Mechanical stretch induces mitochondria-dependent apoptosis in neonatal rat cardiomyocytes and G$_2$/M accumulation in cardiac fibroblasts

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ABSTRACT

Heart remodeling is associated with the loss of cardiomyocytes and increase of fibrous tissue owing to abnormal mechanical load in a number of heart disease conditions. In present study, a well-described *in vitro* sustained stretch model was employed to study mechanical stretch-induced responses in both neonatal cardiomyocytes and cardiac fibroblasts. Cardiomyocytes, but not cardiac fibroblasts, underwent mitochondria-dependent apoptosis as evidenced by cytochrome c (cyto c) and Smac/DIABLO release from mitochondria into cytosol accompanied by mitochondrial membrane potential ($\Delta\psi_m$) reduction, indicative of mitochondrial permeability transition pore (PTP) opening. Cyclosporin A, an inhibitor of PTP, inhibited stretch-induced cyto c release, $\Delta\psi_m$ reduction and apoptosis, suggesting an important role of mitochondrial PTP in stretch-induced apoptosis. The stretch also resulted in increased expression of the pro-apoptotic Bcl-2 family proteins, including Bax and Bad, in cardiomyocytes, but not in fibroblasts. Bax was accumulated in mitochondria following stretch. Cell permeable Bid-BH3 peptide could induce and facilitate stretch-induced apoptosis and $\Delta\psi_m$ reduction in cardiomyocytes. These results suggest that Bcl-2 family proteins play an important role in coupling stretch signaling to mitochondrial death machinery, probably by targeting to PTP. Interestingly, the levels of p53 were increased at 12 h after stretch although we observed that Bax upregulation and apoptosis occurred as early as 1 h. Adenovirus delivered dominant negative p53 blocked Bax upregulation in cardiomyocytes but showed partial effect on preventing stretch-induced apoptosis, suggesting that p53 was only partially involved in mediating stretch-induced apoptosis. Furthermore, we showed that p21 was upregulated and cyclin B1 was downregulated only in cardiac fibroblasts, which may be associated with G$_2$/M accumulation in response to mechanical stretch.

Keywords: apoptosis, mechanical stretch, Bcl-2 and its family proteins, mitochondria, cardiomyocyte.

INTRODUCTION

Abnormal mechanical stretch triggers cardiac remodeling, which is characterized by the loss of contractile tissue, compensatory hypertrophy of cardiomyocytes and increase in fibrous tissue[1]. Increasing evidences suggest that mechanical overload causes apoptotic cell death in heart and vascular system[2,3] and the resultant cardiomyocyte loss is believed to be responsible for the transition from hypertrophy to heart failure[4]. On the other hand, abnormal stretch simultaneously causes cardiac fibroblasts proliferation[5], which will result in increase of fibrous tissue and decrease of contraction power. Both cardiomyocyte apoptosis and cardiac fibroblasts proliferation are crucial events in development of cardiac remodeling and transition from remodeling to heart failure, but the detailed signaling mechanism of stretch-induced responses in cardiac cells has not been fully understood yet.

Mechanical stretch is able to activate multiple signaling pathways, including MAPK, JAK/STAT and PKC, etc[6], which may be responsible for either myocyte hypertrophy or apoptosis, depending on the systems used and the degree of stretch[7]. It was suggested that mechanical stretch induced enhanced autosecretion of Angiotensin II, which led to Bax expression and apoptosis in adult cardiomyocytes in a p53-dependent manner[8, 9], although other reports challenged this view[10]. Another report ascribed mechanical stretch-induced apoptosis of cardiomyocytes to the production of reactive oxygen species during periodic stretch[7]. It seems that a number of

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signaling pathways are responsible for regulating the mechanical stretch-induced apoptotic pathways[11-13]. Despite recent advancing of our understanding of the mechanisms of apoptosis in cardiomyocytes, how the mechanical stretch signal is coupled to mitochondrial apoptosis machinery in cardiomyocytes remains poorly understood.

Another major issue of heart remodeling is the cardiac fibroblasts, which consist of one-third of the overall heart mass, play fundamental roles in the functional integrity of the organ, and are the major source of extracellular matrix production[14]. It has been found that reduction of contractile tissue due to myocytes apoptosis usually occurs in parallel with an increase of interstitial fibrosis[5]. Some reports suggest that mechanical stretch could induce proliferative effects in cardiac fibroblasts by paracrine or autocrine of growth factors[15], although there are other reports that do not support this view[10]. The molecular details of stretch-induced proliferative response in cardiac fibroblasts are still not clear.

In present work, we employed an *in vitro* system to study stretch-induced responses in both cardiomyocytes and cardiac fibroblasts. Our results demonstrated that mechanical stretch induced mitochondria-dependent apoptosis and mitochondrial PTP was involved in mediating stretch-induced cyto c release in cardiomyocytes. In contrast, mechanical stretch induced G_2/M accumulation in cardiac fibroblasts rather than apoptosis, which may be associated with upregulation of p21. A better understanding mechanical stretch induced signaling in heart cells and the associated heart remodeling may offer novel promises for therapeutic interventions.

MATERIALS AND METHODS

Cell culture and treatments

Ventricular myocytes isolated from the hearts of neonatal Wistar rats (1-3 days after birth) were cultured according to a well-established method[6]. Briefly, hearts from neonatal Wistar rats were removed, minced and trypsinized at 37°C with gentle stirring in HEPES buffered Saline A (in mM: 137 NaCl, 5.4 KCl, 4.2 NaHCO$_3$, 5.5 glucose, 1.0 HEPES, pH 7.4) containing 0.1% trypsin (Gibco). Then cells were centrifuged and resuspended in Dulbecco's modified Eagle medium/F-12 (Gibco) containing 10% fetal bovine serum (Hyclone), 100 U/ml penicillin and 100 μg/ml streptomycin. After preplaced at 37°C for 90 min, the unattached cardiomyocytes were seeded at a density of $5×10^4$ cells/cm^2 into the cell stretch system described below. 0.1 mM BrdU (5-Bromo-2'-deoxyuridine, Sigma) was added to the culture for 36 h to inhibit the proliferation of non-myocytes. Over 95% of the cells thus obtained were cardiomyocytes, as identified by anti-α-sarcomeric actin antibody immunostaining (data not shown). Cardiac fibroblasts were prepared from cells that adhered to the culture dish during the preplacing procedure. After 3 passages of subculture, over 95% of cells were identified as cardiac fibroblasts. Cells were grown in DMEM/F-12 containing 10% fetal bovine serum for 72 h and reached 80% confluence. A cell stretch

model was established as Lee et al described with modifications[16]. Clamped silicone elastic membrane (Specialty MFG, MI, USA) in a single well device was uniformly stretched by indentation, resulting in homogeneous equibiaxial strains of the cultured cells. Cells were seeded onto a sheet of membrane finely coated with rat-tail collagen (0.1 mg/ml, in 0.01% acetic acid). Control cells were cultured under identical conditions but were not subject to stretching. After 24 h serum starvation, 20%-sustained stretch was applied when the medium was replaced with fresh complete medium. Extensive and sustained stretch is desirable to investigate the stretch-induced apoptosis signals since moderate cyclic stretch induced more complex response as previously reported[17].

