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Mitochondrial Calpain-1 Disrupts ATP Synthase and Induces Superoxide Generation in Type 1 Diabetic Hearts: A Novel Mechanism Contributing to Diabetic Cardiomyopathy

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Calpain plays a critical role 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 overexpressing calpastatin, in mice with cardiomyocyte-specific *capn4* deletion, or in 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 upregulation of ATP5A1 increased ATP5A1 and ATP synthase activity, prevented mitochondrial ROS generation and oxidative damage, and reduced cardiomyopathic changes in diabetic mice. High glucose concentration induced ATP synthase disruption, mitochondrial superoxide generation, and cell death in cardiomyocytes, all of which were prevented by overexpression of mitochondria-targeted calpastatin or ATP5A1. Moreover, upregulation 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 for 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 patients with diabetes, and ~80% of all patients with diabetes 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. In the early stages, diabetic cardiomyopathy may present with diastolic dysfunction and 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

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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 reported previously that genetic inhibition of calpain by overexpression of calpastatin or deletion of *capn4* prevents cardiomyocyte apoptosis and reduces 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). Hyperhomocysteinemia has been reported to induce the translocation of active calpain-1 from cytosol to mitochondria, which is associated with intramitochondrial oxidative stress in cultured rat heart microvascular endothelial cells (10), suggesting that calpain may regulate mitochondrial reactive oxygen species (ROS) generation. This was supported by our recent study demonstrating that inhibition of calpain prevents mitochondrial ROS generation in endothelial cells upon high glucose stimulation (11). Calpains have been suggested to target some important proteins in mitochondria, including, but not limited to, ATP synthase-α (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 that 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 diabetes models (17–20). Although mitochondrial superoxide generation is not increased in the heart of some type 1 diabetic animals (21,22), selective inhibition of mitochondrial ROS reduces cardiomyopathic changes in type 1 diabetes (23,24). These studies raise an intriguing hypothesis that calpain activation leads 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 type 1 diabetes.

RESEARCH DESIGN AND METHODS

Animals

This investigation conforms to the eighth edition of the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health. All experimental procedures were approved by the Animal Use Subcommittee at the University of Western Ontario (London, ON, 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 (Sacramento, CA). Transgenic mice with overexpression of calpastatin (Tg-CAST; C57BL/6 background) were provided by Laurent Baud (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 other report (7). All 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 months old) by consecutive intraperitoneal injections 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 tail vein and random glucose levels measured using the OneTouch Ultra 2 blood glucose monitoring system (LifeScan, Inc., Milpitas, CA). Mice were considered diabetic and were used for the study only if they had hyperglycemia (≥15 mmol/L) 72 h after STZ injection. Citrate buffer–treated mice were used as a nondiabetic control (blood glucose <12 mmol/L). Two months after induction of diabetes, mice (n = 8–12 per group) were subjected to the following experiments.

Echocardiography

Animals were lightly anesthetized with inhaled isoflurane (1%) and imaged with a 40-MHz linear array transducer attached to a preclinical ultrasound system (Vevo 2100, FUJIFILM VisualSonics, Toronto, ON, Canada) with nominal inplane spatial resolution of 40 μm (axial) × 80 μm (lateral). M-mode and two-dimensional parasternal short-axis scans (133 frames/s) 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, and fractional shortening.

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

Delivery of Adenoviral Vectors Into Mice

Mice were anesthetized with inhaled isoflurane (1–3%). With the guide of echocardiography, adenoviral vectors containing human ATP5A1 gene (Ad-ATP5A1, 2×10^9 plaque-forming units in 100 μL; SignaGen Laboratories, Gaithersburg, MD) or green fluorescent protein (Ad-GFP; SignaGen Laboratories) were injected into mouse LV.

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), or β-gal (Ad-gal; Vector Biolabs, Philadelphia, PA) as a control at a multiplicity of infection of 100 plaque-forming units/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 mitochondria-targeted circularly permuted yellow fluorescent protein by using the cytochrome C oxidase subunit IV–targeting sequence. Twenty-four hours after infection, confocal images were recorded with an Olympus FV1000 laser-scanning microscope equipped with a 63× 1.3 NA oil immersion objective and a sampling rate of 0.7 s/frame. At least 20 cardiomyocytes per culture in each group were analyzed.

Construction of Plasmid With Mitochondria-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) and inserted into *p*CMV/myc/mito, which introduced the mitochondrial signal peptide (Life Technologies Inc., Burlington, ON, 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 by using the jetPRIME DNA transfection reagent (VWR International, Mississauga, ON, Canada) according to the manufacturer's instructions.

Calpain Activity

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

Real-Time RT-PCR

Total RNA was extracted from heart tissues using TRIzol reagent (Life Technologies Inc.), and real-time RT-PCR was performed to analyze mRNA expression for atrial natriuretic peptide (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 β-subunits, and GAPDH were determined by Western blot analysis using respective specific antibodies (Cell Signaling, Danvers, MA, and Santa Cruz Biotechnology, Dallas, TX).

Measurement of ROS Generation in Freshly Isolated Mitochondria

Myocellular mitochondria were isolated from freshly harvested hearts 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 peroxidase (Invitrogen) 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-dichlorodihydrofluorescein diacetate (DCF-DA) (Invitrogen) (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) according to the manufacturer's instructions. The antioxidant capacity was measured based on reduction of copper (II) to copper (I) by using an OxiSelect Total Antioxidant Capacity Assay Kit (Cell Biolabs, Inc.).

Immunofluorescence 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 FV1000 confocal microscope equipped with an IX81 motorized inverted system as described previously (31).

