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

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Kennedy, David J., Sandeep Vetteth, Miaorong Xie, Sankaridrug M. Periyasamy, Zijian Xie, Chi Han, Venkatesha Basrur, Krishna Mutgi, Vladimir Fedorov, Deepak Malhotra, and Joseph I. Shapiro. Ouabain decreases sarco(endo)plasmic reticulum calcium ATPase activity in rat hearts by a process involving protein oxidation. *Am J Physiol Heart Circ Physiol* 291: H3003–H3011, 2006. First published July 21, 2006; doi:10.1152/ajpheart.00603.2006.—The effect of cardiac glycosides to increase cardiac inotropy by altering Ca^{2+} cycling is well known but still poorly understood. The studies described in this report focus on defining the effects of ouabain signaling on sarcoplasmic reticulum Ca^{2+}-ATPase function. Rat cardiac myocytes treated with 50 μM ouabain demonstrated substantial increases in systolic and diastolic Ca^{2+} concentrations. The recovery time constant for the Ca^{2+} transient, $\tau_{Ca^{2+}}$, was significantly prolonged by ouabain. Exposure to 10 μM H_2O_2, which causes an increase in intracellular reactive oxygen species similar to that of 50 μM ouabain, caused a similar increase in $\tau_{Ca^{2+}}$. Concurrent exposure to 10 mM *N*-acetylcysteine or an aqueous extract from green tea (50 mg/ml) both prevented the increases in $\tau_{Ca^{2+}}$ as well as the changes in systolic or diastolic Ca^{2+} concentrations. We also observed that 50 μM ouabain induced increases in developed pressure in addition to diastolic dysfunction in the isolated perfused rat heart. Coadministration of ouabain with *N*-acetylcysteine prevented these increases. Analysis of sarcoplasmic reticulum Ca^{2+}-ATPase protein revealed increases in both the oxidation and nitrotyrosine content in the ouabain-treated hearts. Liquid chromatography-mass spectrometric analysis confirmed that the sarcoplasmic reticulum Ca^{2+}-ATPase protein from ouabain-treated hearts had modifications consistent with oxidative and nitrosative stress. These data suggest that ouabain induces oxidative changes of the sarcoplasmic reticulum Ca^{2+}-ATPase structure and function that may, in turn, produce some of the associated changes in Ca^{2+} cycling and physiological function.

reactive oxygen species; cardiac signaling

CARDIAC GLYCOSIDES have been used for the treatment of congestive heart failure for centuries (64). Heretofore, the molecular mechanism for these cardiac glycosides has been ascribed to their effect on ion concentrations initiated through inhibition of the Na^+-K^+-ATPase (7). Our laboratory has reported that sodium pump inhibition initiates a signal cascade that does not require observable changes in intracellular sodium concentrations that might be anticipated from inhibition of the enzymatic function of the Na^+-K^+-ATPase (35). Furthermore, we have found the generation of reactive oxygen species (ROS) to be an essential part of this signal cascade (59, 68). We also have observed that the administration of either purified digitalis-like substances (DLS) such as ouabain or the serum from uremic patients, which is known to contain increased concentrations of these DLS (20), produces acute diastolic dysfunction in isolated rat cardiac myocytes studied in culture (41). In separate experiments, we also have noted that cardiac myocytes isolated from rats subjected to 5/6th nephrectomy also have diastolic dysfunction that can be attributed to reduced sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA2a) activity; interestingly, the reduction in SERCA2a activity in the 5/6th nephrectomy model occurs in concert with proportional reductions in SERCA2a mRNA and protein expression (26).

The reuptake of Ca^{2+} by the sarcoplasmic reticulum (SR) in myocardial cells is the major determinant of active relaxation in the rat (11). This SR Ca^{2+} reuptake is determined by several factors, including the intrinsic enzymatic activity of SERCA2a and the phosphorylation status of phospholamban (PLB). Because our previous study noted that DLS altered the renormalization of Ca^{2+} very rapidly following electrical stimulation in rat cardiac myocytes (41), we postulated that DLS signaling through the sodium pump might itself alter SERCA function, either directly or through PLB phosphorylation. Because the generation of ROS is an important step in signaling through the sodium pump (68), we further speculated that these ROS might themselves be involved in the molecular alterations induced by DLS stimulation. To test these hypotheses, we performed the following studies.

