Cardiomyocyte mitochondrial oxidative stress and cytoskeletal breakdown in the heart with a primary volume overload

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Yancey DM, Guichard JL, Ahmed MI, Zhou L, Murphy MP, Johnson MS, Benavides GA, Collawn J, Darley-Usmar V, Dell’Italia LJ. Cardiomyocyte mitochondrial oxidative stress and cytoskeletal breakdown in the heart with a primary volume overload. *Am J Physiol Heart Circ Physiol* 308: H651–H663, 2015. First published January 16, 2015; doi:10.1152/ajpheart.00638.2014.—Left ventricular (LV) volume overload (VO) results in cardiomyocyte oxidative stress and mitochondrial dysfunction. Because mitochondria are both a source and target of ROS, we hypothesized that the mitochondrial antioxidants (MitoQ) will improve cardiomyocyte damage and LV dysfunction in VO. Isolated cardiomyocytes from Sprague-Dawley rats were exposed to stretch in vitro and VO of aortocaval fistula (ACF) in vivo. ACF rats were treated with and without MitoQ. Isolated cardiomyocytes were analyzed after 3 h of cyclical stretch or 8 wk of ACF with MitoSox red or 5-(and-6)-chloromethyl-2,7’-dichlorodihydrofluorescein diacetate to measure ROS and with tetramethylrhodamine to measure mitochondrial membrane potential. Transmission electron microscopy and immunohistochemistry were used for cardiomyocyte structural assessment. In vitro cyclical stretch and 8-wk ACF resulted in increased cardiomyocyte mitochondrial ROS production and decreased mitochondrial membrane potential, which were significantly improved by MitoQ. ACF rats demonstrated mitochondrial swelling and disorganization, myofibrillar degeneration and cytoskeletal disruption and mitochondrial damage in the VO of ACF.

A PRIMARY VOLUME OVERLOAD (VO) of the heart increases diastolic load and results in progressive eccentric left ventricular (LV) remodeling and systolic dysfunction, which eventually leads to heart failure (20). An excessive and incessant diastolic load from mitral regurgitation (MR) or aortocaval fistula (ACF) results in adverse eccentric LV remodeling manifested by wall thinning, cardiomyocyte elongation, and a decrease in the LV mass-to-volume ratio (2, 20, 35, 45, 46). In patients with secondary MR, renin-angiotensin system blockade and diuretics are beneficial when MR results from an ischemic or nonischemic cardiomyopathy in which LV dilatation stretches an otherwise normal mitral apparatus geometry (papillary muscle, valve chordae and leaflets, and mitral annulus) causing a secondary or functional MR (7). However, in patients with degenerative mitral valve or in rats with ACF, the VO is the primary stimulus. Currently, there is no medical therapy that halts or attenuates the progression to heart failure in an isolated primary VO of MR (5, 30).

Experimental animal models of isolated VO due to ACF or mitral valvular chordal disruption have emphasized extensive loss of the extracellular matrix in the pathophysiology of adverse LV eccentric remodeling (9, 35, 45, 46). Recently, we have demonstrated increased cardiomyocyte oxidative stress in patients with VO of isolated chronic MR with LV ejection fraction > 60% (2) and in rats with VO of ACF (19, 42). In addition, there is extensive myofibrillar degeneration and mitochondrial dysfunction and damage, despite a well-preserved LV ejection fraction (2). The preservation of LV shortening has been shown to belie underlying cardiomyocyte dysfunction due to ejection into the low-pressure left atrium in MR (2) or into the arteriovenous fistula in ACF (18, 19). Novel drug targets that attenuate oxidative stress and maintain normal cardiomyocyte morphology are an unmet need in arresting the progressive LV dilatation in a pure VO. While the ACF is not a perfect reproduction of MR, it does incorporate the primary hemodynamic stress of VO/stretch with concomitant facilitation of ejection into the arteriovenous fistula.

In both chronic (2) and acute VO (19), there is evidence of increased cardiomyocyte xanthine oxidase (XO) expression and activity. We have demonstrated that cyclical stretch of adult rat cardiomyocytes results in increased XO activity, mitochondrial swelling and disorganization, and myofibrillar degeneration that is similar to the changes identified in the human LV with isolated MR (2). However, chronic XO inhibition in the rat with 8 wk of VO of ACF does not attenuate LV dilatation and LV wall thinning despite an improvement in LV contractile function (18). In our previous in vitro study (19) of stretched cardiomyocytes, the mitochondrially targeted antioxidant mitobiquinone (MitoQ) prevented myofibrillar degener-
METHODS

