Calpain activation contributes to hyperglycaemia-induced apoptosis in cardiomyocytes

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Aims Cardiomyocyte apoptosis contributes to cardiac complications of diabetes. The aim of this study was to investigate the role of calpain in cardiomyocyte apoptosis induced by hyperglycaemia.

Methods and results In cultured adult rat ventricular cardiomyocytes, high glucose (33 mM) increased calpain activity and induced apoptosis, concomitant with the impairment of Na^+/K^+ ATPase activity. These effects of high glucose on cardiomyocytes were abolished by various pharmacological calpain inhibitors, knockdown of calpain-1 but not calpain-2 using siRNA, or over-expression of calpastatin, a specific endogenous calpain inhibitor. The effect of calpain inhibition on cardiomyocyte apoptosis was abrogated by ouabain, a selective inhibitor of Na^+/K^+ ATPase. Furthermore, blocking gp91^{phox}-NADPH oxidase activation, L-type calcium channels, or ryanodine receptors prevented calpain activation and apoptosis in high glucose-stimulated cardiomyocytes. In a mouse model of streptozotocin-induced diabetes, administration of different calpain inhibitors blocked calpain activation, increased the Na^+/K^+ ATPase activity, and decreased apoptosis in the heart.

Conclusion Calpain-1 activation induces apoptosis through down-regulation of the Na^+/K^+ ATPase activity in high glucose-stimulated cardiomyocytes and *in vivo* hyperglycaemic hearts. High glucose-induced calpain-1 activation is mediated through the NADPH oxidase-dependent pathway and associated with activation of L-type calcium channels and ryanodine receptors. Our data suggest that calpain activation may be important in the development of diabetic cardiomyopathy and thus may represent a potential therapeutic target for diabetic heart diseases.

1. Introduction

Cardiovascular disease is one of the major complications of diabetes mellitus and has become the main cause of mortality in the diabetic population.[1] Diabetic patients have a worse outcome after myocardial infarction, with higher risk of heart failure.[2,3] Hyperglycaemia is considered the main cause of chronic diabetic complications.[4] Studies also suggest that hyperglycaemia is associated with significant higher mortality rates in patients hospitalized with acute coronary syndromes.[5] One important consequence of hyperglycaemia in the heart is the induction of cardiomyocyte apoptosis. An increase in cardiomyocyte apoptosis has been reported in diabetic animal models and patients, and causes a loss of contractile tissue that initiates cardiac remodelling.[6–10] The loss of ventricular cardiomyocytes and hypertrophy of the remaining viable cardiomyocytes characterize

the diabetic cardiomyopathy.[11,12] As such, suppression of cardiomyocyte apoptosis results in a significant prevention of the development of diabetic heart diseases.[7] However, the mechanisms by which hyperglycaemia induces apoptosis in cardiomyocytes have not been fully understood.

Calpains belong to a family of calcium-dependent thiol-proteases. Two major isoforms, calpain-1 and calpain-2, are ubiquitously expressed, whereas the remaining isoforms have more limited tissue distribution. All 15 gene products of the calpain family reported in mammals are present in the cytosol as inactive proenzymes. Calcium (Ca^{2+}) is required for calpain-1 and calpain-2 activation. Binding of Ca^{2+} to calpain-1 and calpain-2 induces the release of constraints imposed by domain interactions and results in the release of a regulatory subunit and then the rearrangement of the active site cleft in a catalytic subunit.[13] Both calpain-1 and calpain-2 are specifically countered by the endogenous inhibitor, calpastatin. Over-expression of calpastatin has been shown to inhibit calpain-1 and calpain-2 in *in vitro* and *in vivo* transgenic mice.[14,15] Calpain is involved in

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a variety of biological functions such as cell cycle, cell migration, differentiation, and apoptosis.[13] Both calpain-1 and calpain-2 are expressed in the heart.[16] Activation of calpain is implicated in ischaemia/reperfusion-induced apoptosis in the heart, and plays a role in TNF-α-mediated apoptosis in cardiomyocytes.[17–19] These studies suggest that calpain activation may contribute to the progression of heart failure. This was indeed supported by a recent study which demonstrated that cardiac over-expression of calpain-1 was sufficient to cause heart failure in transgenic mice.[16] However, the role of calpain in cardiac complications of diabetes has not been demonstrated.

In the present study, we employed an *in vitro* model of adult rat cardiomyocyte death induced by high glucose and a mouse model of streptozotocin (STZ)-induced type-1 diabetes to examine the role of calpain in cardiomyocyte apoptosis.

2. Experimental procedures

2.1 Animals and adult rat cardiomyocytes culture

This investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23). 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. Adult male rats (Sprague Dawley, 200 g body weight) were purchased from Charles River Labs. Adult rat ventricular cardiomyocytes were isolated and cultured as described in our recent study.[20]

2.2 Reagents

Streptozotocin, diphenyleneiodonium (DPI), *N*-acetylcysteine (NAC), verapamil, dantrolene, D-glucose, and mannitol were purchased from Sigma. Ouabain, PD150606, calpain inhibitor-I, and calpain inhibitor-III were from Calbiochem. Annexin-V conjugated with FITC and Hoechst 33324 were from Invitrogen. Peptides gp91ds-tat and scramble-tat were synthesized by ProImmune Ltd (UK).

2.3 STZ hyperglycaemic mice

Adult male mice (25–30 g body weight) were intrapenitoneally (i.p.) injected with a single dose of STZ at 150 mg/kg body weight, dissolved in 10 mM sodium citrate buffer (pH 4.5), a well-established agent that destroys pancreatic cells. On Day 3 after STZ treatment, whole-blood was obtained from the mouse tail-vein, and the blood glucose levels were measured using the OneTouch Ultra 2 blood glucose monitoring system (Life Scan, Inc. CA, USA). Non-fasted blood glucose content of 20 mmol/L or greater was chosen as hyperglycaemia for the present study, whereas citrate buffer-treated mice were used as normoglycaemia.

2.4 Calpain knockdown using small interfering RNA

In order to knockdown calpain-1 and calpain-2 expression, a small interfering RNA (siRNA) against rat calpain-1 or calpain-2 was obtained (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a scramble siRNA was employed as control. Transfection was performed using TransMessenger Transfection Reagent (Qiagen) according to the

manufacturer's protocol as described in our previous studies with modifications.[21,22] Briefly, we mixed 3 μL Enhancer, 84 μL Buffer ECR, 4.5 μL siRNA (10 μM), 6 μL TransMessenger, and 220 μL MEM without serum/antibiotics for each well (12-well plates). Four hours after incubation with the siRNA mixture, the medium was changed with a fresh normal culture medium. The cells were then incubated for 24 h before treatment with high glucose.

