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Novel mechanisms for caspase inhibition protecting cardiac function with chronic pressure overload

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Abstract

Myocyte apoptosis is considered a major mechanism in the pathogenesis of heart failure. Accordingly, manipulations that inhibit apoptosis are assumed to preserve cardiac function by maintaining myocyte numbers. We tested this assumption by examining the effects of caspase inhibition (CI) on cardiac structure and function in C57BL/6 mouse with pressure overload model induced by transverse aortic constriction (TAC). CI preserved left ventricular (LV) function following TAC compared with the vehicle. TAC increased apoptosis in non-myocytes more than in myocytes and these increases were blunted more in non-myocytes by CI. Total myocyte number, however, did not differ significantly among control and TAC groups and there was no correlation between myocyte number and apoptosis, but there was a strong correlation between myocyte number and an index of myocyte proliferation, Ki67-positive myocytes. Despite comparable pressure gradients, LV hypertrophy was less in the CI group, likely attributable to decreased wall stress. Since changes in myocyte numbers did not account for protection from TAC, several other CI-mediated mechanisms were identified including: (a) lessening of TAC-induced fibrosis, (b) augmentation of isolated myocyte contractility, and (c) increased angiogenesis and Ki67-positive myocytes, which were due almost entirely to the non-myocyte apoptosis, but not myocyte apoptosis, with CI. CI maintained LV function following TAC not by protecting against myocyte loss, but rather by augmenting myocyte contractile function, myocyte proliferation, and angiogenesis resulting in reduced LV wall stress, hypertrophy, and fibrosis.

Keywords

Apoptosis inhibition; Cardiac hypertrophy; Fibrosis; Angiogenesis; Myogenesis

Introduction

Left ventricular hypertrophy (LVH) in response to LV pressure overload is a double-edged sword; on the one hand, it compensates for the pressure overload, whereas on the other hand LVH impairs LV function. Eventually, LV decompensation develops, leading to heart failure (HF). It is widely held that a major mechanism underlying the reduced LV function with chronic pressure overload and the transition from compensated LVH to HF is myocyte apoptosis with resultant loss of contractile elements and LV mass [9, 16, 26, 29, 30], and that inhibition of caspase will protect apoptosis and LV function in response to chronic pressure overload [1]. However, our recent studies questioned this fundamental concept, by finding that apoptosis predominates in non-myocytes over myocytes during the development of HF [22]. The first goal of the current investigation was to determine if the LV dysfunction that is observed with chronic pressure overload is mediated by apoptosis and loss of contractile elements. The second goal was to determine if inhibiting apoptosis, by administration of a caspase inhibitor, protected the heart from chronic pressure overload and specifically if it caused substantially reduced myocyte apoptosis resulting in greater numbers of myocytes and increased LV mass as compared with animals treated with vehicle. If not, then the third goal was to examine mechanisms by which the caspase inhibitor protected LV function other than through preservation of myocyte numbers and mass. A fourth goal, designed to test further the role of myocyte apoptosis with development of cardiac dysfunction and its rescue by caspase inhibition (CI), was to examine if the mechanism mediating the rescue correlates with either myocyte or non-myocyte apoptosis.

To address the goals of this study, we examined the effects of chronic pressure overload, induced by transverse aortic constriction (TAC) in the presence and absence of the caspase inhibitor. Surprisingly, CI improved LV dysfunction after chronic pressure overload, but not

by protecting myocyte apoptosis, but rather by protecting non-myocyte apoptosis and preserving LV wall stress, isolated myocyte contractility, reduced myocyte hypertrophy, fibrosis and increased angiogenesis and myogenesis, thereby providing a new concept for the mechanism by which CI protects the heart and could be a novel therapeutic approach in the future.

Methods

Chronic pressure overload mouse model

TAC was performed in 3 to 4-month-old male C57BL/6 mice as described previously [18]. Mice were anesthetized with intraperitoneal injection of a mixture of ketamine (65 mg/kg), xylazine (13 mg/kg), and acepromazine (2 mg/kg) before operation. To measure the pressure gradient across the constriction, the pressures in the LV and abdominal aorta were measured simultaneously. Because echo data, hemodynamic data including terminal measurement of pressure gradients, and biochemical and histological data were required at each time point before and after TAC in treated and sham animals, different animals needed to be studied. The following groups were studied for measuring LV function and organ weight: sham with vehicle ($n = 11$), sham with CI ($n = 11$), 1-week TAC with vehicle ($n = 4$), 1-week TAC with CI ($n = 3$), 3-week TAC with vehicle ($n = 12$), and 3-week TAC with CI ($n = 11$). Of these, we measured apoptosis in following animals: sham with vehicle ($n = 8$), sham with CI ($n = 10$), 1-week TAC with vehicle ($n = 4$), 1-week TAC with CI ($n = 3$), 3-week TAC with vehicle ($n = 9$), and 3-week TAC with CI ($n = 8$). These studies were approved by the Institutional Animal Care and Use Committee at the New Jersey Medical School.

