In vivo Ca\(^{2+}\) buffering capacity and microvascular oxygen pressures following muscle contractions in diabetic rat skeletal muscles: fiber-type specific effects

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Eshima H, Poole DC, Kano Y. In vivo Ca\(^{2+}\) buffering capacity and microvascular oxygen pressures following muscle contractions in diabetic rat skeletal muscles: Fiber-type specific effects. Am J Physiol Regul Integr Comp Physiol 309: R128–R137, 2015. First published May 6, 2015; doi:10.1152/ajpregu.00044.2015.—In Type 1 diabetes, skeletal muscle resting intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) homeostasis is impaired following muscle contractions. It is unclear to what degree this behavior is contingent upon fiber type and muscle oxygenation conditions. We tested the hypotheses that: 1) the rise in resting [Ca\(^{2+}\)]\(_i\) evident in diabetic rat slow-twitch (type I) muscle would be exacerbated in fast-twitch (type II) muscle following contraction; and 2) these elevated [Ca\(^{2+}\)]\(_i\) levels would relate to derangement of microvascular partial pressure of oxygen (PmvO\(_2\)) rather than sarcoplasmic reticulum dysfunction per se. Adult male Wistar rats were divided randomly into diabetic (DIA: streptozotocin ip) and healthy (CONT) groups. Four weeks later extensor digitorum longus (EDL, predominately type II fibers) and soleus (SOL, predominately type I fibers) muscle contractions were elicited by continuous electrical stimulation (120 s, 100 Hz). Ca\(^{2+}\) imaging was achieved using fura 2-AM in vivo (i.e., circulation intact). DIA increased fatigability in EDL (P < 0.05) but not SOL. In recovery, SOL [Ca\(^{2+}\)]\(_i\) either returned to its resting baseline within 150 s (CONT 1.00 ± 0.02 at 600 s) or was not elevated in recovery at all (DIA 1.03 ± 0.02 at 600 s, P > 0.05). In recovery, EDL CONT [Ca\(^{2+}\)]\(_i\) also decreased to values not different from baseline (1.06 ± 0.01, P > 0.05) at 600 s. In marked contrast, EDL DIA [Ca\(^{2+}\)]\(_i\) remained elevated for the entire recovery period (i.e., 1.23 ± 0.03 at 600 s, P < 0.05). However, PmvO\(_2\) recovery kinetics were markedly slowed in EDL such that mean PmvO\(_2\) was returned to its resting baseline within 150 s (CONT 1.06 ± 0.03 at 600 s, P > 0.05). Unfortunately, that preparation did not permit resolution of between-fiber-type effects on the profile of [Ca\(^{2+}\)]\(_i\) following contractions (7, 34), and it is therefore possible that the impaired SR-Ca\(^{2+}\) handling in these fibers in diabetes is related to their PmvO\(_2\) profile. Specifically, irrespective of SERCA protein levels and activity, if low O\(_2\) availability compromises SR Ca\(^{2+}\) sequestration via, for example, reduced ATP availability, [Ca\(^{2+}\)]\(_i\) may rise.

Recently, we succeeded in measuring in vivo [Ca\(^{2+}\)]\(_i\) within the mixed-fiber-type rat spinotrapezius muscle (13) following contractions (49, 50). In this preparation, intramyocyte injection of a high Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) bolus revealed a depressed Ca\(^{2+}\)-buffering capability in diabetes that corresponded with the elevated postcontraction [Ca\(^{2+}\)]\(_i\) (17). Unfortunately, that preparation did not permit resolution of between-fiber-type effects on the profile of [Ca\(^{2+}\)]\(_i\) following contraction in Type 1 diabetes, which remain to be resolved.

The greater physiological fragility of fast-twitch fibers under atrophic conditions such as diabetes may relate, in part, to impaired microvascular structure (47) and hemodynamics (26). These effects impact the fine balance of O\(_2\) delivery-to-O\(_2\) utilization, at least within the spinotrapezius muscle, such that very low PmvO\(_2\) values are evident at rest and both during and following contractions (6, 37, 38). It is conceivable that the ~60% fast-twitch fibers that comprise the spinotrapezius (13) are driving these aberrant PmvO\(_2\) profiles.