2.5 Adenoviral infection of cultured adult rat cardiomyocytes

Cardiomyocytes were infected with adenoviral vectors containing either the gene for rat calpastatin (Ad-CAST, University of Buffalo, USA) or beta-gal (Ad-gal, Vector Biolabs) as a control at a multiplicity of infection of 10 PFU/cell. Adenovirus-mediated gene transfer was implemented as described previously.[20]

2.6 Calpain activity

Calpain activity was determined by using a fluorescence substrate *N*-succinyl-LLVY-AMC (Cedarlane Laboratories) as described previously.[20] The calpain inhibitor PD150606 was used to determine the specificity of the assay.

2.7 Na^+/K^+ ATPase activity assay

Cultured cardiomyocytes or heart tissues were homogenized with a lysis buffer containing 10 mM Tris, pH 7.4, 2 mM EDTA, and 250 mM sucrose. After four freeze–thaw cycles, the ouabain-sensitive Na^+/K^+ ATPase activity in homogenates was determined by measuring 3-*O*-methylfluorescein phosphatase (3-*O*-MFPase) activity as described previously.[23] The fluorescence intensity of cleaved 3-*O*-MFP was quantified with a multilabel reader (excitation, 475 nm; emission, 515 nm). The K^+-stimulated 3-*O*-MFPase activity was determined as the difference between the slopes generated from the same sample with or without the addition of KCl, which was ouabain inhibitable.

2.8 Active caspase-3

As described in detail previously,[24] caspase-3 activity in myocardial tissues and cardiomyocytes was measured by using a caspase-3 fluorescent assay kit (BIOMOL Research Laboratories).

2.9 Cell death measurement

Cardiomyocyte death was measured by annexin-V and Hoechst 33342 staining as described in our recent study.[20]

2.10 *In situ* detection of apoptotic cells

To localize cells undergoing nuclear DNA fragmentation in the mouse myocardium, *in situ* terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) was performed using an *in situ* apoptosis detection kit (Roche Biochemicals) as described previously.[24]

2.11 NADPH oxidase activity

NADPH-oxidase activity was assessed in cell lysates by lucigenin-enhanced chemiluminescence (20 μg of protein, 100 μM NADPH, 5 μM lucigenin) as described previously.[20]
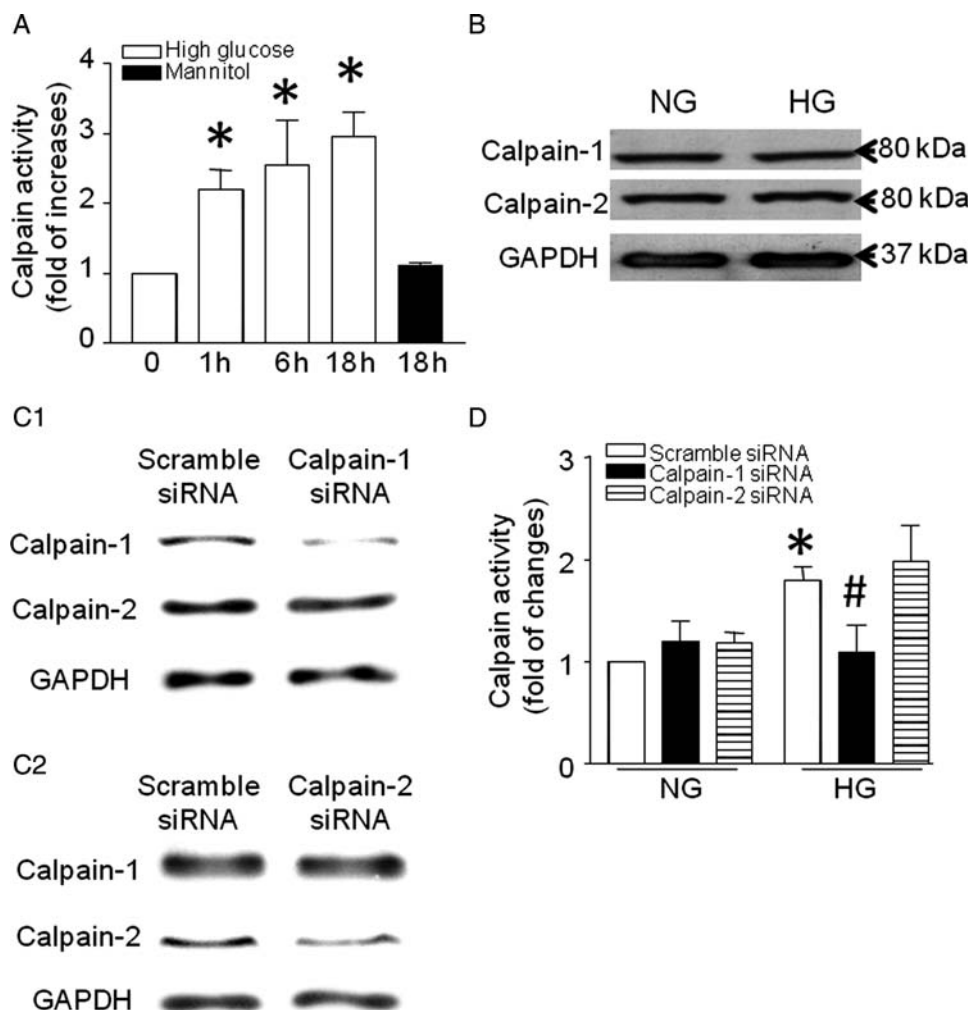
Figure 1 Effects of high glucose on calpain activition in cardiomyocytes. (*A*) Calpain activity in cardiomyocytes-incubated with normal glucose (NG, 5.5 mM), high glucose (HG, 33 mM), or mannitol (33 mM) for different time periods (0, 1, 6, and 18 h). (*B*) A representative western blot from three different cell cultures for the protein levels of calpain-1, calpain-2, and GAPDH in cardiomyocytes incubated with NG or HG for 18 h. (*C*) and (*D*) Cardiomyocytes were transfected with calpain-1 siRNA, calpain-2 siRNA, or scramble siRNA, followed by high glucose. (C) A representative western blot from three different cell cultures for specific down-regulation of calpain-1 and calpain-2 protein in siRNA-transfected cardiomyocytes. (D) Calpain activity in calpain-1 siRNA- and calpain-2 siRNA-transfected cardiomyocytes during high glucose stimulation. Data are mean $\pm$ SD, $n = 3$–5. *$P < 0.05$ vs. time point 0 or scramble siRNA in NG, #$P < 0.05$ vs. scramble siRNA in HG.

2.12 Western blot analysis

Expressions of calpain-1, calpain-2, and GAPDH protein were determined by western blot analysis using specific antibodies against the relevant corresponding proteins, respectively.

2.13 Statistical analysis

All data were given as mean $\pm$ 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.

