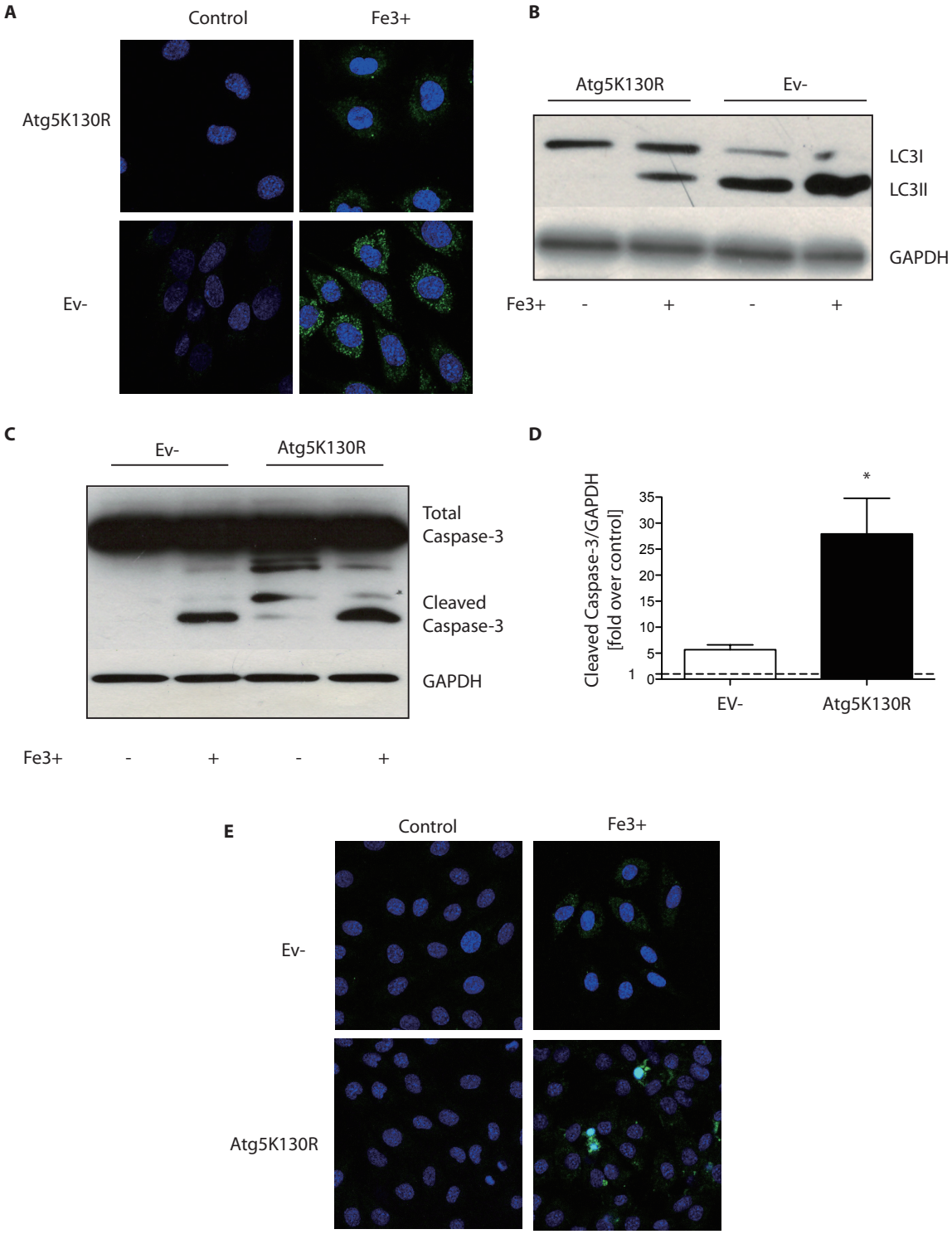
Figure 2.3 Reduction of iron-overload induced apoptosis with autophagy inhibitor. L6 cells were treated with $FeCl_3$ (250uM) with or without chloroquine (CQ, 30uM) for 24 hours. Western blots showing total and cleaved forms of caspase-3 and the loading protein GAPDH (A) and quantification for cleaved casapse-3 over GAPDH (B). no statistical difference in one way ANOVA followed by Dunnett's post-test, but using Student t-test to compare only between control and Fe^{3+}, * indicates significant difference from control $p<0.05$ (n=3).

To further investigate the linkage between autophagy and apoptosis, more experiments comparing autophagy-deficient $Atg5^{K130R}$ and control empty vector (EV) cells were performed. $Atg5^{K130R}$ are dominant negative cell lines in which autophagy is down-regulated, not completely knocked out. To confirm that the cell lines are properly functioning, cytoID immunofluorescence (**Figure 2.4 A**) and LC3 western blots (**Figure 2.4 B**) were performed. There was no autophagosome formation observed when cells were starved. However, when treated with iron, autophagosomes accumulated even in the $Atg5^{K130R}$ cell line. This demonstrated that autophagy was not completely knocked out, but it was significantly down regulated in this line.

After confirming Atg5^{K130R} cell lines are functioning appropriately, I performed the same set of apoptosis analysis performed previously, including Caspase-3 western blot, and immunofluorescence using Cellevent^{TM} Caspase3/7 Green Detection Reagent. In both EV- and Atg5^{K130R} cells the level of cleaved Caspase-3 increased with iron treatment. Even though the absolute difference seemed to be greater in EV- cells, the fold increase between control and iron-treated groups was greater in Atg5^{K130R} cells (**Figure 2.4 C, D**). Comparing control and iron-overloaded Atg5^{K130R} cells, there was a significant increase in activation of Casapse-3/7, as indicated by green signals localized in nucleus. A similar trend was observed with EV- cells; however, the green signal was much weaker with iron treatment as compared to Atg5^{K130R} cells (**Figure 2.4 E**).

2.4.4 Iron overload induces ROS production in L6 skeletal muscle cells

Lastly, to investigate another mechanism through which iron overload may induce cell death, I measured reactive oxygen species (ROS) production in L6 cells with both CellROX green and red reagents (**Figure 2.5**). The images from confocal microscopy showed that intracellular ROS production was increased upon iron treatment in both experiments, and this was then reduced by co-treatment with 10uM MnTBAP, a ROS scavenger.


