Local Nitric Oxide Synthase Inhibition Reduces Skeletal Muscle Glucose Uptake but Not Capillary Blood Flow During In Situ Muscle Contraction in Rats

Renee M. Ross,[1] Glenn D. Wadley,[2] Michael G. Clark,[1] Stephen Rattigan,[1] and Glenn K. McConell[2]

OBJECTIVE—We have previously shown in humans that local infusion of a nitric oxide synthase (NOS) inhibitor into the femoral artery attenuates the increase in leg glucose uptake during exercise without influencing total leg blood flow. However, rodent studies examining the effect of NOS inhibition on contraction-stimulated skeletal muscle glucose uptake have yielded contradictory results. This study examined the effect of local infusion of an NOS inhibitor on skeletal muscle glucose uptake (2-deoxyglucose) and capillary blood flow (contrast-enhanced ultrasound) during in situ contractions in rats.

RESEARCH DESIGN AND METHODS—Male hooded Wistar rats were anesthetized and one hindleg electrically stimulated to contract (2 Hz, 0.1 ms) for 30 min while the other leg rested. After 10 min, the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) (arterial concentration of 5 μmol/l) or saline was infused into the epigastric artery of the contracting leg.

RESULTS—Local NOS inhibition had no effect on blood pressure, heart rate, or muscle contraction force. Contractions increased ($P < 0.05$) skeletal muscle NOS activity, and this was prevented by L-NAME infusion. NOS inhibition caused a modest significant ($P < 0.05$) attenuation of the increase in femoral blood flow during contractions, but importantly there was no effect on capillary recruitment. NOS inhibition attenuated ($P < 0.05$) the increase in contraction-stimulated skeletal muscle glucose uptake by ~35%, without affecting AMP-activated protein kinase (AMPK) activation.

CONCLUSIONS—NOS inhibition attenuated increases in skeletal muscle glucose uptake during contraction without influencing capillary recruitment, suggesting that NO is critical for part of the normal increase in skeletal muscle fiber glucose uptake during contraction. ***Diabetes*** **56:2885–2892, 2007**

From the [1]Menzies Research Institute, University of Tasmania, Hobart, Tasmania, Australia; and the [2]Department of Physiology, University of Melbourne, Parkville, Victoria, Australia.

Address correspondence and reprint requests to Dr. Glenn McConell, Department of Physiology, University of Melbourne, Parkville, Victoria, 3010, Australia. E-mail: mcconell@unimelb.edu.au.

Received for publication 1 June 2007 and accepted in revised form 15 September 2007.

Published ahead of print at http://diabetes.diabetesjournals.org on 19 September 2007. DOI: 10.2337/db07-0745.

[^{14}C]2-DG, 2-deoxy-D-[1-^{14}C]glucose; AMPK, AMP-activated protein kinase; CEU, contrast-enhanced ultrasound; eNOS, endothelial nitric oxide synthase; FBF, femoral blood flow; L-NAME, N^G-nitro-L-arginine methyl ester; L-NMMA, N^G-monomethyl-L-arginine; nNOS, neuronal nitric oxide synthase; NOS, nitric oxide synthase; NOx, nitrate and nitrite.

The uptake and metabolism of glucose by skeletal muscle is the major determinant of whole-body glucose homeostasis (1). People with type 2 diabetes have normal levels of GLUT4 in skeletal muscle but attenuated GLUT4 translocation to the plasma membrane in response to insulin (1). This insufficient GLUT4 translocation is a major contributor to the reduced insulin-stimulated skeletal muscle glucose uptake in people with type 2 diabetes (1). Importantly, however, skeletal muscle GLUT4 translocation (2) and glucose uptake (3) during exercise (i.e., contraction) are normal in people with type 2 diabetes. The intramuscular signaling pathways associated with glucose uptake during exercise, which are not fully understood, differ from the insulin-mediated pathway(s). Possible signaling candidates include calmodulin-dependent protein kinase (CaMK), AMP-activated protein kinase (AMPK), and nitric oxide (NO).

We have evidence in humans that NO is critical for normal increases in skeletal muscle glucose uptake during exercise. In our first study, young healthy men cycled for 30 min at a moderate intensity (60% V_{O_2} peak) with either the NO synthase (NOS) inhibitor N^G-monomethyl-L-arginine (L-NMMA) or saline infused into the femoral artery from 10 min onwards (4). Femoral artery and vein blood samples were obtained, and leg blood flow was determined by thermodilution in the femoral vein. These methodologies are advantageous, as they allow measurements to be determined during the exercise, without the need to stop contractions, and no increases in blood pressure are observed with L-NMMA (4). We allowed exercise to take place for 10 min before starting the infusion of L-NMMA (or saline) so that normal increases in leg glucose uptake (~30-fold) and leg blood flow (~10-fold) could take place before the infusions began. NOS inhibition significantly attenuated the increase in leg glucose uptake during exercise by ~40% at 15 min of exercise compared with the saline infusion without influencing leg blood flow or arterial glucose and insulin concentrations (4). This inhibitory effect appeared to be reversed by infusion of the NOS substrate L-arginine (4). We then showed that L-NMMA attenuated the increase in leg glucose uptake without influencing leg blood flow in people with type 2 diabetes and that this effect of NOS inhibition was significantly greater in people with type 2 diabetes than in control subjects (75% reduction at 15 min in the people with type 2 diabetes vs. 34% in the control subjects) (3). These two studies suggested that NO was critical for normal increases in GLUT4 translocation and therefore glucose uptake during exercise, since blocking NO production substantially reduced leg glucose uptake without affecting leg blood flow. However, changes in leg blood flow can be

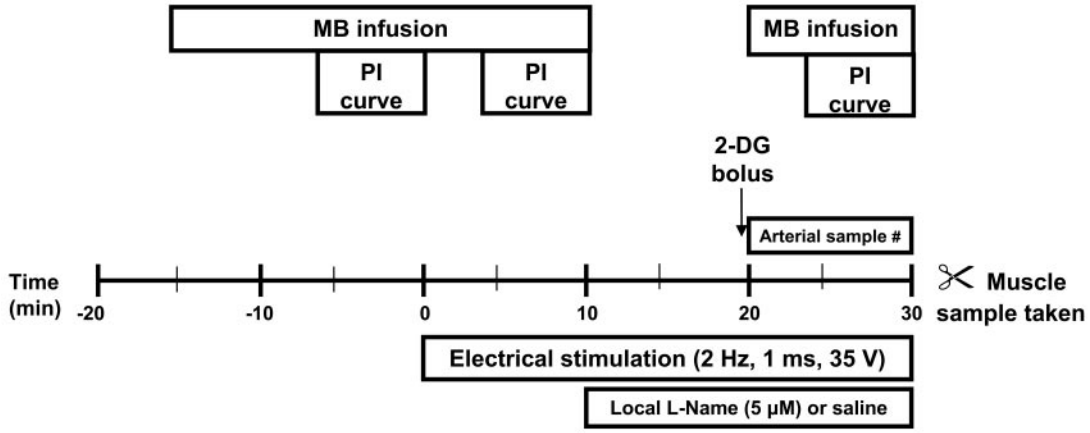

FIG. 1. Experimental protocol. Measurement of capillary recruitment by CEU/microbubbles (MB) (pulsing interval [PI] curve) was conducted immediately before electrical stimulation, immediately before L-NAME, and at $t = 30$ min, requiring microbubble infusion over the periods indicated. Electrical stimulation (0.1-ms impulse at 2 Hz and 35 V) was commenced at $t = 0$ min. Local infusion of L-NAME was commenced at $t = 10$ min via the epigastric artery of the stimulated (test) leg to achieve 5 μmol/l. A bolus of radioactive 2-deoxyglucose (2-DG) was injected at $t = 20$ min, and muscles were removed at $t = 30$ min. Local infusion of L-NAME is via the epigastric artery; #continuous withdrawal (50 μl/min) from the carotid artery. The calf muscle was freeze clamped at 30 min.

independent of muscle microvascular (capillary) perfusion (5), and capillary perfusion may play a critical role in the control of glucose uptake (6).