Animal preparation. Adult male Sprague-Dawley rats (200–250 g) at 12 wk of age were subjected to either sham or ACF, as previously described by our laboratory (9, 18, 19, 35, 42, 45), with and without mitochondrial antioxidant MitoQ treatment (5 mg/kg) for 8 wk. MitoQ treatment was initiated during the week of surgery and delivered in the drinking water as previously described by our laboratory (8). Separate sets of sham and ACF rats were euthanized 8 wk after surgery for experiments of isolated cardiomyocytes for live cell imaging (n = 6/group). Another set of sham and ACF rats was studied for in vivo hemodynamic and echocardiographic measurements before death, and this tissue was used for protein analysis and immunohistochemistry (n = 5/group). The animal use in these experiments was approved by the University of Alabama at Birmingham Animal Resource Program (protocol 130409070).

Hemodynamics and echocardiography. Echocardiography and hemodynamics were performed before death using the Visualsonics imaging system (VIVO 2100, Toronto, ON, Canada) combined with simultaneous high-fidelity LV pressure catheter recordings (Millar Instruments, Houston, TX). With the rats under isoflurane anesthesia, a high-fidelity LV pressure catheter was advanced into the LV cavity via a right carotid cutdown. LV pressure and echocardiography dimensions (wall thickness and chamber diameter) were obtained simultaneously using software included in the Visualsonics system. LV volume was calculated from traced M-mode LV dimensions using the following Teicholz formula: volume = \[ \frac{7}{(2.4 + LVID)} \times (LVID)^3 \], where LVID is LV internal dimension. LV wall stress was calculated from traced M-mode LV dimensions and simultaneous LV pressure data using the following equation: LV wall stress = (LV pressure \times r)/(2 \times LV wall thickness), where r is the LV chamber radius. These LV pressure-volume data were analyzed using the Labscribe2 (iWorx System Dover, NH) software package as previously described by our laboratory (18, 19).

Isolation of LV cardiomyocytes. Cardiomyocytes were isolated from sham and ACF rats as previously described by our laboratory (18, 19, 42). Briefly, hearts were perfused with perfusion buffer (120 mmol/l NaCl, 15 mmol/l KCl, 0.5 mmol/l KH2PO4, 5 mmol/l NaHCO3, 10 mmol/l HEPES, and 5 mmol/l glucose at pH 7.0) for 5 min and digested with perfusion buffer containing 2% collagenase type II (Invitrogen, Carlsbad, CA) for 30 min at 37°C. The right ventricle, atria, and apex were removed before the perfused heart was minced. The digestion was filtered and washed, and cells were pelleted. Only samples with viability (rod-shaped cells) > 80% were used.

Application of stretch to isolated adult rat cardiomyocytes. Cells (50,000 cells/well) were allowed to adhere to laminin-coated Flexcell plates (Flexcell, Hillsborough, NC) in DMEM containing 10% FBS, 2 nM glutamine, 10 U/ml penicillin, and 100 mg/ml streptomycin for 2 h before use. Cells were subjected to cyclical strain (60 cycles/min, 3 h) on the Flexcell strain apparatus (model FX-4000, Flexcell) at a level of distension sufficient to promote an increment of ~20% in surface area at the point of maximal distension on the culture surface as previously described by our laboratory (19, 45). A group of cells stretched for 3 h was also treated with MitoQ (50 nM). Control cells
were prepared on identical culture plates but were not exposed to stretch.

**Live cell imaging.** The cationic potentiometric fluorescent dye tetramethylrhodamine methyl ester (TMRM; 50 nM) was used to monitor changes in mitochondrial membrane potential. ROS production was monitored with 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein (CM-DCF; 5 μmol/l), an H₂O₂-sensitive fluorescent indicator, as previously described by our laboratory (42). The dish containing fluorescent dye-loaded cardiomyocytes was equilibrated at 37°C with unrestricted access to atmospheric O₂ on the stage of an Olympus microscope. CM-DCF and TMRM images were recorded using an Olympus FV1000 confocal laser scanning microscope with excitation at 488 and 543 nm, respectively. Images were analyzed offline using ImageJ software (Wayne Rasband, National Institutes of Health).

**Immunohistochemistry.** Rat hearts were immersion fixed in 10% neutral-buffered formalin and paraffin embedded. Sections (5 μm) were mounted on slides, deparaffinized in xylene, and rehydrated in a gradient series of ethanol. Some sections were stained in basic fuchsin (1:1,000, Abcam, Cambridge, UK), whereas others were stained with a primary antibody to myosin light chain (1:100, Abcam, Cambridge, UK) followed by a secondary antibody conjugated to Alexa Fluor 488 (1:1000, Invitrogen, Carlsbad, CA) and counterstained with DAPI (1:1000, Invitrogen, Carlsbad, CA).

