We tested the hypothesis that increased sarcoplasmic reticulum Ca$^{2+}$ buffering and increased voltage-gated Ca$^{2+}$ channel density underlie coronary smooth muscle intracellular Ca$^{2+}$ (Ca$^{2+}_{i}$) dysregulation in diabetic dyslipidemia and that exercise training would prevent these increases. Yucatan swine were maintained in J control, 2) alloxan-induced hyperglycemic, 3) high fat/cholesterol fed, 4) hyperglycemic plus high fat/cholesterol fed (diabetic dyslipidemic), and 5) diabetic dyslipidemic plus exercise-trained (treadmill running) conditions. After 20 wk, the heart was removed and smooth muscle cells isolated from the right coronary artery. We utilized fura-2 imaging of Ca$^{2+}_{i}$ levels to separate the functional role of the sarcoplasmic reticulum Ca$^{2+}$-ATPase (SERCA) from the Na$^{+}$-Ca$^{2+}$ exchanger and the plasmalemmal Ca$^{2+}$-ATPase, and whole-cell patch clamp to examine voltage-gated Ca$^{2+}_{i}$ channel current density (i.e., Ca$^{2+}$ influx). Results indicated that diabetic dyslipidemia impaired plasmalemmal Ca$^{2+}$ influx, increased basal Ca$^{2+}_{i}$ levels, increased SERCA protein and sarcoplasmic reticulum Ca$^{2+}_{i}$ buffering, and elicited an ~50% decrease in voltage-gated Ca$^{2+}_{i}$ channel current density. Exercise training concurrent with the diabetic dyslipidemic state restored plasmalemmal Ca$^{2+}$ influx, SERCA protein, sarcoplasmic reticulum Ca$^{2+}_{i}$ buffering, and voltage-gated Ca$^{2+}_{i}$ channel current density to control levels. Interestingly, basal Ca$^{2+}_{i}$ levels were significantly lower in the exercise-trained group compared with control. Collectively, these results demonstrate a crucial role for exercise in the prevention of diabetic dyslipidemia-induced Ca$^{2+}_{i}$ dysregulation.

hypercholesterolemia: sarcoplasmic reticulum Ca$^{2+}$-ATPase; voltage-gated Ca$^{2+}_{i}$ channel; Na$^{+}$-Ca$^{2+}$ exchange; intracellular Ca$^{2+}_{i}$ buffering; Ca$^{2+}$ extrusion

AEROBIC EXERCISE TRAINING elicits physiological adaptations that result in an improved quality of life in disease. For example, aerobic exercise is widely accepted to play a role in the prevention of both Type 2 diabetes (20) and coronary artery disease (4). Thus an understanding of the mechanisms by which exercise exerts a beneficial effect on these disease states could greatly enhance efforts to treat these conditions with pharmacological agents.

The combined conditions of hyperglycemia (diabetes) and dyslipidemia have been shown to accelerate the development and progression of vascular disease in both humans (11, 18, 25, 35) and swine (1, 28–31). In swine, this leads to increased coronary artery constriction in response to the proinflammatory molecule prostaglandin F$_{2\alpha}$ in vivo and in vitro (30) and increased constriction in response to endothelin-1 in vitro (30). Previous studies have suggested that the diabetic dyslipidemia-induced impairment in vascular reactivity is largely due to alterations in coronary smooth muscle intracellular Ca$^{2+}$ (Ca$^{2+}_{i}$) regulation. These studies have shown that diabetic dyslipidemia significantly impairs Ca$^{2+}$ influx from the cell (45), increases basal Ca$^{2+}_{i}$ levels (45), and increases sarcoplasmic reticulum Ca$^{2+}_{i}$ buffering (23). Since it is currently thought that the increase in sarcoplasmic reticulum Ca$^{2+}_{i}$ buffering is a compensatory alteration due to the impairment in Ca$^{2+}$ influx and the rise in Ca$^{2+}_{i}$ levels, collectively these results suggest that pro-atherogenic factors present in the diabetic dyslipidemic state negatively impact the functional capacity of plasma membrane Ca$^{2+}$ transporters. However, missing from this model is the impact of diabetic dyslipidemia on plasma membrane Ca$^{2+}$ transporters involved in regulating Ca$^{2+}$ influx, such as the L-type voltage-gated Ca$^{2+}_{i}$ channels. Bowles et al. (6) have shown that hyperlipidemia alone decreased L-type voltage-gated Ca$^{2+}_{i}$ channel density; however, the independent effect of hyperglycemia and synergistic effects of hyperglycemia and dyslipidemia have not been studied. Thus one aim of this study is to examine the effects of diabetes, hyperlipidemia, and diabetic dyslipidemia on coronary smooth muscle L-type voltage-gated Ca$^{2+}_{i}$ channel current density.

Exercise training in healthy/normal animals has direct effects on the coronary vasculature, including enhanced vasodilatory responses to adenosine both in vivo and in vitro (15, 26, 27, 33), and reduced vasoconstrictor responses to norepinephrine and endothelin in vitro (9, 33). Along with these adaptations in coronary vasoreactivity, exercise training has been shown to increase Ca$^{2+}_{i}$ buffering via mechanisms other than the sarcoplasmic reticulum Ca$^{2+}$-ATPase (SERCA) or the Na$^{+}$-Ca$^{2+}$ exchanger (NCX) (19), and to increase Ca$^{2+}$ influx via L-type voltage-gated Ca$^{2+}_{i}$ channels (8). Collectively, these results suggest enhanced coronary smooth muscle Ca$^{2+}_{i}$ regulation with exercise training.

