Activation of apoptotic processes during transition from hypertrophy to heart failure in guinea pigs

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Sharma AK, Dhingra S, Khaper N, Singal PK. Activation of apoptotic processes during transition from hypertrophy to heart failure in guinea pigs, Am J Physiol Heart Circ Physiol 293: H1384–H1390, 2007. First published July 6, 2007; doi:10.1152/ajpheart.00553.2007.—Changes in oxidative stress and apoptotic process were studied during the progression of a compensated hypertrophy to a decompensated heart failure in guinea pigs. Banding of the ascending aorta resulted in heart hypertrophy. At 10 wk, ventricle-to-body weight ratio and thickness of the interventricular septum as well as the left ventricular wall were increased significantly. Although fractional shortening and ejection fraction were decreased, there were no signs of heart failure. Furthermore, there was no increase in wet-to-dry weight ratios for the lungs and liver at this stage. However, at 20 wk, heart failure was characterized by a significant depression in heart function as indicated by a decrease in fractional shortening, and ejection fraction and a lesser increase in wall thickness from diastole to systole. Animals also showed clinical signs of heart failure, and the wet-to-dry weight ratios of the lungs and liver were significantly higher. Cardiomyocyte oxidative stress was significantly higher in the 20-wk aortic-banded group. The ratio of Bax to Bcl-xl showed an increase at 10 wk, and there was a further increase at 20 wk. Mitochondrial membrane potential in the aortic-banded animals was significantly decreased at 10 and 20 wk. Cytochrome c levels were higher in the cytosol compared with the mitochondria, leading to a considerable increase in the expression of p17 subunit of caspase-3. At 20 wk, both early and late stages of apoptosis were observed in isolated cardiomyocytes. It is suggested that an increase in oxidative stress initiates mitochondrial death pathway during the hypertrophic stage, leading to apoptosis and heart failure at a later stage.

METHODS

Animal Model

All experimental protocols including the animal studies were approved by the University of Manitoba Animal Care Committee, following the guidelines established by the Canadian Council on Animal Care. Male guinea pigs, weighing 150 ± 10 g, were maintained on standard chow and water ad libitum. Banding of the ascending aorta was done according to the procedure described earlier (36). Sham-operated animals were treated in an identical manner except that no band was placed around the aorta. The entire surgical procedure was carried out in sterile conditions. Following the surgery, the animals were allowed to recover in a postsurgery recovery room. These animals were monitored on a regular basis, and various parameters were studied at 10 and 20 wk.

Echocardiography

For the study of the heart function, transthoracic echocardiography was performed at 10 and 20 wk. Animals were sedated with 2% isoflurane. Imaging of the heart was done using a 12-MHz (s12) ultrasound probe and a Sonos 5500 echogapher (Agilent Technologies, Andover, MA). For M-mode recordings, the parasternal short-axis view was used to image the heart in two dimensions (2-D) at the level of the papillary muscles. Left ventricular fractional shortening...
and ejection fraction were analyzed using a commercially available software program as described previously (16).

Collection of Tissues

The heart, lungs, and liver from 10- and 20-wk aortic-banded animals were removed and weighed. To obtain the ventricular weight, atria were carefully removed before the ventricles were weighed. For further studies, a portion of the fresh ventricular tissue was used for the isolation of mitochondria, and the other portion from ventricles was immediately frozen in liquid nitrogen and stored for Western blot analysis of Bax, Bel-1x, and caspase-3. Isolated hearts were perfused for the preparation of primary cardiomyocytes. Preweighed lungs and liver were chopped into small pieces. These were placed in an oven at 65°C until a constant weight (usually after 72 h) was obtained for the determination of wet-to-dry weight ratios.

Separation of Mitochondrial and Cytosolic Fractions From Ventricular Tissue

Isolation of mitochondria from fresh ventricles was performed by following the protocol provided with the mitochondria isolation kit (MITO-ISO1; Sigma). In brief, the heart tissue from 10- and 20-wk aortic-banded and sham animals was cut into small pieces and weighed. After treatment with trypsin, the tissue was homogenized in extraction buffer A, provided with the mitochondria isolation kit. The homogenate was centrifuged at 600 g for 5 min, and the supernatant so obtained was then centrifuged at 10,000 g for 10 min. The supernatant obtained in the later step was saved as the cytosolic fraction for cytochrome c study. The pellet containing the mitochondria was suspended in 1× storage buffer. A portion of this mitochondrial fraction was then immediately used for measuring membrane potential as well as protein concentration. The other portion was frozen in liquid nitrogen for cytochrome c measurement.

