N-acetylcysteine reverses diastolic dysfunction and hypertrophy in familial hypertrophic cardiomyopathy

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Wilder T, Ryba DM, Wieczorek DF, Wolska BM, Solaro RJ. N-acetylcysteine reverses diastolic dysfunction and hypertrophy in familial hypertrophic cardiomyopathy. Am J Physiol Heart Circ Physiol 309: H1720–H1730, 2015. First published October 2, 2015; doi:10.1152/ajpheart.00339.2015.—S-glutathionylation of cardiac myosin-binding protein C (cMyBP-C) induces Ca2⁺ sensitization and a slowing of cross-bridge kinetics as a result of increased oxidative signaling. Although there is evidence for a role of oxidative stress in disorders associated with hypertrophic cardiomyopathy (HCM), this mechanism is not well understood. We investigated whether oxidative myofilament modifications may be in part responsible for diastolic dysfunction in HCM. We administered N-acetylcysteine (NAC) for 30 days to 1-mo-old wild-type mice and to transgenic mice expressing a mutant tropomyosin (Tm-E180G) and nontransgenic littermates. Tm-E180G hearts demonstrate a phenotype similar to human HCM. After NAC administration, the morphology and diastolic function of Tm-E180G mice was not significantly different from controls, indicating that NAC had reversed baseline diastolic dysfunction and hypertrophy in our model. NAC administration also increased sarco(endo)plasmic reticulum Ca²⁺ ATPase protein expression, reduced extracellular signal-related kinase 1/2 phosphorylation, and normalized phosphorylation of phospholamban, as assessed by Western blot. Detergent-extracted fiber bundles from NAC-administered Tm-E180G mice showed nearly nontransgenic (NTG) myofilament Ca²⁺ sensitivity. Additionally, we found that NAC increased tension cost and rate of cross-bridge reattachment. Tm-E180G myofilaments were found to have a significant increase in S-glutathionylation of cMyBP-C, which was returned to NTG levels upon NAC administration. Taken together, our results indicate that oxidative myofilament modifications are an important mediator in diastolic function, and by relieving this modification we were able to reverse established diastolic dysfunction and hypertrophy in HCM.

sarcomeres; cardiac myosin-binding protein C; oxidative stress; diastolic dysfunction; S-glutathionylation

NEW & NOTEWORTHY

Novel findings include reversal of established hypertrophy, left atrial dilation, and diastolic dysfunction in a model of HCM treated with the antioxidant N-acetylcysteine. This was associated with a reduction in S-glutathionylation of myofilament proteins, which was increased in untreated controls, a decrease in myofilament Ca²⁺ response, and hastening of cross-bridge kinetics.

* T. Wilder and D. M. Ryba contributed equally to this work.

HYPERTROPHIC CARDIOMYPATHY (HCM) often progresses to heart failure with preserved ejection fraction (HFrEF), an increasingly common diagnosis characterized by severely impaired diastolic function with little or no effect on systolic cardiac function. The etiology of HFrEF remains unclear, but with many risk factors that range from genetic predisposition, as seen in familial HCM, to common acquired conditions, such as hypertension and hyperlipidemia, HFrEF-related diagnoses are likely to rise in the coming years. However, there are few treatments targeted to relieve the diastolic dysfunction observed in HFrEF (4).

Despite strong implications for a role of redox-related modifications in HCM linked to mutations in sarcomeric proteins, there have been no studies investigating the potential role of reactive oxygen species (ROS)-induced exacerbation of the HCM phenotype at the level of oxidative modification of the myofilament proteins. Our previous studies indicate that ROS-related posttranslational modifications in sarcomeric proteins are likely to be a major mechanism in the diastolic abnormalities associated with familial HCM. These studies reported that ROS, associated with a hypertensive model of HFrEF, induced S-glutathionylation of cardiac myosin-binding protein C (cMyBP-C), which slowed cross-bridge kinetics and increased Ca²⁺ sensitivity. Ventricular myocytes from the model demonstrated diastolic abnormalities with no change in the Ca²⁺ transients, suggesting that the diastolic dysfunction was attributed to dysfunction at the level of the sarcomere. Restoring redox balance in this model by relieving NOS uncoupling restored diastolic function to control levels in correlation with a reduction of cMyBP-C S-glutathionylation to basal levels (5, 12).