When exercise training is performed concurrent with the diabetic dyslipidemic state, it has been shown to attenuate vascular dysregulation, including an attenuation of the diabetic dyslipidemia-induced decrease in vasoconstriction in response to...
to endothelin-1 in vitro (30). Consistent with this training-induced attenuation of vascular dysfunction, exercise training prevents the diabetic dyslipidemia-induced decrease in plasma membrane Ca$^{2+}$-efflux (45). Collectively, these results suggest that exercise training would prevent and/or attenuate diabetic dyslipidemia-induced alterations in SERCA, as well as plasma membrane Ca$^{2+}$ transporters, such as the L-type voltage-gated Ca$^{2+}$ channels. Thus we tested the hypothesis that increased Ca$^{2+}$ buffering by the SERCA and increased voltage-gated Ca$^{2+}$ channel density underlie coronary smooth muscle Ca$^{2+}$ dysregulation in diabetic dyslipidemia and that exercise training would prevent these increases.

**MATERIALS AND METHODS**

This study combined established protocols for examining diabetic dyslipidemia-induced coronary artery disease and endurance exercise training in a porcine model (8–10, 16, 37, 38). All procedures involving animals were approved by the Animal Care and Use Committee of the University of Missouri and complied fully with guidelines in the Guide for the Care and Use of Laboratory Animals (32) and the American Veterinary Medical Association Panel on Euthanasia (3).

**Experimental Design**

Male Yucatan swine (aged 8–12 mo, 35–45 kg) were obtained from the Sinclair Research Center (Columbia, MO) and then randomly assigned to one of five treatment groups for 20 wk as follows: 1) low-fat fed, sedentary [control (C), N = 6]; 2) alloxan-induced diabetes [diabetic (D), N = 8]; 2) high-fat, high-cholesterol fed [high fat (H), N = 8]; 4) alloxan-induced diabetes and high-fat, high-cholesterol fed [diabetic dyslipidemic (DD), N = 8]; and 5) alloxan-induced diabetes, high-fat, high-cholesterol fed plus aerobic exercise training [diabetic dyslipidemic plus exercise (DDX), N = 8].

As previously described (16), control and diabetic animals were fed a low-fat diet consisting of Purina mini-pig chow (Purina Mills, St. Louis, MO). All other animals were fed the high-fat, high-cholesterol diet consisting of Purina mini-pig chow supplemented with 2.0% cholesterol, 17% coconut oil, 2% corn oil, and 1% sodium cholate (16). The high-fat, high-cholesterol diet increased the %kcal of the diet from fat by 8 to 46%. All animals were fed once daily and given free access to water.

Hyperglycemia was induced by the intravenous administration of 100 mg/kg of the pancreatic ß-cell toxin alloxan monohydrate (Sigma-Aldrich, St. Louis, MO), as previously described (5, 16). Animals were monitored closely for 24 h after alloxan monohydrate administration to prevent the life-threatening drop in blood glucose levels triggered by the massive insulin release associated with alloxan-induced ß-cell death (34, 36). Fasting blood glucose levels and blood urea nitrogen levels were measured once a week using an Accu-Check blood glucose monitor (Boehringer Mannheim, Ridgefield, CT) and Azostix diagnostic strips (Bayer, Elkhart, IN), respectively. Pigs were maintained with fasting blood glucose levels between 300 and 400 mg/dl for the duration of the study using daily insulin injections as required.

Arterial blood samples were obtained from fasted swine at the time of death. Plasma was assayed for total cholesterol, high-density lipoprotein and low-density lipoprotein cholesterol, and total triglyceride levels as previously described (16, 44).

**Exercise Training Procedures**

To prevent any detrimental cardiac event in diabetic dyslipidemic swine with cardiovascular disease, the endurance exercise training protocol was modified from our early studies (8–10, 37, 38) and performed as previously described (45). This protocol complied fully with guidelines in the APS Resource Book for the Design of Animal Exercise Protocols (14). Briefly, exercise training consisted of treadmill running performed 4 days/wk for 16–18 wk with the last week corresponding to 20 wk of diabetic dyslipidemia. The daily 45-min training bout consisted of four stages: 1) a 5-min warm-up at 2.2 km/h, 2) 5 min at 3.8 km/h (40–50% maximum heart rate), 3) 30 min at 4.8 km/h with a variable grade (65–75% of maximum heart rate), and 4) a 5-min cool-down at 2.2 km/h. Heart rates were taken using a stethoscope just before the exercise bout and at the middle and end of stage 3. Throughout the 20-wk training period, training intensity was maintained in the desired heart rate range by altering the treadmill grade. Efficacy of training was assessed by measurements of resting heart rate and the treadmill grade (i.e., submaximal workload) at which 65–75% of maximal heart rate was achieved.

**Tissue Removal and Isolation of Coronary Smooth Muscle Cells**

The procedure for the isolation of the right coronary artery and the enzymatic dispersal of porcine coronary smooth muscle cells has been previously described (21–23, 37). Briefly, at least 48 h after the completion of the 20-wk training period, swine were anesthetized with isoflurane gas (4%) and the heart surgically removed and rapidly placed in ice-cold physiological salt solution (PSS). The right coronary artery was dissected away from the myocardium, and distal arterial sections (4- to 5-mm lumen diameter) were incubated with a low-Ca$^{2+}$ solution containing 294 U/ml collagenase, 5 U/ml elastase, 2 mg/ml bovine serum albumin, 1 mg/ml soybean trypsin inhibitor, and 0.4 mg/ml DNase. Cells were enzymatically dispersed by incubation in a shaking water bath at 37°C for 60 min. The first fraction, consisting largely of endothelial cells, was removed and discarded. Additional collagenase solution was added, and the artery was incubated in the water bath for an additional 30 min. Smooth muscle cells from this fraction were removed from the collagenase solution and identified morphologically (41). All experiments were performed on freshly dispersed cells within 24 h of death and within 6 h of isolation from the artery.