Caspase inhibitor treatment

The caspase inhibitor (Z-Asp-2,6-dichlorobenzoyloxymethylketone, Z-Asp-CH₂DCB, Enzo Life Science) was chronically administered (10 mg/kg/day) via intra-peritoneal micro-osmotic pumps (Alzet, model 1002, Durect Corp.) in mice with a delivery rate of 0.25 μ l/h. It is well known that Z-Asp-CH₂DCB preferentially inhibits the caspase-3-like activity [2, 14] and caspase-1-like proteases [7, 14]. All mice received either a bolus of drug or vehicle immediately after TAC surgery, followed by chronic administration through the micro-osmotic pumps. The pumps were replaced at 2 weeks after TAC because each micro-osmotic pump contained only 100 μ l.

Measurement of cardiac function

Two-dimensional echocardiography was performed in mice using ultrasonography (Accuson 256, Siemens Medical Solutions) with 13-MHz linear ultrasound transducer, measuring LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), LV fractional shortening (FS), and LV ejection fraction (EF) and LV posterior wall thickness in systole (LVPWs). Cardiac catheterization was performed using a Millar micromanometer in the LV to measure LV systolic pressure (LVSP) and LV end-diastolic pressure (LVEDP). Using a second catheter in the aorta, LV/aortic pressure gradients were measured. LV systolic wall stress (kdyn/cm²) was calculated as: $(1.35 \times \text{LVSP} \times \text{LVESD}) / [4 \times \text{LVPWs} \times (1 + \text{LVPWs} / \text{LVESD})]$. Sodium pentobarbital (100–500 mg/kg) or CO₂ was used for euthanasia after terminal hemodynamic measurements.

Detection of apoptosis

Tissue samples were fixed with 10 % neutral buffered formalin and embedded in paraffin. Apoptosis was detected by TUNEL assay. To discriminate apoptosis in non-myocytes and myocytes, tissue sections were co-stained with rhodamine-conjugated wheat germ agglutinin (WGA) (Vector Laboratories) as previously described [22]. WGA stains all cell membranes

in LV myocytes. The pattern of WGA staining is seen not only at the outer membrane, but also followed a striated pattern in myocytes. Apoptotic rate was expressed as the percentage of TUNEL positive cells per nuclei. To avoid the potential overestimation of apoptotic rate by TUNEL assay, we also evaluated apoptosis by immunohistochemical staining for cleaved caspase-3 (CC3) [22].

Immunohistochemistry and histology

Apoptotic non-myocytes were detected by dual staining with TUNEL assay and cell type specific antibody. For characterization of cell types in apoptotic non-myocytes, serial tissue sections were immunostained with the following antibodies: F4/80 (AbDSerotec) for macrophages; mouse monoclonal anti-HSP47 (Stressgen) for fibroblasts; Isolectin IB₄-Alexa568 (Invitrogen) for endothelial cells. Troponin I (Thermo Scientific) was used as myocyte specific marker. To detect cell-to-cell communication, connexin-43 (Cx43, Millipore), a gap junction protein, was examined. Those primary antibodies were detected by goat anti-rat or goat-anti-mouse IgG conjugates with Alexa555, or 568, or 594 or 488 (Invitrogen). To examine angiogenesis, tissue sections were double immunostained with Ki67, as a proliferation marker (Thermo Scientific) and Isolectin IB₄-Alexa488 (Invitrogen). To examine an index of proliferating myocytes, tissue sections were double immunostained with Ki67 and WGA, or phosphohistone H3 (PHH3, Cell Signaling) and WGA. To examine the collagen deposition during LV remodeling after chronic pressure overload, Masson's trichrome staining was performed. The percentage of interstitial fibrosis and total fibrosis was quantitated by picrosirius red (PSR) staining and ImagePro-Plus software analysis. To detect connective tissue growth factor (CTGF), tissue sections were dual immunostained with rabbit polyclonal CTGF (Abcam) and WGA.

