Ouabain decreases sarco(endo)plasmic reticulum calcium ATPase activity in rat hearts by a process involving protein oxidation

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METHODS

Animals. Male Sprague-Dawley rats weighing 300–350 g were used for all studies. Animals were euthanized, and hearts were removed for isolated isovolumic Langendorff perfusion, determination of SERCA2a enzymatic activity, or Western blot analysis using standard methods (26, 41, 42). In other cases, hearts were removed and cardiac myocytes were isolated for subsequent study. All animal experimentation described in the manuscript was conducted in accordance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals using protocols approved by the Medical University of Ohio Institutional Animal Use and Care Committee.

Isolation and culture of cardiac myocytes. Details of the method of isolation and culture of Ca2+-tolerant adult myocytes may be found in several recent reports from our laboratory (26, 41, 42). This method of isolation produced a good yield of rod-shaped (70–80%) myocytes in each of the experimental groups presented in this report. To make the myocytes Ca2+-tolerant after isolation in Ca2+-free medium, we

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resuspended the pellet in media containing graduated increases in the concentration of Ca\textsuperscript{2+} from 10 μM to 1.8 mM.

**Measurements of Ca\textsuperscript{2+} transient.** The cytosolic Ca\textsuperscript{2+} concentrations during contraction and relaxation (i.e., Ca\textsuperscript{2+} transient) were monitored with the Ca\textsuperscript{2+}-selective fluorescent dye indo-1 (Molecular Probes, Eugene, OR), employing a spectrophotometer (Photon Technology International, Monmouth Junction, NJ) interfaced with an inverted microscope as previously described (26, 41). The time constant τ\textsubscript{C2+}, for recovery of Ca\textsuperscript{2+} following electrical stimulation, was obtained by fitting a least-squares regression line to the log transformation of the fluorescence data as described by Bassani et al. (11) to measure SERCA activity as previously reported by our group (26, 41). Measurements were performed before treatment and 5 min after ouabain exposure.

To determine the role of ROS in Ca\textsuperscript{2+} metabolism, we pretreated cells for 30 min with either 50 mg/ml aqueous green tea extract (GT) or 10 mM N-acetylcysteine (NAC) before ouabain exposure. After 5 min, electrical stimulation was turned off and the cells were allowed to rest for 10 s. Next, caffeine (10 mM) was applied to the chamber with the use of an automatic switching device. The amplitude of the Ca\textsuperscript{2+} transient of the caffeine contracture was then compared with the amplitude of the twitch Ca\textsuperscript{2+} transient (at 5 min). The ratio of caffeine contracture to twitch amplitude was used to assess SERCA activity, which is predominantly SERCA2a activity in rat cardiac tissue, we employed the method of Simonides and van Hardeveld (49) with minor modifications as described previously (26, 27).

**Carboxylation ELISA analysis.** Total protein carboxyl concentration of the LV homogenate was determined by ELISA using the Zentech PC test kit according to the manufacturer’s protocol (Northwest Science Specialties, Vancouver, WA) as described previously (26, 27).

**Immunoprecipitation.** LV homogenate was solubilized in radioimmunoprecipitation buffer containing proteinase inhibitor cocktail (Sigma P-2714) for 2 h at 4°C and then centrifuged at 16,000 g for 20 min to remove insoluble material. The solubilized protein fraction (1 mg/ml) was then immunoprecipitated using polyclonal anti-SERCA2a antibody (Abcam, Cambridge, MA) overnight at 4°C. Immune complexes were collected on protein G-Sepharose (Upstate, Lake Placid, NY) and washed four times in the immunoprecipitation buffer. The immunoprecipitate was dissolved in sample buffer, separated on 10% SDS-PAGE, and blotted with either polyclonal anti-dinitrophenyl (DNP) antibody (Chemicon International) or anti-nitrotyrosine MAb. To confirm the presence of SERCA2a oxidation, some samples were first derivatized with DNP or a derivatization control solution and immunoprecipitated using polyclonal anti-DNP antibody overnight at 4°C. RNS/peroxynitrite modifications were confirmed using anti-nitrotyrosine MAb overnight at 4°C. Immune complexes were processed as described above and blotted with anti-SERCA2a MAb. All gels were run in duplicate so that identical gels could be processed for mass spectrophotometric analysis.