Co-immunoprecipitation and Native Gel Electrophoresis

Co-immunoprecipitation and nondenaturing PAGE were carried out to analyze protein-protein interactions. Briefly, calpain-1 and its interacting proteins were coprecipitated by using an immunoprecipitation kit (Dynabeads Protein G; Life Technologies, Inc.), and ATP synthase complex was isolated by using an ATP synthase immunocapture kit (Abcam, Toronto, ON, Canada) in isolated mitochondria according to the manufacturer's instructions. Both calpain-1/interacting proteins and ATP synthase complex were subjected to nondenaturing PAGE for separation followed by Western blot analysis.

ATP Synthase Activity

ATP synthase activity was measured by 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 are presented as mean ± SD. A one-way or two-way ANOVA followed by Newman-Keuls test was performed for multigroup comparisons as appropriate. For comparison of two groups, unpaired *t* test was used. $P < 0.05$ 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 H_2O_2 generation. As shown in Fig. 1*A*, H_2O_2 generation in isolated mitochondria was increased in a time-dependent manner by 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. 1*B*). 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 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 concentrations for 24 h. Overexpression of calpastatin significantly decreased mitochondrial superoxide generation induced by high glucose concentrations in Tg-CAST cardiomyocytes (Fig. 1*C*). This result provides direct evidence that inhibition of calpain by overexpressing calpastatin blunts high glucose–stimulated superoxide generation in cardiomyocytes.

We then made Tg-CAST and *capn4*-ko and their wild-type mice diabetic by injection of STZ. Sixty days after STZ injection, calpastatin overexpression and *capn4* deletion significantly reduced H_2O_2 generation in mitochondria from STZ-treated Tg-CAST and *capn4*-ko mice, respectively, after the addition of pyruvate/malate (Fig. 2*A* and *B*) or succinate (Supplementary Fig. 1*A* and *B*). Similarly, H_2O_2 formation as determined by using DCF-DA (Fig. 2*C* and *D*) and Amplex Red (Fig. 2*E* and *F*) and the protein carbonyl content (Fig. 2*G* and *H*) were increased in diabetic mouse hearts and abrogated in Tg-CAST and *capn4*-ko mice, respectively. However, total antioxidant capacity was comparable among 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 Diabetic Mouse Hearts

Having shown that inhibition of calpain prevented mitochondrial superoxide generation, we determined whether the calpain levels 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. 3*A*). Consistently, diabetes also increased calpain activities in mitochondria of diabetic versus sham animal hearts (Supplementary Fig. 2). However,

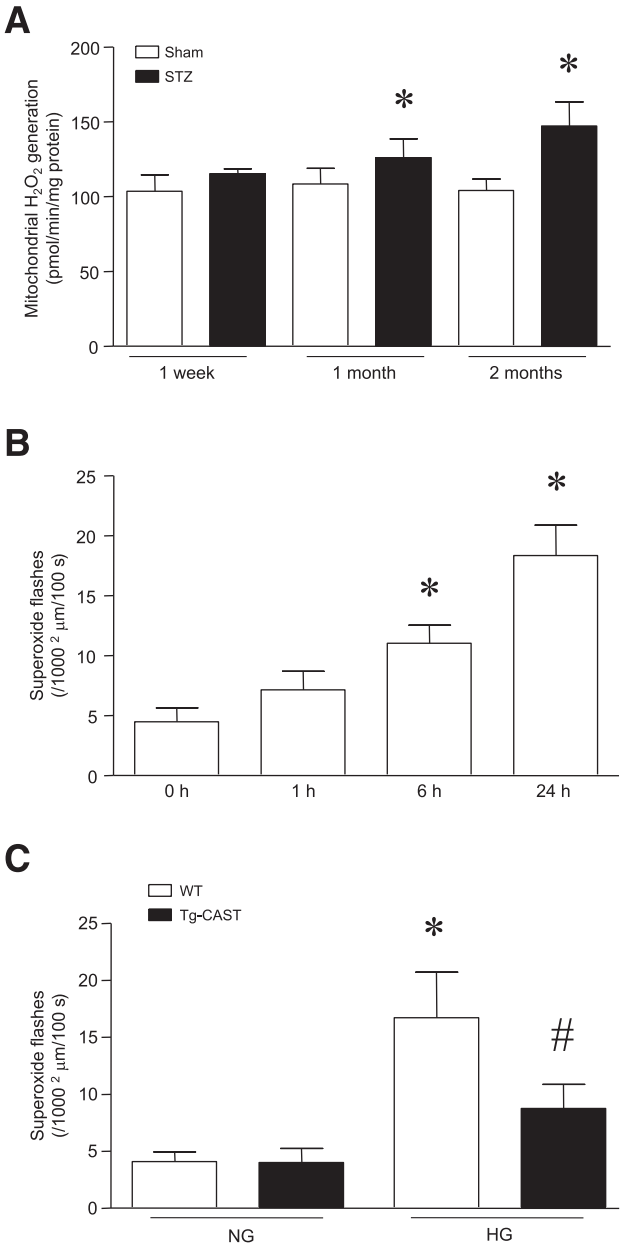

Figure 1—Determination of mitochondrial ROS generation. *A*: Adult wild-type (WT) mice were injected with STZ 50 mg/kg/day i.p. for 5 days. Mice were killed 1 week, 1 month, and 2 months after STZ injection. Mitochondria were isolated from heart tissues. Mitochondrial H_2O_2 generation was determined by using Amplex Red as an indicator after addition of pyruvate/malate. *B* and *C*: Adult cardiomyocytes were isolated and cultured for up to 24 h. *B*: Time course of mitochondrial superoxide flashes after incubation with a high glucose (HG) concentration (30 mmol/L) in WT cardiomyocytes. *C*: Twenty-four hours after incubation with HG (30 mmol/L) or normal glucose (NG) (5 mmol/L), mitochondrial superoxide flashes were analyzed in WT and Tg-CAST mice. Data are mean ± SD (n = 6 or three separate cultures). *P < 0.05 vs. sham, 0 h, or NG in WT; #P < 0.05 vs. HG in WT.