Apoptosis assays

Apoptosis was examined by detecting phosphatidylserine exposure on cell membrane with Annexin V, as described[18]. Cells were simultaneously stained with Annexin V-FITC (25 ng/ml; green fluorescence) and dye exclusion (PI, red fluorescence). This assay discriminates between intact (FITC$^-$/PI$^-$), early apoptotic (FITC$^+$/PI$^-$), and late apoptotic cells (FITC$^+$/PI$^+$). Comparative experiments were performed at the same time by bivariate flowcytometry using a FACScan (BD) and analyzed with CellQuest software on data obtained from the cell population from which debris was gated out.

Nuclear condensation and fragmentation were detected in some experiments using DAPI staining[18]. Cells were washed with PBS and stained with 10 ng/ml DAPI (4, 6-Diamidino-2-phenyindole, Sigma-Aldrich, Inc.) before visualization with fluorescent microscopy. At least 200 cells were counted in each experiment. Caspase 3 protease activity was determined, using the p-nitroanilide-derived chlorometric substrate acetyl-Asp-Glu-Val-Asp-p-nitroanilide (DEVD-pNA, BIOMOL, Inc.)[19]. Enzyme-catalyzed release of pNA was monitored at 405 nm in a 96-well microtiter plate reader. Caspase 3 activity was expressed as relative absorbance at 405 nm. The Caspase inhibitor z-VAD-fmk (Clontech) was used to confirm caspases activation. Caspase 9 activation was determined by detecting its activated fragments by Western blotting. Pro-caspase 9 (48 kD) was cleaved into 35 kD fragment during apoptosis.

Cell cycle assay

Cell cycle was examined by propidium iodide (PI, Sigma-Aldrich, Inc) staining. In brief, $5×10^6$ cells were harvested and washed twice with Ca^{2+}/Mg^{2+} free phosphate-buffered saline (PBS), fixed overnight in 75% cold ethanol, digested with RNase A (Sigma-Aldrich, Inc.) and stained with PI (100 μg/ml). Data was obtained and analyzed by flow cytometry using the CellQuest software on a FACScan (BD) from a cell population from which debris was gated out.

Immunoblotting

Western blotting was performed according to our published method [18]. Rat cardiocytes were prepared, and lysed in lysis buffer (in mM: 25 HEPES, pH 7.4, 5 EDTA, 8.0 EGTA, 1.0 Na$_3$VO$_5$, 0.25 NaF, 0.1 phenylmethylsulfonyl fluoride, 1.0 dithiothreitol; and 1% NP-40, 5 μg/mL aprotinin, 100 μg/ml leupeptin, 50 μg/ml Trypsin Inhibitor). Cellular protein (20 μg) was loaded and separated on sodium dodecyl sulfate polyacrylamide gel (BioRad mini gel, 6-12% according to target protein molecular weight) and transferred to a nitrocellulose membrane (GibcoBRL) by the standard electric transfer protocol. The membrane was blocked at room temperature with

PBS containing 0.1% Tween-20 (PBST) plus 5% non-fat milk for 2 h, probed with antibodies overnight at 4 °C, then incubated with horseradish peroxidase-labeled second antibody (KPL Corp.) in blocking buffer for 2 h at room temperature. The membrane was then exposed to an enhanced chemiluminescent system (Pierce) and autoradiography was used to visualize immuno-reactive bands.

Cell fractionation

Cell fractionation followed our published protocol[19]. Briefly, 1×10^6 cells were washed twice with ice-cold PBS, and resuspended with five volumes of buffer A (in mM: 20 HEPES-KOH, pH 7.2, 10 KCl, 1.5 MgCl$_2$, 1.0 EDTA, 1.0 EGTA, 250 sucrose, 1.0 dithiothreitol, 0.1 phenylmethylsulfonyl fluoride, 10 aprotinin, leupeptin and pepstatin). Cells were kept on ice for 30 min after which cell suspension was gently homogenized with a Dounce homogenizer (5-10 strokes) and cell lysates were checked by trypan blue staining. The homogenate was centrifuged at 750 g for 5 min at 4°C, and the supernatant subjected to further centrifugation at 10,000g for 10 min at 4 °C. This pellet, containing mitochondria, was designated P10. The supernatant, designated S10, was subjected to further ultracentrifugation at 100,000 g for 45 min at 4°C. The resulting pellet and supernatant were designated P100 and S100. Cyto c was not detectable in the P100 fraction. In most case, there is no detectable cyto c in untreated samples. In certain cases, there was a trace amount of cyto c in untreated samples due to leakage of cyto c during manipulation.

Mitochondrial membrane potential ($\Delta\psi_m$) assay

Dψ_m was measured by either laser scan confocal microscopy or flow cytometry, respectively[19, 20]. For confocal microscopy, cells were incubated for 45 min in DMEM containing 5 μM Rhodamin-123 (Molecular Probes), a typical dye for probing $\Delta\psi_m$, and washed with Locke's buffer (in mM: 154 NaCl, 5.6 KCl, 2.3 CaCl$_2$, 1.0 MgCl$_2$, 3.6 NaHCO$_3$, 5.0 glucose, 5.0 HEPES, pH 7.2). Cellular fluorescence in random selected fields was imaged with a laser scan confocal microscope (Zeiss, LSM510) with excitation at 488 nm and emission at 530±30 nm.

$\Delta\psi_m$ was also examined by flow cytometry with DiOC$_6$[3] staining. 20 nM DiOC$_6$[3] (Molecular Probes) was loaded into 4×10^5 cells suspended in 0.5 mL fresh DMEM (pH 7.2) and incubated at 37°C for 5 min. Each examination was performed in duplicate. Data were validated after 5 min of DiOC6[3] loading by the addition of 20 mM CCCP, the $\Delta\psi_m$ uncoupler. DiOC$_6$[3] fluorescence was examined at 530±30 nm. Data were obtained and analyzed with the CellQuest software from a PI negative cell population on a BD FACScan.

