No effect of NOS inhibition on skeletal muscle glucose uptake during in situ hindlimb contraction in healthy and diabetic Sprague-Dawley rats

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EXERCISE (muscle contraction) stimulates uptake of blood glucose into skeletal muscle fibers (41), in part, by inducing an increase in glucose transporter 4 (GLUT4) translocation (26, 40). Importantly, this process is normal in skeletal muscle of insulin-resistant obese Zucker rats (5) and in individuals with Type 2 diabetes (T2D) (28). Indeed, during an acute bout of intense cycling exercise, the elevated plasma glucose levels of individuals with T2D are reduced to normal levels (38). This suggests that individuals with insulin resistance/T2D have the functional machinery to increase glucose uptake during muscle contractions despite an impairment in insulin signaling (59). Ironically, however, many studies investigating new potential pharmaceutical therapies for diabetic individuals and studying the mechanism(s) of contraction-stimulated skeletal muscle glucose uptake employ healthy nondiabetic rodents or humans in their experiments.

Nitric oxide (NO) appears to play an important role in regulating skeletal muscle glucose uptake during contraction and exercise (34, 41). We and others have demonstrated that NO is involved in muscle glucose uptake during ex vivo contraction of mouse muscles (35, 36), during and after in situ muscle contractions (1, 44), during in vivo rat treadmill exercise (43), and during exercise in humans (4, 28). However, some studies show no effect of NO synthase (NOS) inhibition on contraction-stimulated skeletal muscle glucose uptake in rodents (12, 17, 24, 45). Various methodological differences may explain these conflicting results, in particular, that glucose uptake was measured sometime after contraction was completed rather than during contraction in these ex vivo studies (see review in Ref. 33 for a full discussion of these differences). Interestingly, local infusion (femoral artery) of a NOS inhibitor during cycling exercise led to a greater attenuation of the increase in leg glucose uptake in individuals with T2D compared with controls (~75% vs. ~30%, respectively) (28). Thus, this suggests that individuals with T2D have a greater reliance on NO-mediated muscle glucose uptake during exercise, revealing a potential therapeutic target to aid glycemic control in individuals with T2D. It is important to note that the attenuation of glucose uptake occurred independently of femoral artery blood flow (FBF), suggesting a direct intramuscular effect of NO on glucose uptake (28). The intramuscular effects were, however, not investigated as muscle biopsies were not obtained during the experiment. Also, it was not examined whether exercise increases NOS activity to a greater extent in skeletal muscle of individuals with T2D, which could explain the observed greater effect of NOS inhibition on glucose uptake during exercise in individuals with T2D.

Exercise (muscle contraction) increases blood flow to the contracting muscle and can also increase muscle capillary recruitment (7), which contributes to the increase in muscle glucose uptake (8, 41). This capillary response during contraction has been shown to be normal in insulin-resistant rats (49). However, diabetic individuals with microvascular complications have impaired capillary recruitment during hand-grip exercise (58). Although we have previously demonstrated that local NOS inhibition does not affect muscle capillary recruitment during contraction in healthy rats (44), it remains un-
known whether it reduces capillary recruitment during contraction in diabetic rats.

Therefore, the purpose of this study was to examine whether local NOS inhibition results in a greater attenuation of the increase in contraction-stimulated muscle glucose uptake in T2D rats compared with healthy controls, as we have previously shown in individuals with T2D (28). We hypothesized that local NOS inhibition would cause greater attenuation of the increase in muscle glucose uptake during contraction in T2D rats compared with healthy controls and that this effect would be due to intramuscular signaling alterations rather than changes in capillary blood flow.

**MATERIALS AND METHODS**

**Animals.** All procedures were approved by The University of Tasmania Animal Ethics Committee and conformed to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004, 7th ed.). Male Sprague-Dawley rats were obtained from either Monash Animal Services or The University of Tasmania Animal House. Rats were housed in plastic rat cages with 3–5 rats per cage. Rats were maintained at constant temperature of 21 ± 1°C and on a 12:12-h light-dark cycle. Dietary manipulation was started at 6 wk of age, and experimentation occurred at 10 wk of age.

