Carbonylation Contributes to SERCA2a Activity Loss and Diastolic Dysfunction in a Rat Model of Type 1 Diabetes

Chun Hong Shao,1 Haley L. Capek,2 Kaushik P. Patel,3 Mu Wang,4 Kang Tang,3 Cyrus DeSouza,5 Ryoji Nagai,6 William Mayhan,7 Muthu Periasamy,7 and Keshore R. Bidasee1,8,9

OBJECTIVE—Approximately 25% of children and adolescents with type 1 diabetes will develop diastolic dysfunction. This defect, which is characterized by an increase in time to cardiac relaxation, results in part from a reduction in the activity of the sarco(endo)plasmic reticulum Ca2+-ATPase (SERCA2a), the ATP-driven pump that translocates Ca2+ from the cytoplasm to the lumen of the sarcoplasmic reticulum. To date, mechanisms responsible for SERCA2a activity loss remain incompletely characterized.

RESEARCH DESIGN AND METHODS—The streptozotocin (STZ)-induced murine model of type 1 diabetes, in combination with echocardiography, high-speed video detection, confocal microscopy, ATPase and Ca2+ uptake assays, Western blots, mass spectrometry, and site-directed mutagenesis, were used to assess whether modification by reactive carbonyl species (RCS) contributes to SERCA2a activity loss.

RESULTS—After 6–7 weeks of diabetes, cardiac and myocyte relaxation times were prolonged. Total ventricular SERCA2a protein remained unchanged, but its ability to hydrolyze ATP and transport Ca2+ was significantly reduced. Western blots and mass spectroscopic analyses revealed carbonyl adducts on select basic residues of SERCA2a. Mutating affected residues to mimic physio-chemical changes induced on them by RCS reduced SERCA2a activity. Preincubating with the RCS, methyglyoxal (MGO) likewise reduced SERCA2a activity. Mutating an impacted residue to chemically inert glutamine did not alter SERCA2a activity, but it blunted MGO’s effect. Treating STZ-induced diabetic animals with the RCS scavenger, pyridoxamine, blunted SERCA2a activity loss and minimized diastolic dysfunction.

CONCLUSIONS—These data identify carbonylation as a novel mechanism that contributes to SERCA2a activity loss and diastolic dysfunction during type 1 diabetes.
that synthesizes the potent RCS methylglyoxal, also was found to be upregulated as early as 1 week after the onset of diabetes (21). When in excess, RCS reacts with exposed arginine, lysine, and histidine residues on proteins to form carbonyl adducts by a process referred to as carboxylation (22,23). In an earlier study (24), we found elevated levels of carbonyl adducts on select basic residues of SERCA2a isolated from the hearts of streptozotocin (STZ)-induced diabetic rats. However, it was not clear whether these adducts were functionally important or an epiphenomenon of the diabetes. Therefore, the purpose of the current study was to ascertain whether carbonyl adducts formed on SERCA2a during diabetes impair its ability to transport Ca\(^{2+}\).

**RESEARCH DESIGN AND METHODS**

**Antibodies and chemicals.** SERCA2a and PLN antibodies were obtained from Thermo Fisher Scientific (Boulder, CO) and Millipore (Rochester, NY). Arginine, lysine, and histidine antibodies were obtained from JaiCA (Shizuoka, Japan), and VAP-1 (1H-43), actin (\(^{1C}\)), and all secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Advanced glycation end products (AGEs), 3-deoxyglucosone/imidazolone, carboxymethyllysine (CML), carbonyllysine, N-carboxyethyllysine, and pentosidine, and pyralline antibodies on SERCA2a. Site-directed mutagenesis. Wild-type or mutant SERCA2a (10–15 µg) was transfected into human embryonic kidney (HEK)-293T cells (30–40% confluence) using Ca\(^{2+}\)-phosphate and grown in Petri dishes (100 mm) for 36–44 h. After this time, cells were harvested, sonicated (4 × 6 s) in buffer (0.25 mol/L sucrose, 10 mmol/L histidine, pH 7.3) and a protease inhibitor mix (1 mmol/L benzamidine, 2 µg/mL leupeptin, 2 µg/mL pepstatin A, 2 µg/mL apro tin, and 0.5 mmol/L phenylmethylsulfonyl fluoride) and centrifuged at 13,000 rpm for 3 min. Supernatants were collected, and relative SERCA2a content was determined using serial-dilution Western blots. HEK-293T cells were used for expression and chosen because they express low levels of endogenous SERCA2a and do not express PLN.

