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Increased Mitochondrial Calpain-1 Is an Important Mechanism Contributing to Mitochondrial ROS Generation in Cardiac Diseases

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Abstract

Both calpain activation and excessive mitochondrial reactive oxygen species (mtROS) have been implicated in the pathogenesis of cardiac diseases. We investigated whether and how calpain regulates mtROS generation in mediating cardiac diseases.

In mouse models of streptozotocin-induced type-1 diabetes and lipopolysaccharides- induced sepsis, we show that the protein levels of calpain-1 and calpain activities in mitochondria were significantly elevated in diabetic and septic hearts. The elevation of mitochondrial calpain-1 correlated with an increase in mtROS generation and oxidative damage. Importantly, cardiomyocyte-specific deletion of *capns1* disrupted calpain-1 and calpain-2 in the heart and prevented mtROS generation in both septic and diabetic mouse hearts. As a consequence, cardiomyopathic changes (e.g. cardiac apoptosis, hypertrophy and fibrosis) and myocardial dysfunction were attenuated in diabetic or septic *capns1* knockout mice compared with their wild-type littermates. Mechanistically, we demonstrate that mitochondrial calpain-1 directly targeted and cleaved ATP synthase subunit-alpha (ATP5A1), leading to a reduction in ATP synthase activity in diabetic hearts and septic hearts, and that up-regulation of ATP5A1 restored ATP synthase activity, prevented mtROS generation and reduced cardiomyopathic changes in type-1 diabetic mice and in septic mice. In addition, selective inhibition of mtROS with mitochondria-targeted antioxidant mito-TEMPO prevented mtROS production and intracellular oxidative stress, reduced cardiomyopathic changes and improved myocardial function in mouse models of type-1 and type-2 diabetes. These *in vivo* data were recapitulated in cultured cardiomyocytes stimulated with diabetic and septic conditions.

In summary, we have provided strong evidence demonstrating that calpain-1 accumulation in mitochondria disrupts ATP synthase through the proteolysis of ATP5A1 and promotes mtROS generation, both of which contribute to diabetic cardiomyopathy and septic

cardiomyopathy. Given that mitochondrial calpains also increase and contribute to myocardial injury in ischemic hearts, our findings suggest that increased mitochondrial calpain-1 may be a common mechanism contributing to mtROS generation and myocardial injury in the pathogenesis of cardiac diseases. Thus, targeted inhibition of mitochondrial calpain may be a potentially effective therapy for cardiac diseases.

Keywords

ATP synthase, calpain, cardiac disease, cardiomyopathy, diabetes, mitochondria, reactive oxygen species, sepsis

Co-Authorship Statement

The studies in chapters 2, 3 and 4 were performed by Rui Ni in the laboratory of Dr. Tianqing Peng, with assistance of the co-authors named below.

Chapter 2 is adapted from **Ni R**, Zheng D, Wang Q, Yu Y, Chen R, Sun T, Wang W, Fan GC, Greer PA, Gardiner RB, and Peng T. (2015) Deletion of capn4 Protects the Heart Against Endotoxemic Injury by Preventing ATP Synthase Disruption and Inhibiting Mitochondrial Superoxide Generation, *Circ Heart Fail 8*, 988-996. Zheng D, Wang Q, Yu Y, Sun T and Gardiner RB assisted with performing experiments. Chen R contributed to the discussion and reviewed and edited the manuscript. Fan GC reviewed and edited the manuscript. Greer PA and Wang W contributed materials and to the discussion. Peng T contributed to the conception and design of the study, assisted with data analyses, and wrote the manuscript.

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conception and design of the study; Xiong S, Fan GC, Lacefield JC and Peng T assisted with data interpretation and analyses, and revised the manuscript.

Dedication

I dedicate this to my father Ji-Yun Ni and mother Xue-Qun Li, as well as my Ph.D. supervisor Dr.Tianqing Peng

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List of Abbreviations

ACE	Angiotensin-converting enzyme
ADP	Adenosine diphosphate
Ad-ATP5A1	Adenoviral vectors containing human ATP5A1 gene
Ad-gal	Adenoviral vectors containing beta-gal gene
Ad-GFP	Adenoviral vectors containing GFP gene
Ad-mtCAST	Adenoviral vector containing mitochondria-targeted calpastatin
Ad-mt-cpYFP	Adenoviral vector expressing mt-cpYFP
AGEs	Advanced glycation end products
AIF	Apoptosis-inducing factor
AMPK	Adenosine monophosphate-activated protein kinase
ANP	Atrial natriuretic peptide
ARB	Angiotensin receptor blocker
ARE	Antioxidant response element
α-SMA	Alpha-smooth muscle actin
ATP	Adenosine triphosphate
ATP5A1	ATP synthase subunit-alpha
β-MHC	β-myosin heavy chain
BNP	brain natriuretic peptide
cDNA	Complementary deoxyribonucleic acid
CHOP	CCAAT/enhancer-binding protein homologous protein
co-IP	Co-immunoprecipitation
cpYFP	Circularly permuted yellow fluorescent protein
CSS2	Calpain small subunit 2
CuZnSOD	Copper-zinc SOD

CVD	Cardiovascular disease
DCF-DA	2,7-dichlorodihydro-fluorescein diacetate
DHE	Dihydroethidium
DNA	Deoxyribonucleic acid
GFP	Green fluorescent protein
ECM	Extracellular matrix
ECMO	Extracorporeal membrane oxygenation
EF	Ejection fraction
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinase-1/2
ETC	Electron transport chain
FADH2	Flavin adenine dinucleotide
FFAs	Free fatty acids
FS	Fractional shortening
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLP-1	Glucagon-like peptide-1
GPX	Glutathione peroxidase
GR	Glutathione reductase
GRP78	Glucose regulated protein78
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
HRP	Horseradish peroxidase
HSP	Heat shock protein
IABP	Intra-aortic balloon pump
IκB	Inhibitor-κB

IKK	IκB kinase
IL	Interleukin
i.p.	Intraperitoneal
IR	Insulin resistance
I/R	Ischemia/reperfusion
IRS-1	Insulin receptor substrate-1
JNK1/2	c-Jun NH2-terminal kinase-1/2
KEAP1	Kelch ECH associating protein 1
KO	Knockout
LPO	Lipid peroxidation
LPS	Lipopolysaccharides
LV	Left ventricle
LVIDd	LV end-diastolic inner diameter
LVIDs	LV end-systolic inner diameter
MAPK	Mitogen-activated protein kinase
MDF	Myocardial depressant factor
MI	Myocardial infarction
mito-TEMPO	Mitochondrial targeted antioxidant (2-(2,2,6,6-tetramethylpiperidin -1-oxyl-4-ylamino)-2-oxoethyl) triphenylphosphonium chloride
MMPs	Matrix metalloproteinases
MnSOD	Manganese superoxide dismutase
mtDNA	Mitochondrial DNA
mtROS	Mitochondrial reactive oxygen species
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NCX-1	Na^{+}/Ca^{2+} exchanger-1
NF-AT	Nuclear factor of activated T-cells

NF-κB	Nuclear factor-κB
NLRP3	Nucleotide-binding oligomerization domain-like receptor protein 3
NO	Nitric oxide
NOX	NADPH oxidase
Nrf2	Nuclear erythroid-related factor 2
$^{\bullet}O_2^-$	Superoxide radical
$^{\bullet}OH$	Hydroxyl radical
OH^-	Hydroxide anion
$ONOO^-$	Peroxynitrite
Opa-1	Optic atrophy-1
PARP	Poly ADP-ribose polymerase
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PLB	Phospholamban
qRT-PCR	Real-time reverse transcriptase PCR
RAAS	Renin-angiotensin-aldosterone system
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SIRS	Systemic inflammatory response syndrome
SOD	Superoxide dismutase
TIMPs	Inhibitor of metalloproteinase
Tg-CAST	Calpastatin transgenic mice
Tg-mtcpYFP	Transgenic mice over-expressing a circularly permuted yellow fluorescent protein in the mitochondrial matrix of cells
TGF-β1	Transforming growth factor- beta
TLRs	Toll like receptors

TNF-α	Tumor necrosis factor-alpha
SERCA	sarcoplasmic reticulum calcium ATPase
SS	Szeto-Schiller
SS31	6’-dimethyltyrosine-Lys-Phe-NH2
STZ	Streptozotocin
VDAC	Voltage-dependent anion channel
VEGF	Vascular endothelial growth factor
XO	Xanthine oxidase
8-oxoG	8-hydroxyguanine

Chapter 1

1 General introduction [1]

1 Parts of this chapter have been modified and adapted from the following manuscript:

- Ni R, Zheng D, Wang Q, Yu Y, Chen R, Sun T, Wang W, Fan GC, Greer PA, Gardiner RB, and Peng T. (2015) Deletion of capn4 Protects the Heart Against Endotoxemic Injury by Preventing ATP Synthase Disruption and Inhibiting Mitochondrial Superoxide Generation, *Circ Heart Fail 8*, 988-996.
- Ni R, Zheng D, Xiong S, Hill DJ, Sun T, Gardiner RB, Fan GC, Lu Y, Abel ED, Greer PA, and Peng T. (2016) Mitochondrial Calpain-1 Disrupts ATP Synthase and Induces Superoxide Generation in Type 1 Diabetic Hearts: A Novel Mechanism Contributing to Diabetic Cardiomyopathy, Diabetes 65, 255-268.
- Ni R, Cao T, Xiong S, Ma J, Fan GC, Lacefield JC, Lu Y, Le Tissier S, and Peng T. (2016) Therapeutic inhibition of mitochondrial reactive oxygen species with mito-TEMPO reduces diabetic cardiomyopathy, Free Radic Biol Med 90, 12-23.

1.1 Introduction of cardiovascular disease

Cardiovascular disease (CVD) includes cardiac disease and vascular disease, referring to a range of diseases affecting the circulatory system, such as coronary artery disease, congenital heart disease, cardiomyopathy, rheumatic heart disease, atrial fibrillation, hypertensive heart disease, aortic aneurysms, endocarditis, peripheral artery disease and venous thrombosis [1, 2]. CVD remains the major cause of death globally. Each year, more than 17 million people die from CVD which accounts for 30% of all global deaths [3]. It is also anticipated that by 2030, CVD-related deaths will increase to more than 23 million each year [1]. In Canada alone, approximate 70,000 individuals die directly associated with CVD every year [4]. There are 1.3 million Canadians with CVD at a conservative estimate [5]. Approximately 50,000 new patients [6] are diagnosed with heart failure each year and in total about 600,000 Canadians suffering it [7]. Each year, the Canadian economy spends almost $20.9 billion on CVD in healthcare system costs, decreased productivity and lost wages [8].

Risk factors for CVD include hypertension, tobacco use, lack of physical activity, obesity, high blood cholesterol, poor diet, excessive consumption of alcohol, diabetes and inflammation [5] as well as genetic susceptibility [9]. In Canada, about 80% of individuals have at least one of these risk factors, and around 10% are exposed to three or more [5]. The most above risk factors are preventable. According to the report from World Health Organization, for the purpose of disease management, risk factor modification is the first recommendation, which can significantly decrease the number of people with premature cardiac disease globally [10]. This includes weight loss, quitting smoking, more exercise, reductions of lipids, sodium, sugar and alcohol intake, and increase of vegetable and fruit consumption. In addition to the lifestyle changes, pharmacological therapeutic strategies are available to aid in the risk factors prevention,

such as anti-hypertensive therapy and lipid-lowering therapy. Nevertheless, CVD remain one of the biggest health challenges in the world.

Given that diabetes and inflammation are important contributors to cardiac disease, this thesis is focused on diabetic cardiomyopathy and septic cardiomyopathy. As such, I introduce these two types of cardiac disease in more details as follows.

1.1.1 Diabetic cardiomyopathy

1.1.1.1 The definition and characteristics of diabetic Cardiomyopathy

Diabetes represents a major threat to global public health [11]. Globally, the number of adults affected with diabetes is estimated to increase from 135 million in 1995 to 300 million by 2025[12]. Thus, diabetes is projected to become one of the world's main disablers and killers within the next twenty years. The direct costs are extremely high because it is a chronic disease with severe complications [13]. Furthermore, total financial burden includes indirect costs of lost productivity due to sickness, disability and premature retirement of working-age patients [14]. There are two basic types of diabetes [15]. Type 1 diabetes is an autoimmune disease in which the body's immune defense system mistakenly attacks and destroys the insulin-producing beta cells of the pancreas, which are essential for processing sugar (glucose). Patients with type-1 diabetes require exogenous insulin to maintain the blood glucose levels. Approximately, 10 % of patients with diabetes are type-1 diabetes [16]. In type-2 diabetes, the more prevalent form, patients may produce insulin, but they cannot use it effectively, resulting in high levels of glucose in the blood, but low levels of glucose inside cells that need it. Type-2 diabetes is associated with several risk factors, including older age, a family history of diabetes, and obesity. Both type-1 and type-2 diabetes share the same possible complications, including retinopathy, nephropathy, neuropathy, lower limb amputations, sexual dysfunction and

cardiovascular disease, particularly heart attack and stroke that can lead to premature death [17, 18].

Cardiovascular complications are the most common cause of morbidity and mortality in diabetic patients. Approximately 80% of all diabetic patients die of cardiovascular complications [19, 20]. Cardiac complications are mostly due to two pathophysiological processes. First, coronary (ischemic) heart disease is increased as a consequence of accelerated atherosclerosis associated with risk factors such as visceral obesity, hypertension, dyslipidemia, and prothrombotic factors [21-24]. Second, diabetes can affect cardiac structure and function in the absence of changes in blood pressure and coronary artery disease, a condition called diabetic cardiomyopathy [25].

Clinical reports in the 1970s first described such patients, who were considered to have a diabetic cardiomyopathy [26]. Since then, diabetic cardiomyopathy has been defined as ventricular dysfunction that occurs independently of coronary artery disease and hypertension [25, 27]. In addition, diabetic cardiomyopathy may be characterized by diastolic dysfunction [28, 29], which is more apparent in the presence of myocardial ischemia or hypertension [26]. Of far greater epidemiologic importance, however, is the risk when diabetes is combined with coronary artery disease and/or hypertension [23]. Diabetes patients with acute myocardial infarction have approximately twice the incidence of heart failure and death compared to non-diabetic patients [30-32]. The development of heart failure in patients with diabetes is a problem of major clinical and epidemiologic importance [33]. Diabetic cardiomyopathy is patho-physiologically characterized by the loss of cardiomyocytes, cardiac hypertrophy, fibrosis and inflammation [34-37]. The pathogenesis of diabetic cardiomyopathy has not been fully understood (Figure 1-1) but seems multifactorial, which includes, but not limited to, autonomic dysfunction, metabolic disorders, alteration in structural proteins, disturbance of ion homeostasis,

myocardial hypertrophy and interstitial fibrosis [38, 39]. In addition, increased glycation of interstitial proteins such as collagen resulting from sustained hyperglycemia promotes myocardial stiffness and impairs contractility [40-42]. At present, therapeutic approaches are limited.

1.1.1.2 The underlying mechanisms of diabetic cardiomyopathy

1.1.1.2.1 Metabolic dysfunction in cardiomyocytes

1.1.1.2.1.1 Hyperglycemia

Hyperglycemia reduces glucose clearance, increases gluconeogenesis, and plays an important role in the pathogenesis of diabetic cardiomyopathy (Figure 1-1). Chronic hyperglycemia impairs myocardial cells, as well as fibroblasts and endothelial cells, in which multiple mechanisms have been proposed. Hyperglycemia increases the production of reactive oxygen species (ROS) in the heart [43-46]. ROS activates poly ADP-ribose polymerase (PARP) which increases glycosylation and inhibits glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and therefore, the glycolysis process turns into a biochemical cascade of myocardial injury [29, 47]. This includes elevated advanced glycation end products (AGEs), activations of the hexosamine biosynthesis pathway and the polyol pathway, as well as increased production of protein kinases (PKC) [48]. AGEs induce excessive collagen expression and accumulation, and promote collagen cross-linking, leading to myocardial fibrosis and consequently decreased myocardial compliance [49]. Under hyperglycemic state, the glucose transporter-4 activity is decreased, causing reduced transmembrane transportation of glucose to the cardiomyocytes, leading to decreased glucose uptake of myocardial cells, which then affects the energy metabolism of cardiomyocytes [50]. Increase of ROS, PARP, AGEs and aldehyde reductase by hyperglycemia are directly associated with myocardial apoptosis. Hyperglycemia also affects the structure and function of the

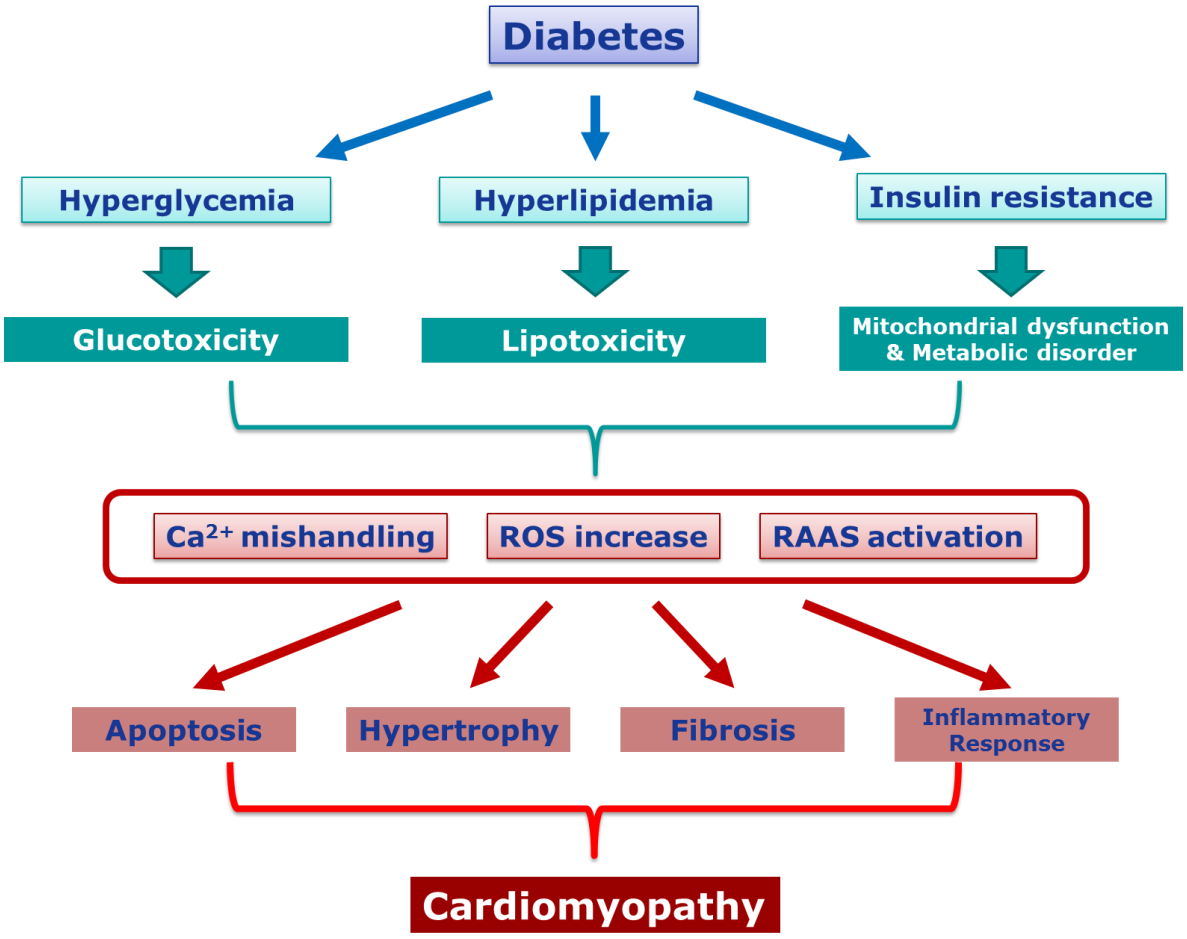

Figure 1-1. Schematic of mechanisms contributing to diabetic cardiomyopathy

The proposed mechanisms and their downstream consequences are summarized: hyperglycemia-induced glucotoxicity, hyperlipidemia-induced lipotoxicity and Insulin resistance-induced mitochondrial dysfunction and metabolic disorder contribute to ROS production, RAAS activation, calcium disorder, etc. all of which promote cardiac apoptosis, hypertrophy, fibrosis and inflammation, leading to cardiomyopathy in diabetes. ROS, reactive oxygen species; RAAS, renin-angiotensin-aldosterone system.

myocardium by post-translational modifications [51]. For example, hyperglycemia induces structural changes in the extracellular matrix, as well as expression and function of ryanodine receptors and sarcoplasmic reticulum calcium ATPase (SERCA), leading to the myocardial systolic and diastolic dysfunction [29].

Hyperglycemia can introduce a number of changes in metabolism, particularly substrate utilization that could be an underlying mechanism leading to cardiomyopathy [52]. Although the normal heart is able to derive energy from multiple substrates including ketones, amino acids, carbohydrates and fatty acids, approximately 70% of adenosine triphosphate (ATP) generation occurs via fatty acid oxidation and the remaining 30% is provided by glucose and lactate [53]. The diabetic myocardium however, uses fatty acid oxidation almost exclusively for energy production.

1.1.1.2.1.2 Increased free fatty acids

Free fatty acids (FFAs) are important energy substances for the heart. Approximately two-thirds of the energy production comes from fatty acid oxidation in the normal heart. In diabetic patients, glucose utilization is significantly decreased and fatty acid β-oxidation increased in cardiomyocytes, while fatty acid synthesis is increased in hepatocytes, and fatty acid catabolism is elevated in adipocytes, leading to high levels of triglyceride glycerol and FFAs in the circulation [54, 55]. Meanwhile, hyperinsulinemia and hyperlipidemia induce accumulation of fatty acids in cardiomyocytes exceeding the cellular fatty acid oxidation capacity, leading to cardiac lipotoxicity [56] (Figure 1-1). Excessive intake and oxidization of fatty acids cause the accumulation of metabolic intermediate products of fatty acids in myocardial cells [57, 58], increase the oxygen demand and elevate mitochondrial membrane potential, resulting in increased ROS generation, decreased ATP synthesis, mitochondrial dysfunction and finally apoptosis in cardiomyocytes [59-61]. Increased oxidized fatty acids also induce production of

ceramide which is a sphingomyelin that can induce cardiomyocyte apoptosis by inhibition of mitochondrial respiratory chain [62, 63].

1.1.1.2.1.3 Disorder of calcium homeostasis

Precise regulation of calcium homeostasis in cardiomyocytes is the core link to ensure cardiac function. In diabetic hearts, increased intracellular fatty acid levels cause potassium channels opening, leading to shortening of action potential duration and L-type calcium channel opening, which eventually affects the calcium storage of sarcoplasmic reticulum, contributing to the disturbance of calcium homeostasis [64-66]. In addition, ROS-induced endoplasmic reticulum stress causes intracellular calcium accumulation leading to reduced myocardial cell contraction [67]. ROS also modulates L-type calcium channel to inhibit Ca^{2+} influx and suppresses SERCA activation [68]. Activities of SERCA and its inhibitor phospholamban (PLB) play important roles in maintaining calcium homeostasis in cardiomyocytes [69]. PLB is a key regulator of SERCA activity and cardiac contractility by modulating sarcoplasmic reticulum calcium sequestration. Study has shown that in diabetic rat hearts, the messenger RNA (mRNA) and protein levels of PLB were significantly increased while SERCA activity and sarcoplasmic reticulum calcium concentration were decreased, leading to cytoplasmic calcium overload, impaired ventricular relaxation and cardiac diastolic dysfunction [68]. Overexpression of SERCA2a in cardiomyocytes improved calcium homeostasis and myocardial contraction in diabetic rats [70].

1.1.1.2.1.4 Insulin resistance

Insulin resistance (IR) is an important risk factor for diabetic cardiovascular complications [71] (Figure 1-1). Cell insulin signals have two key signaling pathways. One is the insulin receptor substrate-1 (IRS-1) pathway, which is the upstream signal of phosphatidylinositol 3-kinase (PI3K)/Akt pathway, responsible for the major metabolic

response. The other is mitogen-activated protein kinase (MAPK) signaling pathway, which is related to vascular remodeling, cardiac hypertrophy, myocardial fibrosis, and cardiomyocyte apoptosis [72]. IR may accumulate fatty acids, inhibit IRS and Akt, thus reducing insulin-mediated uptake of glucose. Studies have shown that endothelial dysfunction caused by tumor necrosis factor-alpha (TNF-α) and excessive generation of ROS is an important mechanism of IR [73]. IR not only disturbs myocardial energy mechanism, but also directly impairs left ventricular structure and function [74]. IR and hyperinsulinemia have been shown to aggravate systemic metabolic disorders, activate the sympathetic nervous system and the renin-angiotensin-aldosterone system (RAAS), and induce oxidative stress, mitochondrial dysfunction, endoplasmic reticulum stress and calcium homeostasis disturbance, all of which contribute to myocardial fibrosis, cardiac hypertrophy, cardiomyocyte apoptosis, and coronary microcirculation dysfunction, eventually leading to heart failure [73].

1.1.1.2.2 Myocardial fibrosis and hypertrophy

1.1.1.2.2.1 RAAS system

The RAAS is well-known in the development of heart failure caused by various stress including diabetes [75, 76]. Studies have shown that RAAS system activation in diabetic patients is closely related to cardiac hypertrophy and fibrosis [77]. Angiotensin II activates angiotensin receptor-1 which is located on cardiomyocytes and cardiac fibroblasts, and induces increased collagen synthesis and reduced collagen decomposition, leading to cardiac hypertrophy and fibrosis which eventually result in decreased ventricular compliance and cardiac dysfunction [78]. At present, RAAS inhibitors such as angiotensin receptor blocker (ARB) and angiotensin-converting enzyme (ACE) inhibitor have been widely used to mitigate diabetic cardiac complications in clinical settings. Both are effective in decreasing morbidity and mortality in diabetic patients with cardiac

complications [79].

1.1.1.2.2.2 Matrix metalloproteinases (MMPs)

The homeostasis of myocardial extracellular matrix is dependent on collagen synthesis and degradation. It is well known that MMPs promote collagen degradation, whereas tissue inhibitor of metalloproteinase (TIMPs) inhibit MMPs and consequently prevent collagen degradation [80]. Thus, the balance between MMPs and TIMPs is important in maintaining collagen homeostasis and subsequent extracellular matrix homeostasis. In fact, up-regulation of MMP-2 and MMP-9 has been shown to play an important role in atherosclerosis, cardiomyopathy and congestive heart failure [81, 82]. Interestingly, studies have found that myocardial expression of MMP-2 is decreased in streptozotocin (STZ)-induced diabetic mice, causing reduced collagen degradation and promoting myocardial fibrosis [83]. It has also been found that in diabetic rat hearts, increased production of ROS interacts with cytoplasmic glutathione leading to activation of MMP-2 which cleaves PARP [84] and then induces apoptosis in cardiomyocytes through a mitochondrial pathway. The long-term high glucose stimulation induces an unbalance between MMPs and TIMPs, which disturbs homeostasis of extracellular matrix collagen synthesis and degradation, leading to myocardial fibrosis and finally ventricular systolic and diastolic dysfunction [80].

1.1.1.2.2.3 Cardiomyocyte apoptosis

Apoptosis is an evolutionarily conserved suicide process that plays critical roles in embryonic development and in the homeostasis, remodeling, surveillance, and host defenses of postnatal tissues [85]. Apoptosis is mediated mainly through two central pathways: the extrinsic (or death receptor) pathway and the intrinsic (or mitochondrial) pathway, which converge via activation of intracellular enzymes called 'caspases'. The caspase cascade eventually leads to the activation of the effector caspases, in particular,

caspase-3. These effector caspases are responsible for the typical morphological changes observed in cells undergoing apoptosis [86].

Cardiac myocyte apoptosis has been observed in the hearts of diabetic patients and mouse models of diabetes and is believed to promote diabetic cardiomyopathy [35, 37, 46, 87-89]. The diabetic heart may in fact be more susceptible to cardiac myocyte apoptosis as reports show that death from myocardial infarction is twice higher in diabetic patients than in non-diabetic patients [30-32]. Further studies have shown that hearts of diabetic patients with dilated cardiomyopathy had a pronounced increase in apoptotic cells than non-diabetic patients.

Zhang et al found that high glucose activated caspase-8 and caspase-9, the key promoters of cardiomyocyte exogenous and endogenous apoptotic pathways followed by activation of downstream apoptotic executioner caspase-3 and induced apoptosis in cultured neonatal mouse cardiomyocytes [90]. Studies from our lab also showed an increase in cardiac apoptosis in cultured cardiomyocytes and mouse hearts under diabetic conditions [91, 92]. In addition, studies have shown that endoplasmic reticulum stress is another mechanism of apoptosis in cardiomyocytes. Li et al found that in STZ-induced diabetic rats, glucose regulated protein78 (GRP78) expression was up-regulated, caspase-12 and CCAAT/enhancer-binding protein homologous protein (CHOP) were activated, which mediated endoplasmic reticulum stress and further induced apoptosis, and eventually leading to diabetic cardiomyopathy [93]. Cardiomyocyte death causes a loss of contractile tissue, directly leading to myocardial dysfunction. The loss of cardiomyocytes induces hypertrophy of the remaining cells, characteristic changes in diabetic cardiomyopathy. Thus, inhibition of cardiomyocyte apoptosis results in a significant prevention of the development of diabetic cardiomyopathy in animal models [94, 95].

1.1.1.2.2.4 Microvascular disease

Diabetic cardiomyopathy has been associated with myocardial microvascular abnormalities [96]. Diabetes impaired the stability of myocardial microvascular vessels in both diabetic human myocardial explants and experimental diabetes, and microvascular endothelial dysfunction was observed in pre-diabetes, which may explain the increased risk of complications of microvascular origin in pre-diabetes and early type-2 diabetes [97]. Of note, coronary microvascular rarefaction frequently occurs in diabetic hearts and contributes to diabetic cardiac complications [98]. Myocardial microvascular rarefaction compromises coronary circulation, which can directly impair myocardial function in diabetes. On the other hand, compromised coronary circulation may induce a condition of sub-ischemia in hearts, which initiates cardiomyocyte death and subsequent myocardial remodeling, characteristic changes in diabetic cardiomyopathy [99]. Thus, prevention of coronary microvascular rarefaction may be a useful strategy to reduce diabetic cardiac complications. However, the mechanism of diabetic microvascular disease in diabetic hearts has not been clear. Hyperglycemia, lipid metabolism disorder, oxidative stress, inflammatory response, reduced nitric oxide (NO) synthesis, endothelial damage, PPAR over-expression, platelet dysfunction and coagulation abnormalities can cause vascular endothelial cell damage or functional disorder, leading to increased vascular endothelial permeability and basement membrane thickening, which ultimately contribute to the development of diabetic cardiomyopathy [100]. Activation of PKC seems an important down-stream signaling mechanism, contributing to decreased nitric oxide (NO) production in the vascular endothelium and increased prostaglandin synthesis, both of which contribute to endothelial dysfunction. In addition, PKC regulates other protein kinase activity, such as PI3K/Akt and MAPK, causing vascular endothelial dysfunction and vasoconstriction [101]. Imbalance of vasoactive hormones causes endothelial damage and the capillary basement membrane thickening, and further leads to myocardial fibrosis

and reduced blood flow which contribute to early diastolic dysfunction and late contraction abnormality in the heart [102, 103].

1.1.1.2.2.5 Nrf2-ARE pathway

Nuclear erythroid-related factor 2 (Nrf2) - antioxidant response element (ARE) pathway is an antioxidant defense system [104]. The activated Nrf2 dissociates from Kelch ECH associating protein 1 (KEAP1) into the nucleus, binds to the ARE sequence, and further activates the ARE-regulated gene transcription. Currently studies have shown that Nrf2-ARE pathway is involved in progress of tumor, stress, apoptosis and inflammatory response. Nrf2 as a redox-sensitive transcription factor regulates the transcription of antioxidant enzymes and plays an important role in inflammatory response, apoptosis, mitochondrial metabolism and stem cell regulation [105]. Meanwhile, Nrf2 inhibits high glucose-induced nuclear factor-κB (NF-κB) activation, improves cardiac diastolic and systolic function, prevents left ventricular end-diastolic pressure increase and ventricular dilatation, and reduces cardiomyocytes apoptosis and interstitial fibrosis. In the diabetic mouse model, sulforaphane restored the expression levels of Nrf2 and Nrf2-dependent antioxidant genes in the aorta, which prevented diabetes-induced ventricular wall thickening, myocardial lipid accumulation, fibrosis, inflammation and apoptosis [106]. In addition, Nrf2 decreases ROS production, prevents oxidative stress - induced insulin resistance, and reduces oxidative stress - induced islet beta cell apoptosis. It has been found that in the heart of diabetic patients, Nrf2 protein was significantly decreased and ROS production was increased. He et al found that high glucose injured cardiomyocytes and induced diabetic cardiomyopathy by ROS [107]. They have also shown that in Nrf2 gene knockout mice, even slightly elevation of blood glucose level caused diabetic myocardial injury in a short period. Therefore, this suggests that Nrf2-ARE pathway may be involved in the development of diabetic cardiomyopathy.

1.1.1.2.2.6 Inflammatory response

In recent years, studies have found that abnormalities in energy metabolism are closely associated with the occurrence of chronic inflammation, which is induced by proinflammatory cytokines and chemokines, and which is believed to be one of the important pathogenesis of diabetic cardiomyopathy [108, 109]. Increased concentrations of inflammatory cytokines such as TNF-α and interleukin-6 in serum of patients with diabetes suggest that chronic inflammation may be associated with cardiac complications [110]. TNF-α induces inflammation and cell apoptosis leading to myocardial fibrosis [111]. In diabetic rats, inhibition of TNF-α reduced myocardial fibrosis and improved cardiac function [112]. NF-κB, as a transcription factor of various inflammatory factors, regulates the expression of proinflammatory, fibrosis and hypertrophy-related genes. In Diabetic patients, non-enzymatic glycation reaction of excessive blood glucose with proteins produces AGEs, which bind the specific receptors on the cell membrane, leading to the release of a large amount of ROS. The ROS activates NF-κB, which further promotes transcriptions of TNF-α, interleukin-6 and other inflammatory factors, and eventually induces vascular endothelial cell injury and fibroblast proliferation, leading to the onset of diabetic cardiomyopathy [113]. In macrophages, NF-κB activation up-regulates the expression of nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3), which interacts with ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) and pro-caspase-1 leading to the formation of an inflammasome. The inflammasome promotes self-cleavage of pro-caspase-1 to form active caspase-1 (p10 / p20), which induces the maturation and secretion of interleukin 1 beta (IL-1β) and IL-18. Luo et al found that both NLRP3 inflammasome and IL-1β were increased in diabetic hearts, leading to the development of diabetic cardiomyopathy, and silencing of NLRP3 gene prevented diabetic heart remodeling and improved cardiac function [114]. Thus, NLRP3 inflammasome has been suggested as a potential target for

clinical treatment of diabetic cardiomyopathy.

1.1.1.3 Treatments of diabetic cardiomyopathy

Metabolic disorders, myocardial fibrosis, cardiomyocyte apoptosis, microvascular disease, oxidative stress, inflammatory response, mitochondrial structure and function changes are all involved in the development of diabetic cardiomyopathy. At present, there is no specific treatment for diabetic cardiomyopathy, and the main treatments include lifestyle Interventions, blood sugar regulation and clinical medications.

1.1.1.3.1 Lifestyle Interventions

Diet and exercise are beneficial for the treatment of diabetic cardiomyopathy and also helpful for blood sugar control, weight control, improving insulin sensitivity, and reducing heart burden [73].

1.1.1.3.2 Blood sugar regulation

Control of blood sugar limits glucose toxicity caused by hyperglycemia, and therefore prevents diabetes cardiomyopathy. Metformin, an oral hypoglycemic which is a medication that lowers blood sugar, is believed to activate adenosine monophosphate-activated protein kinase (AMPK) which plays an important role in cardiac energy metabolism [115]. Thus, metformin treatment may improve diabetic cardiomyopathy. Incretin mimetics, such as glucagon-like peptide-1 (GLP-1) receptor agonists, which are innovative and effective medications to improve blood glucose control, have been shown to have cardioprotective effects [116, 117].

1.1.1.3.3 β-blockers

These drugs can improve autonomic nervous system function in diabetes, restore cardiac remodeling and reduce the incidence of sudden death [118].

1.1.1.3.4 Statins

Statins, a class of lipid-lowering medications, have effect of inhibiting cholesterol synthesis, inflammatory and oxidative stress. Studies have shown that statins improve left ventricular function and inhibit myocardial fibrosis [119]. Large-scale clinical trials confirmed that statins have a protective effect on cardiovascular disease [120].

1.1.1.3.5 RAAS blockers

Studies have shown that RAAS blockers not only lower blood pressure, but also reduce insulin resistance and improve myocardial diastolic function [121, 122].

1.1.1.3.6 Calcium antagonists

Calcium antagonists or calcium channel blockers have antihypertensive effects and also play roles in decrease of excitation-contraction coupling, inhibition of oxidative stress and regulation of vascular smooth muscle proliferation and so on [123]. Cardiomyocyte calcium retention in diabetic patients is associated with depletion of high-energy phosphate compound and ultrastructural disorders, which can be corrected by calcium antagonists [124].

1.1.1.3.7 Insulin and thiazolidinedione derivatives

Insulin and thiazolidinedione derivatives [125] improve endothelial function, reduce vascular smooth muscle hyperplasia, and have protective effects on left ventricular function in diabetic cardiomyopathy patients [126].

1.1.2 Septic cardiomyopathy

1.1.2.1 The definition and characteristics of septic cardiomyopathy

Sepsis, or infection-induced systemic inflammatory response syndrome (SIRS), is a common complication after infection, shock, severe trauma and major surgery, and the

most common cause of death in critically ill patients [127]. According to statistics, about 15,000 patients die from sepsis and its complications every day in the world [128]. The cardiovascular system is usually compromised by sepsis and septic shock. Myocardial injury is a common complication during sepsis, the incidence of which is up to 40% [129]. Meanwhile, myocardial dysfunction also aggravates the sepsis condition, and is an important factor affecting the prognosis of sepsis [130, 131]. Sepsis combined with myocardial dysfunction intensifies the evolution of the disease, increasing the risk of multiple organ failure and death [132], and its mortality rate is as high as 70-90% compared to 40-60% in septic shock patients without myocardial dysfunction [133, 134]. In 1951, Waisbren [135] was the first to report that sepsis could lead to myocardial depression characterized by impaired systolic function, enlarged heart, decreased ejection fraction, poor ventricular contractility in preload and decreased peak systolic pressure/end-systolic volume ratio, and so on [136-138]. Currently, sepsis complicated with cardiac dysfunction is commonly referred to as septic cardiomyopathy.

1.1.2.2 The underlying mechanisms of septic cardiomyopathy

1.1.2.2.1 Myocardial depressant factor (MDF)

As early as the 1960s, many studies have demonstrated the presence of MDF in septic shock [139, 140]. During sepsis, endotoxins or lipopolysaccharides (LPS) of Gram-negative bacteria are important pathogens responsible for myocardial dysfunction [130, 141, 142]. LPS activates mononuclear macrophages, neutrophils, lymphocytes and other immune cells, leading to the release of various myocardial inhibitory factors in the blood [143]. Among myocardial inhibitory factors, TNF-α, IL-1β, and IL-6 have been well addressed to cause cardiac insufficiency. TNF-α has been found to induce myocardial injury by causing cardiomyocyte apoptosis and calcium mishandling [144]. IL-1β is another important MDF, which is generated by monocytes, macrophages and

neutrophils after stimulation by TNF-α. IL-1β has been shown to inhibit myocardial contractility, and has a synergistic effect with TNF-α [145]. Studies have shown that IL-6 is also involved in myocardial injury in acute sepsis. Increased IL-6 expression in monocytes in patients with meningococcal sepsis is associated with cardiac dysfunction [146]. However, the disappointing clinical trials using anti-TNF-α approaches suggest the complexity of and involvement of potential multiple-mechanisms in septic cardiomyopathy [144].

1.1.2.2.2 Mitochondrial dysfunction and ROS generation

Mitochondrion is the major site of myocardial energy metabolism. Sepsis induces myocardial mitochondrial respiratory chain disorders which disturb oxidative phosphorylation and decrease ATP synthesis leading to cardiac dysfunction [143, 147, 148]. In septic animal models and patients, it has been shown that myocardial mitochondria were decreased associate with ultrastructure abnormalities and autophagy [149-151]. Meanwhile, mitochondrial dysfunction caused myocardial energy metabolism disorders and impaired electron transport chain resulting in generation of a large amount of ROS [152]. Mitochondrial ROS production and oxidant damage occur in cultured cardiomyocytes under septic conditions. Mitochondrial ROS production is also increased in septic hearts [153, 154].

An excessive ROS is generated by mitochondria under pathological conditions, leading to mitochondrial oxidative damage, which impairs mitochondrial function and induces cell death via apoptosis and necrosis [155, 156]. ROS production by mitochondria has also been shown to trigger the redox dependent intracellular signaling, leading to pro-inflammatory response [157]. Thus, selective inhibition of mitochondrial ROS prevents proinflammatory cytokine expression in cardiomyocytes under septic conditions and reduces myocardial dysfunction in septic animals, underscoring an important role of

mitochondrial ROS in septic cardiomyopathy.

1.1.2.2.3 Caspase-3 activation and apoptosis

In experimental endotoxemia/sepsis, numerous studies have evaluated the role of apoptotic pathway program activation [158, 159]. In cultured adult cardiomyocytes, LPS directly activates caspase-3 and induces apoptosis [160]. A recent study demonstrated that septic serum also induces caspase-3 activation and apoptosis in cultured human cardiomyocytes [161]. *In vivo* studies have confirmed that caspase-3 activation is significantly increased and apoptosis occurs in the heart in animal models of sepsis and septic patients, which is associated with multiple heart caspase activation and cytochrome c release from mitochondria in sepsis [162, 163]. Although cardiac caspase-3 activity is dramatically increased in these models, the levels of apoptotic cell death in septic heart are very low and may be insufficient to explain the dramatic progression of myocardial dysfunction in sepsis [151]. Interestingly, studies have promisingly shown that either blocking caspase-3 activation by targeting mitochondria-dependent apoptotic pathway or direct inhibition of caspase-3 activity significantly attenuates myocardial dysfunction and improves the survival rate in sepsis [160, 162, 164, 165]. These results suggest that nuclear apoptosis independent pathways are also involved in caspase-3-mediated myocardial dysfunction in sepsis. Indeed, activated-caspase-3 may also directly impair myocardial function via changes in calcium handling and cleavage of sarcomeric myofilaments including α-actin, α-actinin, troponin-T, and myosin light chain cleavage, independent of their modulation of nuclear apoptosis [166].

1.1.2.2.4 Calcium homeostasis and imbalance

Calcium is an important second messenger during cardiac contraction. It participates in cardiac excitation-contraction coupling. The homeostasis of intracellular and extracellular calcium concentration in cardiomyocytes is the basis of maintaining myocardial function

[167]. Sepsis induces considerable inflammatory factors release and ROS production, which may impair calcium regulatory machinery in sarcoplasmic reticulum, leading to calcium leakage and subsequent damage of mitochondrial membrane calcium transport systems [168]. In cardiomyocytes, excessive uptake and accumulation of calcium inside the mitochondria exceed the tolerance range of calcium, leading to the formation of "calcium overload" and finally resulting in irreversible mitochondrial damage and cell death [169]. Calcium overload may also induce excessive ROS production in mitochondria, which signals expression of pro-inflammatory factors.