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 Ca^{2+}-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 Ca^{2+} tolerant after isolation in Ca^{2+}-free medium, we

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

Measurements of Ca^{2+} transient. The cytosolic Ca^{2+} concentrations during contraction and relaxation (i.e., Ca^{2+} transient) were monitored with the Ca^{2+}-selective fluorescent dye indo-1 (Molecular Probes, Eugene, OR), employing a spectrofluorimeter (Photon Technology International, Monmouth Junction, NJ) interfaced with an inverted microscope as previously described (26, 41, 42). The time constant $\tau_{Ca^{2+}}$, for recovery of Ca^{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^{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^{2+} transient of the caffeine contracture was then compared with the amplitude of the twitch Ca^{2+} transient (at 5 min). The ratio of caffeine contracture to twitch amplitude was used to assess SR Ca^{2+} content, where a larger difference between these amplitudes indicates greater Ca^{2+} content as described by Bassani et al. (11). The time constant $\tau_{Ca^{2+}}$, for recovery of Ca^{2+} following application of caffeine, was obtained as described above to measure Na^+/Ca^{2+} exchanger activity as described by Bassani et al. (11). All measurements were performed at 37°C.

Isolated perfused heart. The isolated isovolumic Langendorff preparation was performed in a similar fashion to that previously described by our group in some detail (70). Isovolumic perfusion was performed with a perfusion pressure of 80 cmH_2O by employing Krebs-Henseleit bicarbonate buffer that was oxygenated with 95% O_2-5% CO_2 at 37°C (pH 7.4). A latex balloon-tipped fluid-filled catheter was placed in the left ventricle through the left atrium so that left ventricular (LV) pressure could be measured with a pressure transducer. This latex balloon was filled with fluid at the start of each experiment so that the LV end-diastolic pressure was 8–12 mmHg. Hearts were paced with a Grass stimulator at a rate of 300 beats/min. All experiments consisted of a 30-min period of equilibration (baseline) followed by another 30 min of perfusion with either Krebs-Henseleit (time control), 50 μM ouabain, or 50 μM ouabain and 10 mM NAC. For the experiments involving ouabain/NAC, hearts were pretreated with 10 mM NAC during the equilibration phase. Hemodynamic data were acquired at 500 Hz and stored electronically using a BioPac MP110 acquisition system and AcqKnowledge 4.7.3 software (BIOPAC Systems, Santa Barbara, CA).

Western blot analysis. At the termination of the Langendorff experiments, left ventricles were quickly dissected out, frozen in liquid nitrogen, and stored at −80°C until further analysis. Left ventricles were homogenized in 25 mM imidazole buffer (pH 7.0) containing protease inhibitors (Sigma P-2714), and Western blot analysis was performed as described previously (26, 27) using either 10 or 15% SDS-PAGE gels (Ready Gel; Bio-Rad, Richmond, CA). Immunodetection of SERCA2a was carried out using both anti-SERCA2 monoclonal antibody (MAb) (Affinity Bioreagents, Golden, CO) and anti-SERCA2 polyclonal antibody conjugated directly to horseradish peroxidase (Bethy Laboratories). Immunodetection of PLB was obtained with anti-PLB MAb (Affinity Bioreagents), whereas PLB phosphorylation sites were obtained with goat polyclonal antibodies raised against a PLB peptide phosphorylated at Ser^{16} (Santa Cruz Biotechnology, Santa Cruz, CA). For immunodetection of oxidatively modified proteins, 10 mg/ml of LV homogenate was derivatized with either 2,4-dinitrophenyl hydrazine (DNP) or a derivatization control solution and detected using the OxyBlot protein oxidation detection kit according to the manufacturer's protocol (Chemicon International, Temecula, CA) as previously described (27). As a marker of reactive nitrogen species (RNS) and peroxynitrite ($ONOO^-$), tyrosine nitration was detected using anti-nitrotyrosine MAb (Cayman, Ann Arbor, MI). The immunoreactive products for all Western blots were visualized with secondary antibody conjugated to horseradish peroxidase using either SuperSignal West Pico substrate (Pierce, Rockford, IL) or ECL Plus Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ). The images of the immunoreactive products were quantified with a Molecular Analyst software program (Bio-Rad Laboratories, Hercules, CA) as described previously (26, 27).