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**Fig. 2.** MitoQ protects mitochondrial membrane potential in stretched cardiomyocytes. Isolated adult rat cardiomyocytes underwent cyclical (60 cycles/min) ~20% stretch for 3 h followed by staining with tetramethylrhodamine methyl ester (TMRM; 50 nM) for 15 min. This staining indicated a depolarized mitochondrial membrane and mitochondrial damage that was significantly improved by treatment with MitoQ. Left: live cell TMRM staining of stretched and control nonstretched cardiomyocytes. Right: intensity quantification of live cell imaging. n = 50 cells/group. *P < 0.05 vs. the sham group; ^P < 0.05 vs. the ACF group.

**Fig. 3.** MitoQ does not affect the basal O₂ consumption rate (OCR) in isolated adult cardiomyocytes. Neither MitoQ nor (decyl)triphenylphosphonium (dTPP), the targeting scaffold of MitoQ, changed basal or mitochondrial function in control rat cardiomyocytes. Left: OCR plotted over time. Right: quantification. MitoQ or dTPP (50 and 100 nM) were added at 20 min. Oligomycin (O; 1 μg/ml) was used to determine non-ATP-dependent O₂ consumption by inhibiting complex V of the mitochondria. FCCP (F; 1 μM) was used to uncouple mitochondrial respiration and stimulate the maximal OCR. Rotenone/antimycin A (R/A; 1 μM/10 μM) was used to completely inhibit the mitochondrial electron transport chain, indicating the nonmitochondrial OCR. n = 6–7 cells/group.
used for fluorescent staining. Sections used for fluorescent imaging were blocked with 5% goat serum in 1% BSA in PSA and incubated overnight at 4°C with either desmin antibody (1:100, Abcam), mitochondrial complex IV (1:200, Abcam), or β2-tubulin (1:500, Abcam). Image acquisition and intensity measurements for desmin were performed on a Leica DM6000 epifluorescence microscope with Simple-PCI Imaging software (Compix, Cranberry Township, PA). Alexa fluor-conjugated secondary antibodies (1:500 each, Molecular Probes, Eugene, OR) were applied to visualize desmin (green), β2-tubulin (red), and complex IV (red) in the tissue. Nuclei were stained (blue) with 4',6-diamidino-2-phenylindole (1.5 μg/ml, Vector Laboratories, Burlingame, CA). Image acquisition (×100 objective, ×4,000 video screen magnification) was performed on a Leica DM6000 epifluorescence microscope.

Fig. 4. dTPP has no effect on oxidative stress or mitochondrial membrane potential in stretched adult cardiomyocytes. MitoSox red, indicating ROS production, and TMRM fluorescent staining, indicating mitochondrial membrane potential, in stretched rat cardiomyocytes treated with dTPP, the targeting moiety of MitoQ used as a negative control, were not significantly different from untreated stretched cells, indicating that the therapeutic effects shown in Figs. 1 and 2 were due to the active antioxidant quinone moiety of MitoQ.

Fig. 5. MitoQ decreases oxidative stress and protects mitochondrial membrane potential in cardiomyocytes isolated from 8-wk ACF rats. Analysis of ROS by 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein (CM-DCF), a fluorescent marker of oxidative stress, in cardiomyocytes isolated from 8-wk ACF rat hearts indicated a significant increase in ROS in ACF rats, which was attenuated by treatment with MitoQ (top). Mitochondrial membrane potential, as measured by TMRM, was significantly depolarized in ACF animals and improved by treatment with MitoQ (bottom). n = 50 cells/group. *P < 0.05 vs. the sham group.
with SimplePCI software (Compix). Images were adjusted appropriately to remove background fluorescence.

Transmission electron microscopy of rat tissue. Tissue was fixed overnight in 25% glutaraldehyde, mounted for transmission electron microscopy (TEM), and analyzed by EmLabs (Birmingham, AL) as previously described by our laboratory (19, 42).

Western blot analysis. LV tissue lysates (30 μg protein) were separated on a 4–12% bis-Tris gradient gel (Invitrogen), transferred to membranes, incubated with horseradish peroxidase-conjugated secondary antibody. Membranes were incubated with Chemiluminescent Substrate (Pierce, Cary, NC) for all comparisons among sham, ACF, MitoQ-treated sham, and MitoQ-treated ACF groups. This statistical method was chosen due to the variability between animal parameters in the study. All statistical analyses were two sided, and P values of <0.05 were considered statistically significant. Adjustments for multiple comparisons were not made because there were no repeated comparisons.