**Diet manipulations and streptozotocin injections.** Prior to diet manipulation, rats were allowed ad libitum access to standard rat chow (meat-free rat and mouse diet, 4.8% fat wt/wt; Specialty Feeds, Glen Forest, Western Australia, Australia) and water. A modified protocol from Zhang et al. (60) was followed to generate a cohort of T2D rats. Briefly, at 6 wk of age, rats were randomly divided into two groups: control (control) and high-fat diet with streptozotocin (T2D). The control group continued on the standard chow, while the T2D group received a high-fat diet (SF01-028, Specialty Feeds, 23% fat wt/wt, 43% kcal fat) for 4 wk. Two weeks into high-fat diet feeding, T2D rats were injected intraperitoneally with a low dose of streptozotocin (35 mg/kg) in citrate buffer (pH 4.4), and a second, equivalent dose was administered 3 days later. Control rats were injected with vehicle citrate buffer. Both groups continued on their respective diets until experimentation. This level of high-fat diet feeding causes insulin resistance, possibly due to impairment in insulin receptor substrate-1-associated phosphoinositide 3-kinase activity (62), but not frank hyperglycemia or the diabetic state in animals (29, 51, 52, 61). Multiple low-dose STZ injections induce partial pancreatic β-cell destruction (27), leading to decreased insulin secretion and subsequent hyperglycemia, which mimics the development of a T2D diabetic state (13). These rats have previously been shown to display increases in IL-6 and TNF-α, as typically observed in insulin-resistant and diabetic states (39). This is in contrast to high-dose STZ (~60 mg/kg), which causes massive pancreatic destruction, leading to severe reduction in plasma insulin and resulting in severe hyperglycemia and, therefore, Type 1 diabetes (6, 10). The dose of STZ (35 mg/kg) used in this study has been shown to cause T2D only if in conjunction with high-fat diet-induced insulin resistance but not in Chow-fed rats (48). This resembles the situations in humans in which people with obesity and insulin resistance are predisposed to T2D development (48). Furthermore, Sprague-Dawley rats, in contrast to the inbred spontaneous T2D rodents, are highly heterogeneous in a genetic background (47), making it a better representation of the human situation (i.e., interaction of environmental factors with diverse genetic predispositions). Nonfasting plasma samples were collected for measurement of plasma glucose and insulin levels prior to the contraction experiments.

**Surgical procedure.** The surgical procedures have been described previously (44, 49). Briefly, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg body wt; Ilum, Troy Laboratory, Australia). Body temperature was maintained throughout the surgery and experiment at 37°C using a water-jacketed platform and a heating lamp. A tracheotomy tube was inserted to facilitate respiration. Jugular veins were cannulated (PE-60; Microtube Extrusions, North Rocks, NSW, Australia) for infusion of solutions. Continuous anesthesia was maintained by infusion of pentobarbital sodium (0.6 mg·min⁻¹·kg⁻¹ body wt) via one of the jugular veins. A carotid artery was cannulated for arterial blood pressure and heart rate monitoring (Transpac IV; Abbott Critical Systems, Morgan Hill, CA), as well as for arterial blood sampling. A small incision (~1.5 cm) was made in the overlying skin to expose both femoral arteries to allow for measurement of FBF using ultrasonic probes (VB series 0.5 mm; Transonic Systems, Ithaca, NY) placed around the exposed femoral arteries. The probes were connected to a flow meter (model T106 ultrasonic volume flow meter; Transonic Systems) interfaced with a computer. Blood pressure, heart rate, and FBF were recorded using Windaq Data Acquisition software (DATAQ Instruments, Akron, OH). The epigastric artery in the stimulated (contracted) leg was also cannulated with an insulin needle (30 gauge) connected to PE-30 tubing for local (retrorgrade) infusion of Nω-nitro-L-arginine methyl ester (L-NAME) directly into the femoral artery.

**Experimental protocols.** The experimental protocols were as previously described (Fig. 1) (44). Hindlimb muscle contractions were achieved by attaching electrodes at the knee and the Achilles tendon of the contracting leg, while the contralateral limb served as a resting control. There were two protocols in the experiments. *Protocol A* was designed to assess the effect of contraction on FBF and force development, and *protocol B* was for measurement of the effect of contraction on capillary recruitment and capillary blood flow in the