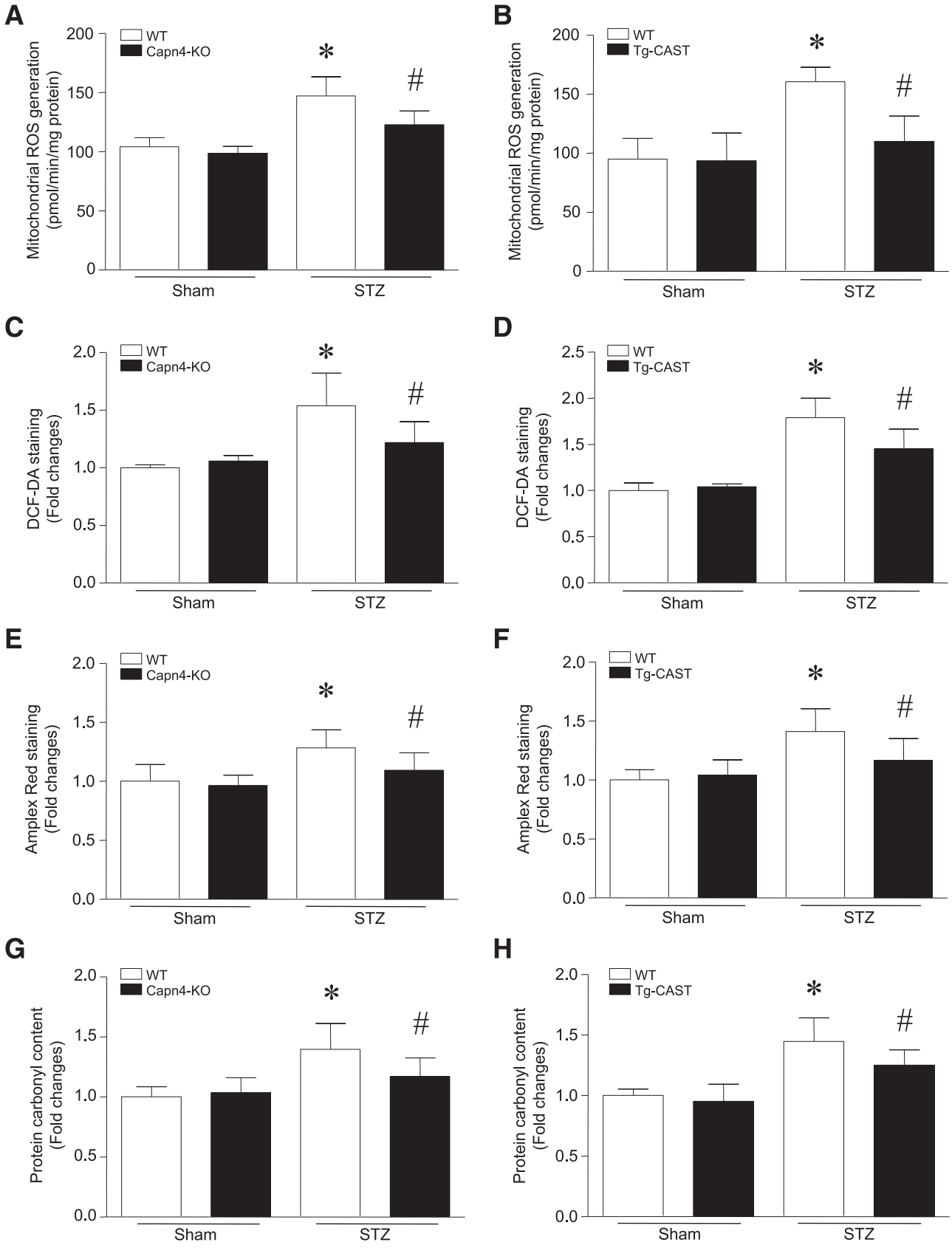

Figure 2—Assessment of mitochondrial ROS generation and oxidative stress. Wild-type (WT), Tg-CAST, or cardiomyocyte-specific *capn4*-ko mice were injected with STZ 50 mg/kg/day i.p. for 5 days. Two months after STZ injection, mitochondria were isolated from heart tissues. Mitochondrial ROS generation was measured using Amplex Red after addition of pyruvate/malate (*A* and *B*). ROS formation in heart tissue lysates was determined by using DCF-DA as an indicator (*C* and *D*) or Amplex Red (*E* and *F*). Oxidative damage was assessed

diabetes did not change the protein levels of capn2 and calpain-10, an isoform well recognized 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), that capn1 was present inside mitochondria (green), and that percentages of capn1-labeled mitochondria were much greater in diabetic versus sham mouse hearts (Fig. 3*B*). Similarly, the protein levels of capn1 were also increased in hearts of *db/db* type 2 diabetic versus $db^{+/-}$ mice (Fig. 3*C*).

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 Ad-mtCAST and incubated them under high glucose conditions for 24 h. Selective overexpression of calpastatin in mitochondria prevented mitochondrial superoxide flashes and cell death induced by high glucose concentrations (Fig. 4*A*–*D*). This result suggests that mitochondrial calpain contributes to superoxide generation and cell death induced by high glucose levels in cardiomyocytes.

