Over-expression of calpastatin aggravates cardiotoxicity induced by doxorubicin

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Aims
Doxorubicin causes damage to the heart, which may present as cardiomyopathy. However, the mechanisms by which doxorubicin induces cardiotoxicity remain not fully understood and no effective prevention for doxorubicin cardiomyopathy is available. Calpains, a family of calcium-dependent thiol-proteases, have been implicated in cardiovascular diseases. Their activities are tightly controlled by calpastatin. This study employed transgenic mice over-expressing calpastatin to investigate the role of calpain in doxorubicin-induced cardiotoxicity.

Methods and results
Doxorubicin treatment decreased calpain activities in cultured neonatal mouse cardiomyocytes and in vivo mouse hearts, which correlated with down-regulation of calpain-1 and calpain-2 proteins. Over-expression of calpastatin or incubation with pharmacological calpain inhibitors enhanced apoptosis in neonatal and adult cardiomyocytes induced by doxorubicin. In contrast, over-expression of calpain-2 but not calpain-1 attenuated doxorubicin-induced apoptosis in cardiomyocytes. The pro-apoptotic effects of calpain inhibition were associated with down-regulation of protein kinase B (AKT) protein and mRNA expression, and a concomitant reduction in glycogen synthase kinase-3β (GSK-3β) phosphorylation (Ser9) in doxorubicin-treated cardiomyocytes. Blocking AKT further increased doxorubicin-induced cardiac injuries, suggesting the effects of calpain inhibition may be mediated by inactivating the AKT signalling. In an in vivo model of doxorubicin-induced cardiotoxicity, over-expression of calpastatin exacerbated myocardial dysfunction as assessed by echocardiography and haemodynamic measurement in transgenic mice 5 days after doxorubicin injection. The 5-day mortality was higher in transgenic mice (29.16%) compared with their wild-type littermates (8%) after doxorubicin treatment.

Conclusion
Over-expression of calpastatin enhances doxorubicin-induced cardiac injuries through calpain inhibition and thus, calpains may protect cardiomyocytes against doxorubicin-induced cardiotoxicity.

Keywords
Doxorubicin • Cardiotoxicity • Calpain • Calpastatin • Protein kinase B

1. Introduction
Doxorubicin is an effective and frequently used chemotherapeutic agent for various malignancies. However, it is well recognized that doxorubicin may cause damage to the heart (cardiotoxicity), which may present as cardiomyopathy. Doxorubicin-induced cardiomyopathy, once developed, is frequently fatal. This side effect of doxorubicin therapy may limit its use. The incidence of acute cardiotoxicity is ~11% and the incidence of chronic doxorubicin cardiotoxicity is about 1.7%. However, the mechanisms by which doxorubicin induces cardiotoxicity remain not fully understood and no effective prevention is available for doxorubicin-induced cardiomyopathy.

Calpains belong to a family of calcium-dependent cysteine proteases. Fifteen gene products of the calpain family have been...
identified in mammals. Among them, calpain-1 (μ-form) and calpain-2 (m-form) are well-studied and ubiquitously expressed. Calcium is usually required for calpain activation. Calpastatin is an endogenous inhibitor of calpain. It specifically inhibits calpain but not other cysteine proteases. As such, over-expression of calpastatin has been successfully used to inhibit calpain in a variety of in vitro and in vivo models. Calpain activation has been implicated in ischaemia/reperfusion-induced myocardial injury. We have demonstrated that calpains are important in apoptosis and inflammatory responses in cardiomyocytes under stresses. Our recent study also showed that calpain significantly contributes to diabetic cardiomyopathy in different mouse models of type-1 diabetes. These studies suggest that calpain activation may play a role in the progression of heart failure. Indeed, cardiac over-expression of calpain-1 induces heart failure in transgenic mice. In contrast, studies have also suggested that calpain may play a beneficial role in the heart. For example, calpain has been shown to be necessary for maintaining protein homeostasis in cardiomyocytes and deficiency of calpain-induced dilated cardiomyopathy. A recent study also suggested that calpain may protect the heart against haemodynamic stress in a mouse model of pressure overload-induced myocardial dysfunction. These previous studies suggest that the diverse roles of calpain may depend on different patho-physiological conditions.