1.1.2.2.5 Activation of the RAAS

In recent years, increased attention has been drawn on the role of RAAS in sepsis-induced cardiomyopathy [170]. During sepsis, cardiac RAAS is highly activated and myocardial ACE activity increases, which up-regulates expression of renin and angiotensin [171]. It is well known that activation of the RAAS promotes myocardial injury under pathological conditions, e.g. ischemic heart disease [172]. It has been found that animal model administrated with ACE inhibitors and angiotensin II receptor blockers in early phase of sepsis showed improved cardiovascular function. This may be associated with RAAS antagonists-induced hemodynamic changes, including shunt reduction, attenuation of sepsis-induced microcirculation dysfunction, tissue edema alleviation, etc., which prevent myocardial damage and reduce mortality [173].

1.1.2.3 Treatments of septic cardiomyopathy

1.1.2.3.1 Fluid resuscitation

In patients with severe infections or septic shock have relative or absolute hypovolemia, rapid and effective fluid resuscitation works well [174]. However, in patients with septic cardiomyopathy, the effect of liquid resuscitation is limited. Normal fluid resuscitation cannot restore left ventricular function, and the fluid overload or hypervolemia is also

harmful for the body. It has been controversial that which kind of fluid should be used for resuscitation in septic cardiomyopathy. Study has shown that in early phase of sepsis, colloid fluid resuscitation improved cardiac perfusion, cardiac output and systolic function [175].

1.1.2.3.2 Levosimendan

Levosimendan, marketed under the trade name Simdax, is a calcium sensitizer. It effects independently of the beta-adrenergic receptor and is used as treatment of decompensated heart failure. Prospective randomized controlled study has found that in patients with septic cardiomyopathy, administration of levosimendan increased cardiac output and left ventricular ejection fraction, reduced pulmonary arterial pressure, lowered blood lactate levels and improved systemic and local tissue perfusion [176]. In patients with severe heart failure, levosimendan significantly reduced the levels of inflammatory mediators in the blood, lowered the blood brain natriuretic peptide (BNP) level and improved the hemodynamic status [177].

1.1.2.3.3 Beta blockers

Beta-adrenergic hyperactivity is an important factor in the pathogenesis of septic cardiomyopathy, and the administration of beta-blockers may benefit [178]. However, the use of β-blockers is controversial, as it may reduce myocardial contractility. Therefore, although β-blockers have been used in sepsis studies for nearly 50 years, they cannot be included in sepsis guidelines. At present, there is still controversy about the use of β-blockers in the treatment of septic cardiomyopathy [179].

1.1.2.3.4 Mechanical assist devices

In the United States, the intra-aortic balloon pump (IABP), a cardiac assist device, has been approved by the U.S. Food and Drug Administration for adjuvant treatment of septic

shock. In a canine model of severe septic shock with a low cardiac index, Solomon et al [180] have reported that IABP prolonged survival and reduced the dose of antihypertensive drugs. Clinical studies have also shown that in patients with septic shock, IABP significantly restored blood pressure, improved cardiac function index, increased urine output and reduced the 30-day mortality [181].

Extracorporeal membrane oxygenation (ECMO) is an extracorporeal assist device that temporarily circulates blood through an artificial lung to take over the function of the lungs, providing prolonged cardiac and respiratory support in patients with acute respiratory failure [182, 183]. ECMO could reduce mortality of severe myocardial depression caused by septic shock [184]. However, there are only few case reports using ECMO to treat sepsis cardiomyopathy.

To date mechanical assist devices such as IABP and ECMO do not appear to be the standard treatment for septic cardiomyopathy, but may be the last resort when serious infections lead to severe myocardial depression, while all else therapies have failed. The administration of IABP or ECMO in the treatment of septic cardiomyopathy still requires more and larger clinical studies for their efficacies.

1.2 ROS and cardiac disease

1.2.1 Oxidative stress in cardiac disease

Numerous studies have demonstrated that increased ROS contributes to cardiac disease progression, such as coronary artery disease, cardiomyopathy, myocardial infarction, ischemia/reperfusion injury, and heart failure [185]. The enhanced generation of ROS and following oxidative stress is evidently a common phenomenon during cardiac disease [186]. Oxidative stress refers to a pathological condition in which toxic effects induced by ROS damage all components of the cell due to an imbalance between ROS production

and antioxidant defense mechanisms [187]. Increased biochemical markers of oxidative stress, such as 8-iso-prostaglandin F2α or lipid peroxides, have been observed both systemically in the plasma and locally in the pericardial fluid or myocardium in patients and animal models with heart failure and also directly correlated with the severity of myocardial injury [188-190]. This notion suggests that ROS are important mediators in cardiac remodeling. In fact, inhibition of ROS-generating oxidases or antioxidant treatment has been shown to be effective to prevent cardiac remodeling [191]. In mice, for example, dimethyl thiourea alleviated oxidative stress and inhibited cardiac remodeling and heart failure after myocardial infarction [192], and the antioxidant N-2-mercaptopropionyl glycine mitigated hypertrophic remodeling in pressure overload model of transverse aortic constriction [193].

1.2.2 Reactive oxygen species (ROS) in cardiac cells

ROS are various highly reactive compounds with unpaired electrons in the outer valence shell [194]. Major intracellular ROS in heart tissues include superoxide radical ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$) [185] (Figure 1-2). As a primary radical, $^{\bullet}O_2^-$ is formed from one-electron reduction of oxygen molecular. $^{\bullet}O_2^-$ is very unstable and its half-life is very short. Its dismutation happens in a few seconds after its formation, and it is finally converted to H_2O_2 either spontaneously or by superoxide dismutase (SOD) [195] (Figure 1-2). As a membrane-impermeant molecule, $^{\bullet}O_2^-$ diffusion capacity is limited, and therefore it mostly induces intracellular compartments damage, such as disassembly of iron-sulphur clusters. In contrast, H_2O_2 is less reactive than $^{\bullet}O_2^-$, but is more stable and more lipophilic, allowing it to permeate membranes and act at remote sites from its original location. H_2O_2 can be dismutated by catalase, or reduced by glutathione peroxidase (GPX) to H_2O (Figure 1-2). In addition, H_2O_2 could be reduced to $^{\bullet}OH$ and hydroxide anion (OH^-) in the presence of transition metal ions such as Fe^{2+} or Cu^+ via the Fenton reaction [195] (Figure 1-2). The $^{\bullet}OH$ can also be formed

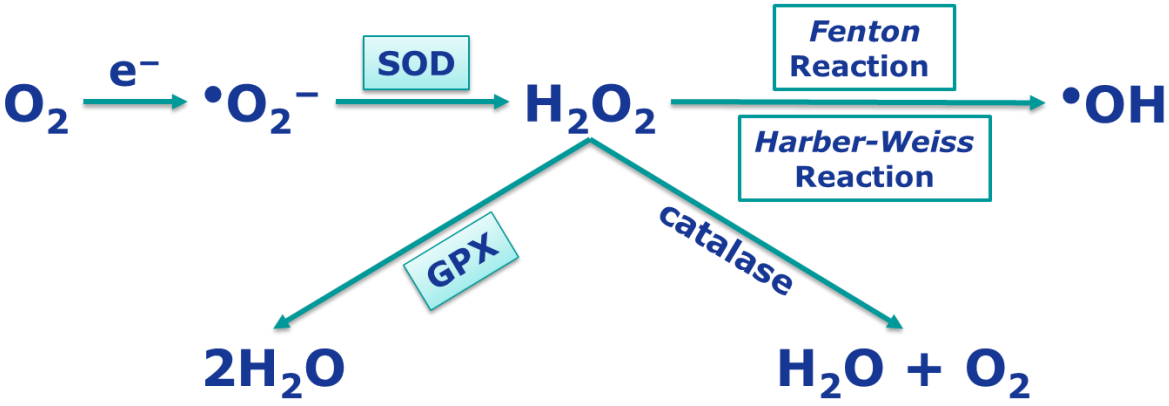

Figure 1-2. Schematic diagram of formation and elimination of ROS

The superoxide ($^{\bullet}O_2^-$) is formed from one-electron reduction of oxygen molecular (O_2) and can be dismutated to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). The H_2O_2 can be converted to $2H_2O$ by glutathione peroxidase (GPX) or to $O_2 + H_2O$ by catalase. The $^{\bullet}OH$ can be formed from H_2O_2 via the Fenton reaction and the Harber-Weiss reaction.

from electron exchange between H_2O_2 and $^{\bullet}O_2^-$ by means of the Harber-Weiss reaction (Figure 1-2). As the most reactive oxidant free radical species, $^{\bullet}OH$ is primarily responsible for oxidative stress-associated cellular injury in cardiac disease. It is normally generated in trace amounts, but in pathologic conditions such as ischemia/reperfusion injury, its formation is markedly increased [196]. Due to its non-diffusible characteristic and extremely short half-life of 10^{-9} s, $^{\bullet}OH$ causes non-specific damage to all cellular macromolecules within a small radius from the site of its generation, including but not limited to protein and deoxyribonucleic acid (DNA) modification, and lipid peroxidation [197].

1.2.3 Sources of ROS in cardiac cells

Cellular ROS are generated from various sources including mitochondrial respiratory chain, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, NO synthases, peroxidases, cytochrome P450, lipoxygenase, and other hemoproteins [198]. Three major cell types in the heart (cardiomyocytes, fibroblasts and endothelial cells) express all these enzymes and are capable of generating ROS. Although the exact proportions of ROS production from individual source are not known, it has been suggested that the predominant sources of ROS in heart tissues are NADPH oxidase, mitochondria and xanthine oxidase, all of which have been demonstrated to play important roles the pathogenesis of cardiac disease. These three major ROS sources in heart are described below:

1.2.3.1 NADPH oxidase (NOX)

NOX is a multi-component enzyme system that catalyzes the NADPH-dependent reduction of oxygen to the $^{\bullet}O_2^-$, which is the precursor of the other ROS [199]. This O_2^- producing system is well characterized in neutrophils where it plays an important role in

bacteria killing. The enzyme complex comprises two membrane subunits (gp91^{phox} and p22^{phox}, which form flavocytochrome b558), at least three cytosolic proteins (p40^{phox}, p47^{phox} and p67^{phox}, which form the cytosolic complex) and the small GTPase Rac1 or Rac2 [200-203]. The catalytic core of NADPH oxidase is the membrane-integrated flavocytochrome b558 [204]. NADPH is a highly regulated enzyme. In resting cells, the cytosolic complex is separated from the membrane-bound catalytic core. Upon stimulation, NADPH oxidase activation appears to be triggered by the phosphorylation of the cytosolic phox proteins and their translocation with Rac to the membrane-bound flavocytochrome b558 to assemble into an active oxidase [204, 205].

Several homologs of gp91^{phox} (also termed Nox2)—Nox1, Nox3, Nox4, and Nox5—have been identified in nonphagocytic cells [206]. In the heart, recent studies showed that the phagocyte-type of NADPH oxidase is expressed in adult cardiomyocytes and is a major source of O_2^- during pathophysiological conditions [207]. In addition, NOX has been demonstrated to significantly contribute to ROS generation in vascular cells (such as adventitia, medial smooth muscle, and endothelium) [208]. It has been shown to be activated and generate excessive $^{\bullet}O_2^-$ in vascular tissue contributing to the pathogenesis of angiotensin II - induced endothelial dysfunction and vascular hypertrophy [209].

1.2.3.2 Mitochondria

The heart is the highest oxygen consuming organ which uptakes about 0.1ml O_2/g/min at basal conditions [210]. Therefore, cardiomyocytes have the highest volume density of mitochondria within human body, to fulfill the demand for generation of the energy source ATP via oxidative phosphorylation reactions. The enzymatic components of oxidative phosphorlyation are consistent in all eukaryotic cells. These components include: NADH-ubiquinone oxidoreductase (complex I), succinate-ubiquinone oxidoreductase (complex II), ubiquinol - ferricytochrome c oxidoreductase (complex III),

ferricytochrome c-oxygen oxidoreductase (complex IV), and F1F0 ATP synthase (complex V). During the process of ATP production, electrons transfer through these multimeric complexes, termed as electron transport chain (ETC). Briefly, in mitochondrial ETC, electrons donated from nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH2) are transferred by complex I and II respectively, to complex III via ubiquinone (Coenzyme Q10) [211]. Electrons are then delivered by cytochrome c to complex IV, and finally passed to O_2, converting to water. During the transfer of electrons, protons are translocated from the mitochondrial matrix to the intermembrane space by complex I, III and IV, and thereby the proton gradient and the mitochondrial membrane potential are formed. At last, complex V via the use of the energy stored in this proton gradient across the inner mitochondrial membrane produces ATP. Complex V using the ETC - driven membrane potential is termed oxidative phosphorlyation. However, not all of the oxygen acts as the terminal electron acceptor. Small amount of oxygen is partially reduced by electrons leaked from the ETC and transformed into $^{\bullet}O_2^-$. Hence, a little oxidative species is generated as byproducts. The complexes I and III are believed as the major sites for electron leakage [211]. Of note, an impairment of complex V not only reduces ATP production but also contributes to mitochondrial ROS generation.

Under normal conditions, mitochondria are capable to eliminate these toxic by-products of ROS by mitochondrial antioxidant systems. These antioxidant enzymes include catalase, SOD and GPX, and several low-molecular-weight antioxidants such as ascorbate, α-tocopherols and thiols and ubiquinol [212]. $^{\bullet}O_2^-$ can be converted to H_2O_2 by manganese SOD (MnSOD) in mitochondrial matrix or copper-zinc SOD (CuZnSOD) in the intermembrane space. Then, H_2O_2 can be readily reduced to water by catalase or GPX, which utilize H_2O_2 to oxidize reduced glutathione (GSH) to oxidized glutathione (GSSG). Meanwhile, GSSG can be converted back to GSH by glutathione reductase

(GR). Under pathological conditions, the ETC is blocked, leading to substantial electrons inappropriately transferring directly to O_2 to form $^{\bullet}O_2^-$, which is beyond the ability of mitochondrial antioxidant systems to scavenge, and thereby a large amount of ROS is produced [213]. However, it remains incompletely understood how mitochondrial ROS generation is regulated in cardiomyocytes under pathological conditions.

1.2.3.3 Xanthine oxidase (XO)

XO plays an important role in purine metabolism pathway under normal physiological conditions [214]. The enzyme generates ROS as a byproduct in the catalytic process of final product uric acid by oxidation of hypoxanthine and xanthine [215]. It has been suggested that the level of XO may be low in the heart and it was even not found in human cardiomyocytes. XO is normally present in the small intestinal mucosa and the liver [216, 217]. However, in disease conditions such as cardiomyopathies or under stress such as inflammation or increased oxidative stress by prolonged hypoxia, it is activated and released into serum [218]. The released XO may subsequently reach to target tissues like heart via circulating system and gather more than thousand-fold at the interstitial matrix of the vasculature or binding to surface of endothelial cells. XO-derived ROS has been implied to contribute to myocardial ischemia/reperfusion injury by many experimental data [219]. It has been shown that XO is highly activated and produces excessive superoxide in mesenteric tissue leading to intensified vascular tone in an essential hypertensive model[220]. Increased activity of XO has also been observed in end-stage failing human heart. It has been suggested that inhibition of XO is a potential therapeutic method to improve cardiovascular injury [221, 222]. For example, treatment with allopurinol, an inhibitor of XO, has been proved to effectively decrease the level of lipid peroxidation and aid postoperative recovery [223].

1.2.4 Oxidative damage in cardiac cells

ROS can induce massive peroxidative damage to phospholipids, proteins and DNA. ROS also lead to protein modifications, such as nitration and carbonylation, and generation of lipid peroxidation adducts (e.g. 4-hydroxynonenal [HNE]) [224]. ROS-mediated protein modifications (carbonylation and nitration) and HNE adduct change or impair protein function and stability [225]. As a consequence, ROS directly influences cellular structure and function, and has been suggested as the integral signaling molecules in myocardial remodeling and failure, leading to cardiomyocyte apoptosis, inflammation, hypertrophy and interstitial fibrosis in the development of heart failure [226, 227].

1.2.4.1 Oxidative damage to proteins

Superoxide induces the release of ferrous iron from Fe-S clusters. Ferrous iron then readily binds polypeptides. Thus, the Fenton reaction results in the formation of $^{\bullet}OH$, which damages proteins at sites [212]. Protein oxidation is assessed by measuring the contents of carbonyls, which can be easily quantified [228]. In addition to sulfur-containing residues, most amino acids are irreversibly oxidized, and thus the oxidatively-modified protein is likely degraded through the proteolysis. Given that hydroxyl radicals readily react with, and H_2O_2 can directly oxidize cysteine and methionine, both residues are targeted by the hydroxyl radical resulting from the Fenton chemistry more than other amino acid residues [212, 229]. However, $H_2O_2^-$ mediated cysteine and methionine oxidation is very slow and thus, H_2O_2 at physiological concentrations is unlikely to induce direct oxidation of amino acids [230, 231]. Particular mitochondrial proteins such as aconitase have been shown to be inactivated by H_2O_2 [232]. In aconitase, superoxide is highly reactive with the [4Fe-S] cluster [233]. Citrate synthase is another target of mitochondrial ROS, and in fact SOD2 mutant mice display a reduction of citrate synthase activity in the heart [234].

1.2.4.2 Oxidative damage to lipids

ROS can cause oxidation of lipids which is one of major mechanisms contributing to lipid peroxidation (LPO). Others include enzymatic oxidation and non-enzymatic oxidation such as ozone. Thus, LPO product can be measured as an indicator of oxidative damage to lipids.

Superoxide itself may not directly oxidize lipids. However, it reacts with NO to form peroxynitrite ($ONOO^-$), which can initiate lipid peroxidation [235]. Superoxide may also induce conversion of Fe^{3+} to Fe^{2+}, which reacts with H_2O_2 and subsequently generates hydroxyl radicals in the vicinity of lipids, contributing to LPO.

The reaction of ROS with lipids can be detrimental or beneficial to the cell depending on their products. Lipid peroxidation can disturb bio-membrane and modify proteins and DNAs, but products of lipid peroxidation may also regulate gene expression and redox signaling, leading to oxidative stress tolerance. In addition, lipid oxidation results in lipid hydroperoxides, which in turn become DNA-adducting electrophiles, leading to propagation of ROS damage [236, 237].

1.2.4.3 Oxidative damage to nucleic acids

As mentioned above, superoxide can release Fe^{2+} from Fe-S clusters, which can damage DNA. H_2O_2 directly oxidizes Fe^{2+} in the Fenton reaction thus generating the extremely reactive $^{\bullet}OH$ [238, 239]. Although $^{\bullet}OH$ reacts readily with all biomolecules, such reactions are limited because $^{\bullet}OH$ only affects them near the site of its production. Thus, when $^{\bullet}OH$ production is present at the site of a ferrous iron-DNA complex, DNA can be damaged [212]. $^{\bullet}OH$ can attack any bases in DNA, with guanine being the particular target [240]. Since 8-hydroxyguanine (8-oxoG) is the major product formed in an $^{\bullet}OH$-DNA reaction, it has used as an indicator of DNA oxidative damage [241]. In

addition to nuclear DNA, mitochondrial DNA (mtDNA) is particularly susceptible to oxygen radicals because of its close proximity to the locations of superoxide generation by the ETC and its lack of histone protection. In fact, studies have shown more mtDNA damage than nuclear DNA damage in cells treated with H_2O_2 at concentrations at 200 μM or less [242-245], indicating the increased ROS vulnerability of mtDNA compared to nuclear DNA.

1.2.5 Mitochondrial ROS and cardiac disease

For a long time, people have been focusing on antioxidant therapies for cardiovascular disease like vitamin E and CoQ based on promising experiments [246, 247]. Unfortunately, clinical trials showed disappointing outcomes. Recently, it has been recognized that such therapies need to be more targeted towards mitochondria and the respiratory chain because they are the main sources of ROS in cardiomyocytes [248]. Indeed, mitochondrial ROS production is increased and oxidative stress occurs in a variety of cardiac diseases [249]. Sustained high levels of ROS in the mitochondria directly damage mitochondrial components [250], including permanent inactivation of mitochondrial proteins, destruction of mitochondrial DNA and phospholipid bilayer of mitochondrial membrane, inhibition of mitochondrial respiratory chain function, and down-regulation of mitochondrial DNA replication, which further accelerate free radicals generation leading to forming a vicious cycle: free radical generation → mitochondrial structure destruction → more free radical generation. As a consequence, excessive mitochondrial ROS compromises energy production and induces cell death. Mitochondrial ROS may also serve as a signaling molecule, leading to pro-inflammatory cytokines expression resulting in inflammation and fibrosis as well as myocardial hypertrophy [251]. All of these changes contribute to cardiac injury, remodeling and heart failure. Thus, targeted inhibition of mitochondrial ROS has been shown to reduce cardiac injury and prevent myocardial remodeling and heart failure in a variety of animal

models [252, 253]. Thus, mitochondrial ROS may represent an excellent target for therapy. Indeed, a number of mitochondrial-targeted anti-oxidants have been commercially developed including mito-E, mito-TEMPO, mitoQ10, SS31 (6'-dimethyltyrosine-Lys-Phe-NH2), etc. with some being under clinical trials for various diseases [254].

1.2.5.1 Mitochondrial ROS and ischemic heart disease

Mitochondria are not only the main targets of oxidative stress but also the major sites of ROS production. Under physiological conditions, about 0.2% -2.0% of molecular oxygen is transformed to superoxide by acquiring electron from complex I and complex III of the electron transport chain in cardiomyocytes [211]. During myocardial ischemia, numerous studies have shown that a small amount of ROS is generated in mitochondria, which may disrupt the electron transport chain [195]. After onset of reperfusion, the immediate restoration of oxygen levels and inefficient oxidative phosphorylation promote a large amount of mitochondrial ROS production and subsequent oxidative stress [255]. Excessive mitochondrial ROS induces cell death and promotes proinflammatory cytokine expression in ischemic heart tissues, leading to the infiltration of inflammatory cells which enhances ischemic heart injury [256]. Thus, targeted inhibition of mitochondrial ROS reduced ischemia/reperfusion (I/R) injury in *in vitro* cardiomyocytes and *in vivo* hearts.

1.2.5.2 Mitochondrial ROS and hypertensive cardiomyopathy

Hypertension is a highly prevalent human disease that imposes a major risk for development of wide spectrum of cardiac and vascular diseases including atherosclerosis, cardiomyopathy, stroke and kidney diseases. Meanwhile, mitochondrial ROS and oxidative stress are increased in heart tissues in response to hypertension and implicated in the development of hypertensive cardiomyopathy [257]. Increased mitochondrial ROS

in cardiomyocytes induces mitochondrial protein oxidative damage and mitochondrial DNA deletions, contributing to the development of cardiac hypertrophy, fibrosis and failure. In an angiotensin II-induced mouse model, Dai et al reported that mitochondrial ROS and oxidative stress were induced in cardiomyocytes and over-expression of catalase targeted to mitochondria prevented myocardial hypertrophy, fibrosis and mitochondrial damage in mice [257]. Over-expression of catalase targeted to mitochondria also prevented heart failure induced by over-expression of Gαq in mice [258]. Similarly, administration of mitochondrial targeted antioxidant peptide SS31 reduced mitochondrial oxidative damage, prevented apoptosis, and ameliorated cardiac hypertrophy, diastolic dysfunction, and fibrosis induced by angiotensin-II in mice without changing blood pressure. The SS31 administration also partially rescued the heart failure phenotype of Gαq overexpressing mice [259]. The protective effects of SS31 were reproduced by McLachlan et al in stroke-prone spontaneously hypertensive rats using another mitochondrial-targeted antioxidant mitoQ10 [260]. These findings suggest the potential clinical application of mitochondrial-targeted antioxidants in treating hypertensive cardiomyopathy.

1.2.5.3 Mitochondrial ROS and metabolic cardiomyopathy

Mitochondrial ROS and consequent oxidative stress are associated with metabolic heart disease [36, 261]. Many studies have shown that elevated glucose and free fatty acids levels present in the diabetic state drive the formation of ROS [262, 263]. It is believed that enhanced substrate flux through the mitochondria enhances electron leak and subsequently ROS formation [264]. Oxidative stress occurs when ROS are generated in excess through the reduction of oxygen and inadequate antioxidant defense [187]. In this regard, studies have shown that anti-oxidant defense system was usually impaired in diabetic hearts [265-267]. Furthermore, analysis of cardiac biopsy on patients diagnosed with diabetes complicated with heart failure revealed mitochondrial swelling and ROS

production in cardiomyocytes. Lastly, restoration of mitochondrial anti-oxidant defense function by over-expression of thioredoxin-2 effectively prevented mitochondrial oxidative stress and consequently attenuated myocardial hypertrophy in mouse models of type-1 diabetes. However, further investigation is needed to determine whether therapeutic scavenging mitochondrial ROS using mitochondrial-targeted anti-oxidants reduces diabetic cardiomyopathy in animal models of both type-1 and type-2 diabetes.

Under other metabolic stress, pooled neutral fatty acids within the mitochondria due to the inability to utilize efficiently are more susceptible to oxidative damage [268]. Sverdlov Al et al observed oxidative posttranslational modifications of cardiac mitochondrial proteins in mice fed a high-fat high-sucrose diet [269, 270]. This mitochondrial oxidative stress in the heart was associated with cardiac diastolic and mitochondrial dysfunction as well hypertrophy. Importantly, transgenic over-expression of catalase in mitochondria ameliorated cardiac mitochondrial dysfunction, hypertrophy and diastolic dysfunction in mice fed a high-fat high-sucrose diet [271, 272]. Thus, increased myocardial mitochondrial ROS generation is an important factor promoting the development of metabolic cardiomyopathy and may represent an important target for therapy.

1.2.5.4 Mitochondrial ROS and septic cardiomyopathy

Mitochondrial ROS production and oxidant damage occur in cultured cardiomyocytes under septic conditions [273]. Mitochondrial ROS production is also increased in septic hearts [274]. It has been demonstrated that excessive mitochondrial ROS induced caspase-3 activation and apoptosis in cardiomyocytes during sepsis, which compromises myocardial function [143]. Interestingly, mitochondrial ROS also serves as a signaling molecule, which induces expression of pro-inflammatory mediators and subsequent infiltration of inflammatory cells, leading to myocardial injury and depression in sepsis

[275, 276]. Our lab demonstrated that mitochondrial ROS increased and mediated TNF-α expression in cardiomyocytes in response to LPS [277]. In a rat model of a pneumonia-related sepsis, Zhang et al reported that mitochondria-targeted vitamin E, another mtROS specific antioxidant, improved cardiac performance and attenuated inflammation [278]. Yao et al further demonstrated that sepsis-induced mitochondrial ROS damage mtDNA, leading to the release of free mtDNA and the activation of a toll like receptor 9 pathway, which contributes to the development of cardiac failure after sepsis [273]. Thus, targeting mitochondrial ROS by mitochondria-targeted antioxidants represents a promising therapeutic approach to protect the heart in sepsis.

1.3 The Calpain family

1.3.1 General information

Calpains belong to a family of calcium-dependent thiol-proteases [279, 280]. The first calpain discovered and purified was calpain-1 in 1964 by Guroff [281]. A total of 15 isoforms of calpain (14 large subunit members and one small subunit) and one endogenous inhibitor (calpastatin) have been discovered in mammals. Among them, calpains 1,2,4, 5, 7, 9 and 10 are ubiquitously expressed members. Calpain-3 (skeletal muscle and retina), calpain-6 (placenta), calpain-8 (smooth muscle), calpain-11 (testes), calpain-12 (skin) and calpain-13 (testes and lung) are tissue specific isoforms [282]. Based on domain IV structure, calpains can be divided into two groups: the typical and atypical calpains. Nine of calpain isoforms (1, 2, 3, 8, 9, 11, 12, 13 and 14) containing a penta-EF hand in domain IV are classified as typical. The penta-EF domain can bind calpastatin, Ca^{2+} or the calpain small subunit (only calpains 1, 2 and 9 have been shown to bind). In contrast, other five isoforms (calpains 5, 6, 7, 10 and 15) are classified as atypical as they do not have a penta-EF hand in domain IV and thus, they are unable to bind calpastatin or the calpain small subunit [283, 284]. (Figure 1-3).

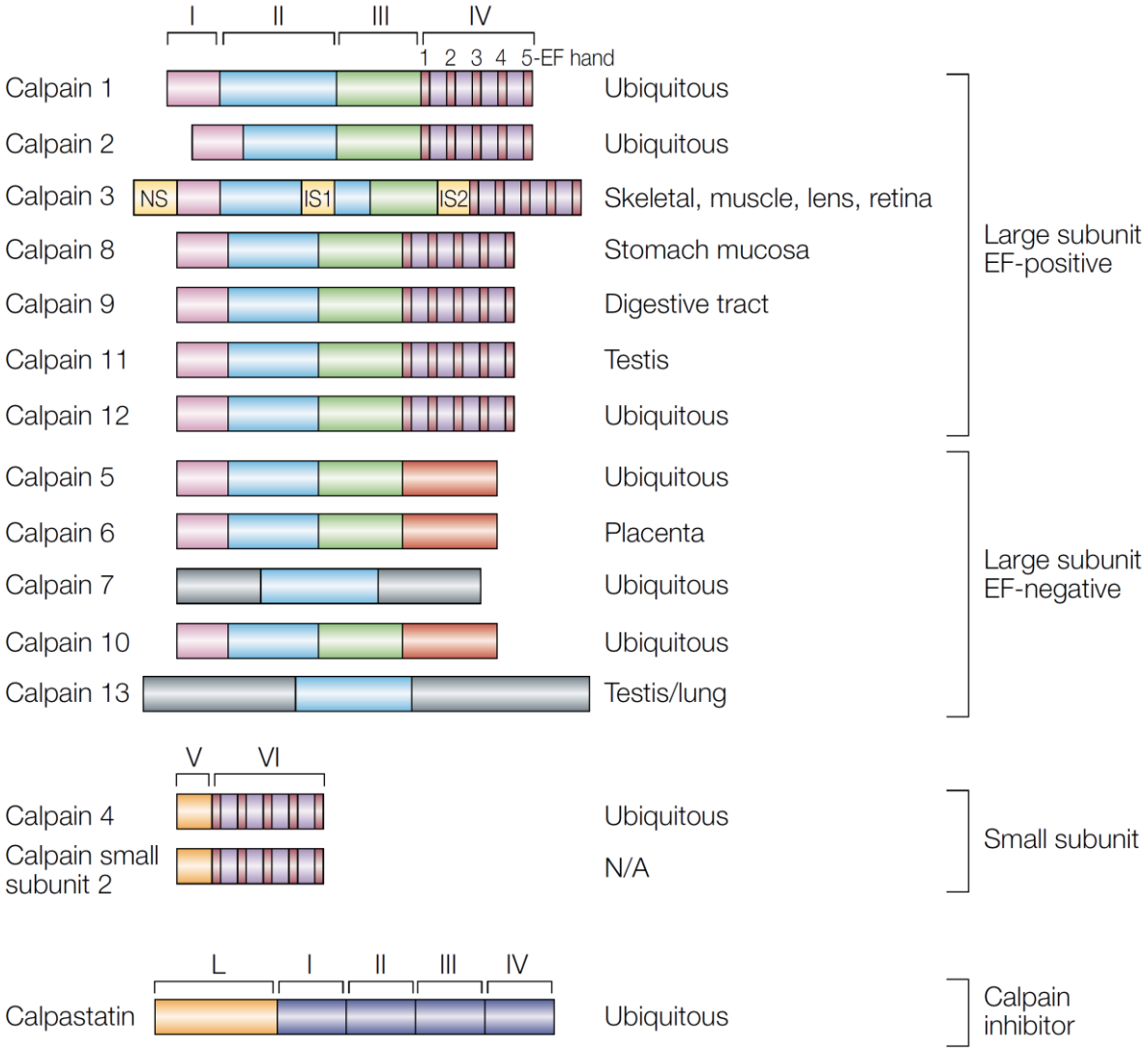

Figure 1-3. Schematic structures of calpain family members in mammals

The main structural domains and localization of the mammalian calpain protein family and their endogenous inhibitor calpastatin are shown. Figure is adapted from Margaret C. Frame, et al. Nature Reviews Molecular Cell Biology, 2002 Apr;3(4): 233-245

Most calpains have four structural domains: I, II, III and IV (Figure 1-3). Upon Ca^{2+} activation (autolysis), domain I is cleaved in calpains 1, 2 and 9 [280]. However, it is unknown if other typical calpains have autolysis of domain I. In atypical calpains, domain I is not cleaved and their function remains largely unknown, except in calpain-10 where a mitochondrial targeting sequence is identified within domain I [285]. The calpain active site resides in domain II, which has a catalytic triad of cysteine, asparagine and histidine. This catalytic triad is present in the entire family except calpain-6, which does not have proteolytic activity [286]. In addition to the catalytic triad, domain II can also have binding sites for Ca^{2+} which assists in calpain activation [287]. There are two Ca^{2+}-binding spots and one C2-like motif which binds phospholipid in domain III [288]. These Ca^{2+}-binding and phospholipid binding residues are conserved in the calpain family except calpain-10. Domain III is important in regulation of calpain activity by management of specific electrostatic interactions and in recognition of their substrates [287]. Domain IV has the penta-EF hand to bind Ca^{2+}, calpastatin or domain VI of the small subunit (calpain 4). These penta-EF hands are important for calpain activation since they contain the most Ca^{2+} binding spots [289].

Calpain-4, also known as a small calpain subunit, is a 28 kDa protein that forms heterodimers with typical calpains, encoded by *capn4 or (capns1)* gene [280]. It only contains domain V and VI (Figure 1-3). The function for domain V is known to bind the C-terminus region of domain IV in large calpain subunits [290]. Both domain VI and IV have a penta-EF hand for Ca2+-binding and heterodimer formation [291]. Calpain small subunit 2 (CSS2) was recently discovered and it dimerizes with calpain-2 [292]. However, CSS2 may not redundant with calpain-4 because global deletion of calpain-4 is embryonic lethal [293].

1.3.2 Calpastatin

Calpastatin is an endogenous protein that specifically inhibits calpains (e.g. calpain-1 and calpain-2), but not any other proteases. Calpastatin has 8 splice variants, ranging from 18.7-85 kDa [294, 295]. Calpastatin has six domains (XL, L, I, II, III and IV) [280]. Domains I-IV are similar and contain inhibitory subdomains A, B and C. None of the subdomains can inhibit calpains when assayed alone, but when subdomains A and B or B and C are assayed together calpain activity is reduced. When subdomains A and C are assayed together there is no inhibition. Subdomains A and C have been shown to bind to domain IV and VI, respectively, in a Ca^{2+}-dependent manner (Figure 1-4), while subdomain B binds domain II in a Ca^{2+}-independent manner. Of the four domains the order of inhibition effectiveness is: domain I>domain IV>domain III>domain II [296]. Little is known about the XL domain other than it contains three PKA phosphorylation sites [297]. The function of domain L is still unknown.

1.3.3 Calpain activation

Calpains require Ca^{2+} concentrations at physiological pH and ionic strength (Figure 1-4). Although calpains 1 and 2 share identical 28 kDa small subunits, their 80 kDa large subunits are different. This discrepancy in structures presumably accounts for the differential Ca^{2+} requirement for activation between them. Calpains 1 and 2 require micromolar and millimolar of Ca^{2+} for their half-maximal activation, respectively [280].

Activation of typical calpains involves a series of conformational changes, which engage the interactions between various domains. In the absence of Ca^{2+}, the interaction between the N-terminal anchor helix and Domain VI of the small subunit imposes constraints on the protease core (Domains I and II) and makes these two Domains (I and II) separate in an inactive conformation [298, 299]. This prevents the active site Cys residue located in Domain I from forming the catalytic triad with His and Asn in Domain II because the Cys

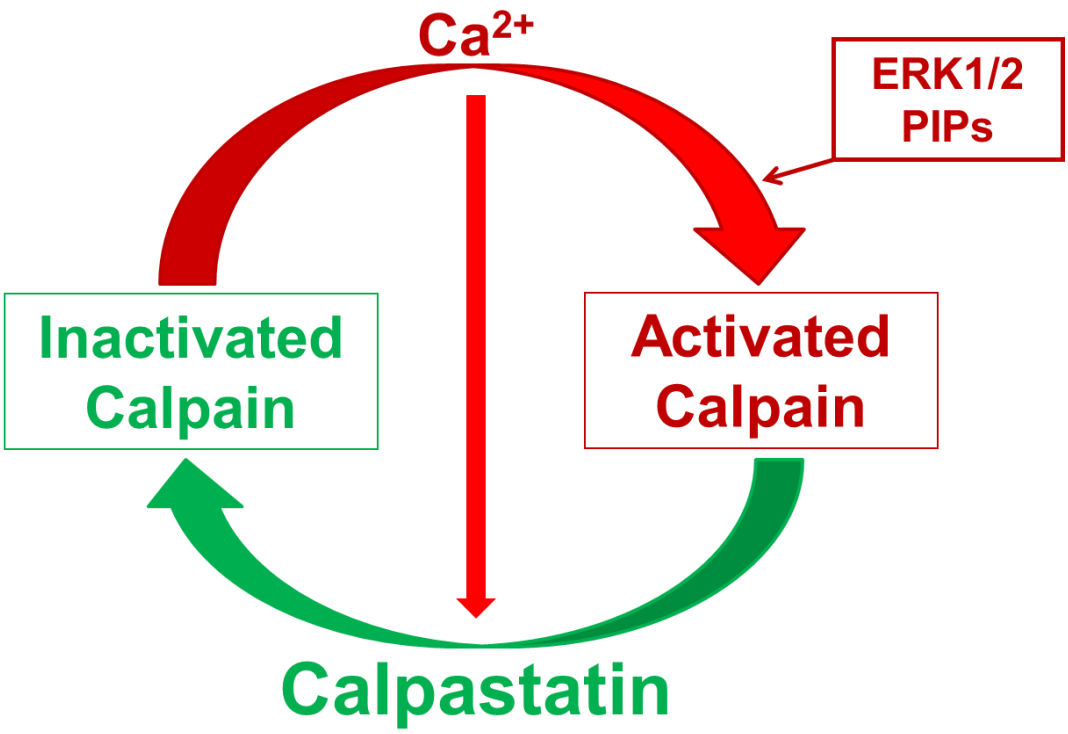

Figure 1-4. Schematic diagram of calpain activation and regulation

Calpain requires Ca^{2+} for activation. Calpain activity can also be regulated by other factors, such as ERK1/2 and PIPs. Calpastatin represents the negative regulator of calpain activity, which is also in a Ca^{2+}-dependent manner. ERK1/2, extracellular signal-regulated kinase-1/2; PIPs, phosphatidylinositol phosphates.

is far away from the His (about 10 Å) [300]. Also, the wedge (tryptophan 288) between Domains I and II also blocks the formation of the catalytic triad. In the presence of sufficient Ca^{2+}, calpain undergoes a series of conformational changes that lift the restraints on the protease core, which includes freeing the anchor helix from its contact with the small subunit. As a consequence, the catalytic triad containing the active site Cys residue located in Domain I and His and Asn in Domain II is formed in a proteolytically active conformation [300-302]. This activating process is reversible because abrogation of Ca^{2+} by adding excess EDTA pushes this reaction back to an inactive form of calpain.

In addition to Ca^{2+}, several other factors have been shown to affect calpain activation (Figure 1-4). For example, phosphorylation of calpain may lower the Ca^{2+} requirement for its activation. It was reported that phosphorylation of calpain-2 by ERK1/2 can directly induce its activation without increasing Ca^{2+} [303-305]. Calpain-2 activation is also regulated by its binding to phosphatidylinositol 4,5-bisphosphate [306, 307]. This may explain why calpain-2 can be activated in cells where there is no way that Ca^{2+} can reaches the millimolar levels.

1.4 Calpain and cardiac disease

Activated calpain cleaves its substrates through limited enzymatic cleavage. Its substrates are numerous, including a variety of regulatory and receptor proteins, cytoskeletal proteins, myofibrillar proteins and protein kinases [308], which are involved in many pathophysiological functions. Thus, calpain activation has been implicated in cardiac apoptosis, hypertrophy, fibrosis and inflammation in the development of cardiac disease.

1.4.1 Calpain and cardiac remodeling

1.4.1.1 Calpain and cardiac apoptosis

Apoptosis is a highly-regulated, energy-dependent form of programmed cell death known

to play an important role in the development and progression of cardiovascular disease [309]. One mechanism by which apoptosis is induced in cardiomyocytes is via calpain activity [310-314].

Calpain has been proved to directly cleave pro-caspase 3, 7, 8, 9 and as such has been implicated in apoptosis [315, 316]. Additionally, apoptosis resulting from alterations in intracellular calpain concentrations may include calpain-2 mediated caspases-12 cleavage and activation [317]. Moreover, calpain has been shown to cleave Bcl-xL, an apoptotic inhibitor [318]. The protease is also implicated in ischaemia-reperfusion induced apoptosis and has been shown to participate in TNF-alpha mediated apoptosis in cardiomyocytes [312]. A recent study demonstrated that cardiac over-expression of calpain-1 is sufficient to cause heart failure in transgenic mice [319]. Our lab recently showed that hyperglycemia-induced calpain-1 activation, mediated through an NADPH oxidase-dependent pathway, can lead to apoptosis through down-regulation of (Na, K)-ATPase activity in cardiomyocytes and *in vivo* diabetic hearts [311]. Calpain inhibition via calpastatin over-expression imparted an anti-apoptotic effect on cardiomyocytes. In this study we also demonstrated that the pro-apoptotic role of calpain is mediated through caspase-3 activation. During high glucose stimulation calpain inhibition via pharmacological calpain inhibitors, calpain-1 siRNA and calpastatin overexpression was able to block calpain activation and consequently prevent apoptosis in cardiomyocytes and hyperglycemic hearts [320].

Calpain inhibition has been found to be protective in ischaemia injury models as well [321-324]. ROS production and the resulting oxidative stress is a key feature of I/R injury [325]. Our lab has shown that in high glucose treatment, ROS mediate calpain activation in cardiomyocytes. Calpain activation may be the outcome of increased intracellular calcium in cardiomyocytes after oxidative activation of L-type calcium channels and

ryanodine receptor by ROS leads to calcium release [311]. This is consistent with reports that calcium is increased under I/R stimulation and high glucose conditions [326-328]. The pro-apoptotic effects of calpain were also on display as once calpain activity was inhibited by calpastatin overexpression, cardiomyocyte apoptosis and necrosis were diminished in diabetic and non-diabetic hearts after I/R and encouraged myocardial functional recovery [329]. Mani and colleagues used a mouse model of myocardial infarction to demonstrate that active caspase-3 increased after myocardial infarction (MI) in the border zone but calpeptin, a calpain inhibitor and caused a decrease in calpain activity that reduced chamber dilation and protected left ventricular pump function and reduced cardiomyocyte loss in the border zone [330]. This was further supported by our recent report in a mouse model of MI [331]. All these previous studies have demonstrated an important role of calpain activation in cardiac apoptosis under stress.

1.4.1.2 Calpain and cardiac hypertrophy

Hypertrophy is an important feature of cardiac remodeling. It is characterized by the individual cardiomyocyte size increase. Pathological cardiac hypertrophy is initiated by biomechanical, stretch-sensitive (mechanical deformation) or neurohumoral mechanisms (release of endothelin-1, catecholamines, angiotensin II, chemokines, cytokines, and growth factors) [72]. Proteins involved in these intracellular signaling pathways would be the targets of calpains [332].

Although the exact mechanism is unclear, calcium is believed to play some role in promoting cardiac hypertrophy [333] Alteration in calcium handling is a common occurrence in some models of cardiac hypertrophy [334]. The clear increase in intracellular calcium may serve as an avenue facilitating the morphological and biochemical changes that lead to a hypertrophic state. One way in which this occurs may be through the action of calcium-activated calpain [335]. Despite limited understanding

of its role in cardiac hypertrophy, elevated calpain activity is an established feature of the diabetic myocardium [336]. In one study, calpain-like activity was increased in the myocardium concurrent with cardiac mass after injection with isoproterenol to stimulate beta-agonist induced hypertrophy [337].