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 mitochondria-targeted *capn1* into cardiomyocyte-like H9c2 cells. Twenty-four hours after transfection, mitochondrial and cytosolic fractions were isolated from H9c2 cells. Overexpressed *capn1* was confirmed in mitochondrial but not in cytosolic fractions (Fig. 5*A*). Of note, mitochondria-targeted overexpression of *capn1* significantly increased mitochondrial superoxide generation as determined by mitochondrial superoxide flashes (Fig. 5*B*) and induced apoptosis (Fig. 5*C* 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

Because 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 (~38 kD) was detected (Fig. 5*E*). Of note, upregulation of calpain-1 selectively in mitochondria led to a similar cleaved fragment of ATP5A1 protein in H9c2 cells (Fig. 5*F*). 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. 6*A*). Likewise, capn1 was detected in immune-captured ATP synthase complex (Fig. 6*B*). 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. 6*C*), which is consistent with previous reports (16,17), whereas the protein levels of ATP synthase β-subunit remained unchanged in diabetic hearts (Fig. 6*C*). However, the reduction in ATP5A1 protein levels was prevented by calpastatin overexpression (Fig. 6*D*). 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 (Fig. 6*E*).

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

Overexpression of ATP5A1 Reduces Mitochondrial Superoxide Generation, Cardiac Hypertrophy, and Myocardial Dysfunction in Diabetic Mice

To investigate whether upregulation of ATP5A1 protects diabetic hearts, we delivered Ad-ATP5A1 into mice 72 h 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 the 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 (Fig. 7*A* and *B*), suggesting that ectopic expression of ATP5A1 integrates into the complex of ATP synthase. Upregulation of ATP5A1 reduced the formation of H_2O_2 (Fig. 7*C* and *D*) and attenuated cardiac hypertrophy as evidenced by decreased cardiomyocyte sectional area (Fig. 7*E*) and downregulation of ANP and β-MHC expression in diabetic mouse hearts (Fig. 7*F* and *G*), leading to an improvement of myocardial function in diabetic mice as determined by the increased fractional shortening and E/A ratio (Fig. 7*H* and *I* and 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

by measuring protein carbonyl contents in heart tissue lysates (*G* and *H*). Data are mean ± SD ($n = 6$). *$P < 0.05$ vs. sham in WT; #$P < 0.05$ vs. STZ-treated WT.