Construction and infection of adenovirus containing p53DN

A dominant negative mutant of the human p53 gene[21], p53DN[175], was constructed into a recombinant Adenovirus which contained GFP in a separate open reading frame using the AdEasy adenovirus system as described by He et al[22]. Cardiomyocytes were infected with Ad-p53DN[175] 12 h after seeding, the total infection period lasting for 24 h before stretch was applied. Adenovirus containing GFP and LacZ gene (Ad-LacZ) was used as vector control.

Statistical analysis

All results are the mean±SD of at least 3 independent experiments unless stated otherwise. Significance was determined using Student's unpaired t test or one-way ANOVA (SPSS software). Difference between groups were considered significant at a value of $p<0.05$.
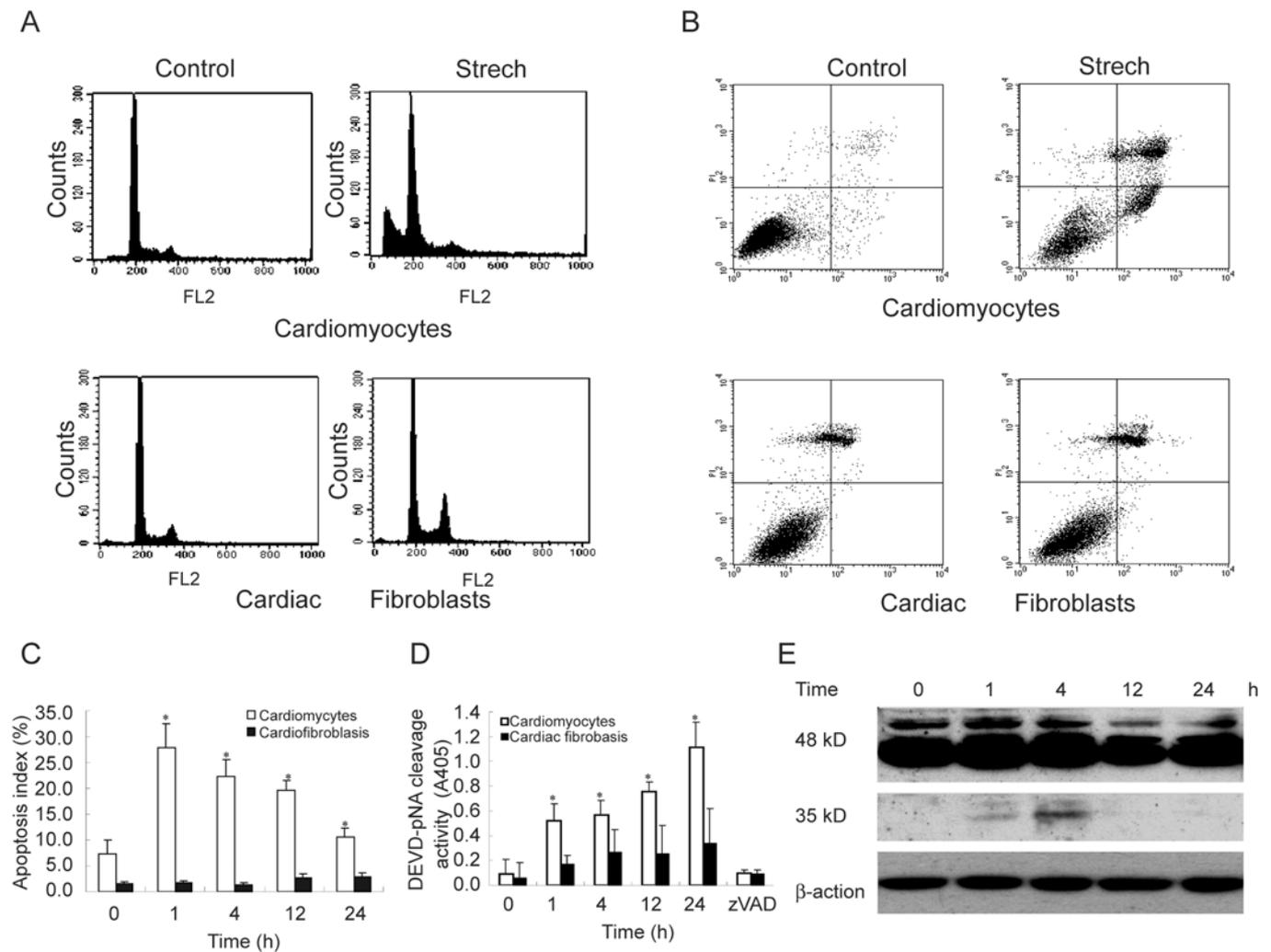
RESULTS

Stretch induced apoptosis and caspase activation in cardiomyocytes, but not in cardiac fibroblasts

Isolated neonatal rat cardiomyocytes and cardiac fibroblasts were placed onto silicone membrane coated with rat-tail collagen. After reaching 80% confluence, the cells were subjected to 20% sustained mechanical stretch up to 24 h. We chose the extensive (20%) and sustained stretch which itself did not damage the cells by membrane rupture as previous suggested[8]. We first determined the cell cycles of these two types of cells in response to mechanical stretch. Interestingly, after 24 h stretch, cardiomyocytes showed appearance of a sub-G$_1$ population, an indicative of cell death, while cardiac fibroblasts showed G$_2$/M accumulation (Fig 1A). The amount of G$_2$/M of cardiac fibroblasts increased from 7.3% to 17.8% 24 h after stretch. This G$_2$/M accumulation was not due to serum starvation since we did not observe this effect in unstretched cardiac fibroblasts.

To confirm cardiomyocytes apoptosis, an Annexin V assay was used to detect exposure of phosphatidylserine, a hallmark of apoptosis. Cardiomyocytes were found to undergo apoptosis as early as 1 h after stretch, while there were no Annexin V positive /PI negative cardiac fibroblasts detected (Fig 1B, C). Apoptosis in cardiomyocytes was also measured by DAPI staining of condensed nuclei observed by fluorescent microscope, which revealed similar results (data not shown). These results also suggest that stretch-induced apoptosis in cardiomyocytes was specific. To understand whether this stretch-induced apoptosis by the activation of caspases, both caspase-3 and caspase-9 activation were determined. There was a significant increase of DEVD cleavage activity following the stretch of cardiomyocytes, indicating the activation of caspase 3 (Fig 1D), a biochemical hallmark of apoptosis. Caspase 9 is a pro-enzyme in the cells and can be processed to smaller fragments during apoptosis mainly through the mitochondrial apoptosis pathway. Western blotting showed that caspases 9 was cleaved in cardio-myocytes after stretch, indicating its activation and suggesting that the mitochondrial dependent apoptosis pathway was involved in stretch-induced apoptosis (Fig 1E).