A proposed method by which calpain indirectly promotes hypertrophy is through the activation of calcineurin, a Ca2+/calmodulin dependent protein phosphatase [338]. Proteolysis of calcineurin by calpain involves removal of the autoinhibitory domain which causes calcineurin to assume its active conformation, no longer requiring calcium and calmodulin for activation [339]. Calpain can also cleave an endogenous inhibitor of calcineurin, cain/cabin 1 [340]. On its way to promoting hypertrophy, calcineurin activates nuclear factor of activated T-cells (NF-AT), a transcription factor that translocates into the nucleus and initiates pro-hypertrophic gene expression [341].

Another transcription factor implicated in hypertrophy is NF-κB [342]. When inactive, NF-κB is complexed to its inhibitor, inhibitor-κB (IκB) and is thus prevented from entering the nucleus [343]. Calpain has been shown to degrade IκB but under normal circumstances activation of IκB kinase (IKK) by extracellular signals initiates the degradation of IκB [344-346]. Phosphorylation of two serine residues of the IκB structure by IKK leads to ubiquitination and subsequent digestion by the proteasome. NF-κB becomes free to translocate into the nucleus and transcribe its associated genes [343]. Specifically, NF-κB is responsible for the activation of pro-inflammatory cytokines some of which, such as TNF-α, are elevated in the diabetic heart [347, 348]. Pro-inflammatory cytokines can subsequently induce expression of other cytokines to enhance cell injury.

Our lab used a cardiac-specific *capn4* knockout model and both an STZ and OVE26 mouse model with calpastatin overexpression to investigate the role of calpain in the hyperglycemic myocardium. We demonstrated that impeding calpain function can reverse

hypertrophy and concurrently found that elevated calpain also increased NF-AT and NF-κB activity [336]. This finding supports the suggestion that calpain plays an important role in the activity of NF-κB and calcineurin, both key activators of hypertrophic pathways. We also observed that limiting calpain activity through *capn4* knockout also decreases TNF-α and TGF-ß1 expression in the diabetic heart [336]. Similarly, in a mouse model of ischemic heart disease using cardiac-specific *capn4* knockout mice, our lab reported that disruption of calpain reduces hypertrophy. Again, this anti-hypertrophic effect of calpain disruption was associated with inhibition of NF-κB activation and subsequent reduction of TNF-α expression in ischemic hearts [331]. Nevertheless, the signaling mechanisms by which calpain promotes hypertrophy in diseased hearts have not been fully understood.

1.4.1.3 Calpain and cardiac fibrosis

Myocardial fibrosis is a maladaptive response to stress characterized by excess collagen accumulation and is believed to occur independently of organ hypertrophy [349]. In the heart, myocytes are bounded by the extracellular matrix (ECM) which is primarily composed of collagen with smaller amounts of elastin, laminin and proteoglycans among others [350]. Collagen is vital to the maintenance of cardiac architecture [351]. Although studies indicate that only 2-4% of the myocardium is collagen, even slight changes in collagen concentration can have substantial effects on the mechanical properties of the heart [352]. The dominant collagens are type I, accounting anywhere from 50-80%, and type III collagen, comprising around 10% of the ECM; the other collagens are present but to a much lesser degree [353]. Collagen I is characterized by tensile strength whereas collagen III has greater elastic potential and for this reason, ratios between collagen types are significant [354]. Collagen may also be involved in the transmission of force generated by cardiac muscle and therefore has a substantial impact on the diastolic and systolic function of the heart.

Increased ventricular stiffness resulting from elevated levels of collagens can inhibit myocyte recoil during cardiac relaxation resulting in aberrant ventricular filling and consequently stroke volume. Increases in collagen content impair sarcomere extension and compromise the heart's ability to generate adequate pressure for systemic perfusion [355]. Taken together, it is clear that fibrosis greatly jeopardizes the heart's ability to function and not surprisingly, fibrosis is a leading cause of heart failure.

We have shown that myocardial collagen deposition is increased in mouse models of Type I diabetes such as the STZ and OVE26 mouse. In these models, cardiac-specific *capn4* knockout or calpastatin overexpression reduced both collagen deposition and gene expression of collagens I and III. Clearly as calpain over-activity is restrained, the amount of total collagen accumulation in the hyperglycemic heart is diminished and the relative amounts of collagens I and III are brought back to homeostatic levels [349]. In a mouse model of myocardial infarction, we similarly demonstrated that cardiac-specific *capn4* knockout attenuated both collagen deposition and gene expression of collagens I and III [331].

Fibrosis is believed to be mediated by fibroblasts [356]. Having established a correlation between calpain activity and collagen content in the myocardium, we isolated and cultured cardiac fibroblast cells. The study aimed to identify fibroblasts as targets of calpain hyperactivity and examine the mechanisms that relate calpain to fibroblast stimulation in the diabetic heart [336]. The precise manner in which hyperglycemia stimulates increased collagen deposition by fibroblasts in cardiac tissue is not clearly understood. Transforming growth factor- beta 1 (TGF-β1) has gained notoriety as a potent fibrogenic cytokine [357]. TGF-ß1 is a key regulator of inflammation and wound healing; it's released at wound sites where it recruits neutrophils, monocytes and fibroblasts to initiate tissue repair [358]. It also plays a central role in the maintenance of

the ECM and can induce the expression of ECM proteins including collagen from fibroblasts [359]. But further research into the mechanisms behind fibrosis has highlighted its ability to promote excess and unnecessary secretion of collagen from fibroblasts. One possibility is that high glucose up-regulates TGF-β1 expression leading to increased fibroblast proliferation. TGF-ß1 knockout models show reduced fibrosis in ageing hearts while TGF-ß1 overexpression in mice caused interstitial fibrosis [360].

TGF-β1 has also been demonstrated to induce differentiation of fibroblasts to myofibroblasts [360]. Although in their resting state, fibroblasts are considered to be sessile they migrate and proliferate upon activation. During the reparative process, tissue needs to be contracted and as a result a subpopulation of the fibroblasts will gain contractile properties that resemble those belonging to smooth muscle cells and this modulation allows the cell, now called a myofibroblast, to secrete greater amounts of collagen [361]. Over the course of their development, myofibroblasts irreversibly acquire proteins, one of which is alpha-smooth muscle actin (α-SMA), a well-accepted marker of myofibroblast differentiation. Calpastatin overexpression imparts an anti-fibrotic advantage to the myocardium as our study showed that α-SMA is elevated in the diabetic heart but diabetic *capn4* knockout hearts showed a significant decrease in α-SMA expression [336].

Maintenance and degradation of the extracellular matrix in the myocardium is regulated by MMPs which are regulated in turn by their inhibitors, TIMPs. Modifications in the activities of either MMPs or TIMPs have marked effects on the architecture of the ECM in the myocardium [362]. In the event of impaired MMP regulation an imbalance in collagen synthesis versus collagen degradation leads to the accumulation of fibrillar collagen and the appearance of myocardial fibrosis [363]. The diabetic heart has been shown to have up-regulated MMP-9 activity [364] and down-regulated MMP-2 activity

[83]. In our lab we found an increase in total MMP activity in fibroblasts in vitro and diabetic hearts in vivo, calpain inhibition reduced MMP activity in both cases suggesting MMP inhibition may protect the diabetic heart against fibrosis [336].

Further, in angiotensin-II induced cardiac remodelling, Letavernier and colleagues demonstrated using microscopy and immunohistochemical analysis of collagen type I that fibrosis can be reduced around the aorta and tissue arteries by inhibiting calpain [365]. An accompanying decrease in MMP activity was also observed suggesting that stimulation by calpain is not limited only to the fibroblast, whether directly or indirectly through fibrogenic factors, but also to the remodelling machinery in the ECM.

1.4.1.4 Calpain and cardiac inflammation

Inflammation occurs frequently in the heart under stress [366]. After an initial injury of the myocardium, the endogenous stress protein levels are up-regulated, such as HSP10 (heat shock protein 10), HSP60, and HSP70. These stress proteins, known as ‘alarmins’, are ligands for toll like receptors (TLRs) which are expressed on the surface of inflammatory/ immune cells and cardiac cells. Binding of HSPs to TLRs induces the expression of pro-inflammatory factors including cell adhesion molecules, chemokines, and chemokine receptors, promoting both the recruitment and the activation of a series of inflammatory cells, such as neutrophils and monocytes. These cells release cytotoxic substance, inducing damage to endothelial cells and cardiomyocytes, which in turn, amplify alarmin expression [367]. The expression of proinflammatory factors are regulated by various transcriptional factors with NF- κB being the most important. At rest state, iκB binds and inhibits NF-κB in cytosol. Upon activation, iκB is degraded and NF-κB translocated into nucleus for activation [368]. Calpain has been shown to target and cleave iκB and thus, promotes NF-κB activation in inducing the expression of proinflammatory factors and inflammation [369]. Using calpastatin transgenic mouse

model, our lab demonstrated that inhibition of calpain by calpastatin over-expression or pharmacological inhibitor of calpain significantly reduced inflammatory cytokine TNF-α expression in cardiomyocytes and hearts [336]. We further reported that cardiac-specific knockout *capn4* restored the protein levels of IκB, prevented NF-κB activation and attenuated TNF-α expression in hearts after myocardial infarction [331]. Clearly, calpain activation promotes inflammation in the heart under stress. In addition, in animal mouse model of angiotensin II-mediated cardiovascular remodeling, over-expression of calpastatin disturbed and delayed the ability to recruit inflammatory cells in mice [365]. Similarly, decreased endothelium adhesiveness to circulating leucocytes has been shown in angiotensin II infusion model with calpastatin overexpressing or calpain-1 deficiency [370]. Thus, calpain is involved in the occurrence of inflammation in cardiovascular system under diseased conditions.

1.4.2 Calpain in cardiac disease

1.4.2.1 Calpain and ischemia-reperfusion

Calpain has been implicated in ischemia/ reperfusion (I/R)-induced injury in the heart [371]. Activation of calpains induced by Ca^{2+} overload during IR, leads to the proteolysis of the cytoskeletal protein fodrin rendering the membrane fragile [372]. IR increases cytosolic calpains activities leading to cardiac injury by cleaving Bid to truncated Bid [373], Ca^{2+} - ATPase [374, 375], Na, K - ATPase [376] and troponin T [372]. Pharmacological inhibitors of calpain decreased myocardial infarction size in I/R models and attenuated the progression of heart failure after MI [330]. The protective effects of calpain inhibitors were then confirmed by over-expression of calpastatin in global whole I/R model and a mouse model of ischemia/reperfusion injury [324, 329]. In addition, cardiac-specific *capn4* knockout reduced myocardial infarct size and remodeling, and improved myocardial dysfunction in a mouse model of myocardial infarction [331].

These promising findings suggest that calpain may be a therapeutic target to limit myocardial ischemia/reperfusion injury. However, further investigation is warranted to understand the underlying mechanisms.

1.4.2.2 Calpain and diabetic cardiomyopathy

Diabetic cardiomyopathy is a serious clinical condition. It is a tremendous personal struggle for Canadians and a significant financial burden for our health care system [4]. However, the mechanisms by which this occurs remain incompletely understood and no cure is available for this disease. In recent years, our lab has been investigating calpain activation in diabetic cardiomyopathy. In cultured cardiomyocytes, our lab reported that incubation with high glucose or high palmitate increased calpain activation and inhibition of calpain with pharmacological inhibitor, siRNAs or over-expression of calpastatin prevented high glucose or palmitate-induced apoptosis [91, 377]. Further studies showed that selective silencing of calpain-1 but not calpain-2 prevented high glucose or palmitate-induced apoptosis in cardiomyocytes [91, 377]. In mouse models of STZ-induced and genetic type-1 diabetes, our lab reported that transgenic over-expression of calpastatin or cardiac-specific *capn4* knockout attenuated cardiac apoptosis, hypertrophy and fibrosis, which are associated with an improvement of myocardial function [336]. These findings provide strong evidence in support of the role of calpain in type-1 diabetes-related cardiomyopathy. In db/db mice, calpain activity was increased in the heart and correlated with myocardial remodeling and dysfunction, which were attenuated by over-expression of calpastatin [378]. This data underlies a role of calpain in type-2 diabetes-related cardiomyopathy. More recently, our lab demonstrated that cardiac-specific *capn4* knockout prevented cardiac apoptosis, reduced hypertrophy and fibrosis, and improved myocardial function in mice fed a high fatty diet, a model of pre-diabetes with impairment of glucose tolerance and insulin resistance [377]. This provides further evidence to support the role of calpain in type-2 related cardiomyopathy.

Mechanistically, our lab showed that calpain might induce ER stress and subsequent apoptosis in diabetic cardiomyopathy [377]. Taken together, calpain may represent a new therapeutic target for diabetic cardiac complications.

1.4.2.3 Calpain and septic cardiomyopathy

As described above, both caspase-3 activation and apoptosis are important in the development of myocardial dysfunction during sepsis [158-160, 162, 164, 165]. Studies have suggested that calpain is an important player in cell death signaling. Partial cleavage of pro- or anti-apoptotic proteins by calpain might activate or inactivate, respectively, putative substrates including caspase-3, caspase-7, -8, and -9 [158, 159], caspase-12 [317], Bcl-2 [318], Bcl-xl [318], Bid [373], Bax [379], and inhibitor of NF-κB [344]. Our lab reported that calpain was activated in cardiomyocytes and hearts under septic conditions. Inhibition of calpain by its pharmacological inhibitors or over-expression of calpastatin protected cardiomyocytes against sepsis-induced apoptosis both in vitro and in vivo, which was associated with improved myocardial dysfunction. Silencing of individual calpain isoforms revealed that calpain-1 but not calpain-2 contributed to sepsis-related apoptosis in cardiomyocytes [320]. Furthermore, a recent study showed that calpain inhibition might be associated with activation of Akt/ GSK-3β (glycogen synthase kinase-3 beta) signaling in preventing cardiac apoptosis during sepsis [380]. Meanwhile, calpain activation was associated with NF-κB activation via degradation of IκB, leading to expression of pro-inflammatory mediates, which in turn elicited myocardial depression in sepsis [381]. Thus, transgenic over-expression of calpastatin or cardiac-specific *capn4* knockout inhibited calpain activation and prevented pro-inflammatory cytokine expression in septic hearts.

1.5 Mitochondrial calpain and cardiac disease

Although calpains have been considered cytoplasmic enzymes [280, 382], studies have

demonstrated that calpain-1, calpain-2 and calpain-10 are also present in mitochondria [285, 314, 383-390]. Further studies reveal that calpain-1 has a mitochondrial targeted signal peptide which induces its translocation to mitochondria [391]. A previous study showed that homocysteine (Hcy) induced the translocation of active calpain from cytosol to mitochondria, leading to matrix MMP-9 activation in cultured rat heart microvascular endothelial cells [392]. In isolated hearts, Chen Q et al showed that a global ischemia/reperfusion induced calpain-1 activation within mitochondrial intermembrane space, leading to the cleavage of apoptosis-inducing factor (AIF) and subsequent apoptosis [313]. Using the same model, Chen Q et al further demonstrated that global ischemia/reperfusion increased calpain activation in mitochondrial matrix and disrupted complex-Ⅰ activity [393]. The increased calpain-2 activity in mitochondrial matrix was also reported in a rat model of ischemia/reperfusion injury by a different lab and contributed to disruption of complex-Ⅰ activity [394]. These studies suggest that increased mitochondrial calpain activity may be implicated in ischemic heart disease. In fact, several key mitochondrial proteins have been suggested to be substrates of calpain including AIF [313, 314, 383, 395], optic atrophy-1 (Opa-1) [396], Na^{+}/Ca^{2+} exchanger [397] and ATP synthase-α [398], etc. Proteolysis of these proteins will compromise mitochondrial function and subsequent cardiac injuries following ischemia/reperfusion. However, it remains to be determined whether mitochondrial calpain is also increased in other pathological conditions. Given that mitochondria are major source of ROS in cardiomyocytes, mitochondrial dysfunction elicits ROS generation which may induce cell death and also serve as a signaling molecule promoting cardiac hypertrophy, fibrosis and inflammation, all of which contribute to cardiac disease and heart failure. Thus, it is highly possible that increased mitochondrial calpain induces ROS generation and cardiac disease under pathological conditions.

1.6 Rationales

CVD are arguably the number-one health problem in the world. Constant prevalence of CVD and its high morbidity and mortality indicate that there are huge gaps in understanding of the underlying mechanisms and that better therapy strategies are urgently needed to control CVD and its clinical manifestations. Amongst various proposed mechanisms, oxidative damage induced by ROS has been critical in this disease. As a major source of ROS in cardiomyocyte, mitochondria are important players in pathogenesis of cardiac disease, while it has been increasingly recognized that calpain activation contributes to cardiac disease [399]. However, it has never been reported whether calpain activation can modulate mitochondrial ROS generation in cardiac disease. A previous study showed that mitochondrial calpains are increased in isolated hearts following global ischemia/reperfusion, leading to apoptosis via cleavage and release of AIF [313]. However, it remains unknown whether increased mitochondrial calpain is a common mechanism contributing to cardiac disease and if yes, how mitochondrial calpain mediates cardiac disease. Since both diabetes and inflammation are critically important factors in promoting cardiac disease, in this thesis, I used diabetic cardiomyopathy and septic cardiomyopathy as disease models to address the above questions.

1.7 Hypotheses

In this work, the following four hypotheses were investigated:

1. Calpain is increased in mitochondria in cardiomyocytes under diabetic and septic conditions;

2. Increased mitochondrial calpain contributes to mitochondrial ROS generation in cardiomyocytes under diabetic and septic conditions;

3. Increased mitochondrial calpain disrupts ATP synthase via cleavage of ATP5A1 leading to excessive mitochondrial ROS generation in cardiomyocytes under diabetic and septic conditions;

4. Administration of mitochondrial-targeted antioxidant reduces diabetic adverse cardiac changes and improves myocardial function in diabetes.

1.8 Reference

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Chapter 2

2 Deletion of capn4 protects the heart against endotoxemic injury by preventing ATP synthase disruption and inhibiting mitochondrial superoxide generation[2]

2 This chapter has been published in the following manuscript:

- Ni R, Zheng D, Wang Q, Yu Y, Chen R, Sun T, Wang W, Fan GC, Greer PA, Gardiner RB, and Peng T. (2015) Deletion of capn4 Protects the Heart Against Endotoxemic Injury by Preventing ATP Synthase Disruption and Inhibiting Mitochondrial Superoxide Generation, *Circ Heart Fail 8*, 988-996.

2.1 Introduction

Sepsis is the leading cause of death among the critically ill [1]. Among the general population, sepsis accounts for 215, 000 deaths per year [2], making it the 10th most common cause of death in the United States [3]. Myocardial dysfunction is a key manifestation contributing to morbidity and mortality among septic patients in intensive care units [4], and 40-50% of patients with prolonged septic shock develop myocardial depression [5]. Estimates of mortality due to sepsis range from 20-30% [6, 7], however, mortality increases to 70-90% when there is accompanying myocardial dysfunction [7]. Thus, myocardial dysfunction is a decisive factor in determining survival or death in sepsis. Lipopolysaccharides (LPS) of gram-negative bacteria are important pathogens responsible for myocardial dysfunction during sepsis [8, 9]. LPS-induced pro-inflammatory cytokines, in particular, tumour necrosis factor-alpha (TNF-α), play a critical role in myocardial dysfunction in animal models of sepsis [8, 10, 11]. However, the mechanisms underlying LPS-induced pro-inflammatory response in septic hearts remain not fully understood and no cure is available to correct this life-threatening condition.

Calpains belong to a family of calcium-dependent thiol-proteases [12, 13]. Fifteen gene products of the calpain family are reported in mammals. Among them, calpain-1 and calpain-2 are ubiquitously expressed, while other calpain family members have more limited tissue distribution. Both calpain-1 and calpain-2 are heterodimers. They consist of distinct large 80-kDa catalytic subunits encoded by *capn1* and *capn2*, respectively, and a common small 28-kDa regulatory subunit encoded by *capn4*. The regulatory subunit is indispensable for calpain-1 and calpain-2 activities. These two calpain isoforms are regulated by the endogenous calpain inhibitor, calpastatin. Our recent study demonstrated that over-expression of calpastatin reduced cardiac TNF-α expression and attenuated

myocardial dysfunction in calpastatin transgenic mice (Tg-CAST) in response to LPS [14]. However, the underlying mechanisms by which calpain participates in the regulation of pro-inflammatory response remain to be defined. Moreover, tissue-specific gene deletion of calpain is generally considered to be more conclusive to clarify the contribution of cardiac calpain since inhibition of systemic inflammation could not be excluded to confer cardiac protection in endotoxemia in Tg-CAST mice.

Although calpain-1 and calpain-2 have been considered mainly cytoplasmic enzymes, recent studies have found that they are also present in mitochondria [15, 16]. Mitochondrial calpains have been shown to play important roles in pathophysiological conditions [16]. However, it has never been shown whether calpains are altered in mitochondria and whether mitochondrial calpains contribute to pro-inflammatory response in septic hearts. Activation of mitochondrial calpains may be involved in mitochondrial dysfunction because several mitochondrial proteins have been suggested to be potential substrates of calpain, including, but not limited to, ATP5A1 [17], optic atrophy-1 (Opa-1) [18], apoptosis-inducing factor [19], and Na^{+}/Ca^{2+} exchanger-1 (NCX-1) [20]. Proteolysis of these mitochondrial proteins will compromise mitochondrial function and may lead to excessive ROS generation. Thus, calpain may regulate mitochondrial ROS production. Our recent study has demonstrated that LPS increases mitochondrial ROS and selectively blocking mitochondrial ROS inhibits TNF-α expression in cardiomyocytes [21]. Taken together, these studies raise an intriguing hypothesis that calpain activation may induce excessive mitochondrial ROS generation, leading to cardiac pro-inflammatory response and myocardial dysfunction in sepsis.

In the present study, we employed cardiomyocyte-specific *capn4* knockout mice to investigate whether and how calpain activation disrupts ATP synthase and induces mitochondrial ROS generation in cultured cardiomyocytes and hearts during LPS

stimulation.

2.2 Methods

2.2.1 Animals

This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011). All experimental procedures were approved by the Animal Use Subcommittee at the University of Western Ontario, Canada. Breeding pairs of C57BL/6 mice were purchased from the Jackson Laboratory. Tg-CAST mice were generously provided by Dr. Laurent Baud (the Institut National de la Santé et de la Recherche Médicale, Paris, France) through the European Mouse Mutant Archive [22]. Mice with cardiomyocyte-specific disruption of *capn4* (*capn4*-ko) were generated as described previously [23]. All of the mice used in this study, including controls, were littermates of the same generation. Adult male mice (aged 2 months, 8-15 mice in each group) were injected with LPS (4 mg/kg, i.p.) or saline as a control.

2.2.2 Echocardiography

Animals were lightly anaesthetized with inhalant isoflurane (1%) and imaged using a 40-MHz linear array transducer attached to a preclinical ultrasound system (Vevo 2100, Visual Sonics, Canada) with nominal in-plane spatial resolution of 40 μm (axial) × 80 μm (lateral). M-mode and 2-D parasternal short-axis scans (133 frames/second) at the level of the papillary muscles were used to assess changes in left ventricle (LV) end-systolic inner diameter (LVIDs), LV end-diastolic inner diameter (LVIDd), LV posterior wall thickness in end-diastole (LVPW;d) and end-systole (LVPW;s), and fractional shortening (FS%). LV end-systolic volume (LV Vol;s) and end-diastolic volume (LV Vol;d) were acquired by $7/(2.4+LVIDs) \times LVIDs^3$ and $7/(2.4+LVIDd) \times LVIDd^3$, respectively.

2.2.3 Delivery of recombinant adenovirus into mice

Mice were anaesthetized with inhaled isofluorane (1%). With the guide of echocardiography, adenovirus containing human ATP5A1 gene (Ad-ATP5A1, 2×10^9 PFU in 100 μL, SignaGen Laboratories) or containing GFP (Ad-GFP, SignaGen Laboratories) was injected into mouse left ventricle.

2.2.4 Isolation and culture of adult mouse cardiomyocytes

Adult mouse ventricle cardiomyocytes were isolated from Tg-CAST and wild-type mice (C57BL/6), and cultured as described in our recent study [24].

2.2.5 Adenoviral infection of cardiomyocytes

Cardiomyocytes were infected with Ad-ATP5A1, adenoviral vector containing mitochondria-targeted rat calpastatin (Ad-mtCAST, SignaGen Laboratories), or beta-gal (Ad-gal, Vector Biolabs) as a control at a multiplicity of infection of 100 PFU/cell as we previously described [25].

2.2.6 Measurement of mitochondrial superoxide generation

Superoxide flashes in single mitochondrion were measured to determine mitochondrial superoxide generation in living cardiomyocytes as described previously [26]. Briefly, cardiomyocytes were infected with an adenoviral vector expressing mt-cpYFP (Ad-mt-cpYFP). Ad-mt-cpYFP expresses a circularly permuted yellow fluorescent protein (cpYFP) in the mitochondrial matrix of cells using the cytochrome C oxidase subunit IV targeting sequence (mt-cpYFP). Twenty-four hours after infection, confocal imaging was recorded using the Olympus FV 1000 laser-scanning microscope equipped with a 63x, 1.3NA oil immersion objective and a sampling rate of 0.7s/frame.

2.2.7 Construction of plasmid with mitochondrial targeted capn1 expression and transfection in cardiomyocyte-like H9c2 Cells

The full coding region of human capn1 cNDA was recovered from *p*CMV6-XL5 containing human *capn1* (Origene) and inserted into *p*CMV/myc/mito following mitochondrial signal peptide (Life Technologies Inc.). The resulting plasmid *p*CMV/myc/mito-*capn1* expresses myc-tagged *capn1* selectively in mitochondria.

Rat cardiomyocyte-like H9c2 cells were cultured and transfected with *p*CMV/myc/mito-*capn1* or *p*CMV/myc/mito as a control using the jetPRIME^{TM} DNA transfection reagent (VWR International) according to the manufacturer's instructions.

2.2.8 Calpain activity

Calpain activity was determined by using a fluorescence substrate N-succinyl-LLVY-AMC (Cedarlane Laboratories) as previously described [14].

2.2.9 Real-time reverse-transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from heart tissues using the Trizol Reagent (Life Technologies Inc.) following the manufacturer's instruction. Real-time RT-PCR was performed to analyze mRNA expression for TNF-α, ATP5A1 and GAPDH as described previously [27].

2.2.10 Western blot analysis

The protein levels of calpain-1, calpain-2, mitochondrial voltage-dependent anion channel (VDAC1), cytochrome c, cyclophidin D, complex Va, ATP synthase-alpha (ATP5A1) and beta subunits, Opa-1, calreticulin, and GAPDH were determined by western blot analysis using respective specific antibodies (Cell Signaling and Santa Cruz Biotechnology).

2.2.11 Measurement of ROS generation in isolated mitochondria

Interfibrillar Mitochondria were isolated from the freshly harvested heart as described previously [28], with minor modifications as follows. Instead of Nagarse, trypsin (5 mg/g wet weight of tissues) was used and after homogenizing and centrifuging, trypsin inhibitor (0.5 mg/ml) was added to the supernatant. The isolated mitochondria were further purified using Percoll density gradient centrifugation [29]. Mitochondrial ROS generation was determined on addition of pyruvate/malate or succinate by using Amplex Red and horseradish peroxidase according to the manufacturer's instruction.

2.2.12 Immuno-fluorescence staining and confocal microscopy

Mitochondrial smears were prepared on slides and fixed with freshly prepared 4% paraformaldehyde. After incubation with appropriate primary antibodies (calpain-1 and VDAC-1) and secondary antibodies conjugated with different fluorescences (Alexa Fluor 488 Donkey anti-mouse IgG and Alexa Fluor 594 Goat anti-rabbit IgG), signals were obtained with an Olympus FluoView^{TM} FV1000 confocal microscope equipped with the IX81 motorized inverted system as described in our recent report [30].

2.2.13 Immuno-electron microscopy

The sections of heart tissues were incubated with primary antibodies against calpain-1 (Cell Signaling), and then labeled with second antibodies tagging with colloidal gold (10nm, Cedarlane Laboratories). Gold particles were identified in mitochondria and quantified as the number of gold particles in given mitochondrial areas.

2.2.14 Co-immunoprecipitation (co-IP) and native gel electrophoresis

Co-IP and non-denaturing polyacrylamide gel electrophoresis were carried out to analyze protein–protein interactions. Briefly, calpain-1 and its interacting proteins were pulled down using Immunoprecipitation kit - dynabeads protein G (Life Technologies Inc.), and

ATP synthase complex was isolated using ATP synthase immunocapture kit (Abcam Inc.) in isolated mitochondria according to the manufacturer's instructions. Both calpain-1/its interacting proteins and ATP synthase complex were subjected to SDS-polyacrylamide gel electrophoresis for separation, followed by western blot analysis for ATP5A1 and calpain-1 using their specific antibodies, respectively.

2.2.15 ATP synthase activity

ATP synthase activity was measured using an assay coupled with pyruvate kinase, which converts ADP to ATP and produces pyruvate from phosphoenolpyruvate, as described previously [31].

2.2.16 Statistical Analysis

All data were presented as mean ± SD. Statistical comparisons between only two groups were done using an unpaired Student's t-test. For comparisons of more than two groups, one-way analysis of variance (ANOVA) or two-way ANOVA was performed as appropriate. Post hoc comparisons were performed using Newman-Keuls or Bonferroni comparison analysis in one-way ANOVA or two-way ANOVA, respectively. One-way repeated-measures ANOVA was performed for time course and dose response studies on mitochondrial superoxide flashes. A value of $P < 0.05$ was considered statistically significant.

2.3 Results

2.3.1 Deletion of *Capn4* Reduces Pro-Inflammatory Response and Attenuates Myocardial Dysfunction in Endotoxemic Mice

To determine whether cardiomyocyte-specific deletion of *capn4* provides beneficial effects in septic hearts, we injected *capn4*-ko and their wild-type mice with LPS or saline. As previously reported [14], LPS induced TNF-α expression and decreased myocardial

function in wild-type mice; however, deficiency of *capn4* significantly attenuated TNF-α expression and myocardial dysfunction in endotoxemic *capn4*-ko mice (Figure 2-1A and B, and Table 2-1). Thus, cardiomyocyte-specific deletion of *capn4* protects the heart against LPS-induced injury.

2.3.2 Genetic Inhibition of Calpain Prevents Mitochondrial Superoxide Generation in Hearts and Cultured Cardiomyocytes during LPS Stimulation

Our recent study has shown that mitochondrial ROS contributes to LPS-induced pro-inflammatory response in cardiomyocytes [32]. In this study, we examined whether there is a link between calpain and mitochondrial ROS generation in endotoxemia. We determined ROS generation in isolated mitochondria of mouse hearts at 4 hours after LPS stimulation. LPS treatment increased ROS generation in mitochondria using either pyruvate/malate or succinate as substrates (Figure 2-1C and D). *Capn4* deletion significantly reduced ROS generation in mitochondria from LPS-stimulated *capn4*-ko mice (Figure 2-1C and D). However, the anti-oxidant capacity slightly increased in response to LPS and there was no difference between wild-type and *capn4*-ko mice (data not shown). These results suggest that calpain induces mitochondrial ROS generation in response to LPS and the increase in mitochondrial ROS production is not due to the disruption of anti-oxidant defence system.

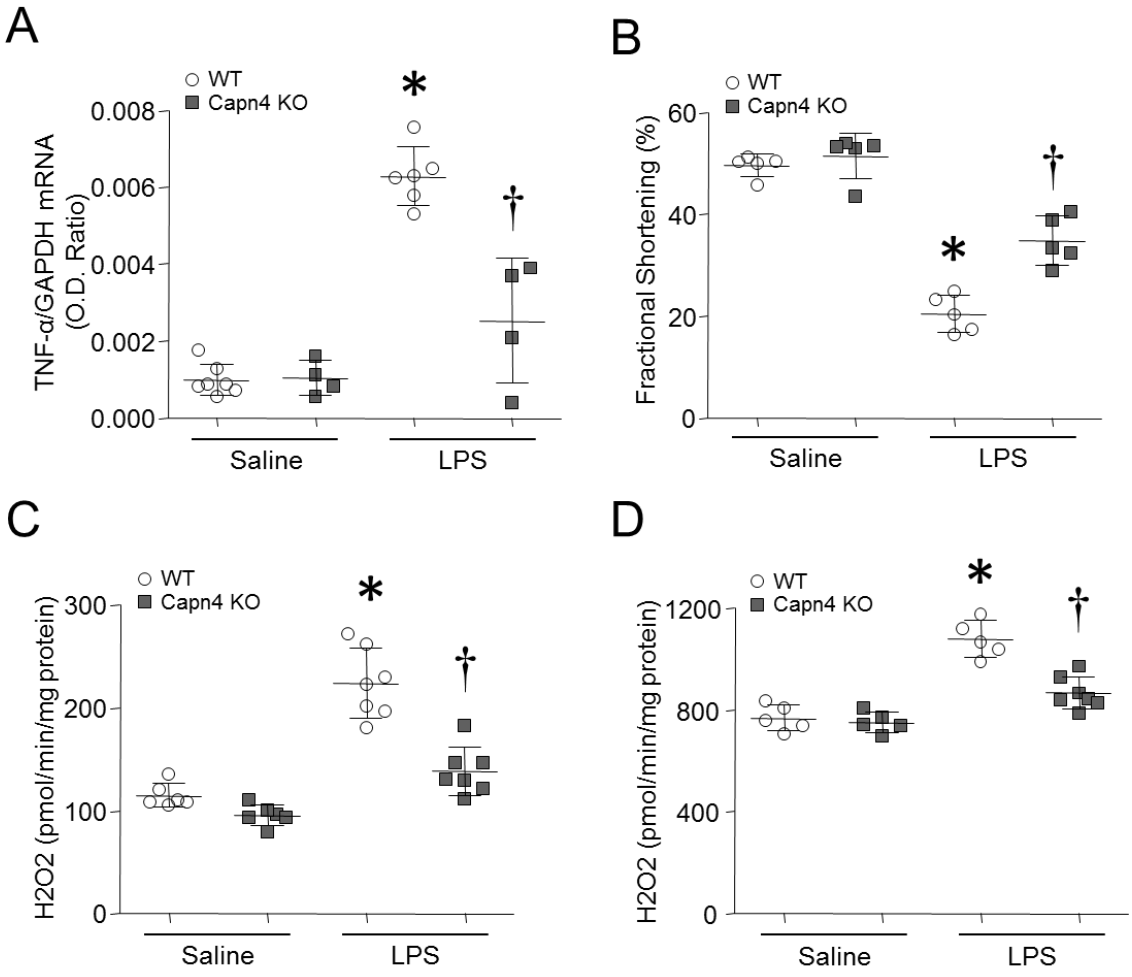

Figure 2-1. Mitochondrial ROS generation, TNF-α expression and dysmyocardial function in capn4-ko mice and their wild-type littermates

Mice were injected with LPS or saline (6-7 mice in each group). Four hours later, myocardial function was assessed (B) and the mRNA levels of TNF-α measured (A). Mitochondrial ROS generation was determined following addition of pyruvate/malate (C) or succinate (D) using Amplex Red. Data are mean ± SD, n = 4-7. *$P < 0.05$ versus saline in WT and $^{\dagger}P < 0.05$ versus LPS in WT.

Table 2-1. Myocardial function in Capn4-KO mice during endotoxemia

	Saline + WT	Saline + Capn4-KO	LPS + WT	LPS + Capn4-KO
ESD (mm)	1.85±0.23	1.89±0.44	3.21±0.37*	2.39±0.69†
EDD (mm)	3.67±0.34	3.86±0.61	4.03±0.34	3.63±0.83
FS(%)	49.55±2.20	51.41±4.46	20.42±3.64*	34.83±4.81†
Heart rate (beats/minute)	430.60±23.53	412.00±16.26	513.20±64.29*	483.60±63.64

* $p<0.05$ versus Saline + WT and # $p<0.05$ versus LPS + WT. ESD: End systolic diameter; EDD: End diastolic diameter; FS: Fractional shortening.

To confirm the role of calpain in mitochondrial ROS generation, we isolated and cultured cardiomyocytes from adult Tg-CAST and wild-type mice. Cardiomyocytes were infected with Ad-mt-cpYFP [26] and followed by incubation with LPS (0.1-5 μg/ml) or saline for up to 4 hours. Although cpYFP is also sensitive to the pH, our recent study showed that mt-cpYFP flash events reflect a burst in electron transport chain-dependent superoxide production that is coincident with a modest increase in matrix pH in cardiomyocytes [33]. Thus, we used cpYFP as a probe to analyze mitochondrial superoxide flashes in cardiomyocytes (Figure 2-2A). Mitochondrial superoxide flashes in cardiomyocytes were inhibited by mitochondria-targeted superoxide dismutase mimetics, mito-TEMPO (Fig. 2B). LPS increased mitochondrial superoxide generation in wild-type cardiomyocytes in a time- and dose-dependent manner (Figure 2-2C and D). However, the increase in mitochondrial superoxide generation by LPS was abrogated in Tg-CAST cardiomyocytes (Figure 2-2E). These results demonstrate that calpain is important in mitochondrial superoxide generation in cardiomyocytes induced by LPS stimulation.

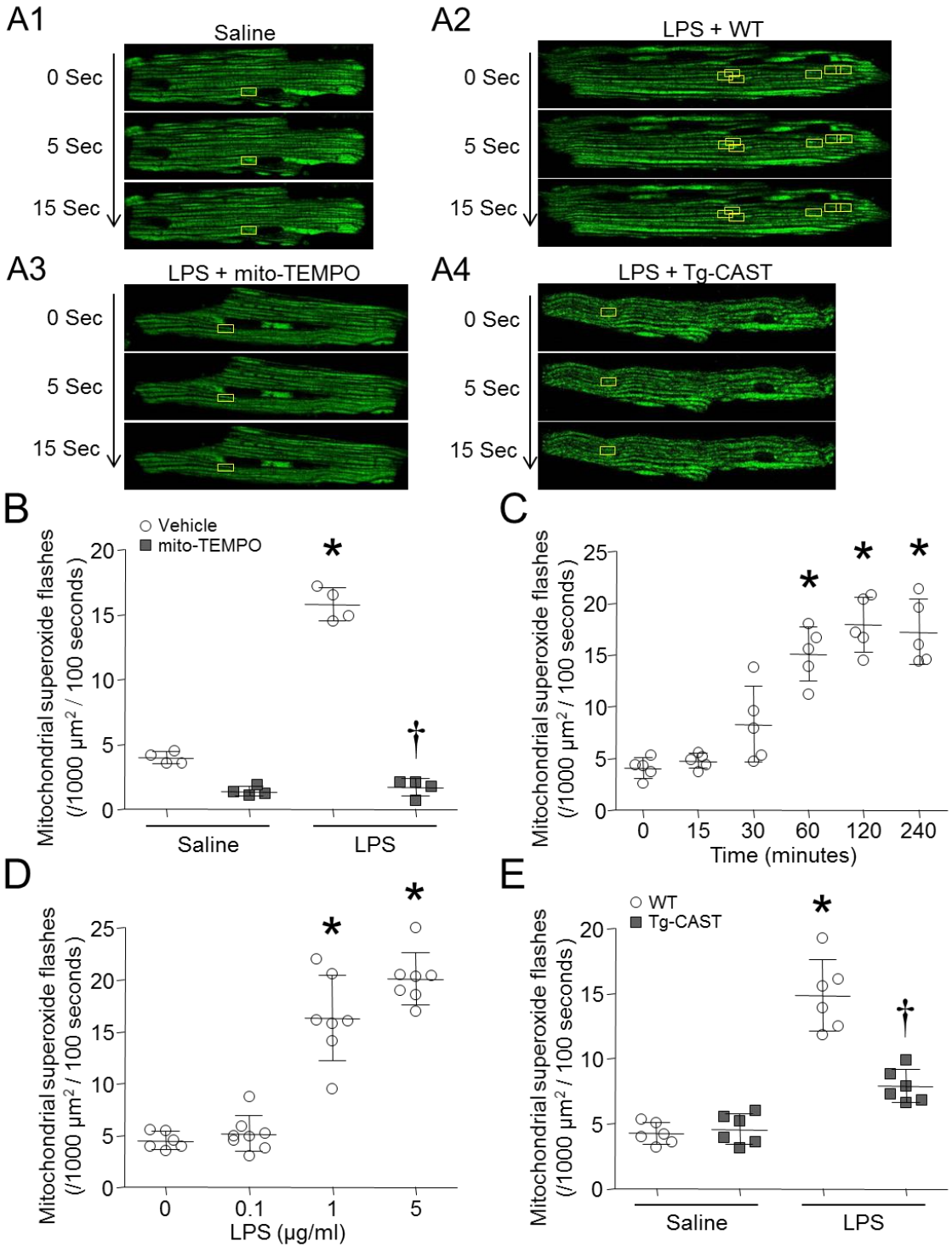
A1
Saline
0 Sec
5 Sec
15 Sec
A2
LPS + WT
0 Sec
5 Sec
15 Sec
A3
LPS + mito-TEMPO
0 Sec
5 Sec
15 Sec
A4
LPS + Tg-CAST
0 Sec
5 Sec
15 Sec
B
Vehicle
mito-TEMPO
Mitochondrial superoxide flashes
(/1000 μm² / 100 seconds)
Saline
LPS
C
Mitochondrial superoxide flashes
(/1000 μm² / 100 seconds)
Time (minutes)
D
Mitochondrial superoxide flashes
(/1000 μm² / 100 seconds)
LPS (μg/ml)
E
WT
Tg-CAST
Mitochondrial superoxide flashes
(/1000 μm² / 100 seconds)
Saline
LPS

Figure 2-2. Measurement of single mitochondrial superoxide flashes in cardiomyocytes

Adult mouse cardiomyocytes were isolated from Tg-CAST mice and their wild-type (WT) littermates. After incubation with LPS or saline for 0-4 hours, mitochondrial superoxide generation was determined. (A1-4) Representative pictures for mitochondrial superoxide flashes in cardiomyocytes. Individual mitochondria display green color in cardiomyocytes. Inside of yellow boxes, the green color increases from 0 to 10 sec and decreases after 15 sec, indicating one superoxide flash in one box. (B) Mitochondrial superoxide flashes were inhibited by mito-TEMPO in cardiomyocytes. (C) Cardiomyocytes were incubated with LPS (1 μg/ml) for up to 240 minutes. Mitochondrial superoxide flashes were measured. (D) Cardiomyocytes were incubated with LPS (0, 0.1, 1 and 5 μg/ml) for 4 hours. Mitochondrial superoxide flashes were quantified. (E) Mitochondrial superoxide flashes in WT and Tg-CAST cardiomyocytes. Data are mean ± SD from 4-7 different cultures. $^{*}P < 0.05$ versus 0, saline + WT or saline + vehicle, and $^{\#}P < 0.05$ versus LPS + WT or LPS + vehicle.

2.3.3 LPS Induces Calpain-1 Accumulation in Mitochondria

To determine whether calpains were altered in mitochondria of LPS-treated mouse hearts, we prepared mitochondrial and cytosolic fractions from sham and LPS-stimulated mouse hearts. An intact set of mitochondrial proteins (VDAC1, cytochrome c, cyclophilin D, complex Va and ATP5A1) was detected in the mitochondrial fraction, whereas GAPDH and calreticulin appeared in cytosolic but not mitochondrial fraction, validating the purity and integrity of isolated mitochondria (Figure 2-3A). The protein levels and activities of calpain-1 and calpain-2 were significantly elevated in mitochondria from LPS- compared with saline-treated hearts (Figure 2-4A-D). LPS also increased calpain activities in cytosol of the heart (Figure 2-3B). However, LPS did not change the protein levels of calpain-10, an isoform widely recognised as a mitochondrial calpain (data not shown). Since our recent study have implicated calpain-1 but not calpain-2 in LPS-induced TNF-α expression in cardiomyocytes [14], we focused on investigating calpain-1 for the following studies.