Measurement of SERCA2a activity. To measure SR Ca^{2+}-ATPase 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).

Carbonylation ELISA analysis. Total protein carbonyl concentration of the LV homogenate was determined by ELISA using the Zentech PC test kit according to the manufacturer's protocol (Northwest Life Science Specialties, Vancouver, WA) as described previously (27).

Immunoprecipitation. LV homogenate was solubilized in radioimmune precipitation buffer containing protease 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 vacufuge. 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 X_{corr} (raw cross-correlation score

between the observed peptide fragment mass spectrum and the theoretically predicted one) and ΔCn (difference between the cross-correlation score (X_{corr}) 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 1SU4_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 Ca^{2+} cycling and contractile function. Myocytes treated with ouabain (50 μM) demonstrated substantial increases in systolic (313 ± 26 vs. 260 ± 11 nM; Fig. 1*A*) and diastolic Ca^{2+} (120 ± 16 vs. 61 ± 10 nM, $P < 0.01$; Fig. 1*B*). The recovery time constant for the Ca^{2+} transient, $\tau_{Ca^{2+}}$, was also prolonged by ouabain exposure (399 ± 35 vs. 288 ± 26 ms, $P < 0.01$; Fig. 1*C*). Exposure to 10 μM H_2O_2, which causes an increase in intracellular ROS similar to that of 50 μM ouabain (68), caused a similar increase in $\tau_{Ca^{2+}}$ (377 ± 35 vs. 288 ± 35 ms, $P < 0.01$; Fig. 1*C*). 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 Ca^{2+} as well as $\tau_{Ca^{2+}}$ (Fig. 1, *A–C*). The ratio between the maximal caffeine and maximal twitch Ca^{2+} was reduced in the ouabain-treated cells, indicating a reduced SR Ca^{2+} load and SERCA2a activity (Fig. 2*A*). Finally, the $\tau_{Ca^{2+}}$ for the caffeine contractures was significantly prolonged in the cardiac myocytes exposed to ouabain (Fig. 2*B*).

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 (+d*P*/d*t* and −d*P*/d*t*). Ouabain also impaired diastolic function, as assessed using the ratio of +d*P*/d*t* to −d*P*/d*t*, 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 H_2O_2 demonstrated the ability of ROS to depress SERCA activity in a dose-dependent manner (Fig. 3).