In experiments reported here, we assessed the redox state and function of a transgenic (Tg) mouse model of familial HCM expressing a mutation in tropomyosin (Tm), where glutamic acid at residue 180 has been exchanged for a glycine (Tm-E180G). Tm-E180G mice develop severe diastolic dysfunction, hypertrophy, and left atrial dilation by 2 wk of age (1). Our previous studies reported that, compared with controls, this model demonstrates a significant increase in myofilament response to Ca²⁺ and diastolic dysfunction that is likely to trigger maladaptive remodeling (21, 26). Here, our current data show that Tm-E180G hearts display early signs of oxidative stress in the form of increased oxidative modifications of cMyBP-C and activation of the MAPK signaling cascade. Therefore, we hypothesized that treatment with the glutathione precursor N-acetylcysteine (NAC) may reverse the oxidative stress in our model and improve hypertrophy and diastolic...
Three parameters of the LV diastolic function were evaluated: E/A Doppler sample volume was moved toward the LVOT, and both the tip of the mitral valve leaflets. To measure time intervals, the long-axis view. The parasternal short-axis view at the level of the (LVOT), and left atrium (LA) were taken from the left parasternal Imaging System with a scan head center frequency of 30 MHz) in formed using a high-resolution transducer (Vevo 770 High Resolution 770 Analytic Software. formations were averaged from three consecutive cycles and per-
mitral annulus in the four-chamber view. All measurements and diastole were obtained with the sample volume at the septal side of the
interstitial fibrosis, and diastolic dysfunction with preserved systolic function (1). Controls were non-Tg (NTg) FVB/N littermates.

MATERIALS AND METHODS

All protocols and procedures involving the use of animals were given prior approval by the Institutional Animal Care and Use Committee of the University of Illinois in Chicago (with Association for Assessment and Accreditation of Laboratory Animal Care International accreditation). Animals were maintained in accordance with the Guide for the Care and Use of Laboratory Animals (8th ed., revised 2011) published by the National Institutes of Health.

Tm-E180G transgenic mice. We employed a transgenic mouse model in a FVB/N background that displayed a phenotype similar to human HCM, in which 65% of wild-type α-Tm is replaced with α-Tm-E180G (21). Previous studies have reported that from 2 wk of age Tg mice begin to exhibit cardiac hypertrophy, myocyte disarray, interstitial fibrosis, and diastolic dysfunction with preserved systolic function (1). Controls were non-Tg (NTg) FVB/N littermates.

Study design and NAC administration. One-month-old male and female NTg and Tm-E180G littermates were administered regular drinking water or NAC (Sigma, St. Louis, MO) dissolved in drinking water administered at 250 mg·kg⁻¹·day⁻¹, based on prior data (14), for 4 wk. All mice were subjected to echocardiography before and after the 1-mo treatment. After 1 mo of NAC administration, mice were euthanized using a ketamine-xylazine mix (200 and 100 mg/kg, respectively), and hearts were extracted for functional and biochemical assessment.

Echocardiography. Echocardiographic measurements were performed using a high-resolution transducer (Vevo 770 High Resolution Imaging System with a scan head center frequency of 30 MHz) in isoflurane-anesthetized 2-mo-old mice, as described previously (22). M-mode images of the left ventricle (LV), left ventricle outflow tract (LVOT), and left atrium (LA) were taken from the left parasternal long-axis view. The parasternal short-axis view at the level of the papillary muscles was used to measure the LV internal dimension, and pulse wave Doppler was performed at the apical four-chamber view. The mitral inflow was recorded with the Doppler sample volume at the tip of the mitral valve leaflets. To measure time intervals, the Doppler sample volume was moved toward the LVOT, and both the mitral inflow and LV outflow were obtained in the same recording. Three parameters of the LV diastolic function were evaluated: E/A ratio, E-wave deceleration time, and LV isovolumic relaxation time. Additional information about the diastolic function was obtained with tissue Doppler imaging. Peak myocardial velocities in the early (Eₑ) diastole were obtained with the sample volume at the septal side of the mitral annulus in the four-chamber view. All measurements and calculations were averaged from three consecutive cycles and performed according to the American Society of Echocardiography guidelines (8, 16). Data analysis was performed offline using the Vevo 770 Analytic Software.

Assessment of β-myosin heavy chain expression. Myofibrils were purified from ~25.0 mg of liquid nitrogen frozen mouse heart tissue and 1-day-old neonatal mouse heart tissue for the analysis of β-myosin heavy chain expression. The tissue was homogenized twice in standard relaxation buffer (10 mM imidazole, pH 7.2, 75 mM KCl, 2 mM MgCl₂, 2 mM EDTA, and 1 mM Na₂ATP, 10 mM Na₂GTP, and 5 mM NaN₃) with 1% (vol/vol) Triton X-100. Myofibrils were centrifuged, and the supernatant fraction was removed. The pellets were then washed once in standard relaxation buffer to remove the Triton X-100. The standard relaxation buffers contained both the protease (Sigma, St. Louis, MO) and phosphatase (Calbiochem, Darmstadt, Germany) inhibitors at a 1:100 dilution. The pellet was solubilized in 2× Laemlli sample buffer (Bio-Rad, Hercules, CA), and the protein concentration of the samples was determined with an RCD assay kit (Bio-Rad). A 6% SDS-PAGE gel was prepared as described previously and run at a constant amperage of 20 mA until the dye front ran off (32). To visualize total protein, Coomassie blue stain was added for 30 min and then destained with 10% methanol and 10% acetic acid and imaged on a Chemidoc XRS+ (Bio-Rad), using a Coomassie blue filter set. Band densities from gels were determined using ImageLab 3.0 software (Bio-Rad).