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The rate of skeletal muscle contraction-relaxation cycling depends on the capacity for Ca\(^{2+}\) release-reuptake of the sarcoplasmic reticulum (SR). In particular, sequestration of Ca\(^{2+}\) from the cytoplasm into the SR via the SR Ca\(^{2+}\)-ATPase (SERCA) permits muscle relaxation (3, 15, 33). There is no indication of impaired SR Ca\(^{2+}\) sequestration capacity in fast-twitch muscle in streptozotocin (STZ) diabetic rats. Indeed, calmodulin, sarcolemmal Ca\(^{2+}\) channel numbers, and SR Ca\(^{2+}\) transport activity are all increased (19, 28, 35, 52, 53). Slow-twitch muscle from STZ rats also demonstrates an absence of reduced SR Ca\(^{2+}\) sequestration capacity (20, 43). If SR Ca\(^{2+}\) handling in diabetes is not impaired, the mechanistic bases for diabetes-induced [Ca\(^{2+}\)]\(_i\) dysregulation must reside elsewhere. Diabetes induces fiber-type-specific effects predominantly targeting fast-twitch fibers/muscles (55). These fibers/muscles are characterized by a very different microvascular partial pressure of oxygen (PmvO\(_2\)) than their slow-twitch counterparts both during and following contractions (7, 34), and it is therefore possible that the impaired SR-Ca\(^{2+}\) handling in these fibers in diabetes is related to their PmvO\(_2\) profile. Specifically, irrespective of SERCA protein levels and activity, if low O\(_2\) availability compromises SR Ca\(^{2+}\) sequestration via, for example, reduced ATP availability, [Ca\(^{2+}\)]\(_i\) may rise.

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Precipitated on the evidence presented above that SERCA activity is preserved/increased in isolated intact (or skinned) single muscle fibers in diabetes, we rationalized that impaired [Ca\(^{2+}\)]\(_i\) homeostasis may relate to impaired microcirculatory hemodynamics (i.e., oxygenation, PmvO\(_2\)) during/following contractions. To address this question, it is crucial to maintain essential tenets of physiological function, including blood flow, O\(_2\) supply, and...
ATP utilization, and assess the predations of diabetes in muscles comprised of disparate fiber types (i.e., slow-twitch soleus vs. fast-twitch extensor digitorum longus). Specifically, we tested the hypotheses that following fatiguing tetanic contractions in diabetics: 1) any rise in resting $[Ca^{2+}]_i$, evident in slow-twitch (type I) would be exacerbated in fast-twitch (type II) muscle and 2) these elevated $[Ca^{2+}]_i$ levels would relate to derangement of PmvO$_2$ rather than SR dysfunction per se.

**METHODS**

**Animals**

Male Wistar rats (10 wk of age; Japan SLC, Shizuoka, Japan) were used in this study. Rats were maintained on a 12:12-h light-dark cycle and received food and water ad libitum. Rats were rendered diabetic by intraperitoneal injection of STZ, as described previously (17). Experiments were conducted 4 wk after confirmation of the diabetic state ($>300$ mg/dl, blood glucose). All experiments were conducted under the guidelines established by the Physiological Society of Japan and were approved by the University of Electro-Communications Institutional Animal Care and Use Committee. The rats were anesthetized using pentobarbital sodium ($60$ mg/kg ip), and supplemental doses of anesthesia were administered as needed. At the end of experimental protocols, animals were killed by pentobarbital sodium overdose.