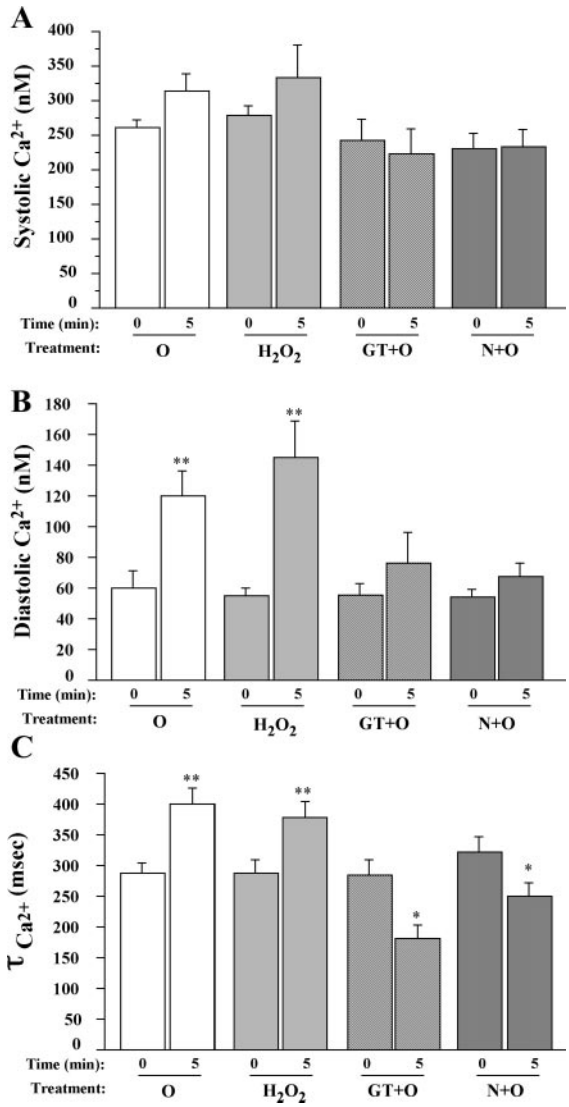

Fig. 1. Effect of ouabain (O), H_2O_2 (H), green tea extract plus ouabain (GT+O), and *N*-acetylcysteine (NAC) plus ouabain (N+O) on systolic Ca^{2+} (*A*), diastolic Ca^{2+} (*B*), and Ca^{2+} removal ($\tau_{Ca^{2+}}$; *C*) in cultured adult rat cardiac myocytes. Data are means ± SE from 6 different preparations. *$P <$ 0.05. **$P < 0.01$ vs. baseline.

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-Ser^{16} 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. 4*A*). There was no significant change with regard to PLB or P-Ser^{16} 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. 5*A*). Given the role of ROS in DLS-induced signal transduction and the biochemical and physiological data pointing to decreased SERCA2a Ca^{2+} handling in this setting, we decide to investigate whether the additional bands were peptide products generated from oxida-

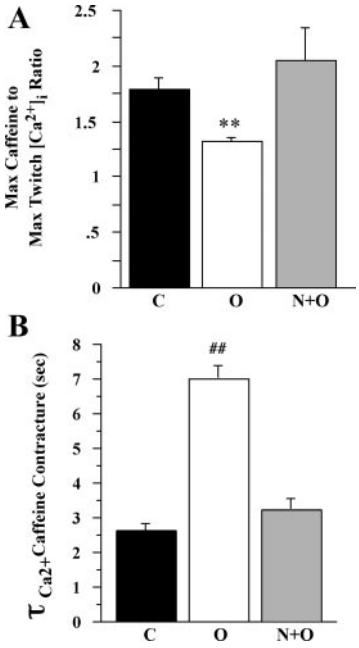

Fig. 2. Effect of ouabain and NAC on the ratio of maximal caffeine to maximal twitch Ca^{2+} (*A*) and recovery of Ca^{2+} transient ($\tau_{Ca^{2+}}$; *B*) after administration of 10 mM caffeine in cultured adult rat cardiac myocytes. Data are means ± SE from 7 different preparations. ** $P < 0.01$; ## $P < 0.0001$ vs. control (C).

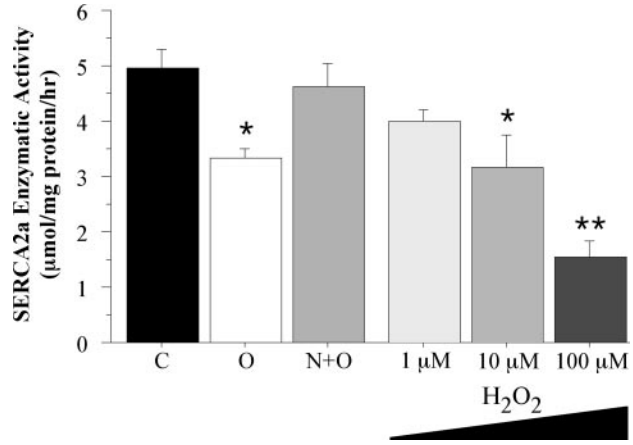

Fig. 3. Sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) enzymatic activity in rat hearts after 30-min treatment with Krebs (control, C; $n = 9$), 50 μM ouabain (O; $n = 6$), 10 mM NAC and 50 μM ouabain (N+O; $n = 5$), 1 μM H_2O_2 ($n = 5$), 10 μM H_2O_2 ($n = 5$), or 100 μM H_2O_2 ($n = 5$). Data are presented as means ± SE. * $P < 0.01$, ** $P < 0.0001$.