### Stretch activated the mitochondrial dependent apoptosis pathway only in cardiomyocytes

Fig 1. Mechanical stretch induces typical apoptosis and caspases activation in neonatal rat cardiomyocytes.   (**A**) Representative of cell cycle assay showing sub G1 population in cardiomyocytes and G_2 accumulation in cardiac fibroblasts. (**B**) Annexin V assay for apoptosis before and after 24 h stretch. And the Apoptosis Index in (**C**) represents the ratio of Annexin V+/PI- cells to the total cell population. D-E: Stretch induced the activation of caspases. Caspase activation was measured either by DEVD-pNA cleavage (**D**: caspase 3) or Western blotting (**E**: caspase 9, NeoMarker RB-1205), respectively. The experiments were performed as described in "Material and Methods". Data were obtained from 4 independent experiments (*p<0.05).

To investigate the mechanisms of stretch-induced caspase activation and apoptosis in cardiomyocytes, we examined stretch-induced release of cyto c and Smac/DIABLO from the mitochondria by Western blotting. A fraction of cyto c and Smac/DIABLO was detected in cytosolic fractions after stretch in a time-dependent manner (Fig 2A). Neither cyto c nor Smac/DIABLO was released from mitochondria in untreated cardiomyocytes and stretched cardiac fibroblasts, confirming that stretch-induced cyto c and Smac/DIABLO release in cardiomyocytes is specific and associated with apoptosis. VDAC1 is a mitochondrial membrane protein used as a marker for mitochondria integrity after homogenization. Western blotting

showed that only the mitochondria fraction (P10) was VDAC1 positive, validating the data on cyto c and Smac/DIABLO release.

Cyto c release is associated with the opening of the permeability transition pore (PTP) of the mitochondrial membrane[23, 24]. The reduction of $\Delta\psi_m$ could be an indicator of the opening of PTP[25]. To test if the permealization of the mitochondrial membrane is associated with cyto c release and apoptosis after stretch, $\Delta\psi_m$ was examined by either flow cytometry or laser scanning confocal microscopy (Fig 2B, C). Initially, there was a slight increase in $\Delta\psi_m$ in cardiomyocytes. This was then followed by a significant decrease of $\Delta\psi_m$ (Fig 2D), which
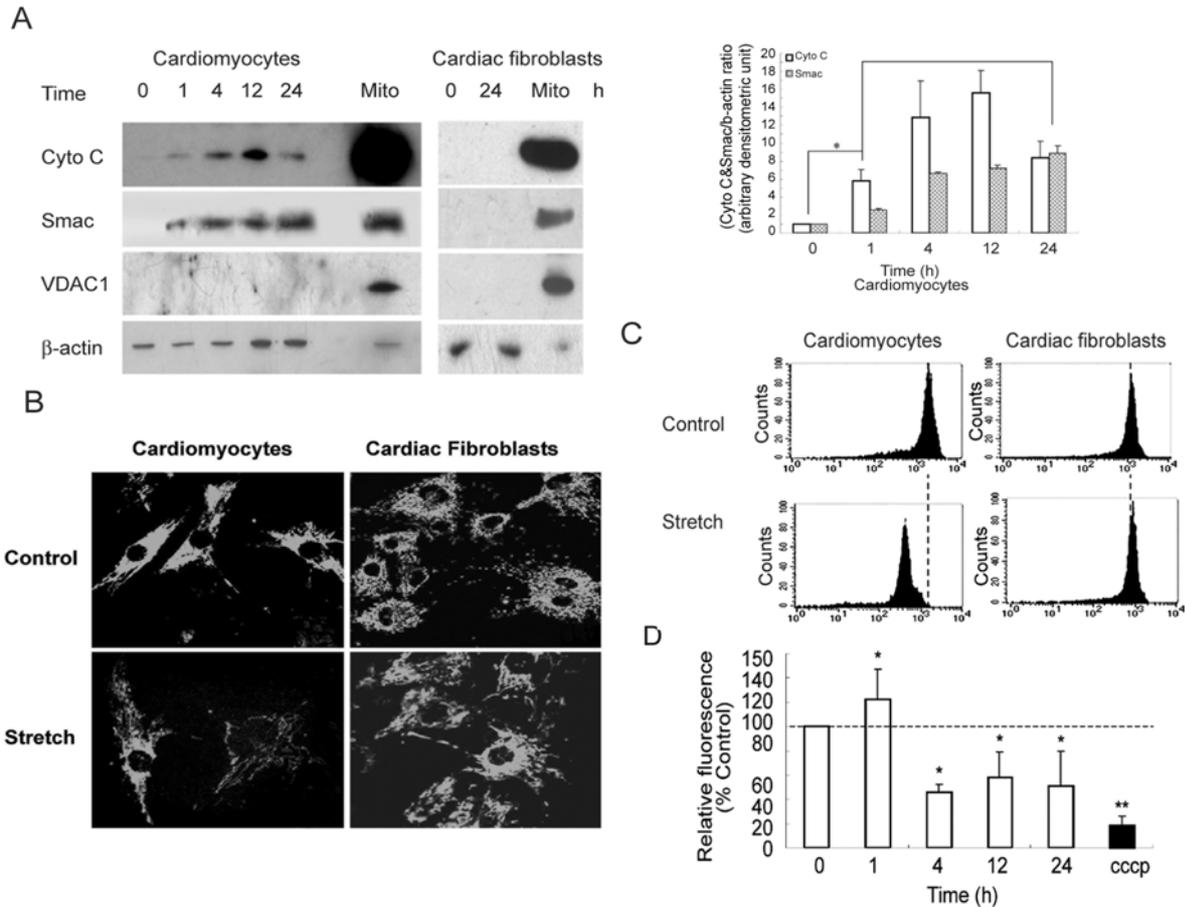
Fig 2. Stretch induces cyto c, Smac/DIABLO release from mitochondria and reduction of $\Delta\psi_m$ in cardiomyocytes, but not in cardiac fibroblasts. (**A**) Western blotting for cyto c and Smac/DIABLO release. Cardiocytes were fractionated as described in "Material and Methods". Cyto c and Smac/DIABLO release was detected by Western blotting using anti-cyto c monoclonal antibody (BD556432) and a rabbit anti-Smac polyclonal antibody (gift from Dr. X Wang) on the same membrane. A polyclonal antibody recognizing mitochondria protein VDAC1 (sc-8828) was employed as a marker for mitochondria fraction. Mitochondria fraction (P10) was loaded as a positive control. β-actin was used as the loading control. Lower panel showing the density of immuno-blot bands, which was analyzed with TotalLab ver1.0 software. Data were representative of 3 independent experiments. (*p<0.05). (**B**) Confocal microscopic assay for $\Delta\psi_m$ using Rhodamin123. (**C-D**) flow cytometric assay of $\Delta\psi_m$ using DiOC6(3). Panel C was the representative of control cells versus 24 h stretched cells, and panel D showed stretch induced loss of $\Delta\psi_m$ of cardiomyocytes. Data were obtained from 4 independent experiments and were normalized against control (0 h) data. (*p<0.05, **p<0.01)