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**Fig. 1.** Protocol for in situ rat hindlimb muscle contraction. Twitch contractions were elicited by electrical stimulation (2 Hz, 0.1 ms, 35 V). MB, microbubbles; P, pulsing interval; 2DG, [1-14C]-2-deoxyglucose; L-NAME: Nω-nitro-L-arginine methyl ester; #Continuous withdrawal (30 µl/min) from the carotid artery. *Arterial blood sampling for blood glucose or insulin levels. Capillary recruitment measurements were done immediately before contraction, during contraction, and during contraction with saline or L-NAME infusion; requiring MB infusion over the durations, as indicated. Microbubbles were only infused in protocol B as described in the METHODS.
muscle using contrast-enhanced ultrasound (CEU). It was necessary to do these as two separate experiments because microbubbles interfere with the Doppler signal of the transonic flow probe; therefore, it is not possible to measure FBF during microbubble infusion. In both protocols, twitch contractions were elicited with 0.1-ms pulses at 2 Hz and 35 V at t = 0 min (Fig. 1) (Grass SD9 Stimulator; Grass Products, Natus Neurology, Middleton, WI). To measure force of contraction, the contracted lig was secured in place at the knee joint, preventing lower limb movement. An isometric force transducer (Harvard Apparatus, Holliston, MA) was attached at the Achilles tendon-ankle joint with a hook, which effectively fixed the ankle joint in place at the same horizontal plane as the knee. During the contraction, no movement at the knee or ankle joints was observed. Contraction force was acquired using Windaq Data Acquisition software. At t = 10 min, local L-NAME infusion was commenced via the epigastric artery of the contracting lig to achieve 5-μM concentration based on the concentration of infused, rate of infusion, and FBF, as per our previous study (44). Infusion of L-NAME was started after 10 min of muscle contractions to maximize the exposure of L-NAME to all vessels involved in exercise hyperemia, which matches the protocol that we have used in human NOS inhibition studies (4, 28). In addition, the contraction-induced femoral blood flow prior to L-NAME infusion is required to calculate the L-NAME infusion rate to achieve the required 5-μM concentration. A bolus of [1-14C]2-deoxyglucose ([14C]2-DG) (20 μCi; American Radiolabeled Chemicals, St. Louis, MO) was injected into the jugular vein at t = 20 min to assess muscle-specific glucose uptake over the final 10 min of muscle contraction. Arterial blood samples were taken as indicated in Fig. 1. In protocol A, FBF was recorded throughout the experiment. These data were later used to guide the adjustment of the infusion rate of L-NAME in protocol B to maintain 5 μM in the hindlimb. In protocol B, capillary blood flow was determined using CEU with microbubble infusion. Microbubbles were infused as indicated in Fig. 1, and capillary blood flow was measured immediately before contraction (basal, t = −1 min), during contraction but before L-NAME infusion (t = 9 min), and during contraction with L-NAME or saline infusion (t = 29 min). Contrast-enhanced ultrasound determination was performed, as previously described (44, 49). Briefly, a linear array transducer (L9-3) interfaced with an iU22 ultrasound machine (Philips Ultrasound, Bothell, WA) was positioned over the midlevel of the lower leg muscles (gastrocnemius, plantaris, and soleus) of the contracting hindlimb. This allowed the estimation of the capillary blood flow of these muscles. Real-time imaging was performed at low-mechanical index (0.08) with the acoustic focus set at the midportion of the muscles. Gain settings were optimized and held constant throughout the experiment. Octafluoropropane-gassed microbubbles were infused through the right jugular vein at 40 μl/min. A steady state of systemic microbubble concentration was achieved before imaging. A high-energy destructive ultrasound pulse was delivered to destroy microbubbles within the volume of muscle tissues being imaged. Immediately after, data were acquired for 30 s in real time to image the replenishment of microbubbles within the muscle vasculature. Three repeated loops were performed, averaged together, and the acoustic intensity was analyzed using QLab (Philips Ultrasound, Bothell, WA). Images were background subtracted for 1.0 s frames to eliminate signal from larger blood vessels. Background subtracted acoustic intensity vs. time was plotted to allow calculation of capillary blood volume (A) and capillary flow rate (A B), according to the equation y = A(1 − e−tB), where y is acoustic intensity at a given time t.