**Liquid chromatography-tandem mass spectrometric analysis.** To obtain positive identification of SERCA as well as to investigate the presence of posttranslational modifications, we analyzed gel slices obtained from duplicate immunoprecipitate gels by liquid chromatography-tandem mass spectrometry (LC-MS) after in-gel proteolysis as described elsewhere (10). Briefly, after the Coomassie-stained gel slices were destained with 30% methanol for 3 h at room temperature, they were digested with 1 μg of sequencing grade, modified trypsin (Promega) in 0.1 M ammonium bicarbonate buffer (pH 8.0) for 12 h at 37°C. Peptides were extracted once each with 60% acetonitrile-0.1% trifluoroacetic acid (TFA) and acetonitrile-0.1% TFA. The extracts were pooled and concentrated down to ~15 μl using vacuum. Two microliters of the digest were separated on a reverse-phase column (75 μm inner diameter × 5 cm × 15 μm Aquasil C18 Picofrit column; New Objectives) using a 1% acetic acid/acetonitrile gradient system (5–75% acetonitrile over 35 min, followed by a 3-min wash with 95% acetonitrile) at a flow rate of ~250 nL/min. The peptide eluent was directly introduced into an ion-trap mass spectrometer (LCQ-Deca XP Plus; ThermoFinnigan) equipped with a nanospray ionization source. The mass spectrometer was operated on a double play mode, in which the instrument was set to acquire a full MS scan (400–2,000 m/z) and a collision-induced dissociation (CID) spectrum on the most abundant ion from the full MS scan (relative collision energy ~30%). Dynamic exclusion was set to acquire three CIDS on the most abundant ion and exclude it for a further 2 min. The CID spectra were searched against a subset of nonredundant, indexed rat protein database using the TurboSEQUEST search program (ThermoFinnigan). Peptide hits with Xcorr (raw cross-correlation score
between the observed peptide fragment mass spectrum and the theoretically predicted one) and ∆ΔCN (difference between the cross-correlation score (Xcorr) between the top two candidate peptides) values of >2 and >0.2, respectively, were considered positive and verified manually. Any uninterpreted spectra were manually searched using the MS-Tag provision of Protein Prospector (http://prospector.ucsf.edu) (17). Cn3D 4.1, a program available in the structure division of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov), was used to display three-dimensional structures of SERCA2a after a gapped-BLAST alignment with SERCA1a was performed. The structure file for SERCA1a was Protein DataBase Id ISU4_A, and the SERCA2a rat sequence aligned with it was from GenBank (accession no. NM_017290).

Statistical analysis. Data presented are means ± SE. Data obtained were first tested for normality. If the data did not pass the normality test, Tukey’s test (for multiple groups) or the Mann-Whitney rank sum test was used to compare the data. If the data did pass the normality test, parametric comparisons were performed. If more than two groups were compared, one-way analysis of variance was performed before comparison of individual groups with the unpaired Student’s t-test, using Bonferroni’s correction for multiple comparisons. If only two groups of normal data were compared, the Student’s t-test was used without correction (63). Statistical analysis was performed by using SPSS software.

RESULTS

Effect of ouabain on isolated cardiac myocyte Ca2+ cycling and contractile function. Myocytes treated with ouabain (50 μM) demonstrated substantial increases in systolic (313 ± 26 vs. 260 ± 11 nM; Fig. 1A) and diastolic Ca2+ (120 ± 16 vs. 61 ± 10 nM; Fig. 1B). The recovery time constant for the Ca2+ transient, τCa2+, was also prolonged by ouabain exposure (399 ± 35 vs. 288 ± 26 ms, P < 0.01; Fig. 1C). Exposure to 10 μM H2O2, which causes an increase in intracellular ROS similar to that of 50 μM ouabain (68), caused a similar increase in τCa2+ (377 ± 35 vs. 288 ± 35 ms, P < 0.01; Fig. 1C). Concurrent exposure to NAC (10 mM) or an aqueous extract from GT (50 mg/ml), maneuvers we have previously used to prevent increased ROS induced by ouabain (42, 58, 59), prevented the increases in systolic and diastolic cytosolic Ca2+ as well as τCa2+ (Fig. 1, A–C). The ratio between the maximal caffeine and maximal twitch Ca2+ was reduced in the ouabain-treated cells, indicating a reduced SR Ca2+ load and SERCA2a activity (Fig. 2A). Finally, the τCa2+ for the caffeine contractures was significantly prolonged in the cardiac myocytes exposed to ouabain (Fig. 2B).