Previous studies showed that calpain may be implicated in doxorubicin-induced cardiomyocyte death. However, no direct evidence is available on the causal relationship between calpain and doxorubicin-induced cardiotoxicity. Thus, in this study, we employed transgenic mice over-expressing calpastatin to investigate the role of calpain in neonatal cardiomyocytes, adult cardiomyocytes, and in vivo mouse models of doxorubicin-induced cardiotoxicity.

2. Methods

2.1 Animals

This investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011). All experimental procedures were approved by the Animal Use Subcommittee at the University of Western Ontario, Canada. Breeding pairs of C57BL/6 mice were purchased from the Jackson Laboratory and transgenic mice with over-expression of calpastatin (Tg-CAST, C57BL/6 background) were kindly provided by Dr Laurent Baud (the Institut National de la Sante´ et de la Recherche Me´dicale, Paris, France) through the European Mouse Mutant Archive. A breeding program was implemented at our animal care facilities.

2.2 Neonatal and adult mouse cardiomyocytes cultures

Neonatal mice (born within 2 days) were euthanized by decapitation and the neonatal cardiomyocytes were prepared and cultured according to methods we described previously. Adult mice were anaesthetized with ketamine (100 mg/kg)/xylazine (5 mg/kg, i.p.) and their hearts were isolated and perfused after ensuring that they did not respond to needle-punch to the skin. Mouse ventricular cardiomyocytes were isolated as previously described with modifications. Cardiomyocytes were re-suspended in a plating buffer (MEM supplemented with 2, 3-Butanedione monoxime 10 mM, penicillin–streptomycin 100 U/mL, and l-glutamine 2 mM/L), and seeded in 12-well laminin-coated dishes. After 2 h incubation at 37 °C (95% air/2% CO2), the cells were maintained in a culture medium (MEM) and were used for experiments after 2 h.

2.3 Reagents

LY294002, Propidium iodide (PI), N-acetyl cysteine (NAC), PD150606, and calpain inhibitor-III were purchased from Sigma or Calbiochem. Annexin-V conjugated with FITC and Hoechst 33324 were from Invitrogen.

2.4 Echocardiography

Animals were lightly anaesthetized with inhaled isoflurane (1%) and imaged in a warm handling platform using a 40 MHz linear array transducer (MS-550D, VisualSonics, Toronto, Canada) attached to a preclinical ultrasound system (Vevo 2100, VisualSonics) with nominal in-plane spatial resolution of 40 μm (axial) × 80 μm (lateral). M-mode and 2-D parasternal short-axis scans (133 frames/s) at the level of the papillary muscles were used to assess changes in left-ventricular (LV) end-systolic inner diameter (LVId), LV end-diastolic inner diameter (LVIDd), LV posterior wall thickness in end-diastole (LVPW;d), and end-systole (LVPW;s). LV volumes at end-diastole (LVEDV) and end-systole (LVESV) were estimated using the formulas: LVEDV = [7/2(LVIDd3)] × [LVIDd] and LVESV = [7/2(LVIDd3)] × [LVIDs3]. LV fractional shortening (FS) and ejection fraction (EF) were used as indexes of cardiac contractile function and were calculated from the inner diameters according to the formula: FS [%] = (LVIDd-LVIDs)/LVIDd × 100, and from LV volumes according to the formula: EF [%] = (LVEDV-LVESV)/LVEDV × 100, respectively.

2.5 In vivo LV pressure–volume measurements

Mice were anaesthetized with ketamine (100 mg/kg)/xylazine (5 mg/kg, i.p.) and ventilated. The chest was opened and a Scisense mouse PV catheter (FTE-1212B-4518, 1.2F) was directly inserted into the LV via the apex to measure LV pressures and volumes as we described recently.