**Muscle glucose uptake.** Muscle glucose uptake (combined gastrocnemius, plantaris, and soleus) was measured for the final 10 min of contraction following the injection of [14C]2-DG. Average plasma [14C]2-DG concentration for the calculation of muscle-specific glucose uptake was determined from arterial blood sample withdrawn continuously at 30 μl/min using an automated syringe pump. At the end of the experiment, the muscles were excised and immediately freeze-clamped. Frozen muscles were ground, and ~100 mg of muscle sample was homogenized with 1.5 ml of water. Phosphorylated [1-14C]2-DG was extracted using an anion exchange resin (AG1-X8; Bio-Rad Laboratories, Hercules, CA). Radioactivity was determined using a β counter (Tri-Carb 2300TR; Perkin Elmer, Chicago, IL). Muscle glucose uptake (Rg) was calculated as previously described (44) on the basis of phosphorylated [14C]2-DG and, therefore, a correction for extracellular space is not necessary.

**Plasma biochemistry.** Plasma glucose and lactate were determined using an automated analyzer (Model 2300 Stat plus; Yellow Springs Instruments, Yellow Springs, OH). Plasma insulin levels were determined using enzyme-linked immunosorbsent assay (Merckodia, AB, Uppsala, Sweden), as per the manufacturer’s instruction.

**Western blot analysis.** Ground frozen muscles (combined gastrocnemius, plantaris, and soleus) were homogenized with 200 times volume of solubilizing buffer (125 mM Tris·HCl [pH 6.8], 4% SDS, 10% glycerol, 10 mM EGTA, 0.1 M DTT, and 0.01% bromophenol blue), as modified from the method previously described (37). Protein concentration of homogenate was determined using the RED 660 protein assay kit (G-Biosciences, St. Louis, MO). Aliquots of whole homogenate containing 5 μg of total protein were separated on SDS-PAGE gels (Bio-Rad) and then wet transferred onto polyvinylidine fluoride (PVDF) membranes. Membranes were probed with the following primary antibodies overnight at 4°C: phospho-AMPKα Thr172 (1:1,000 anti-rabbit), AMPKα (1:1,000 anti-mouse), a-tubulin (1:1,000 anti-rabbit) (Cell Signaling Technology, Danvers, MA); neuronal NOS (1:10,000 anti-mouse) and endothelial NOS (1:1,000 anti-mouse) (BD Biosciences, San Jose, CA); GLUT4 (1:8,000 anti-rabbit) (Thermo Scientific, Rockford, IL); and nitric oxide (1:40,000 anti-rabbit) (Sigma Aldrich, St. Louis, MO). Loading control proteins were always probed using nonstripped membranes. Actin was used for all except GLUT4 where a-tubulin was used as the loading control, as actin and GLUT4 have similar molecular weights and it was not possible to probe both of these proteins without undertaking the stripping process. Following incubation with anti-mouse or anti-rabbit secondary antibodies and a series of washes in Tris-buffered saline with Tween, chemiluminescent signal was developed with enhanced chemiluminescence substrate (SuperSignal West Femto, Pierce). Blot images were taken with a charge-coupled device camera using Quantity One software (Bio-Rad). Prestained molecular weight markers on the membrane were imaged under white light source with the membrane position unchanged. When quantifying both phosphorylated and total protein expression, membranes were first probed with phosphorylation-specific primary antibody; then stripped (62.5 mM Tris·HCl pH 6.8, 2% SDS, 0.8% β-mercaptoethanol), reblocked, and reprobed with primary antibody against the total protein.

**NOS activity assay.** NOS activity was determined on the basis of conversion of radiolabeled l-arginine to radiolabeled l-citrulline, as previously described (31). NOS activity was determined from the difference between samples incubated with and without L-NAME and was expressed as picomoles of l-[14C]citrulline formed per minute per milligram of protein. This assay was validated using skeletal muscles from nNOS−/− knockout mice that had undergone treadmill exercise or contrated ex vivo. A significant increase in NOS activity during exercise or contraction ex vivo was detected in muscles of nNOS−/− mice but no increase was observed in muscles of nNOSα−/− mice (data not shown) using the same protocol to measure NOS activity as was used in this study.