was blocked by a PTP inhibitor, Cyclosporin A (CsA) (Fig 3A). CsA also significantly reduced apoptosis and abrogated cyto c release (Fig 3B, C), suggesting that PTP opening was indeed responsible for stretch-induced cardiomyocytes apoptosis. In contrast, cardiac fibroblasts, which do not undergo apoptosis after stretch, did not show any $\Delta\psi_m$ change (Fig 2B, C).

Distinctly different responses of Bcl-2 family proteins to stretch and their roles in regulating apoptosis in cardiomyocytes and in cardiac fibroblasts

To further understand the mechanisms of stretch induced, PTP mediated cyto c and Smac/DIABLO release in cardiomyocytes, we examined the expression of Bcl-2 family proteins following stretch by Western blotting. In cardiomyocytes, the levels of proapoptotic molecules, Bax and Bad were increased following stretch, while the level

of Bak expression remained unchanged (Fig 4A). Bax appeared to be targeted to mitochondria following stretch (Fig 4B). In contrast, Bad was undetected in cardiac fibroblasts, and the basal levels of both Bax and Bak were significantly lower in cardiac fibroblasts than that in cardiomyocytes. However, Bcl-xL levels were identical in cardiomyocytes and cardiac fibroblasts, and these levels were not affected by stretch. Thus, different types of heart cells had distinct expression pattern of Bcl-2 family protein. It appeared that the increase of the proapoptotic molecules and targeting of Bax into cardiomyocyte mitochondria were associated with cyto c release and apoptosis. The low level of proapoptotic molecules in cardiac fibroblasts seemed to be associated with its resistance to stretch-induced apoptosis.

To further confirm that Bcl-2 family proteins are functionally relevant in stretch-induced myocyte apoptosis, a

A

B

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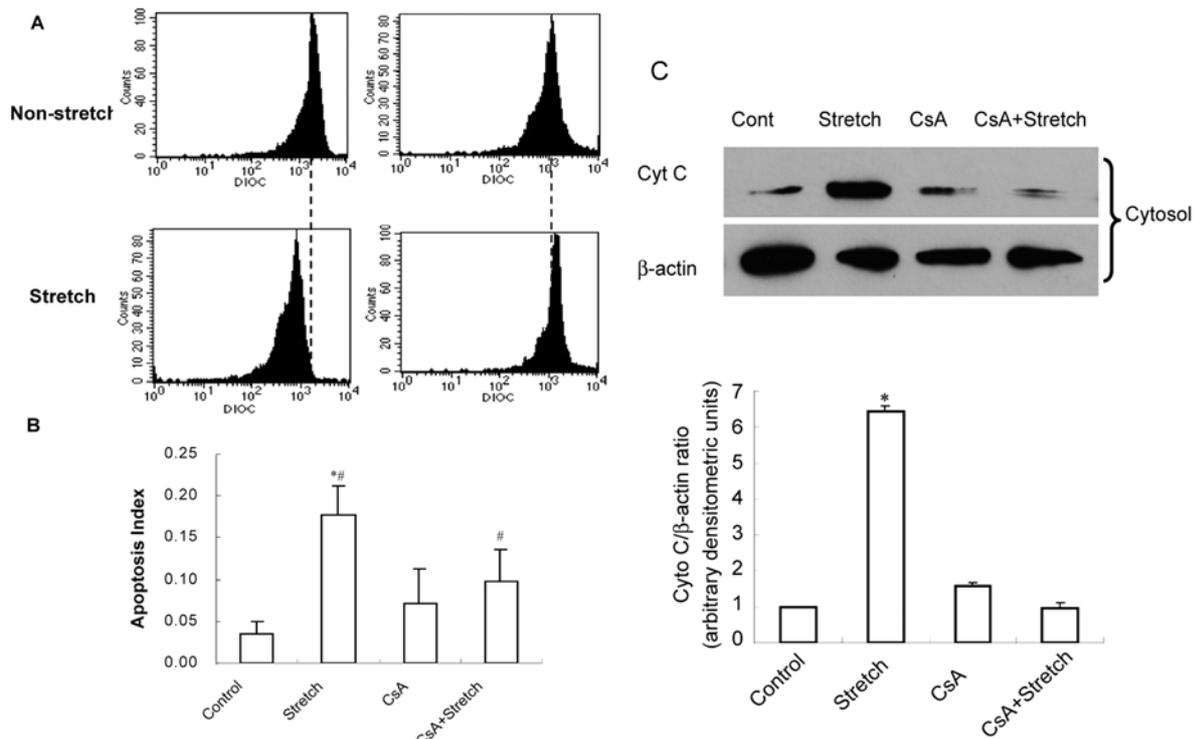
Fig 3. Stretch-induced apoptotic events were prevented by Cyclosporin A, a PTP inhibitor. (**A**): CsA prevented stretch induced redution of $\Delta\psi_m$ as assayed by flow cytometry. Cells were pretreated with 10 μM CsA for 30min before stretch and were then stretched for 12 h in the presence or absence of 10 μM CsA as indicated, and $\Delta\psi_m$ was analyzed as described in "Materials and Methods". (**B**): CsA inhbited stretch-induced apoptosis. Cells were treated as in A and were then stained with DAPI before being counted under fluorescent microscope. At least 200 cells were counted in each experiment. (**C**) CsA blocked stretch-induced Cyto c release as shown by Western blotting. Trace amount of cyto c in untreated sample, maybe due to the leakage of cyto c during manipulation. Data were from 3 independent experiments. (*p<0.05)