2.6 Adenoviral infection of cardiomyocytes

Neonatal cardiomyocytes were infected with adenoviral vectors containing human calpain-1 (Ad-Capn1, SigmaGen Laboratories), calpain-2 (Ad-Capn2, Applied Biological Materials, Inc.), calpastatin (Ad-CAST, Applied Biological Materials, Inc.), or beta-gal (Ad-gal, Vector Biolabs) as a control at a multiplicity of infection of 100 PFU/cell. Adenovirus-mediated gene transfer was implemented as we previously described. Adenoviral vector-mediated transfection efficiency in cardiomyocytes was >90% by determining beta-gal expression.

2.7 Calpain activities

Calpain activities in tissue extracts were detected by using a fluorescence substrate N-succinyl-LLLVY-AMC (Cedarlane Laboratories, Canada) as described in our recent studies. Calpain activities were also determined in cultured cardiomyocytes in situ by using N-succinyl-LLVY-AMC as previously described.

2.8 Active caspase-3

Caspase-3 activity was measured using caspase-3 fluorescence assay kit (Biomol Research Laboratories, USA) as described in our previous reports.

2.9 Measurement of cellular DNA fragmentation

Cardiomyocytes were pre-labelled with BrdU and then incubated with doxorubicin. DNA fragmentation was measured using a Cellular DNA Fragmentation ELISA kit (Roche Applied Science, Canada) according to the manufacturer’s instructions.
2.10 Annexin-V and PI staining
Cardiomyocyte death was measured by annexin-V, PI, and Hoechst 33342 staining as described previously. Briefly, Annexin-V conjugated with FITC, PI, and Hoechst 33342 were added directly to the culture medium, and cardiomyocytes were photographed under both phase contrast and fluorescent conditions. At least 200 cardiomyocytes were examined from each sample, and each condition was measured in triplicate.

2.11 Western blot analysis
Expressions of calpastatin, calpain-1, calpain-2, cleaved caspase-3, protein kinase B (AKT), phosphorylated AKT (Thr308), glycogen synthase kinase (GSK)-3β, and phosphorylated GSK-3β (Ser9) and GAPDH proteins were determined by western blot analysis using specific antibodies (Cell Signalling, 1/1000).

2.12 Real-time reverse-transcriptase–polymerase chain reaction
Total RNA was extracted from cultured cardiomyocytes using Trizol Reagent (Gibco-BRL, USA) following the manufacturer’s instructions. Real-time reverse-transcriptase–polymerase chain reaction (RT–PCR) was performed to analyse mRNA expression for capn1, capn2, AKT1, and GAPDH as described previously.

2.13 Intracellular reactive oxygen species measurement
The formation of reactive oxygen species (ROS) was measured by using 2, 7-dichlorodihydrofluorescein diacetate (DCF-DA, Invitrogen), as an indicator, as described in our recent report. Briefly, cardiomyocytes were homogenized in assay buffer. The homogenates were incubated with DCF-DA at 37°C for 3 h. The fluorescent product formed was quantified by spectrofluorometer at the 485/525 nm. Changes in fluorescence were expressed as arbitrary unit.

2.14 Statistical analysis
All data were presented as mean ± SD. ANOVA followed by Newman–Keuls test was performed for multi-group comparisons. Survival curves were created by the method of Kaplan and Meier, and compared by log-rank test. A value of P < 0.05 was considered statistically significant.