**Statistical analysis.** All data are presented as means ± SE. Data were analyzed by SPSS statistical package using independent Student’s t-test (control vs. T2D), two-factor (between factors: control vs. T2D, saline vs. L-NAME) or three-factor ANOVA (between factors: control vs. T2D, saline vs. L-NAME; within factors: contraction or time). If a significant interaction was detected, specific differences between mean values were located using Fisher’s least significant difference test. The level of significance was set at P < 0.05.
Table 1. Baseline characteristics and skeletal muscle protein expression of control and T2D rats

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>T2D</th>
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<tbody>
<tr>
<td>Body weight, g</td>
<td>340 ± 5</td>
<td>304 ± 4 #</td>
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<tr>
<td>Epididymal fat, g</td>
<td>2.2 ± 0.10</td>
<td>1.7 ± 0.09 #</td>
</tr>
<tr>
<td>Epididymal fat per body weight, %</td>
<td>0.63 ± 0.03</td>
<td>0.57 ± 0.03</td>
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<tr>
<td>Plasma glucose, mmol/l</td>
<td>9.4 ± 0.1</td>
<td>16.7 ± 1.2 #</td>
</tr>
<tr>
<td>Plasma insulin, pmol/l</td>
<td>718 ± 69</td>
<td>424 ± 30 #</td>
</tr>
<tr>
<td>Total AMPKα, AU</td>
<td>0.87 ± 0.04</td>
<td>0.80 ± 0.05</td>
</tr>
<tr>
<td>eNOS, AU</td>
<td>0.97 ± 0.08</td>
<td>1.04 ± 0.05</td>
</tr>
<tr>
<td>GLUT4, AU</td>
<td>0.94 ± 0.12</td>
<td>1.00 ± 0.15</td>
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</tbody>
</table>

Values are expressed as means ± SE; n = 25 to 30 and 35 to 42 in control and Type 2 diabetes (T2D) groups, respectively; n = 8 for all protein expression data in both groups. #P < 0.05 vs. control. AU, arbitrary unit; eNOS, endothelial nitric oxide synthase.

RESULTS

Baseline characteristics of control and T2D rats. Body weight of T2D rats was significantly (P < 0.05) lower than control rats (Table 1), as has been shown previously in high fat-fed rats after low-dose STZ (56). Epididymal fat (a measure of body adiposity) was also significantly lower in T2D rats. However, when epididymal fat was normalized to body weight, it was not significantly different between the two groups (Table 1). Lean body mass data were not available for these rats. T2D rats had significantly elevated plasma glucose concentration and a significantly decreased plasma insulin concentration compared with their control counterparts (Table 1). These features indicated a partial decompensation of pancreatic capacity to secrete higher levels of insulin in response to the insulin-resistant state induced by high-fat feeding and are consistent with the manifestation of T2D and the reported data (60).

Muscle force production. The peak force normalized to muscle weight was similar between control and T2D rats (Fig. 2A). Local infusion of saline or L-NAME into the femoral circulation did not affect the force production in either group (Fig. 2A). Both groups of rats experienced a similar degree of fatigue and a similar reduction in force over time during the contractions, irrespective of the type of infusion received, such that contraction force was significantly reduced to approximately half of the peak force in all groups (Fig. 2B).

Systemic blood pressure during contraction. Systemic blood pressures at rest were not different between the experimental groups. A small (~10 mmHg) but significant increase in systemic blood pressure across all groups was observed during single hindlimb contractions (Fig. 3). Local infusion of L-NAME did not further increase systemic blood pressure, implying that no spillover of L-NAME into the systemic circulation took place (Fig. 3). Systemic blood pressure was not recorded after 20 min, as the arterial line was used for arterial blood withdrawal to estimate average plasma [14C]2-DG levels, used for the calculation of muscle glucose uptake.

Plasma lactate and insulin changes during contraction. Plasma lactate at rest was not different between control and T2D rats (data not shown). There was a small but significant increase in plasma lactate concentrations at the end of the contraction period, with no difference between the two groups and was not affected by L-NAME (data not shown). At basal state, plasma insulin concentrations were significantly lower in T2D rats. A main effect of contraction to modestly increase plasma insulin at the end of contraction with no preferential increase in insulin levels in any of the experimental groups was observed (data not shown). Our group has observed plasma insulin to increase in previous studies involving similar single hindlimb in situ contraction (unpublished observation). Likewise, a tendency of higher plasma insulin levels in exercising mice has also been reported previously (45).
Hindlimb muscle glucose uptake. Contraction greatly increased (>10-fold) hindlimb muscle (gastrocnemius, plantaris, and soleus) glucose uptake, as determined from the uptake and phosphorylation of [14C]2-DG in the muscle (Fig. 4). T2D rats had a greater (37–41%) muscle glucose uptake compared with control rats (Fig. 4). Local infusion of L-NAME did not affect muscle glucose uptake during contraction in either control or T2D groups (Fig. 4). Muscle glucose uptake of the contralateral rested leg was similar between both groups of rats with or without L-NAME infusion (Fig. 4).