**Muscle Preparation**

For all experimental techniques, the extensor digitorum longus (EDL) and soleus (SOL) muscles were used in this study. The EDL is composed primarily of fast-twitch fibers, whereas the SOL is composed of slow-twitch fibers (13). Each muscle was resected at the distal tendon and gently exteriorized with minimal blood loss and tissue/microcirculatory damage. The exposed muscle tissue was kept moist by superfusing with warmed Krebs-Henseleit buffer (KHB; in mM: $132$ NaCl, $4.7$ KCl, $21.8$ NaHCO$_3$, $2$ MgSO$_4$, and $2$ CaCl$_2$) equilibrated with $95\%$ N$_2-5\%$ CO$_2$ and adjusted to pH 7.4, at $37^\circ$C.

**Experimental Protocols**

**Intracellular $Ca^{2+}$ measurement.** Intracellular $Ca^{2+}$ measurements were performed, as described previously (17). The fluorescent $Ca^{2+}$ indicator fura 2-AM (5 mM; Dojindo Laboratories, Kumamoto, Japan) was dissolved in DMSO and Pluronic F-127 and dispersed into KHB solution at a final concentration of $40$ $\mu$M. The muscles were incubated in fura 2-AM/KHB solution for $60$ min on a $37^\circ$C hotplate. After incubation, muscles were rinsed with dye-free KHB solution to remove nonloaded fura 2.

As shown in Fig. 1, the muscles loaded with fura 2-AM were mounted on the $37^\circ$C glass hotplate (Kitazato Supply, Shizuoka, Japan) and observed by fluorescence microscopy using a $\times 4$ objective lens (0.20 numerical aperture; Nikon, Tokyo, Japan). Thereafter, 340- and 380-nm wavelength excitation light was delivered using a Xenon lamp equipped with appropriate fluorescent filters, and pairs of fluorescence images were captured through the 510-nm emission wavelength filter for ratiometry. Fluorescence images were captured by a high-sensitivity charge-coupled device digital camera (ORCA-Flash2.8; Hamamatsu Photonics, Hamamatsu, Japan) using image-capture software (NIS-Elements Advanced Research; Nikon). The scan requirements were set at 0.5–1 s/image, a $1,750 \times 1,310$-$\mu$m ($1.82$ pixel/$\mu$m) imaging field of view. This results in a time frame between 1 and 2 s. The fluorescence intensity of serial ratio images was normalized to the starting point (i.e., precondition, $R_0$) of each experiment ($R/R_0$). Pilot studies confirmed that the $340$-to-$380$-nm ratio value (i.e., resting $[Ca^{2+}]_i$) was unchanged over the $90$-min observation period. This contrasted with the in vitro state

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**Fig. 1.** Schematic showing direct microscopic observation for extensor digitorum longus (EDL) and soleus (SOL) muscles and intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) in vivo. The left rat hindlimb with anterior and posterior crural compartments exposed. The foot was fixed on plate. Knee and ankle angles were kept at $90^\circ$, and the lower leg was held horizontal. Tendons of SOL and EDL muscles were connected to a force transducer. Cuff electrodes were mounted on the sciatic and peroneal nerves. Fluorescence images were captured from same area of muscle tissue. The ratio ($R$: ratio of fluorescence at $340$ to $380$ nm) image was converted, and the mean gray-scale value was measured. CCD, charge-coupled device; TA, tibialis anterior muscle; GA, gastrocnemius muscle.
surgically isolated) where \([\text{Ca}^{2+}]_i\) progressively increased in EDL and SOL muscles (\(n = 5–7\)).

**Microvascular PO2 measurement.** Before the muscle preparation, the carotid artery was isolated and, with the use of an introducer, cannulated with a 22-G needle to provide a route of access for infusion of the phosphorescent probe [palladium mesotetra (4-carboxyphenyl)porphine dendrimer (R2); 15 mg/kg] and blood pressure measurement. The phosphor R2 was infused 15 min before the experimental procedure, and all experiments were conducted in a darkened room to prevent contamination from ambient light. PmvO2 was determined at 1-s intervals at rest, during contractions, and for a few minutes of subsequent recovery.

The theoretical basis for phosphorescence quenching has been detailed previously (46). Briefly, the Stern-Volmer relationship (46) describes quantitatively the \(O_2\) dependence of the phosphorescent probe. R2 is a nontoxic dendrimer (27) that binds completely to albumin at 38°C and pH 7.4, with a quenching constant of 409 Torr\(^{-1}\)·s\(^{-1}\) and lifetime decay in the absence of \(O_2\) of 601 \(\mu\)s (31, 40).