Studies in rodents examining the effect of NOS inhibition on glucose transport/uptake with exercise/contraction have yielded contradictory results (7–12). Three studies found that NOS inhibition reduced skeletal muscle glucose uptake associated with contraction (8,10,12), and three found that it did not (7,9,11). The possible reasons for the discrepant findings in those studies have been discussed in detail in a recent review (13). It is worth noting that in most of these rodent studies, glucose uptake was examined after, rather than during, the exercise bout/contractions (13). In addition, the conflicting results between studies may have been due, at least in part, to the experimental model used (e.g., in vitro vs. in situ, etc.), whether the NOS inhibitor was present during or after the contractions, whether the buffer was capped or continuously gassed during muscle incubations, the muscle(s) being examined, the fiber type of the muscles being examined, the route of delivering of the NOS inhibitor, and whether male or female rats were used (13).

Therefore, in this study, we examined whether local infusion of an NOS inhibitor attenuated the increase in glucose uptake during contraction in situ in rats. We used contrast-enhanced ultrasound (CEU) for measurement of capillary blood volume. The CEU method has revealed that contractions increase both capillary blood volume (capillary recruitment) and capillary flow rate (14). We hypothesized that NOS inhibition would attenuate the normal increase in skeletal muscle glucose uptake during contractions without influencing muscle capillary blood flow.

RESEARCH DESIGN AND METHODS

Male hooded Wistar rats weighing 247 ± 2 g were reared in the University of Tasmania animal house and allowed free access to standard laboratory rat chow (21.4% protein, 4.6% lipid, 68% carbohydrate, and 6% crude fiber with added vitamins and minerals) and water ad libitum. All animals were housed at a constant temperature of 21 ± 1°C and kept on a 12-h light/dark cycle. The experiments and procedures used were approved by the University of Tasmania Animal Ethics Committee. All experiments were conducted using the anesthetized rat model as described previously (5,14,15).

Contraction and NOS inhibition. The experimental protocol is shown in Fig. 1. There were two formats of this protocol, A and B. Both formats A and B shared the following details: electrical stimulation (0.1-ms impulse at 2 Hz and 35 V) commenced at $t = 0$ min and local infusion of N^G-nitri-L-arginine methyl ester (L-NAME) commenced at $t = 10$ min via the epigastric artery of the stimulated (test) leg to achieve 5 μmol/l. In protocol A, a bolus of radioactive 2-deoxyglucose was injected at $t = 20$ min and muscle removed at $t = 30$ min. Femoral blood flow (FBF) was recorded throughout the protocol, and these data were consequently used during protocol B as a guide for the local infusion of L-NAME, which was adjusted according to the FBF to allow a final concentration of 5 μmol/l in the hindlimb. FBF measurements could not be made in protocol B due to microbubble infusion interfering with the Doppler signal of the transonic flow probe. In protocol B, CEU measurements of capillary perfusion were made by microbubble infusion immediately before electrical stimulation (basal, $t = 0$ min), during electrical stimulation (before L-NAME infusion) at $t = 10$ min, and during electrical stimulation and L-NAME infusion at $t = 30$ min; these required microbubble infusion over the periods indicated in Fig. 1. Muscles were removed at $t = 30$ min, frozen in liquid nitrogen, and stored at −80°C until required.

CEU. Details were essentially as described by Dawson et al. (14) with some minor modifications. The lower leg muscles (gastrocnemius, plantaris, and soleus) of the hindlimb were imaged in short axis with a linear-array transducer connected to an ultrasound system (L7-4 transducer, HDI-5000; ATL Ultrasound). Pulse inversion imaging was performed at a mechanical index of 0.8 to destroy and image microbubbles with the same pulse (14,16). The acoustic focus was placed at the mid-muscle level, and gain settings were optimized and held constant throughout the experiment. Octafluoropropane-gassed microbubbles with a phospholipid shell (prepared in-house) were infused through the right jugular vein at 40 μl/min. Intermittent imaging was performed using pulsing intervals from 0.5 to 15 s to allow incremental microvascular (capillary) replenishment with microbubbles between each pulse until the volume within the beam was completely refilled (17). Several frames were acquired at each pulsing interval. Data were transferred to an offline computer and analyzed using QLAB software (version 2.0; Phillips Ultrasound, Bothwell, WA). The ultrasound intensity in decibels within the region of interest was converted to acoustic intensity after background subtraction using 0.5-s ultrasound images (thus removing signals from the large rapid flow vessels), and a pulsing interval (time) versus acoustic intensity curve was plotted (see typical trace, Fig. 3) to allow calculation of capillary volume (A) as well as capillary flow rate ($A \times \beta$) according to the equation $y = A[1 - e^{-\beta(t - 0.5)}]$, where y is the acoustic intensity at a given pulsing interval (16–18).

Muscle glucose uptake. At 10 min before the completion of each experiment (Fig. 1), a 1.85-MBq bolus of 2-deoxy-D-[1-^{14}C]glucose ([^{14}C]2-DG) (specific activity 1.92 TBq/mmol; Amersham Life Science, Castle Hill, NSW, Australia) in saline (isotonic, 154 mmol/l NaCl) was administered via the right jugular vein. Immediately after the administration of the [^{14}C]2-DG, an arterial blood sample (0.5 ml) was withdrawn by an automated syringe pump at 50 μl/min over 10 min. From this blood sample a plasma sample (25 μl) was collected to determine the average plasma specific radioactivity of [^{14}C]2-DG. At the conclusion of the experiment, the lower leg muscles from the test and contralateral legs were freeze clamped in situ using liquid nitrogen–cooled tongs and stored at −80°C. Muscle samples were ground under liquid

nitrogen, and 100 mg muscle tissue were homogenized with 1.5 ml water. Free and phosphorylated [^{14}C]2-DG were separated by ion exchange chromatography using an anion exchange resin (AG1-X8; Bio-Rad, Hercules, CA). Biodegradable Counting Scintillant-BCA (Amersham Life Science) was added to each radioactive sample and radioactivity determined using a liquid-scintillation counter (Tri-Carb 2800TR; Perkin Elmer, Chicago, IL). From this measurement and knowledge of plasma glucose and average plasma radioactive [^{14}C]2-DG, muscle [^{14}C]2-DG, which reflects glucose uptake into the muscle, was calculated.