Measurement of myocyte contractility

Adult cardiac myocytes were isolated from 3 to 4-month-old male C57BL/6 mice using collagenase in a Langendorff perfusion apparatus [12]. Isolated myocytes were placed on the stage of an inverted microscope, superfused with Tyrodes solution containing 1 mM calcium at a flow rate of 1.8 ml/min, and electrically stimulated at 0.5 Hz at 23 °C. Each single myocyte length was monitored from the bright-field image (650–750 nm red light illumination) by an optical edge-tracking method using a video-based edge-detection system (model VED-105, Crescent Electronics) with a 30 ms time resolution. The contraction amplitude was measured as the percentage of shortening of cell length.

Myocyte size and number

To measure myocyte cross-sectional area, tissue sections were co-stained with WGA and DAPI, and quantitated using ImagePro-Plus software. The total number of myocytes of sham and TAC with/without CI was measured by the methods of Kajstura et al. [13].

Endothelial cell tube formation assay on matrigel

Human microvascular endothelial cells from the heart (HMVEC-Cs) were purchased from Lonza (Basel, Switzerland). Matrigel (reduced growth factor, BD Bioscience) was placed in 24-well tissue culture plate (200 µl/well) and allowed to gel at 37 °C for 30 min. HMVEC-Cs were seeded and cultured with vehicle or CI for 20 h. The tube formation of endothelial cells was photographed at 100× and quantified with Image-J software.

Statistics

All data were expressed as mean ± SEM. Comparisons between two groups were made using the Student's *t* test. For statistical analysis of data from multiple groups, we used

ANOVA followed by Bonferroni post hoc analysis. Simple regressions were calculated. $p < 0.05$ was taken as a minimal level of significance.

Results

Caspase inhibition prevented LV dysfunction after chronic pressure overload

Chronic pressure overload in the vehicle group induced progressive LV dysfunction over the 3-week monitoring period. Compared with sham control, LVEF fell (55 ± 3 vs. 70 ± 1 %), LVEDD increased (4.14 ± 0.11 vs. 3.72 ± 0.06 mm) and LVEDP increased (15 ± 2 vs. 5 ± 1 mmHg), whereas lung weight (wt)/tibial length, an index of HF, was just beginning to increase (9.6 ± 0.8 vs. 8.0 ± 0.2). The group with the CI tolerated chronic pressure overload better, i.e., LVEF fell less (66 ± 2 %) and there was no evidence of LV decompensation, i.e., the increases in LVEDP (10 ± 2 mmHg) or LVEDD (3.96 ± 0.08 mm) or lung wt/tibial length (8.2 ± 0.2) were less than in the vehicle group. LV/aortic pressure gradients were similar in the two groups with TAC; however, LV wall stress rose significantly less in the CI-TAC group (Table 1).

Caspase inhibition effects on myocyte apoptosis and proliferation

The first goal of this investigation was to test the hypothesis that the decrease in LV function with chronic pressure overload induced LV hypertrophy may be mediated by excessive myocyte apoptosis resulting in a reduction in total contractile units and LV mass. The corollary of this hypothesis is that the CI prevents myocyte apoptosis, resulting in greater numbers of myocytes and LV mass in the hearts treated with the CI. As expected, chronic pressure overload increased apoptosis in the heart and this was reduced by the CI. The increase in apoptosis occurred at 3 weeks after aortic banding when there was a significant fall in LV function. At an earlier time point (1 week), the increase in apoptosis was minor and there was no difference between CI and vehicle for either apoptosis or LV function (Suppl. Figure 1). When we counterstained the apoptotic cells to determine their cell type, we found that the overwhelming majority of the apoptosis and its correction by the CI occurred in non-myocytes, based on measurements of the numbers of myocytes and non-myocytes undergoing apoptosis, not percentage change (Fig. 1a). The apoptosis in the heart was threefold greater, $p < 0.05$, in non-myocytes after 3-week aortic banding than in myocytes (0.29 ± 0.03 vs. 0.09 ± 0.02 % of apoptosis). CI reduced apoptosis by a similar percentage in non-myocytes and myocytes (33 vs. 29 %). Since the number of apoptotic non-myocytes per field was tenfold greater, the absolute decrease in apoptosis was greater, $p < 0.05$, in non-myocytes than myocytes (0.15 ± 0.01 vs. 0.01 ± 0.002 of apoptosis/field). To characterize the cell types of apoptotic non-myocytes after 3-week TAC, we performed the TUNEL assay followed by immunohistochemical staining with each non-myocyte specific antibody (Fig. 1d). Macrophages were the major cell type protected from apoptosis by CI. Apoptosis assessed by CC3 staining (Fig. 1e) was similar to data described for TUNEL. The results between the TUNEL assay and CC3 immunostaining show a strong positive correlation ($R^2 = 0.84$, $p = 3E-07$) (Suppl. Figure 2).