In addition to binding with albumin, the net negative charge of R2 also facilitates restriction of the compound to the vascular space (41). To determine PmvO2, a PMOD 2000 frequency domain phosphorometer (Oxygen Enterprises, Philadelphia, PA) was used. The common end of the bifurcated light guide was placed 2–3 mm above the medial portion of either muscle. The excitation light was focused on a 2-mm-diameter circle of exposed muscle surface and sampled blood within the microvasculature up to 500 \(\mu\)m deep.

**Modeling of PmvO2 Profiles**

Curve fitting was accomplished using KaleidaGraph software (version 4.1; Synergy software, Reading, PA) and was performed on the PmvO2 data using appropriate exponential models:

<table>
<thead>
<tr>
<th>Table 1. Body weight, blood glucose, and muscle-to-body weight ratio of EDL and SOL muscle in control and diabetic rats</th>
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<tr>
<td><strong>Body Weight, g</strong></td>
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Values are expressed as means ± SE. EDL, extensor digitorum longus; SOL, soleus; CONT, control; DIA, diabetic. **Significant difference between CONT and DIA (\(P < 0.01\)).
PmvO₂(t) = PmvO₂(0) + ΔPmvO₂[1 - e^(-((t/TD)+τ))]

where PmvO₂(t) is PmvO₂ at a given time point (t), PmvO₂(0) is baseline PmvO₂ (i.e., precontraction), ΔPmvO₂ is the decrease in PmvO₂ from baseline to the end-stimulation values, TD is time delay, η is the exponential function, and t is the time constant.

To determine whether the one component would best describe the PmvO₂ response for either muscle, the goodness-of-fit for the model was determined via three criteria: 1) the coefficient of determination (i.e., r²), 2) the sum of the squared residuals term (i.e., χ²), and 3) visual inspection of the model fit to the data. Mean PmvO₂ was determined by integration for 300 s from the end of tetanic contractions (i.e., off-transient).

Stimulation

For the contraction protocols, cuff electrodes were mounted on the sciatic and peroneal nerves. After a 10- to 15-min postsurgery stabilization period, tetanic muscle contractions (100 Hz frequency, 4–8 V, no interval) were elicited by electrical stimulation (SEN-8203; Nihon Kohden, Tokyo, Japan) in either the EDL or SOL muscle (in random order) for 2 min stimulation. This contraction paradigm was designed to produce maximum force in both muscles and significant fatigue and also simulate how muscle(s) might contract isometrically under physiological conditions. Specifically, this protocol may be likened to: 1) the forearm muscles when a person grips the handles of a suitcase while they walk across an airport or to catch a train or bus; 2) the calf muscles when standing on tip toe in a crowded to get a better view of a sporting event, or 3) the thigh muscles when remaining crouched while downhill skiing. After the stimulation period, there was a stimulation-free recovery period for 10–20 min for each muscle. To measure force, the distal tendon of EDL and SOL muscles was connected by fine wire to a strain gauge. Torque was monitored by computer using Mac Lab/8s (AD Instruments, Colorado Springs, CO) via a strain gauge-linked motor device (0–10 mN/m, full-scale deflection, model no. RU-72; NEC Medical Systems, Tokyo, Japan) during all contraction protocols. The muscle force during electrical stimulation was resolved as active force and plotted graphically as an index of fatigue over the 2-min contraction period.

Acute Ischemia Experiment

EDL ischemia (n = 4) was induced by occlusion of the exposed femoral artery with silk surgical suture (Natsume Seisakusho, Tokyo, Japan) immediately following muscle contractions in healthy EDL. [Ca²⁺], was monitored during the ischemic episode. Subsequently, we confirmed complete blockade of flow in the arterioles, venules, and capillaries of the muscle and flow recovery following ischemia by removing the ligation under bright-field microscopy.