Skeletal muscle nitrate and nitrite levels. Frozen muscle was ground under liquid nitrogen into a powder and homogenized (1:20 wt/vol) in freshly prepared ice-cold buffer A (50 mmol/l Tris at pH 7.5 containing 1 mmol/l EDTA, 10% vol/vol glycerol, 1% vol/vol Triton X-100, 50 mmol/l NaF, 5 mmol/l $Na_4P_2O_7$, 1 mmol/l PMSF, 1 μl/ml trypsin inhibitor, and 5 μl/ml protease inhibitor cocktail [P8340, Sigma]). Lysates were spun at 16,000*g* for 20 min at 4°C and purified by spinning the supernatant through 10 kDa molecular weight cutoff filters (Millipore, NSW, Australia) at 16,000*g* for 80 min at 4°C. Total nitrate and nitrite (NOx) levels in the purified samples were determined using Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI). Values are expressed relative to the total protein concentration of the purified samples via the BCA protein assay (Pierce, Rockford, IL), with BSA as the standard.

Preparation of whole muscle lysates. For NOS activity, NOS phosphorylation, and AMPK signaling, frozen powdered muscle was homogenized (1:10 wt/vol) in freshly prepared ice-cold buffer A containing 1 mmol/l dithiothreitol. Tissue lysates were incubated on ice for 20 min and then spun at 16,000*g* for 20 min at 4°C. Protein concentration was determined using the BCA protein assay. An aliquot of the whole muscle lysates was solublized in Laemmli sample buffer and frozen at −20°C for later measurement of AMPK signaling.

NOS was affinity purified from whole muscle lysates using 2′5′-ADP Sepharose 4B beads (Amersham Biosciences, Piscataway, NJ). Following purification, an aliquot of the ADP-Sepharose beads was solublized in Laemmli sample buffer and frozen at −20°C for later measurement of NOS phosphorylation. The remaining ADP-Sepharose beads were used to measure NOS activity.

NOS activity assay. ADP-Sepharose beads were added to 100 μl of preheated (37°C) assay buffer, which contained (in final concentrations) 50 mmol/l Tris-HCl (pH 7.5), 1.15 mmol/l NADPH, 4 μmol/l BH_4, 100 nmol/l CaM, 0.7 mmol/l $CaCl_2$, 10 μmol/l L-arginine, 0.63 μmol/l flavin adenine dinucleotide, and 3 μmol/l L-[U-^{14}C]-arginine (Amersham Biosciences). Samples were incubated in the presence of either H_2O or 1 mmol/l L-NAME for 10 min at 37°C, which was within the predetermined linear range for rat skeletal muscle. The concentration of L-NAME used was sufficient to fully block NOS activity (data not shown), and inter- and intra-assay coefficients of variation were both 9%. NOS activity represents the difference between samples incubated in the absence and presence of L-NAME and is expressed as picomoles of L-[^{14}C]-arginine converted to L-[^{14}C]-citrulline per minute per milligram of protein.

AMPK signaling and NOS phosphorylation. For NOS phosphorylation, equal amounts of purified proteins were subjected to SDS-PAGE. Binding of purified proteins was detected by immunoblotting with either polyclonal rabbit antibodies that recognize the predominant neuronal NOS (nNOS)μ pSer^{1446} variant in skeletal muscle (19) and the endothelial (eNOS) pThr^{1177} antibody (20). For eNOS pThr^{1177}, AICAR (5-aminoimidazole-4-carboxamide-1-ß-D-ribofuranoside)-stimulated rat heart (0.5 mg/g body wt i.p.) was used as a positive control. Membranes were then reprobed with either anti-nNOS or anti-eNOS mouse monoclonal antibodies (BD Transduction Laboratories, NSW, Australia), respectively. For AMPKα threonine^{172} (AMPKα Thr^{172}) and ACCβ serine^{222} (ACCβ Ser^{222}) phosphorylation, 90 μg total protein was subjected to SDS-PAGE and binding detected by immunoblotting with either anti–phospho-ACCβ Ser^{222} polyclonal antibody (19) or AMPKα (Cell Signaling Technology, Hartsfordshire, England). Binding was detected with IRDye 800–conjugated anti-rabbit IgG (Rockland, Gilbertsville, PA) or IRDye 680–conjugated anti-mouse IgG (Molecular Probes, Eugene, OR) secondary antibodies. Direct fluorescence was detected and quantified using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE). For AMPK signaling, membranes were then stripped (2% SDS wt/vol in 25 mmol/l glycine, pH 2.0) and reprobed with IRDye 800–labeled streptavidin (Rockland, Gilbertsville, PA) and anti–phospho-AMPK Thr^{172} antibody (21). Phosphorylation was expressed relative to protein abundance.

Data and statistical analysis. All data are expressed as means ± SEM. Mean FBF, heart rate, and blood pressure were calculated using 5-s subsamples of the data, representing ~500 flow and pressure measurements. Results were analyzed by the SPSS statistical package using two-factor ANOVA (between factor, L-NAME; within factor, contraction) or two-factor repeated-measures ANOVA (between factor, L-NAME; within factors, contraction and time). If the ANOVA revealed a significant interaction, specific differences between mean values were located using the Fisher's least significance difference test. The level of significance was set at $P < 0.05$.

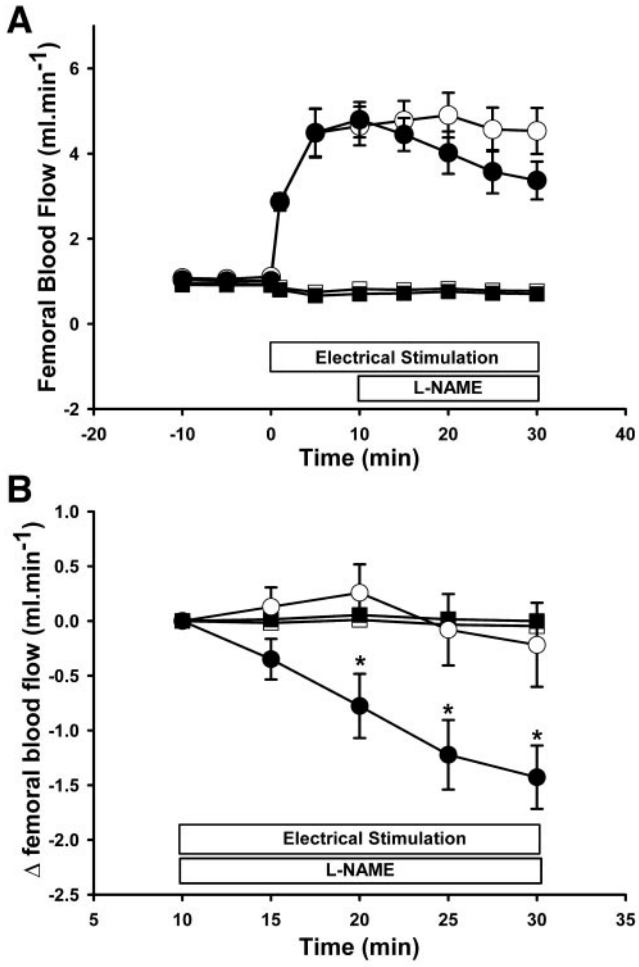

FIG. 2. Effect of local L-NAME (filled symbols) or saline (open symbols) on absolute FBF (*A*) and change in FBF from 10 min (the start of the L-NAME infusion) (*B*) in the contracted test leg (circles) and contralateral control leg (squares). Protocol A of Fig. 1 (see text) was used. Flow probes were positioned around the femoral arteries of each leg. Values are means ± SEM (n = 11). *Significantly different from contraction alone and values for the contralateral leg.