The fundamental question is whether total myocyte numbers in the heart fell due to apoptosis. The technique of Kajstura et al. [13] was used to quantitate the total numbers of myocytes in the LV. Actually myocyte numbers did not fall with TAC, but increased only slightly and non-significantly in vehicle, and significantly increased in CI after 3-week TAC compared to sham (Fig. 1b). From these data, it is not surprising that there was no correlation between myocyte apoptosis and total myocyte numbers in LV ($R^2 = 0.03$, $p = 0.483$) (Fig. 1c). These data suggest that preservation of myocytes was not the mechanism by which the CI protected the heart with chronic pressure overload.

However, CI significantly increased Ki67-positive myocytes, an index of myocyte proliferation, after 3-week TAC. Ki67 positive myocytes were significantly increased with CI compared to vehicle after TAC (0.82 ± 0.2 vs. 0.41 ± 0.12 %) (Fig. 2a, e). Interestingly, the percentage of Ki67-positive myocytes was significantly correlated in the CI group with apoptosis (%) ($R^2 = 0.75$, $p = 0.0003$), but not in the vehicle group after TAC ($R^2 = 0.17$, $p = 0.178$) (Fig. 2b). We reasoned that a better correlation would be observed if the average non-myocyte apoptotic rate in the vehicle group was subtracted from each data point in the CI group. When this was done, the correlation with Ki67-positive myocytes (%) was indeed even more significant ($R^2 = 0.98$, $p = 0.0002$) (Fig. 2c). In contrast, when the same analysis was done for the myocyte apoptosis correlation with Ki67-positive myocytes (%), the correlation was even less significant (Fig. 2d). Thus, the excellent correlation between non-myocyte apoptosis and Ki67-positive myocytes (%) suggests a potential beneficial role of apoptotic non-myocytes on myocardial regeneration after pressure overload in the CI group.

Caspase inhibition reduced LV hypertrophy

If CI improved LV function after TAC by increasing myocyte numbers, it might be expected that LV mass should have been larger in the CI group. Actually the reverse was observed. LV weight/body weight (LVwt/BW) was significantly less, $p < 0.05$, in the CI group (Table 1) and this was confirmed by measuring myocyte cross-sectional area, which was also significantly reduced (Fig. 3a, Suppl. Figure 3). The reduction in myocyte cross-sectional area induced by the CI was observed over the entire distribution of myocyte size after TAC (Fig. 3b). The smaller myocytes with CI after TAC were not because of differences in LV/aortic pressure gradient in CI (84 ± 4 mmHg) versus vehicle (92 ± 4 mmHg), but likely due to protected LV wall stress, which was less ($p < 0.05$) in the CI group (81 ± 4 kdyn/cm²) compared with vehicle (102 ± 6 kdyn/cm²) at 3 weeks after banding before LV decompensation (Fig. 3c; Table 1). Furthermore, myocyte cross-sectional area also correlated significantly with LV systolic wall stress ($R^2 = 0.73$, $p = 8.7E-07$) (Fig. 3d).

Mechanisms by which caspase inhibition protects the heart after chronic pressure overload

Caspase inhibition reduced fibrosis and increased angiogenesis after chronic pressure overload

Next, we determined the extent of fibrosis after chronic pressure overload by measuring interstitial fibrosis in LV myocardium by PSR stain. The percentage of interstitial fibrosis increased less, $p < 0.05$, with CI compared to vehicle (3.3 ± 0.5 vs. 6.8 ± 1.6 %) after TAC (Fig. 4a, b). From Masson's trichrome stain of LV myocardium, we found that collagen deposition also increased less in the CI group compared to the vehicle group. From immunohistochemical staining, connective tissue growth factor (CTGF), a key fibrogenic protein, was also reduced with CI (Suppl. Figure 4). These results suggest that the apoptosis of non-myocytes was involved in fibrosis after chronic pressure overload, either directly or indirectly, since it was also correlated with the preservation of LV systolic wall stress as shown earlier.