Immunoblotting. Whole heart homogenates and enriched myofibril lar proteins were extracted from ventricles of NTg and Tm-E180G hearts from mice treated both with and without NAC, as described above. Protein expression levels and posttranslational modifications were implicated in oxidative phosphorylation. Phosphorylated and total ERK1/2 (Cell Signaling Technology, Danvers, MA), phosphorylated phospholamban (PLN) Ser²⁹, Thr²⁸, and total PLN (Bardilla, Leeds, UK, and Upstate Biotechnology, Darmstadt, Germany), and sarco(endo)plasmic reticulum Ca²⁺ ATPase (SERCA2; Cell Signaling Technology) were assessed as described previously (24). An enriched fraction of sarcomere proteins separated by SDS-PAGE and transferred to PVDF was also probed to determine whether there were altered phosphorylation states of cTnl (Ser²⁹ and Ser²⁴, Cell Signaling Technology), p-cMyBP-C Ser²⁸ (Enzo Life Sciences, Farmingdale, NY) and total cMyBP-C (a generous gift from Richard L. Moss), and S-protein glutathione (Virogen, Watertown, MA). Blots were imaged using a Chemidoc XRS+ imager (Bio-Rad, Hercules, CA), and band densities were analyzed using ImageLab software (Bio-Rad).

Analysis of carbonylated proteins. Carbonylated proteins were detected using the OxyBlot kit (EMD Millepore, Darmstadt, Germany), using enriched myofibrils, according to the manufacturer’s instructions. Myofibrils were obtained as described above. The myofibrils were then derivitized using DNPH solution for 15 min at room temperature in the dark, neutralized, and loaded onto SDS-PAGE gels for Western blotting, as described above.

Simultaneous measurement of the force-Ca²⁺ relationship and cross-bridge kinetics. Left ventricular papillary muscles were isolated, cut into fiber bundles of ~200 μm in width and 3–4 mm in length, and detergent extracted (skinned) in a high-relaxing solution (10 mM EGTA, 119 mM-K·Protein, 25 mM-Na·Protein, 6.73 mM-MgCl₂, 2 mM-Na₂ATP, 10 mM-Na·GTP, and 5 mM-Na·NaN₃) with 1% Triton X-100 for 3–4 h at 4°C. Tension and ATPase rate were measured simultaneously using methods and a previously described experimental apparatus (33). The fiber bundles were mounted between a force transducer and displacement motor using aluminum T-clips, and the sarcomere length set to 2.2 μm using He-Ne laser diffraction. The width and diameter were measured at three points along the fiber bundle. Force per cross-sectional area was used to determine tension. The fiber was contracted initially at a saturating Ca²⁺ concentration (pCa 4.5), and sarcomere length was again adjusted to 2.2 μm. Sarcomere length remained constant throughout the rest of the experiment. ATPase activity was measured at 20°C, as described previously, and calibrated with rapid injections of ADP (0.5 nmol) with a motor-controlled syringe. The fiber was placed in relaxing solution for 2 min and then in the preactivation solution for 2–3 min each time before being placed in the activating solution for 1–2 min (until stabilization of force). The fiber bundle was then quickly returned to the relaxing solution. Various contraction-relaxation cycles were carried out using different ratios of total Ca²⁺ concentration to total EGTA concentration. The final contraction was again carried out at a saturating Ca²⁺ concentration. All experiments were carried out at 20°C. The relation between Ca²⁺ and tension or ATPase activity was fitted using a modified Hill equation (7, 28). We determined pCa values at half-maximum ATPase activity and force generation from mean data normalized to maximum activity. In all experiments, only fiber bundles retaining >80% of their initial maximum tension were included in the analysis.
Statistical analysis. Statistical analysis was performed using Prism 6.0 software (GraphPad Software, La Jolla, CA), using two-way ANOVA followed by Tukey’s post hoc test for multiple comparisons. Factors considered for statistical analysis were genotype and treatment. Data are presented as means ± SE with significance set at \( P < 0.05 \).