Mitochondrial Membrane Potential and Cytochrome c Measurements

Mitochondrial potential was measured by using a fixed-point assay measuring the uptake of JC-1 dye into the matrix with the formation of J-aggregates. The assay was performed by following the protocol provided with the MITO-ISO1 kit (Sigma). Briefly, a known volume of mitochondrial sample was added to 1.9 ml of 1× JC-1 assay buffer in a test tube. The reaction was started by adding 2 μl of JC-1 stain, mixed, and left at room temperature in the dark for 7 min to allow complete uptake of dye into mitochondria. Fluorescence was read in a spectrophuorometer with excitation and emission wavelengths of 490 and 590 nm, respectively. Total protein concentration was determined using the modified Bio-Rad microassay procedure. Fluorescence was expressed as fluorescence units per milligram of mitochondrial protein. Cytochrome c was detected in mitochondrial and cytosolic fractions by Western blotting using anti-cytochrome c primary antibody (Cell Signaling Technology, Danvers, MA) as described below.

Measurement of Caspase-3, Bax, and Bcl-xl Proteins

Protein isolation. Previously frozen whole hearts from 10- and 20-wk aortic-banded and sham control animals were powdered in liquid nitrogen and homogenized in RIPA buffer containing protease inhibitor cocktail (Sigma Aldrich). The lysates were centrifuged at 14,000 rpm for 10 min. The upper layer containing protein fraction was sonicated, frozen in liquid nitrogen, and stored at −75°C.

Western blot analysis. The protein samples were then subjected to one-dimensional 12–15% SDS-PAGE in a discontinuous system. Equal loading of protein (30 μg/lane) was confirmed by Coomassie blue staining and use of anti-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as internal control. Separated proteins were transferred onto 0.45-μm nitrocellulose membrane and incubated overnight with Bax, Bel-1x, and caspase-3 polyclonal antibodies (Santa Cruz Biotechnology). Primary antibodies were detected using a goat anti-rabbit IgG hors eradish peroxidase-conjugated secondary antibody (Bio-Rad) using the BM Chemiluminescence kit (POD substrate; Roche Diagnostics, Laval, QC, Canada). The protein bands were visualized with a Fluor S-Multimeter MAX system (Bio-Rad) and quantified using image analysis software (Quantity One; Bio-Rad).

Oxidative Stress and Apoptosis

Ventricular myocytes were isolated from sham and aortic-banded hearts at 20 wk after banding by using a procedure described earlier (17) with slight modifications. After the excision, hearts were mounted on a modified Langendorff perfusion apparatus. The perfusate consisted of calcium-free modified Krebs buffer containing 110 mM NaCl, 2.6 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 25 mM NaHCO3, and 11 mM glucose (pH 7.4). The perfusion was then switched to recirculating mode with the modified Krebs buffer containing 20 μM calcium, 0.06% (wt/vol) collagenase, and 0.1% (wt/vol) bovine serum albumin (BSA) for 20 min. The collagenase-digested ventricles were gently passed through pipettes with a progressively smaller tip diameter and with an increasing concentration of BSA and CaCl2. The suspension was filtered through a nylon mesh (200 μm) and was allowed to settle for 10 min. The supernatant was discarded, and the cell pellet was suspended in medium M199 containing CaCl2 (1.8 mM). Cardiomyocytes were plated on 4% serum-coated polystyrene tissue culture dishes. Plated myocytes were incubated in serum-free culture medium M199 supplemented with antibiotics (100 μg/ml streptomycin/penicillin) at 37°C under a 5% CO2-95% air atmosphere. Two hours after plating, the culture medium was changed to remove unattached dead cells, and the viable myocytes were incubated overnight.