Angiogenesis is another mechanism by which the chronically pressure overloaded heart can prevent decompensation [8]. With CI after TAC, proliferating endothelial cells, indicating angiogenesis, increased more in the CI group (Fig. 5a, b). This was confirmed by a significant increase of capillary density in the CI group compared to vehicle after TAC (35.2 ± 1.6 vs. 28.1 ± 0.9 %, $p < 0.05$) (Fig. 5c). We evaluated the increase of angiogenesis by CI using a tube formation assay with HMVEC-Cs (human cardiac microvascular endothelial cells). HMVEC-Cs are more sensitive to phenylephrine (PE), which can induce hypertrophy, than human umbilical vein endothelial cells (HUVEC). We observed the trend of increase of

tube formation with treatment with CI (1 μ M) compared to vehicle, and further increase in CI with phenylephrine (100 μ M) even though not significant (Suppl. Figure 5).

Caspase inhibition preserved myocyte contractility and connexin-43

The contractility of isolated myocytes was preserved in the CI group compared to vehicle after aortic banding (Fig. 6a). However, the half width of contraction duration was not significantly changed by CI, suggesting no change in contractile kinetics (data not shown). The preservation of TnI in the CI group after TAC was observed from dual immunohistochemical staining of TnI with WGA. The degradation and destruction of myocyte TnI (yellow arrows) were observed in the vehicle group after TAC, but rarely found in the CI group after TAC (Fig. 6b). From immunohistochemical staining, we observed different re-organization of connexin-43 (Cx43), a gap junction protein, after TAC between the CI and vehicle groups. Cx43 internalization occurred in the vehicle group after TAC, but lateralization was observed in the CI group (Fig. 7).

Discussion

We investigated whether inhibition of myocyte apoptosis by CI could preserve LV function during the transition from compensated hypertrophy to decompensated HF. As expected, inhibition of apoptosis by sustained administration of the CI protected LV function after chronic pressure overload and prevented the decompensation to failure that was observed in the absence of the CI treatment. Results from the literature [2] would argue that this would be expected on the basis that the CI prevented myocyte apoptosis, resulting in preservation of a larger number of contractile units in the CI-treated hearts and preservation of myocardial mass. Not only could we not corroborate this hypothesis, but found the reverse, i.e., total numbers of myocytes were not reduced with chronic pressure overload, and that CI did not affect myocyte apoptosis to a significant extent, but actually increased myocyte numbers, most likely by myocyte proliferation, which was confirmed by measurement of Ki67-positive myocytes, an index of proliferation. With chronic pressure overload, the overwhelming majority of apoptosis occurred in non-myocytes, and although the CI did protect against apoptosis, again the protection was observed almost exclusively in non-myocytes. Interestingly, at 1 week after TAC apoptosis did not increase appreciably and there was no difference between the CI and vehicle groups, most likely because there was no difference in LV function at the 1-week time point. These results are consistent with the previous study by Condorelli et al. [6] demonstrating that cardiac specific overexpression of caspase-3 increases infarct size, but showing a similar level of DNA laddering in Tg and control mice undergoing ischemia–reperfusion. In addition, LV mass, which was hypothesized to be larger with CI after pressure overload, was actually smaller, due to reduced fibrosis and myocyte cross-sectional area. The reduced hypertrophy occurred in the face of identical LV/aortic pressure gradients, and was likely due to the reduced LV wall stress in the CI group. The reduced wall stress is one mechanism by which LVEF was maintained in the CI group, since there is an inverse relationship between LV wall stress and LVEF [31]. The reduced LVH is another mechanism contributing to the preserved LV function, since LVH, per se, can impair cardiac function [17].

Having found that overall LV function following TAC was improved by CI, we then examined function at the level of individual myocytes. As shown in Fig. 6a, isolated myocyte contractility was also preserved following TAC in the CI group. We also found preservation of Tn I in the CI group compared to the vehicle group after TAC. These results are supported by Condorelli et al. [6], showing caspase-3 overexpression depresses cardiac contractility. It is likely that this is due to calcium regulation at the myocyte level, which will be investigated in the future studies. To further confirm that the preserved cardiac function with CI was due in part to an effect on myocyte contractile function, we examined

the expression of Cx43 for gap junction remodeling after chronic pressure overload. Ventricular hypertrophy has been associated with Cx43 translocation from the intercalated disks to intracellular pools [24]. In the current investigation, CI prevented the internalization of Cx43 observed in the vehicle group after TAC, and rather showed lateralization. The beneficial effects of Cx43 lateralization are still controversial; one study reported lateralized Cx43 in the failing heart [10], and another suggested that lateralization of Cx43 may be critical for bone marrow cell differentiation to cardiomyocytes [23].