RESULTS

N-acetylcysteine improves cardiac morphology and diastolic function in hypertrophic cardiomyopathy. Beginning at 1 mo of age, after HCM phenotypic changes were evident, NAC was administered to NTg and Tm-E180G mice (1). We conducted a noninvasive assessment of cardiac morphology and function in Tm-E180G and NTg mice, as summarized in Table 1. M-mode echocardiography demonstrated that Tm-E180G mice have significant left atrial dilation, which was reduced to NTG levels with NAC administration for 1 mo. Hearts of Tm-E180G mice demonstrated an increase in left ventricular mass with no change in internal diameter at diastole, which was characteristic of concentric hypertrophy. Treatment with NAC returned LV mass to NTG levels. Measurements of diastolic function and E/A and E/E’ ratios were also decreased to NTG levels. Systolic function was unchanged in Tm-E180G mice.

Activation of the MAPK pathway is implicated in the development of both physiological and pathological hypertrophy (6). Additionally, these pathways are activated by elevated ROS (15). Compared with controls, there was an increase in ERK1/2 phosphorylation in hearts from Tm-E180G mice, which returned to NTG levels with NAC administration (Fig. 1, A and B). Interestingly, this reversal of MAPK signaling and hypertrophy did not decrease levels of expression of the neonatal myosin heavy-chain isoform β-myosin heavy chain observed in our model (Fig. 1, C and D).

N-acetylcysteine affects SERCA2 expression and phosphorylation of PLN. We have reported previously that despite changes in phosphorylation of PLN and expression of SERCA2 in ventricular myocytes isolated from Tm-E180G hearts compared with NTg controls, there were no changes in Ca\(^{2+}\) transients (26). However, early adenoviral-mediated overexpression of SERCA2a prevented the development of hypertrophy and fibrosis in Tm-E180G mice (27). Therefore, we tested for NAC-induced alterations in Ca\(^{2+}\) regulatory proteins PLN and SERCA2. NAC treatment increased the expression of total cardiac SERCA2 protein expression in both NTg and Tm-E180G hearts (Fig. 2, A and D). NAC treatment induced phosphorylation of PLN at Ser\(^16\) in Tm-E180G hearts to a level close to NTG-levels, but the values failed to reach statistical significance; however, PLN phosphorylation at Thr\(^17\) was significantly elevated in the hearts of Tm-E180G mice and returned to control levels following NAC treatment (Fig. 2, B–D).

N-acetylcysteine decreases Ca\(^{2+}\) sensitivity and improves cross-bridge kinetics in detergent-extracted fiber bundles. We employed detergent-extracted (skinned) fiber bundles from NTg and Tm-E180G hearts to determine the effect of antioxidant treatment on Ca\(^{2+}\) dependence of isometric tension, actomyosin ATPase rate, and the kinetics of tension redevelopment \( (k_{tr}) \), a measurement of the rate of entry of cross-bridges into the force-generating state. Results are summarized in Table 2. Consistent with previous reports (21), there was a significant increase in Ca\(^{2+}\) sensitivity of tension and ATPase. Maximum tension and ATPase rate were unchanged in fibers from both NTg and Tg hearts treated with NAC (Fig. 3A). Compared with NTg controls, the Ca\(^{2+}\) sensitivity of ATPase rate was increased significantly in Tm-E180G fibers, and NAC administration significantly reduced the Ca\(^{2+}\) sensitivity of the rate of ATP hydrolysis in both groups (Fig. 3B). NAC administration also resulted in a significant increase in \( k_{tr} \), the rate of cross-bridge reattachment, measured as a slope of a linear regression of \( k_{tr} \) values plotted against relative tension \( (P/P_0) \). Measurements of \( k_{tr} \) at minimum \( P/P_0 \), revealed no changes between the groups. However, \( k_{tr} \) at maximum \( P/P_0 \) revealed significant changes in NAC-administered NTg and Tm-E180G fibers, suggesting that as tension generation increases, NAC increases the rate of cross-bridge reattachment. The slope of the relation between tension and ATPase rate is a measurement of tension cost, is related to