3. Results

3.1 Effect of doxorubicin on calpain activity
To begin to understand the role of calpain, we measured calpain activity in cultured neonatal cardiomyocytes. Twenty-four hours after doxorubicin incubation, calpain activity was decreased in a dose-dependent manner (0, 0.1, 1, and 2.5 μmol/L) in culture medium with 1% FBS (Figure 1A). Doxorubicin (1 μmol/L) time-dependently decreased calpain activity in cardiomyocytes (Figure 1B). The reduction in calpain activity correlated with down-regulation of calpain-1 and calpain-2 proteins in doxorubicin-incubated cardiomyocytes (Figure 1C). In addition, the protein levels of calpastatin were also reduced in doxorubicin-stimulated cardiomyocytes (Figure 1C). Similarly, injection of doxorubicin (20 mg/kg, i.p.) also decreased calpain activity in the mouse heart (Figure 1D).

It has been shown that doxorubicin induces calpain inactivation in SH-SY5Y human neuroblastoma cells through a mechanism related to oxidative stress. To examine whether it is also operative in cardiomyocytes, we incubated cardiomyocytes with doxorubicin (1 μmol/L) in the presence of NAC (2.5 mmol/L), an antioxidant or vehicle for 24 h. NAC significantly reduced ROS production in cardiomyocytes (Figure 1E); however, there was no change in calpain activity between NAC and vehicle-treated cardiomyocytes (Figure 1F). This result argues that oxidative stress may not contribute to calpain inactivation in doxorubicin-stimulated cardiomyocytes.

3.2 Effects of calpain inhibition on doxorubicin-induced injuries in cardiomyocytes
To examine the effects of calpain inhibition on cardiomyocyte injuries, we incubated neonatal cardiomyocytes with doxorubicin in the presence of pharmacological calpain inhibitors, calpain inhibitor-III (10 μmol/L), PD150606 (5 μmol/L), or vehicle. In agreement with the previous reports, doxorubicin treatment (1 μmol/L) significantly increased caspase-3 activity and DNA fragmentation in cardiomyocytes (Figure 2A and B), indicative of apoptotic cell death. These effects of doxorubicin were significantly enhanced in cardiomyocytes by calpain inhibitor-III and PD150606. The effect of calpain inhibition on apoptosis was further confirmed by detection of cleaved caspase-3 fragment, an active caspase-3 which showed that the levels of active caspase-3 fragment were much greater in calpain inhibitor-III compared with vehicle-treated cardiomyocytes (Figure 2C). These results indicate the detrimental effects of calpain inhibition in doxorubicin-stimulated cardiomyocytes.

Calpastatin is an endogenous calpain inhibitor. Over-expression of calpastatin has been successfully used to inhibit calpain activation in cardiomyocytes and in vivo hearts. To substantiate the effects of calpain inhibition, we isolated and cultured neonatal cardiomyocytes from Tg-CAST and wild-type mice, and then incubated them with doxorubicin or vehicle. Consistently, calpain activity was markedly reduced in Tg-CAST cardiomyocytes (see Supplementary material online, Figure S1). Twenty-four hours after doxorubicin treatment, over-expression of calpastatin significantly enhanced caspase-3 activity, cleaved caspase-3 and DNA fragmentation in Tg-CAST compared with wild-type cardiomyocytes (Figure 2D–F). These results suggest that over-expression of calpastatin augments cardiac injuries and thus, confirm that calpain inhibition exacerbates injuries in cardiomyocytes induced by doxorubicin.

To determine whether the effects of calpain inhibition on doxorubicin-induced injuries were reproducible in adult cardiomyocytes, cardiomyocytes were isolated from adult Tg-CAST mice and their wild-type littermates. After exposure to doxorubicin for 24 h, apoptosis was determined by measuring caspase-3 activity and the percentage of annexin-V positive/PI negative cardiomyocytes. Caspase-3 activity was much greater in Tg-CAST compared with wild-type cardiomyocytes (Figure 3A). Similarly, over-expression of calpastatin further increased the percentage of annexin-V positive/PI negative cardiomyocytes after doxorubicin incubation (Figure 3B and C). Thus, these results indicate that calpastatin over-expression through calpain inhibition also plays an important role in adult cardiomyocytes in terms of apoptosis induced by doxorubicin.