Histology

At the end of the experiments, EDL and SOL muscles were resected under anesthesia. The tissue blocks were frozen rapidly in isopentane cooled in liquid nitrogen. Serial 10-μm sections were made with a cryostat (CM1510; Leica) at −20°C and mounted on polylysine-coated slides. Whole sections were stained for hematoxylin and eosin, succinate dehydrogenase (SDH), and slow and fast myosin

Fig. 3. Expression of SR Ca²⁺-ATPase (SERCA) 1, SERCA2, and ryanodine receptor (RyR) in EDL and SOL muscles from CONT (open bar) and DIA (filled bar) rats. Representative Western blot showing SERCA1 protein (A), SERCA2 protein (B), and RyR protein (C). Significant difference between CONT and DIA (*P < 0.05 and **P < 0.01). Values shown are means ± SE (n = 5–8).
heavy chain (MHC). The SDH activities in individual muscle fibers in histological sections were examined and analyzed as described previously (17). Mouse monoclonal slow MHC antibody (diluted 1:40 in PBS) supplied by Novocastra Laboratories (Leica Biosystems) was used to identify slow-twitch fibers. Mouse fast MHC monoclonal antibody isoforms were used, which specifically react with type IIA (1:1,000; SC-71) and IIB (1:100; BF-F3). These were supplied by Developmental Studies Hybridoma Bank (University of Iowa). The sections were allowed to warm to room temperature and incubated in PBS (pH 7.5) at 25°C before further incubation with the primary antibody in a humidified box overnight at 4°C. Vectastain ABC kit (Vector Laboratories, Funakoshi, Japan) was used to reveal the immunohistochemical reaction according to the manufacturer’s instructions. The cross-sectional areas and SDH activities were measured by tracing fiber outlines of ~220 fibers from the muscle sections. The images were digitized as gray-level pictures. Each pixel was quantified as one of 256 gray levels and then automatically converted to optical density using ImageJ software.

Western Blotting Analysis

For SERCA1, SERCA2, and ryanodine receptor (RyR) protein analysis, dissected EDL and SOL muscles were homogenized and analyzed as described previously (17).

Statistical Analysis

Values are expressed as means ± SE. Statistical analyses were performed in Prism version 5.0 (GraphPad Software, San Diego, CA). Two-way repeated-measures ANOVA were used to identify group differences in [Ca$^{2+}$], levels and muscle force. Bonferroni-adjusted post hoc tests were performed to compare control (CONT) and diabetic (DIA) at each time point. Unpaired t-tests (2-tailed) were used for physical characteristics, relative protein levels, histological data, and modeled parameters for Pmvo$_2$. The level of significance was set at $P < 0.05$.

RESULTS

Body Mass, Muscle Morphology, Fiber Type Characteristics

DIA rats evidenced significantly lower body mass and reduced muscle weight-to-body weight ratio for the EDL but not the SOL (Table 1). Fiber-type composition was unchanged in DIA SOL, whereas in DIA EDL there was an increased percentage of IIx fibers at the expense of IIb, and all type II fibers (a/x/b) were substantially atrophied (Fig. 2).

Oxidative Capacity and SR Ca$^{2+}$ Regulatory Proteins

There was a modest reduction in oxidative capacity (SDH activity) in DIA SOL type I and IIa fibers (Fig. 2F). Both SERCA1 and SERCA2 protein levels increased in EDL but remained either unchanged or decreased in SOL (Fig. 3). RyR protein was decreased 40% in EDL and unchanged in SOL.

Muscle Fatigue

There were significant differences in maximal isometric force between the SOL (CONT 719 ± 109 mN, DIA 456 ± 36 mN, $P < 0.05$) and EDL (CONT 1,436 ± 158 mN, DIA 895 ± 151 mN, $P < 0.05$). During the 120-s tetanic contraction, all muscles exhibited a pronounced fatigue as evident from the precipitous decrease in force to ~10% initial in SOL and ~5% in EDL (Fig. 4). Whereas the force/fatigue profile in SOL was unchanged by the DIA condition, the DIA EDL fatigued far faster than its healthy counterpart.