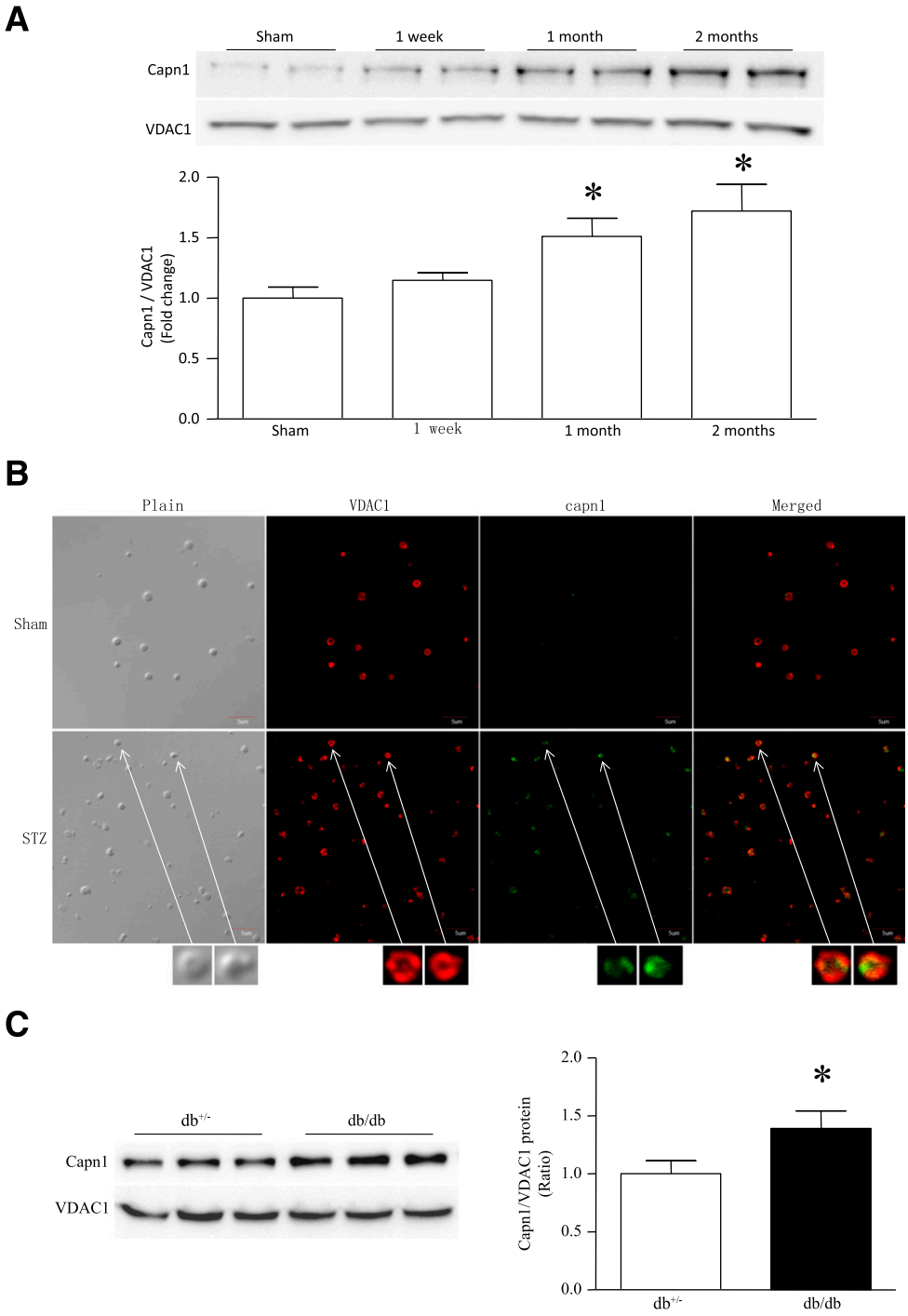

Figure 3—Measurement of calpain-1 in mitochondria. *A*: Adult wild-type mice were injected with STZ 50 mg/kg/day i.p. for 5 days. Mice were killed 1 week, 1 month, and 2 months after STZ injection. Mitochondria were isolated from heart tissues, and the protein levels of calpain-1 and VDAC1 in mitochondria were determined by Western blot analysis. The top panel is the representative Western blot for capn1 and VDAC1 from two of six hearts in each group, and the bottom 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 isolated. After fixation on slides, dual immunofluorescent staining for VDAC1 and capn1 was performed by using their respective antibodies followed by secondary antibodies conjugated with different fluorescent dyes. Representative photomicrographs of confocal microscopy for VDAC1 and capn1 in mitochondria show 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 3.5 months). The protein levels of capn1 and VDAC1 were determined by Western blot analysis. The left panel is a representative Western blot for capn1 and VDAC1 from three of six hearts in each group, and the right panel is the quantification of capn1 protein normalized to VDAC1. Data are mean ± SD from six different heart tissues in each group. *$P < 0.05$ vs. sham.

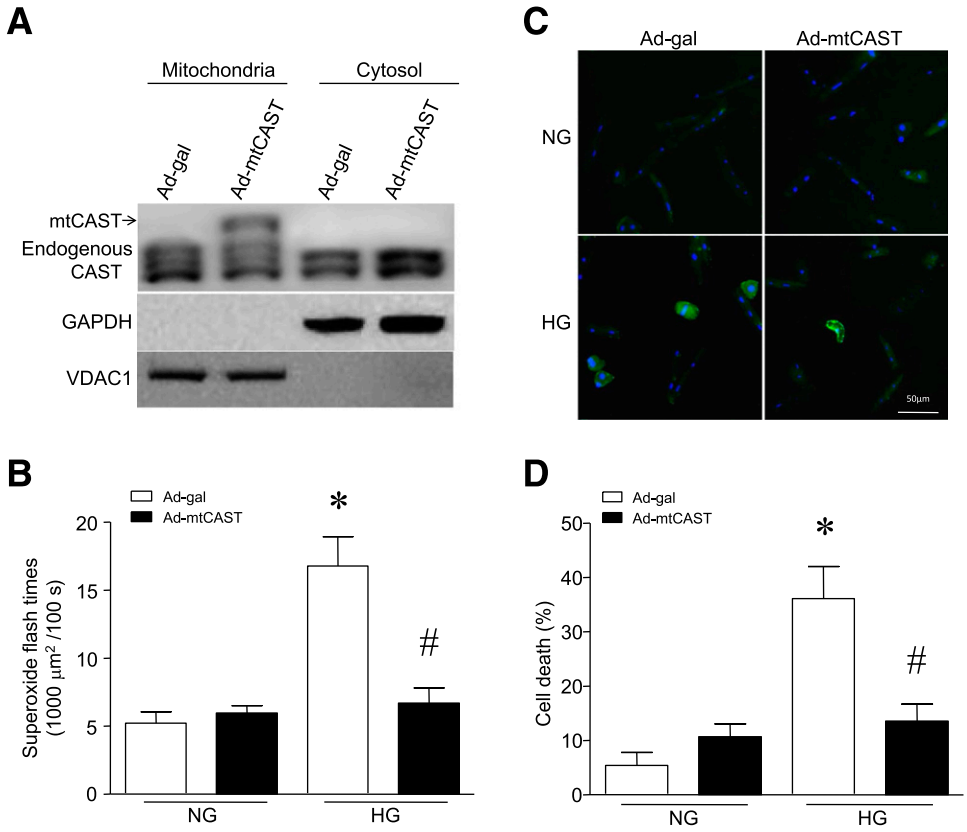

Figure 4— Effects of mitochondria-targeted calpastatin overexpression on mitochondrial superoxide flashes and cell death in high glucose–stimulated cardiomyocytes. *A*: H9c2 cells were infected with Ad-mtCAST or Ad-gal as control. Twenty-four hours later, mitochondrial and cytosolic fractions were prepared, and calpastatin (CAST), GAPDH, and VDAC1 were detected by Western blot analysis. A representative Western blot confirmed that 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 h, and mitochondrial superoxide flashes (*B*) and annexin V staining for cell death (*C* and *D*) were determined. Data are mean ± SD from six different cultures. **P* < 0.05 vs. NG + Ad-gal; #*P* < 0.05 vs. HG + Ad-gal.

or Ad-gal as a control and then incubated them under high glucose conditions for 24 h. Upregulation of ATP5A1 increased ATP synthase activity in high glucose– but not normal glucose–stimulated cardiomyocytes (Fig. 8*A*), reduced mitochondrial superoxide generation (Fig. 8*B*), and prevented cell death induced by high glucose levels (Fig. 8*C* 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 upregulation of calpain-1 specifically in mitochondria induces the cleavage of ATP5A1, superoxide generation, and apoptosis in cardiomyocytes. In a mouse model of type 1 diabetes, upregulation 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 type 1 diabetes models (23,24), supporting a critical role of mitochondrial ROS. The current study 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 signaling pathways (37). Thus, targeted inhibition of mitochondrial ROS by transgenic overexpression of superoxide dismutase 2 and