A
Control
Fe3+
Atg5K130R
Ev-
B
Atg5K130R
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Fe3+ - + - +
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Atg5K130R
Total Caspase-3
Cleaved Caspase-3
GAPDH
Fe3+ - + - +
D
Cleaved Caspase-3/GAPDH [fold over control]
35
30
25
20
15
10
5
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EV-
Atg5K130R
E
Control
Fe3+
Ev-
Atg5K130R

Figure 2.4 Reduced apoptosis activity in autophagy deficient cell line. Confirmed that Atg5K130R and EV- L6 cells were well transfected by comparing starved condition using cyto ID (A) and western blot of LC3B and GAPDH (B). Atg5K130R and EV- L6 cells were treated with $FeCl_3$ (250uM) for 24 hours. Western blot of caspase-3 and loading control protein GAPDH (C) was performed and quantification of iron condition normalized to control, which represented by dotted line, for cleaved caspase-3 over GAPDH is provided (D). Representative images to show increased activation of caspase-3/7 (E).* indicates significant difference from control $p<0.05$ (n=3).

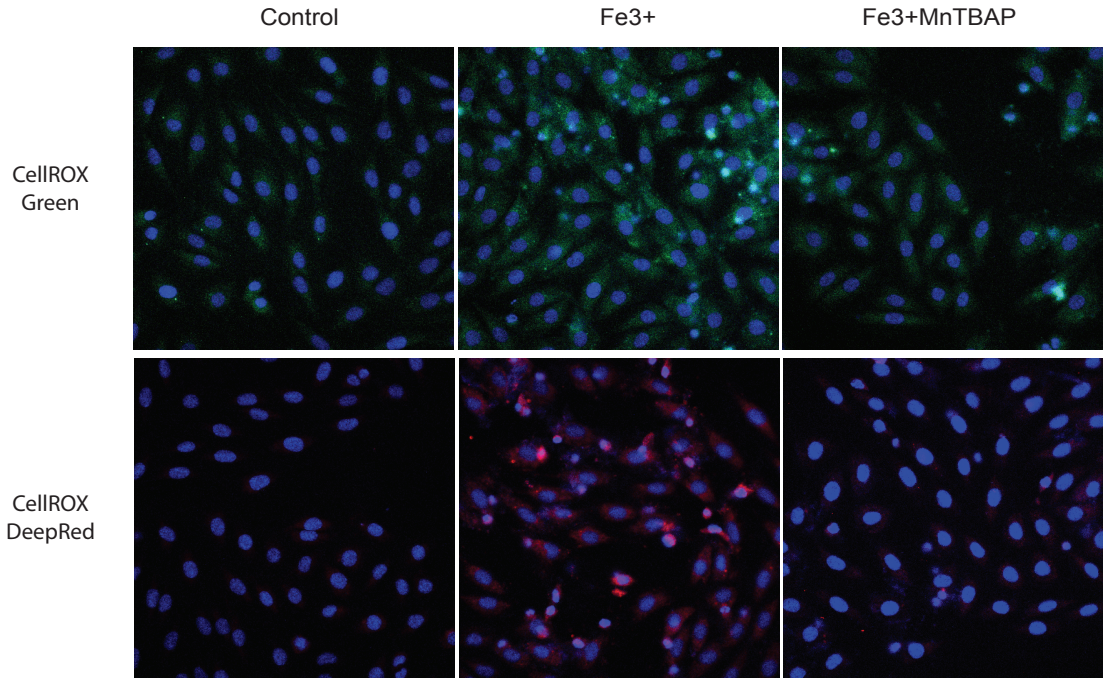

Figure 2.5 Iron overload inducing ROS production in L6 cells. L6 cells were treated with FeCl3 (250uM) with or without MnTBAP (10uM) for 24 hours. Representative images to show increased ROS production with iron treatment and alleviation with ROS scavenger, MnTBAP, using CellROX Green assay and CellROX Deep Red assay.

2.5 Discussion and Future Plan

Excessive iron in the body could result in impairment of cellular activities. In clinical studies, excessive iron in tissues was closely linked to the development of T2D [78]. Although the concentration was lower than other organs such as liver, spleen, and bone marrow, skeletal muscle was one of the largest reservoirs of iron if the total amount was compared due to the large mass of muscle in the body [73]. In the present study, I found that long-term iron treatment resulted in apoptosis, autophagosome accumulation, and excessive oxidative stress in L6 skeletal muscle cells.

Upon iron treatment, cleaved Caspase-3 levels were significantly increased, and cytochrome C was released into the cytosol from damaged mitochondria. Consistent with previous studies, my results showed that iron overload up-regulated apoptosis in L6 cells. Previous studies have shown that iron overload causes cell death in different types of cells, such as cardiomyocytes [49], hepatocytes [79], and bone marrow cells [80]. Apoptosis, also known as programmed cell death, is when a cell commits suicide in response to stimuli [81]. Caspase-3 is an effector protein that carries out apoptosis [82]. This protein was widely used as a marker to check for activation of apoptosis. Additionally, a Cytochrome-c and mitochondria co-localization assay allowed us to visualize whether damaged mitochondria resulted in release of cytochrome-c to the cytosol where it would trigger the intrinsic pathway of apoptosis [16].

Upon treatment with iron for 24 hours there was a significant increase in the number of autophagosomes detected. Autophagy is a survival mechanism which eukaryotes have adapted to resist against various stresses such as limitations in nutrient availability or oxygen levels [24]. There are multiple stages of autophagy progression, but in my study, I

focused on the stage involving autophagosomes. Autophagosome accumulation could be interpreted in two ways, either as increase in induction of autophagy or decrease in autophagosome degradation [29]. CytoID is a novel reagent that was used to visualize the presence of autophagosomes, together with the expression level of LC3II. To better understand autophagic flux, these studies would need to be complemented with further experiments examining the activation of autophagy or blocked degradation of autophagosomes.