RESULTS

MitoQ improves cardiomyocyte oxidative stress and mitochondrial membrane potential in vitro but has no effect on LV dilatation and function in vivo. Cyclical stretch of isolated cardiomyocytes from normal rats caused a significant increase in oxidative stress as measured by MitoSox red, which was significantly decreased by pretreatment with MitoQ (Fig. 1). The mitochondrial membrane potential of isolated cardiomyocytes from normal rats caused a significant increase in oxidative stress as measured by MitoSox red, which was significantly decreased by pretreatment with MitoQ (Fig. 1). The mitochondrial membrane potential of isolated cardiomyocytes from normal rats caused a significant increase in oxidative stress as measured by MitoSox red, which was significantly decreased by pretreatment with MitoQ (Fig. 1).

Table 1. Morphometric data on 8 wk of ACF

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>ACF</th>
<th>Sham + MitoQ</th>
<th>ACF + MitoQ</th>
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</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>475 ± 16</td>
<td>517 ± 11</td>
<td>470 ± 29</td>
<td>489 ± 17</td>
</tr>
<tr>
<td>Heart weight, g</td>
<td>1.15 ± 0.4</td>
<td>2.11 ± 0.06*</td>
<td>1.20 ± 0.04</td>
<td>2.33 ± 0.14*</td>
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<tr>
<td>LV weight, g</td>
<td>0.80 ± 0.2</td>
<td>1.37 ± 0.03*</td>
<td>0.81 ± 0.04</td>
<td>1.37 ± 0.09*</td>
</tr>
<tr>
<td>Right ventricular weight, g</td>
<td>0.23 ± 0.01</td>
<td>0.46 ± 0.03*</td>
<td>0.22 ± 0.01</td>
<td>0.43 ± 0.04*</td>
</tr>
<tr>
<td>Lung weight, g</td>
<td>1.6 ± 0.06</td>
<td>2.2 ± 0.06*</td>
<td>1.6 ± 0.09</td>
<td>2.2 ± 0.17*</td>
</tr>
<tr>
<td>Tibia length, mm</td>
<td>49 ± 0.6</td>
<td>50 ± 0.3</td>
<td>49 ± 0.4</td>
<td>50 ± 0.5</td>
</tr>
<tr>
<td>Heart weight/body weight, g/100</td>
<td>0.24 ± 0.007</td>
<td>0.409 ± 0.009*</td>
<td>0.247 ± 0.007</td>
<td>0.426 ± 0.02*</td>
</tr>
<tr>
<td>Number of rats/group</td>
<td>7</td>
<td>7</td>
<td>8</td>
<td>8</td>
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</table>

Values are means ± SE. Animals were divided into the following groups: sham operation (sham), volume overload of aortocaval fistula (ACF), sham with mitouquinone (MitoQ) treatment (sham + MitoQ), and ACF with MitoQ treatment (ACF + MitoQ). LV, left ventricular. *P < 0.05 vs. the sham group.

Table 2. LV hemodynamic and functional parameters in chronic 8-wk ACF

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>ACF</th>
<th>Sham + MitoQ</th>
<th>ACF + MitoQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, beats/min</td>
<td>343 ± 19</td>
<td>338 ± 12</td>
<td>312 ± 11</td>
<td>330 ± 4</td>
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<tr>
<td>Mean arterial pressure, mmHg</td>
<td>106 ± 6</td>
<td>92 ± 2</td>
<td>100 ± 4</td>
<td>95 ± 2</td>
</tr>
<tr>
<td>+LV dP/dt_max, mmHg/s</td>
<td>7,437 ± 327</td>
<td>7,287 ± 248</td>
<td>6,900 ± 282</td>
<td>7,458 ± 339</td>
</tr>
<tr>
<td>−LV dP/dt_max, mmHg/s</td>
<td>−7,901 ± 405</td>
<td>−6,654 ± 295*</td>
<td>−6,880 ± 394*</td>
<td>−6,494 ± 238</td>
</tr>
<tr>
<td>LV end-systolic pressure, mmHg</td>
<td>79 ± 5</td>
<td>75 ± 2</td>
<td>80 ± 4</td>
<td>75 ± 2</td>
</tr>
<tr>
<td>LV end-diastolic pressure, mmHg</td>
<td>6 ± 2</td>
<td>11 ± 1*</td>
<td>6 ± 1</td>
<td>10 ± 1*</td>
</tr>
<tr>
<td>LV end-diastolic stress, g/mcm</td>
<td>76 ± 5</td>
<td>102 ± 14*</td>
<td>89 ± 11</td>
<td>104 ± 8*</td>
</tr>
<tr>
<td>End-systolic pressure-volume relationship, mmHg/μl</td>
<td>0.36 ± 0.05</td>
<td>0.18 ± 0.02</td>
<td>0.35 ± 0.07</td>
<td>0.29 ± 0.08</td>
</tr>
<tr>
<td>LV end-diastolic volume, mm</td>
<td>8.19 ± 0.18</td>
<td>11.33 ± 0.34*</td>
<td>8.41 ± 0.21</td>
<td>11.09 ± 0.34*</td>
</tr>
<tr>
<td>LV end-diastolic dimension, mm</td>
<td>5.61 ± 0.16</td>
<td>7.69 ± 0.53*</td>
<td>5.74 ± 0.28</td>
<td>7.82 ± 0.30*</td>
</tr>
<tr>
<td>LV ejection fraction, %</td>
<td>57 ± 3</td>
<td>57 ± 2</td>
<td>54 ± 3</td>
<td>59 ± 2</td>
</tr>
<tr>
<td>LV fractional shortening, %</td>
<td>33 ± 2</td>
<td>34 ± 3</td>
<td>34 ± 2</td>
<td>32 ± 1</td>
</tr>
<tr>
<td>Velocity of circumferential shortening, %</td>
<td>6.6 ± 0.4</td>
<td>6.2 ± 0.8</td>
<td>6.7 ± 0.4</td>
<td>5.7 ± 0.2</td>
</tr>
<tr>
<td>Number of rats/group</td>
<td>7</td>
<td>7</td>
<td>8</td>
<td>8</td>
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</table>