Effect of ouabain on isovolumic perfused heart function. The effects of ouabain on LV pressure are summarized in Table 1. We observed that ouabain induced increases in systolic function as assessed by developed pressure and the maximum and minimum rate of pressure development (+dP/dt) and −dP/dt). Ouabain also impaired diastolic function, as assessed using the ratio of +dP/dt to −dP/dt, compared with control hearts at 30 min (Table 1). Coadministration with NAC considerably attenuated the effects of ouabain on systolic and diastolic function (Table 1).

Effect of ouabain and ROS on SERCA activity. Isolated hearts treated with 50 μM ouabain showed substantially decreased SERCA enzymatic activity compared with time controls or hearts in which NAC was administered concurrently with ouabain (Fig. 3). Furthermore, exposure of LV homogenate to various concentrations of H2O2 demonstrated the ability of ROS to depress SERCA activity in a dose-dependent manner (Fig. 3).

Effect of ouabain on SERCA2a and other cardiac proteins. To further examine the mechanisms underlying the alterations in cardiac function seen in both the cardiac myocytes and isolated perfused heart, we examined SERCA2a expression as well as the expression of total PLB and PLB phosphorylation (P-Ser16 PLB). Quantification of protein density with Western blotting, however, showed a small but significant reduction in SERCA2a content in the hearts treated with 50 μM ouabain compared with time controls (Fig. 4A). There was no significant change with regard to PLB or P-Ser16 PLB (Fig. 4, B–E).

It was interesting to note, however, that SERCA2a immunoblots of ouabain-treated hearts showed additional bands below the expected 110-kDa band (Fig. 5A). Given the role of ROS in DLS-induced signal transduction and the biochemical and physiological data pointing to decreased SERCA2a Ca2+ handling in this setting, we decide to investigate whether the additional bands were peptide products generated from oxida-
tion of the SERCA2a protein. Furthermore, because ouabain has been shown to increase levels of nitric oxide (18, 65), which in combination with ROS can yield potent peroxynitrite-mediated protein modification, we also investigated the presence of nitrotyrosine as a marker of such modifications.

To these ends, we first assessed the oxidation status of the whole LV homogenate using an ELISA immunoassay for carbonylated proteins. Total carbonylation was increased with ouabain treatment (0.44 nmol/mg protein) compared with control and NAC plus ouabain-treated hearts (0.21 and 0.13 nmol/mg protein, respectively, P < 0.05). Using a carboxylation immunoblot, we noted that acute ouabain administration did indeed increase both the total oxidation of cardiac proteins and, in particular, the 110-kDa region containing SERCA2a (Fig. 5B). We also noted that ouabain administration increased tyrosine nitration, a marker of peroxynitrite modification, of cardiac proteins, including the same 110-kDa region containing SERCA2a (Fig. 5C).

To carry this analysis further, we performed several coimmunoprecipitations to determine positive identification of the oxidative/nitrosative status of SERCA2a. In the first coimmunoprecipitation, we immunoprecipitated SERCA2a from the LV homogenate and immunoblotted for DNP-derivatized proteins while immunoprecipitating DNP-derivatized proteins from the LV homogenate and immunoblotting for SERCA2a. Not only did this reveal an increase in immunodetected products in the ouabain-treated hearts, but a similar pattern of additional bands was also noted, as in the immunoblots (Fig. 6, A and B). We also immunoprecipitated SERCA2a from the LV homogenate and immunoblotted for tyrosine nitration while immunoprecipitating nitrotyrosine proteins from the LV and immunoblotting for SERCA2a. This coimmunoprecipitation also revealed an increase in immunodetected products in the ouabain-treated hearts (Fig. 6, C and D).