Considering that so many studies implied that myocyte numbers would decrease due to apoptosis with cardiac stress leading to HF [5, 9, 19, 30], we had expected that several of these studies would document that myocyte apoptosis reduced the total numbers of myocytes in the hearts, but did not. Once we found that myocyte apoptosis was not a major feature of chronic pressure overload, we assessed total myocyte numbers in the LV of the hypertrophied heart, and observed that total myocyte numbers were not decreased following pressure overload. It is important that the concept that myocyte loss through apoptosis is responsible for reduced contractile units in the heart subjected to pressure overload was not corroborated by measuring total myocyte numbers.

We found that myocyte numbers were actually elevated after TAC in the CI group, which was most likely due to the increased myocyte hyperplasia, since protection against apoptosis should not cause an increase in myocyte numbers. In support of the concept that CI induced myocyte hyperplasia, an index of this function, the numbers of Ki67-positive myocytes were significantly increased with CI after 3-week TAC, and showed a positive correlation with total myocyte numbers. Since Ki67 protein is present during all active phases of the cell cycle (G1, S, G2, and mitosis), but is absent from resting cells (G0) [25], it is generally recognized that Ki67 can be a proliferation marker [20, 28]. However, there is a limitation for Ki67 as the sole proliferation marker because Ki67-positive myocytes do not necessarily result in cell duplication. Accordingly, we confirmed the Ki67 data with immunostaining with phospho-histone H3 (PHH3), which detects mitosis [3] (Fig. 2f). Thus, in contrast to the widely held belief that inhibiting apoptosis preserves myocytes, in this study with administration of a CI, the major mechanism maintaining total LV myocyte numbers, appeared to be myocyte hyperplasia, which has not been observed by others. There was a striking correlation between the effects of the CI reducing non-myocyte apoptosis, and the increase in myocyte hyperplasia. In contrast, there was a lack of correlation between myocyte hyperplasia and myocyte apoptosis. This demonstrates further the lack of importance of myocyte apoptosis resulting in cardiac dysfunction with chronic pressure overload, but rather the key significance of protection of non-myocyte apoptosis.

Having ruled out the concept that inhibition of myocyte apoptosis protects the heart from failure, we identified several other mechanisms which could account for preservation of LV function with the CI after chronic pressure overload. We observed in the CI group, a marked reduction in fibrosis. First of all, this cannot be attributed to the protection of myocytes from apoptosis with CI, since apoptotic cell death does not lead to fibrosis [27]. It is more likely that the fibrosis was the result of necrosis that occurs with chronic pressure overload, particularly as it progresses to HF [21]. Furthermore, fibrosis, per se, can affect cardiac function adversely, by impairing normal LV contraction [32]. However, this was not the sole mechanism of the improved LV function since isolated myocytes also demonstrated enhanced contractility, which should not be affected by fibrosis.

One potential mechanism mediating the reduction of fibrosis is that CI reduces secretion of CTGF from apoptotic non-myocytes after chronic pressure overload, since it has been demonstrated that CTGF is released from apoptotic endothelial cells mediated by caspase-3 activation in vitro and skin fibrosis in vivo [15]. In this study, we detected less expression of

CTGF in the CI group compared to vehicle by immunohistochemical staining. Another potential mechanism is inhibition of pro-inflammatory caspase-1 followed by reduction of the active form of interleukin-18, i.e., there is blunted hypertrophy after pressure overload in interleukin-18-KO mice [4], and interleukin-18 regulates osteopontin-mediated cardiac fibrosis and diastolic dysfunction [33]. Therefore, caspase-1 may also play a role in compensatory cardiac growth in response to pressure overload through modulation of cytokine activation.

In this connection, the fact that the CI protected non-myocyte apoptosis becomes important. Clearly, the macrophages and other non-myocytes in the heart played a protective role in mediating reduced inflammation, reduced necrosis and reduced fibrosis. Interestingly, the percentage of interstitial fibrosis was significantly correlated with apoptosis in non-myocytes ($R^2 = 0.74$, $p = 1.1E-06$), rather than in myocytes ($R^2 = 0.35$, $p = 0.006$) after chronic pressure overload. Furthermore, there was a significant positive correlation between myocyte cross-sectional area and non-myocyte apoptosis ($R^2 = 0.57$, $p = 0.0001$). These results also support the key role of non-myocytes mediating protection from LVH and fibrosis during the transition from LVH to HF.