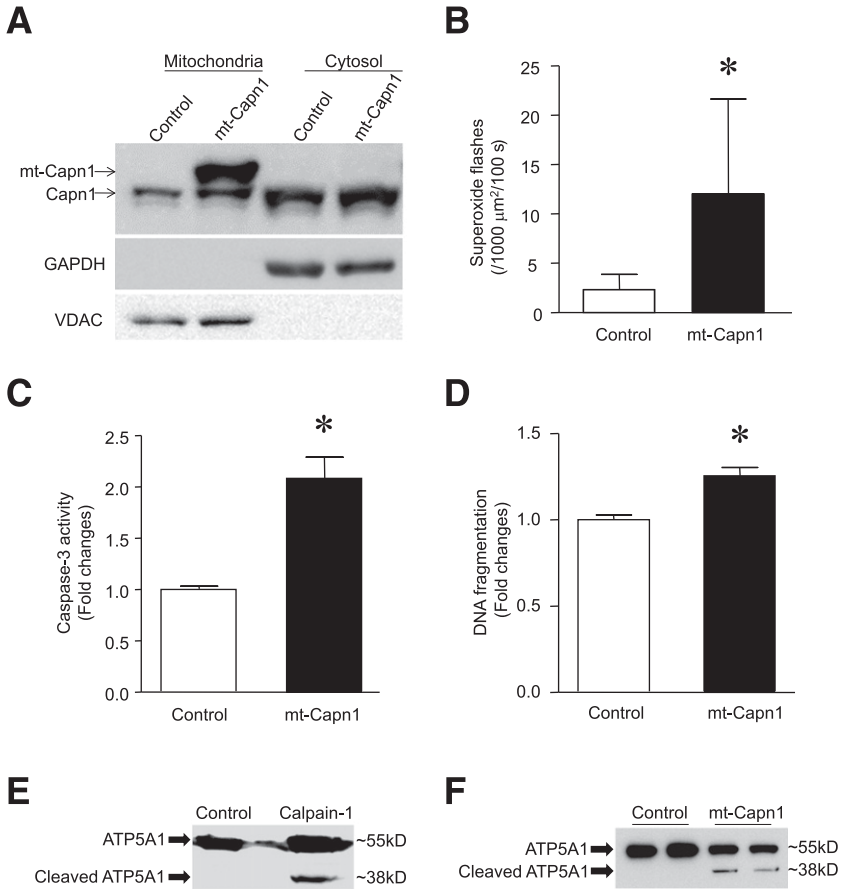

Figure 5—Effects of mitochondrial targeted capn1 on ATP5A1 protein, superoxide generation, and apoptosis in H9c2 cells. H9c2 cells were transfected with *p*CMV/myc/mito-capn1 (mt-Capn1) or *p*CMV/myc/mito as a control. *A*: Twenty-four hours later, mitochondrial and cytosolic fractions were isolated. 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 min. *F*: ATP5A1 immunoblot in H9c2 cells transfected with mt-Capn1. Data are mean ± SD from at least three different experiments. **P* < 0.05 vs. control.

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, the data suggest an important role of calpain in mitochondrial ROS generation in the development of diabetic cardiomyopathy.

It is well known that mitochondria generate superoxide, the primary ROS by-product, when single electrons leak to react with molecular oxygen (38). Although 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 is eliminated by antioxidant defense systems. Superoxide anion dismutates to H_2O_2 spontaneously or by superoxide dismutase 2 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. An increase in superoxide generation and/or a decrease in antioxidant capacity will lead to oxidative stress in mitochondria (39). In this regard, the current data suggest that calpain promotes oxidative damage through increased mitochondrial superoxide generation rather than decreased antioxidant capacity because inhibition of calpain does not affect antioxidant capacity in diabetic hearts.

Multiple mechanisms have been suggested to mediate mitochondrial ROS generation in diabetic hearts. It was reported that high glucose concentrations increased metabolic input into mitochondria, which overwhelms the respiratory chain, causing mitochondrial hyperpolarization and leading to electron backup within the respiratory chain and to ROS overproduction (38). In