**SERCA2a activity**

**Ca\(^{2+}\) uptake assays (steps 1–6 of the post-Elbers cycle \([E_1\rightarrow E_2]\).** Membrane vesicles were resuspended in 1 mL buffer (30 mmol/L Tris-HCl, pH 7.3, 0.1 mmol/L NaCl, 5 mmol/L MgCl₂, 0.15 mmol/L EGTA; 0.12 mmol/L CaCl₂; 1 µM \(^{45}\)Ca\(^{2+}\); and 10 mmol/L potassium oxalate) and were divided into 2 × 500-µL aliquots. One aliquot was incubated with a SERCA2a inhibitor cocktail (10 µmol/L thapsigargin, 100 µmol/L ammonium molybdate, and 0.05 µg bafilomycin) and the other remained untreated. Ca\(^{2+}\) uptake assays were conducted as described by Andersen (30,31) using Na\(_2\)ATP to initiate uptake. In assays involving rat heart membrane vesicles, 50 µmol/L ryanodine was added to the incubation media for 10 min to inhibit or close the ryanodine receptor Ca\(^{2+}\) release channel.

**Ca\(^{2+}\)-ATPase activity (steps 1–3 of the post-Elbers cycle).** Wild-type and mutated SERCA2a (equivalent amounts of proteins) were incubated in 500 µL buffer (10 mmol/L HEPEs, pH 7.3; 0.1 mmol/L KCl; 5 mmol/L Mg\(^{2+}\); 100 µmol/L Ca\(^{2+}\); 100 µmol/L EGTA; and 2.5 mmol/L Na\(_2\)ATP) and incubated for 20 min at 37°C in the presence and absence of the Ca\(^{2+}\) ionophore, A23187 (2 µg/mL) (20–30 min). After this time, reactions were stopped and the inorganic phosphate generated from ATP hydrolysis was assessed using the malachite green colorimetric assay described previously (28).

**Statistical analysis.** Differences among values from each of the five groups (groups C, D, Ins-D, PyD-C, and PyD-D) were evaluated by ANOVA using Prism GraphPad. Data shown are means ± SE. Results were considered significantly different if \(P < 0.05\) (95% CI).

**RESULTS**

**Animal characteristics and conformation of diastolic dysfunction.** The general characteristics of the animals used in this study are listed in Table 1. After 6–7 weeks of
diabetes, echocardiographic analyses showed characteristic reductions in early atrial-phase left-ventricular filling velocity (E) and E-to-A ratio (Fig. 2A). In vivo hemodynamics also showed a parallel increase in time to 50% relaxation ($T_{50}$) (Fig. 2B). Two weeks of insulin therapy initiated 4–5 weeks after the onset of diabetes blunted decreases in blood flow velocity during diastolic filling and the E-to-A ratio (Fig. 2B) and $T_{50}$ (Fig. 2B), establishing that diastolic dysfunction was not a result of STZ toxicity, per se, but rather diabetes.

**Diabetes slows myocyte Ca$^{2+}$ transient decay and myocyte relaxation.** Figure 3A, left side, shows characteristic prolongation in myocyte Ca$^{2+}$ transient decay time induced by diabetes at a low stimulation frequency (0.5 Hz). Rate and amplitude of Ca$^{2+}$ release also were reduced (Table 1, left) probably because of the reduced SR Ca$^{2+}$ load (17). Mean data from >50 cells from three rats per group are shown in Table 1. Parallel reductions in contraction and relaxation velocities as well as myocyte shortening also were seen at 0.5 Hz (Fig. 3A, right side). Mean data are shown in Table 1, right.

At the higher 2-Hz stimulation, ~9% of diabetic myocytes (11 of 120) exhibited Ca$^{2+}$ alternans (Fig. 3B, second panel, red line). In six of these 11 diabetic myocytes, the defect in Ca$^{2+}$ reuptake was limited to a small segment of the scanned region (~15 μmol/L) (Fig. 3B, second panel, white arrows). Treating diabetic animals with insulin blunted the prolongation in Ca$^{2+}$ transient decay time (Table 2). At the