Intracytoplasmic Calcium

The photomicrographs (Fig. 5) and temporal profiles (Fig. 6) capture the dynamics of in vivo [Ca$^{2+}$], at resting baseline and following the 120-s tetanic muscle contraction. In CONT EDL and SOL muscles [Ca$^{2+}$], decreased to baseline levels in 180–210 s and remained there throughout the rest of the 10-min recovery period. This was also the case for DIA SOL. However, in marked contrast, [Ca$^{2+}$] in DIA EDL did not decrease but rather increased during so-called recovery to a peak of 1.23 ± 0.03 at 600 s as seen clearly in Fig. 5, bottom and Fig. 6A.

Pmvo$_2$ Dynamics

Surprisingly, DIA increased baseline Pmvo$_2$ before contractions (Table 2 and Fig. 7). However, during the tetanic contraction, the rate and extent ($\Delta$) of Pmvo$_2$ decrease was accelerated in both SOL and EDL, whereas following contractions the rate of Pmvo$_2$ recovery slowed drastically. The consequences of this slowed recovery differed substantially for the EDL vs. SOL. Specifically, over the first 300 s postcontraction, this phenomenon reduced the mean Pmvo$_2$ from 27.9 ± 2.0 to 18.4 ± 2.0 Torr ($P < 0.05$), whereas that for the SOL was unaffected by DIA ($P > 0.05$, Fig. 8).

Additional information regarding the potential for reduced mean Pmvo$_2$ during the first 300 s of recovery from contraction to impact [Ca$^{2+}$], (at 300 s postcontraction) was derived from the subset of healthy ischemic EDL muscles. As shown in Fig. 8, there was a significant increase in [Ca$^{2+}$], with ischemia.
(1.28 ± 0.03) compared with CONT (normal blood flow) EDL (1.05 ± 0.02). This finding substantiates a relationship between lowered PmvO2 and elevated [Ca2+]i in the absence of any protein modification driven through the chronic DIA condition.

**DISCUSSION**

In Type 1 diabetes, muscle structural and functional aberrations confound to impair contractile performance, and there is substantial evidence that [Ca2+]i dysregulation features center stage in this process. For the first time this investigation has associated fiber-type-specific dysregulation of in vivo [Ca2+]i homeostasis in diabetes with compromised muscle oxygenation following muscle contractions. Specifically, the principal original findings include:

1) diabetic fast-twitch (EDL, type II) but not slow-twitch (SOL, type I) skeletal muscles are more fatigable and, after a prolonged fatiguing tetanic contraction, EDL [Ca2+]i homeostasis cannot be recovered within a prolonged period; 2) SERCA1 and -2 protein expression is not depressed but rather elevated in the EDL in diabetes; and 3) recovery of the postexercise PmvO2 (mean PmvO2) is markedly depressed in the diabetic EDL (but not SOL). These findings together with the calcium dysregulation found in the ischemic healthy EDL support the concept that the mechanistic bases for the dysfunctional [Ca2+]i homeostasis and contractile performance in diabetic muscle may be the consequence of reduced oxygenation rather than downregulation of the SR Ca2+ uptake proteins. However, as discussed in Putative Mechanisms of Elevated [Ca2+]i below, the possibility of non-O2-related dysregulation of Ca2+ regulation cannot be disproven at this time.

**Effect of Diabetes on SR Ca2+ Uptake in Muscles of Contrasting Fiber Types**

Previous evidence demonstrates that SERCA1 is coexpressed with fast type II MHC and SERCA2 with slow type I
MHC (54). In fact, an alteration in fiber-type composition by exercise training induces the change of SERCA1 and SERCA2 proteins, respectively (18). As found herein (Figs. 2 and 3), the compelling weight of evidence from investigations in animals (and humans) supports that SERCA 1 and 2 protein expression can be controlled independently of contractile proteins and thus increase in atrophic fast-twitch muscles in Type 1 diabetes. For example, in diabetic animals, SR Ca2+ uptake is elevated in the fast-twitch hamstring muscles (19, 20, 52, 53), and both Ca2+-ATPase activity and Ca2+ uptake are higher in vastus lateralis muscle from Type 1 diabetic patients vs. matched nondiabetic controls (22).

