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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Running title: NOS inhibition and muscle glucose uptake during contraction
ABSTRACT

Nitric oxide (NO) has been shown to be involved in skeletal muscle glucose uptake during contraction/exercise, especially in individuals with type 2 diabetes (T2D). To examine the potential mechanisms we examined the effect of local NO synthase (NOS) inhibition on muscle glucose uptake and muscle capillary blood flow during contraction in healthy and T2D rats. T2D was induced in Sprague Dawley rats using a combined high-fat diet (23% fat wt/wt for 4 weeks) and low-dose streptozotocin injections (35mg/kg). Anesthetized animals had one hindlimb stimulated to contract in situ for 30 min (2Hz, 0.1ms, 35V) with the contralateral hindlimb rested. After 10 min, the NOS inhibitor, L-NAME (5µM) or saline was continuously infused into the femoral artery of the contracting hindlimb until the end of contraction. Surprisingly, there was no increase in skeletal muscle NOS activity during contraction in either group. Local NOS inhibition had no effect on systemic blood pressure or muscle contraction force but caused a significant attenuation of the increase in femoral artery blood flow in control and T2D rats. However, NOS inhibition did not attenuate the increase in muscle capillary recruitment during contraction in these rats. Muscle glucose uptake during contraction was significantly higher in T2D rats compared with controls but, unlike our previous findings in hooded Wistar rats, NOS inhibition had no effect on glucose uptake during contraction. In conclusion, NOS inhibition did not affect muscle glucose uptake during contraction in control or T2D Sprague Dawley rats and this may have been because there was no increase in NOS activity during contraction.

Keywords: type 2 diabetes, contrast-enhanced ultrasound, nitric oxide synthase activity, femoral blood flow, capillary recruitment
INTRODUCTION

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 non-diabetic rodents or humans in their experiments.

Nitric oxide (NO) appears to play an important role in regulating skeletal muscle glucose uptake during contraction/exercise (34, 41). We and others demonstrated that NO is involved in muscle glucose uptake during ex vivo contraction of mouse muscles (35, 36), during and after in situ rat muscles contraction (1, 44), during in vivo rat treadmill exercise (43) and during exercise in humans (4, 28). However, some studies show no effect of 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 (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% versus ~30% respectively) (28). 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 intra-muscular effect of NO on glucose uptake (28). The intra-muscular 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). While, we have previously demonstrated that local NOS inhibition does not affect muscle capillary recruitment during contraction in healthy rats (44), it remains unknown whether it reduces capillary recruitment during contraction in diabetic rats.

Therefore, the purpose of this study was to examine if 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 Edition). Male Sprague Dawley (SD) rats were obtained from either Monash Animal Services or The University of Tasmania Animal House. Rats were housed in plastic rat cages with 3 to 5 rats per cage. Rats were maintained at constant temperature of 21±1°C and on a 12 h light/dark cycle. Dietary manipulation was started at six weeks of age and experimentation occurred at 10 weeks of age.

Diet/ 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 six weeks 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 four weeks. Two weeks into high-fat diet feeding, T2D rats were injected intraperitoneally with a low dose streptozotocin (STZ) (35 mg/kg) in citrate buffer (pH 4.4) and a second, equivalent dose was administered three 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 interleukin 6 (IL 6) and tumor necrosis factor α (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 where 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 genetic background (47) making it a better representation of the human situation (i.e. interaction of environmental factors with diverse genetic predispositions). Non-fasting 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 sodium pentobarbital (50 mg/kg body weight; Ilium, 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 (PE60, Microtube Extrusions, North Rocks, NSW, Australia) for infusion of solutions. Continuous anesthesia was maintained by infusion of sodium pentobarbital (0.6 mg/min/kg body weight) 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, USA) 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, USA) 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, USA). The epigastric artery in the stimulated (contracted) leg was also cannulated with an insulin needle (30 Gauge) connected to PE30 tubing for local (retrograde) infusion of N-G-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 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, Wisconsin, USA). To measure force of contraction, the contracted leg was secured in place at the knee joint preventing lower limb movement. An isometric force transducer (Harvard Apparatus, Holliston, Massachusetts, USA) 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 leg to achieve $5 \, \mu M$ concentration based on the concentration of infusate, 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 in order to calculate the L-NAME infusion rate to achieve the required $5 \, \mu M$ concentration. A bolus of $[1^{-14}C]2$-deoxy-glucose ($[^{14}C]2$-DG) (20 $\mu Ci$; American Radiolabeled Chemicals, St Louis, MO, USA) 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 Figure 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 $5 \, \mu M$ in the hindlimb. In protocol B, capillary blood flow was determined using CEU with microbubble infusion. Microbubbles were infused as indicated in Figure 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, USA) was positioned over the mid-level 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
mid-portion 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 was 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 (Phillips Ultrasound, Bothell, WA, USA). Images were background subtracted for 1.0 s frames to eliminate signal from larger blood vessels. Background subtracted acoustic intensity versus time was plotted to allow calculation of capillary blood volume (A) and capillary flow rate (A*β) according to the equation $y = A(1 – e^{-βt})$, 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 $[^{14}\text{C}]2$-DG. Average plasma $[^{14}\text{C}]2$-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 $[^{14}\text{C}]2$-DG was extracted using an anion exchange resin (AG1-X8; Bio-Rad Laboratories, CA, USA). Radioactivity was determined using a β counter (Tri-Carb 2800TR; Perkin Elmer, Chicago, IL, USA). Muscle glucose uptake ($R’g$) was calculated as previously described (44) based on phosphorylated $[^{14}\text{C}]2$-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, USA). Plasma insulin levels were determined using enzyme-linked immunosorbent assay (Mercodia, AB, Uppsala, Sweden) as per manufacturer’s instruction.