There have been many studies that have speculated a relationship between autophagy and apoptosis, yet still no definite connection has been made. This is probably due to the multiple proteins involved that could behave differently in different environments and different cell types [47]. In order to characterize the interrelationship between autophagy and apoptosis in skeletal muscle cells, I performed experiments in a setting where autophagy was blocked either chemically or genetically. Chloroquine is one of the commonly used blockers of autophagy, along with bafilomycin A1, which could inhibit degradation of autophagosomes [83]. For genetic inhibition, a special mutant L6 cell line was used. The $Atg5^{K130R}$ cell line expresses a dominant negative Atg5 in which a point mutation replaces arginine (R) to Lysine (K) at position 130 [84]. In the beginning, I hypothesized that blocking autophagy would increase apoptotic activity. Interestingly, co-treatment with iron and chloroquine showed lower activation of apoptosis compared to iron-only treatment. However, taking the results for total caspase-3 into consideration, it was determined that not only the cleaved form but also total form of caspase-3 decreased. Previous research has found that chloroquine could inhibit cell growth, and could also induce cell death through the inhibition of autophagy, however the mechanism is unknown [85] and could possibly involve a caspase-3 independent pathway. This suggests that

chloroquine has a role in blocking autophagy, however, it causes cell death through a different pathway. In autophagy-deficient cells, there was greater increase of casaspe-3 activation with iron treatment compared to control empty vector cells. This suggested that when autophagy was impaired, more apoptosis could be triggered.

My data so far suggests that there is a significant increase in ROS production with iron treatment in both the nucleus and cytoplasm; however, oxidative stress induced by iron overload was abolished by the antioxidant reagent, MnTBAP. It is believed that in many diseases related to iron overload the organs are damaged by excessive ROS production [42]. This can damage mitochondria, which could further initiate apoptotic cell death in many cell types [14]. My findings are consistent with other studies that ROS production is increased by iron treatment.

In summary, skeletal muscle cells were very responsive to iron overload-induced apoptosis. There was a significant induction of autophagosome formation and ROS production. Future studies will focus on looking at autophagosome degradation in autophagic flux, as well as investigating crosstalk between autophagy and oxidative stress in iron overload-induced apoptosis. This, in the future, can provide a new direction of drug discovery for iron overload-associated diseases

Chapter 3: Adiponectin alleviates hyperglycemia-induced cell death via improving the autophagic pathway in cardiomyocytes

3.1 Preface

It is known that diabetes is characterized by elevated blood glucose, a condition called hyperglycemia. Hyperglycemia increases cardiomyocyte apoptosis, which leads to HF. Previous work has demonstrated that adiponectin has a protective effect against apoptosis. Therefore, based on previous studies, my aim is to determine through which pathway adiponectin mediates its beneficial effect on apoptosis. The mechanism I propose is through autophagy, specifically improving autophagic flux. I have found that adiponectin reduces high glucose-induced cell-death. I examined this by using a trypan blue exclusion assay, an annexin V/PI staining assay, a TUNEL assay, and a Caspase-3 activity assay. Also, using a mitotracker and cytochrome C co-localization assay, I verified that hyperglycemia-induced cell death is mediated by the intrinsic pathway of apoptosis. In terms of autophagy, high glucose treatment induced autophagosome formation, and again, adiponectin alleviated this condition. When autophagy was impaired, apoptosis increased even under normal conditions, but adiponectin treatment was able to reduce caspase-3 activation. This suggested that adiponectin may function to reduce apoptosis through a different pathway when autophagy is not fully functioning.

3.2 Introduction

Diabetes mellitus is a metabolic disorder with global prevalence characterized by high blood glucose levels, insulin insensitivity, and/or an inability to produce insulin [1]. It is well known that diabetes is the major risk factor for CVD. Diabetic patients or animals have an elevated glucose level in the blood, a condition called hyperglycemia. HF is characterized by an increased apoptotic rate of cardiomyocytes, which is known to play substantial role in the progression of the disease [19]. Apoptosis has emerged as a potential therapeutic target for treating HF since cardiomyocytes do no proliferate, and even a small reduction in the number of cardiomyocytes is detrimental to cardiac function. It has been discovered that hyperglycemia increases cardiomyocyte apoptosis, which then leads to diabetic cardiomyopathy and HF [72].

It is now known that a circulating hormone secreted from adipocytes, adiponectin, has a protective effect in many cardiac remodeling events such as hypertrophy, fibrosis, metabolism, and cell death [58]. One of the ways that adiponectin exerts its cardio-protective function is through the AMP-activated protein kinase (AMPK)- dependent signaling pathway [63]. AMPK is involved in many cellular regulatory roles, one of which is to act as an inducer of autophagy. This suggest the possibility that adiponectin modulates autophagy. The mechanism underlying adiponectin's role in regulating autophagy and apoptosis needs to be better understood.

Based on previous research, I hypothesized that adiponectin will have a beneficial effect on reducing apoptosis by activating autophagy under hyperglycemic conditions in cardiomyocytes.

3.3 Materials and Methods

3.3.1 Cell culture and treatments

H9c2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) from Gibco and 1% penicillin/streptomycin (v/v). When cell populations reached 80% confluence in 75cm^2 flasks at 37 °C with 5% CO_2 they were either passaged or seeded for experiments. Cultures were exposed to $_D$-glucose at a final concentration of 25mmol/L for *in vitro* treatment with high levels of glucose (HG), and 5.5mmol/L $_D$-glucose as control (NG). To exclude a hyperosmolar effect, we added of 19.5mmol/L mannitol in control cultures. Cells were treated with fAd (5.0ug/mL) for various time points as indicated in figure legends.

3.3.2 Production of fAd

Post-translationally modified fAd was produced in a mammalian expression system (Palanivel, 2007).

3.3.3 Trypan blue exclusion Assay

Cells were seeded in 6-well plates and incubated in NG or HG with or without fAd (5.0ug/mL) for 72 hours. Cells were washed with PBS then trypsinized. 10% FBS DMEM was added to neutralize trypsin. Cells were then suspended and treated with 0.1mL of 0.4% Trypan Blue Stain for 5 minutes. Using a hemocytometer, cells were counted under light microscope. Percentage of trypan blue positive cells over total cell population for each sample was calculated.

3.3.4 Annexin V binding assay

Phosphotidylserine (PS) exposure in apoptotic cells was measured using Annexin V-FITC detection kit (BD Pharmagen, Canada) according to the manufacturer's protocol. Cells were seeded in 12-well plates with coverslips and incubated in NG or HG, with or without fAd (5.0ug/mL) for 24 hours. Cells were washed with PBS and binding buffer (10mM HEPES pH 7.4, 140mM NaCl, 2.5mM CaCl2), followed by a 20 minute incubation with solution containing annexin V-FITC (1:20 dilution) and 1ug/mL propidium iodide, diluted in binding buffer solution.