To provide further evidence in support of calpain-1 accumulation in mitochondria, we determined calpain-1 and VDAC1 proteins in isolated mitochondria of LPS-treated mouse hearts by dual immunofluorescent confocal microscopy. Confocal microscopic analysis demonstrated that VDAC1 was detected in mitochondrial membrane (red) and calpain-1 was present inside of mitochondria (green), and percentages of calpain-1-labelled mitochondria were much greater in LPS-treated versus sham mouse hearts (Figure 2-4E). Immune-electron microscopy confirmed the localization of calpain-1 in mitochondria (Figure 2-4F and G). Consistently, there were much more calpain-1 signals in mitochondria of LPS-treated hearts than those of sham mouse hearts whereas calpain-1 signals in cytosol remained comparable between sham and LPS-treated hearts (Figure 2-4G and Figure 2-5B). As a negative control for primary antibodies, no

signal was observed when calpain-1 antibody was replaced by a negative IgG isotype (Figure 2-5A). These results demonstrate that LPS induces calpain-1 accumulation in mitochondria of the heart. However, inhibition of calpain activity prevented LPS-induced calpain-1 accumulation in mitochondria of mouse hearts (Figure 2-6), suggesting that calpain-1 may re-locate to mitochondria after activation in response to LPS.

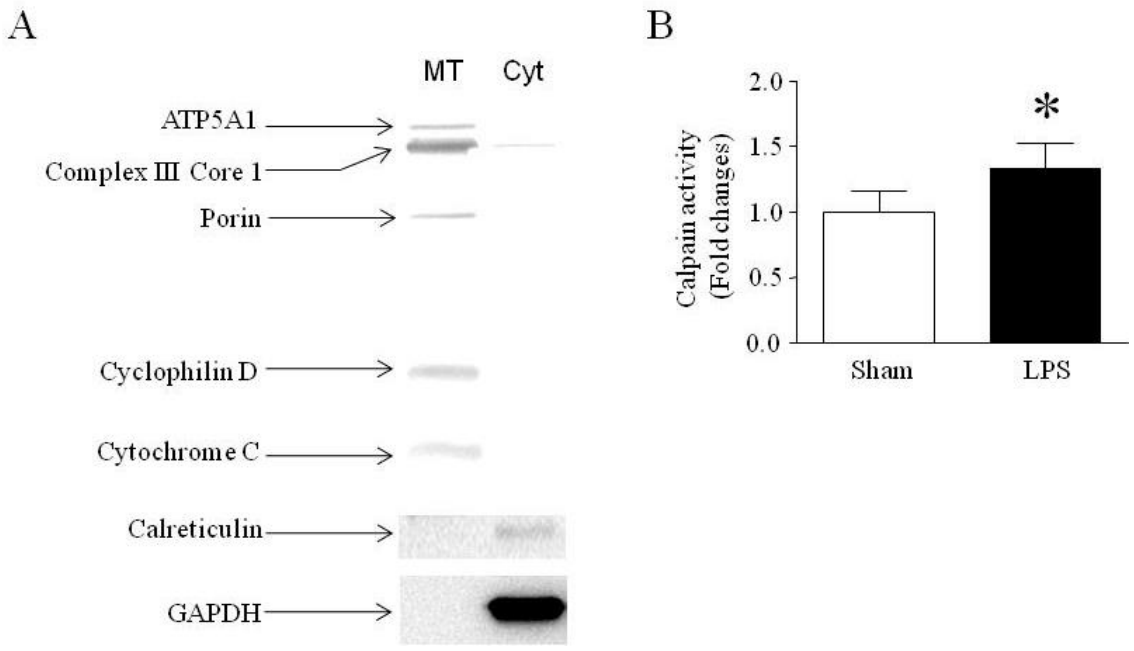

Figure 2-3. (A) Confirmation of isolated mitochondria

Mitochondria were isolated and purified from heart tissues. Western blot analysis was performed to detect a set of mitochondrial proteins, GAPDH and calreticulin in mitochondrial (Mt) and cytosolic fractions (Cyt).

(B) Calpain activities in the cytosol

The cytosol was isolated from heart tissues of sham and LPS-injected mice and calpain activities were measured. Data are mean ± SD, n = 5. *P<0.05.

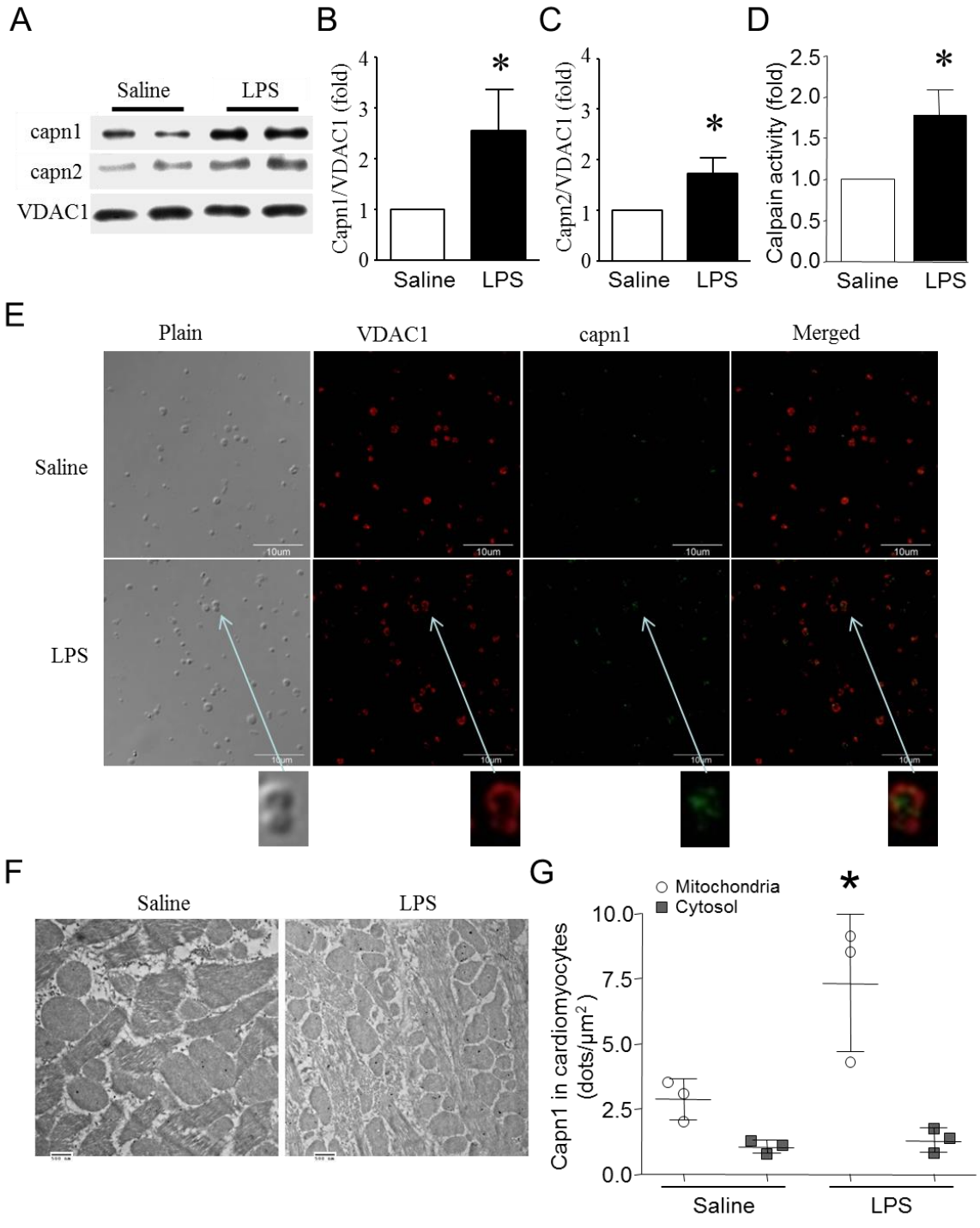
A
Saline
LPS
capn1
capn2
VDAC1
B
Capn1/VDAC1 (fold)
Saline
LPS
C
Capn2/VDAC1 (fold)
Saline
LPS
D
Calpain activity (fold)
Saline
LPS
E
Plain
VDAC1
capn1
Merged
Saline
LPS
10um
F
Saline
LPS
G
Mitochondria
Cytosol
Capn1 in cardiomyocytes (dots/μm²)
Saline
LPS

Figure 2-4. Calpain accumulation in mitochondria

Mitochondrial fractions were prepared from mice treated with saline or LPS (6 mice in each group). (A) A representative western blot for calpain-1, calpain-2, and VDAC1 in mitochondrial fraction from 2 out of 6 different hearts in each group. (B) Quantification of capn1/VDAC1 in mitochondria. (C) Quantification of capn2/VDAC1. (D) Calpain activity in mitochondrial fraction. Data are mean ± SD, $n = 6$. *$P < 0.05$ versus saline. (E) Mitochondria were isolated from sham and LPS-treated mice. Dual immunofluorescent staining for VDAC1 and calpain-1 was performed in isolated mitochondria. Representative microphotographs of confocal microscopy for VDAC1 and calpain-1 shows membrane staining of VDAC1 in mitochondria (Red) and that calpain-1 is located in mitochondria (Green). (F) Representative microphotographs of immune-electron microscopy for calpain-1 in mitochondria (black dots). (G) Quantification of calpain-1 signals in mitochondria and cytosol. Data are mean ± SD from 3 different heart tissues in each group. *$P < 0.05$ versus saline + mitochondria.

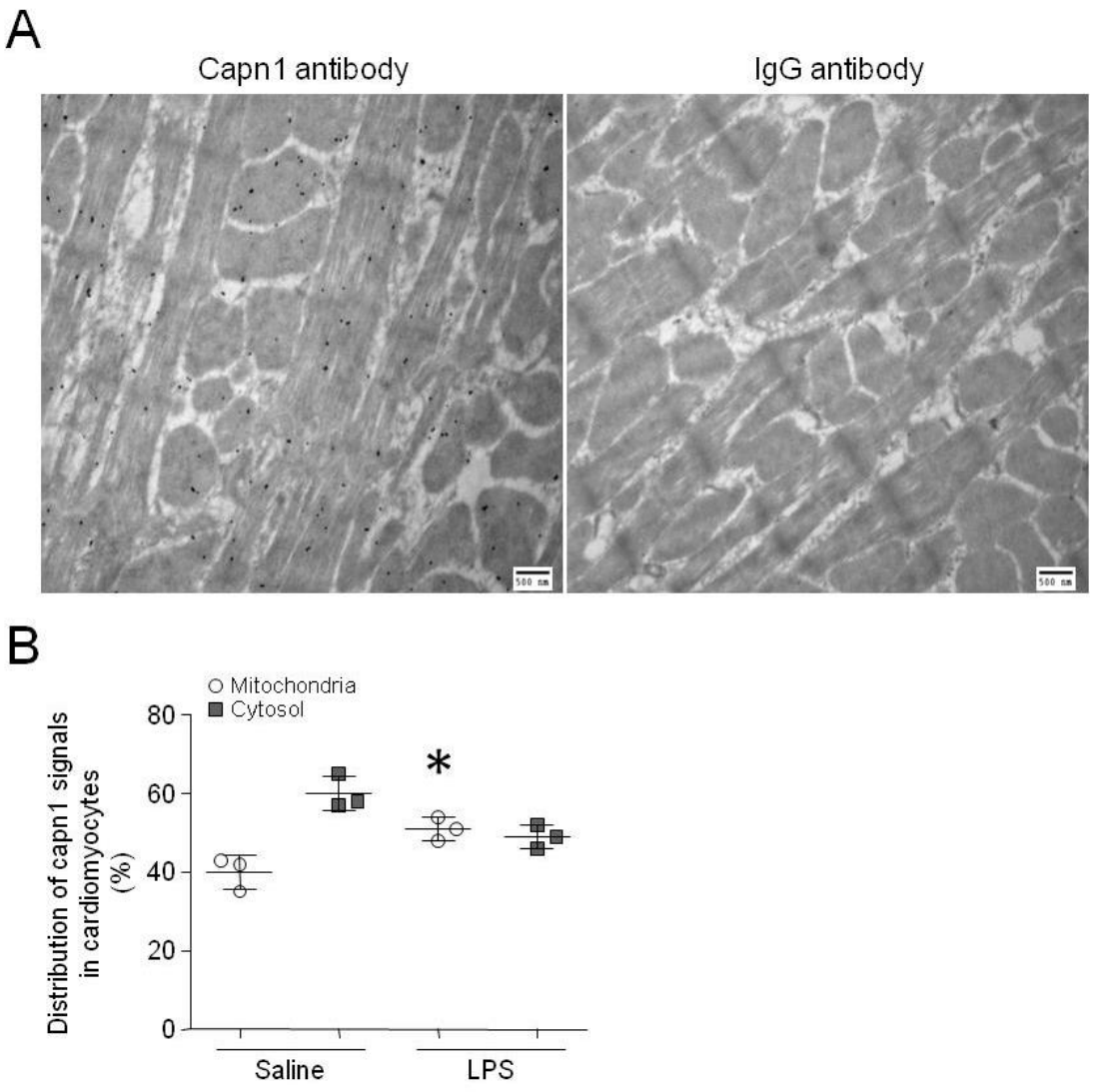

Figure 2-5. (A) Specificity of capn1 staining by immune-electron microscopy

Representative microphotographs of immune-electron microscopy for capn1 (black dots).

(B) Relative distribution of capn1 signals in mitochondria versus cytosol

Data are mean ± SD, n = 3 hearts in each group. * $p < 0.05$ vs mitochondria in Sham.

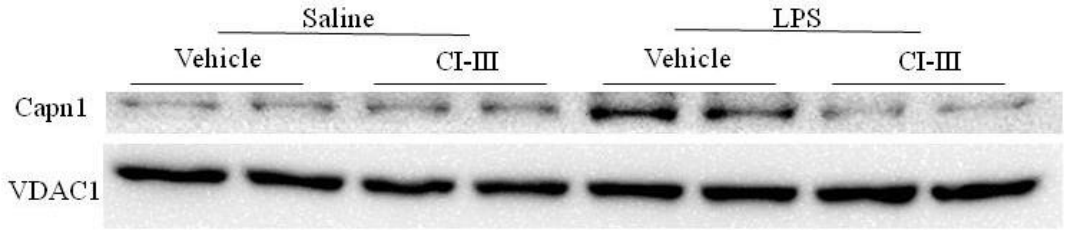

Figure 2-6. The protein levels of capn1 in mitochondria

Mice were injected with LPS or saline in combination with calpain inhibitor-III (CI-III) or vehicle. Four hours later, mitochondria were isolated from the heart and the protein levels of capn1 and VDAC1 were determined by western blot analysis. The representative western blot for capn1 and VDAC1 in mitochondria from 2 out of 6 different hearts in each group is shown.

2.3.4 Targeted Over-expression of Calpastatin in Mitochondria Inhibits Superoxide Generation in Cardiomyocytes during LPS Stimulation

To investigate the role of mitochondrial calpain, we infected cultured cardiomyocytes with adenoviral vector containing mitochondria-targeted calpastatin (Ad-mtCAST) and then incubated them with LPS for 4 hours. Selective over-expression of calpastatin in mitochondria prevented mitochondrial superoxide flashes induced by LPS (Figure 2-7). This result describes a crucial role of mitochondrial calpain in superoxide generation in cardiomyocytes during LPS stimulation.

2.3.5 Up-regulation of Calpain-1 Selectively in Mitochondria Induces Superoxide Generation and Pro-inflammatory Response in Cardiomyocytes

To provide direct evidence to support our hypothesis that the accumulation of calpain-1 in mitochondria contributes to superoxide generation and subsequent pro-inflammatory response, we transfected cardiomyocyte-like H9c2 cells with *p*CMV/myc/mito-*capn1*, a plasmid expressing mitochondrial targeted *capn1*. Twenty-four hours later, mitochondrial and cytosolic fractions were isolated from cardiomyocyte-like H9c2 cells. Over-expressed *capn1* was confirmed as myc-tagged protein in mitochondrial but not in cytosolic fractions (Figure 2-8). Intriguingly, over-expression of *capn1* restricted to mitochondria significantly increased mitochondrial superoxide generation and induced TNF-α expression in cardiomyocyte-like H9c2 cells, both of which were inhibited by mito-TEMPO (Figure 2-9A and B). These results strongly implicate mitochondrial calpain-1 in ROS production and pro-inflammatory response.

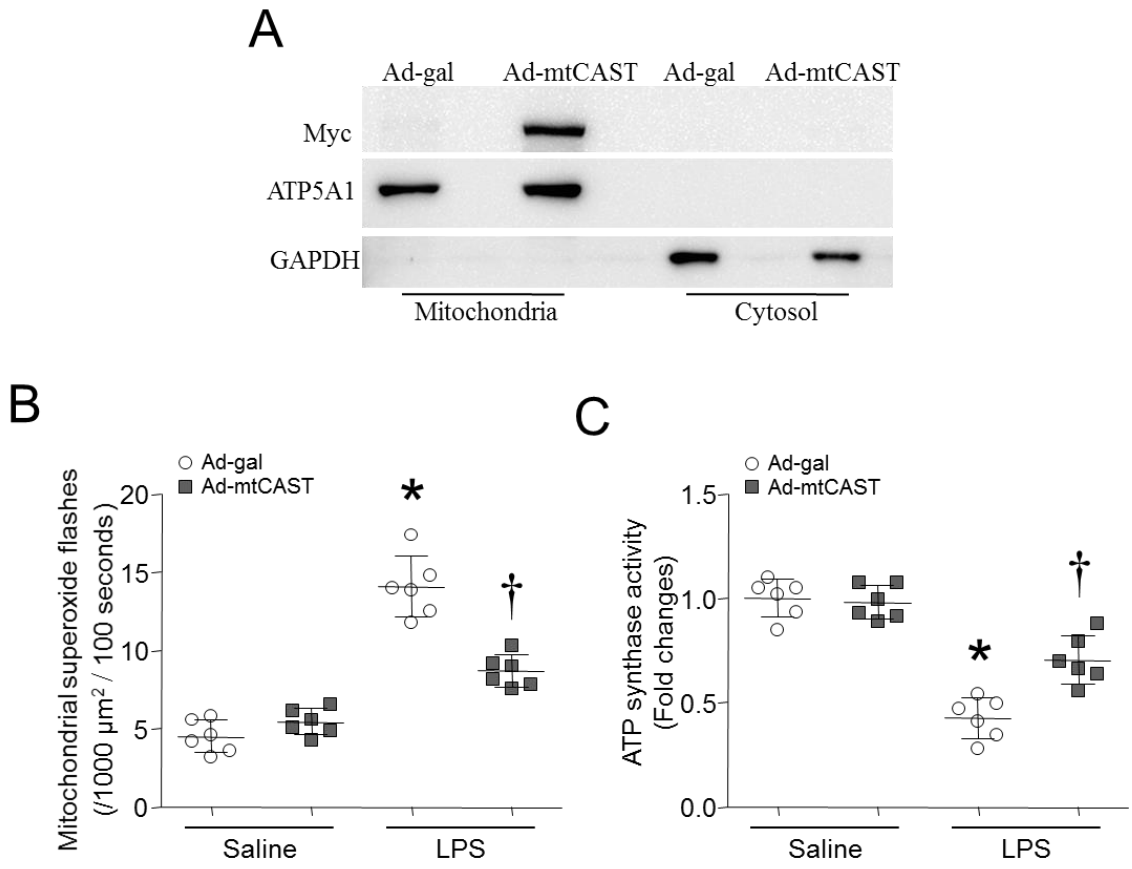

Figure 2-7. Effects of mitochondria-targeted calpastatin over-expression on mitochondrial superoxide flashes and ATP synthase activity in LPS-stimulated cardiomyocytes

(A) A representative western blot confirms myc-tagged CAST is expressed selectively in mitochondria of H9c2 cells after infection with adenoviral vector containing mitochondria-targeted calpastatin (Ad-mtCAST) or Ad-gal as a control. (B and C) After infection with Ad-mtCAST, adult cardiomyocytes were exposed to LPS or saline for 4 hours, mitochondrial superoxide generation (B) and ATP synthase activity (C) were determined. Data are mean ± SD from 6 different cultures. **P* < 0.05 versus saline + Ad-gal, and # *P* < 0.05 versus LPS + Ad-gal.

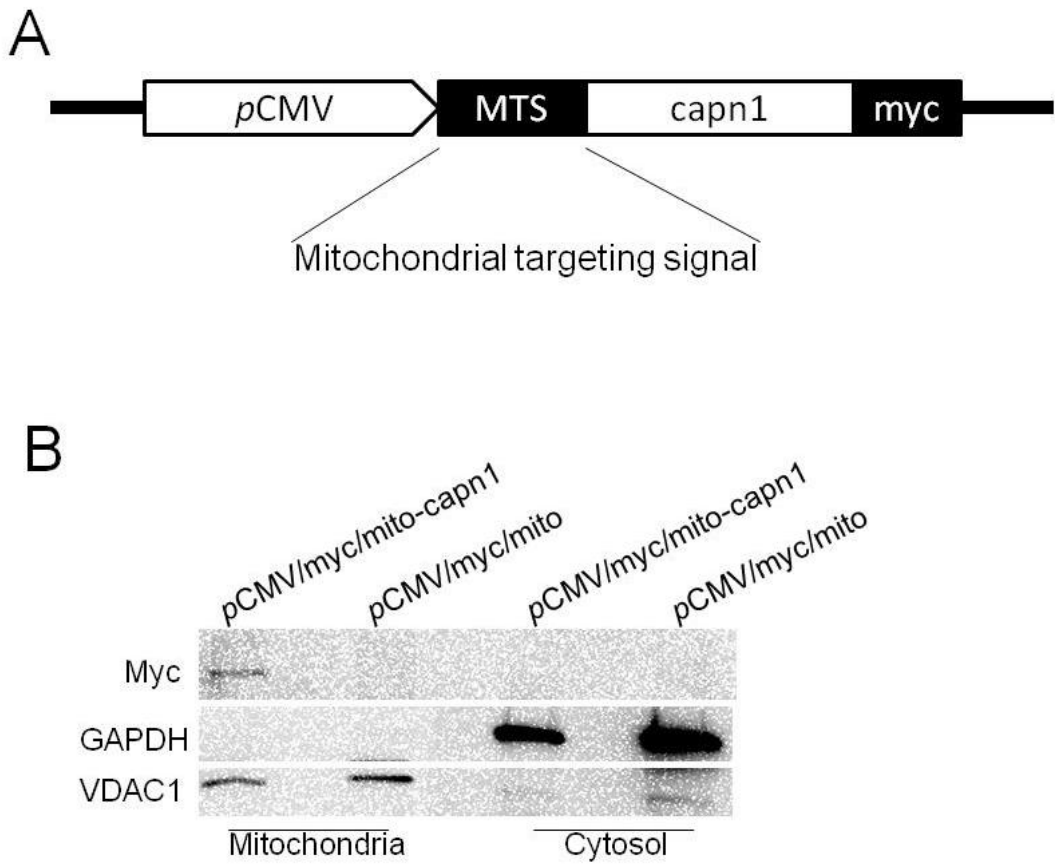

Figure 2-8. Plasmid expressing mitochondria-targeted capn1

(A) Schematic map of the plasmid expressing mitochondrial targeted capn1 (*pCMV*/myc/*mito-capn1*). (B) H9c2 cells were transfected with *pCMV*/myc/*mito-capn1* or an empty plasmid (*pCMV*/myc/*mito*) as a control. Twenty-four hours later, mitochondrial and cytosolic fractions were prepared, and myc, GAPDH and VDAC1 were detected by western blot analysis. A representative western blot confirms myc-tagged calpain-1 is expressed selectively in mitochondria.

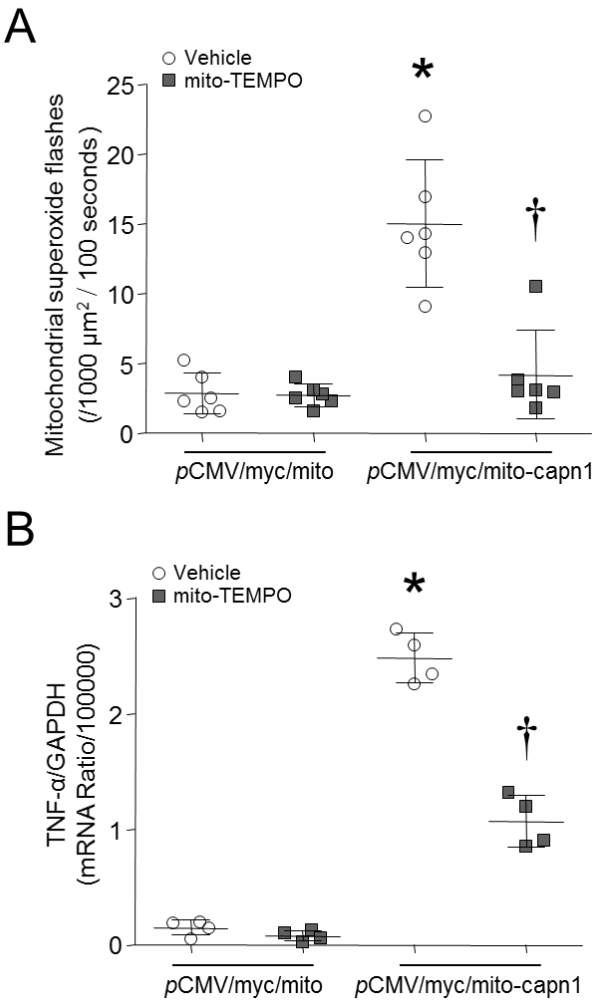

Figure 2-9. Effects of mitochondrial targeted capn1 on superoxide generation and TNF-α expression in H9c2 cells

After transfection with *p*CMV/myc/mito-capn1, H9c2 cells were incubated with mito-TEMPO or vehicle for 24 hours. (A) Mitochondrial superoxide flashes were assessed. (B) TNF-α mRNA was analyzed. Data are mean ± SD from 4-6 different cultures. $*P < 0.05$ versus *p*CMV/myc/mito + vehicle, and $^{\#}P < 0.05$ versus *p*CMV/myc/mito-capn1 + vehicle.

2.3.6 ATP5A1 Is a Direct Target of Calpain-1 in Mitochondria in Response to LPS

To explore the potential targets of calpain-1 in mitochondria, we pulled down the calpain-1 and its interacting proteins from isolated mitochondria of LPS-treated mouse hearts. Western blot analyses for a number of mitochondrial proteins (VDAC1, cytochrome c, cyclophidin D, NCX1, ATP5A1, ATP synthase-β and Opa-1) revealed that only ATP5A1 was pulled down with calpain-1 (Figure 2-10A1). Likewise, calpain-1 was detected in immune-captured ATP synthase complex (Figure 2-10A2). These results demonstrate a physical interaction between calpain-1 and ATP5A1 in mitochondria. Furthermore, ATP5A1 is a direct substrate of calpain-1 since co-incubation of active calpain-1 with recombinant ATP5A1 protein *in vitro* resulted in multiple cleavages of ATP5A1 (Figure 2-11).

We therefore reasoned that calpain-1 cleaved ATP5A1 and disrupted ATP synthase activity in mitochondria of LPS-treated mouse hearts. Accordingly, LPS reduced ATP synthase activity in mitochondria, which is consistent with previous reports [34, 35]. However, the reduction in ATP synthase activity was prevented by *capn4* deletion (Figure 2-10B). In line with the reduction of ATP synthase activity, the protein levels of ATP5A1were markedly decreased in mitochondria from LPS-treated wild-type hearts (Figure 2-10C) and restored in *capn4*-ko mice after LPS stimulation (Figure 2-10D). However, the mRNA levels of ATP5A1 remained comparable among those groups (data not shown). In cultured cardiomyocytes, selective over-expression of calpastatin in mitochondria by infection with Ad-mtCAST significantly attenuated LPS-induced reduction in ATP synthase activity (Figure 2-7C).

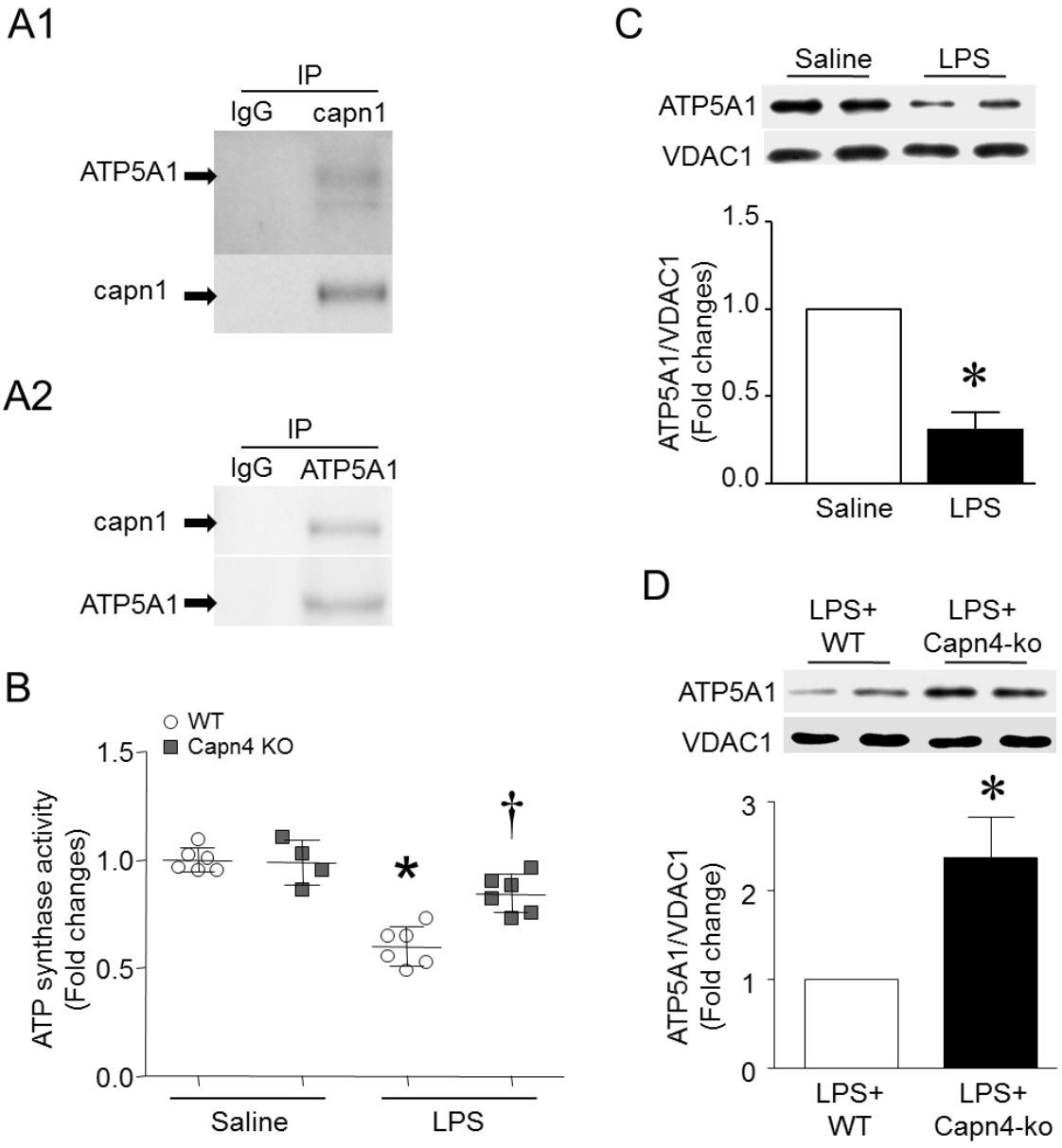
A1
IP
IgG
capn1
ATP5A1
capn1
A2
IP
IgG
ATP5A1
capn1
ATP5A1
B
WT
Capn4 KO
ATP synthase activity
(Fold changes)
1.5
1.0
0.5
0.0
*
†
Saline
LPS
C
Saline
LPS
ATP5A1
VDAC1
ATP5A1/VDAC1
(Fold changes)
1.5
1.0
0.5
0.0
*
Saline
LPS
D
LPS+
WT
LPS+
Capn4-ko
ATP5A1
VDAC1
ATP5A1/VDAC1
(Fold change)
3
2
1
0
*
LPS+
WT
LPS+
Capn4-ko

Figure 2-10. Role of calpain in ATP5A1 expression and ATP synthase disruption in endotoxemic mouse hearts

(A1) A representative western blot shows that ATP5A1 is detected in calpain1 interacting proteins. (A2) A representative western blot shows that calpain-1 is detected in captured ATP synthase complex. (B-D) Myocardial mitochondria were isolated from capn4-ko and their wild-type (WT) mice treated with saline or LPS. (B) ATP synthase activity was measured in mitochondria. (C and D) The upper panels are the representative western blot for ATP5A1 protein from 2 out of 4 hearts in each group and the lower panels are the quantification of ATP5A1 protein relative to VDAC1 in mitochondria. Data are means ± SD, n = 4-6. *P < 0.05 versus saline, saline + WT or LPS+WT and $^{\#}P$ < 0.05 versus LPS + WT.

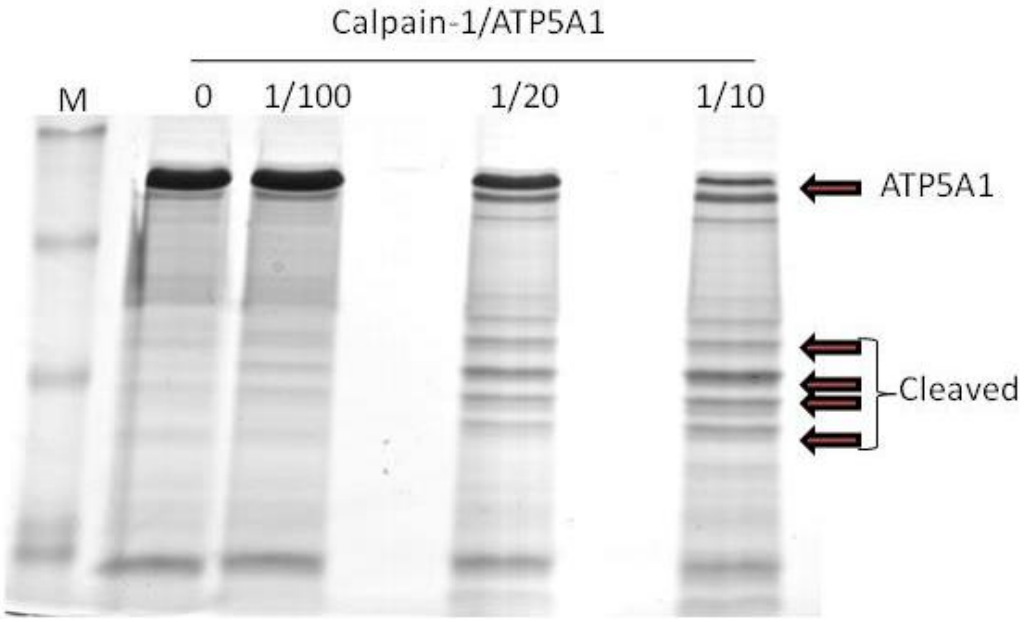

Figure 2-11. Calpain-1 dependent cleavages of ATP5A1

Recombinant protein ATP5A1 was incubated with active calpain-1 in a reaction buffer containing 50 μmol/L of Ca^{2+} at room temperature for 15 minutes. A representative SDS-PAGE gel after coomassie blue staining shows ATP5A1 protein and its multiple cleaved fragments.

2.3.7 Over-expression of ATP5A1 Reduces Mitochondrial Superoxide Generation and TNF-α Expression and Attenuates Myocardial Dysfunction in Endotoxemic Mice

To investigate whether up-regulation of ATP5A1 provides cardiac protection, we delivered Ad-ATP5A1 into mice. Ad-GFP served as a control. Forty-eight hours later, mice received LPS (4 mg/kg, i.p.) or saline. Four hours later, delivery of Ad-ATP51 significantly increased ATP5A1 protein in both sham and LPS-treated hearts (Figure 2-12A and B) and ATP synthase activity in LPS-treated hearts (Figure 2-12C), suggesting that ectopic expression of ATP5A1 integrates into the complex of ATP synthase. Up-regulation of ATP5A1 reduced mitochondrial ROS generation (Figure 2-12D) and TNF-α expression in mouse hearts after LPS stimulation (Figure 2-12E), and improved myocardial function in endotoxemic mice (Figure 2-12F and Table 2-2).

In cultured cardiomyocytes, incubation with ATP synthase inhibitor oligomycin A increased mitochondrial superoxide flash generation (Figure 2-13). To provide direct evidence to support the role of ATP5A1, we infected cardiomyocytes with Ad-ATP5A1 or Ad-gal as a control, and then incubated them with LPS for 4 hours. Up-regulation of ATP5A1 increased ATP synthase activity (Figure 2-14A) and reduced mitochondrial superoxide generation induced by LPS (Figure 2-14B). Similarly, infection with Ad-ATP5A1 attenuated mitochondrial superoxide generation and TNF-α expression induced by mitochondrial-targeted calpain-1 in cardiomyocytes (Figure 2-14C and D).

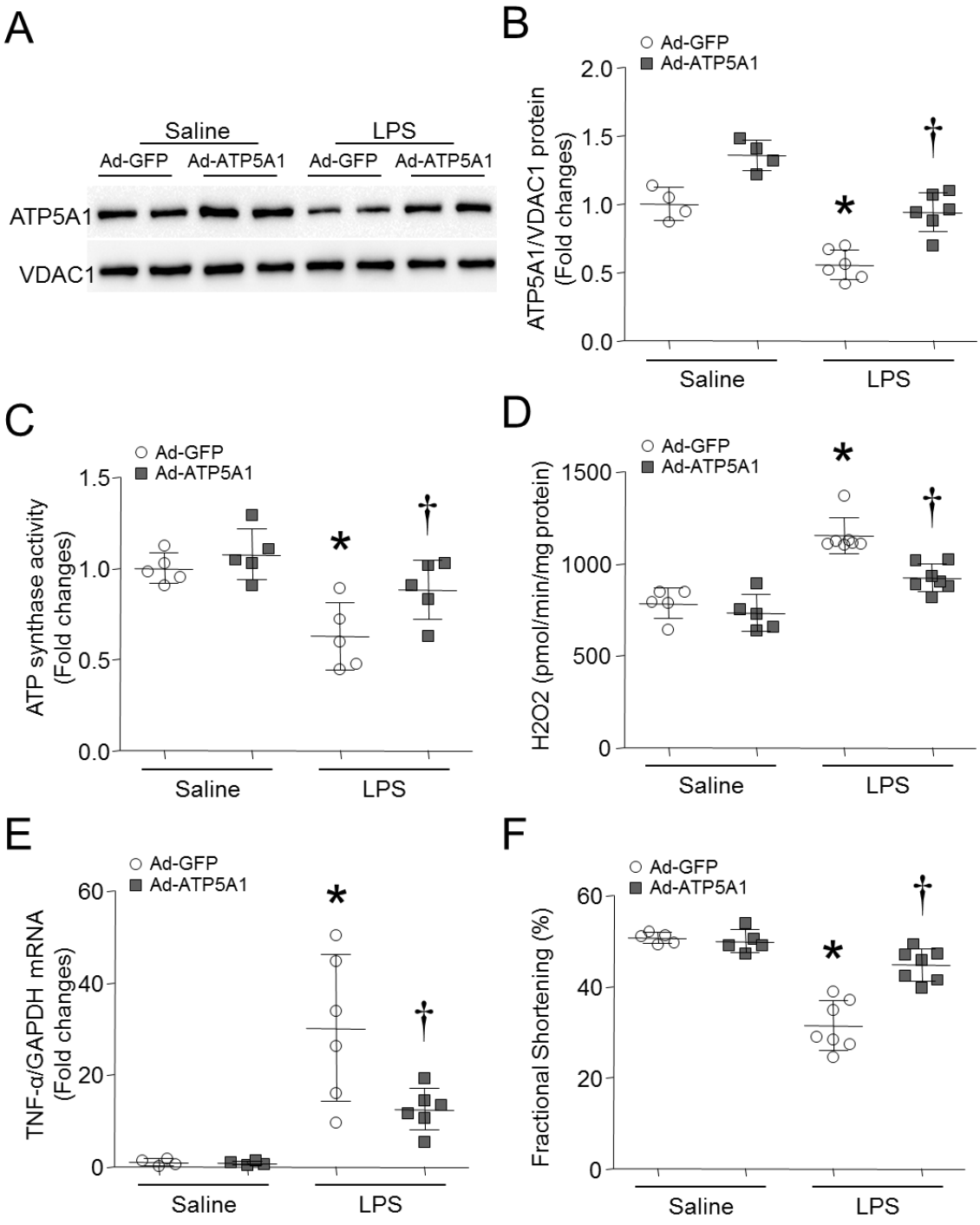
A
Saline
LPS
Ad-GFP
Ad-ATP5A1
Ad-GFP
Ad-ATP5A1
ATP5A1
VDAC1
B
Ad-GFP
Ad-ATP5A1
ATP5A1/VDAC1 protein (Fold changes)
2.0
1.5
1.0
0.5
0.0
*
†
Saline
LPS
C
Ad-GFP
Ad-ATP5A1
ATP synthase activity (Fold changes)
1.5
1.0
0.5
0.0
*
†
Saline
LPS
D
Ad-GFP
Ad-ATP5A1
H2O2 (pmol/min/mg protein)
1500
1000
500
0
*
†
Saline
LPS
E
Ad-GFP
Ad-ATP5A1
TNF-α/GAPDH mRNA (Fold changes)
60
40
20
0
*
†
Saline
LPS
F
Ad-GFP
Ad-ATP5A1
Fractional Shortening (%)
60
40
20
0
*
†
Saline
LPS

Figure 2-12. Effects of ATP5A1 over-expression in endotoxemic mouse hearts

Adult mice were injected with Ad-ATP5A1 or Ad-GFP and then treated with LPS. Four hours later, mitochondria were isolated. (A) A representative western blot from 2 out of 4-6 different hearts for ATP5A1 and VDAC1. (B) Quantification of ATP5A1/VDAC1 protein ratio. (C) ATP synthase activity. (D) Mitochondrial ROS generation was assessed following addition of succinate. (E) TNF-α mRNA was analyzed in heart tissues by real-time RT-PCR. (F) Myocardial function was assessed by echocardiography. Data are means ± SD, n = 4-7. $^{*}P < 0.05$ versus saline+Ad-GFP, and $^{\#}P < 0.05$ versus LPS+Ad-GFP.

Table 2-2. Effect of Ad-ATP5A1 on myocardial function in endotoxemic mice

	Saline + Ad-GFP	Saline + Ad-ATP5A1	LPS + Ad-GFP	LPS + Ad-ATP5A1
ESD (mm)	2.06±0.13	2.07±0.18	2.01±0.29	1.81±0.30
EDD (mm)	4.17±0.22	4.14±0.27	2.93±0.30	3.27±0.50
FS(%)	50.65±1.16	49.91±2.52	31.44±5.43*	44.70±3.54†
Heart rate (beats/minute)	460.20±40.18	532.60±73.78	597.43±49.82*	597.57±59.72

*$p<0.05$ versus Saline + Ad-GFP and # $p<0.05$ versus LPS + Ad-GFP. ESD: End systolic diameter; EDD: End diastolic diameter; FS: Fractional shortening.

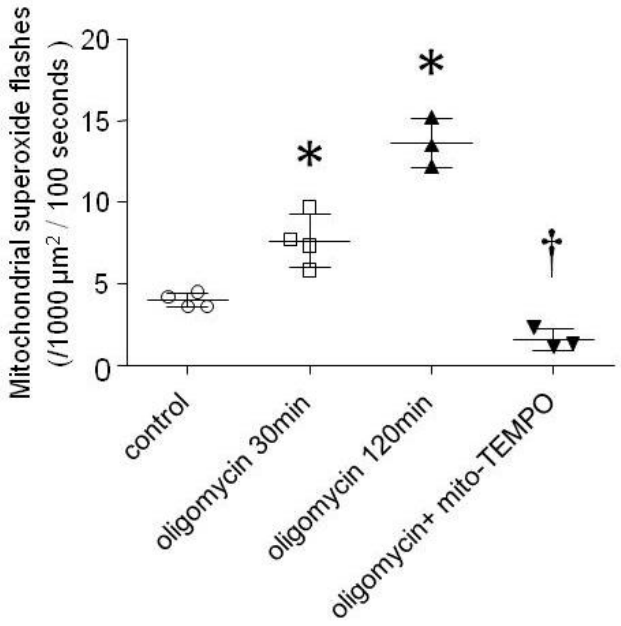

Figure 2-13. Effect of oligomycin A on mitochondrial superoxide flashes in adult cardiomyocytes

Adult mouse cardiomyocytes were incubated with oligomycin A in the presence or absence of mito-TEMPPO. At different time points after addition of oligomycin A, mitochondrial superoxide flashes were determined. Data are MEAN ± SD from 3 different cultures. *$P < 0.05$ versus control and # $P < 0.05$ versus control, oligomycin 30 min, or 20 min.