A

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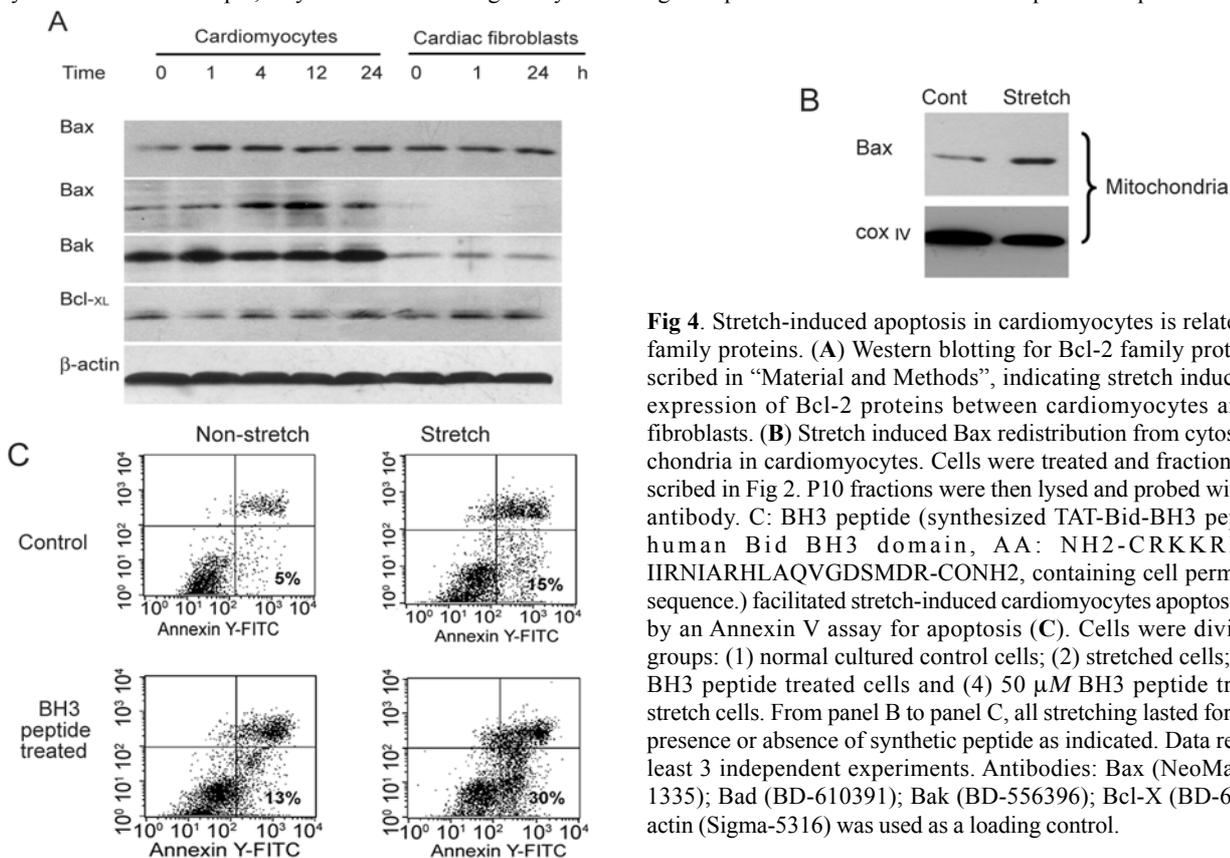
Fig 4. Stretch-induced apoptosis in cardiomyocytes is related to Bcl-2 family proteins. (**A**) Western blotting for Bcl-2 family proteins as described in "Material and Methods", indicating stretch induced distinct expression of Bcl-2 proteins between cardiomyocytes and cardiac fibroblasts. (**B**) Stretch induced Bax redistribution from cytosol to mitochondria in cardiomyocytes. Cells were treated and fractionated as described in Fig 2. P10 fractions were then lysed and probed with anti-Bax antibody. C: BH3 peptide (synthesized TAT-Bid-BH3 peptide from human Bid BH3 domain, AA: NH2-CRKKRRQRRR-IIRNIARHLAQVGDSMDR-CONH2, containing cell permeable TAT sequence.) facilitated stretch-induced cardiomyocytes apoptosis as shown by an Annexin V assay for apoptosis (**C**). Cells were divided into 4 groups: (1) normal cultured control cells; (2) stretched cells; (3) 50 μM BH3 peptide treated cells and (4) 50 μM BH3 peptide treated plus stretch cells. From panel B to panel C, all stretching lasted for 24 h in the presence or absence of synthetic peptide as indicated. Data represents at least 3 independent experiments. Antibodies: Bax (NeoMarkers-MS-1335); Bad (BD-610391); Bak (BD-556396); Bcl-X (BD-610211). β-actin (Sigma-5316) was used as a loading control.
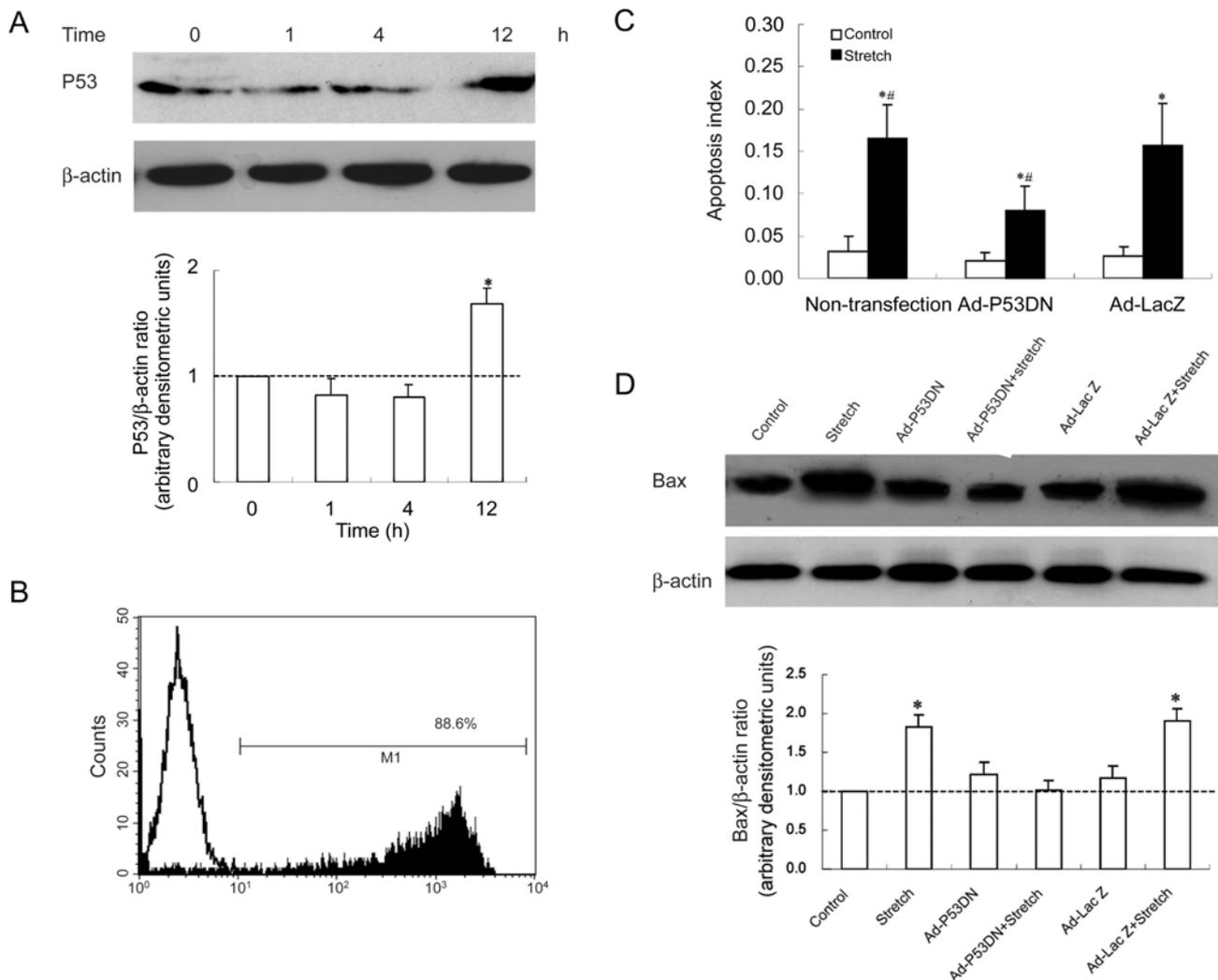
**Fig 5**. p53 is partially involved in mediating stretch-induced apoptosis. (**A**) Stretch induced the increase of p53 expression in cardiomyocytes. Western blotting was performed as described in "Material and Methods". Lower panel shows the Pixel density of immuno-blot bands was analyzed with TotalLab ver1.0 software. Data were from 3 independent experiments ($^*p<0.05$). (**B**) Flow cytometric assay for GFP confirmed successful infection of Ad-p53DN. M1 indicated more than 88.6% cardiomyocytes were infected. (**C**) Ad-p53DN partially prevented stretch-induced apoptosis. Data were calculated from 3 independent DAPI staining assays. At least 200 Nuclei from randomly selected fields (>10 fields counted per dish) were counted and apoptosis was expressed as the Apoptosis Index (AI = Apoptotic nucleus number/total nucleus number). ($^*p<0.05$). (**D**) Ad-p53DN functioned in blocking stretch induced Bax expression. Ad-LacZ (also containing GFP) was used as a control. Stretch in panel C and D is treated for 12 h. Cell fractionation and Western blotting was performed as described above.