Oxidative stress in isolated cardiomyocytes was studied by quantifying the endogenous production of reactive O2− species, utilizing a 5(6)-chloromethyl-2′,7′-dihydrofluorescein diacetate probe (CM-H2DCFDA; Molecular Probes, Eugene, OR). Fluorescence intensity, which was proportional to the level of oxidative stress, was rapidly measured using digital imaging processing software (Image Pro Plus). An excitation wavelength of 485 nm and an emission wavelength of 530 nm were used.

Early apoptosis in isolated cardiomyocytes was detected using a commercially available annexin-V-FLUOS assay kit (Roche Diagnostics, Mannheim, Germany). The cardiomyocytes exhibiting the green fluorescence (annexin-V-FLUOS) were considered to be in the early stage of apoptosis. For the study of late apoptosis, cells were fixed with 4% paraformaldehyde and stained with bisbenzimide 33258 (1 μg/ml) for 10 min to determine nuclear fragmentation in the nuclei.

Statistics Analysis

Data are means ± SE. The data were analyzed using ANOVA followed by Bonferroni’s test for multiple comparisons. A value of P < 0.05 was considered significant. All statistical analysis was performed using Microcal Origin software version 6.

RESULTS

Clinical Observations and Cardiac Function

Aortic-banded and sham control animals were observed daily for 20 wk for their general behavior, food and water intake, and clinical signs of heart failure. At 10 wk postsurgery, the animals did not show any clinical signs of heart failure. However, after about 15 wk, aortic-banded animals began to show signs of heart failure including dyspnea, enlarged abdomen, and cyanosis of limbs and ear lobes. After the assessment of heart function by 2-D echocardiography at 10 and 20 wk postsurgery, the animals were killed and their heart, lungs, and liver were removed for further analysis.

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Table 1. Echocardiographic parameters of heart function at 10 and 20 wk after aortic banding in guinea pigs

<table>
<thead>
<tr>
<th>Parameter</th>
<th>10 Wk</th>
<th>20 Wk</th>
<th>10 Wk</th>
<th>20 Wk</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>Banded</td>
<td>Sham</td>
<td>Banded</td>
</tr>
<tr>
<td>EF, %</td>
<td>81.0±0.02</td>
<td>77.0±0.004*</td>
<td>80.0±0.013</td>
<td>64.0±0.02*†</td>
</tr>
<tr>
<td>FS, %</td>
<td>44.05±1.70</td>
<td>39.41±0.97*</td>
<td>42.15±1.70</td>
<td>28.73±1.15*</td>
</tr>
<tr>
<td>IVS, mm Diastole</td>
<td>2.33±0.01</td>
<td>2.64±0.004*</td>
<td>2.36±0.003</td>
<td>2.92±0.005†</td>
</tr>
<tr>
<td>Systole</td>
<td>3.42±0.02</td>
<td>3.80±0.017*</td>
<td>3.91±0.001</td>
<td>3.97±0.003†</td>
</tr>
<tr>
<td>LVPW, mm Diastole</td>
<td>3.12±0.005</td>
<td>3.95±0.005*</td>
<td>3.32±0.01</td>
<td>4.40±0.018†</td>
</tr>
<tr>
<td>Systole</td>
<td>3.59±0.01</td>
<td>4.89±0.004*</td>
<td>4.02±0.01</td>
<td>4.95±0.002*†</td>
</tr>
</tbody>
</table>

Data are means ± SE of 4–6 animals. *P < 0.05, significantly different from respective sham control, †P < 0.05, significantly different from 10-wk banded animals. EF, ejection fraction; FS, fractional shortening; IVS, interventricular septum thickness; LVPW, left ventricular posterior wall thickness.

At 10 wk, fractional shortening was decreased by 11%, and this was accompanied by a 5% decrease in ejection fraction. These decreases were significant (P < 0.05) compared with their respective sham controls (Table 1). In 20-wk aortic-banded animals, both fractional shortening and ejection fraction showed further decreases by 32 and 20%, respectively. At 10 wk, interventricular septum thickness during diastole and systole was increased by 13 and 11%, respectively, in aortic-banded animals compared with their respective sham controls (Table 1 and Fig. 1). At 20 wk, this increase was 24 and 1.5% during diastole and systole, respectively. Left ventricle posterior wall thickness during diastole and systole at 10 wk showed an increase by 27 and 36%, respectively. At 20 wk, the increase during diastole and systole was 32 and 23%, respectively, compared with their sham counterparts (P < 0.05).