RESULTS

Force development. Electrical stimulation resulted in a maximum tension of ~320 g that decreased to ~250 g after 10 min. This level of force development was sustained for the subsequent 20 min of the experimental period and was unaffected by local L-NAME infusion (data not shown).

Heart rate and blood pressure. Heart rate increased ($P < 0.05$) ~12% over the initial 15 min of one leg contraction, then remained at ~360 bpm for the duration of the experiment (data not shown). Infusion of L-NAME into one leg had no significant effect on heart rate. Electrical stimulation also increased ($P < 0.05$) blood pressure; however, the increase was modest at only ~10 mmHg (data not shown). Infusion of L-NAME into one leg did not further significantly increase the blood pressure, suggesting that any spillover of the NOS blocker was insufficient to cause systemic effects.

Leg blood flow. Electrical stimulation–induced contraction caused a marked ($P < 0.05$) increase in FBF (Fig. 2*A*). L-NAME infusion during contraction tended to decrease FBF, but this was not significant (Fig. 2*A*). However, if

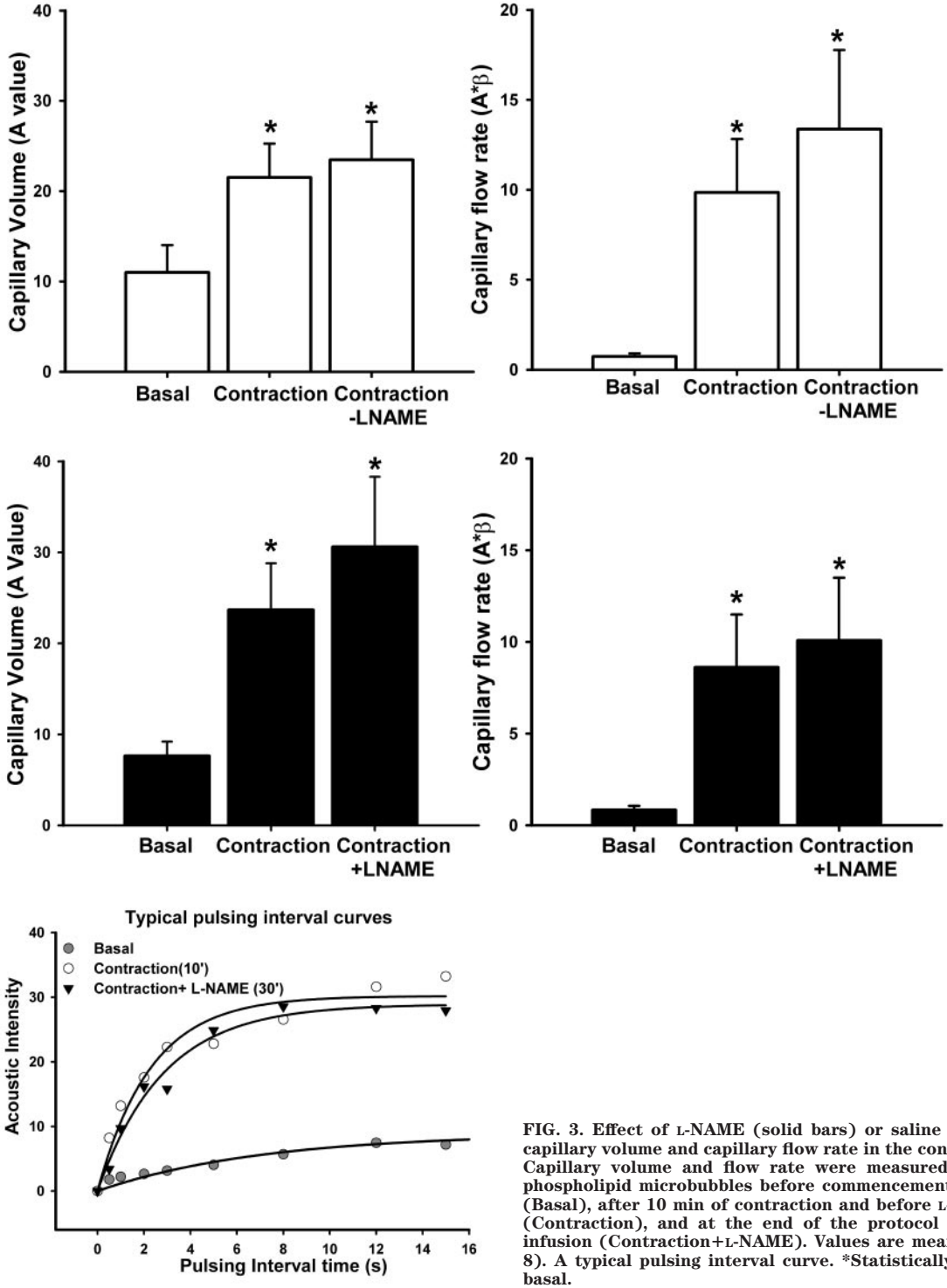

FIG. 3. Effect of L-NAME (solid bars) or saline (open bars) on capillary volume and capillary flow rate in the contracted test leg. Capillary volume and flow rate were measured by CEU using phospholipid microbubbles before commencement of contraction (Basal), after 10 min of contraction and before L-NAME infusion (Contraction), and at the end of the protocol during L-NAME infusion (Contraction+L-NAME). Values are means ± SEM (n = 8). A typical pulsing interval curve. *Statistically different from basal.

FBF was examined from 10 min onwards, i.e., after introduction of the NOS inhibitor, the change (Δ) in FBF from 10 min was significantly reduced with L-NAME infusion during contraction (Fig. 2*B*). Neither electrical stimulation of the test leg nor local infusion of L-NAME into this leg during electrical stimulation had any significant effect on FBF in the contralateral control leg (Fig. 2*A*).

Capillary volume and flow velocity. The ultrasound transducer was positioned over the contracting leg; thus, only values for the contracting leg are shown. Contraction increased both the A value (capillary volume) and the $A \times \beta$ value (capillary flow rate) (Fig. 3). The infusion of L-NAME did not significantly affect capillary volume or capillary flow rate during contraction.

Muscle glucose uptake. Contraction caused a marked increase in muscle glucose uptake of ~16-fold relative to the unstimulated control leg (Fig. 4). L-NAME infusion into the contracting leg significantly inhibited this increase by ~35%. L-NAME had no effect on glucose uptake in the contralateral control leg when infused into the test leg. There was no significant difference in blood glucose concentration between the saline and the L-NAME contraction treatments either immediately before contraction (contraction + saline, 4.9 ± 0.1; contraction + L-NAME,

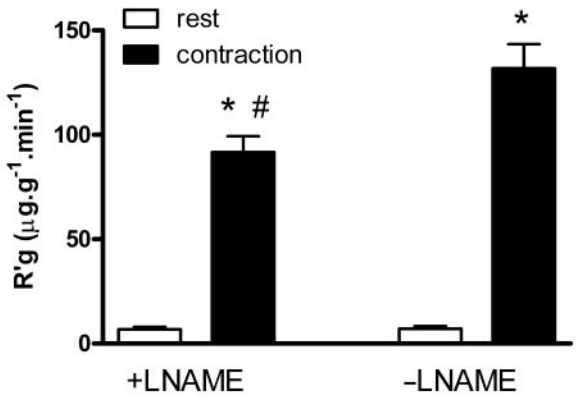

FIG. 4. Effect of L-NAME on 2-deoxyglucose uptake (R′g) in the contracted test leg (contraction) and contralateral control leg (rest). *Significantly different ($P < 0.05$, two-way ANOVA) from control (rest) leg, and #significantly different ($P < 0.05$, two-way ANOVA) from −L-NAME. 2-Deoxyglucose uptake was measured over the final 10 min and the results expressed as means ± SEM ($n = 6$).