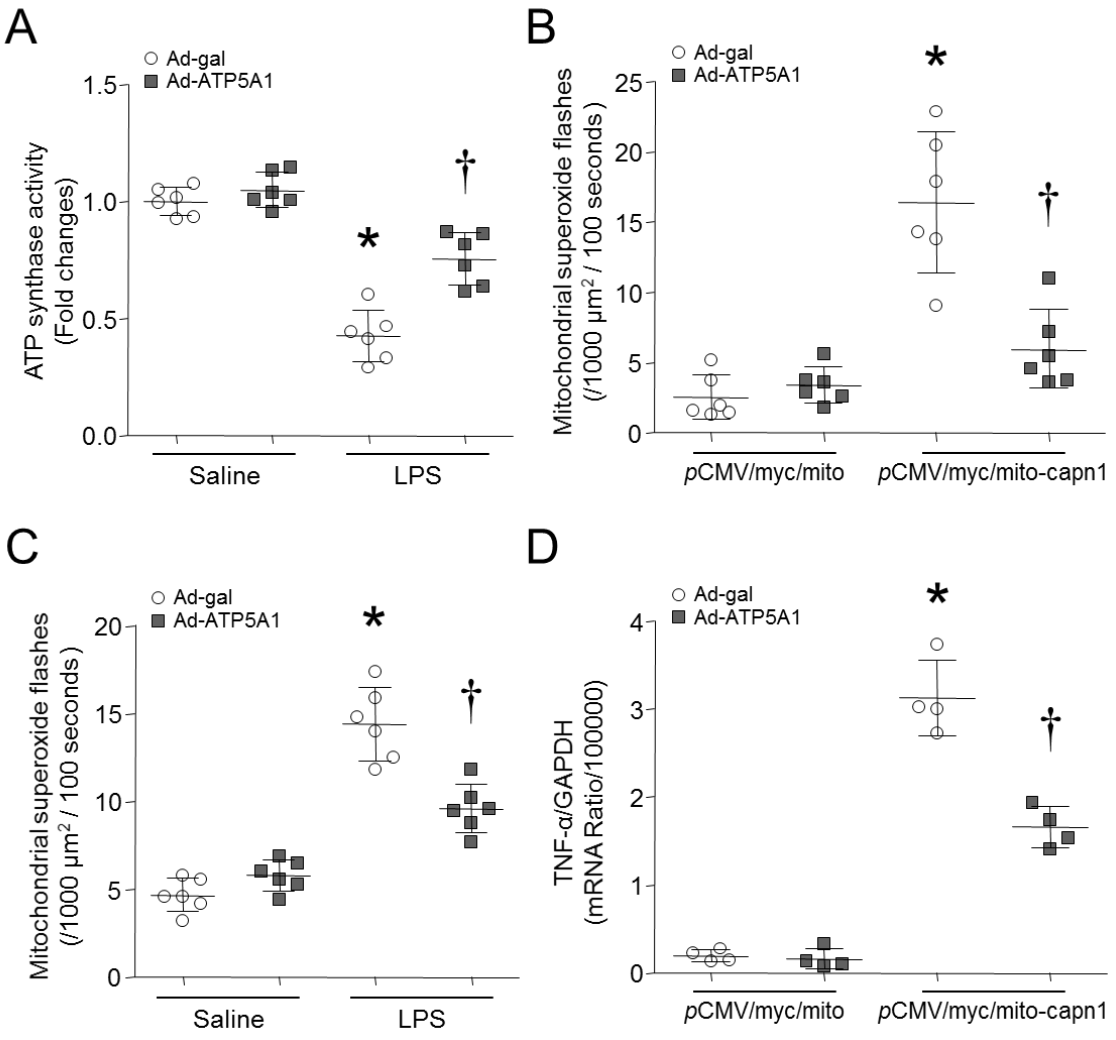

Figure 2-14. Role of ATP5A1 in mitochondrial superoxide generation and TNF-α expression

(A and B) Adult mouse cardiomyocytes were infected with Ad-ATP5A1 or Ad-gal. Twenty-four hours later, the cells were incubated with LPS or saline for 4 hours. Mitochondrial superoxide generation (A) and ATP synthase activity (B) were determined. (C and D) After transfection with *p*CMV/myc/mito-capn1, H9c2 cells were infected with Ad-ATP5A1 or Ad-gal for 24 hours. Mitochondrial superoxide flashes (C) and TNF-α mRNA (D) were analyzed. Data are mean ± SD from 4-6 different experiments. $*P < 0.05$ versus Ad-gal + saline or Ad-gal + *p*CMV/myc/mito, and $^{\#}P < 0.05$ versus Ad-gal + LPS or *p*CMV/myc/mito-capn1 + Ad-gal.

2.4 Discussion

In this study, we demonstrate that LPS treatment increases calpain-1/-2 in mitochondria and the accumulation of calpain in mitochondria correlates with mitochondrial ROS generation in the heart. Deletion of *capn4* reduces mitochondrial ROS production in cardiomyocytes and the hearts in response to LPS. Selective up-regulation of calpain-1 in mitochondria sufficiently induces superoxide generation and TNF-α expression in cardiomyocytes. Furthermore, calpain-1 in mitochondria disrupts ATP synthase through proteolysis of ATP5A1 in response to LPS stimulation. Up-regulation of ATP5A1 inhibits mitochondrial superoxide generation in cardiomyocytes, and attenuates TNF-α expression, leading to the improvement of myocardial function in endotoxemic mice. To our knowledge, this is the first study demonstrating a novel role of calpain-1 in disrupting ATP synthase and promoting mitochondrial superoxide generation in endotoxemic hearts.

In a rat model of endotoxemia, the role of calpain in myocardial dysfunction was suggested by using pharmacological inhibitors of calpain [36]. Further evidence came from our demonstration that over-expression of calpastatin attenuated myocardial dysfunction in Tg-CAST mice during LPS stimulation [14]. In the present study using tissue-specific *capn4* knockout mice, we show that deletion of *capn4* decreases myocardial TNF-α expression, reduces mitochondrial ROS production and attenuates myocardial dysfunction in endotoxemic mice. These findings verify the view that cardiac calpain plays a direct role in myocardial dysfunction in sepsis and may represent an important therapeutic target for sepsis.

Calpains have been shown to relocate to the membrane and nucleus in the heart under stress [37, 38]. They are also present in mitochondria [16, 39, 40]. A recent study has demonstrated that calpain-1 activity is increased in cardiac mitochondria during ischemia-reperfusion [41]. In the present study, we demonstrate that LPS induces the

accumulation of calpain-1 in mitochondria of the heart. Although it is currently unknown whether the accumulation of calpain-1 in mitochondria results from an increase in its translocation into mitochondria or a decrease in their degradation in mitochondria, our previous study showed that the protein levels of calpain-1 in whole heart lysates were not altered after LPS stimulation [14], suggesting that LPS may induce the translocation of calpain-1 into mitochondria. It is worthwhile to mention that immune-electron microscopic analysis shows no significant change in calpain-1 in cytoplasm upon LPS stimulation. Given that interfibrillar mitochondria constitute about 15-20% of cellular volume in cardiomyocytes, we believe that a small portion of calpain-1 protein relocates to mitochondria while the majority of calpain-1 remains in cytoplasm. Thus, relocation of a small portion of calpain-1 may not significantly affect the protein levels of calpain-1 in cytoplasm upon LPS stimulation. Furthermore, the re-location of calpain-1 in mitochondria is dependent on its activation in response to LPS as inhibition of calpain prevents calpain-1 accumulation in mitochondria. Active calpain-1 mitochondrial translocation has been also shown in homocysteine-stimulated microvascular endothelial cells [42]. A recent study has identified a mitochondrial targeting sequence in the N-terminal region of *capn1*, which provides a molecular basis for calpain-1 mitochondrial translocation [43]. It is currently unknown what causes the translocation of active calpain-1 from cytosol to mitochondria in the setting of septic cardiomyopathy. A recent study demonstrated that calpain translocation to sarcolemmal was dependent on Ca^{2+} entry through Na^{+}/Ca^{2+} exchanger in cardiomyocytes [44]. Given that mitochondrial Ca^{2+} is altered in septic cardiomyocytes [45], it is possible that mitochondrial Na^{+}/Ca^{2+} exchanger may facilitate the translocation of calpain-1 from cytosol to mitochondria in cardiomyocytes in response to LPS stimulation, which merits further investigation.

An important finding of this study is that calpain-1 accumulation in mitochondria mediates ROS generation in endotoxemic mouse hearts. Importantly, targeted

over-expression of capn1 in mitochondria mimics the effect of LPS on mitochondrial superoxide generation in cardiomyocytes. Thus, this study provides a novel mechanism that mitochondrial superoxide is induced by calpain-1 in cardiomyocytes during LPS stimulation. Given the importance of mitochondrial ROS in cardiac pathophysiological processes [46], further investigations are needed to clarify whether mitochondrial calpain-1 activation is a common mechanism for mitochondrial superoxide generation in other pathological conditions. Mitochondrial ROS induces the damage to mitochondria which may promote more ROS production in mitochondria, forming the vicious circle, finally leading to mitochondrial dysfunction [47]. Mitochondrial ROS is also an important signaling mechanism in mediating gene expression. In this regard, we have recently demonstrated that mitochondrial ROS mediates TNF-α expression in cultured cardiomyocytes during LPS stimulation [32]. The present study further demonstrates that selective over-expression of *capn1* in mitochondria induces TNF-α expression in cardiomyocytes, which is inhibited by mito-TEMPO, providing direct evidence in support of the view that mitochondrial calpain-1 mediates pro-inflammatory response through superoxide generation.

In an effort to explore the mechanisms by which calpain-1 induces superoxide generation in mitochondria, we demonstrate that ATP5A1 co-localizes with calpain-1 in LPS-treated mouse hearts. We further show that calpain-1 may cleave ATP5A1, substantiating the finding from a recent report that ATP5A1 is a potential substrate of calpain [17]. Disruption of ATP synthase inhibits ATP production, directly contributing to myocardial dysfunction. In septic hearts, ATP synthase activity and ATP production are decreased [34, 35]. Our observations are consistent with a model whereby calpain-1 accumulation in mitochondria compromises ATP synthase in LPS-treated mouse hearts. In fact, we show a significant reduction of ATP5A1 protein and of its activity in mitochondria from LPS-treated mouse hearts, which are prevented by *capn4* deletion. On the other hand,

disruption of ATP synthase within complex V results in excess electron “backup” in the individual electron transfer complexes [47], in particular complexes I and III, promoting mitochondrial superoxide generation. In support of this view, inhibition of ATP synthase activity directly increases mitochondrial superoxide generation in cardiomyocytes, and up-regulation of ATP5A1 attenuates mitochondrial superoxide generation and TNF-α expression in cardiomyocytes induced by LPS and mitochondrial-targeted calpain-1. Furthermore, we show that up-regulation of ATP5A1 increases ATP synthase activity, reduces mitochondrial ROS generation and TNF-α expression, and attenuates myocardial dysfunction in endotoxemic mice. Taken together, our observation argues that calpain-1 mediates mitochondrial superoxide generation, at least partly by disrupting ATP synthase, leading to LPS-induced pro-inflammatory response in the heart. It is worthwhile to mention that over-expression of ATP5A1 did not abrogate TNF-α expression in cardiomyocytes. This suggests that other targets of calpain-1 in mitochondria may exist in regulation of TNF-α expression, which merits further investigation. In fact, a recent study demonstrated that calpain also targets and cleaves apoptosis inducing factor in mitochondria of the heart [41], leading to ischemia/reperfusion injury. Thus, it is possible that calpain-mediated cleavage of apoptosis inducing factor may also contribute to septic cardiomyopathy, which needs further investigation for clarification.

In summary, the present study has provided convincing evidence that calpain activation directly contributes to myocardial pro-inflammatory response to LPS by promoting mitochondrial calpain-1 translocation and ROS generation. ATP5A1 may represent an important target of calpain-1 in mitochondria and its proteolysis disrupts ATP synthase, leading to mitochondrial superoxide generation in endotoxemia. Our study suggests that calpain and mitochondrial ROS may be potential therapeutic targets for myocardial dysfunction in sepsis.

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Chapter 3

3 Mitochondrial calpain-1 disrupts ATP synthase and induces superoxide generation in type-1 diabetic hearts: a novel mechanism contributing to diabetic cardiomyopathy[3]

3 This chapter has been published in the following manuscript:

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3.1 Introduction

Diabetes is a global metabolic disease and will affect nearly 400 million people by 2030 [1]. Cardiovascular complications are the most common cause of morbidity and mortality in diabetic patients, and approximately 80% of all diabetic patients will die of cardiovascular diseases [2, 3]. Both type-1 and type-2 diabetes can directly affect cardiac structure and function in the absence of changes in blood pressure and coronary artery disease, a condition described as diabetic cardiomyopathy. Diabetic cardiomyopathy may present with diastolic dysfunction in the early stages and may subsequently proceed to systolic dysfunction [4]. The pathogenesis of diabetic cardiomyopathy is incompletely understood and limited treatment options exist.

Calpains belong to a family of calcium-dependent thiol-proteases [5]. Fifteen gene products of the calpain family are reported in mammals. Among them, calpain-1 and calpain-2 are ubiquitously expressed and well-studied. Both calpain-1 and calpain-2 consist of distinct large 80-kDa catalytic subunits encoded by *capn1* and *capn2*, respectively, and a common small 28-kDa regulatory subunit encoded by *capn4*. The regulatory subunit is indispensable for calpain-1 and calpain-2 activities. Calpain-1 and calpain-2 are regulated by the endogenous calpain inhibitor, calpastatin. We have recently reported that genetic inhibition of calpain by over-expression of calpastatin or deletion of *capn4* prevented cardiomyocyte apoptosis and reduced cardiomyopathic changes in mouse models of streptozotocin (STZ)-induced type-1 diabetes [6, 7], highlighting a critical role of calpain in diabetic cardiomyopathy. However, the underlying mechanisms remain to be determined.

Although calpain-1 and calpain-2 have been considered as mainly cytoplasmic enzymes, they are also present in mitochondria [8, 9]. It was reported that hyperhomocysteinemia

induced the translocation of active calpain-1 from cytosol to mitochondria, which was associated with intra-mitochondrial oxidative stress in cultured rat heart microvascular endothelial cells [10], suggesting that calpain may regulate mitochondrial ROS generation. This was supported by our recent study, which demonstrated that inhibition of calpain prevented mitochondrial ROS generation in endothelial cells upon high glucose stimulation [11]. It has been suggested that calpains may target some important proteins in mitochondria, including, but not limited to, ATP synthase-alpha (ATP5A1) [12], optic atrophy-1 (Opa-1) [13], apoptosis-inducing factor [14], and Na^+/Ca^{2+} exchanger-1 (NCX-1) [15]. In diabetic hearts, studies have shown that the protein levels of ATP5A1 are reduced and ATP synthase activity decreases [16, 17]. Disruption of these mitochondrial proteins may compromise mitochondrial function, resulting in excessive ROS generation. In fact, mitochondrial ROS production is increased in hearts of type-1 and type-2 diabetic models [17-20]. Although mitochondrial superoxide generation is not increased in the heart of some T1D animals [21, 22], selective inhibition of mitochondrial ROS reduces cardiomyopathic changes in T1D [23, 24]. These studies raise an intriguing hypothesis that calpain activation may lead to excessive mitochondrial ROS generation in diabetic hearts, which contributes to diabetic cardiomyopathy.

In this study, we demonstrate that diabetes induces calpain-1 accumulation in mitochondria of the heart. Increased calpain-1 in mitochondria is associated with ATP synthase disruption, which stimulates mitochondrial ROS generation and thus, promotes diabetic cardiomyopathy in a mouse model of STZ-induced T1D.

3.2 Research design and methods

3.2.1 Animals

This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011).

All experimental procedures were approved by the Animal Use Subcommittee at the University of Western Ontario, Canada in accordance with the guidelines of the Canadian Council for Animal Care. Breeding pairs of C57BL/6 mice and db+/- mice were purchased from the Jackson Laboratory (CA, USA). Transgenic mice with over-expression of calpastatin (Tg-CAST, C57BL/6 background) were generously provided by Dr. Laurent Baud (The Institut National de la Santé et de la Recherche Médicale, Paris, France) through the European Mouse Mutant Archive [25]. Mice with cardiomyocyte-specific disruption of *capn4* (*capn4*-ko) were generated as described in our recent reports [7]. All of the mice used in this study, including controls, were littermates of the same generation.

3.2.2 Experimental protocol

Type-1 diabetes was induced in adult male mice (2-month old) by consecutive peritoneal injection of STZ (50 mg/kg/day) for 5 days [7]. Seventy-two hours after the last injection of STZ, whole blood was obtained from the mouse tail-vein and random glucose levels were measured using the OneTouch Ultra 2 blood glucose monitoring system (Life Scan, Inc. CA, USA). Mice were considered diabetic and used for the study only if they had hyperglycemia (≥15 mM) 72 h after STZ injection. Citrate buffer-treated mice were used as a non-diabetic control (blood glucose < 12 mM). Two months after induction of diabetes, mice (n=8-12 in each group) were subjected to the following experiments.

3.2.3 Echocardiography

Animals were lightly anaesthetized with inhalant isoflurane (1%) and imaged using a 40-MHz linear array transducer attached to a preclinical ultrasound system (Vevo 2100, FUJIFILM VisualSonics, Canada) with nominal in-plane spatial resolution of 40 μm (axial) × 80 μm (lateral). M-mode and 2-D parasternal short-axis scans (133 frames/second) at the level of the papillary muscles were used to assess changes in left

ventricle (LV) end-systolic inner diameter (LVIDs), LV end-diastolic inner diameter (LVIDd), and fractional shortening (FS%).

To assess diastolic function, we obtained apical four-chamber views of the left ventricle. The pulsed wave Doppler measurements of maximal early (E) and late (A) transmitral velocities in diastole were obtained in the apical view with a cursor at mitral valve inflow.

3.2.4 Delivery of adenoviral vectors into mice

Mice were anaesthetized with inhaled isofluorane (1-3%). With the guide of echocardiography, adenoviral vectors containing human ATP5A1 gene (Ad-ATP5A1, 2×10^9 PFU in 100 μL, SignaGen Laboratories, MD, USA) or GFP (Ad-GFP, SignaGen Laboratories, MD, USA) were injected into mouse left ventricle.

3.2.5 Isolation and culture of adult mouse cardiomyocytes

Adult mouse ventricle cardiomyocytes were isolated and cultured as previously described [26].

3.2.6 Adenoviral infection of cardiomyocytes

Cardiomyocytes were infected with Ad-ATP5A1, adenoviral vectors containing mitochondria-targeted rat calpastatin (Ad-mtCAST, SignaGen Laboratories, MD, USA), or beta-gal (Ad-gal, Vector Biolabs, PA, USA) as a control at a multiplicity of infection of 100 PFU/cell as previously described [27].

3.2.7 Measurement of mitochondrial superoxide generation

Superoxide flashes in single mitochondrion were measured to determine mitochondrial superoxide generation in living cardiomyocytes as described previously [28]. Briefly, cardiomyocytes were infected with an adenoviral vector expressing mt-cpYFP (Ad-mt-cpYFP). Ad-mt-cpYFP expresses a circularly permuted yellow fluorescent

protein (cpYFP) in the mitochondrial matrix of cells using the cytochrome C oxidase subunit IV targeting sequence (mt-cpYFP). Twenty-four hours after infection, confocal imaging was recorded using the Olympus FV 1000 laser-scanning microscope equipped with a 63x, 1.3NA oil immersion objective and a sampling rate of 0.7s/frame. At least 20 cardiomyocytes per culture in each group were analyzed.

3.2.8 Construction of plasmid with mitochondrial targeted *capn1* expression and transfection in H9c2 Cells

The full coding region of human *capn1* cDNA was recovered from *p*CMV6-XL5 containing human *capn1* (Origene, Rockville, MD, USA) and inserted into *p*CMV/myc/mito, which introduced the mitochondrial signal peptide (Life Technologies Inc. Burlington, Ontario, Canada). The resulting plasmid *p*CMV/myc/mito-*capn1* expresses myc-tagged *capn1* selectively in mitochondria.

Rat cardiomyocyte-like H9c2 cells were transfected with *p*CMV/myc/mito-*capn1* or *p*CMV/myc/mito as a control using the jetPRIME^{TM} DNA transfection reagent (VWR International, Mississauga, Ontario, Canada) according to the manufacturer's instructions.

3.2.9 Calpain activity

Calpain activity was determined using a fluorescence substrate N-succinyl-LLVY-AMC (Cedarlane Laboratories, Burlington, Ontario, Canada) as previously described [27].

3.2.10 Real-time reverse-transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from heart tissues using the Trizol Reagent (Life Technologies Inc. Burlington, Ontario, Canada) and real-time RT-PCR was performed to analyze mRNA expression for ANP, β-MHC and GAPDH as previously described [7].

3.2.11 Western blot analysis

The protein levels of capn1, capn2, calpastatin, mitochondrial voltage-dependent anion channel (VDAC1), ATP5A1 and beta subunits, and GAPDH were determined by western blot analysis using respective specific antibodies (Cell Signaling, Danvers, MA and Santa Cruz Biotechnology, Dallas, Texas).

3.2.12 Measurement of ros generation in freshly isolated mitochondria

Myocellular mitochondria were isolated from the freshly harvested heart as described previously [29], with minor modifications as follows. Instead of Nagarse, trypsin (5 mg/g wet weight of tissues) was used and after homogenizing and centrifuging, trypsin inhibitor (0.5 mg/ml) was added to the supernatant. The isolated mitochondria were further purified using Percoll density gradient centrifugation [30]. Mitochondrial ROS generation was determined on addition of pyruvate/malate or succinate by using Amplex Red and horseradish peroxidise (Invitrogen, USA) according to the manufacturer's instructions.

3.2.13 Determination of oxidative stress in diabetic hearts

The formation of ROS in heart tissue lysates was measured by using 2,7-dichlorodihydro-fluorescein diacetate (DCF-DA, Invitrogen, USA) [6] and Amplex Red as indicators according to the manufacturer's instructions. The protein oxidation in heart tissues was assessed by measuring protein carbonyl content using a commercial assay kit (Cayman Chemical, USA) following manufacturer's instructions.

The anti-oxidant capacity was measured based on reduction of copper (Ⅱ) to copper (Ⅰ) using OxiSelect^{TM} Total Antioxidant Capacity Assay Kit (Cell Biolabs, Inc., USA).

3.2.14 Immuno-fluorescence staining and confocal microscopy

Mitochondrial smears were prepared on slides and fixed with freshly prepared 4% paraformaldehyde. After incubation with appropriate primary antibodies (capn1 and VDAC-1) and secondary antibodies conjugated with differing fluorescence (Alexa Fluor 488 Donkey anti-mouse IgG and Alexa Fluor 594 Goat anti-rabbit IgG), signals were obtained with an Olympus FluoView^{TM} FV1000 confocal microscope equipped with the IX81 motorized inverted system as described [31].–

3.2.15 Co-immunoprecipitation (co-IP) and native gel electrophoresis

Co-IP and non-denaturing polyacrylamide gel electrophoresis were carried out to analyze protein–protein interactions. Briefly, calpain-1 and its interacting proteins were co-precipitated using an Immunoprecipitation kit - dynabeads protein G (Life Technologies Inc. Burlington, Ontario, Canada), and ATP synthase complex was isolated using ATP synthase immunocapture kit (Abcam Inc, Toronto, Ontario, Canada) in isolated mitochondria according to the manufacturer's instructions. Both calpain-1/interacting proteins and ATP synthase complex were subjected to non-denaturing polyacrylamide gel electrophoresis for separation, followed by western blot analysis.

3.2.16 ATP synthase activity

ATP synthase activity was measured using an assay coupled with pyruvate kinase, which converts ADP to ATP and produces pyruvate from phosphoenolpyruvate, as described previously [32].

3.2.17 Statistical analysis

All data were presented as mean ± SD. A one-way or two-way ANOVA followed by Newman-Keuls test was performed for multi-group comparisons as appropriate. For comparison of 2 groups, unpaired t-test was used. A value of $P < 0.05$ was considered

statistically significant.

3.3 Results

3.3.1 Mitochondrial ros generation is increased in diabetic mouse hearts and high glucose-stimulated cardiomyocytes

To determine mitochondrial ROS generation in cardiomyocytes under diabetic conditions, we made wild-type mice diabetic by injection of STZ. At 0, 7, 28 and 60 days after STZ injection, we isolated mitochondria from mouse hearts and determined mitochondrial H2O2 generation. As shown in Figure 3-1A, H2O2 generation in isolated mitochondria was increased in a time-dependent manner using pyruvate/malate as substrates. Similarly, in cultured adult cardiomyocytes, high glucose (30 mmol/L) incubation increased mitochondrial superoxide generation in a time-dependent manner (Figure 3-1B). These results confirm that mitochondrial ROS generation is increased in cardiomyocytes under diabetic conditions.

3.3.2 Genetic inhibition of calpain prevents mitochondrial ROS generation and reduces oxidative damage in diabetic mouse hearts

We have recently reported that genetic inhibition of calpain reduces diabetic cardiomyopathy in mouse models of type-1 diabetes [6, 7]. To understand the underlying mechanisms, we determined whether calpain plays a role in mitochondrial ROS generation. To this end, we first incubated cultured cardiomyocytes from Tg-CAST and wild-type mice with normal or high glucose for 24 hours. Over-expression of calpastatin significantly decreased mitochondrial superoxide generation induced by high glucose in Tg-CAST cardiomyocytes (Figure 3-1C). This result provides direct evidence that inhibition of calpain by over-expressing calpastatin blunts high-glucose stimulated superoxide generation in cardiomyocytes.

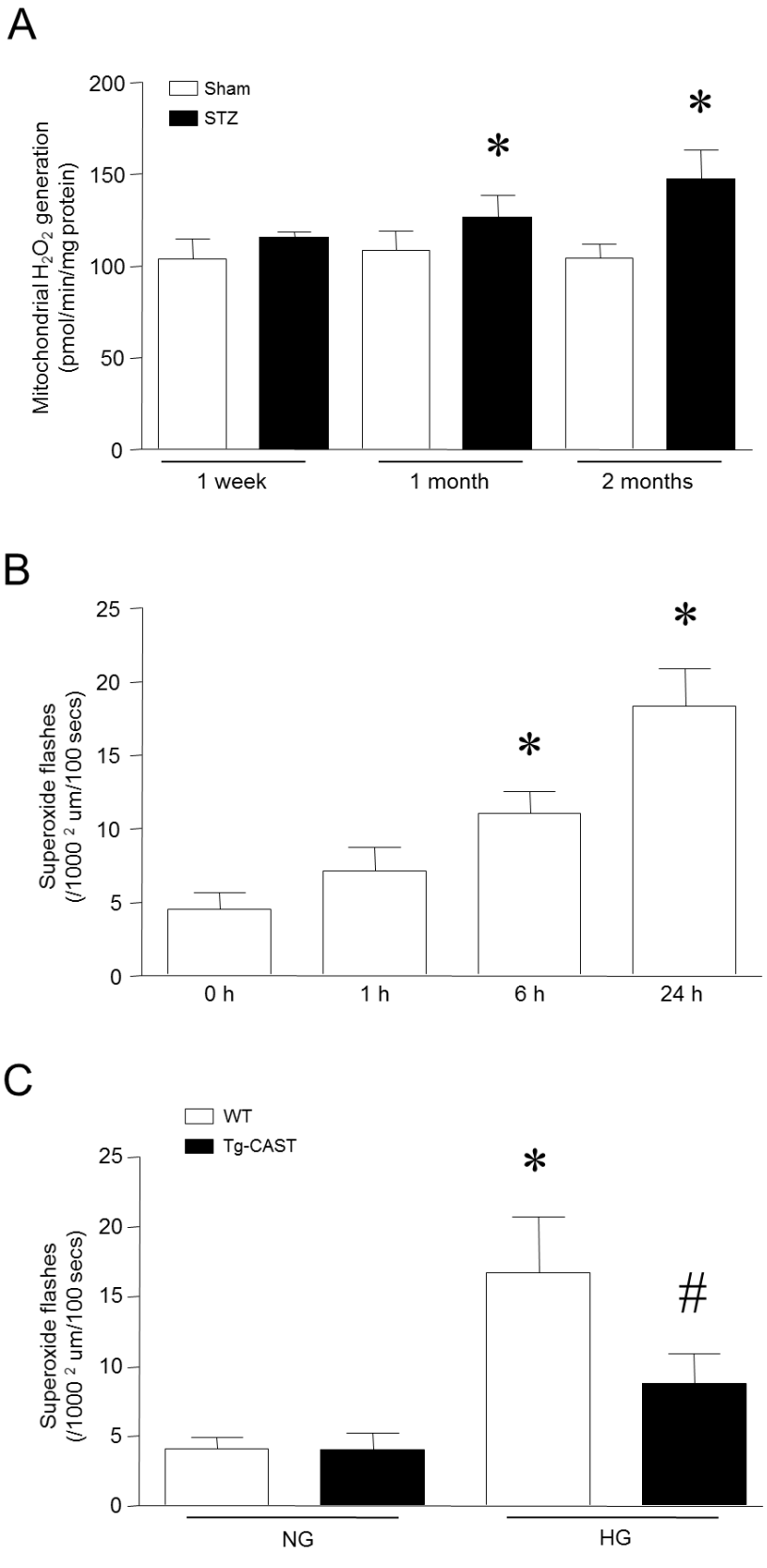

A
Sham
STZ
Mitochondrial H_2O_2 generation (pmol/min/mg protein)
200
150
100
50
0
*
*
1 week
1 month
2 months
B
Superoxide flashes (/1000 ² um/100 secs)
25
20
15
10
5
0
*
*
0 h
1 h
6 h
24 h
C
WT
Tg-CAST
Superoxide flashes (/1000 ² um/100 secs)
25
20
15
10
5
0
*
#
NG
HG

Figure 3-1. Determination of mitochondrial ROS generation

(A) Adult wild-type mice were injected with streptozotocin (STZ, 50 mg/kg/day, i.p.) for 5 days. Mice were killed 1 week, 1 month and 2 months after STZ injection. Mitochondria were isolated from heart tissues. Mitochondrial H2O2 generation was determined using Amplex Red as an indicator after addition of pyruvate/malate. (B and C) Adult cardiomyocytes were isolated and cultured for up to 24 hours. (B) Time course of mitochondrial superoxide flashes following incubation with high glucose (30 mmol/l) in wild-type (WT) cardiomyocytes. (C) Twenty-four hours after incubation with high glucose (30 mmol/l, HG) or normal glucose (5 mmol/l, NG), mitochondrial superoxide flashes were analyzed in WT and transgenic mice over-expressing calpastatin (Tg-CAST). Data are mean ± SD, n = 6 or 3 different cultures. * $P < 0.05$ versus Sham, 0 hr or NG in WT, and # $P < 0.05$ versus HG in WT.

We then made Tg-CAST, *capn4*-ko and their wild-type mice diabetic by injection of STZ. Sixty days after STZ injection, calpastatin over-expression or *capn4* deletion significantly reduced H2O2 generation in mitochondria from STZ-treated Tg-CAST and *capn4*-ko mice, respectively following addition of pyruvate/malate (Figure 3-2A and B) or succinate (Figure 3-3A and B). Similarly, H2O2 formation as determined by using DCF-DA (Figure 3-2C and D) and Amplex Red (Figure 3-2E and F), and the protein carbonyl content (Figure 3-2G and H) were increased in diabetic mouse hearts and abrogated in Tg-CAST and *capn4*-ko mice, respectively. However, total anti-oxidant capacity was comparable between wild-type, Tg-CAST and *capn4*-ko mice after induction of diabetes (data not shown). These results suggest that calpain contributes to mitochondrial ROS generation and oxidative damage in diabetic hearts.

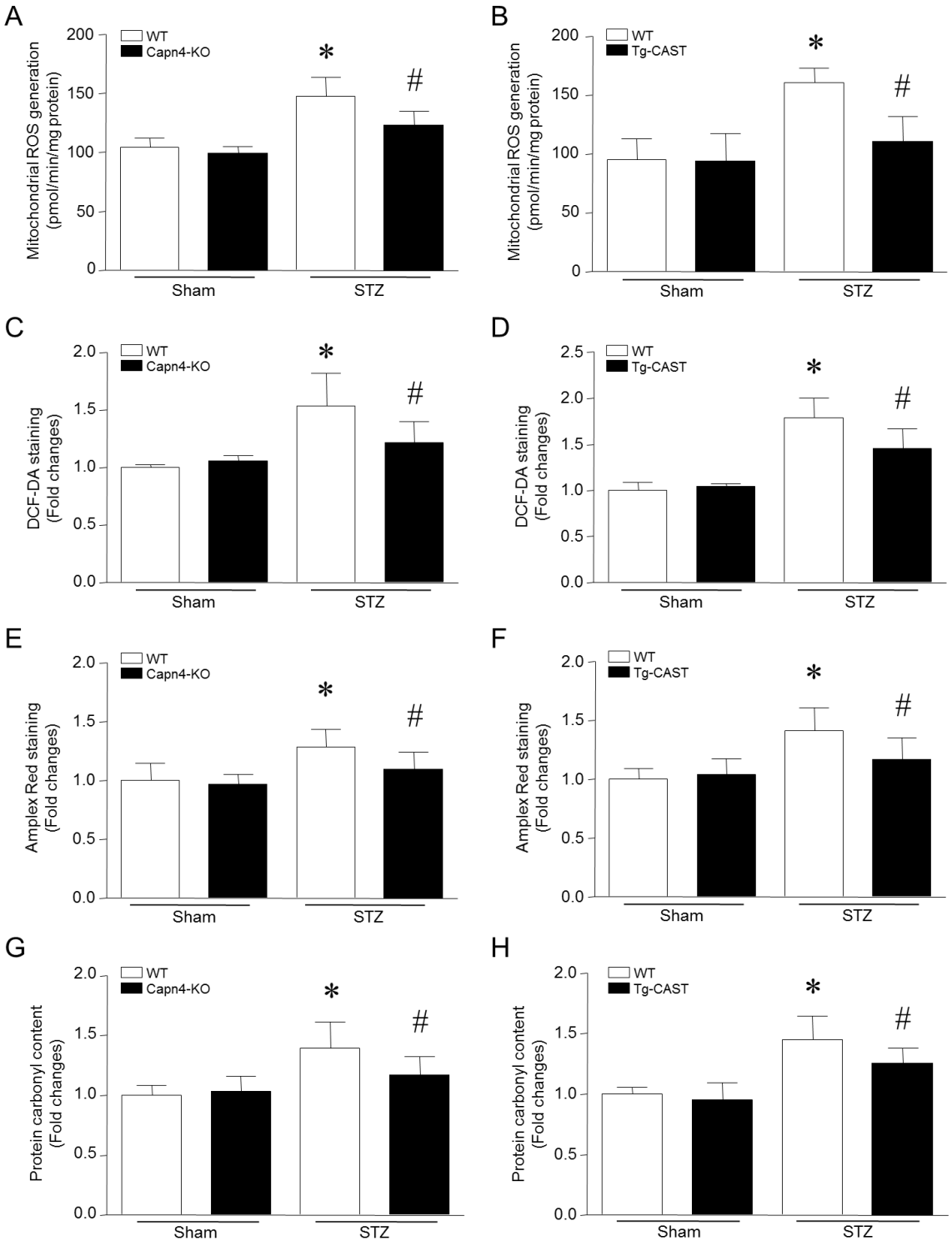

A
Mitochondrial ROS generation (pmol/min/mg protein)
WT
Capn4-KO
Sham
STZ
B
Mitochondrial ROS generation (pmol/min/mg protein)
WT
Tg-CAST
Sham
STZ
C
DCF-DA staining (Fold changes)
WT
Capn4-KO
Sham
STZ
D
DCF-DA staining (Fold changes)
WT
Tg-CAST
Sham
STZ
E
Amplex Red staining (Fold changes)
WT
Capn4-KO
Sham
STZ
F
Amplex Red staining (Fold changes)
WT
Tg-CAST
Sham
STZ
G
Protein carbonyl content (Fold changes)
WT
Capn4-KO
Sham
STZ
H
Protein carbonyl content (Fold changes)
WT
Tg-CAST
Sham
STZ

Figure 3-2. Assessment of mitochondrial ROS generation and oxidative stress

Wild-type (WT), transgenic mice over-expressing calpastatin (Tg-CAST) or cardiomyocyte-specific capn4 knockout mice (Capn4-ko) were injected with STZ (50 mg/kg/day for 5 days, i.p.). Two months after STZ injection, mitochondria were isolated from heart tissues. Mitochondrial ROS generation was measured using Amplex Red after addition of pyruvate/malate (A and B). ROS formation in heart tissue lysates was determined using DCF-DA as an indicator (C and D) or using Amplex Red (E and F). (G and H) Oxidative damage was assessed by measuring protein carbonyl contents in heart tissue lysates. Data are mean ± SD, n = 6. * $P < 0.05$ versus Sham in WT, and # $P < 0.05$ versus STZ-treated WT.

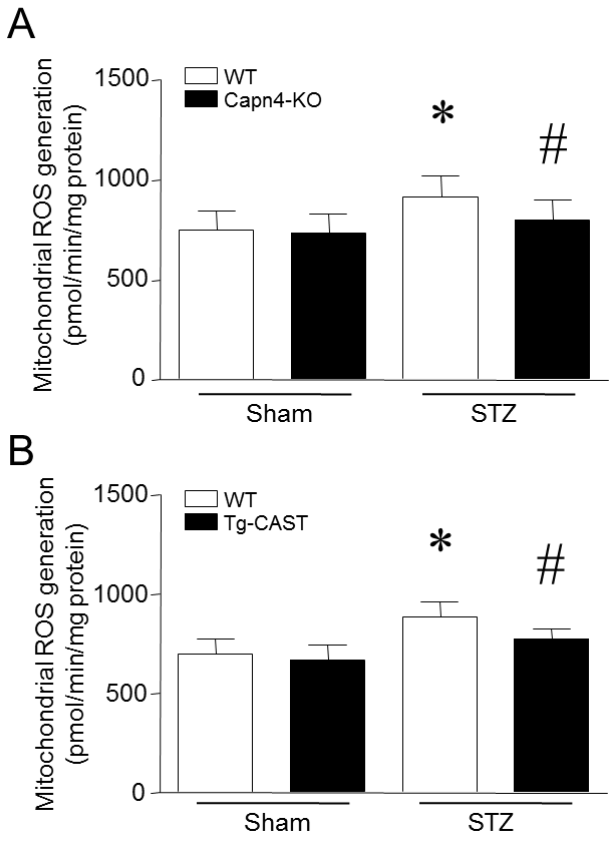


Figure 3-3. Assessment of mitochondrial ROS generation

Wild-type (WT), transgenic mice over-expressing calpastatin (Tg-CAST) or cardiomyocyte-specific capn4 knockout mice (Capn4-ko) were injected with STZ (50 mg/kg/day for 5 days, i.p.). Two months after STZ injection, mitochondria were isolated from heart tissues. Mitochondrial ROS generation was measured using Amplex Red after addition of succinate. Data are mean ± SD, n = 6. * $P < 0.05$ versus Sham in WT, and # $P < 0.05$ versus STZ in WT.

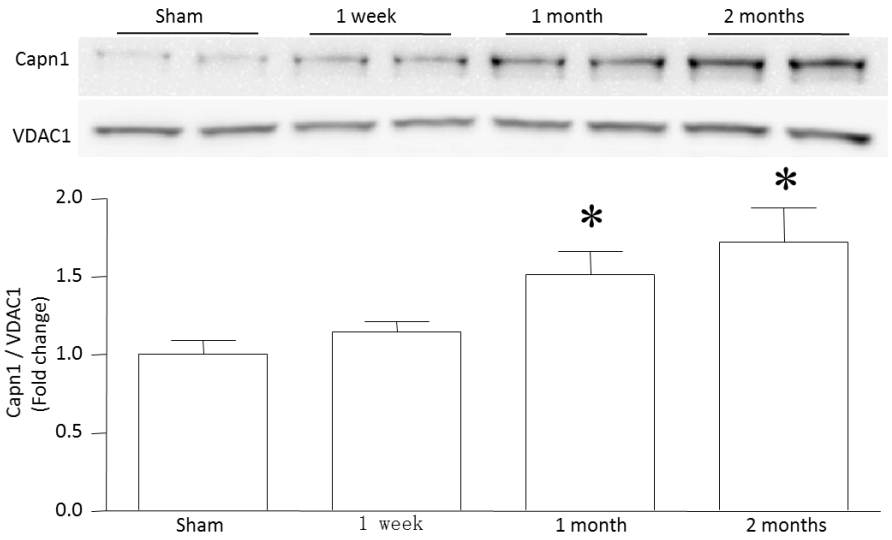
3.3.3 Calpain-1 is increased in mitochondria of stz-induced mouse hearts

Having shown that inhibition of calpain prevented mitochondrial superoxide generation, we determined whether the levels of calpains were altered in mitochondria of diabetic mouse hearts. In line with the increase in mitochondrial ROS generation, the protein levels of capn1 were significantly elevated in mitochondria from diabetic hearts in a time-dependent manner (Figure 3-4A). Consistently, diabetes also increased calpain activities in mitochondria of diabetic compared with sham animal hearts (Figure 3-5). However, diabetes did not change the protein levels of capn2 and calpain-10, an isoform well recognised as a mitochondrial calpain [33] (data not shown).

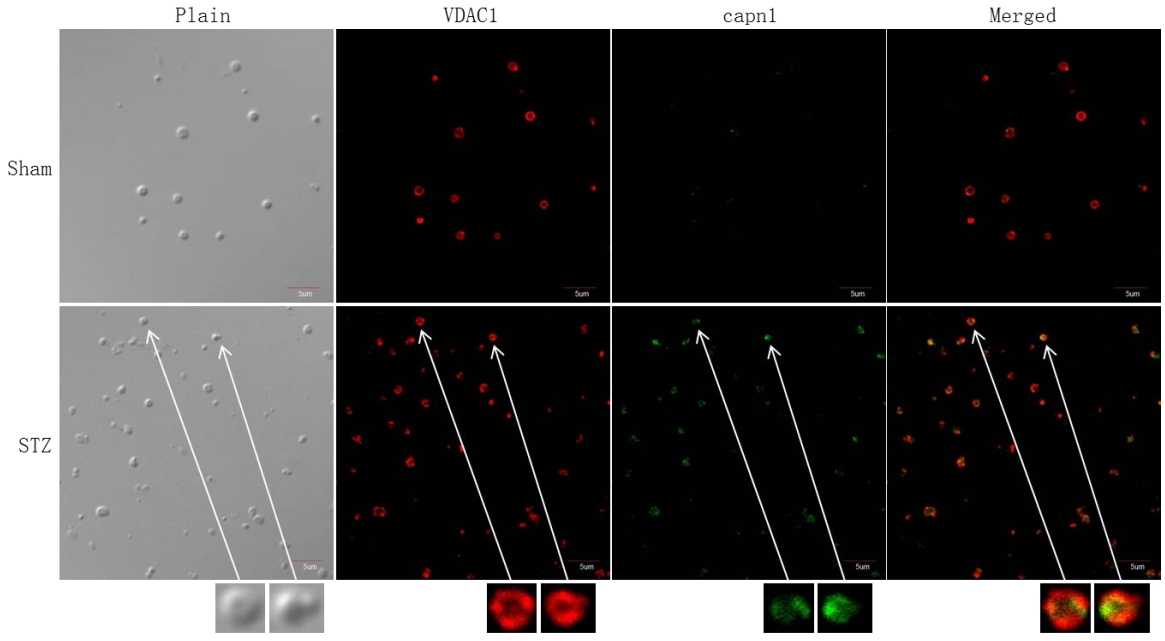
To provide further evidence in support of calpain-1 accumulation in mitochondria, we determined capn1 and VDAC1 proteins in isolated mitochondria of diabetic mouse hearts by dual immunofluorescence confocal microscopy. Confocal microscopic analysis demonstrated that VDAC1 was detected in mitochondrial membranes (red) and capn1 was present inside of mitochondria (green), and that percentages of capn1-labelled mitochondria were much greater in diabetic versus sham mouse hearts (Figure 3-4B).