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 carbonylation 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. 5*B*). We also noted that ouabain administration increased tyrosine nitration, a marker of peroxynitrite modifications, of cardiac proteins, including the same 110-kDa region containing SERCA2a (Fig. 5*C*).

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 carbonylation, nitrotyrosine, or SERCA2a itself from ouabain-treated hearts (Fig. 7). In addition, LC-MS analysis revealed formation of a disulfide bond between Cys^{344} and Cys^{349} of SERCA2a.

DISCUSSION

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

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

	Control		50 μM Ouabain		10 mM NAC + 50 μM Ouabain	
	Baseline	30 min	Baseline	30 min	Baseline	30 min
Systolic pressure, mmHg	126±9	119±7	125±5	168±8*†	125±10	124±10§
Diastolic pressure, mmHg	10±0.4	10±1.3	10±0.4	9±0.6	10±0.4	11±2.0
Developed pressure, mmHg	115±9	110±7	115±5	159±8*†	115±11	113±10§
+dP/d*t*, mmHg/s	2365±193	2292±197	2647±104	3668±192*†	2506±237	2303±234§
−dP/d*t*, mmHg/s	2155±183	2106±180	2398±124	3065±192*†	2278±202	2168±207‡
(+dP/d*t*)/(−dP/d*t*)	1.11±0.02	1.09±0.02	1.11±0.02	1.21±0.02*†	1.10±0.02	1.06±0.02*§

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/d*t*, rate of pressure development; −dP/d*t*, rate of pressure decline.

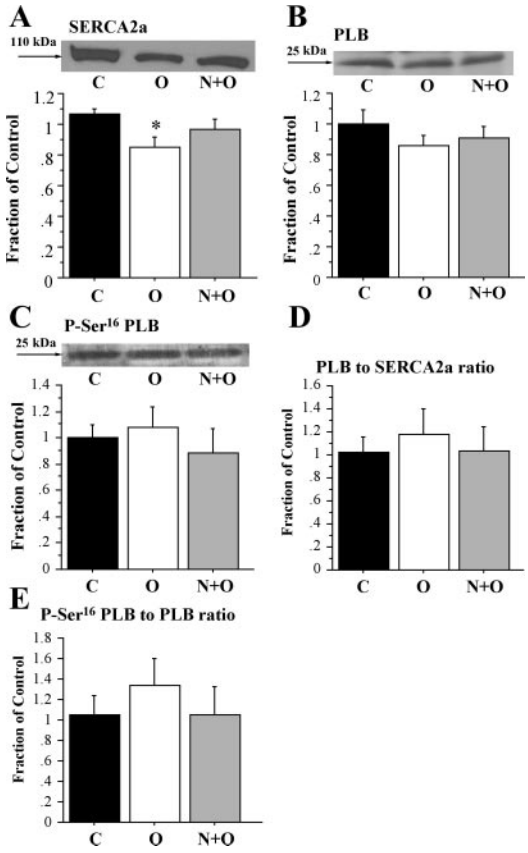

Fig. 4. *A–C*: immunoblots demonstrating levels of SERCA2a (*A*), phospholamban (PLB; *B*), and phosphorylated PLB at Ser^{16} (P-Ser^{16} PLB) in rat hearts after 30 min of Langendorff perfusion with Krebs (C; $n = 5$), 50 μM ouabain (O; $n = 8$), or 10 mM NAC and 50 μM ouabain (N+O; $n = 5$). *D* and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

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+} cycling (41). Although the effect of digitalis to alter Ca^{2+} cycling has been ascribed to changes in cytosolic sodium resulting from the inhibition of the plasmalemmal Na^+-K^+-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^+-K^+-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+} cycling produced by ouabain (30, 37, 46, 47).