Femoral and capillary blood flows. Contraction significantly increased FBF between 3.5-fold and 4.4-fold during the first 5 min, after which, femoral blood flow slowly decreased throughout the contraction and remained at 2.4- to 3.4-fold higher than basal (Fig. 5). Local L-NAME infusion into the contracting hindlimb led to a significantly greater reduction in FBF compared with saline infusion in both control and T2D rats (Fig. 5). This effect was seen within 5 min of L-NAME infusion and continued throughout the rest of the experiment. At rest, capillary blood volume (A value) (1.36 ± 0.23, 0.94 ± 0.20, 1.20 ± 0.27, 0.84 ± 0.24 for control + saline, control + L-NAME, T2D + saline, and T2D + L-NAME, respectively) and capillary flow rate (A·β) (0.14 ± 0.03, 0.14 ± 0.05, 0.18 ± 0.08, 0.08 ± 0.04 for control + saline, control + L-NAME, T2D + saline, and T2D + L-NAME, respectively) were not significantly different (P > 0.05) between experimental groups. In view of the variations in basal values between individual rats, capillary blood volume and capillary flow rate during contraction and during contraction with saline or L-NAME infusions were normalized against its basal values. Contraction significantly increased capillary blood volume (recruitment) (Fig. 6A) and capillary flow rate (Fig. 6B). These indices were similar between control and T2D rats and were not significantly affected by L-NAME infusion (Fig. 6, A and B), indicating that local NOS inhibition did not affect muscle capillary blood flow responses during contraction in both control and T2D rats.

Protein expression and phosphorylation in skeletal muscle. The expression of actin and α-tubulin proteins were not different between control and T2D rat muscles and were used as loading controls. Skeletal muscle protein expression of GLUT4 and eNOS was similar in both control and T2D rats (Table 1). A trend (P = 0.057) for a reduction (~16%) in nNOSα protein in T2D rats was observed (Fig. 7A); while a significant reduction in nNOSβ (a nNOS splice variant) protein expression (~27%) was observed in T2D rats compared with control rats (Fig. 7B). The induction of T2D in these rats did not significantly affect the expression of AMPKα (Table 1). Phosphorylation of the Thr172 site is the major AMPK-activating site. 

Fig. 4. Rest and contracted leg muscle glucose uptake in control and T2D rats with local infusion of saline or L-NAME. Values are expressed as means ± SE; n = 12–14 per group. *P < 0.05 vs. rest of the same group. ‡P < 0.05 main effect for T2D.

Fig. 5. Femoral blood flow of contracted hindlimb during contraction. Values are expressed as means ± SE; n = 8–12 per group. §P < 0.05 main effect for time. γP < 0.05 interaction between L-NAME and saline.

Fig. 6. Changes in capillary blood volume (A) and capillary flow rate (B) of contracted hindlimb during contraction and during contraction with saline or L-NAME infusion relative to resting values. Values are expressed as means ± SE; n = 5, 4, 5 and 3 for control + saline, control + L-NAME, T2D + saline and T2D + L-NAME, respectively. †P < 0.05 main effect for contraction and contraction + infusion.
(50), and our previous studies showed that AMPK activity and AMPKα Thr172 phosphorylation demonstrate an almost identical response during exercise (32, 55). Therefore, AMPKα Thr172 phosphorylation was used as an indicator of AMPK activity. Although there are other sites that are phosphorylated, the functional role of these sites is unclear (50), and we are not aware of any contraction or exercise studies that have measured AMPK phosphorylation sites other than AMPKα Thr172.

Contraction significantly increased AMPKα Thr172 phosphorylation in all groups (more than two-fold), and no difference was observed between control and T2D rats. L-NAME had no effect on AMPK Thr172 phosphorylation during contraction in either group (Fig. 8).

Skeletal muscle NOS activity. T2D rats tended to have a lower NOS activity compared with control rats ($P = 0.051$), in line with their tendency to have lower nNOSμ and nNOSβ protein expressions (Fig. 7, A and B). Unexpectedly, contraction did not increase NOS activity (Fig. 7C). L-NAME did not significantly reduce NOS activity in either control or T2D rats (Fig. 7C).