**Measurement of Ca$^{2+}$, Levels**

Measurements of whole cell Ca$^{2+}$ levels were obtained at room temperature (22–23°C) using the fluorescent Ca$^{2+}$ indicator, fura 2, and the InCa++ Calcium Imaging System (Intracellular Imaging, Cincinnati, OH) as previously described by our laboratory (21–23, 37, 38). Briefly, freshly dispersed cells were incubated with 2.5 μM fura 2-AM (Molecular Probes, Eugene, OR) in a shaking water bath at 37°C for 20 min and washed in a low-Ca$^{2+}$-containing solution supplemented with Eagle’s minimal essential storage media for 20 min. An aliquot of fura 2-loaded cells was placed on a coverslip contained within a constant-flow superfusion chamber that was mounted on an inverted epifluorescent microscope (Nikon, model TMS-F). Fura 2 was excited by light from a 300-W xenon arc lamp that was passed through a computer-controlled filter changer containing 340 ± 10 and 380 ± 10 nm band-pass filters. The fluorescence emission was collected at 510 nm using a monochrome charge-coupled device camera (COHU, San Diego, CA) attached to a 100-MHz Pentium data acquisition computer. Whole cell fura 2 fluorescence was expressed as the ratio of the 340:380-nm ratio of the fura 2 emission.

**Solutions.** The PSS contained (in mM) 138 NaCl, 5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, 10 glucose, and was titrated to a pH of 7.4 with NaOH. The 19 mM Na$^{+}$ solution contained (in mM) 15 NaCl, 124 LiCl, 5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, 10 glucose, and was titrated to a pH of 7.4 with NaOH. The 80 mM K$^{+}$ solution contained (in mM) 65 NaCl, 80 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, 10 glucose, and was titrated to a pH of 7.4 with NaOH. The Ca$^{2+}$-free solution (~100 mM Ca$^{2+}$) contained (in mM) 138 NaCl, 1 MgCl$_2$, 5 KCl, 10 HEPES, 0.01 K$^{+}$-EGTA, 10 glucose, titrated to a pH of 7.4 with

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NaOH. The caffeine solutions consisted of either the PSS or the Ca$^{2+}$-free solution supplemented with 5 mM caffeine.

**Protocol to assess total plasmalemmal Ca$^{2+}$ efflux.** Experimental protocols were designed to assess total Ca$^{2+}$ efflux via both the NCX and the plasma membrane Ca$^{2+}$-ATPase (PMCA). Cells were superfused with PSS for 2 min followed by exposure to an 80 mM K$^+$ (depolarizing) solution for 10 min. The depolarizing step was performed to allow for Ca$^{2+}$ influx via voltage-gated Ca$^{2+}$ channels and the maximal loading of the sarcoplasmic reticulum Ca$^{2+}$ store. Cells were allowed to recover in PSS for 3 min before superfusion with a Ca$^{2+}$-free solution and depletion of the sarcoplasmic reticulum Ca$^{2+}$ store with 5 mM caffeine. The nearly complete absence of Ca$^{2+}$ in the extracellular solution eliminated possible Ca$^{2+}$ influx, whereas caffeine prevented the accumulation of Ca$^{2+}$ into the sarcoplasmic reticulum via SERCA by causing persistent opening of ryanodine receptors (40). (Graphs demonstrating the experimental protocol, the mean ± SE for key levels and times for the caffeine response, and the caffeine response time to half recovery are shown together in RESULTS for clarity.)

**Protocol to assess Ca$^{2+}$ efflux via the NCX and the PMCA.** To separately assess Ca$^{2+}$ efflux via the NCX and/or the PMCA, forward mode activity of the NCX was inhibited by superfusion with a low (19 mM) Na$^+$ solution in the presence or absence of the irreversible SERCA inhibitor, thapsigargin (10 μM). Cells were superfused with PSS for 2 min followed by exposure to an 80 mM K$^+$ (depolarizing) solution for 4 min. Cells were allowed to recover in PSS for 2 min before superfusion with a 5 mM caffeine solution in the absence or presence of thapsigargin. The caffeine emptied the sarcoplasmic reticulum Ca$^{2+}$ store, whereas the thapsigargin prevented reaccumulation of the Ca$^{2+}$ by SERCA. Cells were allowed to recover in PSS for 2 min and then superfused with a low (19 mM) Na$^+$ solution to inhibit Ca$^{2+}$ efflux via NCX activity. The low Na$^+$ solution in the absence of thapsigargin mainly assessed whether the NCX was essential for maintenance of Ca$^{2+}$, after caffeine-induced depletion of the sarcoplasmic reticulum Ca$^{2+}$ store, whereas the low Na$^+$ solution in the presence of thapsigargin mainly assessed the role of the PMCA. Thus the change in the fura 2 signal in response to the 19 mM Na$^+$ with thapsigargin solution primarily represents the balance between Ca$^{2+}$ influx and Ca$^{2+}$ extrusion via the PMCA. (Graphs demonstrating the experimental protocol, the mean ± SE for key levels and times for the 19 mM Na$^+$ responses, and the change in the fura 2 ratio in response to the 19 mM Na$^+$ solutions are shown together in RESULTS for clarity.)