3.3.5 TUNEL assay

Free 3'-OH DNA termini generated during apoptosis were detected by TUNEL staining (Terminal dexoynucleotidyl transferase- mediated dUTP nick end-labeling), with dUTP tagged with fluorescence (FITC). The assay was performed using the *In Situ* Cell death Detection Kit, Fluorescein (Roche Applied Science, Canada) according to the manufacturer's protocol. Cells were seeded in 12-well plates with coverslips and incubated in NG or HG, with or without fAd (5.0ug/mL). Cells were fixed in 4% Paraformaldehyde for 1 hour at room temperature and permeabilized using 0.1% Triton X-10 in 0.1% sodium citrate. Then cells were incubated in the TUNEL reaction mixture for 1 hour at 37°C. Coverslips were mounted on microscope glass slides with DAPI mounting medium (Vector Laboratories Inc., USA) and analyzed using fluorescence microscopy.

3.3.6 Casapse-3 activity

The Caspase-3/CPP32 Colorimetric assay (MBL International Corp., MA, USA) was used to measure the activity of caspase-3 according to the manufacturer's protocol. Cells

were grown in a 6 well plate. After incubation in NG or HG, with or without fAd (5.0ug/mL), floating cells were collected by centrifugation at 12,000 rpm for 1 min, at room temperature. The floating cell pellet combined with adherent cells were lysed in 50uL of chilled lysis buffer on ice for 10 min. Supernatant was transferred to a microtube, and incubated with DEVD-pNA substrate at 37°C for 1.5hrs. After the incubation time, 100ul of each sample was transferred to each well in a 96 well plate, and absorbance was read at 405 nm in a microplate reader (Multiskan Spectrum, Thermo Fisher Scientific Inc.).

3.3.7 Detection of mitochondrial cytochrome c release

Mitochondrion-selective MitoTracker probes were used according to the manufacturer's protocol. Cells were seeded in 12-well plates with coverslips and incubated in NG or HG. Cells were then incubated in MitoTracker solution for 15 minutes at 37°C. Cells were fixed in 4% Paraformaldehyde for 20 minutes at room temperature and permeabilized using 0.1% Triton X-100. Overnight incubation with Cytochrome C primary antibody, followed by 1 hour of anti-goat secondary antibody was performed. Coverslips were mounted on microscope glass slides with DAPI mounting medium (Vector Laboratories Inc., USA) and analyzed using fluorescence microscopy.

3.3.8 Western Blot

After treatment, H9c2 cells were washed in PBS. 120uL of lysis buffer [0.5 M tris-hydrochloride pH 6.8, 2% (vol/vol) SDS, 15% (vol/vol) glycerol, 10% (vol/vol) 2-mercaptoethanol and protease inhibitor cocktail] was added to each well and cells were scraped off. The lysates were then incubated at 95C for 10 minutes. Approximately 35ug of

protein was loaded to each well. Samples were analyzed by SDS-PAGE and transferred to a PVDF membrane. After blocking 1 hour in 3% BSA, specific primary antibodies, LC3B and b-actin (1:1000, Cell Signaling, MA), were used. Then, HRP-linked anti-rabbit (1:5000, Cell Signaling, MA) was used as secondary antibody, and proteins were detected using enhanced chemiluminescence reagents. Band intensity was quantified using ImageJ software.

3.3.9 Statistical analysis

All data are presented as mean ± SEM. Statistical analysis was performed using Student's t-test. Differences between groups were considered statistically significant when $p<0.05$ and are indicated by symbols as described in figure legends.

3.4 Results

3.4.1 High glucose-induced apoptosis and adiponectin alleviation of high glucose-induced apoptosis in H9c2 cells

In order to determine H9c2 cell viability upon high glucose treatment, a series of different assays were used. Cells were treated with either normal glucose (NG; 5.5mM) or high glucose (HG; 25mM) for 24, 48, and 72 hours, and co-treated with adiponectin. The concentration of adiponectin used was based on our preliminary data, as well as those used in a previous publication [86]. First, cells were incubated with either NG or HG media for 72 hours, and it was found that there was significant increase in trypan blue-stained cells in the HG-treated condition (**Figure 3.1-A**). We confirmed this finding in another apoptosis assay by using Annexin V/PI staining. In early apoptosis, phosphatidylserine (PS) is externalized to the outer membrane of the phospholipid bilayer. Cells started to expose PS to the external membrane after 24 hours of incubation with HG media, as was shown by FITC-annexin V binding (**Figure3.1-B**). Cells were counterstained with propidium iodide(PI) to distinguish between apoptosis and necrosis. The viability of H9c2 cells was also confirmed by TUNEL assay, where there were more TUNEL-stained cells observed under HG conditions (**Figure 3.1-C**). Finally, we assessed activation of caspase-3 in order to elucidate the form of cell death induce by hyperglycemia. A significant increase in caspase-3 activity was detected upon 48 hours of treatment with HG media (**Figure 3.1-D**). These results showed that treatment with adiponectin significantly abrogated the effects of hyperglycemia on H9c2 cell viability.