Finally, to verify oxidative and nitrosative modifications of SERCA2a, we performed LC-MS analysis of duplicate SDS-PAGE gels from the experiments outlined above. We obtained positive identification of SERCA2a peptides after the immunoprecipitations for carboxylation, nitrotyrosine, or SERCA2a itself from ouabain-treated hearts (Fig. 7). In addition, LC-MS analysis revealed formation of a disulfide bond between Cys344 and Cys349 of SERCA2a.

**DISCUSSION**

Digitalis has played an important role in the treatment of heart failure for many years. Several laboratories, including our

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**Table 1. Hemodynamics and contractile function of isolated hearts**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>50 μM Ouabain</th>
<th>10 mM NAC + 50 μM Ouabain</th>
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<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>30 min</td>
<td>Baseline</td>
</tr>
<tr>
<td>Systolic pressure, mmHg</td>
<td>126±9</td>
<td>119±7</td>
<td>125±5</td>
</tr>
<tr>
<td>Diastolic pressure, mmHg</td>
<td>10±0.4</td>
<td>10±1.3</td>
<td>10±0.4</td>
</tr>
<tr>
<td>Developed pressure, mmHg</td>
<td>115±9</td>
<td>110±7</td>
<td>115±5</td>
</tr>
<tr>
<td>+dP/dt, mmHg/s</td>
<td>2365±193</td>
<td>2292±197</td>
<td>2647±104</td>
</tr>
<tr>
<td>−dP/dt, mmHg/s</td>
<td>2155±183</td>
<td>2106±180</td>
<td>2398±124</td>
</tr>
<tr>
<td>(+dP/dt)/(−dP/dt)</td>
<td>1.11±0.02</td>
<td>1.09±0.02</td>
<td>1.11±0.02</td>
</tr>
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Values are expressed as means ± SE for 9–16 rats in each group. *P < 0.001 vs. baseline; †P < 0.001 vs. control hearts, 30 min. §P < 0.05; ¶P < 0.001 vs. ouabain-treated hearts, 30 min. +dP/dt, rate of pressure development; −dP/dt, rate of pressure decline.
own, have observed that digitalis and DLS may have acute effects on ventricular relaxation (13–15, 32, 41); our group has connected these changes in diastolic function to alterations in Ca\(^{2+}\)/H\(^{+}\) cycling (41). Although the effect of digitalis to alter Ca\(^{2+}\)/H\(^{+}\) cycling has been ascribed to changes in cytosolic sodium resulting from the inhibition of the plasmalemmal Na\(^{+}\)/H\(^{+}\)-K\(^{+}\)/H\(^{+}\)-ATPase, recent data from our laboratory and others have demonstrated that DLS can signal through the sodium pump without observable changes in cytosolic sodium (2, 5, 33–35, 58) or even in a cell-free system. Our previous work in this area also established that increases in ROS play an important role in the DLS signaling process (35, 59, 68). Of interest, it appears that ROS also may inhibit the Na\(^{+}\)/H\(^{+}\)-K\(^{+}\)/H\(^{+}\)-ATPase and initiate its signaling function, thus creating a potential positive feedback loop (67). Moreover, administration of antioxidants can attenuate both the production of ROS and the cardiac effects of DLS (42, 58, 59). An extensive body of literature has established SERCA2a as the logical focus to better understand alterations in Ca\(^{2+}\)/H\(^{+}\) cycling produced by ouabain (30, 37, 46, 47).

A prolonged tau value in isolated myocytes and increased ratio of \(+\text{dP/dt}\) to \(-\text{dP/dt}\) in isolated hearts indicate impaired relaxation (16) with ouabain administration. These results showing that ouabain impairs relaxation in both whole hearts and isolated myocytes are in agreement with previous reports from our laboratory (41) and others (29, 36, 45, 54). The improvement in active relaxation seen in the NAC plus ouabain-treated hearts and in Ca\(^{2+}\) renormalization following stimulation in the isolated myocytes with NAC plus ouabain and GT plus ouabain further supports the involvement of ROS in ouabain-induced alterations in Ca\(^{2+}\) cycling. The impaired active relaxation observed in our isolated perfused heart studies does not appear to be explained by an increase in the ratio of PLB to SERCA (24) or by a decrease in the ratio of P-Ser\(^{16}\) PLB to total PLB (52, 53), because these ratios remained unchanged in our model. Furthermore, the fact that the expression of PLB was unchanged contrasts with its decreased expression after exposure to ischemia-reperfusion injury (38).