There is also substantial support for Type 1 diabetes either lowering or not changing SERCA protein expression in slow-twitch muscles (as seen in Fig. 3). Also in the SOL of STZ diabetic rats, Racz et al. reported that SR Ca2+-ATPase protein levels declined but in the absence of MHC alterations (43). In addition, SOL SERCA protein contents decrease during regeneration from notexin-induced muscle necrosis (57). From the above it is apparent that fast-twitch muscles overall are more vulnerable to atrophy and a plenitude of dysfunctions compared with their slow-twitch counterparts (present data and Ref. 51). It is fascinating that Type 1 diabetes can evoke dramatic anabolic changes with respect to the calcium protein function while simultaneously evoking catabolism of the contractile proteins in fast-twitch muscles. Resolution of the signaling mechanisms involved and the roles of Ca2+ and oxygen dysregulation in those signaling processes will constitute an important goal in combating diabetes-induced skeletal muscle dysfunction.

Muscle Vascular Oxygenation During Contractions in Fast and Slow Muscles in Diabetes

As exemplified by animal models, the peripheral circulation is profoundly dysfunctional in diabetes (6, 25, 37, 38), raising the distinct possibility that acute and chronic diabetes-induced muscular deficits are driven, at least partially, by impaired oxygen transport. An abundance of evidence demonstrates that contractile function is exquisitely sensitive to increased or decreased blood oxygen content and muscle oxygen supply (1). Intramuscular hypoxia exacerbates intracellular perturbations (e.g., greater decrease in intracellular phosphocreatine, increase in free ADP, enhanced glycogenolysis) needed to support the energetic demand of the muscle (6, 56).
Our previous investigations have established that diabetes impairs PmvO₂ kinetics during muscle contractions in the rat spinotrapezius muscle (38). This is an important observation with respect to the present findings, in part, because disease-associated changes in the on-kinetics often presage derangements in the off-kinetics. For instance, in the spinotrapezius (mixed fiber type) of rats in congestive heart failure, a condition that impacts PmvO₂ on-kinetics (14), the off-kinetics were slowed in proportion to the degree of left ventricular dysfunction (10).

The present investigation focused on the PmvO₂ kinetics following 120 s of a fused tetanic contraction. The kinetics of PmvO₂ during/following contractions provides information regarding the efficacy of the matching between muscle O₂ supply and O₂ demand and may be, to a certain extent, contraction protocol dependent (23, 24). In the present investigation, we used a nonintermittent tetanic contraction model to highlight recovery dynamics after muscle fatigue. Hence, it is expected that the PmvO₂ kinetics after muscle contraction (off-transient) would be an insightful index of the oxygen supply itself as related to the oxygen utilization. The PmvO₂ time constant (τ) in DIA was markedly increased for both EDL and SOL, but this significantly depressed the mean PmvO₂ only in the EDL where the [Ca²⁺]i homeostasis was deranged (Table 2 and Figs. 7 and 8).

In Vivo [Ca²⁺], Homeostasis and Force Production in Diabetic Skeletal Muscle

In diabetes, fast-twitch/glycolytic MHC type II fibers (e.g., EDL) have decreased fiber cross-sectional area and impaired force production, whereas slow-twitch/oxidative/MHC type I fibers (SOL) are affected to a lesser extent or not at all (8, 11, 12, 29, 51). The rise in resting [Ca²⁺]i following muscle contractions found in the present investigation has also been observed in isolated toad and mouse single muscle fibers from healthy animals (9, 44) and is a typical feature of severely fatigued muscle. It was especially interesting that it occurred in the present investigation only in the fast-twitch EDL of diabetic animals, and it did so in the face of increased SERCA protein expression (Fig. 3). The present findings support the possibility that the diabetes-induced peripheral circulatory dysfunction (26), and consequent reduction of recovery PmvO₂ in EDL (Fig. 7), rendered the enhanced SR proteins ineffective.

