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

Running title: Mitochondrial calpain-L1 in diabetic cardiomyopathy

Rui Ni\textsuperscript{1,2,3,4}; Dong Zheng\textsuperscript{1,2,3,4}; Sidong Xiong\textsuperscript{1}; David J Hill\textsuperscript{2,3}; Tao Sun\textsuperscript{2,3}; Richard B Gardiner\textsuperscript{5}; Guo-Chang Fan\textsuperscript{6}; Yanrong Lu\textsuperscript{7}; E. Dale Abel\textsuperscript{8}; Peter A Greer\textsuperscript{9}; Tianqing Peng\textsuperscript{1,2,3,4*}

\textsuperscript{1}From Institutes of Biology and Medical Sciences, Soochow University, Suzhou, Jiangsu Province, China 215123. \textsuperscript{2}Lawson Health Research Institute, Departments of \textsuperscript{3}Medicine, \textsuperscript{4}Pathology, \textsuperscript{5}Biology, University of Western Ontario, London, Ontario, Canada N6A 4G5. \textsuperscript{6}Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, Cincinnati, OH, USA 45267-0575. \textsuperscript{7}Key Laboratory of Transplant Engineering and Immunology, Ministry of Health; Regenerative Medicine Research Center, West China Hospital, Sichuan University, Chengdu, China 610041. \textsuperscript{8}Fraternal Order of Eagles Diabetes Research Center, Division of Endocrinology and Metabolism, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, IA 52242. \textsuperscript{9}Division of Cancer Biology and Genetics, Queen's University Cancer Research Institute, Department of Pathology and Molecular Medicine, Queen's University, Kingston, Ontario, Canada K7L 3N6.

\textsuperscript{*}To whom correspondence should be addressed: Tianqing Peng MD, VRL 6\textsuperscript{th} Floor, A6L140, 800 Commissioners Road, London, Ontario, Canada N6A 4G5. Tel. (519) 685-8300 Ext. 55441. Fax (519) 685-8341. E-mail: tpeng2@uwo.ca.

Work count: 4244
Figures: 8
Tables: 0
Abstract

Our recent study reported a critical role of calpain in cardiomyopathic changes in type-1 diabetes (T1D). This study investigated how calpain regulates mitochondrial reactive oxygen species (ROS) generation in the development of diabetic cardiomyopathy. T1D was induced in transgenic mice over-expressing calpastatin, in mice with cardiomyocyte-specific capn4 deletion or their wild-type littermates by injection of streptozotocin. Calpain-1 protein and activity in mitochondria were elevated in diabetic mouse hearts. The increased mitochondrial calpain-1 was associated with an increase in mitochondrial ROS generation and oxidative damage, and a reduction in ATP synthase-α (ATP5A1) protein and ATP synthase activity. Genetic inhibition of calpain or up-regulation of ATP5A1 increased ATP5A1 and ATP synthase activity, prevented mitochondrial ROS generation and oxidative damage, and reduced cardiomyopathic changes in diabetic mice. High glucose induced ATP synthase disruption, mitochondrial superoxide generation and cell death in cardiomyocytes, all of which were prevented by over-expression of mitochondria-targeted calpastatin or ATP5A1. Moreover, up-regulation of calpain-1 specifically in mitochondria induced the cleavage of ATP5A1, superoxide generation and apoptosis in cardiomyocytes. In summary, calpain-1 accumulation in mitochondria disrupts ATP synthase and induces ROS generation, which promotes diabetic cardiomyopathy. These findings suggest a novel mechanism and may have significant implications in diabetic cardiac complications.
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 \textit{capn1} and \textit{capn2}, respectively, and a common small 28-kDa regulatory subunit encoded by \textit{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 \textit{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'/Ca²⁺ 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.

**Research Design and Methods**

**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.
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 ($\geq 15$ 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.

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) $\times$ 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.

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, $2 \times 10^9$ PFU in 100 μL, SignaGen Laboratories, MD, USA) or GFP (Ad-GFP, SignaGen Laboratories, MD, USA) were injected into mouse left ventricle.

Isolation and Culture of Adult Mouse Cardiomyocytes

Adult mouse ventricle cardiomyocytes were isolated and cultured as previously described (26).

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).