![FIG. 1. Schematic showing the locations of carbonyl adducts found on SERCA2a. The structure of SERCA2a was adapted from Dode L, Andersen JP, Leslie N, Dhitavat J, Vilsen B, Hovnanian A. Dissection of the functional differences between sarco(endoplasmic reticulum Ca$^{2+}$-ATPase (SERCA) 1 and 2 isoforms and characterization of Darier disease (SERCA2) mutants by steady-state and transient kinetic analyses. J Biol Chem 2003;278:47877–47889. (A high-quality digital representation of this figure is available in the online issue.)](https://www.diabetesjournals.org)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (C)</th>
<th>STZ-induced diabetic (D)</th>
<th>STZ-induced diabetic (Ins-D)</th>
<th>PyD-treated control (PyD-C)</th>
<th>PyD-treated STZ-induced diabetic (PyD-D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$n$</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>Body mass (g)</td>
<td>370.0 ± 13.3</td>
<td>290.1 ± 17.3*</td>
<td>324.0 ± 7.5</td>
<td>397.6 ± 12.9</td>
<td>273.5 ± 15.6</td>
</tr>
<tr>
<td>Glycosylated hemoglobin (%)</td>
<td>4.1 ± 0.1</td>
<td>7.2 ± 0.1*</td>
<td>4.7 ± 0.2</td>
<td>4.2 ± 0.2</td>
<td>7.6 ± 0.3</td>
</tr>
<tr>
<td>Blood glucose (mmol)</td>
<td>5.0 ± 0.5</td>
<td>21.1 ± 1.4*</td>
<td>8.1 ± 2.1</td>
<td>4.9 ± 1.1</td>
<td>20.1 ± 1.6</td>
</tr>
<tr>
<td>Serum insulin (ng/mL)</td>
<td>1.02 ± 0.2</td>
<td>0.31 ± 0.03*</td>
<td>0.91 ± 0.02</td>
<td>1.10 ± 0.07</td>
<td>0.28 ± 0.04</td>
</tr>
<tr>
<td>Serum SSAO activity</td>
<td>0.32 ± 0.02</td>
<td>0.56 ± 0.03*</td>
<td>0.35 ± 0.06</td>
<td>0.22 ± 0.01</td>
<td>0.39 ± 0.02†</td>
</tr>
<tr>
<td>Serum T3 (ng/dL)</td>
<td>218.9 ± 13.1</td>
<td>171.8 ± 17.4*</td>
<td>ND</td>
<td>209.7 ± 20.4</td>
<td>174.8 ± 10.4</td>
</tr>
</tbody>
</table>

Data are means ± SE ($n$ ≥ 16). *Significantly different from control rats ($P < 0.05$). †Significantly different from STZ-induced diabetic rats ($P < 0.05$). ND, not done.
2-Hz stimulation, 10 of 123 diabetic myocytes also exhibited contraction alternans (Fig. 3B, second panel, red line), which was likely the consequence of Ca\(^{2+}\) alternans. Contraction kinetics of myocytes from euglycemic animals were similar to that of control animals (Table 2). SERCA2a activity is reduced independent of protein levels. Minimal change in steady-state SERCA2a and monomeric PLN proteins were found in rat ventricular tissues after 6–7 weeks of diabetes (Fig. 4A). There was slight decrease (<10%, \(P > 0.05\)) in pentameric (phospho-Ser16) PLN. The ability of SERCA2a to hydrolyze ATP and transport Ca\(^{2+}\) (\(E_1 \rightarrow E_2\)) were 30.1 ± 6.3% and 35.2 ± 6.4% lower in diabetic animals (Figs. 4B and C). Treating diabetic animals with insulin did not alter steady-state SERCA2a or PLN proteins, but it blunted SERCA2a activity loss.

Assessment of argpyrimidine and other carbonyl adducts on SERCA2a during diabetes. The MGO-derived argpyrimidine adduct was threefold higher on SERCA2a from diabetic animals compared with controls (Fig. 5A, upper autoradiogram). Other adducts such as AGEs, CML, pentosidine, and pyralline also were increased 1.2- to 5-fold during diabetes (Fig. 5A). The amount of immunoreactive 3-deoxyglucosone/imidazolone and carboxyethyllysine did not change on SERCA2a after 6–7 weeks of diabetes.

Mass spectrometry (MALDI-TOF and tandem) were then used to identify and confirm the locations of carbonyl adducts on SERCA2a. As shown in Fig. 5B, alignment of MALDI-TOF data revealed the presence of an M+1 peak at 2485.12 Da in the diabetic sample but not in control or insulin-treated SERCA2a samples. Our Perl algorithm (32) suggests that this mass could arise from a pentosidine adduct cross-linking K460 (452MNVFDTELKGLSK464) in the nucleotide (N) domain and R636 (629GTAVAILRR637) in the phosphorylation (P) domain. Fragmentation of the 2,485.12 Da peak afforded M+1 peaks at 950.4286, 708.3023, 607.2547, 492.2273, 347.2286, 246.0902, and M+1 peaks at 230.1132, 329.1814, 400.2190, and 513.3027, corresponding to b and y M+1 ions from MNVFDTELKGLSK and M+1 b ions from GTAVAILRR, respectively (Fig. 5C). The combination MALDI-TOF followed by tandem mass spectrometry confirmed argpyrimidine on R164, pyralline on K476 and K481, and CML adduct on R636.