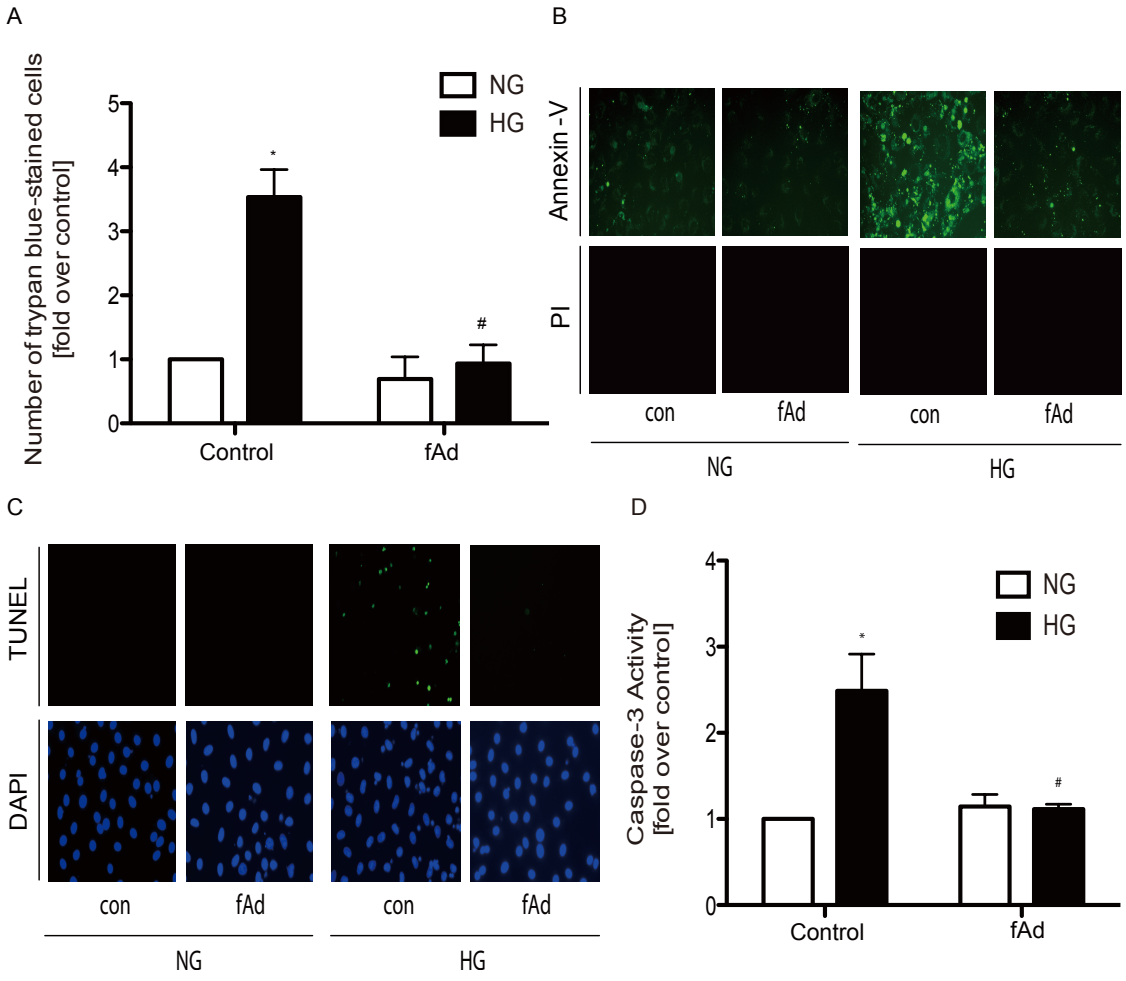

Figure 3.1: Adiponectin treatment attenuated high glucose-induced apoptosis in H9c2 cells H9c2 cells were grown in 10% FBS, then treated with either NG or HG in 0.5% FBS with or without fAd (5.0ug/mL) when 80% confluence reached. Then (A) Cell viability was assessed by trypan blue exclusion assay for 72 hr treatment. Number of dead cells for HG are normalized to NG incubation. (B) Annexin V binding assay shows PS exposure to the outer membrane. Annexin V is shown in green and PI is shown in red fluorescence. (C) TUNEL assay with DAPI were performed for DNA fragmentation analysis (D)Activity of caspase-3 were performed using colorimetric assay. (n=>3) * indicates $p<0.05$ vs NG.

3.4.2 High glucose- induced H9c2 cells undergo apoptosis through the intrinsic pathway

Cytochrome C is considered to be a pro-apoptotic marker when found outside of mitochondria. To examine whether HG induced the intrinsic pathway of the apoptosis, co-localization of cytochrome C and mitochondria was performed. As seen in merged images, brighter yellow appeared in NG conditions compared to dominant orange to red in HG (**Figure 3.2-A**). The results were quantified by Pearson's coefficient and there was a significant decrease in the HG condition, which means there was less co-localization (**Figure 3.2-B**). These results suggest that apoptosis is activated through intrinsic pathway upon hyperglycemia in H9c2 cells.

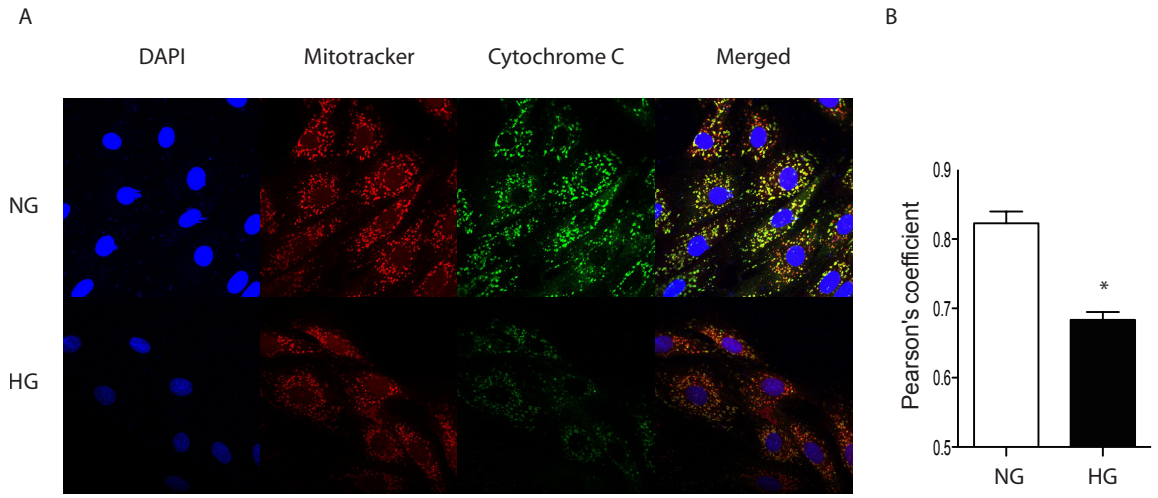

Figure 3.2: High glucose inducing intrinsic apoptotic pathway H9c2 cells were grown in 10% FBS, then treated with either NG or HG when 80% confluence reached. Representative images to show single slice image for DAPI (Blue), mitotracker (Red), Cytochrome C (Green), and Merged. In NG there are more co-localization of cytochrome C with mitochondria represented by dominant yellow puncta compared to HG with dominant orange to red signals. Images were analyzed using Pearson's coefficient for their co-localization (B). * indicates significant difference from NG (n=3) $p<0.05$.