Values are means ± SE. *P < 0.05 vs. the sham group.
improved by pretreatment with MitoQ. MitoSox red and TMRM fluorescent staining were performed in rat cardiomyocytes treated with (decyl)triphenylphosphonium (dTPP), which contains the linker group but not the pharmacologically active redox component of MitoQ. High concentrations of MitoQ or dTPP can disrupt mitochondrial function in cell culture (34). Neither dTPP or MitoQ (50 nM) at the concentration used in this study (50 nM) had any effect on cellular bioenergetics (Fig. 3). In addition, dTPP did not prevent the stretch-dependent loss of mitochondrial membrane potential or increased MitoSOX staining, indicating that the redox active component of MitoQ was essential for its protective effects (Fig. 4).

The beneficial effects of MitoQ on cardiomyocyte stretch led us to test its effects in chronic VO using the same live cell imaging in cardiomyocytes isolated from 8-wk ACF rats treated with or without MitoQ. Sham hearts had a punctate CM-DCF signal from the mitochondria (Fig. 5). In contrast, ACF resulted in both an increased CM-DCF signal and a more diffuse pattern, which was reversed in MitoQ-treated ACF rats. As in the cardiomyocyte stretch experiments, ACF cardiomyocytes had mitochondrial depolarization that was preserved in cardiomyocytes from MitoQ-treated ACF rats (Fig. 5). However, these beneficial effects on ACF cardiomyocytes in vitro did not translate into improved LV remodeling or function after 8 wk of VO in vivo. Heart, LV, right ventricular, and lung weights as well as heart weight-to-body weight ratios were increased in ACF compared with sham rats and did not improve in MitoQ-treated ACF rats (Table 1). Mean arterial pressure did not differ between ACF groups (Table 2). LV end-diastolic dimension, LV end-diastolic volume, and LV end-diastolic wall stress were increased as the LV end-systolic pressure-volume relationship was decreased in both ACF and MitoQ-treated ACF groups versus the sham group (Table 2).

ACF causes mitochondrial changes and breakdown of the cytoskeleton. We previously showed extensive cardiomyocyte ultrastructural breakdown with cell stretch that was improved by MitoQ (19). Thus, we examined the cardiomyocyte cytoskeleton in chronic VO. Normal mitochondrial distribution, as

Fig. 6. Eight-week ACF rats have disorganized cytoskeletal elements and loss of mitochondrial registry. Transmission electron microscopy (TEM) of the left ventricular (LV) myocardium in sham (top) and 8-wk ACF (bottom) hearts demonstrated pathological changes in mitochondrial morphology in ACF hearts compared with sham hearts. ACF mitochondria exhibited a loss of linear registry, clustering, disassociation with sarcomeres, and decreased electron density in ACF. The ACF myocardium also showed a breakdown in myofilbrils, a decrease in Z-line electron density, and large gaps between sarcomeric units. Arrow in the top left image indicates normal Z-line electron density, whereas arrow in the bottom left image indicate the loss of electron density at the Z-lines due to ACF. Insets in the top left and bottom left images are expanded in the top right and bottom right images, respectively.