**Western blotting**

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, USA). Aliquots of whole homogenate containing 5 µg of total protein were separated on SDS-PAGE gels (Bio-Rad) and then wet transferred onto polyvinylidene fluoride (PVDF) membranes. Membranes were probed with the following primary antibodies overnight at 4 °C: phospho-AMPKα Thr172 (1: 1000 anti-rabbit), AMPKα (1: 1000 anti-mouse), α-tubulin (1: 1000 anti-rabbit) (Cell Signaling Technology, Danvers, MA, USA); nNOS (1: 10000 anti-mouse) and eNOS (1: 1000 anti-mouse) (BD Biosciences, San Jose, California, USA); GLUT4 (1: 8000 anti-rabbit) (Thermo Scientific, Rockford, IL, USA); and actin (1: 40000 anti-rabbit) (Sigma Aldrich, St Louis, MO, USA). Loading control proteins were always probed using non-stripped membranes. Actin was used for all except GLUT4 where α-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 ECL substrate (SuperSignal West Femto, Pierce). Blot images were taken with a charge-coupled device (CCD) camera using Quantity One software (Bio-Rad). Pre-stained molecular weight markers on the membrane were imaged under white light source with the membrane position unchanged. When quantifying both phosphorylated and total protein abundance, 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), re-blocked and re-probed with primary antibody against the total protein.

**NOS activity assay**

NOS activity was determined based on the 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-[\(^{14}\)C]citrulline formed per min, per mg of protein. This assay was validated using skeletal muscles from nNOSμ knockout mice that had undergone treadmill exercise or contracted *ex vivo*. A significant increase in NOS activity during exercise or contraction *ex vivo* was detected in muscles of nNOSμ\(^{+/+}\) mice but no increase was observed in muscles of nNOSμ\(^{-/-}\) 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 (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.

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 was 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 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 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 $[^{14}\text{C}]$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 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, determined from the uptake and phosphorylation of $[^{14}\text{C}]$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 to 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 blood flow rate (Fig. 6B). These indices were similar between control and T2D rats and were not significantly affected by L-NAME infusion (Fig. 6A & Fig. 6B) 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 were similar in both control and T2D rats (Table 1). A trend ($P = 0.057$) for a reduction (~16%) in nNOSμ 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 Thr$^{172}$ site is the major AMPK activating site (50) and our previous studies showed that AMPK activity and AMPKα Thr$^{172}$ phosphorylation demonstrate an almost identical response during exercise (32, 55). Therefore, AMPKα Thr$^{172}$ 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 Thr$^{172}$. Contraction significantly increased AMPKα Thr$^{172}$ phosphorylation in all groups (> 2 fold) and no difference was observed between control and T2D rats. L-NAME had no effect on AMPK Thr$^{172}$ 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. 7C). 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, like 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 approximately 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 previously been optimized to elicit local effects without a systemic spillover effect on blood pressure (3, 44). We observed no 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 regards 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/ 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 non-diabetic 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 if there was a compensatory up-regulation 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 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 were, however, not examined in this study. Phosphorylation of eNOS was not determined as skeletal muscle eNOS has been shown to be not involved in muscle NO production (18) or activation of muscle NO downstream signaling (30) during contraction.