4.8 ± 0.1 mmol/l) or at the end of the contractions (contraction + saline, 5.4 ± 0.2; contraction + L-NAME, 5.1 ± 0.1 mmol/l). Similarly, there was no significant difference in plasma insulin concentration either immediately before contraction (contraction + saline, 209 ± 28; contraction + L-NAME, 211 ± 58 pmol/l) or at the end of the contractions (contraction + saline, 246 ± 43; contraction + L-NAME, 218 ± 46 pmol/l).

AMPKα and ACCβ phosphorylation. Contraction caused a two- to threefold increase in AMPKα phosphorylation and ACCβ phosphorylation, and L-NAME infusion during contraction had no effect on the extent of this phosphorylation (Fig. 5). L-NAME infusion had no effect on AMPKα phosphorylation or ACCβ phosphorylation in the contralateral control (rest) leg when infused into the (contracting) test leg (Fig. 5).

NOS activity, NOx, and nNOS and eNOS phosphorylation. Contraction significantly increased skeletal muscle NOS activity, and this increase was prevented by L-NAME infusion during contractions (Fig. 6*A*). A similar response was observed with skeletal muscle NOx content, although the increase with exercise was not significant (Fig. 6*B*). Contraction caused a two- to threefold increase in nNOS phosphorylation, and as was the case for AMPKα phosphorylation, L-NAME infusion during contraction had no effect on the extent of nNOS phosphorylation (Fig. 6*C*). L-NAME infusion into the contracting test leg had no effect on NOS activity, NOx content, or nNOS phosphorylation in the contralateral resting leg (Fig. 6). We were able to observe substantial eNOS phosphorylation in rat heart following intraperitoneal AICAR administration (as a control), but we could not detect any skeletal muscle eNOS phosphorylation at rest or following contractions (data not shown).

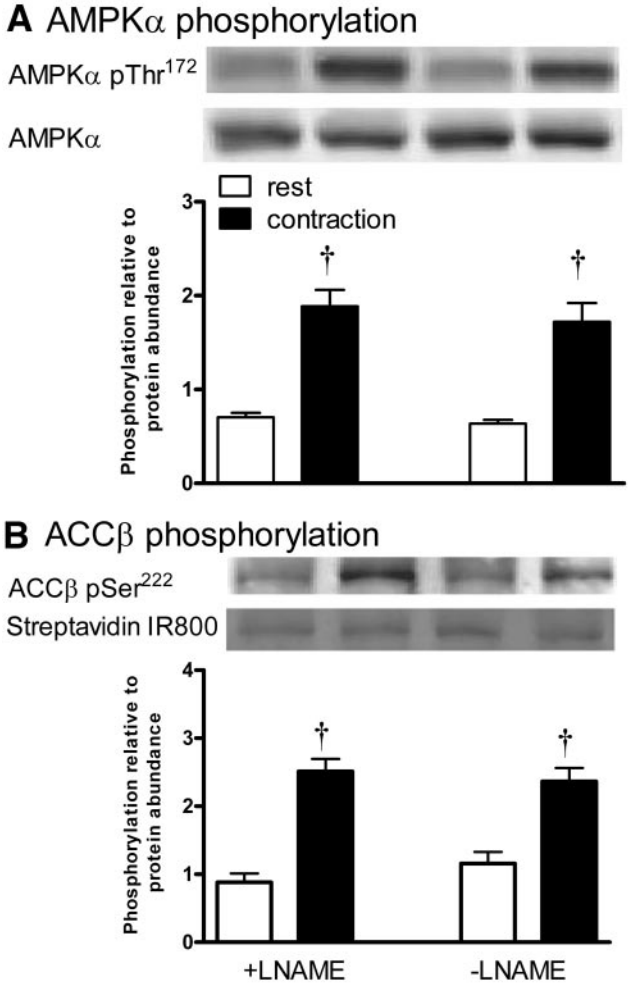

FIG. 5. Effect of L-NAME on Thr^{172} phosphorylation of AMPKα (*A*) and Ser^{222} of ACCβ (*B*) in the contracted test leg and contralateral control leg. Values are means ± SEM. †Main effect ($P < 0.05$, two-way ANOVA) for contraction.

DISCUSSION

The major finding of this study was that local NOS inhibition attenuated the increase in skeletal muscle glucose uptake during in situ contractions without influencing muscle capillary recruitment. In addition, AMPK activation was not altered by NOS inhibition, suggesting that the reductions in glucose uptake were specific to NO reduction and not secondary to effects on AMPK. Importantly, local NOS inhibition had no effect on blood pressure or heart rate during contractions, suggesting that these effects were due to local and not systemic effects of the NOS inhibitor. In definitively separating capillary blood flow effects from metabolic effects, the important implication is that an NO-producing mechanism is activated in muscle by contraction independent of vascular endothelial NO. As such, these findings lend support to other earlier reports of NO donors acting to increase glucose uptake in isolated incubated muscle (7–9,22), where the vascular system has no involvement on nutrient and hormone delivery.

Previous rodent studies examining the effect of NOS inhibition on contraction-stimulated glucose uptake have yielded contradictory results (7–12), but the current study has clearly shown that NO is required for a portion of glucose uptake during contractions (Fig. 4). This is the first study in rats in which NOS inhibition was administered during the contractions and at the same time glucose uptake was determined. Most rat studies have either contracted isolated muscles during NOS inhibition or contracted muscles in situ with no NOS inhibition and then measured glucose uptake 20–70 min after the contractions ceased (with NOS inhibitors present) (see Table 1 of ref. 13). Therefore, these studies actually have little relevance to the regulation of glucose uptake during