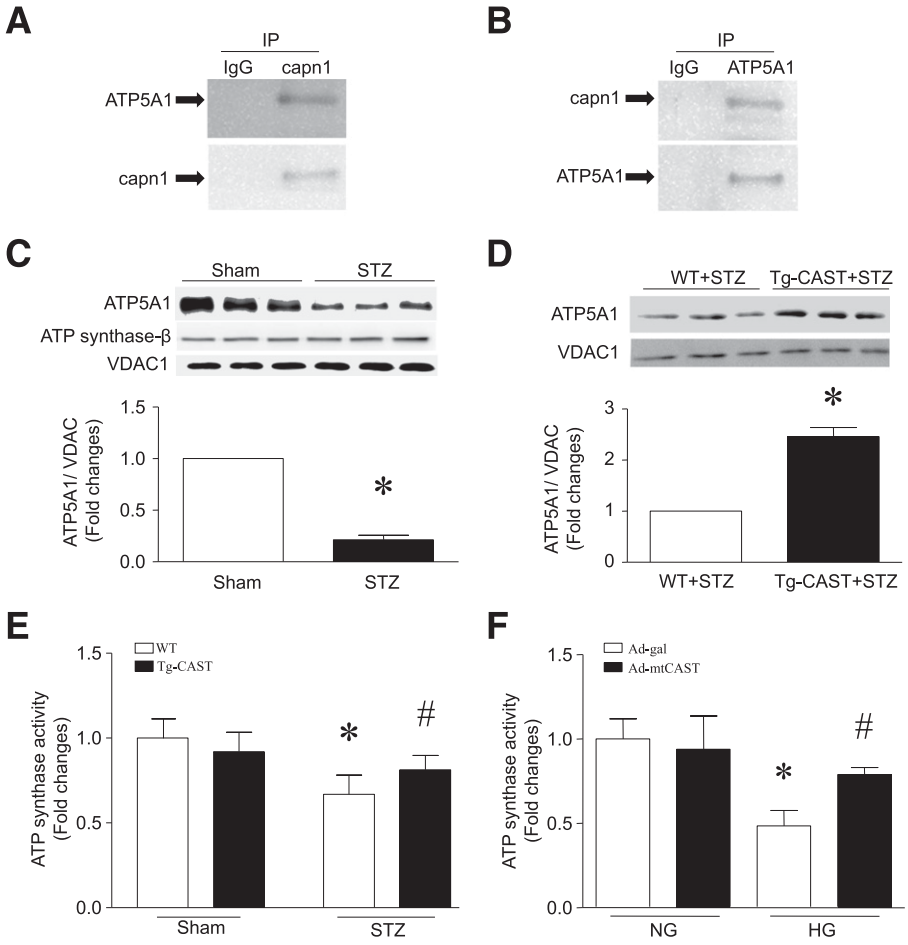

Figure 6—Role of calpain in ATP5A1 expression and ATP synthase disruption in diabetic hearts. *A*: Interaction between ATP5A1 and capn1. Capn1-interacting proteins were co-immunoprecipitated by using capn1 antibody. A representative Western blot shows that ATP5A1 is detected in capn1-interacting proteins. *B*: ATP synthase complex and its interacting proteins were captured with an 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 top panels are the representative Western blots for ATP5A1 protein from three of six hearts in each group, and the bottom panels are the quantification of ATP5A1 protein relative to VDAC1 in mitochondria. *E*: ATP synthase activity was measured in mitochondria. Data are mean ± SD ($n = 6$). *$P < 0.05$ vs. sham or STZ + WT; #$P < 0.05$ vs. STZ + WT. *F*: Adult cardiomyocytes were isolated and cultured from WT mice. After infection with Ad-mtCAST or Ad-gal, the cells were incubated with high glucose (HG) (30 mmol/L) or normal glucose (NG) (5 mmol/L) concentrations for 24 h. ATP synthase activity was determined in cell lysates. Data are mean ± SD ($n = 6$). *$P < 0.05$ vs. NG + Ad-gal; #$P < 0.05$ vs. HG + Ad-gal. IP, immunoprecipitation.

addition, elevated circulating lipid levels 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 current study, we show that diabetes increases calpain-1 in mitochondria, and calpain-1 accumulation in mitochondria correlates with ROS generation in diabetic mouse hearts. Selective inhibition of mitochondrial calpain also reduces superoxide generation in cardiomyocytes under diabetic conditions, whereas targeted overexpression 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 comprises two regions: the F_o portion and the 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 subunits (α, β, γ, δ, and ε) in the matrix of the mitochondria. Downregulation 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