The effect of calpastatin over-expression on cardiomyocyte death was also assessed by PI nuclear staining alone. Doxorubicin increased the percentage of PI nuclear positive cardiomyocytes from wild-type mice, which was further increased by calpastatin over-expression (Figure 3D). Although PI does not enter cells with intact membrane, apoptotic cells lose membrane integrity in the late stage. As such, both necrotic and apoptotic cells may contain PI. Thus, these data provide further evidence which demonstrates that calpastatin plays a detrimental role in doxorubicin-stimulated cardiomyocytes.
3.3 Effects of calpain-1 and calpain-2 over-expression on doxorubicin-induced apoptosis

Having shown that doxorubicin reduced calpain activity and inhibition of calpain enhanced apoptosis, we hypothesized that over-expression of calpain might protect cardiomyocytes against doxorubicin-induced apoptosis. To test this hypothesis, we up-regulated calpain-1 and calpain-2 in neonatal cardiomyocytes by infection with Ad-Capn1 or Ad-Capn2. Ad-gal served as a control. Twenty-four hours after infection, cells were incubated with doxorubicin (1 μmol/L) or saline for another 24 h. Over-expression of calpain-1 further increased doxorubicin-induced apoptosis (Figure 4A and B), suggesting a detrimental role of calpain-1. In contrast, over-expression of calpain-2 significantly reduced doxorubicin-induced apoptosis in cardiomyocytes (Figure 4A and B). These data suggest that calpain-2 protects cardiomyocytes against apoptosis in doxorubicin-stimulated cells.

3.4 Effect of calpain inhibition on the AKT signalling in doxorubicin-induced cardiotoxicity

It has been shown that AKT activation protects cardiomyocytes against doxorubicin-induced toxicity.26–29 This was also demonstrated in the present study in which treatment with the AKT inhibitor LY294002 enhanced caspase-3 activity and DNA fragmentation in doxorubicin-stimulated cardiomyocytes (Figure 5A and B). Calpain has been demonstrated to negatively30,31 and positively32,33 regulate the AKT signalling. To investigate whether calpain inhibition
compromises the AKT signalling in response to doxorubicin, we first measured total and phosphorylated AKT in cardiomyocytes by western blot analysis. Over-expression of calpastatin reduced AKT protein in doxorubicin-stimulated cardiomyocytes \((\text{Figure 5C and D})\). Similarly, the levels of phosphorylated AKT were also reduced in calpastatin over-expressed cardiomyocytes after exposure to doxorubicin \((\text{Figure 5C and E})\). Furthermore, over-expression of calpastatin decreased the mRNA levels of AKT1 in doxorubicin-stimulated cardiomyocytes \((\text{Figure 5F})\), suggesting that calpain may regulate AKT1 transcription in response to doxorubicin. Similarly, incubation with calpain inhibitor-III reduced AKT protein and phosphorylation in doxorubicin-stimulated cardiomyocytes (see Supplementary material online, \text{Figure S2A–C}).

It is well known that AKT phosphorylates glycogen synthase kinase-3beta (GSK-3\textit{\beta}) at Ser9, leading to its inactivation. Inactivation of GSK-3\textit{\beta} has been shown to block cardiomyocyte apoptosis induced by doxorubicin.\textsuperscript{34} To further address the effect of calpain inhibition on the AKT signalling, we also analysed GSK-3\textit{\beta} phosphorylation as a downstream target. In line with the alteration of AKT, calpain inhibitor-III significantly decreased the levels of phosphorylated GSK-3\textit{\beta} \((\text{Ser9})\) in doxorubicin-incubated cardiomyocytes (see Supplementary material online, \text{Figure S2D–F}), whereas the total GSK-3\textit{\beta} protein was comparable (see Supplementary material online, \text{Figure S2D–F}). Thus, these results suggest that calpain inhibition exacerbates doxorubicin-induced injuries at least in part by negatively modulating the AKT/GSK-3\textit{\beta} signalling in cardiomyocytes.