**Protocol to assess sarcoplasmic reticulum Ca$^{2+}$ buffering.** To assess sarcoplasmic reticulum Ca$^{2+}$ buffering capacity, we utilized a protocol that has previously been shown in our laboratory to primarily assess sarcoplasmic reticulum Ca$^{2+}$ uptake when Ca$^{2+}$ influx is known (23). Briefly, cells were superfused with PSS for 2 min followed by exposure to an 80 mM K$^+$ (depolarizing) solution for 10 min. The depolarizing step was performed to allow for Ca$^{2+}$ influx via voltage-gated Ca$^{2+}$ channels and the maximal loading of the sarcoplasmic reticulum Ca$^{2+}$ store. Cells were allowed to recover in PSS for 3 min before superfusion with a nominally Ca$^{2+}$-free solution and depletion of the sarcoplasmic reticulum Ca$^{2+}$ store with 5 mM caffeine. Cells were then superfused with a second 80 mM K$^+$ solution for 10 min to allow for Ca$^{2+}$ influx and the time to reach the half-maximal response measured. The time to reach the half-maximal Ca$^{2+}$ level in response to the second 80 mM K$^+$ exposure is largely indicative of SERCA activity, since the juxtaposition of the sarcoplasmic reticulum creates a buffer barrier (i.e., the superficial buffer barrier), whereby a large fraction of the Ca$^{2+}$ entering the cell through voltage-gated Ca$^{2+}$ channels is sequestered into the sarcoplasmic reticulum Ca$^{2+}$ store (12, 13, 24). The quantification of Ca$^{2+}$ influx through voltage-gated Ca$^{2+}$ channels with direct patch-clamp measures (see below) provides more confidence regarding the contribution of sarcoplasmic reticulum Ca$^{2+}$ buffering to the time to reach the half-maximal Ca$^{2+}$, response. (Graphs demonstrating the experimental protocol, the mean ± SE for key levels and times for the 80 mM K$^+$ responses, and the time to reach a half-maximal response for the 80 mM K$^+$ solutions are shown together in RESULTS for clarity.)

**Immunoblot Analysis of SERCA2b Protein Expression**

Immunoblots for the Ca$^{2+}$-transporter, SERCA2b, were performed according to methods designed in our laboratory for porcine coronary arteries (23). Briefly, frozen proximal segments of the circumflex artery were homogenized on ice in a hypotonic homogenization buffer containing (in mM) 1 NaHCO$_3$, 5 CaCl$_2$, and 10 Tris supplemented with the protease inhibitors (in mM) 1 phenylmethlysulfonyl fluoride, 0.002 leupeptin, 0.002 aprotinin, and 0.002 pepstatin SC. The homogenate was centrifuged at 14,000 g for 15 min, the supernatant removed, and the pellet resuspended in Laemmli buffer (Bio-Rad Laboratories, Hercules, CA). The homogenate was centrifuged at 14,000 g for 15 min, and the supernatant (membrane fraction) was removed. Total membrane protein content was assessed using the Bicinchoninic acid method (Pierce, Rockford, IL) and the absorbance measured at 562 nm. A 20-μg sample of total membrane protein was loaded onto a 4–20% Bio-Rad ready gel (Bio-Rad Laboratories) and run at 110 V for ~60 min. Proteins were transferred onto a polyvinylidene difluoride Hybond-P membrane at 75 V for 75 min. The membrane was blocked with 5% nonfat dry milk, 1 Tris-buffered saline, and 0.01% Tween solution for 60 min to prevent nonspecific antibody binding. The anti-SERCA2b Ca$^{2+}$-ATPase (1:1,000) primary antibody (Affinity Bioreagents, Golden, CO) was added into the blocking solution and allowed to bind to the proteins at room temperature overnight. A horseradish peroxide-conjugated mouse secondary antibody (1:5,000) was incubated with the membrane for 60 min at room temperature. The secondary antibody was detected using electrochemiluminescence detection reagents (Amersham Biosciences, Piscataway, NJ). Densitometric analysis of immunoblots was performed using Scion software (Fredrick, MD). The protein expression of SERCA2b was assessed as the ratio of the density of the treatment group to the control group.

**Whole Cell Voltage Clamp Analysis to Assess Functional L-Type Voltage-Gated Ca$^{2+}$ Channel Expression**

Whole cell currents were measured at room temperature (22–23°C) using a standard whole cell voltage clamp technique as used routinely by our laboratory (2, 7, 8, 10, 19, 38, 39). Cells were initially superfused with PSS during gigaseal formation with 2–5 MΩ heat-polished glass pipette containing pipette solution containing (in mM) 120 CsCl, 10 tetraethylammonium chloride, 1 MgCl$_2$, 20 HEPES, 5 Na$_2$ATP, 0.5 Tris GTP, 10 EGTA, and then titrated to a pH of 7.2. After whole cell configuration, the superfuse was switched to PSS with tetraethylammonium chloride substituted for NaCl and either 2 mM Ca$^{2+}$ or 10 mM Ba$^{2+}$ as the charge carrier. Whole cell currents were amplified with a List EPC-7 patch clamp with a 0.5–50 GΩ capacitance feedback resistance headstage and filtered through an eight-pole low-pass filter with a cutoff frequency of 400 Hz. Capacity currents were filtered at low-pass cutoff frequency of 8.4 kHz. Leak subtraction was not performed. Calcium channel currents were expressed as pA/pF of membrane capacitance to normalize to plasma-lemmal area. Data acquisition and analyses were performed with a Labmaster analog-to-digital converter and AxoBASIC 1.0 data acquisition software (Axon Instruments, Sunnyvale, CA). Cells were constantly superfused at ~1–2 ml/min.