3. Results

3.1 High glucose induces calpain activation in cardiomyocytes

To investigate the effect of high glucose on calpain activation, adult rat cardiomyocytes were incubated with normal glucose (5.5 mM), high glucose (33 mM), or mannitol (33 mM) for various time periods (0, 1, 6, 18 h). High glucose increased

calpain activity starting at 1 h and the activity of calpain reached the maximal levels around 18 h in cardiomyocytes (*Figure 1A*). As an osmotic control, equal amount of mannitol did not alter calpain activity (*Figure 1A*), excluding the possible osmotic effect on calpain activation. Thus, the 18 h time point was chosen for the present study. The up-regulation of calpain activity was not due to alterations of calpain-1 and calpain-2 expression since the protein levels of calpain-1 and calpain-2 were similar in between normal and high glucose-incubated cardiomyocytes (*Figure 1B*).

To clarify the isoform-specific activation of calpain, we transfected cardiomyocytes with calpain-1 siRNA, calpain-2 siRNA, or a scramble siRNA for 24 h and then incubated them with high glucose. Eighteeen hours later, transfection with calpain-1 siRNA significantly decreased calpain-1 but not calpain-2 protein (*Figure 1C*1), suggesting a successful knockdown. Similarly, calpain-2 siRNA downregulated calpain-2 but not calpain-1 protein (*Figure 1C*2). In response to high glucose stimulation, calpain activity was significantly reduced by 80% in calpain-1 siRNA compared with scramble siRNA-transfected cardiomyocytes (*Figure 1D*); whereas calpain-2 siRNA did not change calpain activity (*Figure 1D*). This result strongly
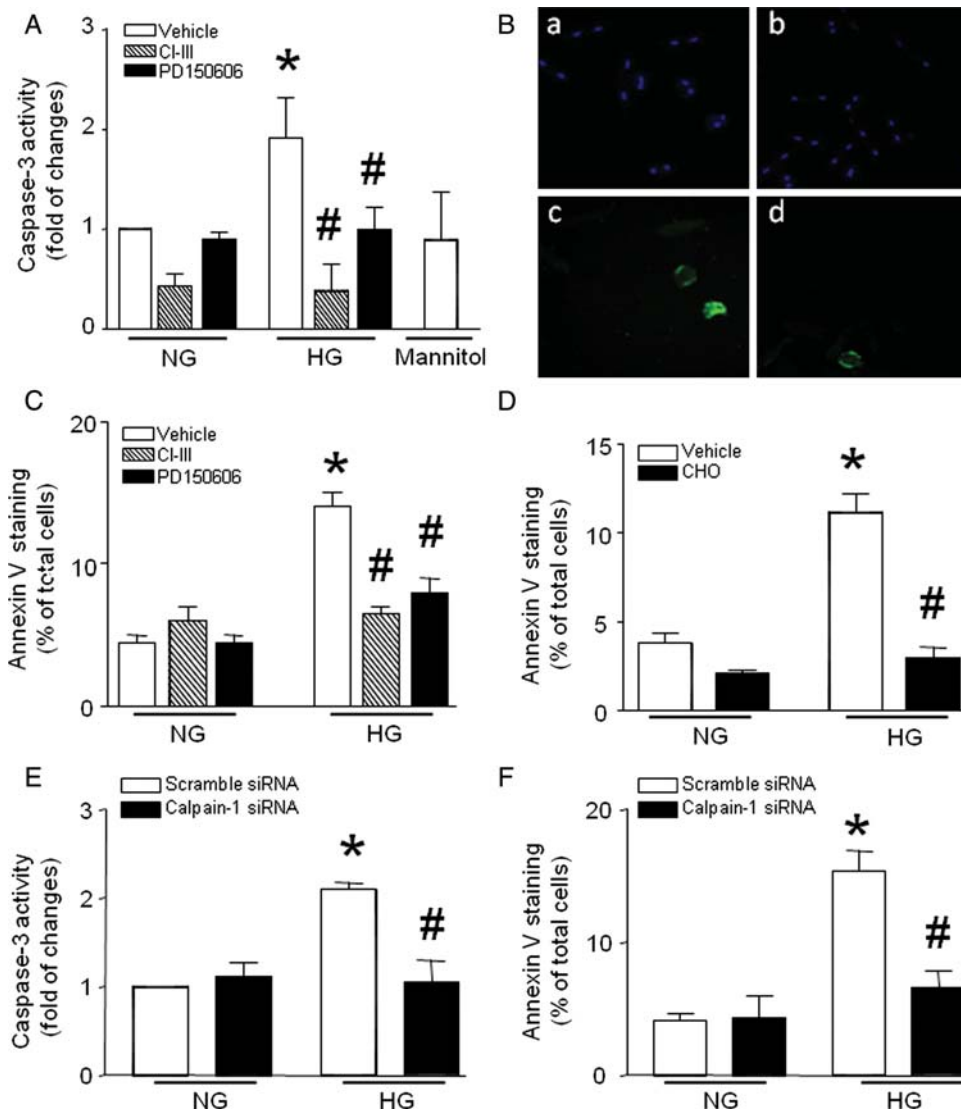
Figure 2 Effects of calpain inhibition on apoptosis in high glucose-stimulated cardiomyocytes. Cardiomyocytes were incubated with NG, HG, or mannitol (33 mM) in combination with CI-III or PD150606 for 18 h. (*A*) Caspase-3 activity was measured in cardiomyocytes. (*B*) A representative staining for Hoechst33342 (blue signal) and annexin-V (green signal) is shown from HG (*a* and *c*) and HG+PD150606 (*b* and *d*) treated cardiomyocytes. (C) Quantification of the percentage of annexin-V positive cells. (D) Cardiomyocytes were incubated with NG or HG in the presence of AC-DEVD-CHO (CHO, 50 μM) or vehicle for 18 h. The percentage of annexin-V positive cells was quantified. (*E*) and (*F*) Cardiomyocytes were transfected with calpain-1 siRNA or scramble siRNA and followed by high glucose. Caspase-3 activity (E) and the percentage of annexin-V positive cells (F) were determined in HG-stimulated cardiomyocytes. Data are mean ± SD, $n = 3–5$. *$P < 0.05$ vs. Vehicle in NG or scramble siRNA in NG, #$P < 0.05$ vs. Vehicle in HG or scramble siRNA in HG.

suggests that the calpain activity induced by high glucose mainly results from calpain-1 in cardiomyocytes.