Fig. 7. ACF causes changes in mitochondrial morphology. Basic fuchsin staining of the LV myocardium in 8-wk sham (left) and ACF (right) hearts demonstrated pathological changes in mitochondrial morphology in ACF hearts compared with sham hearts. Arrows indicate disorganization of the registry of mitochondria in the ACF myocardium compared with sham myocardium.
demonstrated by TEM in sham rats (Fig. 6), shows the typical orderly linear array of one mitochondrion per sarcomere with close proximity to the myofibrils. These mitochondria are electron dense with tightly packed cristae. The VO of ACF caused a complete disruption of this highly organized structure of interfibrillar mitochondria such that mitochondria lost their linear register and were no longer in close proximity to the sarcomere, with large spaces between the mitochondria and individual sarcomeric units. In addition, mitochondria became crowded with several small, round mitochondria per sarcomere by TEM and in a more global representation of the myocardium by basic fuscin staining (Fig. 7). ACF mitochondria also demonstrated a loss of electron density as well as a disruption and loss of cristae. Evidence of mitochondrial structural disruption was also evident by a marked decrease in mitochondrial complex IV (subunit IV) staining by immunohistochemistry (Fig. 8).

The mitochondrial disorganization in ACF was directly paralleled by disruption and breakdown of the cytoskeletal intermediate filament desmin by immunohistochemistry (Fig. 8). Desmin is the major intermediate filament in the heart and provides a structural framework that extends from the subsarcolemma to the nuclear membrane. Desmin is an important structural protein that is essential in the mitochondrial-sarcomere connection, especially along the Z-lines between adjacent sarcomeres. In sham rats, desmin can be clearly seen along the Z-lines of cardiomyocytes in a highly regular linear array and was extensively disrupted in ACF rats (Fig. 8). The loss of desmin by immunohistochemistry is consistent with the loss of electron density of the Z-line in ACF hearts by TEM (Fig. 6, arrow). However, the loss of desmin was extremely patchy throughout the LV myocardium. For example, some cardiomyocytes were completely devoid of desmin staining, whereas neighboring cardiomyocytes had intact desmin staining along Z lines. A similar desmin staining pattern has been previously described by Di Somma and coworkers (13) in humans with end-stage heart failure due to ischemic cardiomyopathy. Quan-

![Fig. 8.](image)

Fig. 8. ACF changes desmin distribution as well as mitochondrial organization. Immunohistochemical (IHC) analysis of desmin (green) and mitochondrial complex 4 (COX4; red) in sham and 8-wk ACF rats demonstrated that the normal desmin distribution along Z-lines was completely disrupted along with a loss and disruption of COX4 staining in ACF. 4',6-Diamidino-2-phenylindole (DAPI; blue) indicates nuclei. Left: desmin and DAPI. Middle: COX4 and DAPI. Right: merge.

![Fig. 9.](image)

Fig. 9. MitoQ prevents decreases in desmin protein expression. Western blot analysis of desmin indicated a significant decrease in desmin whole-length protein in 8-wk ACF rats, corresponding to the loss of desmin shown in Fig. 5. The appearance of a lower-molecular-weight breakdown product accompanied this decrease in full-length protein at 53 kDa. The decrease in the 53-kDa desmin band in ACF was rescued by treatment with MitoQ, leading to a decrease in the breakdown product band.
tification of desmin intensity from immunohistochemistry revealed a mean decrease in ACF versus the sham group (4,773 ± 1,946 vs. 12,580 ± 825, P = 0.03). The rather large SE in the ACF group reflects this marked variability in desmin loss throughout each LV section. The representative desmin Western blot (Fig. 9) demonstrated desmin loss manifested by a decrease in the single whole-length desmin band and breakdown evidenced by the lower-molecular-weight band in ACF rats, which has been previously reported as a desmin breakdown product in heart failure (1). The preservation of whole-length desmin and decreased breakdown product in MitoQ-treated ACF rats are consistent with a MitoQ protective effect, which was reflected in the improved intensity score in MitoQ-treated ACF versus ACF rats (9,939 ± 326 vs. 5,786 ± 1,883 P < 0.05). However, recovery of desmin in individual cardiomyocytes was variable throughout the myocardium.

β2-Tubulin was also disrupted in ACF rats. β2-Tubulin is a cytoskeletal support protein involved in anchoring the mitochondria to the sarcomeres (21, 24). In sham rats, β2-tubulin was located in a linear array along the Z-lines but did not colocalize with desmin. The coincident loss of β2-tubulin and desmin in ACF (Fig. 10) matches the overall loss and disruption of mitochondria revealed by complex IV staining.