**Statistical Analysis**

Statistical significance was defined as $P < 0.05$, and the statistical analyses used to test significance for each measurement are described below. All data are presented as the mean of the group ± SE. For the body weight, blood measurements, and resting heart rate measurements presented in Table 1, statistical significance was assessed by
EXERCISE, DIABETES, AND INTRACELLULAR Ca\(^{2+}\) REGULATION

Table 1. In vivo indicators of diabetes, hyperlipidemia, diabetic dyslipidemia, and exercise training

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<thead>
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<th>Table 1. In vivo indicators of diabetes, hyperlipidemia, diabetic dyslipidemia, and exercise training</th>
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<tr>
<td><strong>Animals</strong></td>
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<tr>
<td><strong>Body weight</strong> (%Change from week 1 to 20)</td>
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<td><strong>Diabetes</strong></td>
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<td>Fasting blood glucose, mg/dl</td>
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<td>Average daily insulin, U/day</td>
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<td><strong>Renal function</strong></td>
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<td>Blood urea nitrogen, mg/dl</td>
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<td>Lipid profile (fasting levels)</td>
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<td>Total triglycerides, mg/dl</td>
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<td><strong>Exercise training</strong></td>
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<tr>
<td>Resting heart rate (beats/min)</td>
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<tr>
<td>Treadmill incline to maintain 65–75% maximal heart rate</td>
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<tr>
<td>Week 1</td>
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<td>Week 16–18</td>
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Values are means ± SE. NS, measurement was not significantly different between the treatment groups. C, control; D, diabetic; H, hyperlipidemic; DD, diabetic dyslipidemic; DDX, diabetic dyslipidemic plus exercise training.

one-way analysis of variance and Fisher’s least significant difference analysis. For the treadmill incline to maintain 65–75% maximal heart rate measurements, statistical significance between the diabetic dyslipidemic and exercise-trained diabetic dyslipidemic groups was assessed by t-tests. The number of animals in each group is indicated in the body of the table below the treatment group. For the Ca\(^{2+}\)i measurements and whole cell patch clamp analysis, statistical significance between treatment groups was determined by one-way analysis of variance and Student-Newman-Keuls post hoc analysis. The number of animals and cells for groups in each experiment are indicated in the body of the figure captions as N = pigs/cells. The number of cells was utilized as the N value for statistical analysis. For the immunoblot analysis, statistical significance between treatment groups was determined by one-way analysis of variance and Student-Newman-Keuls post hoc analysis. The number of animals utilized in each group is indicated in the body of the figure captions as N = pigs.

RESULTS

In Vivo Indicators of Diabetic Dyslipidemia and Exercise Training

Table 1 contains a summary of the in vivo data obtained in this study (30). The change in body weight over the 20-wk study was significantly lower in the diabetic group compared with all other groups, and the exercise-trained swine had a greater increase in body weight percentage compared with the sedentary, diabetic dyslipidemic swine. Fasting blood glucose levels in the diabetic, diabetic dyslipidemic, and exercise-trained diabetic dyslipidemic swine were maintained at levels approximately sixfold higher than control. The average daily insulin dose was not different between these groups. Blood urea nitrogen levels were not significantly elevated in any of the hyperglycemic groups, demonstrating that the pancreatic β-cell toxin alloxa did not elicit renal toxicity.

Fasting total cholesterol levels were elevated approximately fivefold in all groups fed the high-fat, high-cholesterol diet. The ratio of low-density lipoprotein to high-density lipoprotein (LDL:HDL) cholesterol was significantly higher in both diabetic dyslipidemic groups compared with control. Total fasting plasma triglyceride levels were elevated ~3.5-fold with diabetic dyslipidemia, and exercise training prevented this rise.

Efficacy of the exercise training protocol was verified by two measures: 1) resting heart rate and 2) treadmill grade to elicit 65–75% of maximal heart rate. Similar to previous studies from our group, after 20 wk of training, the exercise-trained diabetic dyslipidemic swine had significantly lower resting heart rates compared with the sedentary, diabetic dyslipidemic swine (5, 30, 43). The exercise-trained swine were also able to tolerate running at an increased workload, as evidenced by a significant increase in the treadmill grade at which they achieved 65–75% of their maximal heart rate.

Exercise Training Prevented the Diabetic Dyslipidemia-Induced Rise in Basal Ca\(^{2+}\)i Levels

Basal Ca\(^{2+}\)i levels were assessed by the 340:380-nm ratio of the fura 2 emission (fura 2 ratio) in PSS before any experimental perturbation. As shown in Fig. 1, basal Ca\(^{2+}\)i levels were significantly increased in cells from diabetic dyslipidemic swine over the control, diabetic, and hyperlipidemic swine.

Fig. 1. Exercise training significantly decreased the diabetic dyslipidemia-induced rise in basal intracellular Ca\(^{2+}\) (Ca\(^{2+}\)i) levels. Basal Ca\(^{2+}\)i levels were significantly increased (*P < 0.05) in cells from diabetic dyslipidemic swine (DD; N = 8 pigs and 304 cells) over the control (C; N = 6 pigs and 264 cells), diabetic (D; N = 5 pigs and 226 cells), and hyperlipidemic (H; N = 5 pigs and 253 cells) swine. Exercise training concurrent with diabetic dyslipidemic (DDX; N = 8 pigs and 315 cells) significantly lowered (#P < 0.05) the basal Ca\(^{2+}\)i levels below all groups.
Exercise training concurrent with the diabetic dyslipidemic state significantly lowered the basal $\text{Ca}^{2+}\text{;}_i$ levels below all groups.