Steady-state levels of VAP-1, MGO, and glyoxalase-1 in rat heart during diabetes. Because the argpyrimidine adduct arises from elevation in MGO, we then sought to
A

\[ \text{Fluorescence Change (F/F_0)} \]

\[ \begin{array}{c}
\text{Control} \\
50 \mu M \\
\text{STZ-diabetic}
\end{array} \]

\[ \text{Time (seconds)} \]

\[ \begin{array}{c}
0.0 \\
1.0 \\
2.0 \\
3.0
\end{array} \]

**FIG. 3.** *A*, left side: Representative line-scan images of a single electrically evoked global Ca\(^{2+}\) transient and contraction kinetics (*right side*) of ventricular myocytes from control (C) and STZ-induced diabetic (D) rats. Values shown in the *lower panel* are means ± SE for \( n > 50 \) cells.

*Significantly different from control rats (*P* < 0.05). *B*, left side: Consecutive evoked Ca\(^{2+}\) transients (≥20) in ventricular myocytes isolated from control (C), STZ-induced diabetic (D), and insulin-treated STZ-induced diabetic (Ins-D) rat hearts stimulated at 2 Hz. *B*, right side: Consecutive evoked contractions (≥20) of ventricular myocytes isolated from control (C), STZ-induced diabetic (D), and insulin-treated STZ-induced diabetic (Ins-D) rat hearts stimulated at 2 Hz. Black arrows indicate application of field stimulation (2 Hz), and red lines indicate abnormal Ca\(^{2+}\) transients and contractions. White arrows (*left panel*) represent variation in Ca\(^{2+}\) decay time along the scanned segment. (A high-quality digital representation of this figure is available in the online issue.)
TABLE 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>C</th>
<th>D</th>
<th>Ins-D</th>
<th>PyD-C</th>
<th>PyD-D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate of Ca(^{2+}) rise (fu/s)</td>
<td>78.8±9.4</td>
<td>50.4±1.1*</td>
<td>76.4±2.1†</td>
<td>73.6±6.2</td>
<td>64.0±2.3†</td>
</tr>
<tr>
<td>Peak Ca(^{2+}) amplitude (fu)</td>
<td>3.9±0.2</td>
<td>2.5±0.1*</td>
<td>4.1±0.1†</td>
<td>3.4±0.2</td>
<td>3.3±0.2†</td>
</tr>
<tr>
<td>T(_{50}) decay (ms)</td>
<td>286.5±10.1</td>
<td>730.1±12.5*</td>
<td>320.1±15.5†</td>
<td>229.6±10.8</td>
<td>402.4±20.1†</td>
</tr>
</tbody>
</table>

Myocyte Ca\(^{2+}\) transients: data are means ± SE (n = 100 cells). Myocyte contractility: Data are means ± SE (n = 94 cells). Contractile properties of myocytes from C, D, Ins-D, PyD-C, and PyD-D rats stimulated at 2 Hz. *Significantly different from control rats (P < 0.05). †Significantly different from diabetic rats (P < 0.05).

determine whether expression of the enzyme that synthesizes and the enzyme that degrades MGO (i.e., VAP-1 and glyoxalase-1) were altered in ventricular tissues during diabetes. Consistent with the increase in activity of its soluble form SSAO (Table 1), the steady-state level of membrane-bound VAP-1 was threefold higher in ventricular homogenates and myocytes from STZ-induced diabetic rats (Fig. 6A). Only the monomeric form of VAP-1 (84 kDa) was detected in these studies, probably because of denaturing conditions used to run polyacrylamide gels. Glyoxalase-1 also was increased threefold in left-ventricular myocytes during diabetes (Fig. 6B). MGO levels were twofold higher in ventricular homogenates from STZ-induced diabetic rats (8.0±1.2 μmol/L per 200 mg tissue in STZ-induced diabetic rats vs. 3.8±0.5 μmol/L per 200 mg in controls).

Assessing the importance of amino/azido moieties on amino acid residues of SERCA2a found to be carbonylated during diabetes. Because SERCA2a undergoes a series of timed conformational changes to hydrolyze ATP and transport Ca\(^{2+}\) (33), adducts like pentosidine, which cross-link intra- and interdomain residues, will likely impair the rate of conformation change and the ability of SERCA2a to translocate Ca\(^{2+}\) from the cytoplasm to the lumen of the sarcoplasmic reticulum (SR). What remains uncertain is whether non-cross-linking adducts, such as argpyrimidine, CML, and pyralline, also will do the same; if they do, are their effects on SERCA2a function residue and adduct dependent?