**DISCUSSION**

In this study, single hindlimb in situ contraction increased capillary recruitment and skeletal muscle glucose uptake in healthy and T2D rats. It was reported for the first time that, as in healthy rats (44), local NOS inhibition did not attenuate capillary recruitment during contraction in T2D rats. Unexpectedly, local NOS inhibition had no effect on muscle glucose uptake and skeletal muscle NOS activity during contraction in control or T2D rats. This finding does not correspond with our previous study in hooded Wistar rats, in which we showed that local NOS inhibition significantly attenuated the increase in muscle glucose uptake during contraction by ~35% (44).

The reasons for the lack of effect of NOS inhibition on glucose uptake during contraction in the present study are unclear. Skeletal muscle glucose uptake is dependent on the frequency of stimulation and force of contraction (21–23); however, these parameters were comparable in the present and our previous studies (44). Also, the same concentration of L-NAME (5 μM) was used in both studies, which had previ-
reduction in FBF during contraction following L-NAME infusion. However, consistent with our previous data in hooded NAME infusion rate could also be inadequate to bring about an further increase in systemic blood pressure induced by L-NAME during contraction in the present study, supporting that L-NAME effect was confined to the specific hindlimb. L-NAME infusion rate could also be inadequate to bring about an effect. However, consistent with our previous data in hooded Wistar rats (44), there was a small but significantly greater reduction in FBF during contraction following L-NAME infusion compared with saline infusion (with similar results in both control and T2D rats). This indicates that the dose of NOS inhibition used was sufficient to have effects, despite it having no effect on muscle glucose uptake. Similarly, the muscle forces and the rate of fatigue were comparable to our previous study (44). This suggests that the conduction of the experiments was similar between the present and our previous studies, despite different results being observed with regard to the effects of NOS inhibition on skeletal muscle glucose uptake during contraction.

Capillary blood flow, which could affect glucose delivery to the muscle and, therefore, affect muscle glucose uptake (41), can change independently of FBF (7, 54). In this study, we showed that capillary recruitment and capillary blood flow increased similarly during muscle contraction in T2D and control rats. These findings are in line with previous studies showing that insulin-resistant rats (49) and individuals with T2D without microvascular complications (58) have normal capillary recruitment and capillary blood flow in response to muscle contraction and exercise. We also found that L-NAME infusion had no effect on capillary recruitment and capillary blood flow during contraction in either group of rats, which is in line with our (44) and others’ (24) previous findings in nondiabetic rats. To the best of our knowledge, we show for the first time that L-NAME infusion has no effect on capillary recruitment and capillary blood flow during contraction in T2D rats.

Two puzzling results were found in this study in that T2D rats had higher skeletal muscle glucose uptake during contraction than controls, and there was no effect of NOS inhibition on skeletal muscle glucose uptake during contraction. By means of mass action, higher plasma glucose concentration leads to greater glucose transport across the membrane, as has been demonstrated by several studies showing higher muscle glucose uptake or glucose disappearance rates during exercise in individuals with T2D compared with healthy people (9, 15, 20, 25). We also asked whether there was a compensatory upregulation of other signaling proteins involved in contraction-mediated skeletal muscle glucose uptake. However, we did not find any difference in total AMPK, AMPKα Thr172 phosphorylation, GLUT4 content, or eNOS protein expression, and thus, we have no evidence of a compensatory reaction to explain the lack of NOS inhibition effect, or the higher skeletal muscle glucose uptake during contraction in the T2D group compared with controls. Nevertheless, it might be that the subcellular compartmentalization of proteins rather than total expression is more important in determining their role in a physiological process (16), such as muscle glucose uptake during contraction. For example, GLUT4 needs to be translocated from intracellular vesicles to the sarcolemma to facilitate glucose uptake across the membrane. Subcellular compartmentalization of GLUT4, AMPK, and eNOS was, however, not examined in this study. Phosphorylation of eNOS was not determined as skeletal muscle eNOS has been shown not to be involved in muscle NO production (18) or activation of muscle NO downstream signaling (30) during contraction.