cell permeable synthetic Bid BH3 peptide (TAT-BidBH3) [26] was employed to block Bcl-2 activity. Previous work showed that TAT-BidBH3 peptide can antagonize Bcl-xL to induce apoptosis via a mechanism of PTP[27]. Indeed, we found that TAT-BidBH3 alone could induce apoptosis in cardiomyocytes (Fig 4C) and reduction of $\Delta\psi_m$, but neither the TAT peptide nor TAT-BH3-mu (mutant BH3 peptide: G94E) had this effect (data not shown). These data indicate that Bcl-2 family proteins are indeed involved in regulating apoptosis via a mechanism of PTP in

cardiomyocytes. Moreover, TAT-BidBH3 could enhance apoptosis induced by stretch (Fig 4C).

p53 is partially involved in mechanical stretch-induced apoptosis

Previous work suggests that p53 is a major modulator of cardiomyocyte apoptosis after mechanical stretch [8; 9]. We therefore examined p53 levels by Western blotting following stretch. To our surprise, we found that p53 was increased 12 h after stretch (Fig 5A), far behind the ap-
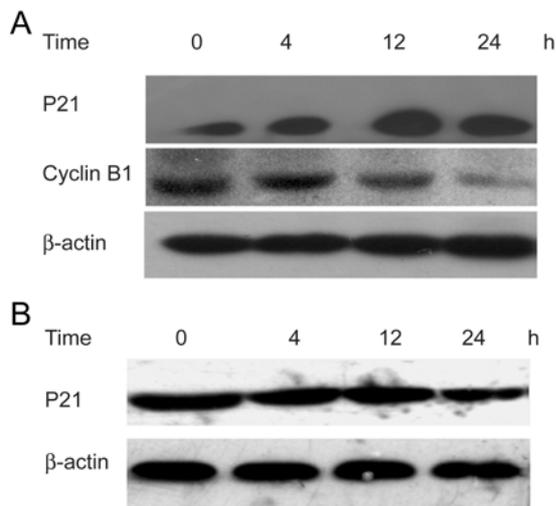
Fig 6. Stretch induces changes of cell cycle modulators only in cardiac fibroblasts (**A**), but not in cardiomyocytes (**B**). p21 and cyclin B1expression was detected by Western blotting as described in " Material and Methods". Antibodies used: anti-rat-p53 (pab246); anti-p21 (sc-6246); anti-Cyclin B1 (sc-595) and anti-β-actin (Sigma-5316). Data represents at least 3 independent experiments.

pearance of apoptosis and the increase of Bax protein observed as early as 1 h. To further understand the role of p53 in stretch-induced cardiomyocyte apoptosis, we employed adenovirus to deliver dominant negative p53 (p53DN^{175}) into cardiomyocytes to block endogenous p53 effects on stretch-induced apoptosis. About 90% of the cells were found GFP-positive 24 h after infection (Fig 5B). We found that p53DN could reduce stretch-induced apoptosis by about 40% percent (Apoptosis Index: Control 3.2±1.8%; Stretch 16.6±4.0%; p53DN 2.0±1.1%; Stretch plus p53DN 6.8±2.4%), and prevent stretch induced Bax upregulation (Fig 5C, D). p53DN has little effect on stretch induced cyto c release (data not shown). These results suggest that p53 is partially involved in stretch-induced apoptosis and there may exist other p53-independent signals.