To date, chemical methods to insert a specific carbonyl adduct onto a specific amino acid without disrupting the tertiary structure of SERCA2a are unavailable. Because

![Representative autoradiogram showing relative levels of SERCA2a, monomeric, and pentameric (Ser16) PLN protein in whole-heart homogenate from control (C), STZ-induced diabetic (D), and insulin-treated STZ-induced diabetic (Ins-D) rats. The graph below shows the relative levels of SERCA2a protein. B: The ability of SERCA2a from control (C), STZ-induced diabetic (D), and insulin-treated STZ-induced diabetic (Ins-D) rats to transport Ca\(^{2+}\) (E1–E2). The graph shows means ± SE in n ≥7 different preparations. C: Ca\(^{2+}\)-ATPase activity of SERCA2a from control (C), STZ-induced diabetic (D), and insulin-treated STZ-induced diabetic (Ins-D) rats. The graph shows means ± SE in n ≥5 different preparations. *Significantly (P < 0.05) different from control rats. **Significantly (P < 0.05) different from STZ-induced diabetic rats.](image-url)
carbonylation 1) neutralizes the basic charge, 2) increases bulk, and 3) in some cases changes the free basic group to an acidic moiety, we reasoned that site-directed mutagenesis could provide insights into the role these basic amino acids are playing in the overall functioning of SERCA2a and, by extension, the impact physio-chemical changes induced on them by carbonylation will have on the ability of SERCA2a to transport Ca^{2+}. Three of four residues investigated in this study (K476 and K481 within the N domain and R636 within the P domain) are exposed to the aqueous medium, whereas the other, R164, is located within the actuator (A domain) and away from the aqueous environment ([34], also see Fig. 1).

Neutralizing basic charges on R164 and K481 by converting them to glycines significantly reduced the ability of SERCA2a to transport Ca^{2+} at all time points, indicating that these amino groups play crucial roles in SERCA2a function (Fig. 7A and C). Neutralizing basic charges on K476 and R636 had minimal impact on the ability of SERCA2a to transport Ca^{2+} within the first 10 min (Fig. 7B and D). However, as time progressed, the activities of these mutants were reduced, suggestive of “run down.” Neutralizing the basic charge and increasing hydrophilic bulk on R164 and R636 by converting them to tyrosines significantly reduced the ability of SERCA2a to transport Ca^{2+} at all time points (Fig. 7A and D). Mutating K476 and...
K481 to tyrosines minimally impacted the ability of SERCA2a to transport Ca\(^{2+}\) within the first 10 min (Fig. 7B and C). However, as pumping time increased, Ca\(^{2+}\) transport rates declined. Mutating residues R164, K476, K481, and R636 to tryptophan to mimic charge neutralization and an increase in hydrophobic bulk significantly reduced the ability of SERCA2a to transport Ca\(^{2+}\) (Fig. 7A–D). Inverting the amino/azide groups on R164, K476, K481, and R636 to acidic moieties significantly reduced the ability of SERCA2a to transport Ca\(^{2+}\) (Fig. 7E). HEK-293T cells expressed full-length wild-type and SERCA2a proteins (Fig. 7F).

To gain further insights into the mechanisms responsible for their altered Ca\(^{2+}\) transport, we also assessed the ability of SERCA2a to hydrolyze ATP. Altering the basic moiety and/or increasing bulk on K476 reduced its ability to hydrolyze ATP, in agreement with reduced Ca\(^{2+}\) uptake data (Fig. 8G). Mutating K481 to tyrosine did not affect the ability of SERCA2a to transport Ca\(^{2+}\) (Fig. 7E). HEK-293T cells expressed full-length wild-type and SERCA2a proteins (Fig. 7F).

**Protecting SERCA2a from the actions of methylglyoxal.**

If the amino/azido groups on R164, K476, K481, and R636 are indeed important for SERCA2a activity, then preventing them from undergoing carboxylation should blunt SERCA2a activity loss.

**Mutating impacted residues to unreactive glutamines.**

Glutamine mutations initially were created and confirmed on R164 and R636 because the arginine moieties on these residues are known to readily form argpyrimidine adducts with MGO. However, we were unable to express significant quantities of R164Q and R636Q in either HEK-293T, CV-1 (simian) in origin and carrying the SV40 (COS) and Chinese hamster ovary (CHO) cells for functional studies. MGO also can form argpyrimidine adducts by utilizing adjacent amine moieties (35). Because K480 and K481 are adjacent lysines (36), this prompted us to synthesize the K481Q mutant to disrupt the adjacent amine configuration and assess whether this change can blunt the action of MGO.

When transfected into HEK-293T cells, we found that both wild-type and the K481Q mutant expressed similar amounts of full-length SERCA2a protein (Fig. 8A, upper autoradiogram). The K481Q mutant also transported Ca\(^{2+}\) (Fig. 8A, graph below) and hydrolyzed ATP (Fig. 8B) in a manner similar to that of wild-type SERCA2a, establishing that the Q mutation did not affect the normal function of SERCA2a.