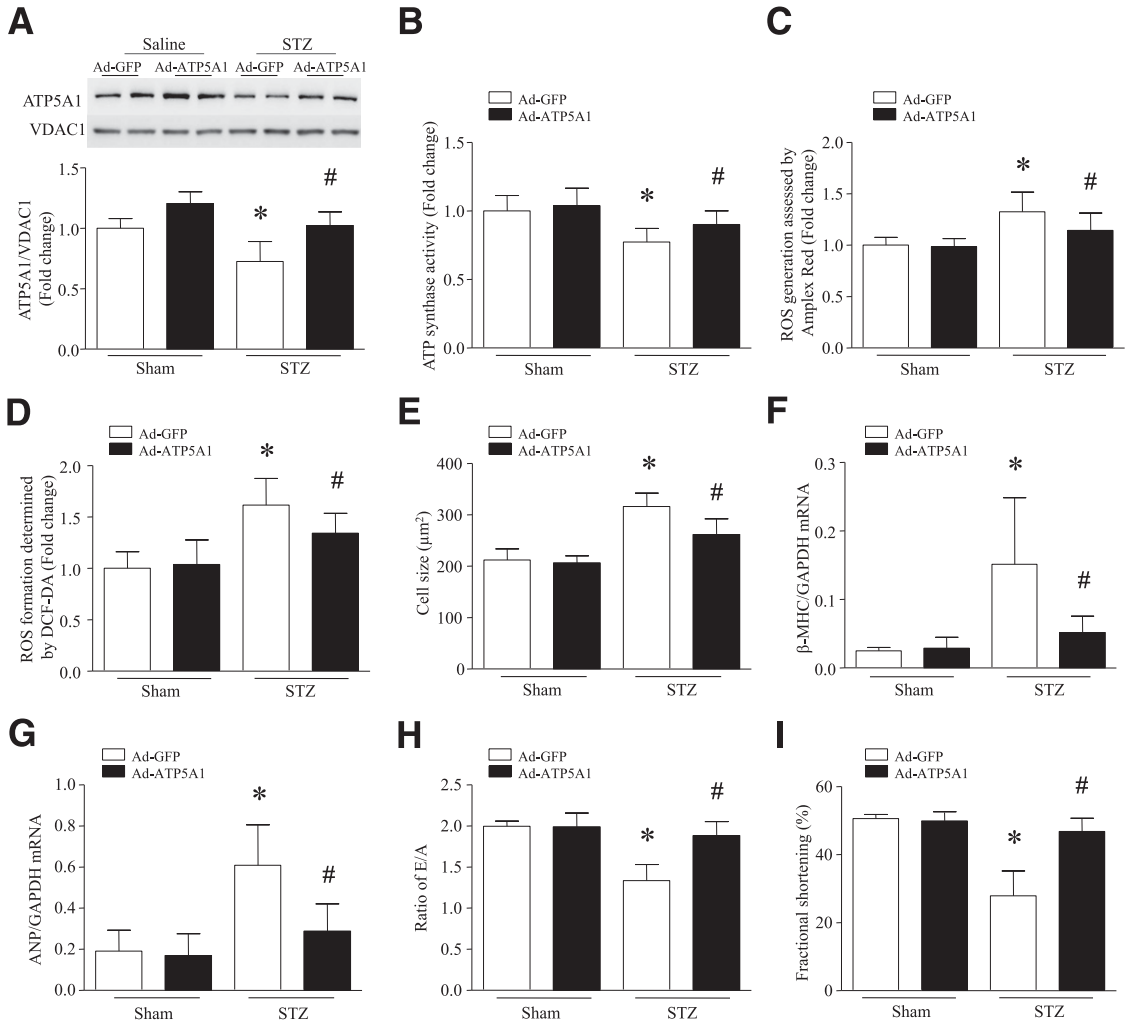

Figure 7—Effects of ATP5A1 overexpression in diabetic cardiomyopathy. Adult mice were injected with Ad-ATP5A1 or Ad-GFP and then treated with STZ. *A*: Upregulation of ATP5A1 protein was confirmed by Western blot analysis. Top panel is a representative Western blot from two of six hearts for ATP5A1 and VDAC1, and the bottom panel is the quantification of the ATP5A1/GAPDH ratio for all hearts. *B*: ATP synthase activity. *C* and *D*: H_2O_2 formation was determined in heart tissue lysates by using Amplex Red (*C*) and DCF-DA as indicators (*D*). *E*: Cardiomyocyte size in heart sections. *F*: The mRNA levels of β-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 vs. sham + Ad-GFP; #P < 0.05 vs. STZ + Ad-GFP.

its activity in mitochondria from diabetic mouse hearts. Diabetes-induced downregulation of ATP5A1 and ATP synthase activity are prevented by both calpastatin overexpression 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 upregulation 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 NCX-1 protein in calpain-1 immunoprecipitates (data not shown), 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 multiple targets of calpain-1 exist 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), particularly 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 the main mechanism 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 upregulation of ATP5A1 increases ATP synthase activity, decreases mitochondrial ROS generation, and mitigates diabetic cardiomyopathy. Taken together, we observed that calpain-1 mediates mitochondrial superoxide generation, at least

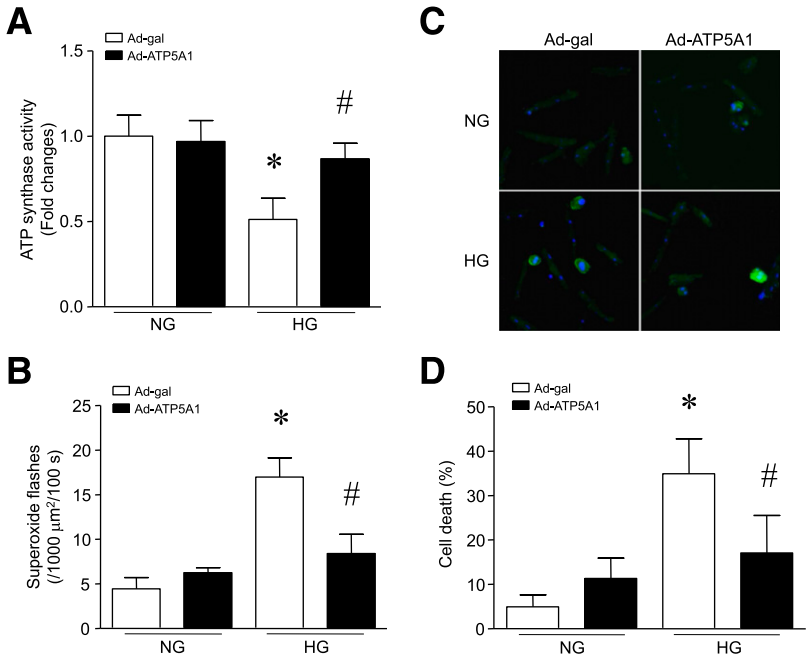

Figure 8— Role of ATP5A1 in ATP synthase activity, mitochondrial superoxide generation, and cell death in cardiomyocytes. Adult mouse cardiomyocytes were isolated from wild-type mice. After attachment to the culture dish, cells were infected with Ad-ATP5A1 or Ad-gal. Twenty-four hours later, cells were incubated with normal glucose (NG) or high glucose (HG) concentrations for 24 h. *A*: ATP synthase activity. *B*: Mitochondrial superoxide generation. *C*: Representative pictures for annexin V–positive cells as an indicator of cell death (green). *D*: Quantification of annexin V–positive cells. Data are mean ± SD from at least three different experiments. *$P < 0.05$ vs. Ad-gal + NG; #$P < 0.05$ vs. Ad-gal + HG.

partly by downregulation of ATP5A1 and disruption of ATP synthase, leading to cardiomyopathic changes in diabetic mice. Overexpression of ATP5A1 per se is not sufficient to increase ATP synthase activity, but it prevents a diabetes/hyperglycemia-induced decrease in its activity in cardiomyocytes.

In the current study, STZ was given in multiple low doses to induce type 1 diabetes 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 type 1 diabetes (44). Because 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 current study focuses on mitochondrial calpain-1 and ROS generation, other mechanisms may also be involved in calpain-mediated diabetic cardiomyopathy. In particular, calpain activation may induce the cleavages of important cytosolic proteins, including signaling molecules (protein kinase C and nuclear factor-κB) (45,46), calcium regulatory proteins (47,48), and myofibril proteins (49,50), which may contribute to myocardial dysfunction in diabetes.

In summary, this study demonstrates that mitochondrial calpain-1 stimulates mitochondrial ROS generation through downregulation 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.

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