3.2 Inhibition of calpain activation blocks apoptosis in high glucose-stimulated cardiomyocytes

High glucose has been demonstrated to induce apoptosis in cardiomyocytes.[8–10] To examine the contribution of calpain to high glucose-induced apoptosis, we incubated cardiomyocytes with normal glucose, high glucose or mannitol in the presence of PD150606 (5 μM), calpain inhibitor-III (10 μM), or vehicle for 18 h. Cardiomyocyte apoptosis was determined by caspase-3 activity and annexin-V staining. High glucose significantly increased caspase-3 activity when compared with normal glucose, whereas, as an osmotic control, equal amount of mannitol did not induce caspase-3 activity (*Figure 2A*), excluding the possible osmotic effect on apoptosis. Incubation with PD150606 or calpain inhibitor-

III significantly decreased caspase-3 activity (*Figure 2A*). In line with the up-regulation of caspase-3 activity, high glucose increased the percentage of annexin-V positive cells, which was significantly reduced by PD150606 or calpain inhibitor-III (*Figure 2B* and *C*). The increase in the percentage of annexin-V positive cells was also abrogated by a selective caspase-3 inhibitor, AC-DEVD-CHO (*Figure 2D*). These results demonstrate that calpain activation induces apoptosis by the caspase-3-dependent pathway in cardiomyocytes during high glucose stimulation.

The role of calpain in high glucose-induced apoptosis was further investigated by using calpain-1 siRNA. Transfection with calpain-1 siRNA significantly decreased high glucose-induced caspase-3 activity and the percentage of annexin-V positive cells compared with the scramble siRNA (*Figure 2E* and *F*). These results demonstrate that calpain-1 activation induces apoptosis in cardiomyocytes during high glucose stimulation.
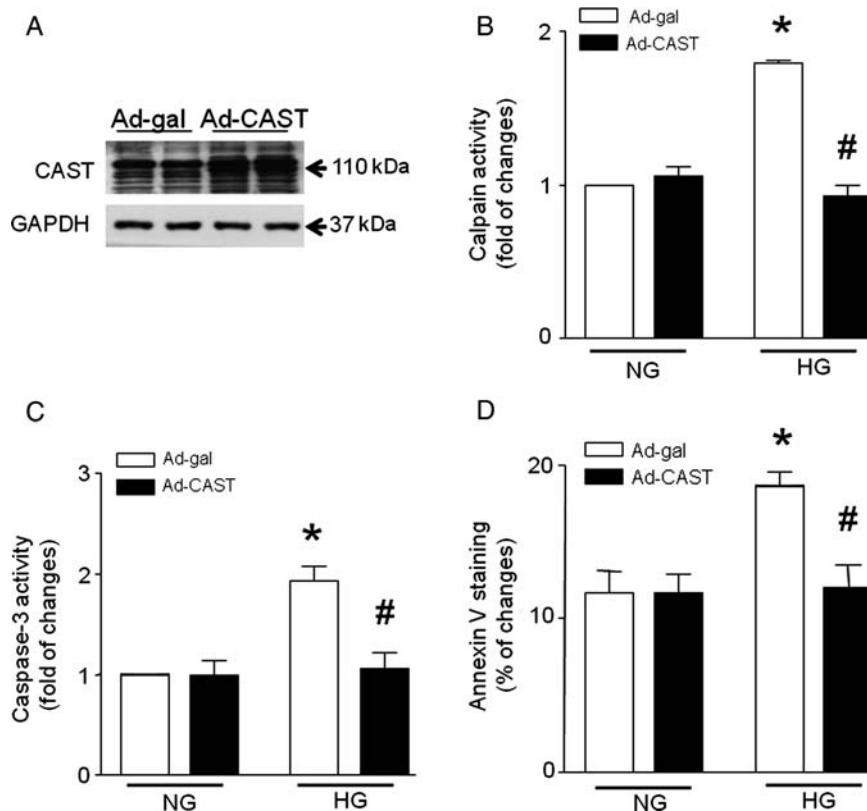
Figure 3 Role of calpastatin in calpain activity and apoptosis in cardiomyocytes. Cardiomyocytes were infected with Ad-CAST or Ad-gal for 24 h, followed by incubation with NG or HG for 18 h. (*A*) A representative western blot for up-regulation of calpastatin protein in Ad-CAST-infected cardiomyocytes. Infection of Ad-CAST inhibited calpain activity (*B*), blocked caspase-3 activation (*C*), and abrogated the increases in the percentage of annexin-V positive cells (*D*) in HG-stimulated cardiomyocytes. Data are mean ± SD, *n* = 3–5. *$P < 0.05$ vs. Ad-gal in NG, #$P < 0.05$ vs. Ad-gal in HG.

3.3 Over-expression of calpastatin blocks calpain activation and inhibits apoptosis during high glucose stimulation

Calpastatin is a highly specific inhibitor of calpain-1 and calpain-2 that does not inhibit the activity of any other protease tested.[13] Thus, calpastatin expression is an effective means of globally inhibiting cellular calpain-1 and calpain-2 activities. To further confirm the role of calpain in apoptosis, we over-expressed calpastatin in cardiomyocytes by using Ad-CAST. Cardiomyocytes were infected with Ad-CAST or Ad-gal for 24 h, followed by incubation with normal or high glucose for 18 h. The over-expression of calpastatin was verified in Ad-CAST-infected cardiomyocytes by western blot analysis (*Figure 3A*). Under normal glucose condition, Ad-CAST infection did not alter calpain activity and apoptosis. In response to high glucose stimulation, over-expression of calpastatin significantly decreased calpain activity, caspase-3 activity, and the percentage of annexin-V positive cells (*Figure 3B–D*). Thus, over-expression of calpastatin inhibits calpain activation and prevents apoptosis in high glucose-stimulated cardiomyocytes.

3.4 Calpain induces the impairment of Na$^+$/K$^+$ ATPase which contributes to apoptosis in high glucose-stimulated cardiomyocytes

It has been shown that calpain impairs Na$^+$/K$^+$ ATPase activity in the heart.[25] We therefore examined the role of calpain in Na$^+$/K$^+$ ATPase activity. In agreement with a previous report,[26] high glucose also reduced Na$^+$/K$^+$ ATPase activity by about 51% in cardiomyocytes. PD150606 treatment restored Na$^+$/K$^+$ ATPase activity during high glucose stimulation (*Figure 4A*), suggesting that calpain activation results in the Na$^+$/K$^+$ ATPase impairment. Since inhibition of Na$^+$/K$^+$ ATPase leads to apoptosis in cardiomyocytes,[27] we investigated if the role of calpain in apoptosis was related to the Na$^+$/K$^+$ ATPase impairment. Under normal glucose condition, incubation with ouabain (200 μM), a selective Na$^+$/K$^+$ ATPase inhibitor, increased caspase-3 activity and the percentage of annexin-V staining cells in cardiomyocytes (*Figure 4C*). In response to high glucose, concurrent treatment with ouabain abolished the inhibitory effect of PD150606 on apoptosis in cardiomyocytes (*Figure 4C*). Thus, calpain induces apoptosis, at least in part, through the Na$^+$/K$^+$ ATPase impairment in cardiomyocytes during high glucose stimulation.