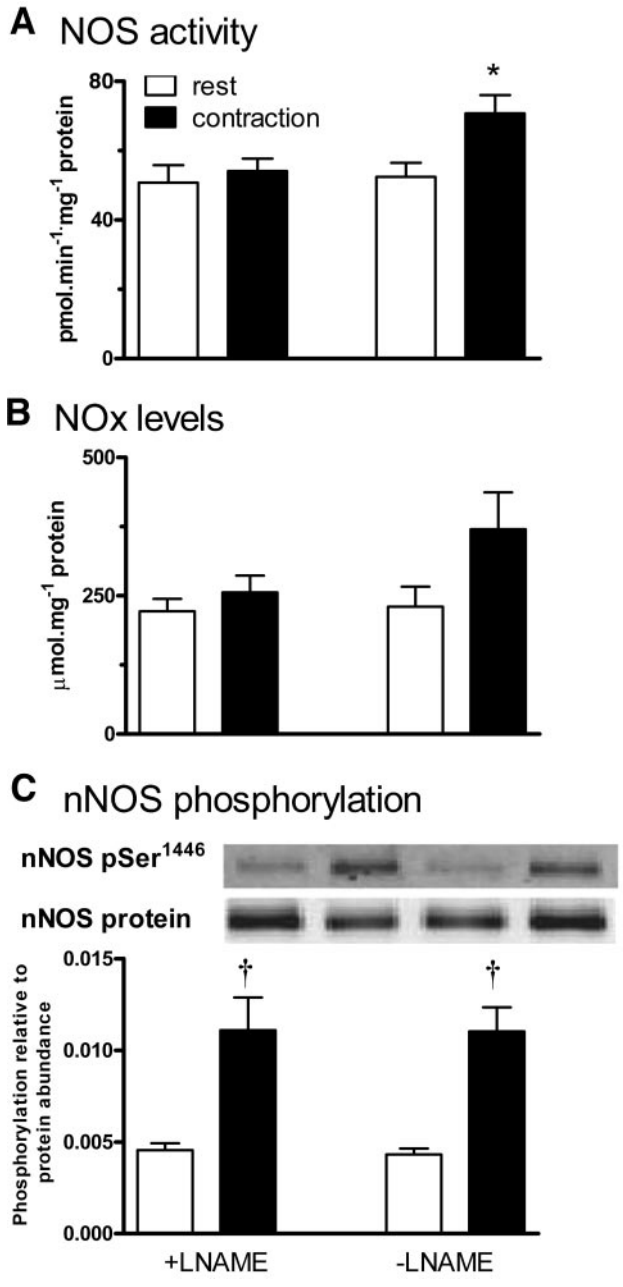

FIG. 6. Effect of L-NAME on muscle NOS activity (*A*), NOx (*B*), and Ser[1446] phosphorylation of nNOS (*C*) in the contracted test leg and contralateral control leg. Values are means ± SEM. *Significantly different ($P < 0.05$, two-way ANOVA) from control leg. †Main effect ($P < 0.05$, two-way ANOVA) for contraction.

exercise and are more related to the possible role of NO in the higher glucose disposal observed following exercise versus rest. The protocol that we used during the current study closely replicated our previous human exercise studies, where we obtained findings similar to those of the current study (3,4). A study demonstrated that ingestion of L-NAME in the drinking water for 3 days before exercise had no effect on glucose uptake during exercise in mice (11). However, L-NAME crosses the blood brain barrier and affects blood pressure and sympathetic outflow; therefore, a model based on that approach must be considered with caution.

The magnitude of increase in NOS activity that we observed during contractions (Fig. 6*A*), although relatively modest, was identical to that observed previously following treadmill running in rats (23). The NOS activity and NOx (Fig. 6*B*) results indicate that the L-NAME infusion completely prevented the activation of skeletal muscle NOS during contractions. Because NOS inhibition attenuated the increase in skeletal muscle glucose uptake during contractions by ~35%, factors in addition to NO must also play a role in contraction-stimulated glucose uptake.

Over the past few years, there has been enormous interest in AMPK as a potential regulator of contraction-stimulated glucose uptake (24–26). Recently, there has been evidence that NOS may lie both upstream and downstream of AMPK. Peroxynitrite, which is formed when NO combines with superoxide, activates AMPK in endothelial cells (27). In addition, high levels of the NO donor sodium nitroprusside activate AMPKα1 in rat skeletal muscle (9). However, in the present study, AMPK activation during contractions, as indicated by AMPKα Thr172 phosphorylation (28,29) and ACCβ phosphorylation, was unaffected by L-NAME infusion (Fig. 5). This indicates that NO was not acting upstream of AMPK. Since AMPK activation was normal during exercise with L-NAME and a substantial portion of contraction-stimulated glucose uptake was intact during NOS inhibition, it is possible that AMPK activation may have contributed to a portion of the increase in glucose uptake during contraction. Although there is some support for a role of AMPK in contraction-stimulated glucose uptake (24,30–32), we (33) and others (34–36) have demonstrated dissociations between AMPK activation and contraction-stimulated glucose uptake; therefore, further research is required to clarify this.

AMPK phosphorylates nNOSμ in skeletal muscle during exercise in humans (21,25). In the current study, the increase in skeletal muscle nNOSμ phosphorylation during contractions was unaffected by L-NAME (Fig. 6*C*). This is not surprising, since AMPK activation during contractions was unaffected by L-NAME (Fig. 5). We have found that AMPK phosphorylation of nNOSμ at Ser1446 has little effect on nNOSμ activity (R. Lee-Young, G.D.W., Z.-P. Chen, B. Kemp, G.K.M., unpublished observations). However, the question is then, how is it that L-NAME prevented the increase in NOS activity with contractions if it did not effect nNOSμ phosphorylation at Ser1446? Since L-NAME is a competitive inhibitor, it would have been diluted and lost during the extraction and purification steps used for the NOS activity assay. Therefore, there must have been alterations in phosphorylation of sites other than Ser1446 and/or other covalent events with L-NAME infusion during contraction. Since rodent muscle expresses both nNOSμ and eNOS (37) and AMPK phosphorylates and activates eNOS (20), we also examined eNOS phosphorylation. However, we were unable to detect any eNOS phosphorylation either at rest or following contractions. This likely reflects that nNOSμ plays a more important role in exercise metabolism than eNOS, since downstream NOS signaling (i.e., increased cGMP levels) occurs during contractions in normal muscle and $eNOS^{-/-}$ muscle but not in $nNOS\mu^{-/-}$ muscle (37). It is also possible that L-NAME affected other covalent events on nNOSμ. Irrespective of the mechanism, this study has shown that NOS inhibition reduces NOS activity during contractions and attenuates increases in skeletal muscle glucose uptake, which is an important finding.

The current study clearly showed that in situ contractions increase both total blood flow (Fig. 2) and capillary

recruitment (Fig. 3). The general finding that exercise invokes a marked increase in capillary recruitment (at least threefold) (Fig. 3*A*) adds support to the view that muscle at rest is only partly perfused (38,39) and detracts from the opposite view of full perfusion at rest (40). Both of the methods that have been developed to specifically assess the extent of muscle capillary perfusion, 1-MX metabolism and contrast-enhanced ultrasound, have shown that exercise and insulin both increase capillary recruitment (14) (Fig. 3). Thus, these findings imply a capillary reserve at rest and that the unperfused capillaries are likely to be those recruited when contractions commence or insulin is infused. By increasing the available capillary surface area, delivery of insulin and glucose, as well as other nutrients, is increased. Capillary recruitment is the initial vascular response for both insulin and exercise, and changes in total muscle blood flow are secondary and independent to the capillary recruitment (41,42).

Infusion of L-NAME during contractions caused a modest but significant reduction in total blood flow (femoral blood flow, Fig. 2), but the contrast-enhanced ultrasound results indicate that there was no alteration to the microcirculation perfusion (Fig. 3). The finding that contraction-mediated capillary recruitment was not blocked by L-NAME contrasts with insulin-mediated capillary recruitment, which is blocked by L-NAME (41,43). For insulin, the increase in capillary perfusion or capillary recruitment plays a crucial role in mediating muscle glucose uptake in vivo. For example, in a number of previous studies it has been shown that blockade of the increase in capillary recruitment due to insulin action was always accompanied by a 50% inhibition of glucose uptake (rev. in 44). Importantly, insulin-mediated glucose uptake correlates with the degree of capillary recruitment and not total blood flow (45). Thus, the decrease in flow during contraction with L-NAME is unlikely to be the cause of the decreased glucose uptake, especially as the extraction of glucose across the leg was only 15% (data not shown), indicating that the delivery of glucose was not limited by the total flow. Additionally, the CEU data (Fig. 3) indicated that L-NAME treatment did not reduce the microvascular filling rate; thus, the reduction in total flow has occurred in the rapid flow non-nutritive route and not in the nutritive flow routes (6). What is clear is that the two stimuli, exercise and insulin, use quite different mechanisms. For example, in the obese Zucker rat, insulin-mediated capillary recruitment is totally impaired but exercise-mediated capillary recruitment appears essentially normal, as does the increase in muscle glucose uptake in response to exercise (46,47).