Mitochondria represent another putative source of Ca²⁺ uptake that must be considered. Mitochondrial [Ca²⁺] is increased within a few milliseconds of the cytosolic [Ca²⁺] rise for both twitch and sustained tetanic contraction in vivo (42, 45). This process can be compromised by loss of mitochondrial connectivity, at least in skeletal myotubes (16). Further evidence supporting a crucial role for mitochondrial Ca²⁺ buff-
ering emerges from mitochondrial Ca\(^{2+}\) uniporter expression knockout mice (39). Muscles from these animals have impaired isometric contraction force in the absence of detectable changes in skeletal muscle composition. It is clear that both mitochondrial Ca\(^{2+}\) permeability and Ca\(^{2+}\) handling are deregulated in fast-twitch muscles in diabetic rats (32). In the present investigation, the primary evidence for mitochondrial changes with diabetes was found in the SOL where a substantial decrease of SDH activity occurred (Fig. 2). What was not appreciated from this analysis, however, is that significant atrophy occurred in the EDL fast-twitch fibers (~40–60%). Had the mitochondrial reticulum remained intact throughout this process, a proportional increase in SDH activity (and therefore staining) would have been expected. That this did not happen provides direct evidence that mitochondrial absorption/impaired oxidative capacity occurred consequent to diabetes. It is quite likely that this process was associated with functional damage to the mitochondrial Ca\(^{2+}\) buffer capacity, although this hypothesis remains to be tested.

Relevant Mechanisms for the Elevated [Ca\(^{2+}\)]. Found in DIA EDL After Contraction

The mechanisms involve the following processes: 1) reduced PmvO\(_2\) consequent to STZ-DIA or ischemia in healthy EDL muscle are both associated with elevated [Ca\(^{2+}\)]. However, the possibilities that a) elevated [Ca\(^{2+}\)], if it occurs initially in artiolar smooth muscle, may precipitate vasoconstriction and thus the reduction of PmvO\(_2\), and b) reduced PmvO\(_2\) in-and-of itself, may cause dysfunction of SERCA and/or RyR must be considered; 2) SERCA1 and SERCA2 protein levels may increase in EDL, but this gives little indication of their activity per se; 3) RyR (2, 48) may become leaky in STZ diabetes; and 4) mitochondrial [Ca\(^{2+}\)], uptake may be impaired (17).

It should also be acknowledged, that, insofar as processes 2–4 are ATP dependent, lowered PmvO\(_2\) might precipitate their impaired ability to regulate [Ca\(^{2+}\)], by compromising intracellular energetics.

Perspectives and Significance

In conclusion, compromised Ca\(^{2+}\) buffering and elevated [Ca\(^{2+}\)], following an in vivo fatiguing tetanic contraction occur preferentially in the fast-twitch EDL rather than the slow-twitch SOL muscle. This process is associated with a substantial reduction in microvascular oxygen pressure that may be responsible, in part, for the dysfunctional Ca\(^{2+}\) buffering and greater fatigue. If dysfunction of SERCA1 and SERCA2 does occur, it is possible that this relates to oxygen levels (PmvO\(_2\)), since their protein levels are up- and not downregulated in EDL. Because the EDL muscle fibers undergo significant diabetes-induced atrophy, it is also feasible that the mitochondrial reticulum undergoes damage, and its capacity to buffer [Ca\(^{2+}\)] is degraded. That eventuality remains to be experimentally tested.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: H.E., D.C.P., and Y.K. conception and design of research; H.E. and Y.K. performed experiments; H.E. and Y.K. analyzed data; H.E., D.C.P., and Y.K. interpreted results of experiments; H.E. prepared figures; H.E., D.C.P., and Y.K. drafted manuscript; H.E. and D.C.P. edited and revised manuscript; H.E., D.C.P., and Y.K. approved final version of manuscript.

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