3.5 Gp91^{phox}-NADPH oxidase contributes to calpain activation in high glucose-induced cardiomyocytes

Our recent study has shown that reactive oxygen species (ROS) produced from gp91^{phox}-NADPH oxidase induces calpain activation in cardiomyocytes during norepinephrine stimulation.[20] To examine if this is also operative in high glucose-induced cardiomyocytes, cardiomyocytes were incubated with normal or high glucose in the presence of gp91ds-tat (2.5 μM), scramble-tat as a control, DPI (10 μM), NAC (2.5 mM), or vehicle. gp91ds-tat is a cell-permeable peptide, which specifically blocks the assembly
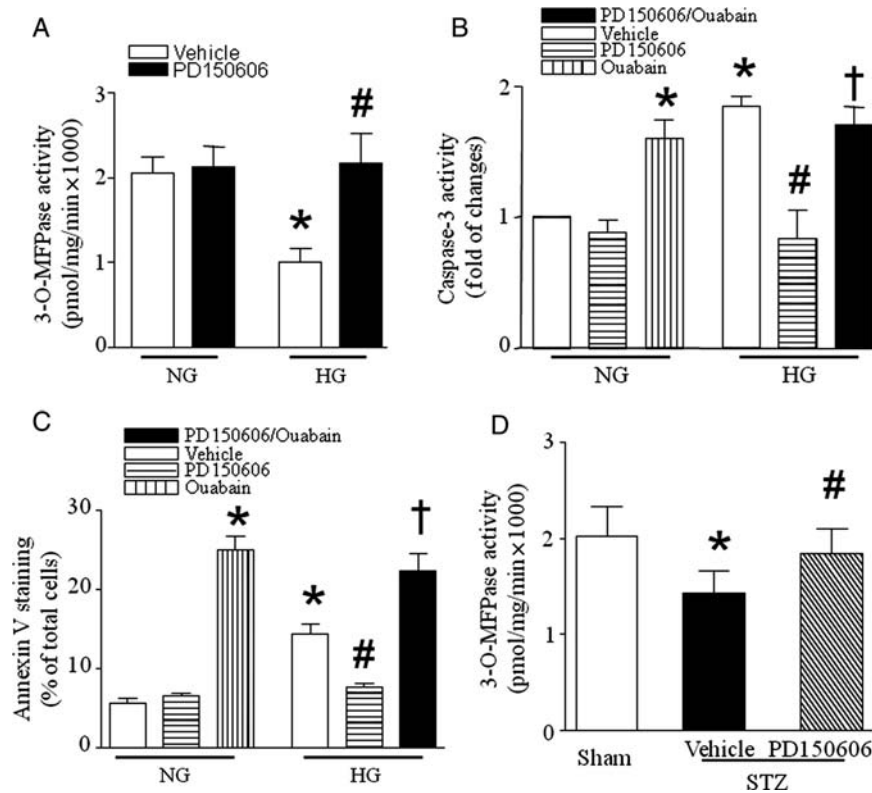
Figure 4 Effects of PD150606 and ouabain on Na^+/K^+ ATPase activity and apoptosis. Cardiomyocytes were incubated with NG or HG in the presence of PD150606 or ouabain alone, or in combination for 18 h. (*A*) Na^+/K^+ ATPase activity was assessed. (*B*) and (*C*) Caspase-3 activity and the percentage of annexin-V positive cells were determined. (*D*) Hyperglycaemia was induced by STZ. On Day 3 after STZ injection, PD150606 or vehicle was given for 7 days. Na^+/K^+ ATPase activity was measured in hyperglycaemic hearts. Data are mean $\pm$ SD, $n = 5$–6. *$P < 0.05$ vs. vehicle in NG or Sham, $^\#P < 0.05$ vs. Vehicle in HG or vehicle in STZ, $^\dagger P < 0.05$ vs. PD150606 in HG.

and activation of the gp91^{phox}-NADPH oxidase.[28] Eighteen hours later, NADPH oxidase activity, calpain activity, caspase-3 activity, and annexin-V positive cells were determined. High glucose significantly increased NADPH oxidase activity, which was inhibited by gp91ds-tat (*Figure 5A*). Gp91ds-tat or DPI also reduced calpain activity, caspase-3 activity, and annexin-V positive cells in high glucose-induced cardiomyocytes (*Figure 5B*, *C*, and *D*). Similarly, scavenging ROS by NAC blocked calpain activation and apoptosis in high glucose-stimulated cardiomyocytes (*Figure 5E* and *F*). Thus, ROS produced from gp91^{phox}-NADPH oxidase mediates calpain activation, resulting in apoptosis in cardiomyocytes during high glucose stimulation.

3.6 Blockade of calcium channels inhibits calpain activation and apoptosis induced by high glucose

Reactive oxygen species has been suggested to modify Ca^{2+} channels and transporters such as L-type calcium channels and ryanodine receptors.[29] Since Ca^{2+} is required for calpain activation, we examined whether blocking calcium channels would inhibit calpain activation and apoptosis in high glucose-stimulated cardiomyocytes. Cardiomyocytes were incubated with normal or high glucose in combination with the L-type calcium channel blocker, verapamil (100 μM) or a pharmacological inhibitor of ryanodine receptors, dantrolene (20 μM). Eighteen hours later, calpain activity, caspase-3 activity, and annexin V staining were analysed.

As shown in *Figure 6A–C*, co-incubation with either verapamil or dantrolene abrogated calpain activation, and blocked caspase-3 activity and annexin-V staining. These results suggest that activation of L-type calcium channels and ryanodine receptors triggers calpain activation in high glucose-stimulated cardiomyocytes. To further confirm the role of L-type calcium channels and ryanodine receptors in ROS-mediated calpain activation, we treated cardiomyocytes with H_2O_2 in the presence of verapamil or dantrolene. Consistent with our recent study,[20] H_2O_2 significantly increased calpain activity, which was blocked by either verapamil or dantrolene (*Figure 6D*). Thus, our data demonstrate that ROS induces calpain activation via L-type calcium channels and ryanodine receptors in cardiomyocytes.