**Exercise Prevents the Diabetic Dyslipidemia-Induced Impairment in Plasmalemmal $\text{Ca}^{2+}$ Extrusion**

Fura 2 imaging was used to assess the contribution of plasmalemmal $\text{Ca}^{2+}$ efflux via both the NCX and the PMCA to the alterations in basal $\text{Ca}^{2+}\text{;}_i$ levels. Figure 2A depicts the response of a cell from a control pig to the experimental protocol. Total plasmalemmal $\text{Ca}^{2+}$ efflux was assessed by examining the time to restore $\text{Ca}^{2+}\text{;}_i$ levels to half of the peak caffeine response in $\text{Ca}^{2+}$-free solution. In Fig. 2B, the symbols and error bars demonstrate (from left to right) the initial rise, the half-maximum, the peak, the half-recovery and the full recovery of the caffeine response. (In Fig. 2B, the lines are only provided to demonstrate the general shape of the response and do not represent the average response of all of the cells in that treatment group.) In cells from diabetic dyslipidemic swine, the time to half-recovery following the caffeine exposure was significantly prolonged compared with control, suggesting an impaired capacity to remove $\text{Ca}^{2+}$ from the cell (Fig. 2, B and C). Exercise training restored the diabetic dyslipidemia-induced impairment in plasmalemmal $\text{Ca}^{2+}$ efflux to control. Hyperglycemia and hyperlipidemia independently had no significant effect on the recovery time following the caffeine exposure.

**Exercise Training Prevents the Diabetic Dyslipidemia-Induced Decrease in $\text{Ca}^{2+}$ Extrusion Via the Plasmalemmal $\text{Ca}^{2+}$-ATPase**

Experimental protocols were designed to more specifically examine the relative contribution of $\text{Ca}^{2+}$ extrusion via the PMCA to the altered regulation of basal $\text{Ca}^{2+}\text{;}_i$ levels seen in coronary smooth muscle from diabetic dyslipidemic and exercise-trained diabetic dyslipidemic swine (Fig. 3, A and B). In Fig. 3, C and D, the symbols and error bars demonstrate (from left to right) the initial rise, the half-maximum, the peak, the start of the recovery, the half-recovery, and the full recovery of the 19 mM Na$^+$ responses. (In Fig. 3, C and D, the lines are only provided to demonstrate the general shape of the response and do not represent the average response of all of the cells in that treatment group.) Figure 3E shows that NCX inhibition alone is not sufficient to increase basal $\text{Ca}^{2+}\text{;}_i$ in diabetic dyslipidemia. This indicates that the NCX alone does not regulate basal $\text{Ca}^{2+}\text{;}_i$ and that either the SERCA and/or the PMCA can compensate for NCX inhibition. In the exercise-trained group, the decreased change in the fura 2 response
when only the NCX is inhibited (Fig. 3E), together with the exercise-induced improvement of total Ca\(^{2+}\) extrusion, is consistent with increased Ca\(^{2+}\) extrusion via the PMCA in exercise-trained pigs. On inhibition of the SERCA and the NCX, Ca\(^{2+}\) buffering was slightly impaired in cells from diabetic and hyperlipidemic swine and significantly impaired in cells from diabetic dyslipidemic swine (Fig. 3F). These results implicate deficits in the PMCA as at least one early factor in the dysregulation of Ca\(^{2+}\) levels in proatherogenic states. Exercise training concurrent with diabetic dyslipidemia prevented the diabetic dyslipidemia-induced impairment in plasmalemmal Ca\(^{2+}\) efflux via the PMCA, suggesting that exercise training enhanced Ca\(^{2+}\) transport by the PMCA. Diabetes and hyperlipidemia independently had no significant effect on the
change in the Ca$^{2+}$ levels in response to the 19 mM Na$^+$ solution.

**Exercise Prevents the Diabetic Dyslipidemia-Induced Increase in Sarcoplasmic Reticulum Ca$^{2+}$ Buffering and SERCA2b Protein Expression**

A previous study has shown that sarcoplasmic reticulum Ca$^{2+}$ buffering is increased by diabetic dyslipidemia (23). To assess sarcoplasmic reticulum Ca$^{2+}$ buffering capacity in this study, we utilized fura 2 imaging and a protocol that has previously been shown in our laboratory to assess sarcoplasmic reticulum Ca$^{2+}$ uptake (23) (Fig. 4A), assuming known Ca$^{2+}$ influx. In Fig. 4B, the symbols and error bars demonstrate (from left to right) the initial rise, the half-maximum, the peak, and the start of the recovery for the 80 mM K$^+$ responses. (In Fig. 4B, the lines are only provided to demonstrate the general shape of the response and do not represent the average response of all of the cells in that treatment group.) As shown in Fig. 4C, in cells from diabetic dyslipidemic swine, the time to reach the half-maximal level in response to the second 80 mM K$^+$ solution was significantly increased compared with control. This result, using a protocol different from our laboratory’s previous studies, was consistent with those earlier findings demonstrating an increase in sarcoplasmic reticulum Ca$^{2+}$ buffering capacity with diabetic dyslipidemia (23). Since enhanced SERCA activity would be expected to lower basal Ca$^{2+}$ levels, although in diabetic dyslipidemia basal Ca$^{2+}$ is increased, this finding is consistent with the hypothesis that SERCA activity is increased to compensate, albeit inadequately, for decreased Ca$^{2+}$ extrusion and/or increased Ca$^{2+}$ influx. Exercise training completely prevented the diabetic dyslipidemia-induced increase in sarcoplasmic reticulum Ca$^{2+}$ buffering. The time to reach the half-maximal response was slightly increased in the diabetic and hyperlipidemic groups compared with control, but this was not significant.

In addition to the assessment of sarcoplasmic reticulum Ca$^{2+}$ buffering, immunoblot analysis was performed to assess SERCA2b protein expression. Previous findings in porcine coronary smooth muscle had demonstrated an increase in the protein expression of SERCA2b with diabetic dyslipidemia (23). The present study demonstrates that the rise in the protein expression of SERCA2b in diabetic dyslipidemia was prevented by exercise training (Fig. 5). There was no significant change in the protein expression of SERCA2b in the diabetic or hyperlipidemic groups.