**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.

**Construction of Plasmid with Mitochondrial Targeted Capn1 Expression and Transfection in H9c2 Cells**

The full coding region of human capn1 cDNA was recovered from pCMV6-XL5 containing human capn1 (Origene, Rockville, MD, USA) and inserted into pCMV/myc/mito, which introduced the mitochondrial signal peptide (Life Technologies Inc. Burlington, Ontario, Canada). The resulting plasmid pCMV/myc/mito-capn1 expresses myc-tagged capn1 selectively in mitochondria.

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

**Calpain Activity**

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

**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).

**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).

**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.

**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™ Total Antioxidant Capacity Assay Kit (Cell Biolabs, Inc., USA).

**Immuo-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™ FV1000 confocal microscope equipped with the IX81 motorized inverted system as described (31).

**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.

**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).

**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.
Results

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 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 (Fig. 1B). These results confirm that mitochondrial ROS generation is increased in cardiomyocytes under diabetic conditions.

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 (Fig. 1C). This result provides direct evidence that inhibition of calpain by over-expressing calpastatin blunts high-glucose stimulated superoxide generation in cardiomyocytes.

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 (Figs. 2A and B) or succinate (Supplementary Figs. 1A and B). Similarly, H2O2 formation as determined by using DCF-DA (Figs. 2C and D) and Amplex Red (Figs. 2E and F),
and the protein carbonyl content (Figs. 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.

**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 (Fig. 3A). Consistently, diabetes also increased calpain activities in mitochondria of diabetic compared with sham animal hearts (Supplementary Fig. 2). 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 (Fig. 3B).

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

**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 (Figs. 4A-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 pCMV/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 (Fig. 5A). Intriguingly, mitochondria-targeted over-expression of capn1 significantly increased mitochondrial superoxide generation as determined by mitochondrial superoxide flashes (Fig. 5B) and induced apoptosis (Figs. 5C and D). These results strongly support a causal role of mitochondrial calpain-1 in superoxide generation and apoptosis in cardiomyocytes.

**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 (Fig. 5E). Interestingly, up-regulation of calpain-1 selectively in mitochondria led to a similar cleaved fragment of ATP5A1 protein in H9c2 cells (Fig. 5F). 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 (Fig. 6A). Likewise, capn1 was detected in immune-captured ATP synthase complex (Fig. 6B). 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 (Fig. 6C), which is consistent with previous reports (16; 17), whereas the protein levels of ATP synthase β subunit remain unchanged in diabetic hearts (Fig. 6C). However, the reduction in ATP5A1 protein levels was prevented by calpastatin over-

11
expression (Fig. 6D). In line with a reduction in ATP5A1 protein, ATP synthase activity was markedly decreased in mitochondria from diabetic hearts and restored in diabetic TgL-CAST mice (Fig. 6E).

In cultured cardiomyocytes, over-expression of calpastatin selectively in mitochondria by infection with Ad-mtCAST significantly increased ATP synthase activity during high glucose stimulation (Fig. 6F). This result provides further evidence to support that calpain activation disrupts ATP synthase activity in diabetic hearts.

**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 (Supplementary Fig. 3). As a result, delivery of Ad-ATP5A1 significantly increased ATP5A1 protein and ATP synthase activity in diabetic mouse hearts (Figs. 7A and B), suggesting that ectopic expression of ATP5A1 integrates into the complex of ATP synthase. Up-regulation of ATP5A1 reduced the formation of H2O2 (Figs. 7C and D), and attenuated cardiac hypertrophy as evidenced by decreased cardiomyocyte sectional area (Fig. 7E) and down-regulation of ANP and β-MHC expression in diabetic mouse hearts (Figs. 7F and G), leading to an improvement of myocardial function in diabetic mice as determined by increased fractional shortening and E/A ratio (Figs. 7H and I, Supplementary Table-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 (Fig. 8A), reduced mitochondrial superoxide generation (Fig. 8B) and prevented cell death induced by high glucose (Figs. 8C and D).
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 $\text{H}_2\text{O}_2$ spontaneously, or by SOD2 in mitochondria. $\text{H}_2\text{O}_2$ 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 $\alpha$-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 ($\alpha$, $\beta$, $\gamma$, $\delta$, and $\varepsilon$) 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.
Acknowledgments

We thank Dr. Wang Wang from the University of Washington for providing Ad-mt-cpYFP and technical support for measurement of mitochondrial superoxide flashes in cardiomyocytes.