MitoQ improves cardiomyocyte mitochondrial organization and the cytoskeleton in chronic VO rats. MitoQ treatment improved mitochondrial organization and structure by TEM analysis after ACF. Although not completely normalized, MitoQ-treated cardiomyocytes had more electron-dense mitochondria with more dense cristae and a somewhat improved linear registry of mitochondria (Fig. 11) as well as restored complex IV staining (Fig. 12). In a similar but not complete fashion, MitoQ-treated ACF rats also improved desmin and β2-tubulin degradation by immunohistochemistry (Figs. 12 and 13, respectively), consistent with the Western blot analysis (Fig. 9).

Treatment of sham rats with MitoQ caused a proliferative response with many more mitochondria in the interfibrillar spaces but preservation of the Z-line by TEM (Fig. 11). Immunohistochemistry of MitoQ-treated sham rats exhibited disruption of desmin and β2-tubulin along the Z-lines and in some regions decreased complex IV staining (Figs. 12 and 13).

DISCUSSION

In the present study, we show that in a pure cardiac VO, where there is a well-established increase in cardiomyocyte oxidative stress, MitoQ improves cytoskeletal breakdown and mitochondrial damage but has no effect on LV dilatation and systolic function. The results from the present investigation underscore that, in addition to a loss of interstitial collagen, there is a breakdown and disorganization of the cardiomyocyte cytoskeletal-myofibrillar-mitochondrial architecture, suggesting the existence of an important balance between mitochon-
Mitochondria-derived oxidants and cytoskeletal-mitochondrial structure in the VO cardiomyocyte that may be a causative factor in the progression to heart failure.

The cytoskeleton is composed of three components: actin microfilaments, tubulin microtubules, and intermediate filaments (6). Desmin is the main intermediate filament protein expressed in the heart and interacts with other structural proteins at the Z-disk, extending from the subsarcolemmal membrane to the nucleus. Desmin thus forms a continuous cytoskeletal network that maintains the spatial relationship between the sarcomeric contractile apparatus and the mitochondria that provides cellular integrity, mechanochemical signaling, and force transmission in the cardiomyocyte (3, 6, 24–28, 36). Currently, 49 mutations have been identified in the desmin gene (12), which largely alters the desmin filament assembly process and interactions with its protein partners. This results in disorganization of the desmin network with cardiac and/or skeletal myopathies that are characterized by disruption of the Z-line and the accumulation of desmin-containing aggregates in the cells. A transgenic mouse model inducing protein aggregation of αβ-crystallin, a heart-specific chaperone for desmin, has recapitulated the desmin cardiomyopathy disease phenotypes observed in humans (26). In this model, there is a formation of perinuclear aggregates and progression to a dilated cardiomyopathy that results in death from heart failure in transgenic mice by 5–7 mo of age (44). In the present study, the loss of desmin by immunohistochemistry and its decrease and degradation by Western blot analysis (Fig. 9) coincided with mitochondrial disorganization and loss of electron density of the Z-line in TEM of VO rats.

In rats with VO, MitoQ improves desmin breakdown and the swelling and disorganization of the interfibrillar mitochondria, suggesting that mitochondrial oxidative stress and subsequent activation of intracellular proteases may be a causative factor. TNF-α overexpression in transgenic mice is associated with desmin cleavage by caspase 6, resulting in sarcomere degeneration, desmin protein aggregates, and a dilated cardiomyopathy (31). In the present study, loss of desmin with VO is associated with decreased mitochondrial complex IV staining. In fact, some cardiomyocytes exhibit a near-complete loss of desmin in conjunction with decreased mitochondrial complex IV staining. A patchy, near-total loss of desmin in cardiomyocytes has also been reported in patients with idiopathic dilated cardiomyopathy (13). Disorganization of desmin and mitochondrial functional complexes with myofibrils and the sarcoplasmic reticulum have been shown to be present before the onset of cardiac and skeletal muscle failure (3, 6, 24–28, 36, 38). The relation of desmin breakdown to long-term survival and to the severity of LV dysfunction in human heart failure (13, 29, 32, 33) provides further evidence for a nongenetic cause of desmin degradation in the pathophysiology of LV functional deterioration.

Similar to desmin, tubulin is a critical cytoskeletal protein. The most common members of the tubulin family are α- and β-tubulin, which dimerize to form the microtubular cytoskeletal network. There are many isoforms of β-tubulin, including β1, β2, β3, β4, and β5. In the cardiomyocyte, β2-tubulin is involved in interactions between the mitochondria and cytoskeleton and participates in the regulation of mitochondrial respiration (21, 24). Saks and coworkers (21, 24) have shown that different isoforms of β-tubulin have varied intracellular distribution and organization and play different roles in mitochondrial respiration. β2-Tubulin has a regular arrangement in rows along the long axis of the cell and colocalizes with the mitochondria. In VO, we find a degradation of β2-tubulin associated with decreased mitochondrial density. As with desmin, MitoQ preserves β2-tubulin in ACF but also causes a
minor degree of β2-tubulin disruption in sham animals, possibly due to the apparent proliferation in mitochondrial number.