Having noted all of the above, there may be another important mechanism by which fibrosis was reduced and function was preserved. Reduced subendocardial coronary reserve is a major mechanism mediating the impaired LV function with pressure overload hypertrophy [11], which will lead to necrosis and fibrosis when there is an imbalance between myocardial oxygen demand and supply. This major mechanism resulting in LV dysfunction and HF can be ameliorated by angiogenesis [8]. Indeed, we observed evidence for increased angiogenesis in the group with chronic pressure overload and CI treatment, which could have favorably affected subendocardial coronary reserve and thereby helped preserve cardiac function. It will be important to find the precise mechanism by which angiogenesis is induced by CI, which will require identifying which vascular growth factor is responsible and whether blocking the angiogenesis reverses the beneficial effects of CI on the protection of cardiac function with chronic pressure overload.

In summary, since it is widely held that reduction of myocyte apoptosis is a major mechanism leading LV decompensation [9, 30], the most novel finding of our investigation is that the reduction of myocyte apoptosis by CI does not mediate the protection of the heart from developing HF following chronic pressure overload, but rather it is due to other mechanisms. These mechanisms are all inter-related to the preservation of LV wall stress and individual myocyte contractility, which reduces increased myocyte size and at the same time enhances myocyte hyperplasia and angiogenesis, which prevents impaired subendocardial reserve and protects individual myocyte contractility, as well as global LV function. This resulted in less myocardial fibrosis, which also protects LV function. Thus, there appears to be little argument that CI could potentially be indicated therapeutically, but the mechanism of its salutary effect is most likely not that which has been previously conjectured, i.e., protection of apoptotic myocytes [1, 9, 16, 26, 29, 30], but rather from the data of our study it is more likely due to angiogenesis and myocyte hyperplasia and consequent protection of LV wall stress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Apoptosis and total myocyte number after chronic pressure overload. **a** Caspase inhibition (*CI*) significantly reduced apoptosis compared to vehicle (*veh*) at 3 weeks after transverse aortic constriction (*TAC*). However, the majority of apoptotic cells rescued by *CI* were non-myocytes (*left panel*). * $p < 0.05$ (vs. sham), † $p < 0.05$ (*CI* + *TAC* vs. *veh* + *TAC*). $n = 5-9$ /each group. Pie graph shows cell types of apoptotic non-myocytes after 3-week *TAC* with or without *CI*. *M ϕ* macrophages, *EC* endothelial cells, *FB* fibroblasts (*right panel*). **b** Total myocyte number was not significantly changed at 3 weeks after *TAC* with or without *CI*. **c** The total myocyte number was not correlated with myocyte apoptosis (%). *CI* (*open circles*), *veh* (*closed circles*). **d** Representative pictures of apoptotic non-myocytes. The tissue

sections were dual immunostained with TUNEL assay and each cell type-specific antibody (*white arrows*). **e** Immunohistochemical staining of cleaved caspase-3 (CC3) in LV myocardium at 3 weeks after TAC. CC3-positive non-myocyte (*green, yellow arrow*). The outlines of myocytes were shown by wheat germ agglutinin (WGA) staining (*red*). The nuclei were co-stained with DAPI (*blue*)

Fig. 2.

Correlation between apoptosis and Ki67-positive myocytes, an index of myocyte proliferation after chronic pressure overload. **a** The proliferation of myocytes was identified by double immunostaining with Ki67 and wheat germ agglutinin (WGA). Ki67-positive myocyte number was significantly increased in the caspase inhibition (CI) group compared to vehicle (veh) group after 3-week transverse aortic constriction (TAC). $^*p < 0.05$ (vs. sham), $^{\dagger}p < 0.05$ (CI + TAC vs. veh + TAC), $n = 5-7$ /each group. **b** The number of Ki67-positive myocytes was significantly correlated with apoptosis only in the CI group, but not veh group after 3-week TAC. CI (open circles), veh (closed circles). **c, d** A significant negative correlation is shown between the number of Ki67-positive myocytes (%) and reduction of non-myocyte apoptosis in the CI group after 3-week TAC, but not in reduction of myocyte apoptosis in the CI group after 3-week TAC. Non-myocytes with CI (closed diamonds), myocytes with CI (closed circles). **e** Representative photographs of Ki67-positive myocytes. Yellow arrows indicate Ki67-positive myocytes in the CI group after TAC; Ki67-positive cells (red), WGA (green). **f** Representative photographs of mitotic cells; phospho-histone H3 (PHH3) (red), WGA (gold), mitotic myocytes (yellow arrow in upper panel), and mitotic non-myocytes (white arrow in lower panel). The nuclei were co-stained with DAPI (blue)

Fig. 3.