Studies have shown that muscle contraction and exercise activate NOS in skeletal muscles and lead to an increase in NO production (2, 31, 42, 44, 53). Inhibition of NOS using pharmacological agents has been shown previously to attenuate this increase in NO activity and NO production induced by muscle contraction (35, 44). In the present study, however, skeletal muscle NOS activity was not increased in the contracted muscles of either control or T2D rats. T2D muscles tended (P = 0.051) to have overall lower NOS activity compared with controls, and this could be due to the reduced nNOS expression in these rats. It is hard to reconcile why NOS activity was not increased during muscle contraction in the present study. Nevertheless, it fits with the present observation that NOS inhibition had no effect on skeletal muscle glucose uptake during contraction, most likely because NOS/NO signaling was not increased to stimulate skeletal muscle glucose uptake during the present contraction protocol in these rats. In our previous study (44), muscle contraction was elicited by the same electrical stimulation protocol as the present study and led to a significant, albeit small, increase in NOS activity and NO production (2, 31, 42, 44). In our previous study, it was found that the NOS inhibition attenuated the increase in NOS activity and NO production. This increase in NOS activity and NO production has been shown to stimulate muscle glucose uptake during exercise in healthy and T2D individuals, and we have shown here that NOS inhibition attenuated the increase in NOS activity and NO production. This increase in NOS activity and NO production has been shown to stimulate muscle glucose uptake during exercise in healthy and T2D individuals (4, 28). The differences observed between our present and previous rat in situ contraction studies may be attributed to inadequate con-
traction stimulation and rat strain differences, as will be discussed below.

The activation of muscle NOS during contraction and exercise appears to be influenced by the contraction-exercise intensity and by the strain of rats involved. It was shown that higher exercise and stimulation intensities are required to activate NOS in mice running on a treadmill (31) and to increase NO production in electrically stimulated primary rat skeletal muscle cells (46). Nevertheless, it was previously shown that higher stimulation intensity during ex vivo contraction (200-ms trains, 100 Hz, and 10 V) did not necessarily activate NOS in muscles of Sprague-Dawley rats (12), while, lower stimulation intensity (15-ms trains, 2 Hz and 20 V) increased NOS activity in muscles of Wistar rats (53). We also observed that a similar stimulation protocol led to an increase in NOS activity in hooded Wistar rats (previous study) but not in Sprague-Dawley rats (present study). Collectively, these results suggest that there may be an interplay between contraction and exercise intensity and the strains of rats in the activation of NOS during contraction and exercise, in which Sprague-Dawley and Wistar rats may require different stimulation intensities for activation of NOS in skeletal muscle. Similar differential effects of NOS activity between these strains of rats have been previously observed in synaptic plasticity, renal function recovery following an insult, and mechanical hypersensitivity following a nerve injury with no prior information from skeletal muscles (11, 19, 57). Furthermore, insulin-stimulated glucose uptake in extensor digitorum longus muscles was found to be lower in Wistar rats compared with Sprague-Dawley rats (14). These results suggest that there may be rat strain differences, in particular, in skeletal muscle glucose metabolism, which may explain the differences in NO-mediated skeletal muscle glucose uptake that we have observed between Sprague-Dawley rats (present study) and hooded Wistar rats (44).

In conclusion, NOS inhibition did not attenuate muscle capillary recruitment and the increase in glucose uptake during contraction in both control and T2D SD rats. The inability of the contraction protocol, which increased NOS activity in hooded Wistar rats (44), to activate NOS in the Sprague-Dawley rats suggests that strain differences in the regulation of contraction-stimulated muscle glucose uptake between Sprague-Dawley and hooded Wistar rats likely exist.

Perspectives and Significance

Skeletal muscle glucose uptake during exercise and contraction is an important process that can be exploited to serve as a potential therapeutic target to aid glycemic control in individuals with diabetes. This is especially the case as individuals with T2D have normal skeletal muscle glucose uptake during exercise, and NO appears to play a greater role in mediating muscle glucose uptake during exercise in individuals with T2D than matched controls (28). Therefore, understanding the mechanism(s), whereby NO mediates muscle glucose uptake, in particular, in the setting of diabetes, may provide knowledge required for the development of new therapeutics for the management of diabetes. Our data indicate that unlike in humans and hooded Wistar rats, NO is unlikely to be essential for the increase in muscle glucose uptake during contraction in control and T2D Sprague-Dawley rats. Our data suggest that differential regulation of skeletal muscle glucose uptake during contraction by NO may exist between hooded Wistar and Sprague-Dawley rats, which is an important consideration for future experiments in the area.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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