3.4.3 High glucose blocked autophagy, but adiponectin restored autophagy

Autophagy was examined using Western blotting to look at the expression level of proteins that are involved in autophagy flux. The level of the lipidated form of LC3, LC3II, is a hallmark of autophagosome formation. In HG conditions, the level of LC3II increased compared to the NG condition. This level was then reduced when adiponectin was supplemented (**Figure 3.3 A,B**).

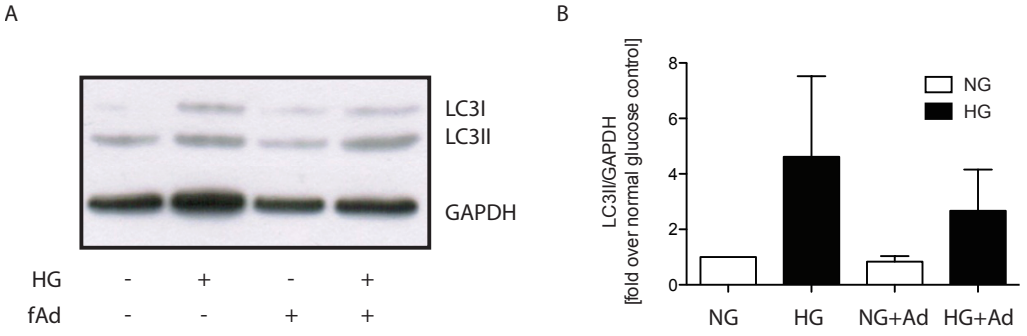

Figure 3.3: High glucose disturbing autophagy flux H9c2 cells were grown in 10% FBS, then treated with either NG or HG with or without fAd (5ug/mL) when 80% confluence reached. Western blot showing LC3B and loading control GAPDH (A) and its quantification for lipidated form of LC3B, LC3II over GAPDH (B). (n=3) No significant difference observed.

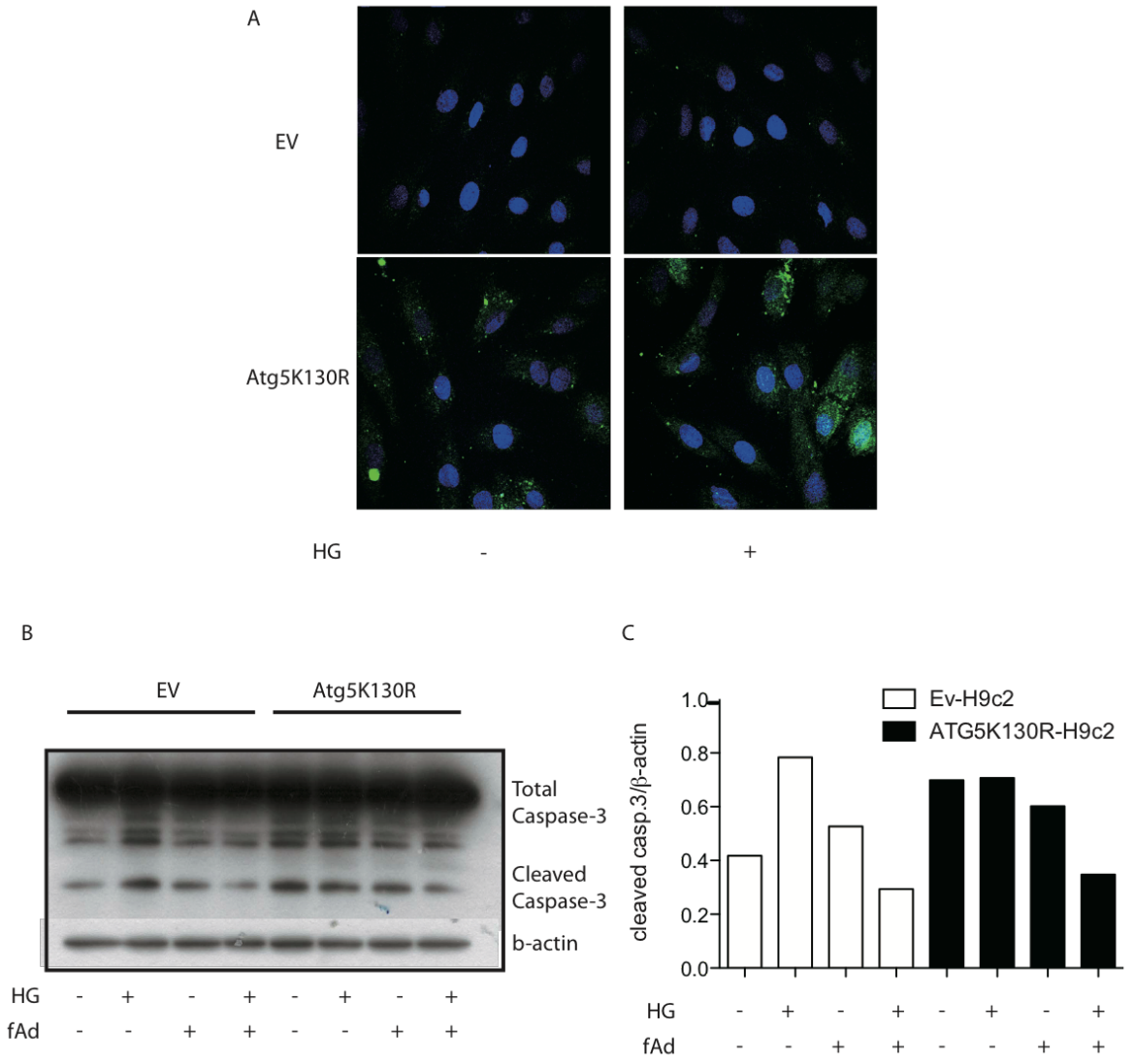

Figure 3.4: Adiponectin reduced HG-induced apoptosis even in autophagy deficient cells. Atg5K130R and EV- H9c2 cells were grown in 10% FBS, then treated with either NG or HG when 80% confluence reached. Representative images to show increased activation of caspase-3 (Green) in HG condition, using CellEvent Caspase-3/7 immunofluoresence (A). Atg5K130R and EV- H9c2 cells were grown in 10% FBS, then treated with either NG or HG with or without fAd (5ug/mL). Western blot showing caspase-3 and loading control beta actin (B) and its quantification for cleaved caspase-3 over beta-actin (C) (n=1).