A prolonged tau value in isolated myocytes and increased ratio of $+dP/dt$ to $-dP/dt$ 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

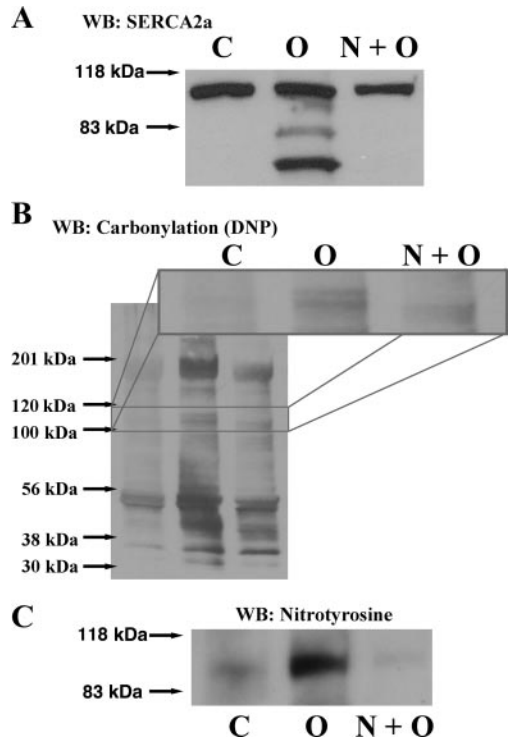

Fig. 5. Acute ouabain administration increases oxidation of cardiac proteins after 30 min of Langendorff of perfusion with Krebs (C; $n = 5$), 50 μM ouabain (O; $n = 8$), or 10 mM NAC and 50 μM ouabain (N+O; $n = 5$). *A*: immunoblot for SERCA2a, overexposed to highlight 2 additional SERCA bands [confirmed 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.

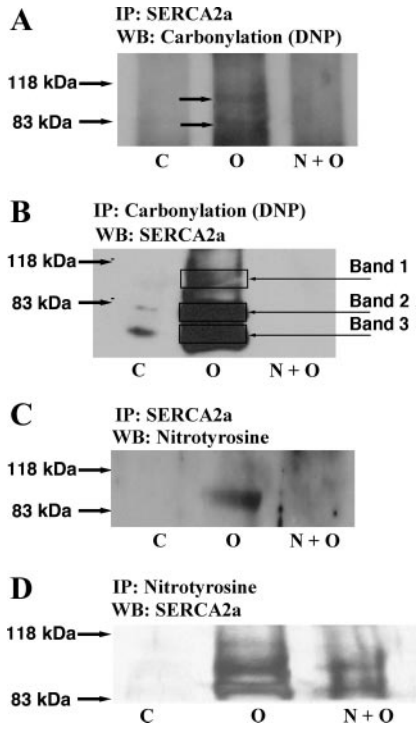

Fig. 6. Acute ouabain administration increases oxidation (carbonylation) and tyrosine nitration of cardiac proteins. *A*: immunoprecipitation of SERCA2a with immunoblot for DNP. *B*: immunoprecipitation of carbonylated proteins (derivatized with DNP) with immunoblot for SERCA2a. *C*: immunoprecipitation of SERCA2a with immunoblot for nitrotyrosine. *D*: immunoprecipitation of nitrotyrosine with immunoblot for SERCA2a. IP, immunoprecipitate.

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 sulfhydryl 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 disulfides 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 carbonylation 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 *S*-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 transfection of