<table>
<thead>
<tr>
<th>Parameter (Sample Size)</th>
<th>NTg ( (n = 6) )</th>
<th>NTg NAC ( (n = 9) )</th>
<th>Tm-E180G ( (n = 6) )</th>
<th>Tm-E180G NAC ( (n = 9) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males/females ( (n) )</td>
<td>3/3</td>
<td>3/6</td>
<td>3/3</td>
<td>5/4</td>
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<tr>
<td>Body weight, g</td>
<td>23.0 ± 1.73</td>
<td>21.7 ± 0.60</td>
<td>25.1 ± 1.57</td>
<td>21.8 ± 0.95</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>500 ± 51</td>
<td>473 ± 15</td>
<td>428 ± 8</td>
<td>542 ± 21</td>
</tr>
<tr>
<td>LA size, mm</td>
<td>1.26 ± 0.06</td>
<td>1.36 ± 0.08</td>
<td>2.19 ± 1.10*,††</td>
<td>1.58 ± 0.06*</td>
</tr>
<tr>
<td>LV mass, mg</td>
<td>43.90 ± 5.27</td>
<td>49.17 ± 5.27</td>
<td>65.69 ± 5.21††</td>
<td>51.39 ± 3.95</td>
</tr>
<tr>
<td>LVIDd, mm</td>
<td>3.73 ± 0.09</td>
<td>3.69 ± 0.11</td>
<td>3.75 ± 0.14</td>
<td>3.47 ± 0.10</td>
</tr>
<tr>
<td>LVIDd, mm</td>
<td>2.28 ± 0.09</td>
<td>2.46 ± 0.10</td>
<td>2.26 ± 0.08</td>
<td>2.19 ± 0.10</td>
</tr>
<tr>
<td>Relative wall thickness, mm</td>
<td>0.34 ± 0.03</td>
<td>0.29 ± 0.03</td>
<td>0.37 ± 0.03</td>
<td>0.38 ± 0.04</td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td>69.95 ± 1.44</td>
<td>63.77 ± 2.61</td>
<td>70.98 ± 0.56</td>
<td>71.01 ± 1.47</td>
</tr>
<tr>
<td>Fractional shortening, %</td>
<td>38.89 ± 1.22</td>
<td>34.33 ± 1.94</td>
<td>39.65 ± 0.45</td>
<td>39.72 ± 1.18</td>
</tr>
<tr>
<td>E-wave, mm/s</td>
<td>774 ± 34</td>
<td>779 ± 38</td>
<td>831 ± 91</td>
<td>846 ± 38</td>
</tr>
<tr>
<td>A-wave, mm/s</td>
<td>516 ± 32</td>
<td>490 ± 26</td>
<td>308 ± 64*††</td>
<td>540 ± 35§††</td>
</tr>
<tr>
<td>Em-wave, mm/s</td>
<td>26.23 ± 2.77</td>
<td>26.74 ± 2.24</td>
<td>20.34 ± 1.15††</td>
<td>35.85 ± 4.50*§</td>
</tr>
<tr>
<td>E/A ratio</td>
<td>1.54 ± 1.14</td>
<td>1.63 ± 0.09</td>
<td>3.03 ± 0.32**,††</td>
<td>1.64 ± 0.15§</td>
</tr>
<tr>
<td>E/Em ratio</td>
<td>30.94 ± 3.10</td>
<td>30.40 ± 2.20</td>
<td>41.44 ± 2.49††</td>
<td>28.05 ± 3.30§</td>
</tr>
</tbody>
</table>

Values are means ± SE. NAC, N-acetylcysteine; NTg, nontransgenic; Tm, tropomyosin; LA, left atrium; LV, left ventricular; LVIDd, LV internal dimension at diastole; LVIDs, LV internal dimension at systole. *\( P < 0.05 \) and **P < 0.001 vs. NTG; §\( P < 0.05 \) and §§P < 0.0001 vs. Tm-E180G; †P < 0.01 and ††P < 0.001 vs. NTg NAC.
the rate of cross-bridge detachment, and followed the same trend as $k_w$ values, where tension cost was increased in fibers excised from NAC-administered mice. (Fig. 4, A and B). Thus, NAC administration results in desensitization of the myofilaments to $\text{Ca}^{2+}$ and increase in cross-bridge kinetics.

$N$-acetylcysteine alters phosphorylation and oxidative modifications of myofilament proteins. Regulatory redox mechanisms that involve posttranslational modifications may account for the alterations in the cross-bridge kinetics found in Tm-E180G hearts. Therefore, we assessed the phosphorylation and $\text{S}$-glutathionylation levels of myofilament proteins (5, 12). Previously, we identified three $\text{S}$-glutathionylation sites on cMyBP-C (19).

At 2 mo of age, phosphorylation of cMyBP-C Ser282 was increased in Tm-E180G mice, which was reduced upon NAC administration (Fig. 5, A and C). Phosphorylation of cTnI at Ser23/24 was decreased in Tm-E180G mice compared with NTG NAC-administered mice. NAC was associated with increased cTnI phosphorylation in Tm-E180G mice administered NAC; however, this value was not significant from controls (Fig. 5, A and C). No other differences in myofilament protein phosphorylation were found using ProQ diamond phosphoprotein stain (data not shown).