To further assess heart hypertrophy, the body and ventricular weights were examined at 10 and 20 wk postsurgery (Table 2). There was no significant difference in gain in body weight between the aortic-banded animals and their respective sham controls at 10 and 20 wk. The ventricular weight was higher in aortic-banded animals compared with their respective sham controls at both stages. The ratio of ventricle to body weight at 10 and 20 wk was higher in aortic-banded animals by 66 and 86% compared with their sham controls, a difference that was statistically significant (P < 0.05).

The wet and dry weights of lungs and liver were also examined. At 10 wk, the ratio of wet to dry weight of lungs showed a slight increase (4%) compared with sham controls. However, at 20 wk, the ratio was ~19% in aortic-banded animals, and this change was statistically significant (P < 0.05). Similarly, the wet-to-dry weight ratio for liver showed a significant increase only at 20 wk (31%) compared with a 4% increase at 10 wk (Table 3).

Apoptotic Processes

Bax/Bcl-xl ratio. Bax (proapoptotic) and Bcl-xl (antiapoptotic) proteins were assessed in heart tissue of aortic-banded and sham animals at 10 and 20 wk. There was a significant increase (P < 0.05) in the Bax-to-Bcl-xl ratio in aortic-banded animals compared with their sham controls at 10 and 20 wk (Fig. 2). The ratio at 20 wk was significantly higher (P < 0.05) compared with the 10-wk aortic-banded animals (Fig. 2).

Mitochondrial membrane potential. Mitochondrial membrane potential dissipation is known to be an early event in the...
process of apoptosis. This was measured by the uptake of cationic carbocyanin dye JC-1 into the mitochondrial matrix. The membrane potential of mitochondria isolated from heart tissue of aortic-banded and sham animals was examined at 10 and 20 wk. The results for the aortic-banded group were normalized by setting the value of membrane potential of sham controls at 1. At both stages, mitochondrial membrane potential of aortic-banded animals was significantly lower \( (P < 0.05) \) compared with their respective sham controls (Fig. 3).

**Cytochrome c levels.** In this study, the levels of myocardial cytochrome c were significantly higher \( (P < 0.05) \) in the cytosolic fraction compared with the mitochondrial fraction isolated from aortic-banded animals at 10 and 20 wk (Fig. 4). Furthermore, the mitochondrial cytochrome c content in aortic-banded animals was significantly lower \( (P < 0.05) \), whereas the cytosolic content of cytochrome c was significantly higher, than in their respective sham controls at both the stages.

**Caspase-3.** Caspase activation has been suggested to play an important role in apoptosis. In intrinsic pathway of apoptosis, cytochrome c, released from mitochondria, functions as an activator of caspase-3 zymogen, and activated caspase 3 cleaves a subset of proteins leading to a chain of events and apoptosis. In this study, we tested whether the zymogen form of caspase-3 is processed to its active p17 subunit in the hearts of aortic-banded animals. There was significant increase \( (P < 0.05) \) in p17 subunit expression in aortic-banded animals at 10 and 20 wk (Fig. 5). This expression in the 20-wk aortic-banded group was significantly higher \( (P < 0.05) \) compared with that in 10-wk aortic-banded animals.

**Isolated cardiomyocytes.** To study the apoptotic changes at the cell level, cardiomyocytes were isolated from aortic-banded animals at 20 wk. For the detection of apoptotic cells, phosphatidylserine externalization (annexin staining) and DNA fragmentation (Hoechst staining) were examined. Annexin staining of the rod-shaped cardiomyocytes suggested early apoptosis (Fig. 6A). Late apoptosis was indicated by the fragmented blue nuclei with Hoechst stain (Fig. 6B).

**Oxidative Stress**

The level of oxidative stress was examined by measuring the optical density (OD) of green fluorescence by using the CM-H2DCFDA assay in isolated cardiomyocytes in 20-wk aortic-banded animals and in age-matched sham controls. This intensity was expressed per unit area of the cells examined (Fig. 7).