Caspase inhibition decreased myocyte hypertrophy. **a** Myocyte cross-sectional area (μm^2) was increased less in the caspase inhibition (CI) group compared to vehicle (veh) group. **b** The distribution of myocyte cross-sectional area in three groups is shown. **c** LV systolic wall stress was increased less in CI than veh after 3-week transverse aortic constriction (TAC). **d** Positive correlation between myocyte cross-sectional area (μm^2) and LV systolic wall stress (kdyn/cm²) in sham and TAC with/without CI is shown. CI (open circles), veh (closed circles). * $p < 0.05$ (vs. sham), † $p < 0.05$ (CI + TAC vs. veh + TAC). $n = 5-11$ /each group

Fig. 4.

Caspase inhibition reduced interstitial fibrosis and increased angiogenesis after chronic pressure overload. **a** The percentage of interstitial fibrosis was detected by picrosirius red (PSR) staining. The interstitial fibrosis (%) was increased less with caspase inhibition (*CI*) compared to vehicle (*veh*) after transverse aortic constriction (*TAC*). **b** Representative photographs of PSR-stained tissue section of heart with 3-week TAC. Collagen deposition was shown in red (*upper panel*) and viewed with circularly polarized light (*lower panel*)

Fig. 5.

Caspase inhibition increased angiogenesis after chronic pressure overload. **a** Caspase inhibition (*CI*) significantly increased Ki67-positive endothelial cells after 3-week transverse aortic constriction (*TAC*). **b** Representative photographs of Ki67-positive endothelial cells (*EC*) in *CI* group at 3-week after *TAC*. Ki67-positive myocytes of endothelial cells (*yellow arrows*) was detected by dual immunohistochemical staining with Ki67 (*red*) and isolectin-IB₄-Alexa 488 (*green*). The nuclei were co-stained with DAPI (*blue*). **c** Capillary density was significantly increased in the *CI* group after *TAC*. * $p < 0.05$ (vs. sham), † $p < 0.05$ (*CI* + *TAC* vs. veh + *TAC*), $n = 5-7$ /each group

Fig. 6.

Caspase inhibition improved myocyte contractility. **a** The amplitude of myocyte contraction is shown as the percentage of shortening of cell length (*upper panel*). The percentage of contractility from isolated myocytes was higher in caspase inhibition (*CI*) than vehicle (*veh*) group after transverse aortic constriction (*TAC*) (*lower panel*). **b** Representative merged image of immunohistochemical double staining with TnI (*red*) and wheat germ agglutinin (WGA) (*green*). The nuclei were co-stained with DAPI (*blue*)

Fig. 7.

Protein expression and localization of connexin-43. Representative photographs of immunohistochemical double staining with connexin 43 (Cx43, *green*) and rhodamine-conjugated wheat germ agglutinin (WGA) (*red*). The nuclei were co-stained with DAPI (*blue*). Cx43 gap junction disorganization was observed in vehicle (*veh*) after transverse aortic constriction (*TAC*) (*white arrows*), and lateralization in caspase inhibition (*CI*) after TAC (*yellow arrows*)

Table 1

Physiological parameters in mice with caspase inhibitor (CI) after TAC

Group	LVwt/BW	LVEDD	EF (%)	FS %	LV wall stress	HR	LVEDP	LVSP	PG (mmHg)
Sham									
Vehicle (<i>n</i> = 11)	3.1 ± 0.1	3.72 ± 0.06	69.0 ± 0.7	32.4 ± 0.5	51 ± 2	432 ± 9	5 ± 1	91 ± 2	4 ± 1
CI (<i>n</i> = 11)	3.1 ± 0.1	3.85 ± 0.10	70.4 ± 1.0	33.5 ± 0.8	57 ± 3	437 ± 18	6 ± 1	98 ± 1	3 ± 0
3-week TAC									
Vehicle (<i>n</i> = 12)	4.9 ± 0.2 [*]	4.14 ± 0.11 [*]	54.6 ± 2.6 [*]	23.5 ± 1.5 [*]	102 ± 6 [*]	500 ± 14	15 ± 2 [*]	165 ± 4 [*]	92 ± 4 [*]
CI (<i>n</i> = 11)	4.3 ± 0.2 ^{*†}	3.96 ± 0.08	65.6 ± 2.3 [†]	30.3 ± 1.6 [†]	81 ± 4 ^{*†}	495 ± 18	10 ± 2 ^{*†}	164 ± 4 [*]	84 ± 4 [*]

^{*} *p* < 0.05 (vs. sham),

[†] *p* < 0.05 (CI + TAC vs. vehicle + TAC)