Functionally, $\text{S}$-glutathionylation results in increased $\text{Ca}^{2+}$ sensitivity with depressed cross-bridge kinetics. Therefore, we investigated whether $\text{S}$-glutathionylation of cMyBP-C contributes to diastolic dysfunction in HCM (5, 12). We found that Tm-E180G mice exhibit a significant increase in $\text{S}$-glutathionylation of cMyBP-C. NAC reduced the levels of $\text{S}$-glutathionylation of cMyBP-C to NTg control levels (Fig. 6). Further analysis revealed that $\text{S}$-glutathionylation of Tm was significantly reduced in NAC-administered NTG and Tm-E180G mice. Additionally, we performed dinitrophenol (DNP) derivitization to determine the extent of carbonylation of myofilament proteins. Western blot analysis revealed that there was a significant increase in carbonylation of myosin heavy chain

![Fig. 1](image-url). $N$-acetylcysteine (NAC) reduces ERK1/2 phosphorylation but not $\beta$-myosin heavy chain expression. A: tropomyosin (Tm)-E180G mice display increased ERK1/2 phosphorylation, which is reduced to nontransgenic (NTg) levels with NAC administration. B: representative Western blot images of phosphorylated (p)-ERK1/2 and total ERK1/2. C: Tm-E180G mice have increased $\beta$-myosin heavy chain expression, which is not reduced with NAC administration. D: representative 6% SDS-PAGE gel image showing $\beta$-myosin heavy chain expression. Data were analyzed by 2-way ANOVA, followed by Tukey’s post hoc test for multiple comparisons and represented as means ± SE; n = 3–6 hearts/group. *$P$ ≤ 0.05; **$P$ ≤ 0.01; ***$P$ ≤ 0.001.
(MHC) in Tm-E180G mice compared with NTG and NTG NAC mice, which was reversed to NTG levels upon NAC administration (Fig. 7). No other changes in myofilament protein carbonylation were observed.

**DISCUSSION**

Our data are the first to report that NAC administration is able to reverse an increase in myofilament Ca\(^{2+}\) sensitivity and diastolic dysfunction associated with S-glutathionylation of cMyBP-C in HCM. Moreover, we report novel evidence that administration of NAC alters expression of SERCA2 as well as phosphorylation of PLN. Previous studies reported that exogenous NAC treatment of models with HCM linked to a cTnT mutation is able to decrease fibrosis and hypertrophy (5, 12, 14). Additional studies were completed in rabbits containing an HCM-linked mutation on cardiac myosin heavy chain, where NAC administration was shown to have similar beneficial effects as in the cTnT murine model (10). Our studies significantly extend these findings in that in addition to reversing the hypertrophy and increase in left atrial size, we report evidence for a mechanistic basis for the reversal of diastolic abnormalities in HCM at the level of the myofilaments. The improvement of the diastolic abnormality was associated with the effects of NAC treatment on reducing the elevated Ca\(^{2+}\) sensitivity of Tm-E180G skinned fiber bundles and the hastening of cross-bridge kinetics.

A novel finding related to the improvement of diastolic function is a reversal of S-glutathionylation of cMyBP-C. Our previous studies reported a strong correlation of increased cMyBP-C S-glutathionylation with slowing of cross-bridge kinetics and an increase in myofilament response to Ca\(^{2+}\) in a model of diastolic heart failure (5, 12). In the present study, we