3.4.4 Adiponectin reduced HG-induced apoptosis even in autophagy deficient cells

To investigate the role of autophagy in cardiomyocyte cell death, autophagy deficient H9c2 cells were generated. Cells were transduced with a retrovirus to stably overexpress a mutant Atg5 gene. Empty vector (EV) was used as control for the experiment. Caspase-3/7 activity assay using immunofluorescence showed that there was an increased level of activation in $Atg5^{K130R}$ cells compared to EV. In the response HG, there was a further increase in activity. To study the mechanism behind the effect of adiponectin activating autophagy to reduce apoptosis, I compared caspase-3 levels by western blot. After 24 hour HG treatment there was an increased level of capsase-3 activity observed in EV but not in $Atg5^{K130R}$ cells. In response to adiponectin, HG-induced caspase-3 activation was reduced in both cell types.

3.5 Discussion

Hyperglycemia is a key feature in diabetic patients and animals. This can be problematic, as it is known that hyperglycemic conditions cause cell death in cardiomyocytes, as well as other cell types. However, this cell death could potentially be prevented by a hormone that is secreted by adipocytes, adiponectin [58]. In this project, I confirmed that HG conditions trigger cell death in H9c2 cells, but this was attenuated with adiponectin co-treatment. My overall goal was to determine the mechanism behind this phenomenon, and so far, I have found that this occurs through the induction of autophagy.

My experiments showed that adiponectin could significantly reduce hyperglycemia-induced cell death in cardiomyocytes. First of all, H9c2 cells responded to HG conditions with increased cell death. Specifically, it induced the intrinsic pathway of apoptosis. Consistent with my data, previous research in mesangial cells as shown that HG induced the intrinsic pathway of apoptosis by stimulating cytochrome c release from mitochondria [87]. A large number of mitochondria can be found in cardiomyocytes [29]. This might be the reason why HG-stimulated damage to mitochondria leads to activation of the intrinsic pathway of apoptosis. However, this HG-induced apoptosis was attenuated when cells were co-treated with adiponectin. Adiponectin is known to have cardioprotective effects against ischemia-reperfusion injury [88], atherosclerosis, and inflammation [63]. However, it was not directly proven that adiponectin has a beneficial effect under hyperglycemic conditions. My research shows that adiponectin significantly reduced cell death in cardiomyocytes exposed to chronic HG conditions.

One of the possible mechanisms underlying this phenomenon is autophagy. I found that it was impaired by HG treatment, but restored upon co-treatment with adiponectin. Autophagy is a very important mechanism to maintain cardiac homeostasis. Adiponectin is

known to stimulate AMPK, a critical protein for the maintenance of cellular homeostasis known to be involved in the initiation of autophagy. I hypothesized that there might be link between autophagy and adiponectin function in the prevention of apoptosis. Even though I did not find direct activation of AMPK by adiponectin in H9c2 cells, I found that autophagy was restored by adiponectin treatment under HG conditions.

To investigate the link between autophagy and apoptosis, an autophagy-deficient cardiomyocyte cell line, $Atg5^{K130R}$, was used. There was an increase in casapse-3 activity in $Atg5^{K130R}$ cells at baseline, and especially more death was observed when cells were exposed to HG. This was confirmed by an increased number of punctae in $Atg5^{K130R}$ cells using caspase-3/7 activity immunofluorescence. It has been previously shown that if autophagy is impaired, it could lead to cardiomyopathy [29]. However, strikingly, even in autophagy-deficient cells, adiponectin reduced hyperglycemia-induced caspase-3 activation. This suggests that adiponectin may function through a different pathway when autophagy is not fully functioning.

In conclusion, apoptosis induced by hyperglycemic conditions in H9c2 cells was attenuated with adiponectin treatment. However, further studies on the effect of adiponectin and autophagy on reducing apoptosis are definitely warranted.

For future studies, more autophagy markers need to be studied such as pAMPK, pULK1, and p62. In order investigate initiation of the autophagy pathway, it is important to determine the related status of phosphorylated AMPK and mTOR activity.

Modulating autophagy can be a therapeutic target for CVDs. Through my research, I propose that adiponectin is one of the key hormones that can mediate this pathway.

Chapter Four: General Conclusion and Future Direction

4.1 Summary

Metabolic syndrome is a group of risk factors that collectively contribute to increased risk of serious diseases such as CVD. High glucose and high lipid levels in the blood, and high blood pressure are some of examples of risk factors that fall into this category. Additionally, studies now show that iron overload can also be a new factor to predict the development of CVD and diabetes. Generally, the level of iron storage in the body is correlated with development of T2D [40]. And patients with T2D have limitation in glucose metabolism in heart and skeletal muscle [89]. Previously it was focused on oxidative stress in skeletal muscle and cardiac muscle because those play important role carrying and utilizing oxygen for glucose metabolism especially during exercise. Both in skeletal muscle and cardiac muscle, there are high demand for mitochondria due to metabolic demand for their function [90].

During my master's study, using two different types of muscle cells, I investigated the mechanisms involved in metabolic syndrome-induced cell death. There are three types of specialized myocytes- cardiac, skeletal, and smooth muscle cells. Among them, cardiomyocytes and skeletal muscle cells have in common that they are both striated cells. This striation is important for cells to have independent, but harmonized function where some cells contract, and the others relax.

My study proved that upon the stimuli, iron overload and high glucose, the apoptosis activation increased with mitochondria-dependent pathway. Mitochondria are important in terms of maintaining homeostasis in cellular level such as ROS signaling, intrinsic pathway of apoptosis regulation, and energy production [91]. Especially, the importance of mitochondria in oxidative phosphorylation and ATP production is widely acknowledged.