A novel component incorporated in this rodent study was the use of locally infused (via the epigastric artery of the contracting leg [15]) NOS inhibitor, thus avoiding problems associated with systemic infusion. Previous studies by others have shown that systemic NOS inhibitors cause a marked rise in blood pressure, which in turn triggers compensatory responses (48). In addition, systemic infusion is likely to increase entry into the brain, where intracerebroventricular-administered NOS inhibitor has been shown to induce a state of insulin resistance (49). A second distinct advantage of locally infused NOS inhibitor is that the contralateral leg can be used as control. Any spillover would be detected by a rise in blood pressure or increase in vascular resistance of the control leg. In so far that this did not occur, we concluded that the effects were limited to the test leg.

In conclusion, this study demonstrated that local L-NAME infusion prevents contraction-induced increases in skeletal muscle NOS activity and NOx content and attenuates the increase in skeletal muscle glucose uptake during contractions without influencing skeletal muscle capillary recruitment. These effects were independent of skeletal muscle AMPK activation during contraction. These results suggest that NO is critical for part of the normal increase in skeletal muscle glucose uptake during contraction.

ACKNOWLEDGMENTS

This work was supported by grants from the National Health and Medical Research Council of Australia (to G.K.M., S.R., and M.G.C.) and Diabetes Australia (to G.K.M.). S.R. is a Career Fellow of the Heart Foundation of Australia.

REFERENCES

1. Richter EA, Derave W, Wojtaszewski JF: Glucose, exercise and insulin: emerging concepts. *J Physiol* 535:313–322, 2001
2. Kennedy JW, Hirshman MF, Gervino EV, Ocel JV, Forse RA, Hoenig SJ, Aronson D, Goodyear LJ, Horton ES: Acute exercise induces GLUT4 translocation in skeletal muscle of normal human subjects and subjects with type 2 diabetes. *Diabetes* 48:1192–1197, 1999
3. Kingwell B, Formosa M, Muhlmann M, Bradley S, McConell G: Nitric oxide synthase inhibition reduces glucose uptake during exercise in individuals with type 2 diabetes more than in control subjects. *Diabetes* 51:2572–2580, 2002
4. Bradley SJ, Kingwell BA, McConell GK: Nitric oxide synthase inhibition reduces leg glucose uptake but not blood flow during dynamic exercise in humans. *Diabetes* 48:1815–1821, 1999 [Erratum in *Diabetes* 48:2480, 1999]
5. Rattigan S, Clark MG, Barrett EJ: Hemodynamic actions of insulin in rat skeletal muscle: evidence for capillary recruitment. *Diabetes* 46:1381–1388, 1997
6. Clark MG, Wallis MG, Barrett EJ, Vincent MA, Richards SM, Clerk LH, Rattigan S: Blood flow and muscle metabolism: a focus on insulin action. *Am J Physiol Endocrinol Metab* 284:E241–E258, 2003
7. Etgen GJ Jr, Fryburg DA, Gibbs EM: Nitric oxide stimulates skeletal muscle glucose transport through a calcium/contraction- and phosphatidylinositol-3-kinase-independent pathway. *Diabetes* 46:1915–1919, 1997
8. Balon TW, Nadler JL: Evidence that nitric oxide increases glucose transport in skeletal muscle. *J Appl Physiol* 82:359–363, 1997
9. Higaki Y, Hirshman MF, Fujii N, Goodyear LJ: Nitric oxide increases glucose uptake through a mechanism that is distinct from the insulin and contraction pathways in rat skeletal muscle. *Diabetes* 50:241–247, 2001
10. Roberts CK, Barnard RJ, Scheck SH, Balon TW: Exercise-stimulated glucose transport in skeletal muscle is nitric oxide dependent. *Am J Physiol* 273:E220–E225, 1997
11. Rottman J, Bracy D, Malabanan C, Yue Z, Clanton J, Wasserman D: Contrasting effects of exercise and NOS inhibition on tissue-specific fatty acid and glucose uptake in mice. *Am J Physiol Endocrinol Metab* 283:E116–E123, 2002
12. Stephens TJ, Canny BJ, Snow RJ, McConell GK: 5′-aminoimidazole-4-carboxyamide-ribonucleoside-activated glucose transport is not prevented by nitric oxide synthase inhibition in rat isolated skeletal muscle. *Clin Exp Pharmacol Physiol* 31:419–423, 2004
13. McConell GK, Kingwell BA: Does nitric oxide regulate skeletal muscle glucose uptake during exercise? *Exerc Sport Sci Rev* 34:36–41, 2006
14. Dawson D, Vincent MA, Barrett EJ, Kaul S, Clark A, Leong-Poi H, Lindner JR: Vascular recruitment in skeletal muscle during exercise and hyperinsulinemia assessed by contrast ultrasound. *Am J Physiol Endocrinol Metab* 282:E714–E720, 2002
15. Mahajan H, Richards SM, Rattigan S, Clark MG: Local methacholine but not bradykinin potentiates insulin-mediated glucose uptake in muscle in vivo by augmenting capillary recruitment. *Diabetologia* 47:2226–2234, 2004
16. Lindner JR, Wei K: Contrast echocardiography. *Curr Probl Cardiol* 27:454–519, 2002
17. Wei K, Jayaweera AR, Firoozan S, Linka A, Skyba DM, Kaul S: Quantification of myocardial blood flow with ultrasound-induced destruction of microbubbles administered as a constant venous infusion. *Circulation* 97:473–483, 1998