**Table 2. NAC decreases Ca\(^{2+}\) sensitivity while hastening cross-bridge kinetics**

<table>
<thead>
<tr>
<th>Parameter (Sample Size, Fibers)</th>
<th>NTg (n = 7)</th>
<th>NTg NAC (n = 6)</th>
<th>Tm-E180G (n = 7)</th>
<th>Tm-E180G NAC (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum tension, nN/mm(^2)</td>
<td>36.07 ± 1.84</td>
<td>27.78 ± 4.18</td>
<td>33.06 ± 2.27</td>
<td>31.03 ± 3.54</td>
</tr>
<tr>
<td>pCa(_{50}) of tension</td>
<td>5.78 ± 0.01</td>
<td>5.55 ± 0.06*</td>
<td>6.01 ± 0.05**,††</td>
<td>5.87 ± 0.05†,§</td>
</tr>
<tr>
<td>Max ATPase, pmol·s(^{-1})·mg(^{-1})</td>
<td>379 ± 0.07</td>
<td>364 ± 14.02</td>
<td>351 ± 12.91</td>
<td>377 ± 0.71</td>
</tr>
<tr>
<td>pCa(_{50}) of ATPase</td>
<td>5.73 ± 0.05</td>
<td>5.68 ± 0.03</td>
<td>6.17 ± 0.07**,††</td>
<td>5.86 ± 0.09§</td>
</tr>
<tr>
<td>Tension cost</td>
<td>9.78 ± 0.74</td>
<td>12.63 ± 0.80**</td>
<td>9.41 ± 0.74††</td>
<td>11.13 ± 0.53**,§§</td>
</tr>
<tr>
<td>(k_\text{o,slope})</td>
<td>14.57 ± 0.81</td>
<td>22.98 ± 2.54**</td>
<td>14.15 ± 0.91††</td>
<td>23.47 ± 1.21**,§§</td>
</tr>
<tr>
<td>(k_o) (s(^{-1})) at minimum P/P(_o)</td>
<td>3.76 ± 0.30</td>
<td>3.62 ± 0.88</td>
<td>3.17 ± 0.50</td>
<td>4.90 ± 1.02</td>
</tr>
<tr>
<td>(k_o) (s(^{-1})) at maximum P/P(_o)</td>
<td>17.63 ± 0.74</td>
<td>28.20 ± 4.55**</td>
<td>13.77 ± 0.92††</td>
<td>27.93 ± 1.77*,§§</td>
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</tbody>
</table>

Values are means ± SE. pCa\(_{50}\), pCa at half-maximal tension; \(k_o\), kinetics of tension redevelopment. *P < 0.05 and **P < 0.001 vs. NTg; §P < 0.05 and §§P < 0.001 vs. Tm-E180G; †P < 0.05 and ††P < 0.001 vs. NTg NAC.
did not find a significant decrease in $k_{tr}$ in skinned fiber bundles from Tm-E180G mice compared with NTg controls. We did find that NAC administration hastened $k_{tr}$ and increased tension cost in both NTg and Tm-E180G skinned fiber bundles, suggesting that the improvement in diastolic function is related to a reduction in oxidative stress and an associated increase in cross-bridge kinetics.

The functional significance of cMyBP-C has generally been considered as a “brake” on the reaction of cross-bridges with the thin filament and as a desensitizer of the myofilaments to $Ca^{2+}$ (23). In Tm-E180G mice, we report differential phosphorylation of cMyBP-C, where phosphorylation of Ser282 is increased. Although we observed a trend toward normalization in phosphorylation of Ser282 in Tm-E180G mice, it was not significant. Additionally, this $Ca^{2+}$-desensitizing phosphorylation of cMyBP-C is present despite the constitutive increase in myofilament $Ca^{2+}$ responsiveness we observed, suggesting that $S$-glutathionylation of cMyBP-C is dominant in its $Ca^{2+}$-
sensitizing effect. Previously, we have identified that S-glutathionylation of cMyBP-C occurs at cysteine residues (C479, C627, C655) in the C3, C4, and C5 domains, whereas phosphorylation is known to occur at the M domain of the COOH terminus (19). Similar to phosphorylation, S-glutathionylation imparts a negative charge (27). Phosphorylation is thought to induce a Ca$^{2+}$-desensitizing effect by inducing steric hindrance of the myosin SI catalytic unit (18). S-glutathionylation may exert its dominant effect by performing the opposite, modifying the radial disposition of cMyBP-C toward the thick filament, where the C5 domain has been shown to interact away from myosin (19). A direct effect may be exerted by the increase in MHC carboxylation in Tg mice that was reversed with NAC administration. Reports have suggested MHC carboxylation is involved in the desensitization of the myofilament to Ca$^{2+}$ and depression in cardiac function (25). However, there is no consensus as to the functional effects of myofilament protein carboxylation, and we note that many studies are not extended to other oxidative modifications of myofilament proteins. Combinational control of cross-bridge
kinetics and Ca\(^{2+}\) sensitivity by various posttranslational modifications is likely to play an effect, as it has been demonstrated in other thin filament proteins under scenarios of phosphorylation (17). Such an investigation in oxidative modifications is likely to be difficult, as approaches involving mutagenesis to mimic these posttranslational modifications are not available. Ultimately, future studies will shed light on the structural/functional changes due to cMyBP-C and myosin posttranslational modification.