There are many mechanisms by which ROS might modify SERCA2a quantity, structure, and function, and several of these could involve decreases in SERCA2a translation. Oxygen radicals have been implicated in depressing the SERCA2a gene expression in the ischemic-reperfused heart (55). It is possible that in our model, ROS acted pretranslationally to decrease SERCA2a mRNA half-life and thus expression (44). We have previously observed decreases in mRNA for SERCA2a in hearts isolated from rats with experimental renal failure (26). Along these lines, ROS could act transcriptionally to decrease SERCA2a expression by acting on the SERCA2a gene. This idea seems to be supported by various models that have indirectly linked ROS to transcriptional control of SERCA2a.

![Fig. 4. A–C: immunoblots demonstrating levels of SERCA2a (A), phospho-lamban (PLB; B), and phosphorylated PLB at Ser\(^{\text{16}}\) (P-Ser\(^{\text{16}}\) PLB) in rat hearts after 30 min of Langendorff perfusion with Krebs (C; n = 5), 50 μM ouabain (O; n = 8), or 10 mM NAC and 50 μM ouabain (N+O; n = 5). D and E: relative ratios of PLB to SERCA2a (D) and P-Ser\(^{\text{16}}\) PLB to total PLB levels (E). Data are presented as means ± SE. * P < 0.05.

![Fig. 5. Acute ouabain administration increases oxidation of cardiac proteins after 30 min of Langendorff perfusion with Krebs (C; n = 5), 50 μM ouabain (O; n = 8), or 10 mM NAC and 50 μM ouabain (N+O; n = 5). A: immunoblot for SERCA2a, overexposed to highlight 2 additional SERCA bands [confirmed by liquid chromatography-mass spectrometry (LC-MS) analysis] in ouabain-treated left ventricles. B: immunoblot for carbonylated proteins [derivatized with dinitrophenyl (DNP)], demonstrating increased oxidation status in ouabain-treated left ventricles. C: immunoblot for nitrotyrosine. WB, Western blot.]
SERCA2a (4, 23, 57). However, the rapid changes observed in Ca$^{2+}$ handling, physiological function, and SERCA2a protein expression argue strongly against these possibilities in our system.

Viewed within the time constraints of our model, it appeared that ROS-induced posttranslational modifications of SERCA2a were the most reasonable explanation for our physiological observations, and this is where we focused our subsequent examinations. Because ROS convert some amino acid residues into derivatives of aldehydes and ketones, the accumulation of carbonyl groups has proven to be a useful measure of oxidative protein modification associated with various conditions of oxidative stress (reviewed in Refs. 19 and 51). We demonstrated that ouabain treatment was associated with marked increases in carbonylated SERCA2a. This specificity of these changes was confirmed by Western blotting, immunoprecipitation, and LC-MS. Peroxynitrite, formed by the combination of superoxide anion and nitric oxide (12), is another potential molecular modifier of SERCA2a structure and function, given that it is a potent effector of oxidation of thiols such as cysteine (43). Ouabain treatment is quite likely to increase peroxynitrite formation, because it has been demonstrated to increase levels of both nitric oxide (18, 65) and ROS (66, 68). Oxygen radicals also have been shown to modify SERCA activity (9, 21, 22, 69). Thiol-containing peptides present particularly sensitive targets for oxidative modifications (3, 50). Because SERCA2a contains 29 cysteine residues, it is very possible that the ouabain-induced changes in SERCA function may be due to the effect on free sulphydryl groups. Indeed, LC-MS analysis of ouabain-treated hearts identified oxidation of cysteines at positions 344 and 349 with the formation of a disulfide bond (Fig. 7). Interestingly, other groups have found that a peroxynitrite-mediated process is capable of oxidizing free cysteines to sulfides in the SERCA1 isoform (60) and that the same Cys$^{344}$ and Cys$^{349}$ residues are affected (61). Furthermore, Viner and colleagues noted that modification of Cys$^{349}$ was sufficient to significantly decrease SERCA1 activity in skeletal muscle (62) and that the SERCA2a isoform was up to four times more susceptible to oxidative modification by peroxynitrite than SERCA1 (61). The significant trend toward increased levels of nitrotyrosine in ouabain-treated hearts warrants closer examination of this potentially potent modification. Last, we should also point out that the oxidative modifications we observed might make SERCA2a more likely to be targeted for proteolysis within the cell, because such modifications are well known to mark proteins for degradation in a multitude of biological systems (31). In this regard, we observed degradation bands of SERCA2a on Western blot that were confirmed on LC-MS, as well as small but significant reductions in SERCA2a content by densitometry. Whether decreases in proteolysis might preserve SERCA2a function in this setting is still unclear, but further studies addressing this important possibility must certainly be pursued.