Similarly, the protein levels of capn1 were also increased in hearts of db/db type-2 diabetic versus db+/- mice (Figure 3-4C).

A


Sham
1 week
1 month
2 months
Capn1
VDAC1
Capn1 / VDAC1
(Fold change)
2.0
1.5
1.0
0.5
0.0
*
*
Sham
1 week
1 month
2 months

B


Plain
VDAC1
capn1
Merged
Sham
STZ

C

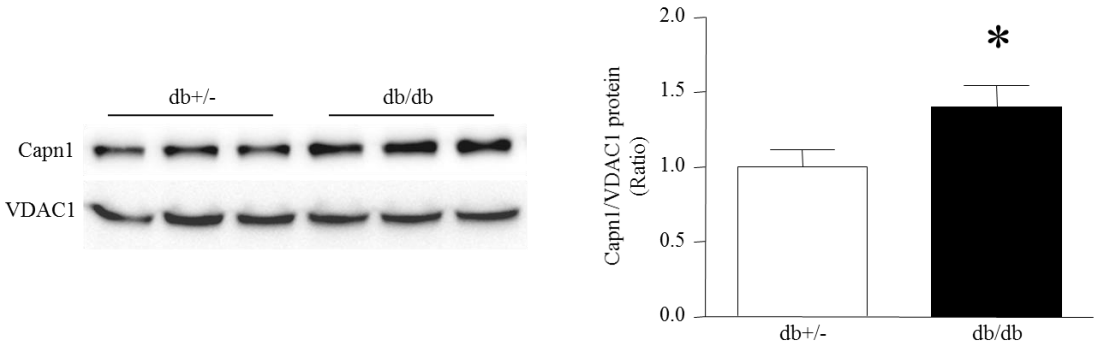

db+/-
db/db
Capn1
VDAC1
Capn1/VDAC1 protein
(Ratio)
2.0
1.5
1.0
0.5
0.0
*
db+/-
db/db

Figure 3-4. Measurement of calpain-1 in mitochondria

(A) Adult wild-type mice were injected with streptozotocin (STZ, 50 mg/kg/day, i.p.) for 5 days. Mice were killed 1 week, 1 month and 2 months after STZ injection. Mitochondria were isolated from heart tissues and the protein levels of calpain-1 and VDAC1 in mitochondria were determined by western blot analysis. Upper panel is the representative western blot for capn1 and VDAC1 from 2 out of 6 different hearts in each group and lower panel is the quantification of capn1/VDAC1 in all animals. (B) Adult wild-type mice were injected with STZ (50 mg/kg/day, i.p.) for 5 days. Two months after STZ injection, heart tissues were collected and mitochondria were isolated. After fixation on slides, dual immunofluorescent staining for VDAC1 and capn1 was performed using their respective antibodies followed by secondary antibodies conjugated with different fluorescent dyes. Representative photomicrographs of confocal microscopy for VDAC1 and capn1 in mitochondria shows membrane staining of VDAC1 (Red) and that capn1 is located in mitochondria (Green). (C) Mitochondria were isolated from db/db type-2 diabetic and db+/- mouse hearts (male and age of 3.5 months). The protein levels of capn1 and VDAC1 were determined by western blot analysis. Left panel is a representative western blot for capn1 and VDAC1 from 3 out of 6 different hearts in each group and right panel is the quantification of capn1 protein normalized to VDAC1. Data are mean ± SD from 6 different heart tissues in each group. *$P < 0.05$ versus Sham.

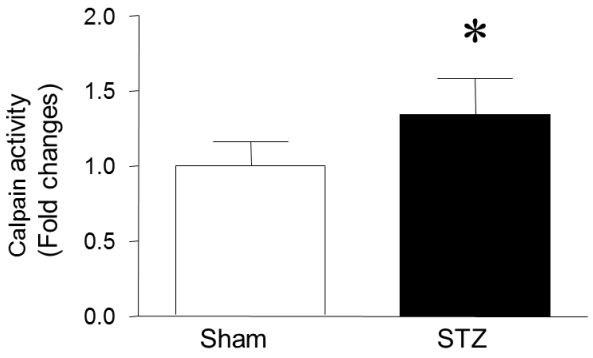


Figure 3-5. Measurement of calpain activity in isolated mitochondria

Wild-type mice were injected with STZ (50 mg/kg/day for 5 days, i.p.) or citrate buffer as a sham control. Two months after STZ injection, mitochondria were isolated from heart tissues. Calpain activity was determined. Data are mean ± SD, n = 6. * $P < 0.05$.

3.3.4 Mitochondrial calpain-1 contributes to superoxide generation and cell death in high glucose-stimulated cardiomyocytes

To determine whether mitochondrial calpain-1 contributes to superoxide generation in cardiomyocytes, we infected cultured cardiomyocytes with an adenoviral vector containing mitochondria-targeted calpastatin (Ad-mtCAST) and then incubated them with high glucose for 24 hours. Selective over-expression of calpastatin in mitochondria prevented mitochondrial superoxide flashes and cell death induced by high glucose (Figure 3-6A-D). This result suggests that mitochondrial calpain contributes to superoxide generation and cell death in cardiomyocytes induced by high glucose.

To provide direct evidence to support our hypothesis that the accumulation of calpain-1 in mitochondria induces superoxide generation and apoptosis, we introduced *p*CMV/myc/mito-*capn1*, a plasmid expressing mitochondrial targeted *capn1* into cardiomyocyte-like H9c2 cells. Twenty-four hours after transfection, mitochondrial and cytosolic fractions were isolated from H9c2 cells. Over-expressed *capn1* was confirmed in mitochondrial but not in cytosolic fractions (Figure 3-7A). Intriguingly, mitochondria-targeted over-expression of *capn1* significantly increased mitochondrial superoxide generation as determined by mitochondrial superoxide flashes (Figure 3-7B) and induced apoptosis (Figure 3-7C and D). These results strongly support a causal role of mitochondrial calpain-1 in superoxide generation and apoptosis in cardiomyocytes.

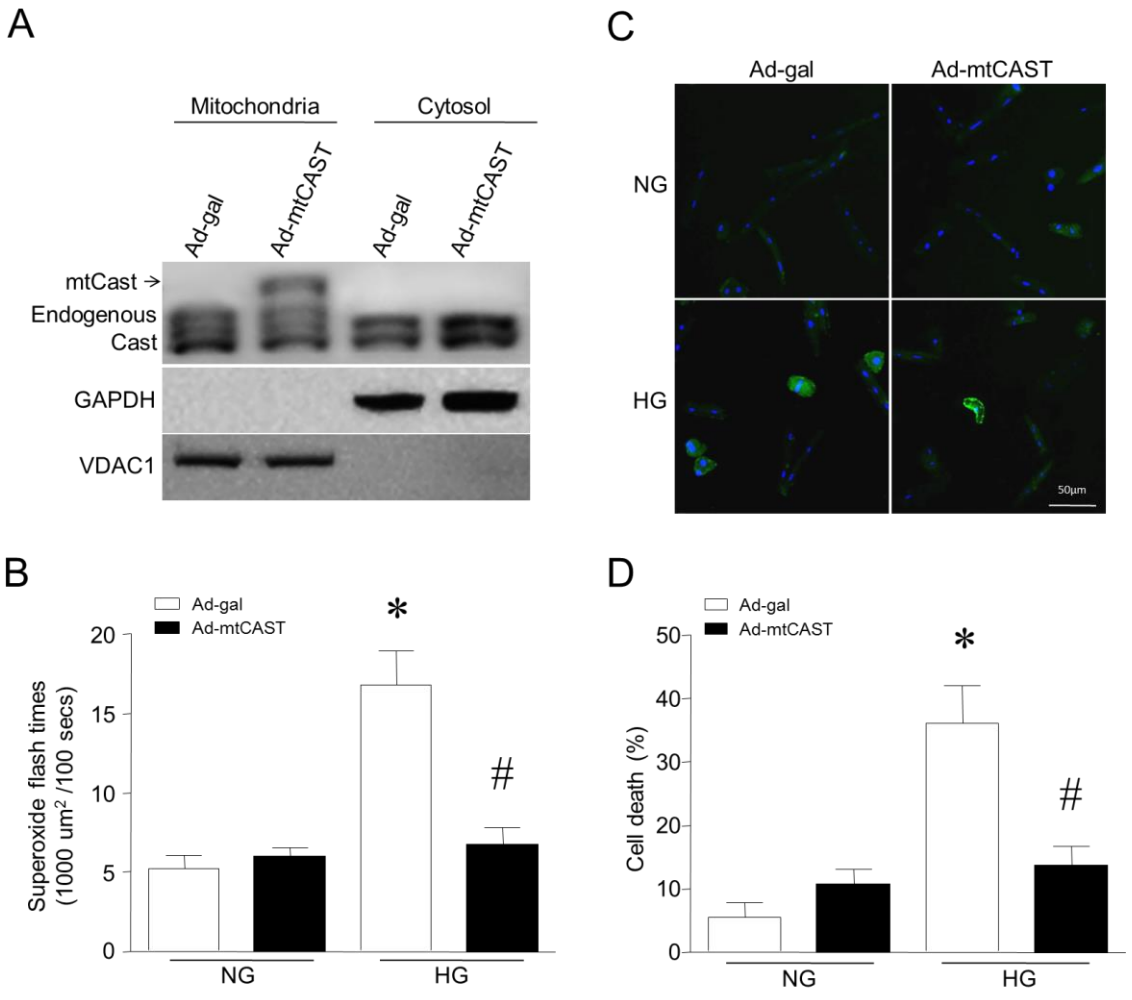


Figure 3-6. Effects of mitochondria-targeted calpastatin over-expression on mitochondrial superoxide flashes and cell death in high glucose-stimulated cardiomyocytes

(A) H9c2 cells were infected with an adenoviral vector containing mitochondria-targeted calpastatin (Ad-mtCAST) or Ad-gal as a control. Twenty-four hours later, mitochondrial and cytosolic fractions were prepared, and calpastatin (CAST), GAPDH and VDAC1 were detected by western blot analysis. A representative western blot confirms myc-tagged CAST is expressed selectively in mitochondria. (B-D) Adult cardiomyocytes were isolated from mice. After infection with Ad-mtCAST, cardiomyocytes were exposed to normal glucose (NG) or high glucose (HG) for 24 hours, mitochondrial superoxide flashes (B), and Annexin V staining for cell death (C and D) were determined. Data are mean ± SD from 6 different cultures. *$P < 0.05$ versus NG + Ad-gal, and # $P < 0.05$ versus HG + Ad-gal.

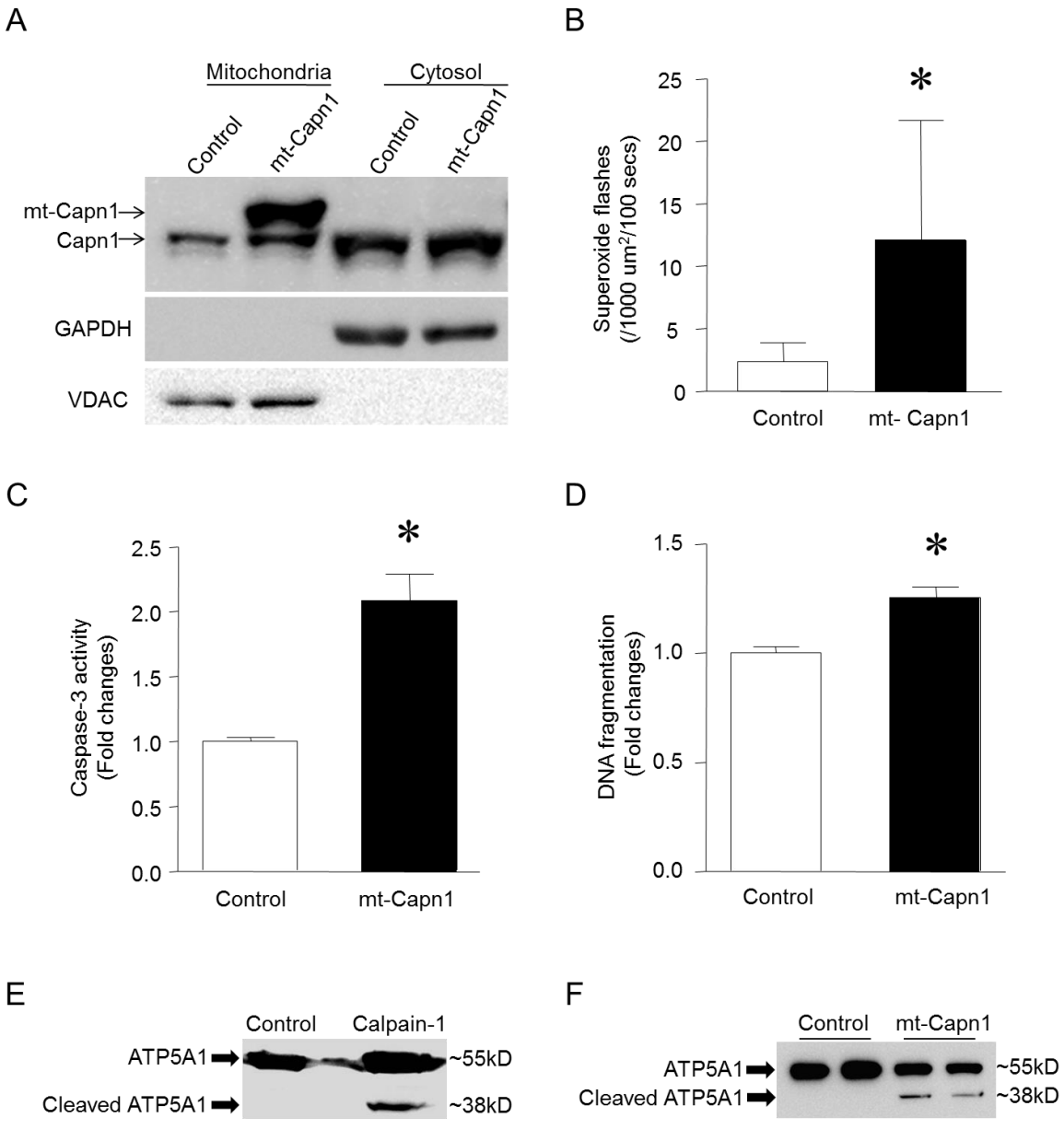

A
Mitochondria
Cytosol
Control
mt-Capn1
Control
mt-Capn1
mt-Capn1
Capn1
GAPDH
VDAC
B
Superoxide flashes
(/1000 um2/100 secs)
25
20
15
10
5
0
*
Control
mt- Capn1
C
Caspase-3 activity
(Fold changes)
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
mt-Capn1
D
DNA fragmentation
(Fold changes)
1.5
1.0
0.5
0.0
*
Control
mt-Capn1
E
Control
Calpain-1
ATP5A1
Cleaved ATP5A1
~55kD
~38kD
F
Control
mt-Capn1
ATP5A1
Cleaved ATP5A1
~55kD
~38kD

Figure 3-7. Effects of mitochondrial targeted capn1 on ATP5A1 protein, superoxide generation and apoptosis in H9c2 cells

H9c2 cells were transfected with *p*CMV/myc/mito-capn1 (mt-Capn1) or *p*CMV/myc/mito as a control. Twenty-four hours later, (A) Mitochondrial and cytosolic fractions were isolated. Western blot analysis was performed to determine the protein levels of capn1, GAPDH and VDAC1. (B) Mitochondrial superoxide flashes were assessed. (C and D) Apoptosis was determined by caspase-3 activity and DNA fragmentation. (E and F) ATP5A1 and its cleaved fragment were determined by western blot analysis. (E) Mitochondrial lysates (100 μg) were incubated with active calpain-1 (5 μg) for 15 minutes. (F) ATP5A1 immunoblot in H9c2 cells transfected with mitochondrial-targeted calpain-1 (mt-capn1). Data are mean ± SD from at least 3 different experiments. *$P < 0.05$ versus control.

3.3.5 ATP5A1 is a target of calpain-1 in diabetic hearts

Since studies have shown that the protein levels of ATP5A1 are reduced and ATP synthase activity decreases in diabetic hearts [16, 17], our initial effort was focused on ATP5A1. After incubation of mitochondrial lysates from the heart with active calpain-1, a cleaved fragment of ATP5A1 protein (about 38KD) was detected (Figure 3-7E). Interestingly, up-regulation of calpain-1 selectively in mitochondria led to a similar cleaved fragment of ATP5A1 protein in H9c2 cells (Figure 3-7F). These results strongly indicate that ATP5A1 protein is a direct substrate of calpain-1.

We further revealed that ATP5A1 was co-immunoprecipitated with capn1 in diabetic hearts (Figure 3-8A). Likewise, capn1 was detected in immune-captured ATP synthase complex (Figure 3-8B). These results demonstrate a potential interaction between calpain-1 and ATP5A1 in mitochondria of diabetic hearts. We also measured the protein levels of ATP5A1 in isolated mitochondria of diabetic hearts. Diabetes significantly reduced ATP5A1 protein levels in mitochondria (Figure 3-8C), which is consistent with previous reports [16, 17], whereas the protein levels of ATP synthase β subunit remain unchanged in diabetic hearts (Figure 3-8C). However, the reduction in ATP5A1 protein levels was prevented by calpastatin over-expression (Figure 3-8D). In line with a reduction in ATP5A1 protein, ATP synthase activity was markedly decreased in mitochondria from diabetic hearts and restored in diabetic Tg-CAST mice (Figure 3-8E).

In cultured cardiomyocytes, over-expression of calpastatin selectively in mitochondria by infection with Ad-mtCAST significantly increased ATP synthase activity during high glucose stimulation (Figure 3-8F). This result provides further evidence to support that calpain activation disrupts ATP synthase activity in diabetic hearts.

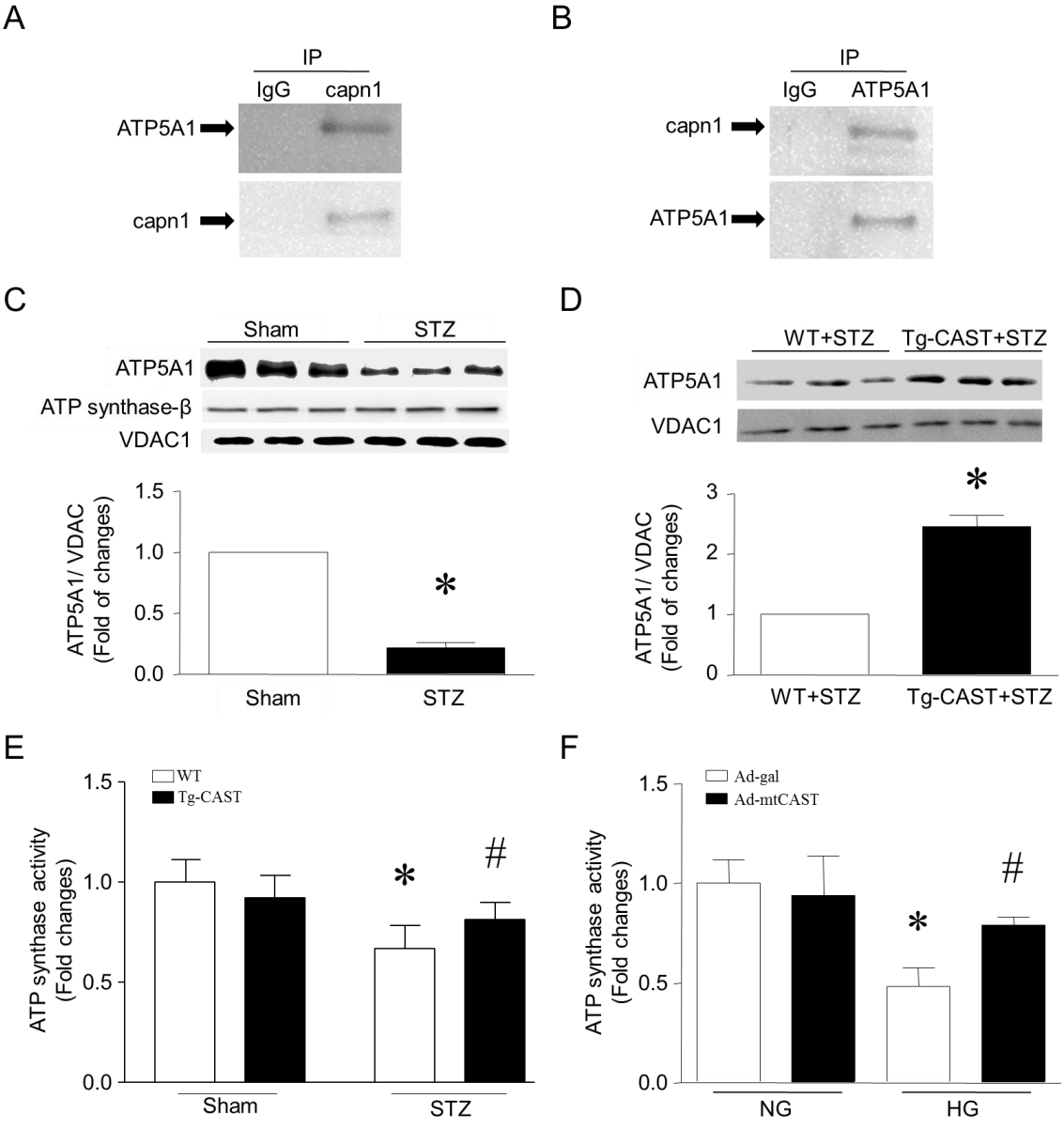

A
IP
IgG
capn1
ATP5A1
capn1
B
IP
IgG
ATP5A1
capn1
ATP5A1
C
Sham
STZ
ATP5A1
ATP synthase-β
VDAC1
ATP5A1/ VDAC
(Fold of changes)
1.5
1.0
0.5
0.0
*
Sham
STZ
D
WT+STZ
Tg-CAST+STZ
ATP5A1
VDAC1
ATP5A1/ VDAC
(Fold of changes)
3
2
1
0
*
WT+STZ
Tg-CAST+STZ
E
WT
Tg-CAST
ATP synthase activity
(Fold changes)
1.5
1.0
0.5
0.0
*
#
Sham
STZ
F
Ad-gal
Ad-mtCAST
ATP synthase activity
(Fold changes)
1.5
1.0
0.5
0.0
*
#
NG
HG

Figure 3-8. Role of calpain in ATP5A1 expression and ATP synthase disruption in diabetic hearts

(A) Interaction between ATP5A1 and capn1. Capn1 interacting proteins were co-immunoprecipitated using capn1 antibody. A representative western blot shows that ATP5A1 is detected in capn1 interacting proteins. (B) ATP synthase complex and its interacting proteins were captured using ATP synthase immune-capture assay kit. A representative western blot shows that capn1 is detected in captured ATP synthase complex. (C-E) Myocardial mitochondria were isolated from sham and STZ-injected Tg-CAST and their wild-type (WT) mice. (C and D) The upper panels are the representative western blot for ATP5A1 protein from 3 out of 6 hearts in each group and the lower panels are the quantification of ATP5A1 protein relative to VDAC1 in mitochondria. (E) ATP synthase activity was measured in mitochondria. Data are mean ± SD, $n = 6$. $^{*}P < 0.05$ versus sham or STZ + WT, and $^{\#}P < 0.05$ versus STZ + WT. (F) Adult cardiomyocytes were isolated and cultured from wild-type mice. After infection with Ad-mtCAST or Ad-gal, the cells were incubated with high glucose (30 mmol/l, HG) or normal glucose (5 mmol/l, NG) for 24 hours. ATP synthase activity was determined in cell lysates. Data are mean ± SD, $n = 6$. $^{*}P < 0.05$ versus NG + Ad-gal, and $^{\#}P < 0.05$ versus HG + Ad-gal.

3.3.6 Over-expression of ATP5A1 reduces mitochondrial superoxide generation, cardiac hypertrophy and myocardial dysfunction in diabetic mice

To investigate whether up-regulation of ATP5A1 protects diabetic hearts, we delivered Ad-ATP5A1 into mice 72 hours after the last STZ injection. Ad-GFP served as a control. Two weeks later, mice received the second dose of Ad-ATP5A1. Two months after STZ injection, mice were subjected to various experiments. The efficient delivery of adenoviral vectors into the heart was confirmed by GFP signal in heart tissues (Figure 3-9). As a result, delivery of Ad-ATP5A1 significantly increased ATP5A1 protein and ATP synthase activity in diabetic mouse hearts (Figure 3-10A and B), suggesting that ectopic expression of ATP5A1 integrates into the complex of ATP synthase. Up-regulation of ATP5A1 reduced the formation of H_2O_2 (Figure 3-10C and D), and attenuated cardiac hypertrophy as evidenced by decreased cardiomyocyte sectional area (Figure 3-10E) and down-regulation of ANP and β-MHC expression in diabetic mouse hearts (Figure 3-10F and G), leading to an improvement of myocardial function in diabetic mice as determined by increased fractional shortening and E/A ratio (Figure 3-10H and I, Table 3-1). However, delivery of Ad-ATP5A1 slightly elevated ATP5A1 protein levels in sham mouse hearts but did not increase ATP synthase activity.

To provide further evidence to support the role of ATP5A1, we infected adult cardiomyocytes with Ad-ATP5A1 or Ad-gal as a control, and then incubated them with high glucose for 24 hours. Up-regulation of ATP5A1 increased ATP synthase activity in high glucose- but not normal glucose-stimulated cardiomyocytes (Figure 3-11A), reduced mitochondrial superoxide generation (Figure 3-11B) and prevented cell death induced by high glucose (Figure 3-11C and D).

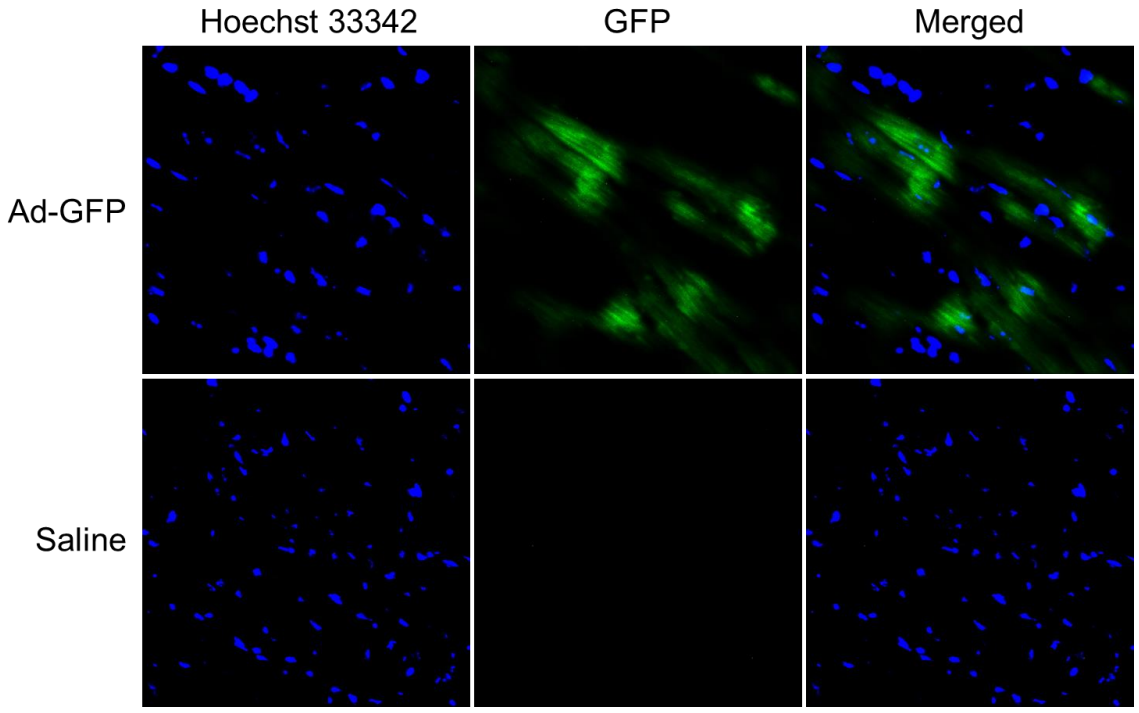


Figure 3-9. GFP signal in heart tissues after delivery of Ad-GFP

Adult mice were injected with Ad-GFP via tail vein. Five days later, heart tissues were collected and cryosections were prepared. The sections were stained with Hoechst 33342 for nuclei. The signals for GFP (green color) and Hoechst 33342 (blue) were captured.

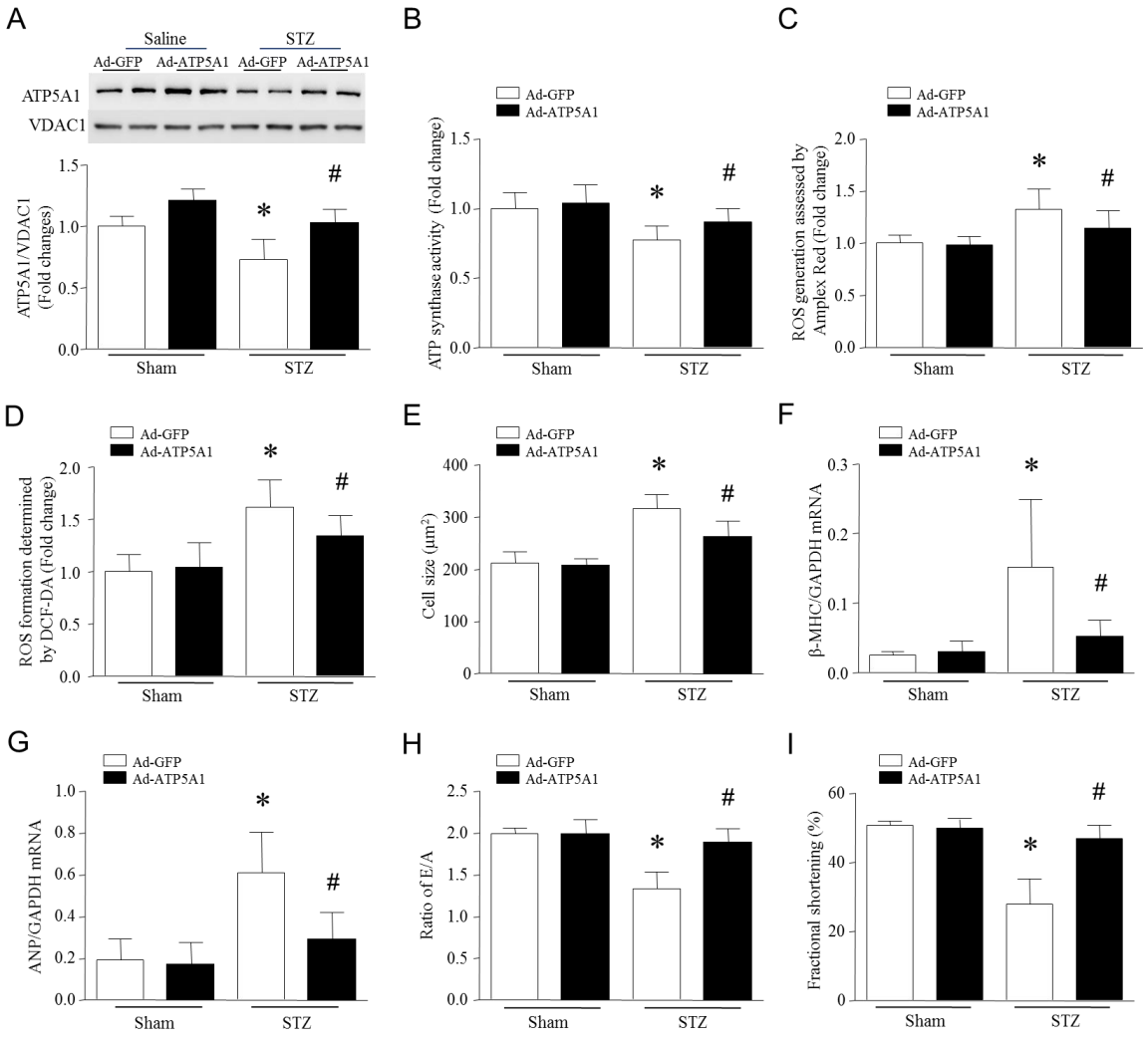


Figure 3-10. Effects of ATP5A1 over-expression in diabetic cardiomyopathy

Adult mice were injected with Ad-ATP5A1 or Ad-GFP and then treated with STZ. (A) Up-regulation of ATP5A1 protein was confirmed by western blot analysis. Upper panel is a representative western blot from 2 out of 6 different hearts for ATP5A1 and VDAC1, and lower panel is quantification of ATP5A1/GAPDH ratio for all hearts. (B) ATP synthase activity. (C and D) H2O2 formation was determined in heart tissue lysates using Amplex Red (C) and DCF-DA as indicators (D). (E) Cardiomyocyte size in heart sections. (F) The mRNA levels of beta-MHC. (G) The mRNA levels of ANP. (H and I) Echocardiographic analysis was performed to assess myocardial function. Data are mean ± SD, n = 6-8. *P < 0.05 versus sham + Ad-GFP, and $^{\#}P$ < 0.05 versus STZ + Ad-GFP.

Table 3-1. General information in mice after receiving Ad-GFP or Ad-ATP5A1

	BW (g)	HW (mg)	HW/BW (mg/g)	BG (mmol/l)	LVESD (mm)	LVEDD (mm)	HR (beats/min)
Sham+Ad-GFP	29.0±3.0	133±14	4.6±0.22	6.3±1.6	2.05±0.14	4.13±0.24	469±40
Sham+Ad-ATP5A1	28.7±2.7	134±12	4.68±0.15	6.6±1.2	2.11±0.19	4.12±0.3	514±69
STZ+Ad-GFP	28.6±6.7	138±24	4.89±0.42	31.2±1.9*	3.04±0.61*	4.19±0.44	421±66
STZ+Ad-ATP5A1	29.3±3.2	136±10	4.64±0.25	31.4±1.8*	2.1±0.27†	3.95±0.47	464±71

(Data are mean ±SD, n = 6. * $P < 0.05$ versus Sham+Ad-GFP or Sham+Ad-ATP5A1 and † $P < 0.05$ versus STZ+Ad-GFP. BW, body weight; HW, heart weight; BG, blood glucose; LVESD, left ventricle end systolic diameter; LVEDD, left ventricle end diastolic diameter; HR, heart rate)

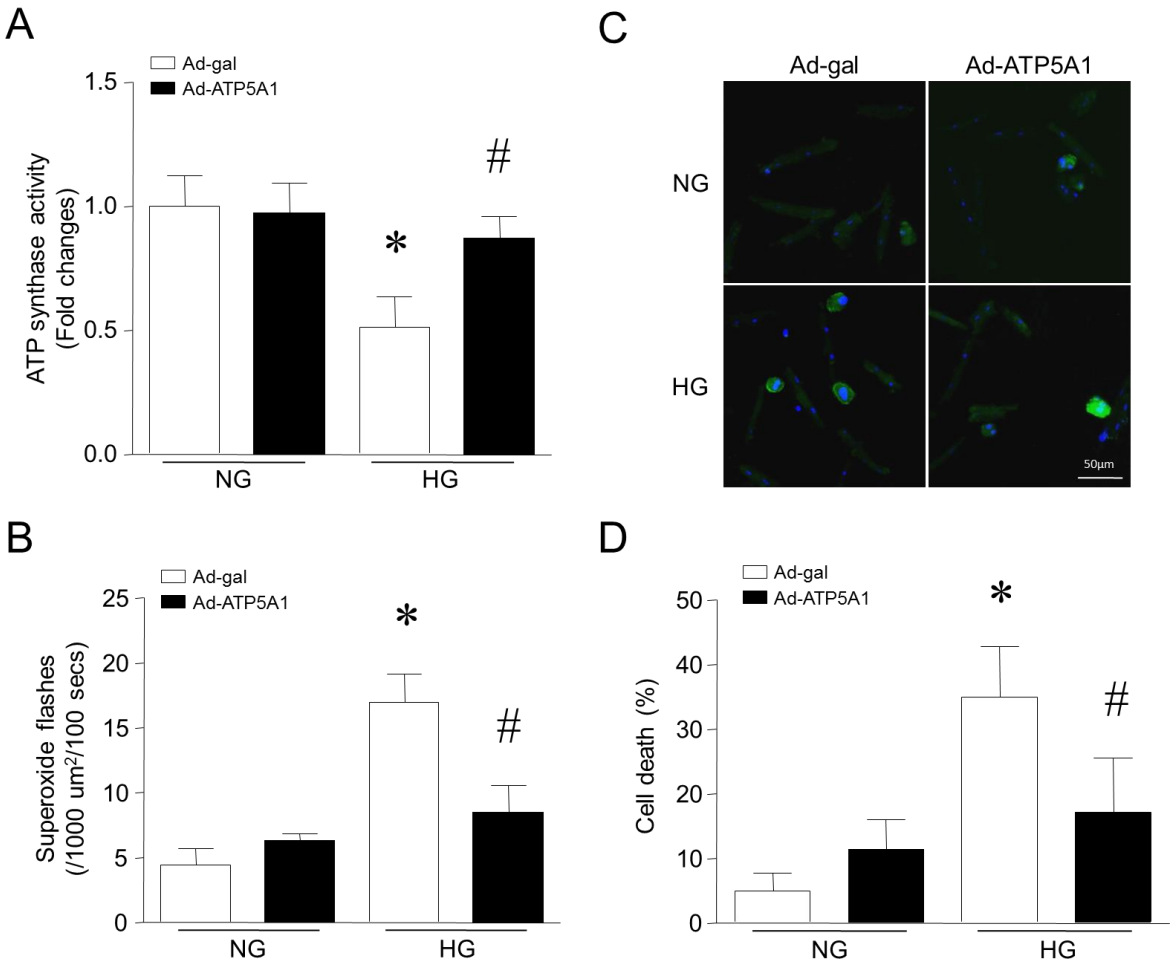


Figure 3-11. Role of ATP5A1 in ATP synthase activity, mitochondrial superoxide generation and cell death in cardiomyocytes

Adult mouse cardiomyocytes were isolated from wild-type mice. After cell attachment to the culture dish, they were infected with Ad-ATP5A1 or Ad-gal. Twenty-four hours later, cells were incubated with normal glucose (NG) or high glucose (HG) for 24 hours. (A) ATP synthase activity. (B) Mitochondrial superoxide generation. (C) Representative pictures for annexin V staining positive cells as an indicator of cell death (green color). (D) Quantification of annexin V staining positive cells. Data are mean ± SD from at least 3 different experiments. *P < 0.05 versus Ad-gal + NG, and $^{\#}P$ < 0.05 versus Ad-gal + HG.

3.4 Discussion

The major findings of this study are that genetic inhibition of calpain increases the protein levels of ATP5A1 and ATP synthase activity, and decreases mitochondrial ROS generation and oxidative damage in diabetic hearts. Both type-1 and type-2 diabetes induce calpain-1 accumulation in mitochondria of the heart. Selective inhibition of mitochondrial calpain attenuates ATP synthase disruption, reduces mitochondrial superoxide generation and prevents apoptosis in cardiomyocytes under diabetic conditions, whereas targeted up-regulation of calpain-1 specifically in mitochondria induces the cleavage of ATP5A1, superoxide generation and apoptosis in cardiomyocytes. In a mouse model of T1D, up-regulation of ATP5A1 restores ATP synthase activity and decreases mitochondrial ROS generation in diabetic hearts, and reduces diabetic cardiomyopathy. Thus, ATP synthase disruption and mitochondrial ROS generation are important mechanisms by which calpain activation promotes diabetic cardiomyopathy.

Accumulating evidence indicates that mitochondrial ROS production is increased and oxidative stress occurs in type-1 and type-2 diabetic hearts [17-20]. Although some type-1 diabetic animals did not exhibit increased mitochondrial superoxide generation in the heart [21, 22], selective inhibition of mitochondrial ROS production reduces adverse cardiac changes in T1D models [23, 24], supporting a critical role of mitochondrial ROS. The present study provides further evidence that demonstrates that diabetic conditions induce mitochondrial superoxide generation in cultured cardiomyocytes and hearts *in vivo*. ROS produced by mitochondria not only directly contributes to mitochondrial dysfunction [34], cell death and hypertrophy in cardiomyocytes and hearts under stress [35, 36], but also serves as “second messengers” in cellular signalling pathways [37]. Thus, targeted inhibition of mitochondrial ROS by transgenic over-expression of superoxide dismutase-2 (SOD2) and mitochondrial catalase reduces cardiac hypertrophy,

preserves cardiac structures and improves function in a mouse model of type-1 diabetes [23] and in insulin-resistant and obese Ay mice [24], respectively. We further show that genetic inhibition of calpain significantly attenuates mitochondrial superoxide generation and subsequent oxidative damage in diabetic mouse hearts, which are associated with reduced myocardial injury and improved myocardial function in diabetic mice. Thus, our data suggest an important role of calpain in mitochondrial ROS generation in development of diabetic cardiomyopathy.

It is well-known that mitochondria generate superoxide, the primary ROS as by-products, when single electrons leak to react with molecular oxygen [38]. While many mitochondrial enzymes have been reported to produce ROS, the respiratory chain is the major source of ROS in mitochondria. Within the respiratory chain, Complexes Ⅰ and Ⅲ have been identified as major ROS generators. On the other hand, mitochondrial ROS are eliminated by antioxidant defence systems. Superoxide anion dismutates to H_2O_2 spontaneously, or by SOD2 in mitochondria. H_2O_2 can be readily converted to water by catalase and glutathione peroxidase. In addition to these antioxidant enzymes, mitochondria possess several low-molecular-weight antioxidants, including α-tocopherol and ubiquinol, *etc*. An increase in superoxide generation and/or a decrease in antioxidant capacity will lead to oxidative stress in mitochondria [39]. In this regard, our data suggest that calpain may promote oxidative damage through increased mitochondrial superoxide generation rather than decreased antioxidant capacity because inhibition of calpain does not affect the anti-oxidant capacity in diabetic hearts.

Multiple mechanisms have been suggested to mediate mitochondrial ROS generation in diabetic hearts. It was reported that high glucose concentrations result in increased metabolic input into mitochondria, which overwhelms the respiratory chain causing mitochondrial hyperpolarization, leading to electron backup within the respiratory chain

and ROS overproduction [38]. In addition, elevated circulating lipids and hyperinsulinemia together increase fatty acid delivery to cardiomyocytes, which rapidly adapt by promoting fatty acid utilization. High rates of fatty acid oxidation increase mitochondrial membrane potential, leading to the production of ROS in mitochondria [40, 41]. In the present study, we show that diabetes increases calpain-1 in mitochondria and calpain-1 accumulation in mitochondria correlates with ROS generation in diabetic mouse hearts. Importantly, selective inhibition of mitochondrial calpain reduces superoxide generation in cardiomyocytes under diabetic conditions whereas targeted over-expression of *capn1* in mitochondria sufficiently induces superoxide generation in cardiomyocytes. Thus, mitochondrial calpain-1 may represent a novel mechanism underlying mitochondrial ROS generation in cardiomyocytes under diabetic conditions.