Having established that the K481 mutation did not alter SERCA2a activity, we then sought to determine whether it can blunt MGO action. Preincubating wild-type SERCA2a for 20 min at 37°C with 1 μmol/L freshly synthesized MGO (equivalent to the amount found in the serum of healthy individuals) (26) potentiated the ability of SERCA2a to transport Ca\(^{2+}\) (Fig. 8C). Preincubation with higher MGO (5–500 μmol/L, equivalent to the amount found in the serum of diabetic patients) (26,37), dose-dependently reduced the ability of SERCA2a to transport Ca\(^{2+}\). Interestingly, the K481Q mutant was resistant to the action of MGO (Fig. 8C), and Western blots indicate that this protection resulted directly from reduced formation of argpyrimidine (Fig. 8C, middle autoradiogram).

**Scavenging RCS in diabetic animals using PyD.**

The vitamin B6 congener PyD is a potent scavenger of RCS (26), and it recently was shown to clinically reduce nephropathy in patients with type 1 and type 2 diabetes (38). We tested whether this compound also is capable of reducing carboxylation of SERCA2a and minimizing diastolic dysfunction. Treatment of STZ-induced diabetic animals with PyD did not alter blood serum glucose levels, but it blunted serum SSAO activity (Table 1). PyD treatment also blunted increases in left-ventricular pressure and cardiac relaxation times (Fig. 9A) and increases in

---

**FIG. 6.** A: Representative autoradiograms for VAP-1 in whole-heart homogenates (upper panel) and ventricular myocytes (lower panel) from control (C) and STZ-induced diabetic (D) rats. The graph below shows means ± SE in n ≥ 5 different preparations. B: Representative autoradiogram for glyoxalase-1 in ventricular myocytes isolated from control (C) and STZ-induced diabetic (D) rat hearts. The graph below shows means ± SE for n ≥ 7 preparations. *Significantly (P < 0.05) different from control.
myocyte Ca\textsuperscript{2+} transient decay and relaxation times (Fig. 9B; Table 2). PyD treatment also reduced expression of VAP-1 (Fig. 9C), formation of argpyrimidine and other carbonyl adducts on SERC2a (Fig. 9D), and blunted its activity loss without altering its expression (Fig. 9E).

**DISCUSSION**

In the current study, we demonstrate for the first time that by changing the charge and/or size of multiple critical basic moieties, carbonylation reduces the ability of SERCA2a to transport Ca\textsuperscript{2+} (E1→E2). E: The impact of simultaneously neutralizing charge and increasing bulk on multiple residues on the ability of SERCA2a to transport Ca\textsuperscript{2+} (E1→E2). F: Representative autoradiograms for relevant SERCA2a mutants (upper band), emphasizing that changes in activities observed were not a result of degradation of the SERCA2a protein. The lower band represents β-actin. G–I: The impact of neutralizing charge and/or increasing bulk on amino acid residues 476, 481, and 636 on the ability of SERCA2a to hydrolyze ATP (steps 2–3 of the post-Elber’s cycle), respectively. Graphs are means ± SE from n = 4 experiments. *Significantly (P < 0.05) different from wild-type.
SR. This mechanism is independent of steady-state levels of SERCA2a. In fact, in this study SERCA2a (and PLN) proteins remained essentially unchanged even though serum the T3 level was reduced by 21% during diabetes. Data from the present and our earlier study (24) also suggest that RCS does not react indiscriminately with all available basic moieties on SERCA2a. Some residues (e.g., R164, K476, K481, and R636) are more susceptible to carbonylation than others. Although the reason for this selectivity is unclear at this time, the electronic environment of the specific amino acid residue or groups of amino acids may be a contributing factor.

We also found that the amount and location of carbonylation dictates the impact it will have on SERCA2a function. With low levels of carbonylation (incubation with 1 μmol/L MGO), SERCA2a activity is actually enhanced. Adachi et al. (39) also showed that low levels of peroxynitrite oxidation resulted in gain of function of smooth-muscle SERCA. However, which residues of SERCA2a are hypersensitive to this low level of MGO remain unknown at this time. Concentrations of MGO ≥5 μmol/L dose-dependently reduced the ability of SERCA2a to transport Ca$^{2+}$, which is consistent with the notion that carbonylation at multiple sites is needed to reduce its activity.