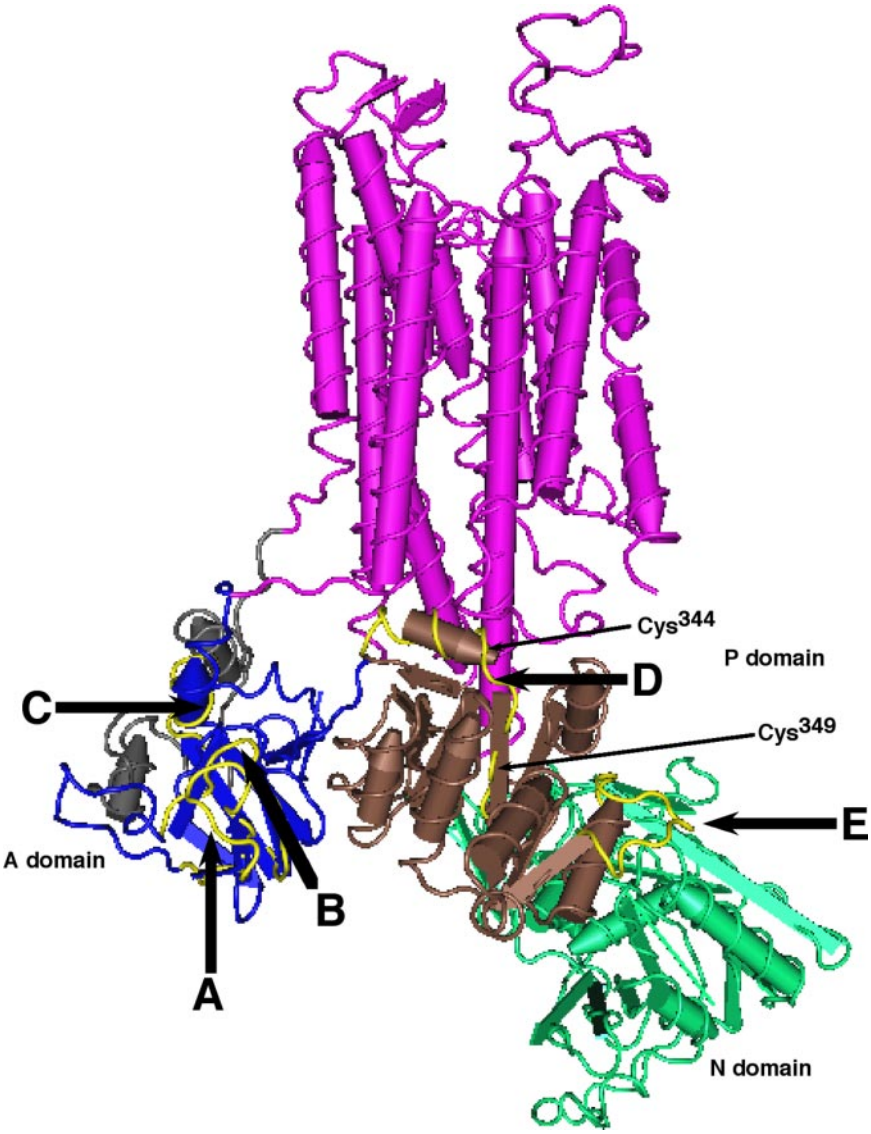

Fig. 7. SERCA2a three-dimensional structure with yellow highlighting identifying *peptides A–E* (arrows) from LC-MS analysis of duplicate gels from ouabain-treated left ventricular homogenate, carbonylated (DNP derivatized) proteins, and nitrotyrosine immunoprecipitate. *Peptide A*, peptide $175^{VDQSILTGESVSVIK}189$, identified from postnitrotyrosine immunoprecipitation, *band 3* of post-DNP immunoprecipitation, and post-SERCA2a immunoprecipitation of ouabain-treated heart (see Fig. 6). *Peptide B*, peptide $206^{NMLFSGTNIAAGK}218$, identified from post-SERCA2a immunoprecipitation of ouabain-treated heart (see Fig. 6). *Peptide C*, peptide $219^{AMGVVVATGVNTEIGK}234$, identified from *band 1* post-DNP immunoprecipitation and post-SERCA2a-immunoprecipitation ouabain-treated heart (see Fig. 6). *Peptide D*, peptide $335^{SLPSVETLGCTSVICSDK}352$ containing cysteine-cysteine disulfide bond, identified from *top* (110 kDa) band of ouabain-treated heart (see Fig. 5*A*). *Peptide E*, peptide $638^{IGIFGQDEDVTSK}650$, identified from post-SERCA2a immunoprecipitation of ouabain-treated heart (see Fig. 6). Other peptides identified from the 110-kDa band corresponding to SERCA2a included $111^{NAENAIEALK}120$, $234^{IRDEMVATEQER}245$, $252^{LDEFGEQLSK}261$, $515^{GAPEGVIDR}523$, $573^{EEMHLEDSANFIK}585$, $712^{KSEIGIAMGSGTAVAK}727$, and $971^{ISLPVILMDETL}982$. Activation domain (A domain) is highlighted in blue; phosphorylation domain (P domain) is highlighted in brown; membrane-spanning domain is highlighted in pink; nucleotide-binding domain (N domain) is highlighted in green.

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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