Another important finding is that mitochondrial calpain-1 negatively regulates ATP5A1 protein, leading to ATP synthase disruption in diabetic hearts. ATP synthase, also called Complex V, is an enzyme that uses the energy created by the proton electrochemical gradient to synthesize ATP from ADP [42]. It is located within the mitochondria. ATP synthase consist of 2 regions: the F_o portion and F_1 portion. The F_o region of ATP synthase is a proton pore located within the inner membrane of mitochondria, which transfers the energy created by the proton electrochemical gradient to F_1, where ADP is phosphorylated to ATP. The F_1 region of ATP synthase comprises five different subunits (α, β, γ, δ, and ε) in the matrix of the mitochondria. Down-regulation of ATP synthase has been shown in both type-1 and type-2 diabetic hearts [16, 17]. Similarly, we show a significant reduction of ATP5A1 protein and of its activity in mitochondria from diabetic mouse hearts. Importantly, diabetes-induced down-regulation of ATP5A1 and ATP synthase activity are prevented by both calpastatin over-expression and *capn4* deletion. Thus, our observations are consistent with a model whereby calpain-1 accumulation in mitochondria compromises ATP synthase through the proteolysis of ATP5A1 protein in

diabetic mouse hearts. In fact, selective up-regulation of calpain-1 in mitochondria induces the cleavage of ATP5A1 protein, mitochondrial superoxide generation and apoptosis in cultured cardiomyocytes. Although we could not detect Opa-1 and NCX1 protein in calpain-1 immunoprecipitates (data not shown), it is worthwhile to mention that calpain-1 may also target other substrates in mitochondria. For example, calpain-1 has been reported to cleave apoptosis inducing factor, leading to apoptosis during ischemia/reperfusion injury in the heart [14]. Thus, it is possible that there may be multiple targets of calpain-1 in mitochondria of diabetic hearts, which merits further investigation.

Disruption of ATP synthase within Complex V results in excess electron “backup” in the individual electron transfer complexes [34], in particular Complex I and III, promoting mitochondrial superoxide generation. Indeed, an increase in reverse electron flow and electrons leaking from Complex I and III of the respiratory chain has been suggested to be main mechanisms promoting mitochondrial ROS generation in diabetes [40, 41]. Disruption of ATP synthase also induces insufficient ATP production, which directly contributes to myocardial dysfunction. In support of this view, we show that up-regulation of ATP5A1 increases ATP synthase activity, decreases mitochondrial ROS generation and mitigates diabetic cardiomyopathy. Taken together, our observation argues that calpain-1 mediates mitochondrial superoxide generation, at least partly by down-regulation of ATP5A1 and disruption of ATP synthase, leading to cardiomyopathic changes in diabetic mice. It is important to mention that over-expression of ATP5A1 per se is not sufficient to increase ATP synthase activity but it prevents diabetes/hyperglycemia-induced decrease in its activity in cardiomyocytes.

In the present study, STZ was given in multiple low doses to induce T1D in mice. In this model, an inflammatory response occurs in the β-cells, leading to lymphocytic infiltrates

and cell death [43], which effectively models the autoimmune T cell-mediated destruction and hypoinsulinemia observed in human T1D [44]. Since mitochondrial capn1 protein is also elevated in db/db type-2 diabetic mouse hearts, similar mechanisms may be operating in type-2 diabetic cardiomyopathy, which requires further study for clarification. Future study is also needed to determine whether mitochondrial calpain is increased and contributes to diabetic cardiomyopathy in humans.

Although the present study focuses on mitochondrial calpain-1 and ROS generation, other mechanisms may be also involved in calpain-mediated diabetic cardiomyopathy. In particular, calpain activation may induce the cleavages of important cytosolic proteins including signaling molecules (PKC and NF-κB) [45, 46], calcium regulatory proteins [47, 48] and myofibril proteins [49, 50], which may contribute to myocardial dysfunction in diabetes.

In summary, we have provided evidence to demonstrate that mitochondrial calpain-1 stimulates mitochondrial ROS generation through down-regulation of ATP5A1 and disruption of ATP synthase, which promotes diabetic cardiomyopathy. These findings uncover a novel mechanism underlying diabetic cardiomyopathy, which may have significant implications in diabetic cardiac complications.

3.5 References

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Chapter 4

4 Therapeutic inhibition of mitochondrial reactive oxygen species with mito-TEMPO reduces diabetic cardiomyopathy[4]

4 This chapter has been published in the following manuscript:

- Ni R, Cao T, Xiong S, Ma J, Fan GC, Lacefield JC, Lu Y, Le Tissier S, and Peng T. (2016) Therapeutic inhibition of mitochondrial reactive oxygen species with mito-TEMPO reduces diabetic cardiomyopathy, Free Radic Biol Med 90, 12-23.

4.1 Introduction

Globally, the number of adults affected with diabetes is rapidly growing and it is estimated to increase to nearly 400 million by 2030 [1]. Both type-1 and type-2 diabetes induce complications including visual impairments and blindness, nerve and kidney damage [2]. However, the greatest challenges lie in cardiovascular complications, accounting for up to 80% diabetes-related morbidity and mortality [3]. Diabetes can directly affect the heart, a condition described as diabetic cardiomyopathy. Diabetic cardiomyopathy has been defined as ventricular dysfunction that occurs in the absence of changes in blood pressure and coronary artery disease [4]. So far, there is no specific therapy available for this disease.

Oxidative damage induced by reactive oxygen species (ROS) has been implicated in the pathogenesis of diabetic cardiomyopathy [5-7]. ROS is mainly produced by mitochondria, NADPH oxidase and xanthine oxidase in the heart [8, 9]. Inhibition of xanthine oxidase or NADPH oxidase reduces diabetic cardiomyopathy [10-12]. There is convincing evidence that mitochondrial ROS production is increased in type-1 and type-2 diabetic hearts [13-17]. Transgenic over-expression of manganese superoxide dismutase (MnSOD) and mitochondrial catalase inhibit mitochondrial ROS and reduce cardiac hypertrophy, preserves cardiac structures and improves function in a mouse model of type-1 diabetes and in insulin-resistant and obese Ay mice, respectively [18, 19]. These studies suggest a critical role of mitochondrial ROS in diabetic cardiomyopathy. Given the fact that commonly employed antioxidants have proven ineffective in clinical trials, it is possible that these agents may not be adequately delivered to the sub-cellular sites of ROS production. Because the mitochondria are important sources of ROS, we hypothesized that therapeutic inhibition of mitochondrial ROS by a mitochondrial targeted antioxidant (2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl) triphenylphosphonium

chloride, monohydrate (mito-TEMPO) might be beneficial in the setting of diabetic cardiomyopathy.

Mito-TEMPO is a physicochemical compound as one of SOD mimics. It has an ability to pass through lipid bilayers easily and accumulate selectively in mitochondria [20]. Both in vitro and in vivo studies have confirmed that mito-TEMPO is a mitochondria-targeted antioxidant with superoxide and alkyl radical scavenging properties [20-22]. In vitro study showed that incubation with mito-TEMPO prevented cell death in adult cardiomyocytes induced by a pharmacological MnSOD inhibitor [23]. In vivo studies revealed that administration of mito-TEMPO improved cardiac function in a mouse model of pressure over-load heart failure [24], and reduced diabetes-attributable cardiac injury and mortality after myocardial infarction [25]. In this study, we demonstrated that therapeutic inhibition of mitochondrial ROS using mito-TEMPO prevented oxidative stress and reduced cardiomyopathic changes in mouse models of type-1 and type-2 diabetes. Our data strongly indicate that mitochondria-targeted antioxidants have therapeutic effects on diabetic cardiac complications.

4.2 Material and methods

4.2.1 Animals and cardiomyocytes culture

This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011). All experimental protocols were approved by the Animal Use Subcommittee at the University of Western Ontario, Canada (2008-079). Breeding pairs of C57BL/6 mice and db+/- mice were purchased from the Jackson Laboratory. Transgenic mice over-expressing a circularly permuted yellow fluorescent protein in the mitochondrial matrix of cells (Tg-mtcpYFP) were kindly provided by Dr. Wang Wang (University of Washington, Seattle) [26]. A breeding program for mice was implemented at our animal

care facilities.

Adult mouse ventricle cardiomyocytes were isolated from C57BL/6 mice, and cultured as described in our recent studies [27].

4.2.2 Experimental protocol

Type-1 diabetes was induced in adult male mice (2-month old) by consecutive intraperitoneal injection of streptozotocin (STZ, 50 mg/kg/day) for 5 days [11]. Seventy-two hours after the last injection of STZ, random blood glucose levels were measured using the OneTouch Ultra 2 blood glucose monitoring system (Life Scan, Inc. CA, USA). Mice were considered diabetic and used for the study only if they had hyperglycemia (≥15 mM) 72 h after STZ injection. Citrate buffer-treated mice were used as non-diabetic control (blood glucose < 12 mM). Thirty days after diagnosis, diabetic mice (6-8 in each group) received daily injection of mito-TEMPO (0.7 mg/kg/day, i.p., Enzo Life Sciences, Inc., the product number: ALX-430-150) [28] or vehicle for 30 days.

Type-2 diabetic db/db mice were produced by breeding db+/- mice. Male db/db mice and their littermate db+/- mice received daily injection of mito-TEMPO (0.7 mg/kg/day, i.p.) starting at age of 2.5 months for 30 days.

4.2.3 Echocardiography

Animals were lightly anaesthetized with inhalant isoflurane (1%) and imaged using a 40-MHz linear array transducer attached to a preclinical ultrasound system (Vevo 2100, FIJIFILM VisualSonics, Canada) with nominal in-plane spatial resolution of 40 μm (axial) × 80 μm (lateral). M-mode and 2-D parasternal short-axis scans (133 frames/second) at the level of the papillary muscles were used to assess changes in left ventricle (LV) end-systolic inner diameter, LV end-diastolic inner diameter, LV posterior wall thickness in end-diastole and end-systole, fractional shortening (FS%) and ejection fraction (EF%).

An apical four chamber view of the left ventricle was obtained and the pulsed wave Doppler measurements were performed in the apical view with a cursor at mitral valve inflow: maximal early (E) and late (A) transmitral velocities in diastole. The diastolic function was determined by the ratio of E to A peak.

4.2.4 Histological analyses

For cardiomyocyte cross-sectional area, several sections of the heart (5 μm thick) were prepared and stained for membranes with fluorescein isothiocyanate–conjugated wheat germ agglutinin (Invitrogen) and for nuclei with Hochest. A single cardiomyocyte was measured by using an image quantitative digital analysis system (NIH Image version 1.6) as described [11]. The outline of at least 200 cardiomyocytes was traced in each section.

4.2.5 Measurement of ROS generation in isolated mitochondria

Interfibrillar mitochondria were isolated from freshly harvested hearts as described previously [29]. The isolated mitochondria were further purified using Percoll density gradient centrifugation. The freshly isolated mitochondria (10 μg) was incubated with pyruvate/malate (5/5 mmol/l) in a reaction buffer containing Amplex Red (0.05 mmol/l, Life Technologies Inc. Burlington, Ontario, Canada) and horseradish peroxidase (0.1 units/ml) at 37ºC. The fluorescent signals were monitored by spectrofluorometer at the 520/580 nm in every 10 minutes.

4.2.6 Determination of oxidative stress levels in diabetic hearts

The oxidative stress levels in heart tissue lysates were measured by using 2,7-dichlorodihydro-fluorescein diacetate (DCF-DA, Invitrogen, USA) as an indicator [11]. Briefly, fresh heart tissues were homogenized in an assay buffer. The homogenates (50 μg protein) were incubated with DCF-DA at 37ºC for 3 hours. The fluorescent product formed was quantified by spectrofluorometer at the 485/525 nm. Changes in

fluorescence were expressed as arbitrary unit.

The protein oxidation in heart tissue lysates was assessed by measuring protein carbonyl content using a commercial assay kit (Cayman Chemical, USA) following manufacturer's instructions.

4.2.7 Measurement of mitochondrial superoxide generation in cardiomyocytes

Superoxide flashes in single mitochondrion were measured to determine mitochondrial superoxide generation in living cardiomyocytes as described [30]. Briefly, cardiomyocytes were infected with an adenoviral vector expressing mt-cpYFP (Ad-mt-cpYFP, kindly provided by Dr. Wang Wang from the University of Washington, Seattle, USA). Ad-mt-cpYFP expresses a circularly permuted yellow fluorescent protein (cpYFP) in the mitochondrial matrix of cells using the cytochrome C oxidase subunit IV targeting sequence (mt-cpYFP). Twenty-four hours after infection, confocal imaging was recorded using the Olympus FV 1000 laser-scanning microscope equipped with a 63x, 1.3NA oil immersion objective and a sampling rate of 0.7s/frame. At least 20 cardiomyocytes per culture in each group were analyzed.

Mitochondrial superoxide generation was also assessed in living cardiomyocytes from Tg-mtcpYFP mice by using the MitoSOX^{TM} Red mitochondrial superoxide indicator (Molecular Probes) and oxidant levels measured by using Dihydroethidium (DHE, Molecular Probes). The cpYFP signals were used to identify mitochondrial MitoSOX^{TM} Red and DHE staining in cardiomyocytes.

4.2.8 Determination of apoptotic cell death

Caspase-3 activity in myocardial tissues and cardiomyocytes was measured by using a caspase-3 fluorescent assay kit (BIOMOL Research Laboratories).

Cell death was also determined in cardiomyocytes by annexin V/ Hochest staining as described [31].

4.2.9 Real-time RT-PCR

Total RNA was extracted from heart tissues using the Trizol Reagent (Life Technologies Inc. Burlington, Ontario, Canada). Real-time RT-PCR was performed to analyze mRNA expression for β-myosin heavy chain (β-MHC), atrial natriuretic peptide (ANP), $gp91^{phox}$, $p47^{phox}$ and GAPDH as previously described [11].

4.2.10 Western blot analysis

The protein levels of Bcl-2, phosphorylated and total extracellular signal-regulated kinase-1/2 (ERK1/2), c-Jun NH2-terminal kinase-1/2 (JNK1/2), p38 kinase, and GAPDH were determined by western blot analysis using their specific antibodies (Cell Signaling, Danvers, MA).

4.2.11 Statistical analysis

All data were given as means ± SD. Differences between two groups were compared by unpaired Student's t test. For multi-group comparisons, ANOVA followed by Newman-Keuls test was performed. A value of $P < 0.05$ was considered statistically significant.

4.3 Results

4.3.1 Mito-TEMPO inhibited high glucose-induced mitochondrial superoxide generation and cell death in cardiomyocytes

To determine the effects of mito-TEMPO on mitochondrial superoxide generation and cell death, we incubated adult cardiomyocytes with normal glucose (5 mmol/l) or high glucose (30 mmol/l) in the presence of mito-TEMPO (25 nmol/l) or vehicle for 24 hours. This dose of mito-TEMPO was chosen because it has been shown to increase

mitochondrial superoxide dismutation by 3-fold while not affecting cytoplasmic dismutation in cultured cells [20]. As shown in Figure 4-1A, high glucose increased mitochondrial flashes in cardiomyocytes, which were abrogated by mito-TEMPO, indicating an increase in mitochondrial superoxide generation. This was further confirmed by the MitoSOX^{TM} Red or DHE staining in living cardiomyocytes during high glucose stimulation (Figure 4-1B and C).

High glucose induced cardiomyocyte death as determined by annexin-V/Hochest staining. Co-incubation with mito-TEMPO prevented high glucose-induced cell death in cardiomyocytes (Figure 4-1D and E). These results demonstrate that inhibition of mitochondrial superoxide generation prevents high glucose-induced cardiomyocyte death.

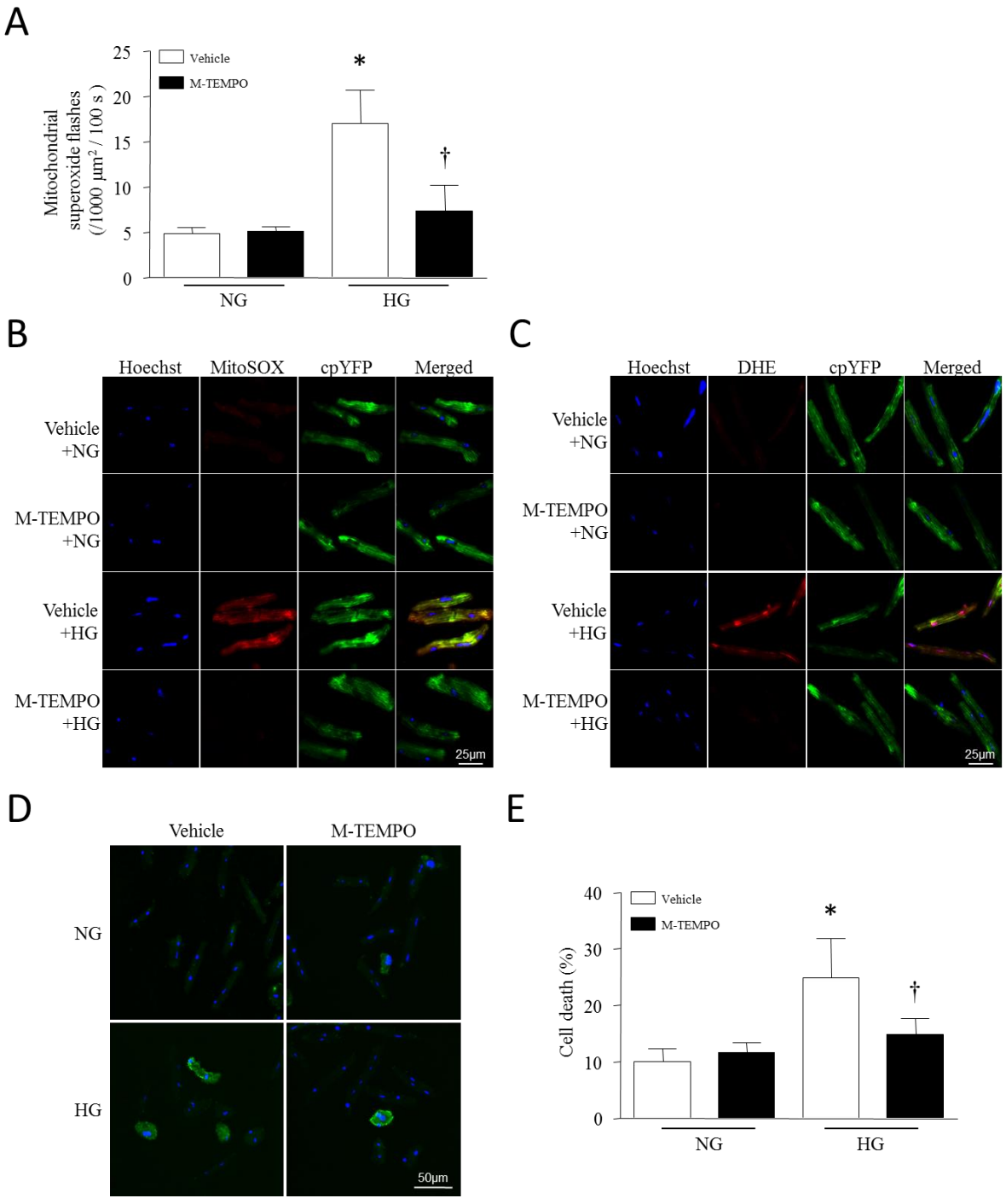
A
Vehicle
M-TEMPO
Mitochondrial superoxide flashes (/1000 μm² / 100 s)
25
20
15
10
5
0
*
†
NG
HG
B
Hoechst
MitoSOX
cpYFP
Merged
Vehicle +NG
M-TEMPO +NG
Vehicle +HG
M-TEMPO +HG
25μm
C
Hoechst
DHE
cpYFP
Merged
Vehicle +NG
M-TEMPO +NG
Vehicle +HG
M-TEMPO +HG
25μm
D
Vehicle
M-TEMPO
NG
HG
50μm
E
Vehicle
M-TEMPO
Cell death (%)
40
30
20
10
0
*
†
NG
HG

Figure 4-1. Effects of mito-TEMPO (M-TEMPO) on mitochondrial superoxide generation and cell death in cardiomyocytes

Adult mouse cardiomyocytes were incubated with normal glucose (NG) or high glucose (HG) for 24 h in the presence of M-TEMPO or vehicle. (A) Single mitochondrial superoxide flashes were determined. (B) Representative microphotographs from 5 different cultures for the MitoSOX staining, cpYFP signals and nuclei (Hoechst staining) show overlap of MitoSOX staining and cpYFP signals in cardiomyocytes. (F) Representative microphotographs from 5 different cultures for the DHE staining, cpYFP signals and nuclei (Hoechst staining) show overlap of DHE staining and cpYFP signals in cardiomyocytes. (D) A representative staining for annexin V (green color) and nuclei (blue color) and (E) Quantification of death cells (%). Data are mean ± SD from 5 different cell cultures. *$P < 0.05$ versus vehicle + NG and †$P < 0.05$ versus vehicle + HG.

4.3.2 Administration of mito-TEMPO abolished mitochondrial ROS generation and oxidative stress in hearts of diabetic mice

Injection of mito-TEMPO did not affect the blood glucose levels, heart weight and body weight in both sham and diabetic mice (Tables 4-1 and 2). Neither abnormal behaviors or health problems including myocardial function and death nor changed intake of food and water was observed due to the use of mito-TMEPO in sham mice (Tables 4-3), suggesting there might be no obvious toxic side-effects of mito-TEMPO.

Mitochondrial ROS generation was significantly increased in both db/db and STZ-induced mouse hearts. Administration of mito-TEMPO abolished mitochondrial ROS generation in diabetic mouse hearts (Figure 4-2A and B).

To assess oxidative stress in diabetic hearts, we first measured oxidative stress levels in heart tissue lysates. The oxidative stress levels were increased in db/db and STZ-induced mouse hearts, which was inhibited by mito-TEMPO (Figure 4-2C and D). The total antioxidant capacity (including small molecule and protein antioxidants) was also increased in diabetic hearts (Figure 4-3). We then determined the oxidative damage in diabetic mice hearts by measuring the protein carbonyl content. The protein carbonyl content was elevated in db/db and STZ-induced mouse hearts. However, injection with mito-TEMPO abrogated diabetes-induced protein carbonyl content (Figure 4-2E and F). These results suggest that mito-TEMPO effectively blocks mitochondrial ROS production and prevents oxidative stress in diabetic mouse hearts.

Table 4-1. General information in db/db and db+/- mice receiving mito-TEMPO

	Body weight (g)	Heart weight (mg)	Tibia length (mm)	Heart weight/Tibia length (mg/mm)	Blood glucose (mM)
db+/- + Vehicle	25.84±3.71	117.67±25.42	17.65±0.51	6.64±1.25	8.17±1.19
db+/- + mito-TEMPO	24.92±3.13	114.01±19.64	17.32±0.45	6.58±0.93	7.73±2.71
db/db + Vehicle	52.61±4.83	124.67±12.26	17.14±0.58	7.28±0.79	29.08±4.07
db/db + mito-TEMPO	55.34±5.82	125.86±14.40	16.82±0.64	7.47±0.69	24.00±4.23

Table 4-2. General information in STZ-injected mice receiving mito-TEMPO

	Body weight(g)	Heart weight(mg)	Heart/Body weight (mg/g)	Blood glucose (mM)
Sham	29.89±2.96	132.28±15.36	4.42±0.43	5.82±2.67
STZ + Vehicle	26.33±3.48	125.50±18.56	4.77±0.29	28.02±5.02
STZ + mito-TEMPO	24.78±2.73	120.20±10.18	4.87±0.31	30.34±2.81

Table 4-3. Intake of food and water in mice receiving mito-TEMPO

	Food Intake (g)	Water Consumption (ml)
Vehicle	4.2±0.2	12.6±2.5
Mito-TEMPO	4.5±0.5	14.1±2.2

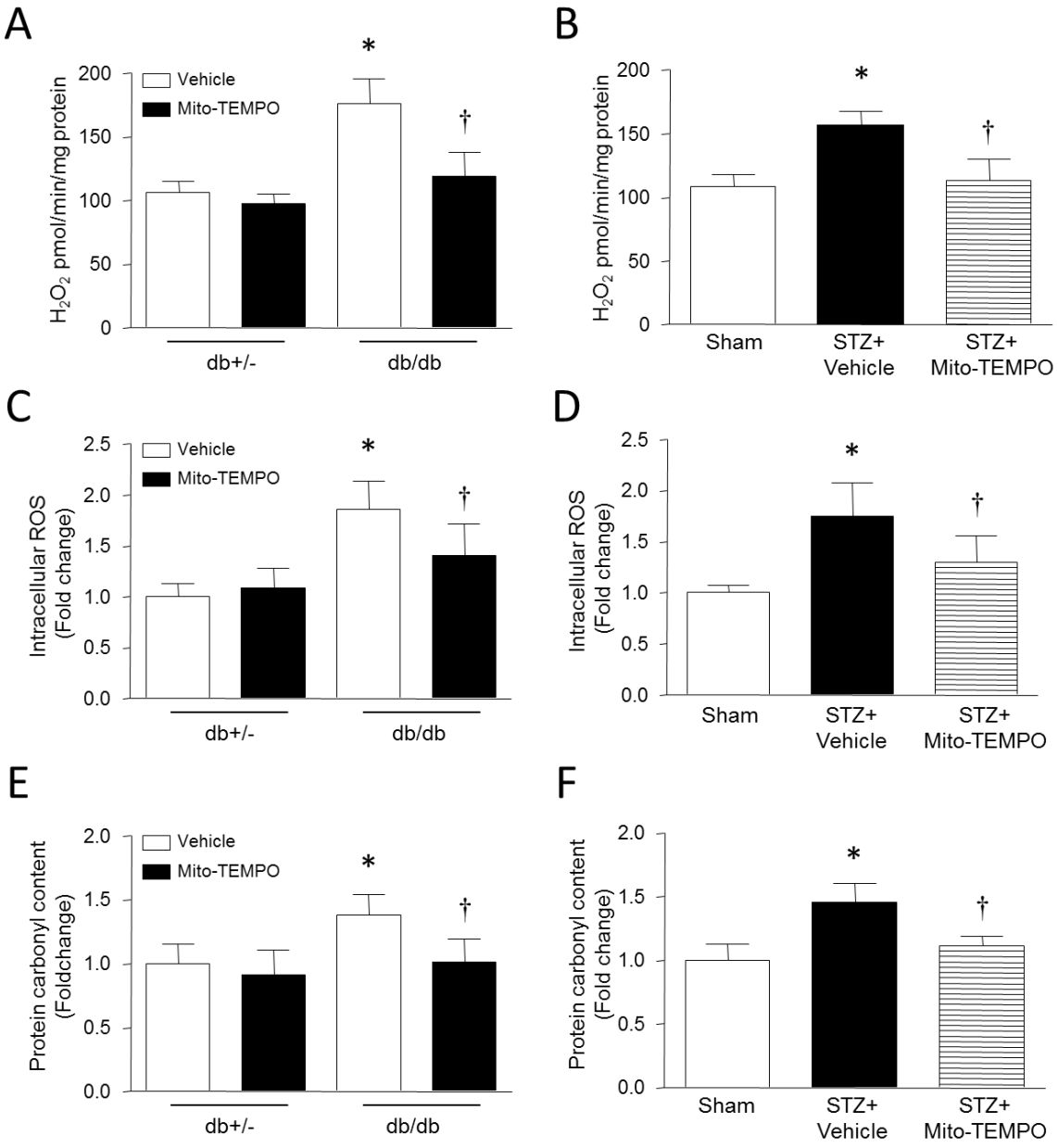

Figure 4-2. Effects of mito-TEMPO (M-TEMPO) on mitochondrial ROS generation and oxidative stress

Mitochondrial oxidant levels were assessed using Amplex Red in db+/- and db/db hearts (A) or sham and STZ-treated hearts (B). Oxidative stress levels were determined using DCF-DA in db+/- and db/db hearts (C) or sham and STZ-treated hearts (D). Protein carbonyl content was measured in db+/- and db/db hearts (E) or sham and STZ-treated hearts (F). Data are mean ± SD; n = 6-8. *P < 0.05 versus vehicle in db+/- or sham and †P < 0.05 versus vehicle in db/db or STZ.

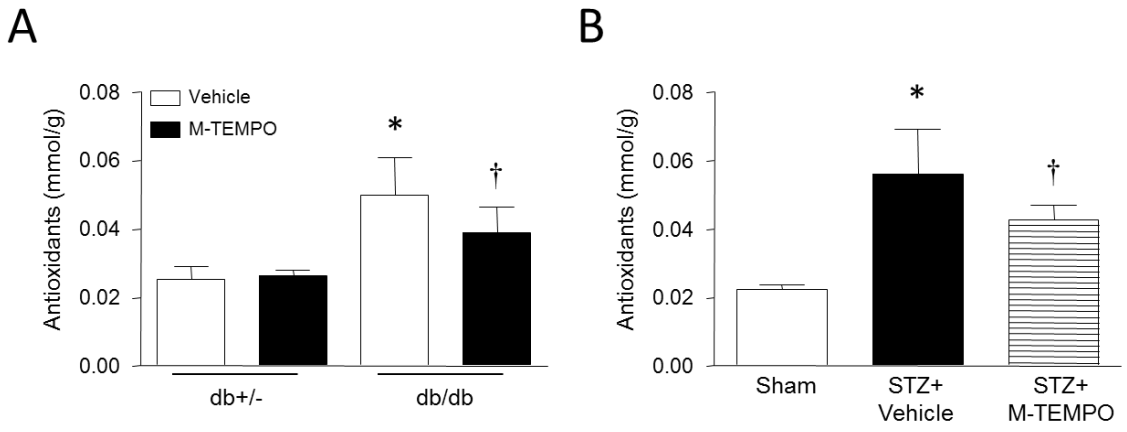

Figure 4-3. Effect of mito-TEMPO on total antioxidant capacity in db/db mice and STZ-injected mice

The total antioxidant capacity was increased in db/db (A) and STZ-treated mouse hearts (B), but was reduced by mito-TEMPO (M-TEMPO). Data are means ± SD; n = 6-8. *P < 0.05 versus vehicle in db+/- or sham and †P < 0.05 versus vehicle in db/db or STZ.

Since NADPH oxidase is another important source of ROS in the heart, we measured NADPH oxidase expression. Real-time PCR revealed that the mRNA levels of gp91^{phox} and p47^{phox} were significantly increased in STZ-induced type-1 diabetic hearts but not in db/db mouse hearts. Administration of mito-TEMPO decreased the mRNA levels of gp91^{phox} and p47^{phox} in STZ-induced mouse hearts whereas it did not have significant impact on their expression in db/db mouse hearts (Figure 4-A-D).

To examine whether mitochondrial superoxide interacts with gp91^{phox}-containing NADHP oxidase (Nox2) activation, we used gp91ds-tat peptide to inhibit Nox2 activation in cardiomyocytes [32]. Cardiomyocytes were incubated with normal or high glucose in the presence of gp91ds-tat (10 μmol/l, AnaSpec, Inc., Fremont, CA, USA), mito-TEMPO (25 nmol/l) and vehicle, either alone or in combination for 24 hours. Gp91ds-tat and mito-TEMPO alone or in combination prevented mitochondrial superoxide production and oxidant levels induced by high glucose as determined by the MitoSOX Red and DHE staining, respectively (Figure 4-E and F). Similarly, high glucose-induced cell death was inhibited by gp91ds-tat or mito-TEMPO; however, a combination of gp91ds-tat and mito-TEMPO did not provide further protection in cardiomyocytes (Figure 4-5), suggesting a potential interaction between mitochondrial superoxide and Nox2 activation.

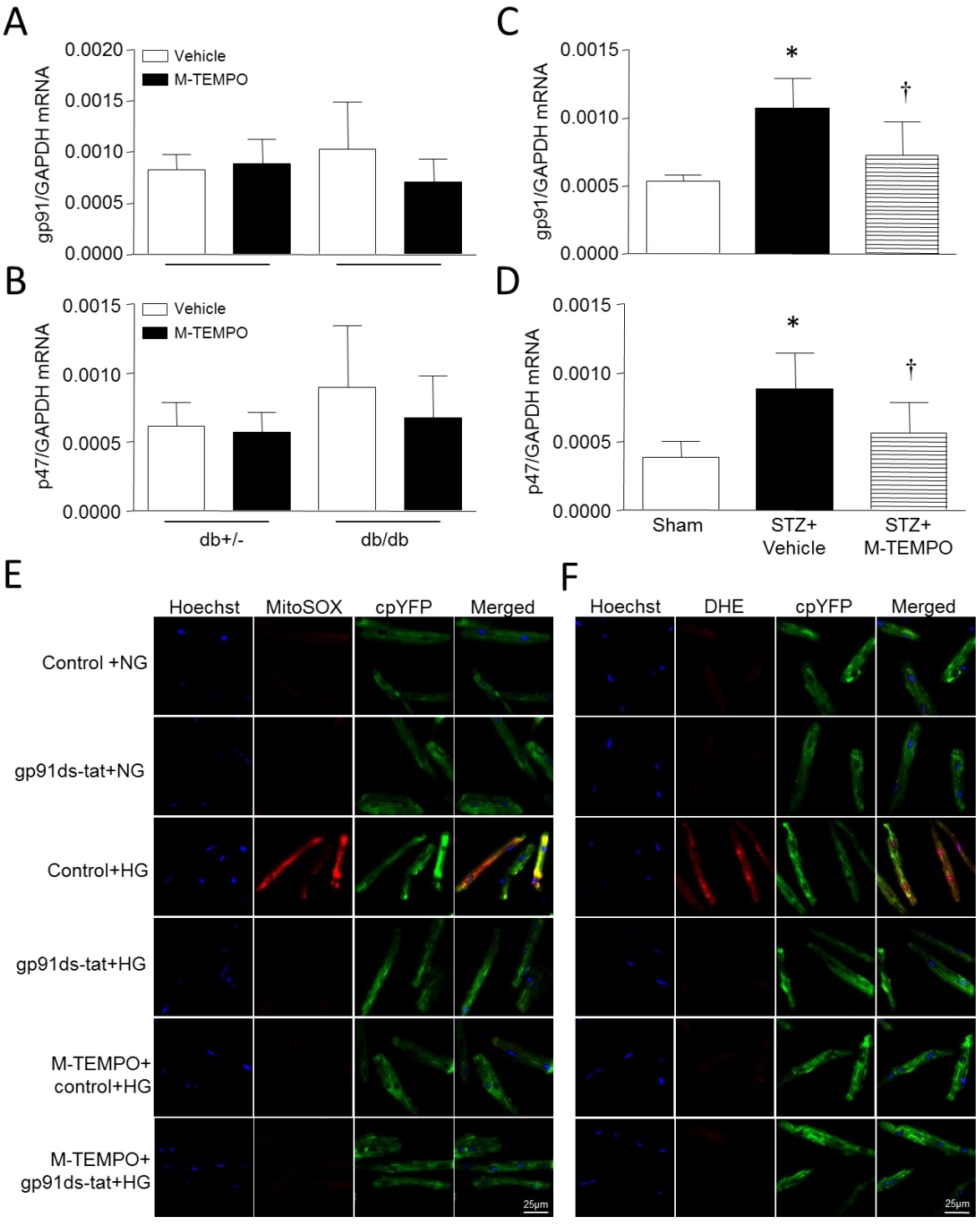
A
gp91/GAPDH mRNA
0.0020
0.0015
0.0010
0.0005
0.0000
Vehicle
M-TEMPO
B
p47/GAPDH mRNA
0.0015
0.0010
0.0005
0.0000
Vehicle
M-TEMPO
db+/-
db/db
C
gp91/GAPDH mRNA
0.0015
0.0010
0.0005
0.0000
*
†
D
p47/GAPDH mRNA
0.0015
0.0010
0.0005
0.0000
*
†
Sham
STZ+ Vehicle
STZ+ M-TEMPO
E
Hoechst
MitoSOX
cpYFP
Merged
Control +NG
gp91ds-tat+NG
Control+HG
gp91ds-tat+HG
M-TEMPO+ control+HG
M-TEMPO+ gp91ds-tat+HG
25μm
F
Hoechst
DHE
cpYFP
Merged
25μm

Figure 4-4. (A-D) Effects of mito-TEMPO (M-TEMPO) on NADPH oxidase expression in diabetic mice

The mRNA levels of gp91^{phox} and p47^{phox} mRNA in db+/- and db/db mouse hearts (A and B) or sham and STZ-treated mouse hearts (C and D) were determined by real-time RT-PCR. Data are mean ± SD; n = 6-8. *P < 0.05 versus sham and †P < 0.05 versus vehicle + STZ.

(E and F) Effects of gp91ds-tat and mito-TEMPO (M-TEMPO) on mitochondrial superoxide production in cardiomyocytes

Cultured cardiomyocytes isolated from Tg-mtcpYFP mice were incubated with normal (NG) or high glucose (HG) in the presence of gp91ds-tat, M-TEMPO or vehicle, either alone or in combination for 24 hours. Mitochondrial superoxide production was then determined. (E) Representative microphotographs for the MitoSOX staining, cpYFP signals and nuclei (Hoechst staining) show overlap of MitoSOX staining and cpYFP signals in cardiomyocytes. (F) Representative microphotographs for the DHE staining, cpYFP signals and nuclei (Hoechst staining) show overlap of DHE staining and cpYFP signals in cardiomyocytes. Data are from 5 different cultures.

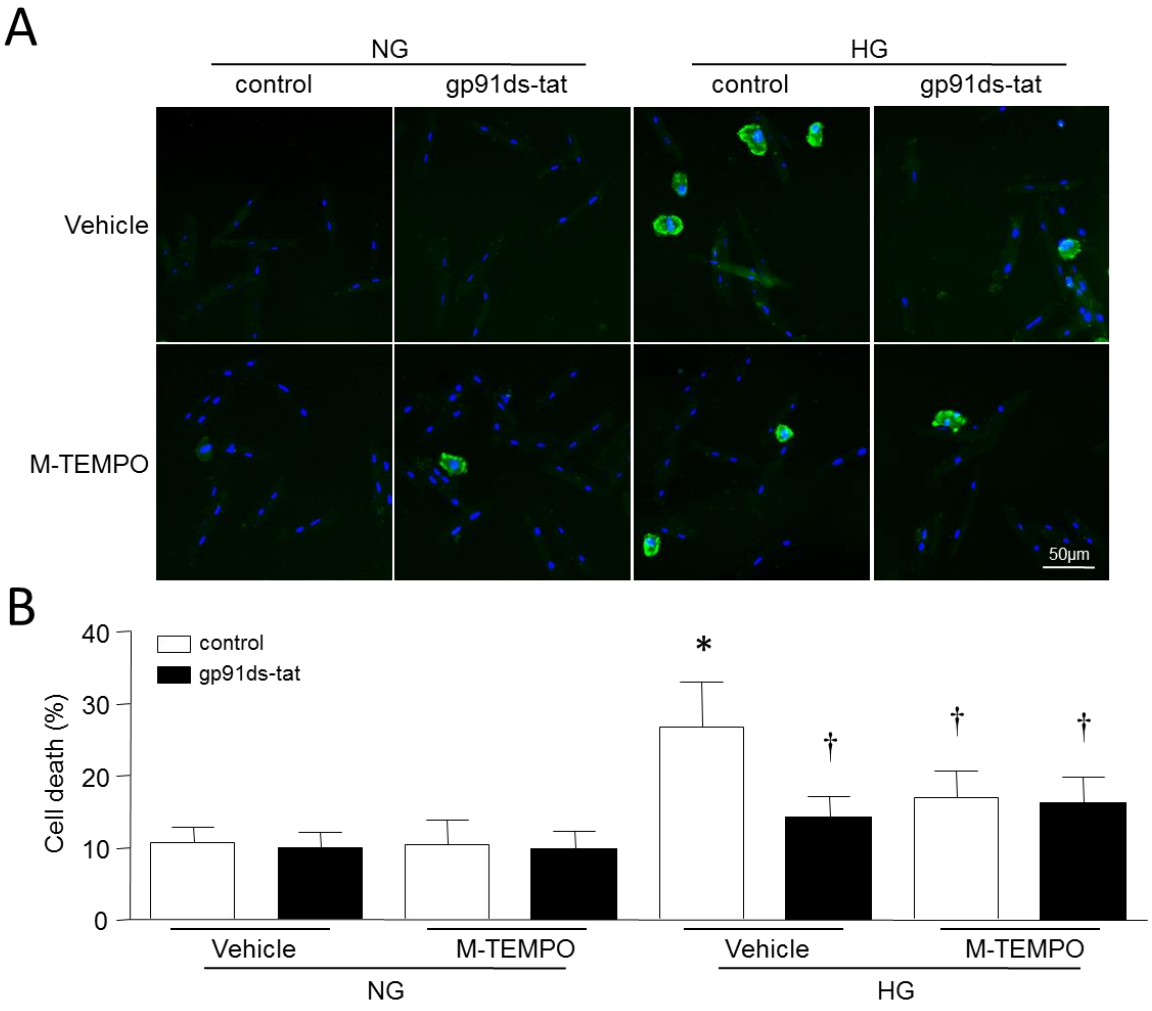

Figure 4-5. Effects of gp91ds-tat and mito-TEMPO (M-TEMPO) on cell death

Cultured cardiomyocytes isolated from Tg-mtcpYFP mice were incubated with normal (NG) or high glucose (HG) in the presence of gp91ds-tat, M-TEMPO or vehicle, either alone or in combination for 24 hours. Cell death was determined by annexin V staining. (A) Representative staining for annexin V (green color) and nuclei (blue color). (B) Quantification of death cells (%). Data are mean ± SD from 5 different cultures. *P < 0.05 versus vehicle+control+NG and †P < 0.05 versus vehicle+control+HG.

4.3.3 Mito-TEMPO reduced cardiomyopathic changes in type-2 diabetic db/db mice

Histological analysis of cardiomyocyte cross-sectional areas showed an increase in cardiomyocyte size from db/db mouse hearts. However, administration of mito-TEMPO did not affect cardiomyocyte size in sham animals but reduced cardiomyocyte size in db/db mice hearts (Figure 4-6A). Similarly, the mRNA levels of ANP and β-MHC were elevated in diabetic db/db mouse hearts and significantly reduced in db/db mice receiving mito-TEMPO (Figure 4-6B and C). These results demonstrate that mito-TEMPO prevents myocardial hypertrophy in type-2 diabetic db/db mice.

Apoptosis plays an important role in development of diabetic cardiomyopathy. We examined the effects of mito-TEMPO on apoptosis in db/db mouse hearts. Diabetes increased caspase-3 activity, an indicator of apoptosis, and decreased the protein levels of BCL-2, an important anti-apoptotic factor in db/db mouse hearts. Administration of mito-TEMPO prevented caspase-3 activity and increased BCL-2 protein expression in db/db mouse hearts (Figure 4-6D and E). These results suggest that inhibition of mitochondrial ROS protects the heart against apoptosis in diabetic mice.

Echocardiographic analysis revealed a decline of E/A ratio in db/db mice (Figure 4-6F), indicating myocardial diastolic dysfunction, while myocardial systolic function was preserved in db/db mice as determined by FS% and EF% (Figure 4-6G and H, and Table 4-4). Injection of mito-TEMPO significantly increased the E/A ratio in db/db mice (Figure 4-6F). Taken together, administration of mito-TEMPO reduces cardiomyopathy in type-2 diabetic db/db mice.