Less clear is the effect of S-glutathionylation on tropomyosin. Previously, we have shown that Tm is able to be S-glutathionylated after in vitro incubation with oxidized glutathione (19). To our knowledge, this work is the first to suggest S-glutathionylation of Tm in vivo. The Tm-E180G mutation results in decreased thermal stability and increased flexibility, and it has been suggested that this could contribute to the increase in Ca\(^{2+}\) sensitivity by allowing the interaction of strongly bound cross-bridges with actin in the absence of Ca\(^{2+}\) (9, 11, 13). Tm contains one cysteine at residue 190, which is important for the maintenance of the coiled-coil structure by stabilizing ionic interactions throughout the molecule. Experiments performed in vitro where 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) was used to disrupt the disulfide bond created at C190, which is important in maintaining the coiled-coil structure of Tm, result in a decreased affinity of Tm for actin. The structural consequences of this “cross-linking”, or disruption of

Fig. 6. NAC decreased S-glutathionylation of cMyBP-C and tropomyosin. cMyBP-C glutathionylation was significantly increased in Tm-E180G mice, which was reduced to NTg control and NTg NAC-levels after NAC administration. Tropomyosin S-glutathionylation was significantly reduced in NTg NAC- and Tm-E180G NAC-administered mice compared with NTg and Tm-E180G controls. Data were analyzed by 2-way ANOVA followed by Tukey’s post hoc test for multiple comparisons and represented as means \(\pm\) SE; \(n = 4–6\) hearts/group. *\(P \leq 0.05\); **\(P \leq 0.01\).

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the disulfide bond, induce partial unfolding of the molecule at physiological temperatures and increased regional flexibility, most likely through a destabilization of the structure, similar to and in the same region as the Tm-E180G mutation (31). S-glutathionylation at C190 may also disrupt the disulfide bond, resulting in the same effect as DTNB, causing a local destabilization of the molecule and promoting Ca\(^{2+}\)/H\(^{+}\) sensitivity. This hypothesis could explain the decrease in Ca\(^{2+}\) sensitivity observed in NTg NAC-administered mice, as we report a significant decrease in S-glutathionylation of NTg NAC mice compared with NTg controls, although unlike the transgenic groups, no change was seen in S-glutathionylation of cMyBP-C. The same effect on S-glutathionylation of Tm was seen in Tm-E180G NAC mice.

Peña et al. (20) showed that early adenoviral overexpression of SERCA2a is able to delay the development of fibrosis and hypertrophy in Tm-E180G mice. However, SERCA2a overexpression was unable to reverse myofilament modifications and baseline diastolic dysfunction in this model. Additionally, mRNA levels of heart failure markers ANP, BNP, and β-myosin heavy chain were unchanged as the SERCA2a-overexpressing Tm-E180G mice aged. Although we also found that β-myosin heavy chain expression was unchanged with NAC, in our present work we found both a functional and morphological improvement with NAC administration. Unexpectedly, we found a significant increase in SERCA2 protein expression in both groups treated with NAC along with reversal of hypertrophy in Tm-E180G mice. The mechanism by which NAC increases SERCA2 expression is unclear. Supporting our findings, in a model of inflammatory signaling-mediated diastolic dysfunction, SERCA2a expression was reduced. The authors who made this observation attributed this reduction in SERCA2a to activation of NF-κB. Treating cells and rats with NF-κB blockers, as well as statins, improved SERCA2a expression in these models (30). Additionally, NAC administration prevented the activation of NF-κB in the hearts of brain-dead Ba-Ma miniature pigs (34).

In summary, we have demonstrated that antioxidant administration improves diastolic dysfunction, hypertrophy, Ca\(^{2+}\)/H\(^{+}\)-regulatory protein expression, cross-bridge cycling kinetics, and posttranslational modification of myofilament proteins to relieve maladaptation in HCM. Although much work has been done toward generating drugs acting as Ca\(^{2+}\) sensitizers or sarcomere activators, no Ca\(^{2+}\)-desensitizing therapies exist (3, 29). Our work provides novel evidence for a Ca\(^{2+}\)-desensitizing therapy of an approved drug. We hypothesize that desensitization of the myofilaments to Ca\(^{2+}\) and improvement of diastolic dysfunction can be attributed to the reduction in
S-glutathionylation of myofilament proteins. Therefore, our work suggests that reducing the oxidative load on the cell may be an effective treatment for HCM. We look forward to the results of a clinical trial that is underway investigating the effect of NAC on human populations with HCM induced by sarcomeric mutations (clinical trial identifier NCT01537926).

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REFERENCES
7. Lang RM, Bierig M, Devereux RB, Flachskampf FA, Foster E, Pellikka PA, Picard MH, Roman MJ, Seward J, Shaeuws IS, Solomon SD, Spencer KT, Sutton MS, Stewart WJ; Chamber Quantification Writing Group; American Society of Echocardiography’s Guidelines and Standards Committee; European Association of Echocardiography. Recommendations for chamber quantification: a report from the American Society of Echocardiography’s Guidelines and Standards Committee and the Chamber Quantification Writing Group, developed in conjunction with the European Association of Echocardiography, a branch of the European Society of Cardiology. J Am Soc Echocardiogr 18: 1440–1463, 2005.