3.7 *In vivo* effects of calpain inhibitors in the hyperglycaemic heart

To investigate the role of calpain in *in vivo* hearts, adult mice were injected with STZ (150 mg/kg, i.p.) or citrate buffer. When hyperglycaemia was confirmed on Day 3 after STZ injection, mice were treated with calpain inhibitor-I (3 mg/kg/day, i.p.), calpain inhibitor-III (10 mg/kg in every other day, i.p.), PD150606 (10 mg/kg in every other day, i.p.), or vehicle. On Day 9 after STZ injection, blood glucose content, calpain activity, caspase-3 activity, and apoptosis were then assessed. To eliminate possible confounding effects of STZ on the early cell death, insulin was immediately given using a long-term insulin preparation
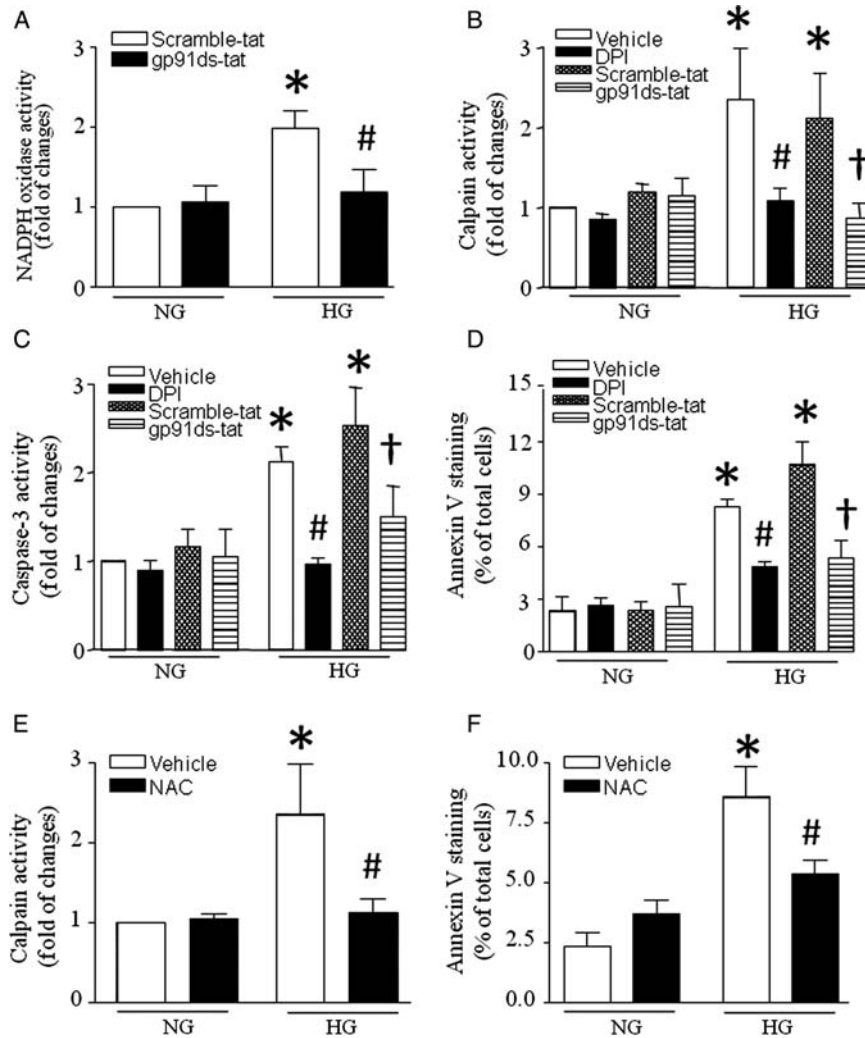
Figure 5 Role of gp91^{phox}-NADPH oxidase in calpain activation and apoptosis in cardiomyocytes. Cardiomyocytes were incubated with NG or HG in combination with gp91ds-tat, scramble-tat, DPI or NAC for 18 h. NADPH oxidase activity (*A*), calpain activity (*B* and *E*), caspase-3 activity (*C*), and the percentage of annexin-V positive cells (*D* and *F*) were assessed in cardiomyocytes. Data are mean ± SD, $n = 3$–5. *$P < 0.05$ vs. vehicle in NG or scramble-tat in NG, #$P < 0.05$ vs. vehicle in HG, †$P < 0.05$ vs. scramble-tat in HG.

(Lantus Insulin glargine injection, Sanofi Aventis Canada Inc. Laval Canada) at a dose of 5–15 U/mouse once a day to maintain the blood glucose levels between 5 and 12 mmol/L when hyperglycaemia was diagnosed on Day 3 after STZ treatment. Insulin-treated mice were also examined on Day 9 after STZ treatment.

Compared with citrate buffer-treated mice, 7 days of hyperglycaemia significantly increased calpain activity in STZ-treated compared with citrate buffer-treated hearts (*Figure 7A*). In contrast, myocardial calpain activity was not increased in insulin-supplemented mice (*Figure 7A*). These data suggest that hyperglycaemia activates calpain in the heart.

Hyperglycaemia significantly increased myocardial caspase-3 activity and the number of TUNEL-positive cardiomyocytes (*Figure 7B* and *C*), whereas myocardial caspase-3 activity was not increased in insulin-supplemented mice (*Figure 7B*). These results exclude the direct possible effects of STZ on myocardial apoptosis, which is in agreement with a recent report.[7] Administration of calpain inhibitor-I, calpain inhibitor-III, or PD150606 decreased calpain activity, caspase-3 activity, and apoptosis

in hyperglycaemic hearts (*Figure 7B* and *C*), while the levels of blood glucose were similar between calpain inhibitors- and vehicle-treated mice (data not shown). Thus, inhibition of calpain blocked cardiomyocyte apoptosis in hyperglycaemic hearts.

Hyperglycaemia also significantly decreased Na$^+$/K$^+$ ATPase activity in the heart by about 30%. Inhibition of calpain by PD150606 preserved Na$^+$/K$^+$ ATPase activity in hyperglycaemic hearts (*Figure 4D*). This result further supports the inhibitory role of calpain in Na$^+$/K$^+$ ATPase activity during hyperglycaemia.