An important finding of the present study is that the breakdown of desmin and β2-tubulin with VO is in direct contrast to their increase in either compensated or decompensated pressure overload in multiple animal species, including humans (10, 11, 40, 41, 43, 47, 48), which may be a consequence of divergent hemodynamic loads of pressure versus VO. The finding that treatment with MitoQ attenuates the breakdown of cytoskeletal proteins in vitro and in vivo suggests that oxidative stress activates intracellular proteases and provides an important underpinning mechanism in cardiomyocyte remodeling in VO. To further support this contention, we have demonstrated that increased superoxide production, H₂O₂ formation, and XO activity in cardiomyocytes is associated with cardiomyocyte matrix metalloproteinase activation after 24 h of VO of ACF in the rat (19, 42).

MitoQ treatment in sham rats resulted in mitochondrial clustering along with a disruption of tubulin and desmin, which did not cause desmin degradation or loss of Z-line integrity. These findings suggest a different mechanism of cytoskeletal disruption than that observed in ACF hearts. MitoQ has a beneficial but still a very patchy effect on desmin and tubulin preservation in ACF hearts. One possibility is that the delivery at the same dosage of MitoQ to the mitochondria is higher in the normal mitochondria but lower in ACF animals due to the lower mitochondrial membrane potential in ACF (39). In MitoQ-treated sham rats, there appears to be a mitochondrial proliferative response that has a secondary reorganization effect on desmin and β2-tubulin but with Z-line preservation. Nevertheless, MitoQ did not affect LV function in sham rats even at 12 wk of treatment (data not shown).

Current guidelines do not recommend any specific medical therapy for a primary VO (30). Furthermore, LV ejection fraction can decrease after mitral valve repair even when LV ejection fraction and LV end-systolic dimension are within recommended guidelines of 60% and LV end-systolic dimension of 4.0 cm, respectively (2, 15, 37). The failure of LV shortening indexes to reflect underlying myocardial pathology has driven the controversial recommendation of early surgical therapy in the asymptomatic patient with primary MR (14, 17). It is of great interest that Hutchinson and coworkers (23) recently reported a decrease in LV contractility after correction of a 4-wk ACF, despite a reversion of LV end-diastolic dimension/volume to normal, as seen in patients after mitral valve repair for primary MR (2, 15, 37). Since the hemodynamic stress of a primary VO can only be corrected by surgery, future studies should assess the ability of prior drug therapy to protect the myocardium and improve LV functional recovery after correction of the ACF. In addition to extracellular matrix loss (4, 9, 42, 45, 46), the present study demonstrated a

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Fig. 12. MitoQ maintains desmin distribution and protects against mitochondrial disorganization in 8-wk ACF rats. IHC analysis of desmin (green) and mitochondrial COX4 (red) in sham and ACF rats treated with MitoQ is shown. In MitoQ-treated sham rats, there was a loss of the normal desmin distribution along the Z-lines (see Fig. 5) that was paralleled by a similar disruption of COX4 staining. In MitoQ-treated ACF rats, the desmin and COX4 loss and disruption were improved compared with ACF rats (Fig. 5). In MitoQ-treated sham rats, there was a disruption of desmin and distribution of mitochondria within cardiomyocytes. Left: desmin and DAPI. Middle: COX4 and DAPI. Right: merge.
matching cytoskeletal breakdown associated with loss of mitochondrial connection to sarcomeric units in a primary VO. The critical relevance of this finding in the rat is underscored by a similar pathology in the human with isolated MR and preserved LV ejection fraction (2). Understanding the pathological imbalance of oxidative stress and other signaling pathways that cause cytoskeletal breakdown and mitochondrial dysfunction in the pathophysiology of a pure VO may lead to the development of novel drug targets to prevent the progression to clinical heart failure and improve surgical outcomes.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


Fig. 13. MitoQ protects against desmin and β2-tubulin disruption in 8-wk ACF rats. IHC analysis of desmin (green) and β2-tubulin (red) indicated attenuation of the disruption of β2-tubulin in ACF by treatment with MitoQ (see Fig. 8). Treatment with MitoQ in sham rats caused a disruption in the distribution of β2-tubulin. Left: desmin and DAPI. Middle: β2-tubulin and DAPI. Right: merge.