**Figure 2** Effects of pharmacological calpain inhibitor and over-expression of calpastatin on cell death in cardiomyocytes. Neonatal cardiomyocytes were incubated with Doxorubicin (DOX) or saline in the presence of calpain inhibitor-III (CI-III), PD150606 or vehicle. (A) Caspase-3 activity in cell lysates. (B) DNA fragmentation in cardiomyocytes. (C) A representative western blot for cleaved caspase-3 fragment and GAPDH in duplication from three different cultures. Cardiomyocytes were isolated from Tg-CAST and wild-type (WT) neonates. Twenty-four hours after Doxorubicin incubation (1 \(\mu\text{mol/L}\)), caspase-3 activity \((\text{D})\) and DNA fragmentation \((\text{E})\) were measured. (F) A representative western blot for cleaved caspase-3 fragment and GAPDH in duplication from three different cultures. Data are mean ± SD from three independent cell cultures. *\(P < 0.05\) vs. saline; #\(P < 0.05\) vs. DOX+vehicle or DOX+WT.
3.5 Role of calpastatin in myocardial function of doxorubicin-induced cardiotoxicity

To determine the in vivo significance of calpastatin over-expression, we injected doxorubicin (20 mg/kg, i.p.) or saline into adult calpastatin transgenic mice and their wild-type littermates. Five days later, myocardial function was assessed by echocardiography. Both calpain-1 and calpain-2 expressions were not altered in transgenic mouse hearts compared with their wild-type littermates (see Supplementary material online, Figure S3). The heart rate remains unchanged between Tg-CAST mice and their wild-type littermates (both saline- and doxorubicin-treatment). The systolic function indexes per cent fractional shortening and ejection fraction showed a significant decrease in doxorubicin-treated Tg-CAST mice and their wild-type littermates compared with their sham animals (Figure 6A and B). However, Tg-CAST mice exhibited relatively worse performance in per cent fractional shortening and ejection fraction compared with their wild-type littermates after doxorubicin treatment (Figure 6A and B), suggesting that over-expression of calpastatin further compromises myocardial function after doxorubicin injection. Similar to a previous report, the LV diastolic dimension was significantly lower in doxorubicin-treated than in saline-treated wild-type mice, and there was no change of LV systolic dimension between them; however, both diastolic and systolic dimensions were significantly increased in doxorubicin-treated compared with saline-treated Tg-CAST mice (see Supplementary material online, Figure S4).

Myocardial function was further determined by haemodynamic measurement. Similar to the alteration in echocardiography, the maximal positive and negative first derivative of LV pressure (+dP/dt_max and −dP/dt_min) were significantly reduced in doxorubicin-treated compared with sham animals. Over-expression of calpastatin further decreased LV +dP/dt_max and −dP/dt_min in Tg-CAST mice.
compared with their wild-type littermates after doxorubicin treatment (Figure 6C and D). These data confirm that over-expression of calpastatin enhances myocardial dysfunction in doxorubicin-cardiotoxicity.

The mortality was also monitored for 5 days after doxorubicin. As shown in Figure 6E, the 5-day mortality was greater in Tg-CAST mice compared with their wild-type mice (29.16% vs. 8%); however, the difference was not statistically significant.

4. Discussion

Calpains have been implicated in a variety of cardiovascular diseases.6–8,11–15,24 In the present study, we elucidated the effects of calpain inhibition by calpastatin over-expression on doxorubicin-induced cardiotoxicity. Our major finding is that over-expression of calpastatin or incubation with calpain inhibitors enhances, whereas up-regulation of calpain-2 reduces doxorubicin-induced apoptosis in cultured cardiomyocytes. This effect of calpain inhibition correlates with the suppression of the AKT signalling in cardiomyocytes. Transgenic over-expression of calpastatin exacerbates myocardial dysfunction and increases the mortality in doxorubicin-treated mice. Thus, this study reveals a novel effect of calpain inhibition in promoting doxorubicin-induced cardiotoxicity.