Neutralizing the charge and increasing hydrophilic bulk on residues exposed to the aqueous environment (K476, K481, and R636) minimally impacted the ability of SERCA2a to transport Ca$^{2+}$ during short-duration pumping. However, neutralizing the charge and increasing hydrophilic bulk at R164, which resides away from the aqueous environment, negatively impacted the ability of SERCA2a to transport Ca$^{2+}$. Based on the work of Andersen (30,31) and Clausen et al. (40), carbonylation of A domain R164 could be uncoupling ATP hydrolysis from calcium transport. Switching the polarity of the charge on R164, K476, K481, and R636 from basic to acidic resulted in SERCA2a activity loss, indicating that these amino/azide moieties are integral for the function of SERCA2a.

Using membrane vesicles, we found an aggregate two- to fivefold increase in carbonyl adducts on SERCA2a during diabetes. However, these data do not reveal the extent to which a specific SERCA2a molecule becomes carbonylated during diabetes and whether a few defective SERCA2a molecules are sufficient to negatively impact the overall Ca$^{2+}$ transient integrity. To address this question, we resorted to using confocal microscopy in the line-scan mode. In ~10% of diabetic myocytes investigated, the increase in time to Ca$^{2+}$ transient decay was not prolonged throughout the entire scanned region but limited to a small segment (Fig. 3B, left side, second panel, white arrows). We interpret this data to mean that some SERCA2a molecules are defective, whereas others are not. Interestingly, when these myocytes were stimulated at 2 Hz, Ca$^{2+}$ alternans were observed (Fig. 3B, left side, second panel, red line). In some diabetic animals we also observed cardiac ventricular fibrillation following isoproterenol injection (data not shown), but the specific causes of this remain unclear. Because Ca$^{2+}$ alternans is an underlying cause for ventricular arrhythmias, these data also suggest that increased carbonylation of SERCA2a may be contributing to tachycardia-induced ventricular arrhythmias during diabetes. However, it should be pointed out that defects in other SR Ca$^{2+}$-cycling proteins, such as type 2 ryanodine receptor and type 2 calsequestrin also may be contributors (41,42). Based on the data from this and an earlier study (17), we speculate that perturbation in myocyte intracellular

![FIG. 8. A: Relative expression levels of wild-type SERCA2a and the K481Q SERCA2a mutant in HEK-293T cells (upper autoradiogram) and the ability of these proteins to transport Ca$^{2+}$ (A, graph) and hydrolyze ATP (B). C: K481Q mutation blunted the ability of MGO to reduce the ability of SERCA2a to transport Ca$^{2+}$ (E1–E2). For the latter, wild-type and K481Q SERCA2a proteins were incubated in buffer with varying concentrations of MGO (0–500 μmol/L) for 20 min at 37°C. At the end of this time, 45Ca$^{2+}$ and ATP were added, and after 20 min samples were filtered. The amount of 45Ca$^{2+}$ remaining on the filter papers was used as an index of the ability of SERCA2a to transport Ca$^{2+}$. Western blot analyses also were conducted on wild-type and K481Q samples incubated with 100, 300, and 500 μmol/L MGO to determine the extent of the argpyrimidine adduct (C, middle autoradiogram). Western blots also were performed using SERCA2a and actin primary antibodies to ensure equivalent protein load. Values are means ± SE in n ≥3 different preparations. *Significantly (P < 0.05) different from wild-type.](image-url)
FIG. 9. A: Mean -dP/dt (rate of left-ventricular pressure decline) and time to 50% relaxation obtained from in vivo hemodynamic studies of STZ-induced diabetic (D), PyD-treated control (PyD-C), and PyD-treated STZ-induced diabetic (PyD-D) rats before and after isoproterenol stimulation. Values shown are means ± SE (n ≥ 8). *Significantly different from diabetic (P < 0.05) rats. B, left side: Consecutive evoked Ca2+ transients (≥20) in ventricular myocytes isolated from D, PyD-C, and PyD-D rat hearts stimulated at 2 Hz. B, right side: Consecutive contractions (≥20) of ventricular myocytes isolated from D, PyD-C, and PyD-D rat hearts stimulated at 2 Hz. Black arrows indicate the application of field stimulation (2 Hz), and red lines indicate abnormal Ca2+ transients and contractions. White arrows (left panel) represent the variation in Ca2+ decay time along the scanned segment. C: Autoradiogram for relative levels of VAP-1 in hearts from D, PyD-C, and PyD-D rats. D: Representative autoradiograms for carbonyl adducts on SERCA2a from D, PyD-C, and PyD-D rats. Standard Western blots were used for these studies using 75–100 μg of membrane vesicles. D also shows alignment of a segment of MALDI-TOF mass spectra obtained following trypsin digestion of SERCA2a from D, PyD-C, and PyD-D rats. M+1 peak at 2,485.12 Da seen in diabetic samples was not present after PyD treatment. E: Relative levels of SERCA2a expression in ventricular tissues from D, PyD-C, and PyD-D rats. The graph below shows the ability of SERCA2a from D, PyD-C, and PyD-D rat hearts to transport Ca2+. Values shown are means ± SE in n ≥ 7 different preparations. *Significantly (P < 0.05) different from diabetic rats. (A high-quality digital representation of this figure is available in the online issue.)
Ca$^{2+}$ cycling arising from defects in SERCA2a and type 2 ryanodine receptor activities may be contributing to nocturnal hypoglycemia–induced ventricular arrhythmia and the resultant dead-in-bed in young type 1 diabetic patients.