### Table 3. Wet-to-dry weight ratio of lungs and liver at 10 and 20 wk after aortic banding in guinea pigs

<table>
<thead>
<tr>
<th>Group</th>
<th>Wet/Dry Weight</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Lungs</td>
</tr>
<tr>
<td>10 Wk</td>
<td>5.89±0.06</td>
</tr>
<tr>
<td>Sham</td>
<td>5.16±0.08</td>
</tr>
<tr>
<td>Banded</td>
<td>6.12±0.21*</td>
</tr>
<tr>
<td>20 Wk</td>
<td>6.15±0.12</td>
</tr>
</tbody>
</table>

Data are mean ± SE of 4–6 animals. *\( P < 0.05 \), significantly different from respective sham control. †\( P < 0.05 \), significantly different from 10-wk banded animals.

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**Fig. 2.** Bax/Bcl-xl protein ratio in the myocardium of sham and aortic-banded animals at 10 and 20 wk. Data are means ± SE from 5 experiments. Data are normalized with sham control values taken as 1. *\( P < 0.05 \), significantly different from respective sham control. †\( P < 0.05 \), significantly different from 10-wk animals. Western blot bands for Bax and Bcl-xl are shown at top.

**Fig. 4.** Cytochrome c in the mitochondrial (Mit) and cytosolic (Cyt) fractions isolated from hearts in sham and aortic-banded animals at 10 and 20 wk. Data are means ± SE from 5 experiments. Data are normalized with sham control values taken as 1. *\( P < 0.05 \), significantly different from respective sham control. Western blot bands for cytochrome c are shown at top.

**Fig. 3.** Mitochondrial membrane potential in hearts from sham and aortic-banded animals at 10 and 20 wk. Data are means ± SE from 5 experiments. *\( P < 0.05 \), significantly different from respective sham control. FLU, fluorescence units.

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There was a 60% increase in the OD per unit area in aortic-banded animals compared with the sham animals.

DISCUSSION

In the present study, the progression of a compensated hypertrophied heart to a decompensated stage of heart failure was established. Heart hypertrophy at 10 and 20 wk was indicated by a significant increase in ventricle-to-body weight ratio. At 10 wk, there was a significant increase in the interventricular septum as well as left ventricular wall thicknesses during the diastolic phase. Although there was some depression in heart function at 10 wk as indicated by a decrease in fractional shortening as well as in ejection fraction, these changes were quantitatively small and were not associated with signs of heart failure. In this regard, wet-to-dry weight ratios of the lungs and liver were not changed, and the animals did not show any dyspnea or cyanosis of the extremities. Thus we arbitrarily characterized this stage as compensated hypertrophy stage.

Although at 20 wk there was a significant increase in the ventricle-to-body weight ratio, the change was associated with a lesser increase in the wall thickness from diastole to systole in the aortic-banded animals compared with sham controls. These observations suggested that the enlarged hearts had poor ability to develop force. The cardiac dysfunction in the aortic-banded animals at 20 wk was further indicated by a decrease in fractional shortening as well as in ejection fraction. Furthermore, these animals showed clinical signs of heart failure, including dyspnea. An increase in the fluid content of tissues such as the lungs and the liver at 20 wk, subsequent to the banding of the aorta, also supported the fact that these animals were in a congestive heart failure stage. Thus, at 20 wk postbanding, the animals were in decompensated congestive heart failure. Similar findings in this model of hypertrophy and heart failure have been reported previously (36).

Increased oxidative stress has been reported to be associated with transition from hypertrophy to heart failure in the pressure overload model of guinea pig (8) and rat (15). An excessive increase in reactive oxygen species has been suggested to play an important role in the development and progression of heart failure and in cardiac remodeling. The latter is associated with the induction of myocyte hypertrophy and apoptosis and with interstitial fibrosis by activating matrix metalloproteinases (19). In guinea pigs, an increase in endogenous antioxidant

Fig. 5. Caspase p17 subunit in myocardium in sham and aortic-banded animals at 10 and 20 wk. Data are means ± SE from 5 experiments. Data are normalized with sham control values taken as 1. *P < 0.05, significantly different from respective sham control. #P < 0.05, significantly different from 10 wk animals. Western blot bands for caspase-3 are shown at top.

Fig. 6. Early and late apoptosis in cardiomyocytes isolated from 20-wk aortic-banded animals. Green stain with annexin (A) shows early apoptosis, and fragmented nuclei stained blue with Hoechst (B) show late apoptosis.