Calpain activity is mainly regulated by altering the Ca\(^{2+}\) concentration required for calpain activity. It has been demonstrated that certain phospholipids lower the Ca\(^{2+}\) concentration required for autolysis of calpain-1 and calpain-2.36 Mitogen-activated protein kinase ERK1/2,37 protein kinase C,38 and protein kinase A39 can phosphorylate calpain thereby regulating its activity. In cultured rat cardiomyocytes and the in vivo rat heart, two previous studies showed that doxorubicin increased calpain activity.16,17 In striking contrast to those reports, our data demonstrate that doxorubicin decreases calpain activity in cultured mouse cardiomyocytes in situ and in cell lysates from both cultured mouse cardiomyocytes and the in vivo mouse heart. This reduction is associated with down-regulation of calpain proteins but not transcriptional expression in doxorubicin-treated cardiomyocytes. It is currently unknown what causes this discrepancy. However, this is not due to the different species used (mouse vs. rat) because doxorubicin also decreased calpain activity in rat myoblasts H9c2 cells (data not shown). In an early report, calpain was inactivated in SH-SYSY human neuroblastoma cells in response to doxorubicin.22 It was further demonstrated that calpain inactivation was attributed to oxidative stress induced by doxorubicin as calpain activity was recovered by antioxidants. However, our recent studies have demonstrated that NADPH oxidase signalling through reactive oxygen species production promotes calpain activation in cardiomyocytes under different stresses.6,7,12, suggesting oxidation-induced calpain inactivation is unlikely to be operative in cardiomyocytes. Indeed, the present study shows that inhibition of ROS does not have any effect on calpain activities in doxorubicin-treated cardiomyocytes.

Calpain has been suggested to play a pro-apoptotic or anti-apoptotic role in a variety of cell types. The role of calpain in cell death is likely dependent on cell types and insults.40 In this study, we demonstrate that over-expression of calpastatin enhances cardiomyocyte death in response to doxorubicin and thus, aggravates doxorubicin-induced cardiotoxicity. Several lines of evidence support this conclusion. First, over-expression of calpastatin enhances doxorubicin-induced apoptosis in neonatal cardiomyocytes. Secondly, apoptotic cell death is increased in adult cardiomyocytes from Tg-CAST compared with wild-type mice after doxorubicin treatment. Lastly, over-expression of calpastatin significantly aggravates myocardial dysfunction and increases mortality in Tg-CAST mice compared with their wild-type littermates after doxorubicin injection, suggesting a functional significance of calpastatin over-expression in doxorubicin cardiotoxicity. These effects of calpastatin may be mediated through calpain inhibition because over-expression of calpastatin inhibits calpain activity and pharmacological inhibition of calpain phenocopies the effects of calpastatin over-expression in doxorubicin-incubated mouse neonatal and adult cardiomyocytes. Since calpastatin is an endogenous calpain inhibitor, which does not inhibit any other proteases being tested so far, our data provide convincing evidence to support the view that inhibition of calpain aggravates doxorubicin-induced cardiotoxicity. Furthermore, up-regulation of calpain-2 reduces doxorubicin-induced apoptosis, providing direct evidence to support the protective role of calpain-2 in doxorubicin-induced cardiotoxicity. However, our findings are apparently different from the two previous reports which showed that inhibition of calpain reduced doxorubicin cardiotoxicity in cultured rat cardiomyocytes.16,17 It remains unknown whether this is due to the use of different calpain inhibitors. In previously reported studies,16,17 different pharmacological calpain inhibitors were employed, which may complicate the interpretation of the results as those inhibitors may not be specific for calpain and may have other effects.40
AKT is a potent regulator of cell proliferation and cell survival, and prevents cell death in a variety of settings. Adenoviral transfer of the constitutively active form of AKT to cardiomyocytes restored cell survival after doxorubicin treatment, suggesting a beneficial role of AKT in cardiotoxicity. This was then supported by an in vivo study which demonstrated that elevated myocardial AKT signalling ameliorated doxorubicin-induced congestive heart failure and promoted heart growth. Thus, AKT is an important mechanism mediating protection against doxorubicin-induced cardiotoxicity in doxorubicin cardiotoxicity. It is known that phosphoinositide 3-kinase (PI3K) activates the AKT signalling, whereas protein phosphatase 2A (PP2A) interacts with and suppresses the AKT signalling by de-phosphorylating AKT. A recent study has shown that calpain disrupts the interaction of AKT and PP2A thereby activating AKT signalling.