18. Wei K, Le E, Bin JP, Coggins M, Thorpe J, Kaul S: Quantification of renal blood flow with contrast-enhanced ultrasound. *J Am Coll Cardiol* 37:1135–1140, 2001
19. Chen ZP, McConell GK, Michell BJ, Snow RJ, Canny BJ, Kemp BE: AMPK signaling in contracting human skeletal muscle: acetyl-CoA carboxylase and NO synthase phosphorylation. *Am J Physiol Endocrinol Metab* 279:E1202–E1206, 2000
20. Chen ZP, Mitchelhill KI, Michell BJ, Stapleton D, Rodriguez-Crespo I, Witters LA, Power DA, Ortiz de Montellano PR, Kemp BE: AMP-activated protein kinase phosphorylation of endothelial NO synthase. *FEBS Lett* 443:285–289, 1999
21. Wadley GD, Lee-Young RS, Canny BJ, Wasuntarawat C, Chen ZP, Hargreaves M, Kemp BE, McConell GK: Effect of exercise intensity and hypoxia on skeletal muscle AMPK signaling and substrate metabolism in humans. *Am J Physiol Endocrinol Metab* 290:E694–E702, 2006
22. Young ME, Leighton B: Evidence for altered sensitivity of the nitric oxide/cGMP signalling cascade in insulin-resistant skeletal muscle. *Biochem J* 329:73–79, 1998
23. Roberts CK, Barnard RJ, Jasman A, Balon TW: Acute exercise increases nitric oxide synthase activity in skeletal muscle. *Am J Physiol* 277:E390–E394, 1999
24. Hayashi T, Hirshman MF, Kurth EJ, Winder WW, Goodyear LJ: Evidence for 5′ AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport. *Diabetes* 47:1369–1373, 1998
25. Chen Z-P, Stephens TJ, Murthy S, Canny BJ, Hargreaves M, Witters LA, Kemp BE, McConell GK: Effect of exercise intensity on skeletal muscle AMPK signaling in humans. *Diabetes* 52:2205–2212, 2003
26. Richter EA, Nielsen JN, Jorgensen SB, Frosig C, Birk JB, Wojtaszewski JF: Exercise signalling to glucose transport in skeletal muscle. *Proc Nutr Soc* 63:211–216, 2004
27. Zou MH, Hou XY, Shi CM, Kirkpatick S, Liu F, Goldman MH, Cohen RA: Activation of 5′-AMP-activated kinase is mediated through c-Src and phosphoinositide 3-kinase activity during hypoxia-reoxygenation of bovine aortic endothelial cells: role of peroxynitrite. *J Biol Chem* 278:34003–34010, 2003
28. Park SH, Gammon SR, Knippers JD, Paulsen SR, Rubink DS, Winder WW: Phosphorylation-activity relationships of AMPK and acetyl-CoA carboxylase in muscle. *J Appl Physiol* 92:2475–2482, 2002
29. Lee-Young RS, Palmer MJ, Linden KC, LePlastrier K, Canny BJ, Hargreaves M, Wadley GD, Kemp BE, McConell GK: Carbohydrate ingestion does not alter skeletal muscle AMPK signaling during exercise in humans. *Am J Physiol Endocrinol Metab* 291:E566–E573, 2006
30. Sandstrom ME, Zhang SJ, Bruton J, Silva JP, Reid MB, Westerblad H, Katz A: Role of reactive oxygen species in contraction-mediated glucose transport in mouse skeletal muscle. *J Physiol* 575:251–262, 2006
31. Musi N, Hayashi T, Fujii N, Hirshman MF, Witters LA, Goodyear LJ: AMP-activated protein kinase activity and glucose uptake in rat skeletal muscle. *Am J Physiol Endocrinol Metab* 280:E677–E684, 2001
32. Mu J, Brozinick JT Jr, Valladares O, Bucan M, Birnbaum MJ: A role for AMP-activated protein kinase in contraction- and hypoxia-regulated glucose transport in skeletal muscle. *Mol Cell* 7:1085–1094, 2001
33. McConell GK, Lee-Young RS, Chen ZP, Stepto NK, Huynh NN, Stephens TJ, Canny BJ, Kemp BE: Short-term exercise training in humans reduces AMPK signalling during prolonged exercise independent of muscle glycogen. *J Physiol* 568:665–676, 2005
34. Jorgensen SB, Viollet B, Andreelli F, Frosig C, Birk JB, Schjerling P, Vaulont S, Richter EA, Wojtaszewski JF: Knockout of the alpha2 but not alpha1 5′-AMP-activated protein kinase isoform abolishes 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranosidebut not contraction-induced glucose uptake in skeletal muscle. *J Biol Chem* 279:1070–1079, 2004
35. Fujii N, Hirshman MF, Kane EM, Ho RC, Peter LE, Seifert MM, Goodyear LJ: AMP-activated protein kinase alpha2 activity is not essential for contraction- and hyperosmolarity-induced glucose transport in skeletal muscle. *J Biol Chem* 280:39033–39041, 2005
36. Wojtaszewski JF, MacDonald C, Nielsen JN, Hellsten Y, Hardie D, Kemp BE, Kiens B, Richter EA: Regulation of 5′AMP-activated protein kinase activity and substrate utilization in exercising human skeletal muscle. *Am J Physiol Endocrinol Metab* 284:E813–E822, 2003
37. Lau KS, Grange RW, Isotani E, Sarelius IH, Kamm KE, Huang PL, Stull JT: nNOS and eNOS modulate cGMP formation and vascular response in contracting fast-twitch skeletal muscle. *Physiol Genomics* 2:21–27, 2000
38. Krogh A: August Krogh-Nobel Lecture: A contribution to the physiology of the capillaries. In *Nobel Lectures*, *Physiology or Medicine 1901–1921*. Amsterdam, Elsevier Publishing Company, 1967, p. 1–8
39. Lindbom L, Tuma RF, Arfors KE: Influence of oxygen on perfused capillary density and capillary red cell velocity in rabbit skeletal muscle. *Microvasc Res* 19:197–208, 1980
40. Kindig CA, Richardson TE, Poole DC: Skeletal muscle capillary hemodynamics from rest to contractions: implications for oxygen transfer. *J Appl Physiol* 92:2513–2520, 2002
41. Vincent MA, Clerk LH, Lindner JR, Klibanov AL, Clark MG, Rattigan S, Barrett EJ: Microvascular recruitment is an early insulin effect that regulates skeletal muscle glucose uptake in vivo. *Diabetes* 53:1418–1423, 2004
42. Vincent MA, Clerk LH, Lindner JR, Price WJ, Jahn LA, Leong-Poi H, Barrett EJ: Mixed meal and light exercise each recruit muscle capillaries in healthy humans. *Am J Physiol Endocrinol Metab* 290:E1191–E1197, 2006
43. Vincent MA, Barrett EJ, Lindner JR, Clark MG, Rattigan S: Inhibiting NOS blocks microvascular recruitment and blunts muscle glucose uptake in response to insulin. *Am J Physiol Endocrinol Metab* 285:E123–E129, 2003
44. Rattigan S, Bradley EA, Richards SM, Clark MG: Muscle metabolism and control of capillary blood flow: insulin and exercise. *Essays Biochem* 42:133–144, 2006
45. Rattigan S, Clark MG, Barrett EJ: Acute vasoconstriction-induced insulin resistance in rat muscle in vivo. *Diabetes* 48:564–569, 1999
46. Wheatley CM, Rattigan S, Richards SM, Barrett EJ, Clark MG: Skeletal muscle contraction stimulates capillary recruitment and glucose uptake in insulin-resistant obese Zucker rats. *Am J Physiol Endocrinol Metab* 287:E804–E809, 2004
47. Wallis MG, Wheatley CM, Rattigan S, Barrett EJ, Clark AD, Clark MG: Insulin-mediated hemodynamic changes are impaired in muscle of Zucker obese rats. *Diabetes* 51:3492–3498, 2002
48. Frandsen U, Bangsbo J, Sander M, Hoffner L, Betak A, Saltin B, Hellsten Y: Exercise-induced hyperaemia and leg oxygen uptake are not altered during effective inhibition of nitric oxide synthase with N(G)-nitro-L-arginine methyl ester in humans. *J Physiol* 531:257–264, 2001
49. Shankar R, Zhu JS, Ladd B, Henry D, Shen HQ, Baron AD: Central nervous system nitric oxide synthase activity regulates insulin secretion and insulin action. *J Clin Invest* 102:1403–1412, 1998