Fig. 7. Reactive oxygen species generation in cardiomyocytes isolated from sham and aortic-banded animals at 20 wk. Top: representative fluorescent microscope images for myocytes stained with 5(6)-chloromethyl-2',7'-dihydrodmoxy fluorescein diacetate probe. Bottom: fluorescence intensity data expressed as OD per unit area. *P < 0.05, significantly different from respective sham control.
enzymes and a decrease in lipid peroxidation during hypertrophy stage and a significant decline in antioxidant enzymes during heart failure stage have been reported (8). Since cardiomyocytes isolated from heart failure stage in the present study showed significantly higher levels of oxidative stress, it is suggested that oxidative stress indeed is at the cardiomyocyte level. These data are in agreement with earlier reports on 20-wk aortic-banded groups, and clearly these failing hearts had higher oxidative stress. An increase in oxidative stress during heart failure is not only limited to the pressure overload model; other types of heart failure also have been shown to be associated with an increase in oxidative stress (18, 22, 26, 29, 43).

Cardiomyocyte apoptosis is implicated in the pathogenesis of heart failure (27, 41, 44). Biopsy samples from failing human hearts that were explanted from the patients undergoing cardiac transplantation showed a significant increase in apoptosis (32, 33). Apoptotic pathways also have been reported to be activated in cardiomyocytes in the context of many etiologies, such as ischemia-reperfusion, hypoxia, calcium excess, oxidative stress, gene induction, rapid pacing, stretching of myocytes, and Adriamycin administration (4, 10, 12, 41, 44, 45).

The present study documents an activation of mitochondrial death pathways, which include such steps as an increase in the ratio of Bax to Bcl-xl, lowering of mitochondrial membrane potential, cytochrome c release, and activation of caspase-3 leading to apoptosis during the transition to heart failure stage subsequent to chronic pressure overload. In this regard, the pressure overload resulted in a significant increase in the expression of Bax-to-Bcl-xl ratio, which is reported to precede apoptosis (30). Furthermore, an increase in Bax/Bcl-2 during heart failure also has been shown in rats and the bovine model of pressure overload (5, 30). An increase in Bax/Bcl-2 promotes homodimerization of Bax. An exposure of isolated cardiomyocytes to oxidative stress was found to cause the incorporation of Bax into mitochondrial membranes (6). In the present study, an increase in oxidative stress in cardiomyocytes isolated from failing hearts also may have resulted in the incorporation of Bax into the mitochondrial membrane, thereby lowering membrane potential, resulting in the leakage of cytochrome c from mitochondria to cytosol as also has been reported by others (28, 46). Since decreases in membrane potential and cytochrome c release at 10 and 20 wk of aortic banding were similar with different pathophysiological consequences, it is likely that there also are other factors that influence the ultimate end effect.

In the cytoplasm, the cytochrome c binds and activates an adaptor, the apoptotic protease-activating factor (Apaf)-1, to recruit and activate caspase-9, forming apoptosome, which activates the initiator caspase-9 (25, 48). This in turn leads to the activation of downstream effector caspases; zymogen caspase-3 is converted into an active form (p17) of caspase-3 and causes apoptosis. In our study, p17 subunit was indeed significantly elevated in the heart failure stage, suggesting an activation of caspase-3. Similar results exhibiting release of cytochrome c from mitochondria into the cytoplasm also have been shown in explanted human hearts with end-stage cardiomyopathy that was accompanied by an activation of caspase-3 (32). Mitochondrial cytochrome c release and caspase activation also have been reported during hypoxia-mediated apoptosis of adult ventricular myocytes (7), hyperglycemia-induced apoptosis in mouse myocardium (3), and colchicine-induced apoptosis of cerebellar granule cells (9).

In conclusion, the present study provides evidence that an increase in oxidative stress occurs at the cardiomyocyte level and that the apoptotic processes are initiated during the transition from hypertrophy to heart failure. The list includes an increase in proapoptotic factors, a decrease in mitochondrial membrane potential, release of cytochrome c from mitochondria, triggering of caspase activation, and apoptosis. Inhibition of this process, at any of the steps, can be seen to interfere with the pathogenesis of heart failure subsequent to pressure overload.

REFERENCES