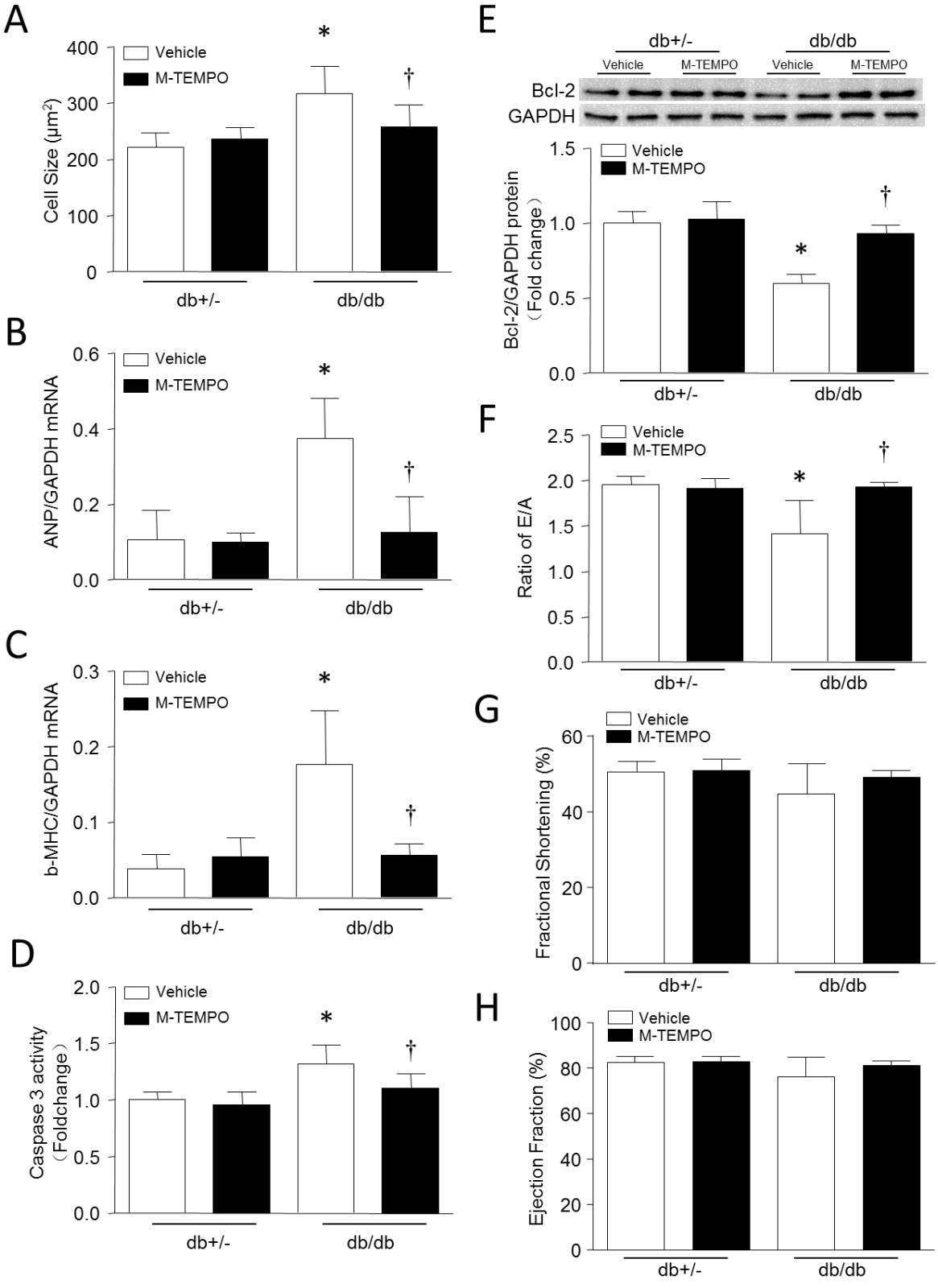
A
Cell Size (μm²)
400
300
200
100
0
Vehicle
M-TEMPO
*
†
db+/-
db/db
B
ANP/GAPDH mRNA
0.6
0.4
0.2
0.0
C
b-MHC/GAPDH mRNA
0.3
0.2
0.1
0.0
D
Caspase 3 activity
（Foldchange）
2.0
1.5
1.0
0.5
0.0
E
db+/-
db/db
Vehicle
M-TEMPO
Bcl-2
GAPDH
Bcl-2/GAPDH protein
（Fold change）
1.5
1.0
0.5
0.0
F
Ratio of E/A
2.5
2.0
1.5
1.0
0.5
0.0
G
Fractional Shortening (%)
60
40
20
0
H
Ejection Fraction (%)
100
80
60
40
20
0

Figure 4-6. Effects of mito-TEMPO (M-TEMPO) on cardiomyopathic changes in db/db mice

(A) Cardiomyocyte cross-sectional areas. (B and C) the mRNA levels of ANP and β-MHC in db/db mouse hearts. (D) Caspase-3 activity in db/db hearts. (E) The upper panel is a representative western blot for BCL-2 in myocardial tissues, and the lower panel is the quantitative data from western blot analysis for BCL-2/GAPDH. (F) Myocardial diastolic function (E/A). (G) Myocardial systolic function (FS%). (H) Left ventricular ejection fraction (EF%). Data are mean ± SD; n = 6-8. *P < 0.05 versus vehicle in db+/- and †P < 0.05 versus vehicle in db/db.

Table 4-4. Parameters of echocardiographic analysis in db/db and db+/- mice receiving mito-TEMPO

	Ratio of E/A	Fractional Shortening (%)	Ejection Fraction (%)	End-systolic diameter (mm)	End-diastolic diameter (mm)
db+/- + Vehicle	1.95±0.09	50.5±2.61	82.4±2.66	1.88±0.3	3.79±0.43
db+/- + mito-TEMPO	1.92±0.1	50.89±2.82	82.67±2.56	1.9±0.28	3.88±0.48
db/db + Vehicle	1.41±0.39*	44.75±6.91	76.16±7.43	2.05±0.33	3.71±0.26
db/db + mito-TEMPO	1.93±0.06†	49.14±1.79	81.23±1.87	1.88±0.2	3.69±0.29

(* $P < 0.05$ vs db+/- + Vehicle and † $P < 0.05$ vs db/db + Vehicle)

4.3.4 Mito-TEMPO mitigated diabetic cardiomyopathy in STZ-induced mice

We also examined the effects of mito-TEMPO on diabetic cardiomyopathy in STZ-induced mice. Consistently, cardiomyocyte cross-sectional areas and gene expression of ANP and β-MHC were significantly increased in STZ-induced type-1 diabetic hearts (Figure 4-7A-C). These hypertrophic changes were attenuated by mito-TEMPO (Figure 4-7A-C). Injection of STZ increased caspase-3 activity (Figure 4-7D) and reduced anti-apoptotic protein BCL-2 in the heart (Figure 4-7E), which were prevented by mito-TEMPO (Figure 4-7D and E). Finally, myocardial diastolic and systolic functions were decreased in STZ-induced mice as determined by the E/A ratio (Figure 4-7F), FS% and EF% (Figure 4-7G and H, and Table 4-5), respectively. However, administration of mito-TEMPO restored both myocardial diastolic and systolic functions in STZ-induced type-1 diabetic mice (Figure 4-7F-H and Table 4-4).

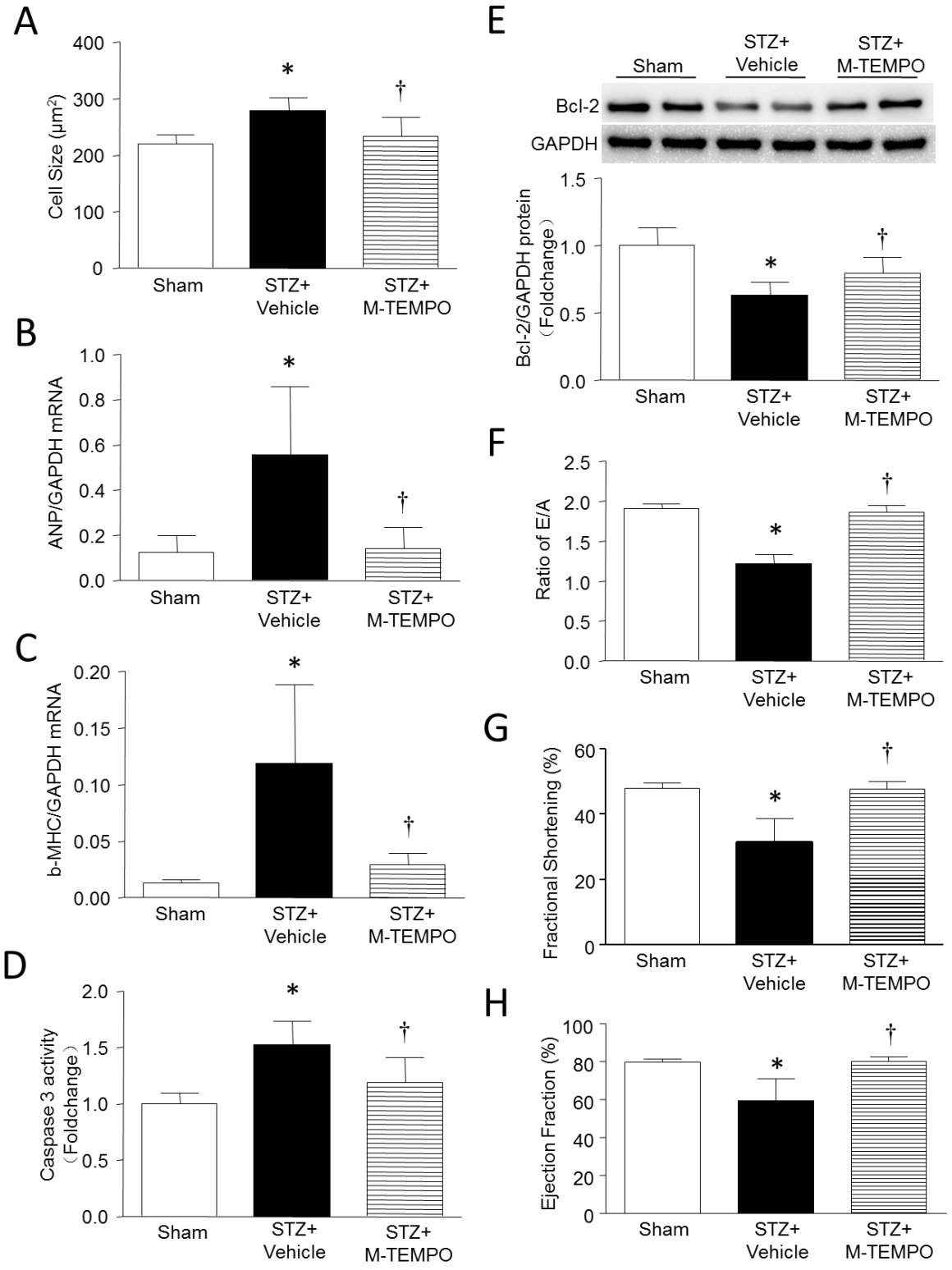
A
Cell Size (μm²)
400
300
200
100
0
*
†
Sham
STZ+ Vehicle
STZ+ M-TEMPO
B
ANP/GAPDH mRNA
1.0
0.8
0.6
0.4
0.2
0.0
C
b-MHC/GAPDH mRNA
0.20
0.15
0.10
0.05
0.00
D
Caspase 3 activity (Foldchange)
2.0
1.5
1.0
0.5
0.0
E
Bcl-2
GAPDH
Bcl-2/GAPDH protein (Foldchange)
1.5
1.0
0.5
0.0
F
Ratio of E/A
2.5
2.0
1.5
1.0
0.5
0.0
G
Fractional Shortening (%)
60
40
20
0
H
Ejection Fraction (%)
100
80
60
40
20
0

Figure 4-7. Effects of mito-TEMPO (M-TEMPO) on cardiomyopathic changes in STZ-injected mice

(A-D) Cardiomyocyte cross-sectional areas (A), the mRNA levels of ANP (B) and β-MHC (C) and caspase-3 activity (D) were determined in STZ-treated hearts. (E) The upper panel is a representative western blot for BCL-2, and the lower panel is the quantification of BCL-2 protein levels relative to GAPDH. (F) Echocardiographic analysis (E/A). (G) Myocardial systolic function (FS%). (H) Left ventricular ejection fraction (EF%). Data are mean ± SD; n = 6-8. *P < 0.05 versus sham and †P < 0.05 versus vehicle + STZ.

Table 4-5. Parameters of echocardiographic analysis in STZ-injected mice receiving mito-TEMPO

	Ratio of E/A	Fractional Shortening (%)	Ejection Fraction (%)	End-systolic diameter (mm)	End-diastolic diameter (mm)
Sham	1.91±0.06	48±1.58	80.03±1.53	2.03±0.19	3.91±0.34
STZ + Vehicle	1.21±0.12*	31.26±7.34*	59.35±11.41*	2.56±0.58*	3.69±0.47
STZ + mito-TEMPO	1.86±0.09†	47.68±2.28†	80.05±2.47†	1.8±0.23†	3.43±0.37

(* $P < 0.05$ vs Sham and † $P < 0.05$ vs STZ + Vehicle)

4.3.5 Mito-TEMPO attenuated ERK1/2 activation in diabetic mouse hearts and high glucose-stimulated cardiomyocytes

Since activation of mitogen-activated protein kinase has been implicated in diabetic cardiomyopathy and they are sensitive to oxidative stress, we therefore examined the phosphorylation of its three family members: ERK1/2, JNK1/2 and p38. Western blot analysis showed that phosphorylated ERK1/2 was increased in both diabetic mouse hearts (Figure 4-8A and B) while no change in phosphorylated JNK1/2 and p38 was observed (data not shown). Mito-TEMPO significantly reduced ERK1/2 phosphorylation in db/db and STZ-induced mouse hearts (Figure 4-8A and B).

To further address the involvement of ERK1/2, we incubated adult cardiomyocytes with normal (5 mmol/l) or high glucose (30 mmol/l) in the presence of mito-TEMPO (25 nmol/l) or vehicle for 24 hours. High glucose increased phosphorylated ERK1/2, which was abolished by mito-TEMPO (Figure 4-8C). In addition, co-incubation with ERK1/2 inhibitor PD98059 (10 μmol/l) prevented high glucose-induced cell death (Figure 4-8D).

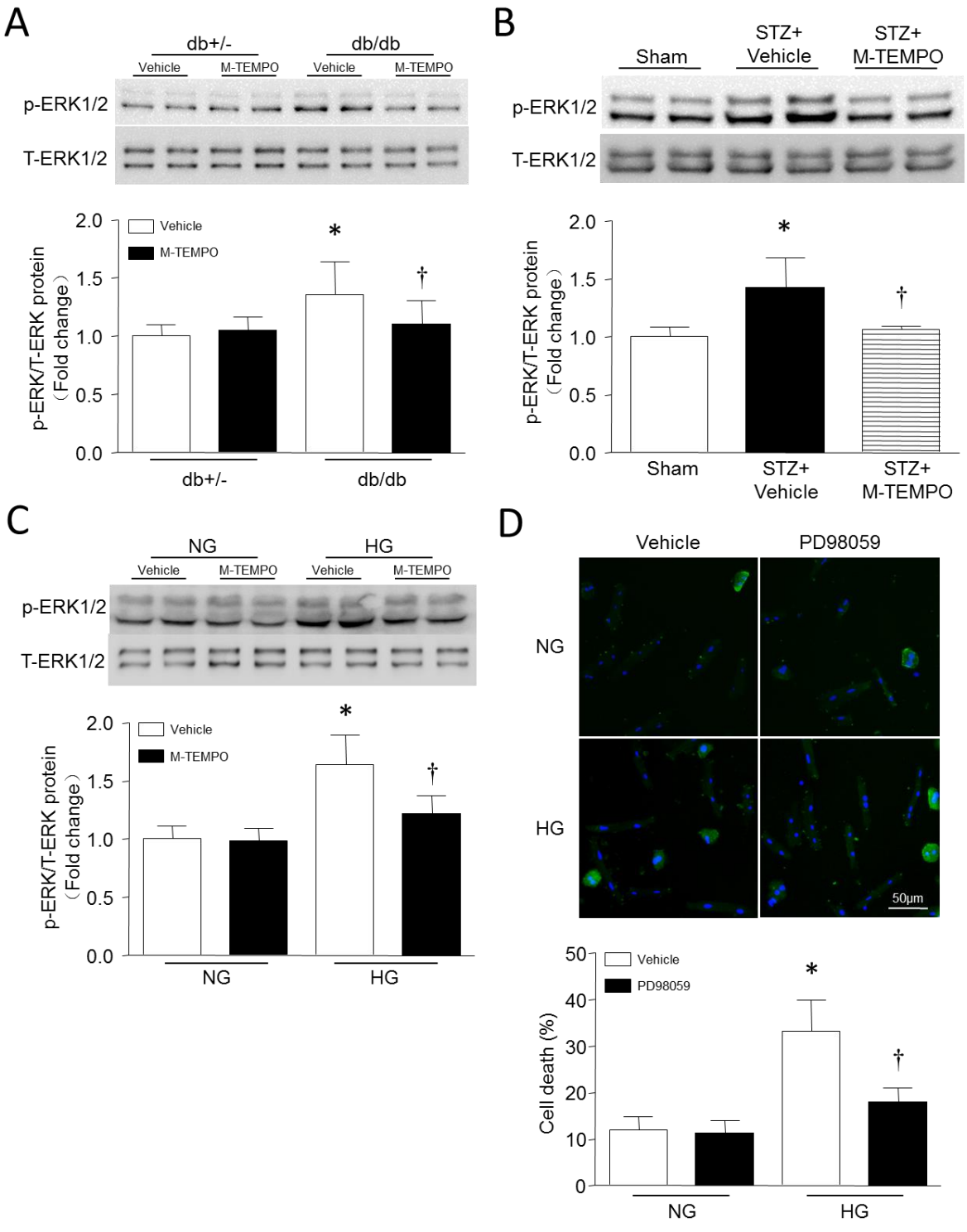
A
db+/-
db/db
Vehicle
M-TEMPO
Vehicle
M-TEMPO
p-ERK1/2
T-ERK1/2
p-ERK/T-ERK protein (Fold change)
2.0
1.5
1.0
0.5
0.0
Vehicle
M-TEMPO
*
†
db+/-
db/db
B
Sham
STZ+ Vehicle
STZ+ M-TEMPO
p-ERK1/2
T-ERK1/2
p-ERK/T-ERK protein (Fold change)
2.0
1.5
1.0
0.5
0.0
*
†
Sham
STZ+ Vehicle
STZ+ M-TEMPO
C
NG
HG
Vehicle
M-TEMPO
Vehicle
M-TEMPO
p-ERK1/2
T-ERK1/2
p-ERK/T-ERK protein (Fold change)
2.0
1.5
1.0
0.5
0.0
Vehicle
M-TEMPO
*
†
NG
HG
D
Vehicle
PD98059
NG
HG
50μm
Cell death (%)
50
40
30
20
10
0
Vehicle
PD98059
*
†
NG
HG

Figure 4-8. Effects of mito-TEMPO(M-TEMPO) on ERK1/2 phosphorylation in hearts and cardiomyocytes

Upper panels are representative western blots for phosphorylated ERK1/2 (p-ERK) and total ERK1/2 (T-ERK), and lower panels are quantitative data of p-ERK/T-ERK ratio in db+/- and db/db hearts (A), sham and STZ-treated hearts (B) or high glucose-stimulated cardiomyocytes (C). (D) Cell death was determined by annexin V staining. Upper panel is a representative staining for annexin V (green color) and nuclei (blue color) and lower panels are the quantification of death cells (%). Data are mean ± SD; n = 6-8 or 5 different cultures. *P < 0.05 versus vehicle in db+/-, sham or normal glucose and †P < 0.05 versus vehicle in db/db, STZ or high glucose.

4.4 Discussion

The present study provides the first evidence that therapeutic inhibition of mitochondrial ROS by mito-TEMPO reduces cardiomyopathic changes and improves myocardial function in type-1 and type-2 diabetic mice. Our study also provides direct evidence that high glucose induces mitochondrial superoxide generation, which is prevented by mito-TEMPO. We further suggest that ROS amplified in mitochondria subsequently activates downstream ROS-sensitive signaling pathways (e.g. ERK1/2) implicated in pathological cardiac changes in diabetes (Figure 4-9).

This study demonstrates that both type-1 and type-2 diabetes promoted mitochondrial ROS generation, increased intracellular oxidative stress levels and induced oxidative damage in the heart, which are consistent with previous reports [13-17]. The total antioxidant capacity was also increased in diabetic hearts as well. Furthermore, our in vitro study provides direct evidence that high glucose induced mitochondrial superoxide generation in cultured cardiomyocytes. Thus, the elevation of oxidative stress levels and oxidative damage may be caused by increased ROS generation rather than decreased antioxidant capacity. Whereas we used cpYFP as a probe to measure mitochondrial superoxide flashes in cardiomyocytes, we are aware there has been a controversy concerning the use of the cpYFP as a superoxide probe [33, 34]. To validate mitochondrial superoxide generation in cardiomyocytes, we also assessed mitochondrial superoxide production by using the MitoSOX^{TM} Red mitochondrial superoxide indicator. In addition, DHE was employed to measure oxidant levels in cardiomyocytes. Both MitoSOX^{TM} Red and DHE staining showed an increase in mitochondrial oxidant levels during high glucose stimulation, which was inhibited by mitochondrial-targeted antioxidant mito-TEMPO, similar to the changes in mitochondrial flashes.

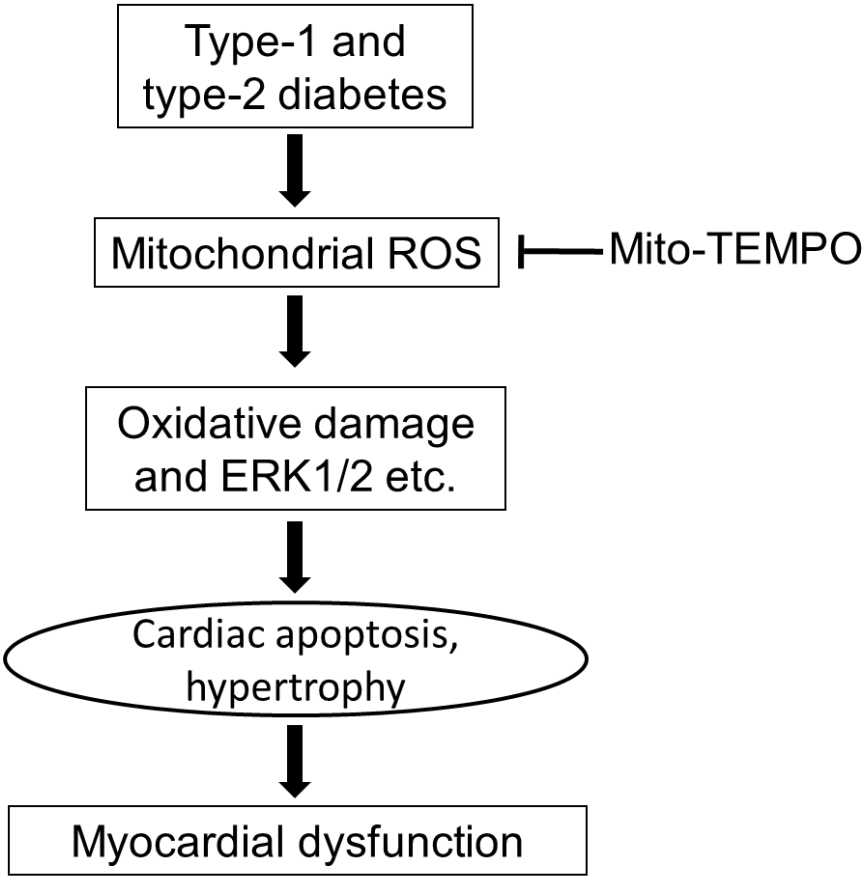

Figure 4-9. Diagrammatic illustration of the proposed mechanisms of mito-TEMPO protection in type-1 and type-2 diabetic cardiomyopathy

Diabetes induces mitochondrial ROS generation. ROS-induced oxidative damage and ROS-mediated activation of signaling contribute to cardiac apoptosis, hypertrophy and dysfunction.

It is well known that excessive mitochondrial ROS causes mitochondrial dysfunction in cardiomyocytes [35], compromising ATP production and inducing cell death [36], both of which directly contribute to myocardial dysfunction [37]. In fact, ATP product is reduced and apoptosis is induced in diabetic hearts [38, 39]. Mitochondrial ROS over-production also promotes adverse myocardial hypertrophy, an important cellular hallmark of diabetic cardiomyopathy [40, 41]. Thus, targeted inhibition of mitochondrial ROS by transgenic over-expression of SOD2 and mitochondrial catalase prevents cardiac adverse changes and dysfunction in a mouse model of type-1 diabetes and in insulin-resistant and obese Ay mice, respectively [18, 19]. The present study extends these previous findings to investigate the therapeutic potentials of targeted inhibition of mitochondrial ROS in both type-1 and type-2 diabetic mice. We show that incubation with mito-TEMPO efficiently inhibited mitochondrial superoxide generation in high glucose-stimulated cardiomyocytes and treatment of mito-TEMPO after diabetes onset abolished diabetes-induced mitochondrial ROS production and oxidative damage in hearts. Consequently, administration of mito-TEMPO prevented hypertrophy and attenuated myocardial dysfunction in both type-1 and type-2 diabetic mice. Thus, antioxidant strategies specifically targeting mitochondria may have therapeutic benefit in diabetic cardiac complications.

Gp91^{phox}-containing NADPH oxidase is another important source of ROS in cardiomyocytes [42]. It consists of cytosolic subunits (p67^{phox}, p47^{phox}, p40^{phox} and Rac1) and membrane subunits (gp91^{phox} and p22^{phox}). We have recently demonstrated that gp91^{phox} containing NADPH oxidase significantly contributes to diabetic cardiomyopathy [11, 31]. The present study shows that gp91^{phox} and p47^{phox} were up-regulated in STZ-induced mouse hearts, which is consistent with our recent report [11]. In contrast, NADPH oxidase expression was not altered in type-2 diabetic db/db mice, suggesting a differential expression of NADPH oxidase in type-1 and type-2 diabetes. Inhibition of mitochondrial ROS by mito-TEMPO prevented up-regulation of NADPH oxidase expression in type-1 diabetic hearts. This suggests that mitochondrial ROS signaling may promote NADPH oxidase expression in type-1 diabetic hearts. In fact, this has been recognized that mitochondrial ROS positively regulates NADPH oxidase subunits expression and activation under pathological conditions including diabetes [43]. On the

other hand, we have recently reported that deletion of Rac1 or pharmacological inhibition of NADPH oxidase activation reduces mitochondrial ROS generation in diabetic hearts, suggesting that NADPH oxidase promotes mitochondrial ROS generation [31]. Thus, it is most likely that cross-talks between mitochondria and NADPH oxidase form a positive feedback loop in favor of ROS production and oxidative damage in type-1 diabetic hearts, and disruption of this feedback loop by inhibiting either of them provides beneficial effects in diabetes. This is indeed supported by our findings that inhibition of either mitochondrial superoxide or $gp91^{phox}$-containing NADPH oxidase prevents mitochondrial superoxide production and attenuates apoptosis in high glucose-stimulated cardiomyocytes.

Apoptosis has been implicated in the pathogenesis of diabetic cardiomyopathy [4, 39, 44]. Cardiomyocyte apoptosis has been reported to occur in diabetic animal models and patients [44]. Apoptotic cell death in the heart causes a loss of contractile tissue which initiates cardiac remodeling. The loss of cardiomyocytes and hypertrophy of the remaining viable cardiomyocytes characterize the diabetic cardiomyopathy. As such, inhibition of cardiomyocyte apoptosis has been shown to prevent the development of diabetic heart diseases [45]. In the present study, administration of mito-TEMPO prevented caspase-3 activation in type-1 and type-2 diabetic mouse hearts. This effect of mito-TEMPO was associated with up-regulation of BCL-2 protein in diabetes. In cultured cardiomyocytes, we provide direct evidence in support of our hypothesis that inhibition of mitochondrial superoxide generation protects cardiomyocytes under diabetic conditions. Thus, inhibition of apoptotic cell death may be one of important mechanisms by which mito-TEMPO reduces diabetic cardiomyopathy.

ROS production and subsequent oxidative damage have been demonstrated to activate a variety of signaling pathways, among which ERK1/2, p38 and JNK1/2 have been

implicated in apoptosis and hypertrophy in the heart [46, 47]. Studies have shown that ERK1/2 is activated in diabetic hearts whereas p38 and JNK1/2 activities are either increased or decreased in diabetes [48, 49]. Activation of ERK1/2 and p38 contributes to apoptosis and hypertrophy in cardiomyocytes under diabetic conditions [50, 51]. The present study shows that the levels of phosphorylated ERK1/2 were increased in type-1 and type-2 diabetic hearts; however, phosphorylation of p38 and JNK1/2 remained unaltered. Importantly, administration of mito-TEMPO prevented ERK1/2 activation in diabetic hearts and cardiomyocytes under diabetic conditions, and inhibition of ERK1/2 prevented cell death in high glucose-stimulated cardiomyocytes. Thus, blocking ERK1/2 signaling may represent a potential mechanism underlying the cardiac protection of mito-TEMPO in diabetes.

Although mito-TEMPO is a mitochondria-targeted antioxidant with superoxide and alkyl radical scavenging properties [20], it is currently unknown whether there are any off-target effects of mito-TEMPO when it is accumulated in mitochondria, and it is also unclear how much of mito-TEMPO has actually gone to the heart. The present study shows that administration of mito-TEMPO did not display any effects on blood glucose, body weight, activity and dietary ingestion in both sham and diabetic mice, suggesting no significant side-effects of this reagent. In addition, systemic administration of mito-TEMPO may provide protective effects on other organs in diabetes, which may benefit the heart.

4.5 Conclusions

Administration of mitochondria-targeted antioxidants may be an effective therapy for diabetic cardiac complications.

4.6 References

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Chapter 5

5 General discussion, limitations and future directions

5.1 General Discussion

Calpains have been implicated in a variety of cardiac diseases and may represent an important target for therapy [1]. However, the mechanisms by which calpains play a role in the pathogenesis of cardiac diseases remain incompletely understood. The major findings of this thesis are that calpain-1 is increased in mitochondria of the hearts under septic (Chapter 2) and diabetic conditions (Chapter 3), and that increased mitochondrial calpain-1 correlates with mitochondrial ROS generation, myocardial pathological changes and myocardial dysfunction. Targeted inhibition of mitochondrial ROS prevents apoptosis in cardiomyocytes and reduces cardiomyopathic changes in both type-1 and type-2 diabetes (Chapter 4). Importantly, we demonstrate for the first time that mitochondrial calpain-1 targets and cleaves ATP5A1 subunit, leading to a reduction in ATP synthase activity, and that disruption of ATP synthase promotes mitochondrial ROS generation in cardiomyocytes and hearts under septic and diabetic conditions. These findings reveal a novel role of calpain-1 in septic and diabetic cardiomyopathy, identify ATP5A1 as a new target of calpain-1 in mitochondria and provide a new mechanism underlying calpain-mediated cardiac diseases, specifically mitochondrial ROS generation. Thus, this study significantly increases our understanding of the role of calpain in cardiovascular diseases.

Calpains have been considered as a cytoplasmic enzyme [2, 3]. However, it has been shown that calpains are trans-located from the cytosol to sarcolemma following myocardial ischemia/reperfusion (I/R) [4]. Translocation of calpain-2 to nuclei of cardiomyocytes was also observed in tail-suspended rats [5]. Some isoforms including calpain-1, calpain-2 and calpain-10 have been demonstrated to be also present in mitochondria [6-15]. An important finding of this thesis is that both septic and diabetic conditions significantly increase the protein levels and activities of calpain-1 in

mitochondria of mouse hearts, whereas the protein levels of calpain-2 are slightly elevated in both conditions. In contrast, the protein levels of calpain-10 are not changed in septic and diabetic hearts. Our present data further show that the majority of mitochondrial calpain-1 is located in mitochondrial matrix as well as the intermembrane space of septic hearts. An early study reported that I/R condition increases calpain-1 in mitochondrial intermembrane space in cardiomyocytes [16]. More recent studies from two independent laboratories demonstrated that both calpain-1 and calpain-2 are increased in mitochondrial matrix from heart tissues following I/R [17, 18]. Thus, increased calpains in mitochondria may be a common phenomenon in the heart under stress. However, it is currently unknown how calpains are elevated in mitochondria of the heart under stress. Our data indicate that increased calpains in mitochondria may be due to translocation from the cytosol because (1) the protein levels of total intercellular calpains are comparable between normal and diseased hearts (including sepsis and diabetes), and (2) inhibition of calpain activity prevents the accumulation of calpain-1 in mitochondria of septic hearts. The latter also suggests that calpain activation is necessary for its translocation into mitochondria. Further evidence in support of the mitochondrial translocation is that the N-terminus of calpain-1 contains a mitochondrial targeting sequence [19]. Nevertheless, it is still unclear how calpains relocate to mitochondria after activation in diseased hearts. A recent study revealed that Ca^{2+} overload induced translocation of calpain to sarcolemma through reverse mode of Na^{+}/Ca^{2+} exchanger in the heart during ischemia. They showed that inhibition of Na^{+}/Ca^{2+} exchanger markedly attenuated calpain-1 translocation, suggesting that Na^{+}/Ca^{2+} exchanger may be important for calpain translocation [20]. Interestingly, similar Na^{+}/Ca^{2+} exchanger exists on mitochondrial membrane [21]. It is possible that Na^{+}/Ca^{2+} exchanger may mediate relocation of calpains from the cytosol to mitochondria in cardiac disease, which merits future investigation.

Increased calpains in mitochondria was reported to be associated with cardiac apoptosis. Following I/R, mitochondrial calpain-1 increased in the intermembrane space and induced cleavage of apoptosis inducible factor (AIF), leading to apoptosis in cardiomyocytes [16]. Our data demonstrate for the first time that calpain-1 increases in mitochondrial matrix and cleaves ATP5A1, leading to disruption of ATP synthase in both septic and diabetic hearts. Disruption of ATP synthase results in a reduction in ATP production, which directly contributes to myocardial dysfunction. Furthermore, compromised ATP synthase within mitochondrial complex V results in "back-up" of electrons and thus promotes electron leak from the individual complexes on the respiratory chain, especially complexes I and III, leading to mitochondrial superoxide generation. In fact, inhibition of calpain or restoration of ATP synthase by up-regulation of ATP5A1 prevents mitochondrial ROS generation in cardiomyocytes and hearts under septic and diabetic conditions. Furthermore, selective up-regulation of calpain-1 in mitochondria sufficiently induces mitochondrial ROS generation in cardiomyocytes. Thus, our data provide a novel mechanism that mitochondrial calpain-1 induces mitochondrial superoxide generation by disruption of ATP synthase through proteolysis of ATP5A1 in cardiomyocytes under septic and diabetic conditions. Increased calpains in mitochondrial matrix were also reported to cleave and compromise complex 1 activity in ischemic hearts, leading to defect in ATP production, which directly contributes to myocardial dysfunction and may also promote superoxide generation [17]. It is important to mention that mitochondrial complex-Ⅰ activity was also compromised in diabetic and septic hearts [22, 23]. Thus, it is likely that increased calpains in mitochondrial matrix may disrupt the respiratory chain by targeting both ATP synthase and complex-Ⅰ, and may represent a new mechanism underlying calpain-mediated mitochondrial ROS generation in cardiac diseases. However, it remains to be determined whether calpains have any other targets in mitochondrial matrix in diseased hearts. Taken together,

calpains in mitochondrial intermembrane space cleaves AIF, leading to apoptosis and calpains in mitochondrial matrix disrupts energetic metabolism and promotes mitochondrial ROS generation in diseased hearts, which contribute to myocardial dysfunction and pathological changes as discussed below.

It is well-known that excessive mitochondrial ROS have been implicated in cardiac pathophysiological processes [24]. First, mitochondrial ROS impairs mitochondria which may induce more ROS generation in mitochondria and further mitochondrial dysfunction, causing a vicious circle [25]. Second, mitochondrial ROS also promotes cell death in the heart [26]. Third, mitochondrial ROS act as a “second messenger” in cellular signaling pathways, leading to pro-inflammatory response and hypertrophy in cardiomyocytes [27]. Thus, selective inhibition of mitochondrial ROS has been protective in a variety of cardiac diseases. For example, pressure-overload induced heart failure was significantly ameliorated by treatment of mitochondrial-targeted antioxidant peptide, Szeto-Schiller (SS) 31 in a mouse model of transverse aortic constriction [28]. Transgenic over-expression of mitochondrial catalase and superoxide dismutase-2 (SOD2) attenuated cardiac hypertrophy, protected cardiac structures and improved myocardial function in angiotension-II induced hearts and type-1 diabetic mice [26, 29, 30]. In this work, we extend these previous findings to investigate the therapeutic effects of mitochondria-targeted antioxidant mito-TEMPO on diabetic cardiomyopathy in both type-1 and type-2 diabetic mice. We show that injection of mito-TEMPO efficiently inhibits mitochondrial superoxide generation, reduces hypertrophy and attenuates cardiac dysfunction in type-1 and type-2 diabetic mice. Given the evidence that mitochondrial calpain-1 induces mitochondrial ROS generation in diseased hearts, excessive mitochondrial ROS production may be an important mechanism by which calpain-1 mediates cardiac diseases (Figure 5-1).

In summary, this thesis study has provided evidence in support that accumulation of calpain-1 in mitochondria disrupts ATP synthase through the proteolysis of ATP5A1, leading to a reduction in ATP production and promoting mitochondrial ROS generation. Mitochondrial ROS not only induces apoptosis, hypertrophy and pro-inflammatory response, but also promotes mitochondrial dysfunction, leading to ATP depletion, all of which directly contribute to septic cardiomyopathy and diabetic cardiomyopathy. Considering that calpains are also increased in ischemic heart diseases, where mitochondrial ROS production are increased, it is most likely that increased calpains in mitochondria and subsequent mitochondrial ROS generation may be a common mechanism contributing to cardiac diseases (Figure 5-1). Thus, targeted inhibition of mitochondrial calpains may represent a novel therapeutic approach for cardiac diseases.

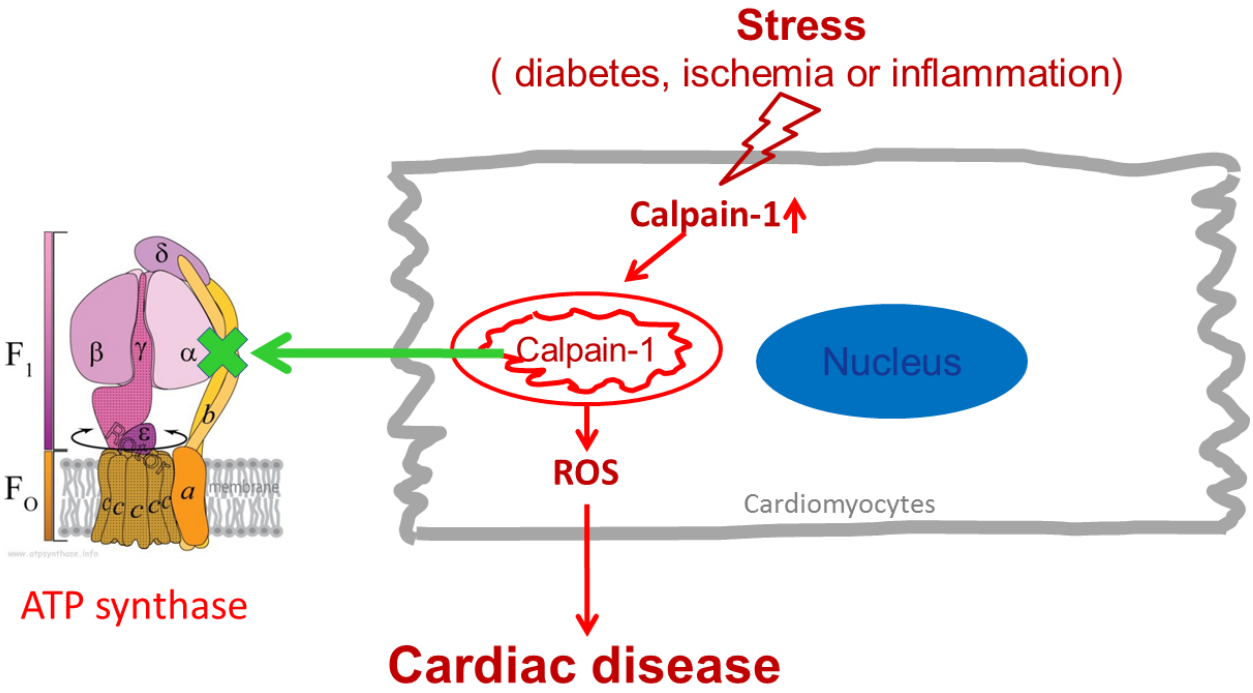

Figure 5-1. Illustration of the proposed mechanism for increased mitochondrial calpain 1-induced ROS generation in cardiac disease

The proposed mechanism in present study is summarized: increased mitochondrial calpain 1 disrupts ATP synthase via cleavage of ATP5A1 leading to excessive mitochondrial ROS generation, which contributes to the development of cardiac disease.

5.2 Limitations

This study is mainly focused on the role of mitochondrial calpain-1; however, mitochondrial calpain-2 may also play a role in septic and diabetic cardiomyopathy as increased calpain-2 in mitochondria was reported to compromise complex-Ⅰ activity in ischemic hearts [18]. Although our data indicate that increased mitochondrial calpain-1 is closely associated with mitochondrial ROS generation in septic and diabetic hearts, direct evidence from *in vivo* hearts is lacking. It is also unknown whether targeted inhibition of mitochondrial calpains provides beneficial effects in diseased hearts.

As for the substrates of calpains in mitochondrial matrix, other proteins may be also targeted by calpain-1 and calpain-2 in diseased hearts. This thesis study did not examine the role of increased calpains in mitochondrial intermembrane space whereas they may play a role in cardiac diseases.

So far, all data regarding the role of mitochondrial calpains in cardiac diseases were obtained from cultured cardiomyocytes and animal models. Studies are needed to determine whether calpains are also increased in mitochondria of human diseased hearts. If yes, whether increased calpains in mitochondria are associated with compromised ATP synthase and complex-Ⅰ activities, as well as mitochondrial ROS generation in human diseased hearts.

5.3 Future directions

Future directions should include the following items:

First, to provide direct *in vivo* evidence to support the role of increased mitochondrial calpains in mitochondrial ROS generation and myocardial injury, we are investigating whether forced up-regulation of calpain-1 and calpain-2 restricted to cardiomyocyte

mitochondria induces mitochondrial ROS generation, ATP synthase activity, complex-Ⅰ activity, cardiac structural and morphological changes, and myocardial function by generating transgenic mice with cardiac-specific and mitochondrial-targeted up-regulation of calpain-1 and calpain-2.

Second, we will generate transgenic mice with cardiac-specific and mitochondrial-targeted over-expression of calpastatin, an endogenous inhibitor of calpain-1 and calpain-2, and investigate whether selective inhibition of mitochondrial calpain-1 and calpain-2 prevents mitochondrial ROS generation and reduces myocardial injury under pathological conditions including ischemia/reperfusion, diabetes and sepsis.

Third, we will investigate additional targets of calpains in mitochondria. To do this, we will use proteomic approaches (including 2D gel and MASS) to identify potential substrates of calpains in mitochondria of diseased hearts.

Finally, it is important to determine whether calpain-1 and calpain-2 in mitochondrial are increased in human diseased hearts and whether these changes correlate with cleavages of calpain targets in mitochondria. We will plan to compare the protein levels of calpains in mitochondria between human diseased hearts (e.g. transplanted diseased hearts) and healthy hearts (brain trauma).

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Appendices

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Author: Rui Ni,Dong Zheng,Qiang Wang,Yong Yu,Ruizhen Chen,Tao Sun,Wang Wang,Guo-Chang Fan,Peter A. Greer,Richard B. Gardiner,Tianqing Peng

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Appendix III. Copyright of Chapter 4.

Curriculum Vitae

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- **Ni R**, Zheng D, Xiong S, Hill DJ, Sun T, Gardiner RB, Fan GC, Lu Y, Abel ED, Greer PA, and Peng T. (2016) Mitochondrial Calpain-1 Disrupts ATP Synthase and Induces Superoxide Generation in Type 1 Diabetic Hearts: A Novel Mechanism Contributing to Diabetic Cardiomyopathy, Diabetes 65, 255-268.

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