Distinct responses of p21$^{(WAF1)}$ to stretch in cardiac fibroblasts from that in cardiomyocytes

Next, we sought to address how stretch induced G_2/M accumulation in cardiac fibroblasts. It is well known that p21$^{(WAF1)}$ not only inhibits Cdk4/Cyclin 1 and Cdk6/Cyclin D2 in the G_1 checkpoint, but also inhibits Cdc2/Cyclin B1 during G_2/M transition, at least *in vitro*[28]. Expression of p21 is known to induce cell cycle arrest at the G_2 phase in a number of cell systems[29]. Thus, we examined the expression of p21$^{(WAF1)}$ in both cardiac fibroblasts and myocytes in response to stretch. Consistent with our data on G_2/M accumulation, p21$^{(WAF1)}$ was increased following mechanical stretch in cardiac fibroblasts (Fig 6A). In

contrast, p21$^{(WAF1)}$ was not changed in cardiomyocytes (Fig 6B). We also observed that cyclin B1, a critical component for cell cycle progression was downregulated in cardiac fibroblasts following stretch (Fig 6A).

DISCUSSION

Increasing evidences suggest that apoptosis is a major mechanism in the development of a number of cardiovascular diseases, such as hypertension, acute myocardial infarction, ischemia-reperfusion injury and heart failure [30-33]. Abnormal mechanical load triggers significant apoptosis in cardiomyocytes, which is believed to be a dominant factor leading to heart failure. However, the detailed signal pathways responsible to mechanical stretch-induced apoptosis are not fully examined. Here, we present data to demonstrate the pivotal role of mitochondria and Bcl-2 in mechanical stretch-induced apoptosis in cardiomyocytes. We showed that mitochondrial PTP is involved in mediating stretch-induced cyto c release since we found that mechanical stretch induced the reduction of $\Delta\psi_m$, indicative of PTP opening. Importantly, PTP inhibitor CsA could potently inhibit mechanical stretch-induced reduction of $\Delta\psi_m$ and cyto c release. We showed that Bax and Bak were induced by stretch in cardiomyocytes and extended previous findings by showing that Bax can target cardiomyocyte mitochondria. We also showed that synthetic cell permeable BidBH3 peptide itself, but not its mutant which lacks interaction with Bcl-xL, promotes apoptosis and reduction of $\Delta\psi_m$ (data not shown) and facilitates stretch-induced apoptotic events. We suggest that these results link stretch-induced proapoptotic Bcl-2 family proteins to apoptosis machinery via PTP mechanism in cardiomyocytes. Our data are in good agreement with previous reports suggesting that Bcl-2 and its family of proteins function as an apoptosis "check-point" at the level of mitochondria in many other systems (see review papers[24, 34-36]).

Cardiac fibroblasts play fundamental roles in the functional integrity of myocardium and development of heart remodeling as well. We found that cardiac fibroblasts were resistant to the extensive and sustained stretch in our experiment system performed in parallel with myocytes and showed that there were no cyto c or Smac/DIABLO release from cardiac fibroblast mitochondria, although they underwent apoptosis when challenged with oxidative stress (data not shown). Interestingly, cardiac fibroblasts express low level of pro-apoptotic molecules and there are no changes in expression following stretch. These results reinforce the notion that Bcl-2 family proteins are important for regulating apoptosis. We first showed that the expression of p21$^{(WAF1)}$ was induced and cyclin B1 was downregulated only in cardiac fibroblasts, which were

associated with G_2/M accumulations. The G_2/M accumulation, rather than cell proliferation as others reported[15,37], could be due to the different degree and duration of stretch employed. Previous work showed that oxidative stress induced p21 upregulation and G_2/M arrest in cardiac fibroblasts[38]. We found that mechanical stretch induced intracellular free radical generation only in cardiomyocytes (data not shown), suggesting that p21 and G_2/M accumulation in fibroblasts were not mediated by oxidative stress in our system. It is reported that stretch-induced effects on cardiac fibroblasts may be mediated by autocrine of Angiotensin II[39]. However, recent evidence suggests that stretch does not increase Ang II secretion in cultured cardiomyocytes or cardiac fibroblasts [10]. The role Ang II in mechanical stretch induced p21 (WAF1) upregulation and G_2/M accumulation remains to be defined.

An important intracellular mediator to these distinct responses could be p53 since both Bax and p21 are transcriptionally regulated by p53[8, 40]. Using a similar *in vitro* cultural system, Leri et al showed that mechanical stretch induced p53 dependent apoptosis by activating Bax expression or enhancing the secretion of Ang II and p53 DN could completely abrogate mechanical stretch-induced apoptosisin adult myocytes[9]. We noticed that Bax was upregulated as early as 1 h and p53 is upregulated at relatively late time. These may indicate that the regulatory effect of p53 on Bax could happen without changes of p53 levels. Alternatively, some additional factors may play a role in regulating Bax upregulation independent of p53. Nevertheless, We found that p53-DN could only partially block stretch-induced apoptosis without affecting cyto c release (data not shown), although p53-DN inhibited Bax upregulation at 12 h (Fig 5D). Our data support that p53 is involved in stretch-induced cardiac cell response although there may be p53-independent mechanisms of apoptosis in cardiomyocytes. It was reported that cardiomyocytes from p53 knock-out mice still undergo apoptosis under hypoxic stresses[41]. Indeed, our preliminary data showed that NO produced by NOS was responsible to stretch-induced apoptosis in cardiomyocytes via a p53-independent manner (manuscript in preparation). In addition, our work seems not to be in favor of Ang II in mediating cardiomyocyte apoptosis since apoptosis occurs within 1 h (Fig 1C) although we do not rule out its involvement in regulating cardiomyocyte apoptosis. Further work is required to examine the role of p53 and Ang II in mechanical stretch-induced apoptosis in knockout mice.

Taken together, our results demonstrate that mechanical stretch induces distinct intrinsic cellular mechanisms, as exemplified by their distinct expression of cell cycle and apoptosis regulators, resulting in cell death in

cardiomyocytes and G_2/M accumulation in cardiac fibroblasts. Mechanical stretch can activate programmed cell death in cardiomyocytes via proapoptotic molecules of Bcl-2 family proteins. Proapoptotic Bax and/or Bad act at mitochondrial levels, probably via a mechanism of PTP, to induce cyto c and Smac/DIABLO release to activate caspases. On the other hand, mechanical stretch induces cell cycle alteration by modulations of p21 and cyclin B1expression. It is intriguing how the mechanical signal is sensed by different types of cardiac cells. Our recent data suggest Ca^{2+} is important for mediating the conversion of mechanical signals to biological signals in cardiomyocytes [42]. Further study of the distinct molecular signaling in cardiomyocytes and cardiac fibroblasts could be helpful for better understanding and treatment of heart remodeling and associated diseases.

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