4. Discussion

The present study investigated the role of calpain in apoptosis in high glucose-stimulated cardiomyocytes and hyperglycaemic hearts. The major findings are as follows: first, high glucose induces calpain-1 activation via gp91^{phox}-NADPH oxidase-dependent ROS production. Second, ROS modulation of L-type calcium channels and ryanodine receptors is involved in high glucose-induced calpain activation. Third, calpain-1 activation contributes to apoptosis in
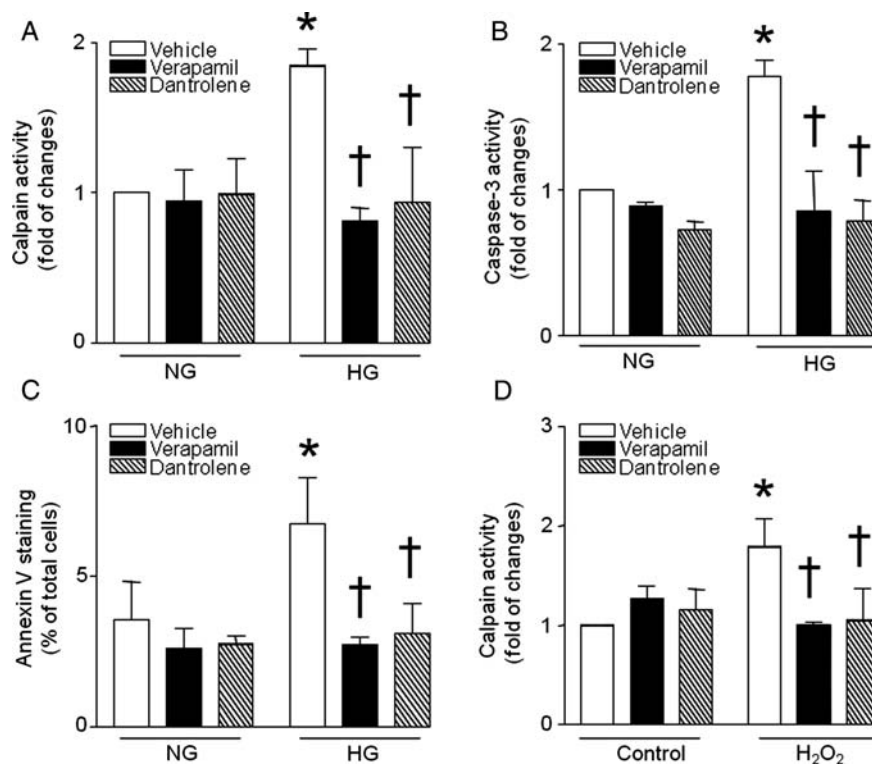
Figure 6 Effects of verapamil and dantrolene on calpain activation and apoptosis. Cardiomyocytes were incubated with NG or HG in the presence of verapamil, dantrolene, or vehicle for 18 h. (A) Calpain activity was determined. (B) and (C) Caspase-3 activity and the percentage of annexin-V positive cells were analysed. (D) Cardiomyocytes were incubated with H_2O_2 in the presence of verapamil, dantrolene, or vehicle for 18 h. Calpain activity was then measured. Data are mean $\pm$ SD, $n = 5$–6. *$P < 0.05$ vs. vehicle in NG or vehicle in control, †$P < 0.05$ vs. vehicle in HG or vehicle in H_2O_2.

cardiomyocytes and hearts during hyperglycaemia. Fourth, over-expression of calpastatin inhibits calpain and prevents apoptosis in cardiomyocytes during hyperglycaemia. Finally, the pro-apoptotic role of calpain in cardiomyocytes is mediated through caspase-3 activation and associated with the impairment of Na^+/K^+ ATPase activity during hyperglycaemia. To our knowledge, this is the first demonstration that calpain-1 mediates apoptosis induced by hyperglycaemia in cardiomyocytes.

Myocardium contains modest amounts of ubiquitous calpain-1 and calpain-2.[16] Calpain activity is mainly regulated by altering the Ca^{2+} concentration required for its proteolytic activity, normally with approximately micromolar concentration of Ca^{2+} for the maximal calpain-1 and approximately millimolar concentration for calpain-2 activities.[13] Although these levels of Ca^{2+} are not likely achieved in cardiomyocytes, the Ca^{2+} requirement for calpain activity is modulated by several mechanisms. For example, certain phospholipids would lower the Ca^{2+} concentration required for autolysis of calpain-1 and calpain-2.[30] Calpain activity can also be regulated through phosphorylation by MAPK, protein kinase C (PKC), and protein kinase A (PKA).[31–33] As such, a slight rise of Ca^{2+} concentration activates calpain in intact cardiomyocytes.[34] Previous studies have demonstrated that the cytosolic Ca^{2+} level in cardiomyocytes is increased in diabetes or high glucose stimulation,[11,35] in addition to an increase in MAPK, PKC, and PKA activation.[36] It is therefore possible that high glucose may induce calpain activation. In this regard, the present study showed that calpain activity was increased in both high glucose-stimulated cardiomyocytes and hyperglycaemic hearts. High glucose-induced calpain activity resulted from calpain-1 activation

since it was inhibited by calpain-1 knockdown. Action of calpain-1 was also shown in endothelial cells during hyperglycaemia.[37] Furthermore, the present study suggests that NADPH oxidase is the upstream signalling in calpain activation in response to high glucose. NADPH oxidase-mediated calpain activation was also shown in cardiomyocytes during NE stimulation in our recent study.[20] Thus, NADPH oxidase may be a universal mechanism for calpain activation in cardiomyocytes. The exact mechanisms by which NADPH oxidase-produced ROS induces calpain activation are currently unknown. The present study found that calpain activity induced by both high glucose and H_2O_2 was abolished by blocking L-type Ca^{2+} channels or ryanodine receptors, suggesting a critical role of both Ca^{2+} channels. Since the Ca^{2+} influx through L-type Ca^{2+} channels in the plasma membrane activates ryanodine receptors in the sarcoplasmic reticulum to amplify Ca^{2+} signals, it is possible that ROS modulates L-type Ca^{2+} channels and subsequently ryanodine receptors to release more Ca^{2+},[29] which increases calpain activity. However, future studies will be needed to further clarify the signalling pathways in regulating calpain activation in cardiomyocytes during hyperglycaemia.