Studies have shown that muscle contraction/ exercise activates NOS in skeletal muscles and leads 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 NOS 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. In that study, local L-NAME infusion into the contracting leg prevented the activation of NOS and led to a significant attenuation in glucose uptake during contraction. It is also important to highlight that NOS inhibition attenuated the increase in NOS activity and glucose uptake in mouse skeletal muscle contracted \textit{ex vivo} (35, 36). Leg
glucose uptake during cycling exercise in healthy and T2D individuals was also attenuated during local infusion of NOS inhibitor (4, 28). The differences observed between our present and previous rat in situ contraction studies may be attributed to inadequate contraction stimulation and rat strain differences as will be discussed below.

The activation of muscle NOS during contraction/exercise appears to be influenced by the contraction/exercise intensity and by the strain of rats involved. It was shown that higher exercise/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 & 10 V) did not necessarily activate NOS in muscles of SD rats (12); while, lower stimulation intensity (15 ms trains, 2 Hz & 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 SD rats (present study). Collectively, these results suggest that there may be an interplay between contraction/exercise intensity and the strains of rats in the activation of NOS during contraction/exercise where SD 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 EDL muscles was found to be lower in Wistar rats compared with SD 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 SD 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 SD rats suggests that strain differences in the regulation of contraction-stimulated muscle glucose uptake between SD and hooded Wistar rats likely exists.

Perspective and significance

Skeletal muscle glucose uptake during exercise/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 SD rats. Our data suggest that differential regulation of skeletal muscle glucose uptake during contraction by NO may exist between Hooded Wistar and SD rats, which is an important consideration for future experiments in the area.

ACKNOWLEDGEMENTS

The authors would like to thank the staff and students, in particular Eloise Bradley and Helena Ng of the Muscle Research Group, Menzies Research Institute, The University of Tasmania for their technical assistance in the experiments.
The authors would like to acknowledge funding from the National Health and Medical Research Council (NHMRC) of Australia for the support for this study.

**AUTHOR CONTRIBUTIONS**

REFERENCES


FIGURE LEGENDS

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-deoxy-glucose; L-NAME: N-G-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 measurement 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 method section.

Fig. 2: Peak contraction force normalized to muscle weight (A) and percent of decrease in contraction force from the initial maximum force over time (B). Values are means ± SE, n = 5 – 7 per group. § P < 0.05 main effect for time. Ctrl = control rats; T2D = type 2 diabetic rats

Fig. 3: Systemic blood pressure changes at rest and during single hindlimb contraction. Note: Blood pressure measurement could not be obtained during the final 10 min because the same carotid artery line was necessary for the continuous arterial withdrawal for glucose uptake measurement. Values are means ± SE, n = 8 – 12 per group. § P < 0.05 main effect for time.

Fig. 4: Rest and contracted leg muscle glucose uptake in control and T2D rats with local infusion of saline or L-NAME. Values are 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 means ± SE, n = 8 – 12 per group. § P < 0.05 main effect for time, γ P < 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 means ± SE, n = 5, 4, 5 & 3 for control + saline, control + L-NAME, T2D + saline and T2D + L-NAME, respectively. † P < 0.05 main effect for contraction and contraction + infusion.

Fig. 7: nNOSμ (A) and nNOSβ (B) protein expressions in control and T2D rats relative to actin abundance. Rest and contracted muscle NOS activity in control and T2D rats with local infusion of saline or L-NAME (C). Data are means ± SE, n = 8. # P < 0.05 vs control; ψ trend (P = 0.051) for main effect of T2D.

Fig. 8: AMPKα Thr172 phosphorylation relative to AMPKα abundance in control and T2D rats during in situ contraction with saline or local L-NAME infusion. Data are means ± SE, n = 8 per group. * P < 0.05 vs rest of the same genotype.
Table 1: Baseline characteristics and skeletal muscle protein expression of control and T2D rats

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control</th>
<th>T2D</th>
</tr>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>340 ± 5</td>
<td>304 ± 4 #</td>
</tr>
<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>
</tr>
<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>
</tr>
</tbody>
</table>

Values are means ± SE, n = 25 to 30 and 35 to 42 in control and T2D groups, respectively; n = 8 for all protein expression data in both groups. # P < 0.05 versus control. T2D: type 2 diabetes; AU: arbitrary unit.
Figures

Fig. 1

Completion of surgical procedures

Time (min) -40 -15 -10 0 10 20 30

Electrical stimulation (2 Hz, 0.1 ms, 35 V)

Local L-NAME (5 μM) or saline

Muscle sample taken

Bolus 2DG (20 μCi)

Arterial sample #
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Fig. 6
Fig. 7
Fig. 8