**Fig. 4.** Exercise training prevented the diabetic dyslipidemia-induced increase in sarcoplasmic reticulum Ca$^{2+}$ buffering. A: protocol to assess total plasmalemmal Ca$^{2+}$ efflux. The tracing depicts the response of a control cell to the experimental protocol. The solid horizontal lines below the tracings indicate the solution and exposure duration. Ca$^{2+}$-free = −100 mM Ca$^{2+}$ solution; 80 mM K$^+$ = 80 mM K$^+$ solution; CAF = 5 mM caffeine. B: group averages for landmark points of the 80 mM K$^+$ responses. For all groups depicted, the average group response ± SE for the initial rise, half-maximum, peak, and start of the recovery for the 80 mM K$^+$ responses are shown as symbols with error bars. Lines are only provided to demonstrate the general shape of the response and do not represent the average response of all of the cells in that treatment group. For simplicity, only average responses are shown for the control, diabetic dyslipidemic, and diabetic dyslipidemic plus exercise-trained groups. C: 80 mM K$^+$ time to half-maximum. Data represent the time (in seconds) to reach the half-maximal response to 80 mM K$^+$-induced Ca$^{2+}$ influx. For the first 80 mM K$^+$ exposure (80K1), there was no significant difference in the time to reach the half-maximal response. The time to reach the half-maximal response for the second 80 mM K$^+$ exposure (80K2) was significantly prolonged (*P < 0.05) in cells from the DD (N = 8 pigs and 79 cells) above the C (N = 5 pigs and 109 cells), but not the D (N = 5 pigs and 54 cells) or H swine (N = 5 pigs and 71 cells). Exercise training (DDX; N = 7 pigs and 96 cells) prevented (#P < 0.05) the increase in the diabetic dyslipidemic response time.
Fig. 5. Exercise prevented the diabetic dyslipidemia-induced increase in SERCA2b protein expression. Each band represents 20 μg of sample protein. Data represent the ratio of the band densities for the diabetic (D; N = 5 pigs), hyperlipidemic (H; N = 4 pigs), diabetic dyslipidemic (DD; N = 6 pigs), and diabetic dyslipidemic plus exercise-trained (DDX; N = 7 pigs) swine to control (C; N = 6 pigs). *P < 0.05 vs. all groups.

Fig. 6. Exercise prevented the decrease in voltage-gated Ca\(^{2+}\) channel current density elicited by diabetic dyslipidemia. Top: representative Ca\(^{2+}\) current traces. Bottom: representative current-voltage relationships. For clarity, only the peak inward current for the diabetic and hyperlipidemic groups at +20 mV were plotted and not the entire current-voltage relationship. Mean current densities at −20 mV were decreased −50% in cells from diabetic (N = 5 pigs and 24 cells); hyperlipidemic (N = 4 pigs and 15 cells), and diabetic dyslipidemic swine (N = 6 pigs and 26 cells) from control (N = 6 pigs and 35 cells). Exercise training prevented the diabetic dyslipidemia-induced decrease in voltage-gated current density (N = 6 pigs and 26 cells) and restored currents to control levels. *Statistically significant difference (P < 0.05).

L-Type Voltage-Gated Ca\(^{2+}\) Channel Current Density Was Decreased by Diabetic Dyslipidemia and Exercise Prevented This Decrease

To determine whether exercise training prevented diabetic dyslipidemia-induced alterations in Ca\(^{2+}\) influx via L-type voltage-gated Ca\(^{2+}\) channels, whole cell patch-clamp analysis was performed on freshly isolated cells. Figure 6, top, shows representative 10 mM Ba\(^{2+}\) current traces from coronary smooth muscle cells elicited by test pulse to +10 mV from the holding potential of −80 mV. The magnitude of both the peak and sustained inward current was decreased in cells from the diabetic, hyperlipidemic, and diabetic dyslipidemic groups compared with control. Thus hyperglycemia and hyperlipidemia independently decrease whole cell Ca\(^{2+}\) channel currents, and these decreases were not additive, as evidenced by the similar decrease in peak current in the diabetic dyslipidemic group compared with control. In contrast, the magnitude of both the peak and sustained inward current was returned to control levels in diabetic dyslipidemic animals that were endurance exercise trained for 16–18 wk.

The effects of diabetes, hyperlipidemia, diabetic dyslipidemia, and the combined effects of diabetic dyslipidemia plus exercise training on the current vs. voltage (I–V) relationships for coronary smooth muscle cells using 10 mM Ba\(^{2+}\) as the charge carrier are shown in Fig. 6, bottom. Hyperglycemia, hyperlipidemia, and diabetic dyslipidemia significantly decreased peak voltage-gated Ca\(^{2+}\) channel current density by 50% compared with control, whereas exercise-trained diabetic dyslipidemic swine maintained peak voltage-gated Ca\(^{2+}\) channel current density at control levels. In control and exercise-trained diabetic dyslipidemic swine, inward current was significantly different from diabetic, hyperlipidemic, and diabetic dyslipidemic at −10-, −0-, +10-, +20-, and +30-mV test potentials (Fig. 6, bottom). There was no difference in steady-state inactivation of the currents nor in cell capacitance (data not shown).

DISCUSSION

The main findings from this study are in support of the overall hypothesis that aerobic exercise training prevents the diabetic dyslipidemia-induced impairment of coronary smooth muscle Ca\(^{2+}\) regulation. Using freshly isolated porcine coronary smooth muscle cells, we demonstrated that exercise training prevents diabetic dyslipidemia-induced decreased plasma-membrane Ca\(^{2+}\) influx via the PMCA, increased basal Ca\(^{2+}\) levels, increased SERCA activity and protein expression, and decreased voltage-gated Ca\(^{2+}\) channel current density (i.e., Ca\(^{2+}\) influx).