This study was supported by grants from the Canadian Institutes of Health Research (MOP-133657), the National Natural Science Foundation of China (81470499) and in part by Western Department of Medicine Program of Experimental Medicine (POEM) Research Award. The research in Dr. Guo-Chang Fan’s lab is supported by NIH R01 grant [grant number HL-087861]. T.P. is a recipient of a New Investigator Award from the Canadian Institutes of Health Research. T.P. is the guarantor for this manuscript.

No conflicts exist.

R.N. researched data. D.Z. researched data. S.X. contributed to discussion and reviewed/edited manuscript. D.J.H. contributed to discussion and reviewed/edited manuscript. T.S. researched data. R.B.G. researched data. G.F. reviewed/edited the manuscript. Y.L. researched data. E.D.A. contributed to the experimental design and reviewed/edited manuscript. P.A.G. contributed to materials and discussion. T.P. designed the study, analyzed data and wrote the manuscript.
References
21. Bugger H, Boudina S, Hu XX, Tuinei J, Zaha VG, Theobald HA, Yun UJ, McQueen AP, Wayment B, Litwin SE, Abel ED: Type 1 diabetic akita mouse hearts are insulin sensitive but manifest structurally abnormal mitochondria that remain coupled despite increased uncoupling protein 3. Diabetes 2008;57:2924-2932
**Figure Legends**

**Figure 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.

**Figure 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. 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 4. 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.

**Figure 5. Effects of mitochondrial targeted capn1 on ATP5A1 protein, superoxide generation and apoptosis in H9c2 cells.** H9c2 cells were transfected with pCMV/myc/mito-capn1 (mt-Capn1) or
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.

Figure 6. 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.

Figure 7. 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.

Figure 8. 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.
Fig. 3

A

Capn1 / VDAC1 (Fold change)

Sham 1 week 1 month 2 months

* *

B

Plain VDAC1 capn1 Merged

Sham STZ

C

Capn1 / VDAC1 protein (Ratio)

db+/- db/db

*
Fig. 4

A

<table>
<thead>
<tr>
<th>Mitochondria</th>
<th>Cytosol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad-gal</td>
<td>Ad-mtCAST</td>
</tr>
<tr>
<td>mtCast →</td>
<td>Endogenous Cast</td>
</tr>
<tr>
<td>Cast</td>
<td>GAPDH</td>
</tr>
<tr>
<td>VDAC1</td>
<td></td>
</tr>
</tbody>
</table>

B

![Bar chart showing superoxide flash times](image)

C

![Images showing Ad-gal and Ad-mtCAST](image)

D

![Bar chart showing cell death](image)
Fig. 5

A

Mitochondria
Control | mt-Capn1
---|---
mt-Capn1 | Capn1
GAPDH
VDAC

B

Superoxide flashes (/1000 um²/100 secs)

Control | mt-Capn1
---|---

C

Caspase-3 activity (Fold changes)

Control | mt-Capn1
---|---

D

DNA fragmentation (Fold changes)

Control | mt-Capn1
---|---

E

ATP5A1
Cleaved ATP5A1

Control | Calpain-1
---|---

F

ATP5A1
Cleaved ATP5A1

Control | mt-Capn1
---|---

~55kD
~38kD
**Fig. 6**

**A**

![Western blot images showing ATP5A1, capn1, and IgG.]

**B**

![Western blot images showing capn1 and ATP5A1.]

**C**

![Western blot images showing ATP synthase-β and VDAC1 under Sham and STZ conditions.]

**D**

![Bar graph showing ATP synthase activity (Fold of changes) for WT+STZ and Tg-CAST+STZ.]

**E**

![Bar graph showing ATP synthase activity (Fold of changes) for WT and Tg-CAST under Sham and STZ conditions.]

**F**

![Bar graph showing ATP synthase activity (Fold of changes) for Ad-gal and Ad-mtCAST under NG and HG conditions.]

**Diabetes**
**Supplementary Fig. 1. 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.
Supplementary Fig. 2. 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.
Supplementary Fig. 3. 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.
Supplementary table 1. General information in mice after receiving Ad-GFP or Ad-ATP5A1.

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

(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)