Interestingly, we showed that inhibition of calpain down-regulated the levels of AKT mRNA and protein, leading to a decrease in phosphorylated AKT in doxorubicin-induced cardiomyocytes. This suggests that calpain may promote AKT transcriptional expression in doxorubicin cardiotoxicity; however, this needs to be addressed in future studies. The down-regulation of AKT signalling correlated with a further increase in cardiac injuries after doxorubicin treatment. This suggests that calpain may protect cardiomyocytes against doxorubicin cardiotoxicity by promoting the AKT survival signalling. AKT phosphorylates GSK-3β (Ser9), leading to its inactivation. Previous study has shown that inhibition of GSK-3β reduces doxorubicin-induced cardiomyocyte apoptosis, mirroring the role of AKT activation. Accordingly, our data demonstrate that calpain inhibition further decreases the phosphorylation of GSK-3β in cardiomyocytes.

Figure 5 Effects of AKT inhibitor on cell death and over-expression of calpastatin on AKT signalling. (A and B) Neonatal cardiomyocytes were incubated with saline or Doxorubicin (DOX, 1 μmol/L) in the presence of LY294002 (2 μmol/L) or vehicle. Caspase-3 activity (A) and DNA fragmentation (B) were measured. (C–F) Neonatal cardiomyocytes were infected with Ad-CAST or Ad-gal and then incubated with DOX (1 μmol/L) or saline. Twenty-four hours after DOX incubation, total AKT and phosphorylated AKT (pAKT) protein, and AKT1 mRNA were determined by western blot and real-time RT–PCR, respectively. (C) A representative western blot for AKT, phosphorylated AKT, and GAPDH in duplication from three different cultures. (D and E) Quantification of AKT/GAPDH and pAKT/GAPDH ratio, respectively. (E) Quantification of AKT1/GAPDH mRNA ratio. Data are mean ± SD from three indifferent cell cultures. *P < 0.05 vs. saline and #P < 0.05 vs. DOX+vehicle or DOX+Ad-gal.
doxorubicin-treated cardiomyocytes, providing further evidence to support the view that calpain inhibition enhances doxorubicin cardiotoxicity through suppression of the AKT signalling. In contrast to our findings, calpain activation has been shown to cleave HSP90 and thus inhibit AKT signalling.30 Calpain also activates GSK-3β by removing its N-terminal small fragment.46 It remains unknown about the differential modulation of AKT and GSK-3β signalling by calpain, which merits further investigations.

In summary, we have shown that over-expression of calpastatin exacerbates cardiac injuries induced by doxorubicin through calpain inhibition and up-regulation of calpain-2 provides a protective effect. This effect of calpain inhibition may be mediated at least in part by down-regulating AKT expression in cardiomyocytes. The present study suggests that calpain inhibition may be detrimental in doxorubicin-mediated cardiotoxicity. Given the fact that calpains have been implicated in processes crucial for cancer development and progression47 and thus, they may represent important potential anti-cancer targets, our finding suggests that potential cardiac complications of calpain inhibition should be considered when designing new calpain inhibitors as a novel approach to limiting development of primary tumours and formation of metastases.48

**Supplementary material**

Supplementary material is available at *Cardiovascular Research* online.

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