Having established that carbonyl adducts are functionally important, we then proceeded to assess whether preventing the formation of these adducts could minimize SERCA2a activity loss and, by extension, diastolic dysfunction, in vitro and in vivo. As indicated above, in vitro studies MGO (5–500 μmol/L) dose-dependently reduced the ability of SERCA2a to transport Ca$^{2+}$. Mutating K481 to a glutamine to prevent MGO from forming argpyrimidine with the adjacent amino residues on K480 and K481 blunted the ability of MGO to reduce SERCA2a activity loss. We also showed that treating STZ-induced diabetic animals with the RCS scavenger PyD blunted carbonylation of SERCA2a and the diastolic dysfunction induced by diabetes. These data further strengthen the notion that carbonylation is an important mechanism underlying diabetic cardiomyopathy.

Another novel finding of the current study is that although expression of the MGO-degrading enzyme glyoxalase-1 is elevated in rat ventricular myocytes during diabetes, this increase was not sufficient to reduce formation of argpyrimidine adducts on SERCA2a. In fact, we measured and found MGO levels to be twofold higher in ventricular tissues from STZ-induced diabetic rats compared with ventricular tissues from control animals. It should also be pointed out that although this study focused on the impact of MGO on SERCA2a, MGO also is likely to react with and alter the function of other intracellular, long-lived proteins, including type 2 ryanodine receptors (43). Another interesting finding of the current study is that treatment with PyD blunted the increase in cardiac VAP-1 induced by diabetes. The latter is extremely exciting because it suggests, for the first time, that MGO and other RCS may be potentiating the expression of VAP-1/SSAO by a feed-forward mechanism.

In conclusion, this study demonstrates, for the first time, that carbonylation is an important mechanism that contributes to SERCA2a activity loss and diastolic dysfunction in a rat model of type 1 diabetes. These findings also have implications beyond that of type 1 diabetes. Elevated levels of carbonylated proteins also are found in patients with type 2 diabetes, congestive heart failure, and renal failure (44–50). Increasing expression of proteins including SERCA2a without lowering carbonyl stress is likely to only transiently blunt diastolic dysfunction during diabetes because newly expressed SERCA2a proteins will subsequently undergo carbonylation (51). We propose scavenging RCS as an adjunct therapeutic strategy for slowing diastolic dysfunction during type 1 diabetes.

ACKNOWLEDGMENTS

This work was supported in part by grants from the American Diabetes Association (to K.R.B.), the Edna Ittner Pediatric Research Foundation (to K.R.B.), the National Institutes of Health (NS-59751 to K.P.P.; HL-090657 and AA-01128 to W.M., and HL-085061 to K.R.B.), and a grant-in-aid from the Ministry of Education, Science, Sports, and Cultures of Japan (Scientific Research Grant no. 15790619, to R.N.). No potential conflicts of interest relevant to this article were reported.

C.H.S., H.L.C., K.P.P., M.W., K.T., C.D., W.M., and M.P. performed experiments, analyzed data and assisted with manuscript editing. R.N. provided critical reagents that were not commercially available. K.R.B. came up with the hypothesis, designed experiments, performed data analyses, and wrote the manuscript.

The authors thank Dr. David Mellenman, University of Toronto, for human SERCA2a cDNA, and Caronda J. Moore and Matthew Dale, University of Nebraska Medical Center, Omaha, for editorial assistance.

REFERENCES

11. Kim HW, Ch YS, Lee HK, Park SY, Kim YH. Diabetic alterations in cardiac sarcoplasmic reticulum Ca2+-ATPase and phospholamban protein expression. Life Sci 2001;70:367–379


41. Marks AR, Priori S, Mennu M, Kontula K, Laitinen PJ. Involvement of the cardiac ryanodine receptor/calcium release channel in catecholaminergic polymorphic ventricular tachycardia. J Cell Physiol 2002;190:1–6


46. Kilhovd BK, Berg TJ, Birkenland KI, Thorby S, Hanssen KP. Serum levels of advanced glycation end products are increased in patients with type 2 diabetes and coronary heart disease. Diabetes Care 1999;22:1543–1548