In this study, alloxan treatment elicited approximately sixfold increases in fasting blood glucose levels in all diabetic groups (i.e., the diabetic, diabetic dyslipidemic, and exercise-trained diabetic dyslipidemic groups), and these levels were tightly maintained even in the exercise group. Thus, even though exercise is widely known to increase skeletal muscle insulin sensitivity, which would act to lower blood glucose levels, close monitoring of food intake and exogenous insulin supplementation allowed for us to conclude that exercise train-
ing has effects on the vasculature that are independent of glycemic status in this study (5).

Consistent with previous findings from our laboratory (45), in this study, we observed a diabetic dyslipidemia-induced impairment in total plasmalemmal Ca\(^{2+}\) influx that was restored to control levels by exercise training (Fig. 2, B and C). Although we cannot yet completely dismiss the possible contribution of Ca\(^{2+}\) buffering via mitochondria and/or Ca\(^{2+}\)-binding proteins (e.g., calmodulin), these data, taken collectively with other findings from this paper, strongly suggest that exercise training prevents the diabetic dyslipidemia-induced impairment in Ca\(^{2+}\) regulation via enhanced PMCA activity or protein expression. Immunoblot analyses to assess the protein expression of the NCX and/or the PMCA are currently hindered by the lack of commercially available antibodies that are reactive with these proteins in porcine coronary smooth muscle.

Exercise training not only completely reversed the diabetic dyslipidemia-induced rise in basal Ca\(^{2+}\) levels but significantly lowered it compared with control, suggesting a significant beneficial effect of exercise training on impaired Ca\(^{2+}\) regulation associated with diabetes (21, 23, 28, 44). This finding is in contrast with a previous study in our laboratory demonstrating no effect of exercise on the diabetic dyslipidemia-induced rise in basal coronary smooth muscle Ca\(^{2+}\) levels (45). One possible explanation for the differences is that the swine in the present study were not allowed to gain as much weight as the swine in the previous study. In the previous study, the diabetic dyslipidemic and exercise-trained diabetic dyslipidemic swine increased their body weights by ~60% over the 20-wk treatment period (45), whereas in the current study the weight gain was limited to ~20%. It seems reasonable that this large difference in body weight hindered the ability of the swine in the previous study to fully benefit from the effects of the exercise training bouts.

In diabetic dyslipidemia, enhanced Ca\(^{2+}\) binding by SERCA has been consistently observed in smooth muscle studies performed in our laboratory and others (17, 21, 23). The findings from this study (Fig. 5) were in agreement with the previous studies. Since the diabetic dyslipidemic condition is associated with impaired plasmalemmal Ca\(^{2+}\) influx and elevated basal Ca\(^{2+}\) levels, the increase in sarcoplasmic reticulum Ca\(^{2+}\) buffering is interpreted as a compensatory alteration. The enhanced SERCA activity seen in diabetic dyslipidemia increased coronary atheroma development (30), a potential yet undefined mechanism linking Ca\(^{2+}\) dysregulation to smooth muscle cell phenotypic modulation in atherosclerosis.

In summary, the main findings from this study demonstrate that exercise training is able to prevent diabetic dyslipidemia-induced alterations in coronary smooth muscle Ca\(^{2+}\) regulation. This includes a restoration of the diabetic dyslipidemia-induced decrease in plasmalemmal Ca\(^{2+}\) influx via the PMCA, increase in SERCA activity and protein expression, and decrease in Ca\(^{2+}\) influx via L-type voltage-gated Ca\(^{2+}\) channels to control levels. In addition, exercise training significantly lowered basal Ca\(^{2+}\) levels and enhanced non-NCX-induced Ca\(^{2+}\) buffering compared with control cells, suggesting that exercise training is doing more than just preventing the diabetic dyslipidemia-induced alterations in Ca\(^{2+}\), handling.

In view of these findings, we propose a model for the diabetic dyslipidemia-induced impairment of coronary smooth muscle Ca\(^{2+}\) regulation and the mechanism by which exercise prevents the detrimental alterations. We speculate that the initial defect in Ca\(^{2+}\) regulation is an impairment in plasmalemmal Ca\(^{2+}\) efflux that then results in an increase in whole cell Ca\(^{2+}\) levels. In response to this rise, the cell activates a compensatory response that includes increasing Ca\(^{2+}\) uptake into the sarcoplasmic reticulum and decreasing Ca\(^{2+}\) entry into the cell. However, as evidenced by the increased basal Ca\(^{2+}\) levels observed in the cells from the diabetic dyslipidemic swine, the compensatory responses do not completely prevent the rise in basal Ca\(^{2+}\) levels. Exercise training concurrent with the diabetic dyslipidemic state restored most of the diabetic dyslipidemia-induced alterations in Ca\(^{2+}\), transporters to control levels, suggesting that exercise training prevents the initial defect in plasmalemmal Ca\(^{2+}\) efflux. Alternatively, since exercise training significantly lowered basal Ca\(^{2+}\) levels below control, and this was observed concurrent with enhanced Ca\(^{2+}\) buffering via non-NCX mechanisms, it suggests that exercise training may be enhancing Ca\(^{2+}\) efflux via the PMCA. Future studies should focus on elucidating which of these possibilities...
are responsible for the beneficial effects of exercise training on coronary smooth muscle Ca$^{2+}$ regulation.

ACKNOWLEDGMENTS

We thank Dr. Joseph Dixon for plasma lipid assays.

GRANTS

This work was supported by grants from the National Institutes of Health (RR-13223 and HL-62552) to M. Sturek and Fellowships from the American Heart Association to B. R. Wamhoff.

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