Although high glucose/hyperglycaemia induces calpain activation, the pathophysiological significance of calpain activation remains not fully elucidated in diabetic hearts. In the present study, direct exposure to high glucose induced apoptotic cell death in cardiomyocytes, which is in agreement with previous reports.[8–10] Pharmacological inhibition of calpain prevented apoptotic cell death in high glucose-stimulated cardiomyocytes. The anti-apoptotic effect of calpain inhibition was further confirmed by calpain-1 siRNA and over-expression of calpastatin in
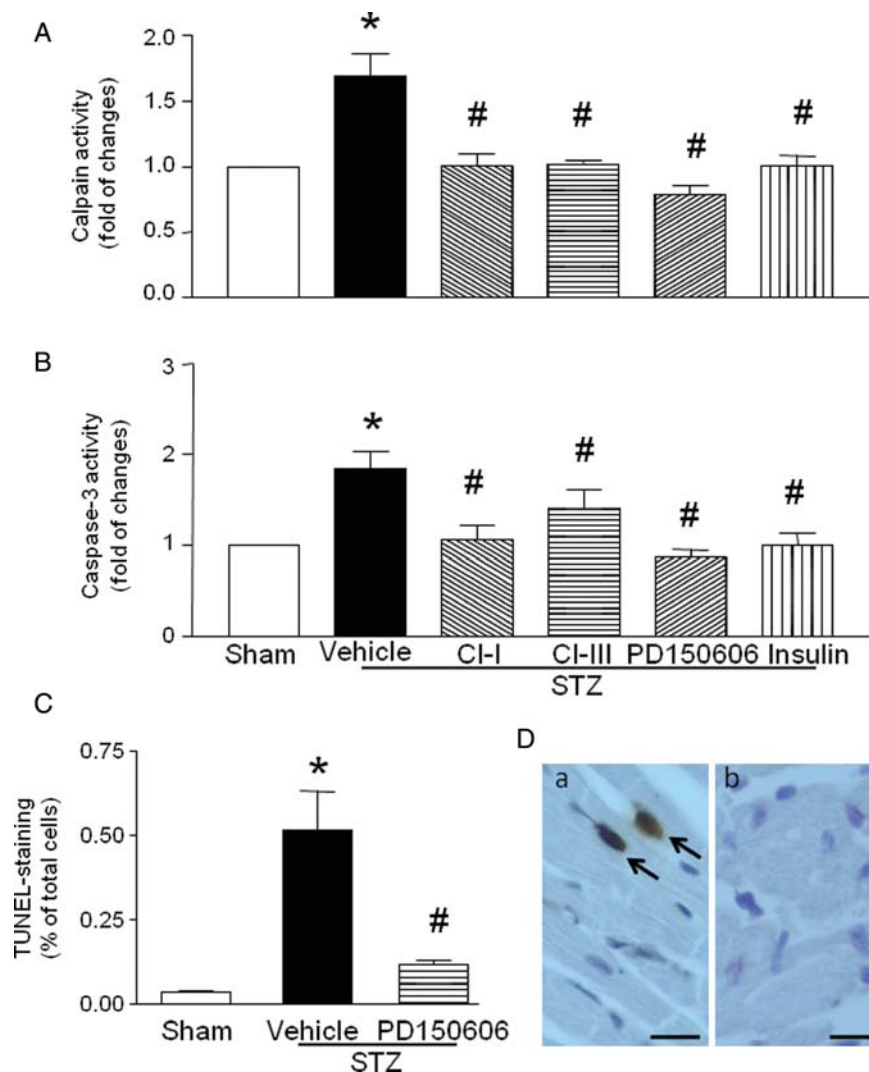
Figure 7 Effects of calpain inhibitors on apoptosis in hyperglycaemic hearts. Hyperglycaemia was induced by STZ. On Day 3 after STZ treatment, mice were treated with insulin, calpain inhibitor-I (CI-I), calpain inhibitor-III (CI-III), or PD150606 for 7 days. Calpain activity (*A*) and caspase-3 activity (*B*) were measured in hyperglycaemic hearts. (*C*) Quantification of TUNEL staining positive cardiomyocytes. Data are mean $\pm$ SD, $n = 5$–6. *$P < 0.05$ vs. sham and #$P < 0.05$ vs. Vehicle in STZ. (*D*) Representatives of TUNEL staining in cardiomyocytes (yellow-blown signal, arrows showing nuclear localization) from vehicle-treated (a) or PD150606-treated hyperglycaemic hearts (b). (Magnification: $\times 40$; scale bar: 10 μm.)

cardiomyocytes, both of which specifically blocked calpain activation and prevented apoptosis in cardiomyocytes during high glucose stimulation. To examine the role of calpain in cardiomyocyte apoptosis *in vivo*, we extended our analysis to diabetic models. In a mouse model of type-1 diabetes, administration of different calpain inhibitors (calpain inhibitor-I, calpain inhibitor-III, and PD150606) blocked calpain activation and inhibited apoptosis in hyperglycaemic hearts. These data strongly support the fact that inhibition of calpain activation protects cardiomyocytes from apoptotic death in diabetic conditions. Since apoptosis in cardiomyocytes significantly contributes to cardiac complications of diabetes,[11,12] modulating calpain/calpastatin system may be a potential therapeutic strategy to treat diabetic heart diseases. Because basal calpain activity is required for physiological functions of the heart[16] and overexpression of calpastatin appears to limit calpain activation without interfering with basal calpain activity,[15] strategies focusing on up-regulation of calpastatin may have significant therapeutic potentials for diabetic heart diseases.

Calpain has been demonstrated to modulate various pro- and anti-apoptotic molecules including caspases, Bcl-2, Bcl-xl, Bid, Bax, NF-κB, and p53.[13,38] The present study found that inhibition of calpain blocked caspase-3 activation in both high glucose-stimulated cardiomyocytes and hyperglycaemic hearts and inhibition of caspase-3 activity prevented apoptosis in cardiomyocytes. Thus, the pro-apoptotic action of calpain may be mediated by the caspase-3-dependent pathway in hyperglycaemia. However, the present study could not exclude the involvement of caspase-3-independent pathways in calpain-mediated apoptosis in diabetic hearts. In addition to promoting apoptosis, calpain activation may also contribute to necrosis in cardiomyocytes.[39]

It has recently been shown that calpain inhibits Na$^+$/K$^+$ ATPase activity in the heart,[25] which may induce apoptosis via Ca^{2+} overload.[27] In the present study, we showed that high glucose down-regulated Na$^+$/K$^+$ ATPase activity in cardiomyocytes. Although we could not exclude the possibility that the down-regulation of Na$^+$/K$^+$ ATPase activity was the result of cell death in cultured cardiomyocytes during

high glucose stimulation, data from STZ-induced mice strongly suggest that hyperglycaemia induces the impairment of Na^+/K^+ ATPase in the heart. This was indeed supported by a previous report in STZ-diabetic rats.[26] We further showed that inhibition of calpain activation preserved Na^+/K^+ ATPase activity in both high glucose-induced cardiomyocytes and hyperglycaemic hearts. Interestingly, ouabain, a specific Na^+/K^+ ATPase inhibitor, abolished the protective effect of calpain inhibitors in cardiomyocytes. These results suggest that the protective effect of calpain inhibition on cardiomyocytes may result from the normalization of Na^+/K^+ ATPase. However, future studies will be needed to elucidate the signalling mechanisms downstream of Na^+/K^+ ATPase in calpain-mediated apoptosis in hyperglycaemic hearts.

In summary, we have provided definite evidence showing that ROS produced from gp91^{phox}-NADPH oxidase induces calpain-1 activation, and L-type calcium channels and ryanodine receptors are involved in ROS-mediated calpain activation in high glucose-stimulated cardiomyocytes. Inhibition of calpain-1 activation by pharmacological calpain inhibitors, calpain-1 siRNA or over-expression of calpastatin blocks apoptosis in both high glucose-induced cardiomyocytes and hyperglycemic hearts. These effects of calpain on apoptosis are mediated through the caspase-3-dependent pathway and associated with the impairment of Na^+/K^+ ATPase activity. Given cardiac apoptosis significantly contributes to diabetic heart diseases,[6,7,9,12] these findings suggest that calpain activation may be important in the development of diabetic cardiomyopathy and thus, calpain-1 may serve as a potential therapeutic target for the treatment of cardiac complications in diabetes.

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