They utilize fatty acid or glucose as energy source. However, under detrimental stimuli, such as excessive generation of ROS, they can be damaged and cause mitochondrial dysfunction [92]. In my first project, I observed that prolonged iron overload conditions induced cell death mediated by the intrinsic apoptosis pathway in skeletal muscle cells. In this model, disturbed mitochondrial membrane potential resulted in release of cytochrome C into the cytosol, acting as pro-apoptotic molecule. Similarly, in cardiac and neuronal cells, iron overload-induced apoptosis has been shown to be mediated through a mitochondria-caspase-3 dependent pathway as well [50]. Therefore, iron overload can definitely trigger the intrinsic pathway of apoptosis not only in skeletal muscle cells, but many different cell types. In my second project, I also added on potential therapeutic target, adiponectin, on HG- induced cell death in cardiac muscle cells. Adiponectin is an adipokine that has been shown to have beneficial roles in many cardiac remodelling events such as inflammation, atherosclerosis, and diabetes [58]. I determined that exposure to prolonged HG conditions resulted in increased cell death at different time points, and adiponectin significantly reduced this event. I also demonstrated that this activation of cell death was possibly through the intrinsic pathway of apoptosis by showing reduced co-localization of cytochrome C and mitochondria.

Sarcopenia and heart failure are common outcome of decline in quality of skeletal muscle cells and cardiac muscle cells, respectively. Sarcopenia is a condition of reduced muscle mass [21] and heart failure is a condition of loss of contractility [93]. In skeletal muscle, this muscle dysfunction will be resulted by reduced oxidative phosphorylation [94]. Malfunctioning heart will have reduced oxidative phosphorylation, as well as decreased number of mitochondria and their enzyme [95, 96]. Further these losses of cellular function are closely related to aging. Aging can be defined many different ways. In terms of cells, it

can be defined as changes in metabolism [92] or imbalance in homeostasis.

Because autophagy is now a well-known cell survival mechanism, I proposed that iron overload and high glucose could disrupt normal autophagy to cause cell death. Especially in skeletal and cardiac myocytes, autophagy is the crucial process to prevent cells from accumulating damaged or dysfunctional proteins and organelles [97]. Having impaired autophagy in muscle and heart can cause sarcopenia and cardiomyopathy [98]. In the context of autophagy, I observed that significant autophagosome induction. Increase in autophagosomes can be reflective of either an increase in autophagy or reduction of autophagosomal degradation; however, I witnessed that many have found increased autophagosome level was indication of blockage of autophagic flux [99, 100]. This is one of limitation to my study, that in both projects, autophagic degradation was not measured. One might argue no definite conclusion can be made at this point, so this can be elucidated by further study. Benefit to learn autophagy in skeletal muscle and cardiac muscle is that it can be applied to exercise training, potentially change lifestyle to overcome metabolic syndrome. [101].

Lastly, in order to examine other possible pathways that cause cell death in skeletal muscle cells with iron overload oxidative stress was studied. Previously researchers have found that production of ROS through the Fenton reaction was mediated by iron, and levels of ROS were elevated in iron-overload patients. Overproduction of ROS causing apoptosis is extensively studied, especially in relation to damage to mitochondria. Also, cells can induce autophagy to remove damaged proteins and organelles to protect the cell against oxidative stress. However, crosstalk between autophagy and oxidative stress to induce apoptosis in iron overload conditions is not yet well studied. Therefore, I propose that future studies should investigate their relationship and mechanism(s) to induce cell

death.

4.2 Future direction

Further studies can be conducted in order to have better understanding of the mechanisms responsible for myocyte apoptosis in metabolic syndrome. For future studies, the relationship between oxidative stress and iron overload-induced cell death can be studied. Previous literature has shown that co-treating with iron and the antioxidant reagent N-Acetyl-Cysteine (NAC) for 120 hours prevented cell death in osteoblasts [102]. My expectation is that a similar phenomenon will be observed in skeletal muscle cells, where preventing oxidative stress results in the prevention of cell death. Then, crosstalk between autophagy and oxidative stress to trigger apoptosis by iron overload can be investigated. The effect of iron overload on oxidative stress when autophagy is deficient can be studied using the same cell line, $Atg5^{K130R}$.

In terms of studying the effect of adiponectin on alleviating cell death via controlling the autophagic pathway, detailed analysis of the autophagic pathway can be performed. There are many steps in autophagy that can be regulated. Some studies have shown that adiponectin regulates in initiation step when AMPK phosphorylation occurs [103]. Other studies have pointed out that beclin-1 is important protein to regulate crosstalk between apoptosis and autophagy [67]. Additionally, other pathways that adipoenctin can activate to reduce apoptosis can also be investigated.

Also, *In vivo* experiments can be done for future studies to complement *in vitro* studies. In my lab, we have designed autophagy knockout model mice, which is specific to heart. Further study can be conducted using heart sample from that model, to look at the apoptotic activity when the adiponectin is supplemented. With inducing iron overload in

this specific mice model, skeletal muscle samples can be obtained to measure apoptotic activity. And further study to learn mitochondria specific characteristic such as mitochondria biogenesis and mitophagy since as mentioned previously, mitochondria play important role in metabolic syndrome induced cell death.

Taken together, results from my *in vitro* studies and previously published data suggest the importance of autophagy in regulating apoptosis. Therefore, this provides potential therapeutic target to reduce apoptosis and protect different types of muscles in optimal condition. Iron overload and hyperglycemic condition induced mitochondria-dependent intrinsic apoptotic pathway and reduced autophagy in skeletal muscle cells and cardiomyocytes respectively. Meanwhile, co-treatment of adiponectin in hyperglycemia condition altered this event by inhibiting apoptosis. It is not clear which is the cause or effect, as there is a correlation between iron metabolism and glucose metabolism. Iron can modulate glucose metabolism and glucose metabolism can impact the iron pathway [40]. For this reason, thorough investigation of how to stimulate or inhibit underlying mechanisms as therapeutic target to reduce disease associated with myocyte apoptosis, sarcopenia and heart failure in metabolic syndrome would be appreciated.

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Appendix B: List of Contributions

In chapter 2, majority of the research and thesis writing were performed by myself, Hee Ho Cho (HC) with exception of preliminary screening to show iron overload inducing apoptosis and oxidative stress was contributed by James Jahng (JJ).

Figure 2.1C: performed by JJ

Figure 2.4E: performed by JJ

Figure 2.5: performed by JJ

In chapter 3, majority of the research and thesis writing were performed by myself, Hee Ho Cho (HC) based on preliminary evidence to show beneficial effect of adiponectin HG induced cell death was performed and quantified by Megumi Eguchi (ME), a previous member of our lab planned and started the project but left unfinished.

Figure 3.1 A, C, E: performed by ME