With our focus on the SERCA2a protein, we should also point out that some of our observations might be attributable to oxidative changes in SR lipids, because oxygen radicals have been shown to promote lipid peroxidation in SR membranes (28). Unfortunately, we were not able to address this issue further in the current study. However, the fact that ouabain-treated hearts and myocytes had increased levels of carboxylation and tyrosine nitration, decreased SR Ca$^{2+}$ content, lower SERCA2a activity, and slower diastolic function certainly indicate that the observed changes in SERCA2a structure are likely to have pathophysiological relevance. Furthermore, the fact that virtually all of the physiological effects of ouabain as well as the observed changes in SERCA2a structure could be prevented with concomitant antioxidant therapy suggests the novel idea that oxygen radicals may play a central role in the physiological effects of ouabain and other cardiotonic steroids. Oxidative modifications present an attractive signaling mechanism for changes in Ca$^{2+}$ handling, because a variety of these modifications, including that of peroxynitrite (8, 25), is potentially reversible.

Interestingly, systolic as well as diastolic function was altered by antioxidant administration; in fact, all of the increases in inotropy as well as systolic and diastolic Ca$^{2+}$ concentrations induced by ouabain could be prevented by administration of antioxidants. These data are seemingly at odds with recent reports linking increases in SERCA activity to improved systolic function as well as a large body of work, some from our own laboratory, linking decreases in SERCA activity with decreased inotropy in chronic models (6, 26, 27, 48, 56). Adachi et al. (1) proposed a physiological/pathophysiological scheme in which ROS such as peroxynitrite can actually increase SERCA activity by $\delta$glutathiolation at a reactive Cys$^{674}$ residue but proposed that this process is impaired in the setting of atherosclerosis by irreversible oxidation of the key reactive thiol(s) on SERCA. Other insight into this issue also may be derived from the recent report of Teucher et al. (56), who performed various levels of transfec
SERCA into rabbit myocytes. These workers found that whereas active relaxation was improved in a dose-dependent manner, the lower transfection dose of SERCA increased systolic function whereas the higher amount actually attenuated systolic function. Because the rat depends more on SERCA for active relaxation than most other species, including the rabbit (11), a modest impairment of SERCA in this system also may explain the increases in inotropy. These concepts are supported by our previous work in hearts of animals subjected to experimental renal failure, where we observed impaired SERCA activity correlated with increases in systolic and diastolic Ca
\(^{2+}\) in isolated myocytes (26) as well as increased fractional shortening by echocardiography (27). At this point we also should mention that the prolonged time constant for recovery after caffeine treatment leaves open the possibility for involvement of the Na
\(^{+}/Ca^{2+}\) exchanger, a line of investigation we did not pursue in these studies, given the Na
\(^{+}/Ca^{2+}\) exchanger’s diminished contribution to Ca
\(^{2+}\) handling in the rat (11). Whereas Nishio et al. (40) determined that the cellular effects of ouabain were independent of the Na
\(^{+}/Ca^{2+}\) exchanger, this same group has implicated a role for the ryanodine receptor in ouabain’s inotropic effects (39). It is clear that additional work examining the effects of ouabain and the ROS/RNS generated by ouabain signaling on this and other proteins important in Ca
\(^{2+}\) cycling and contractile function is necessary to completely examine this important issue.

In summary, these data suggest that ouabain impairs SERCA activity in a ROS/RNS-dependent manner. Oxidative and nitrosative modifications of the SERCA protein suggest molecular mechanisms by which ouabain may alter cardiac Ca
\(^{2+}\) cycling and physiological function.

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