Local NOS inhibition impairs vascular and metabolic actions of insulin in rat hindleg muscle in vivo

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First published July 30, 2013; doi:10.1152/ajpendo.00289.2013.—Insulin stimulates microvascular recruitment in skeletal muscle, and this vascular action augments muscle glucose disposal by ~40%. The aim of the current study was to determine the contribution of local nitric oxide synthase (NOS) to the vascular actions of insulin in muscle. Hooded Wistar rats were infused with the NOS inhibitor N^ω-nitro-l-arginine methylester (l-NNAME, 10 μM) retrogradely via the epigastric artery in one leg during a systemic hyperinsulinemic-euglycemic clamp (3 mU·min⁻¹·kg⁻¹ × 60 min) or saline infusion. Femoral artery blood flow, microvascular blood flow (assessed from 1-methylxanthine metabolism), and muscle glucose uptake (2-deoxyglucose uptake) were measured in both legs. Local l-NNAME infusion did not have any systemic actions on blood pressure or heart rate. Local l-NNAME blocked insulin-stimulated changes in femoral artery blood flow (84%, P < 0.05) and microvascular recruitment (98%, P < 0.05), and partially blocked insulin-mediated glucose uptake in muscle (reduced by 34%, P < 0.05). l-NNAME alone did not have any metabolic effects in the hindleg. We conclude that insulin-mediated microvascular recruitment is dependent on local activation of NOS in muscle and that this action is important for insulin’s metabolic actions.

muscle blood flow; microvascular blood flow; hyperinsulinemic-euglycemic clamp; nitric oxide; nitric oxide synthase

INSULIN STIMULATES TOTAL LIMB blood flow and recruits flow to the microvascularity in skeletal muscle (21, 31, 32, 34). These vascular actions occur in both humans (6, 33) and experimental animals (21, 31, 32, 34). We (4), and others (1, 2), have proposed that insulin-mediated vascular responses facilitate insulin and glucose delivery to the myocyte and thereby enhance the rate of glucose disposal. Insulin-mediated increases in skeletal muscle blood flow and microvascular responses are blunted in insulin-resistant rats (26, 36) and humans (6, 13), suggesting that reduced muscle perfusion may contribute to the insulin resistance. Recently, we demonstrated that insulin resistance induced by increased dietary fat can originate from impaired microvascular insulin responses and occur before the development of myocyte insulin resistance (18). Therefore, it is important to understand the mechanism by which insulin stimulates microvascular recruitment under healthy circumstances to identify the basis of vascular-derived insulin resistance.

Studies by Steinberg et al. (27) and Scherrer et al. (23) have demonstrated that local infusion of a nitric oxide synthase (NOS) inhibitor completely abolishes insulin-induced increases in total limb blood flow in humans. However, insulin’s actions on total blood flow and microvascular recruitment are independent events, and insulin-mediated glucose uptake is modified by microvascular recruitment but not total blood flow (32). The studies that have reported decreased insulin-mediated glucose uptake by NOS inhibition have all involved systemic infusion of the inhibitor (31, 32). Systemic infusions of NOS inhibitors are known to increase mean arterial blood pressure (31) and therefore may trigger homeostatic responses that could impact on skeletal muscle insulin sensitivity. Additionally, acute intracerebroventricular administration of the NOS inhibitor NG mono-methyl-l-arginine (l-NAME) in healthy rats during insulin infusion causes peripheral insulin resistance (3, 24) and impairs insulin-stimulated microvascular perfusion (3), and similar central responses may occur as a consequence of systemic infusion of NOS inhibitors (31, 32). Therefore, it is presently unknown whether insulin-mediated microvascular recruitment is dependent on local nitric oxide (NO) production in skeletal muscle. The aim of the current study was therefore to determine the contribution of local NO in the rat hindleg to the insulin-mediated microvascular recruitment and associated muscle glucose uptake.

MATERIALS AND METHODS

Animal care. All procedures adopted and experiments undertaken were approved by the University of Tasmania Animal Ethics Committee (in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 2004, 7th edition). Male Hooded Wistar rats weighing 253 ± 2 g at the time of the experiments were obtained from the University of Tasmania Animal House (Hobart, Australia). Animals were raised on a commercial diet (Pivot, Launceston, Australia) containing 21.4% protein, 4.6% lipid, 68% carbohydrate, and 6% crude fiber with added vitamins and minerals together with water ad libitum and were housed at 21 ± 1°C on a 12:12-h light-dark cycle.

Surgical preparation. Rats were anesthetized with pentobarbital sodium (50 mg/kg ip). Polyethylene cannulas (PE-58, Intramedic; Becton Dickinson, Parsippany, NJ) were inserted in the carotid artery for arterial blood sampling and measurement of arterial blood pressure (pressure transducer Transpac IV; Abbott Critical Care Systems, Morgan Hill, CA) and heart rate and in both jugular veins for intravenous infusions. Animals were allowed to spontaneously breathe room air through a tracheostomy tube. A small incision was made in the skin overlying the femoral vessels of both hindlegs. The femoral artery, femoral vein, and nerve were all carefully separated. In one leg (test leg), the epigastric artery was cannulated, and an ultrasound flow probe (VB series; Transonic Systems, Ithaca, NY) was positioned around the femoral artery, before the point where the epigastric artery leaves the femoral artery. In the contralateral leg (control leg), the epigastric artery was ligated, and a second ultrasound flow probe was positioned around the femoral artery as above. Probes were connected to flowmeters (Transonic systems, T402 ultrasonic volume flowmeter). Continuous measurement of femoral artery blood flow, heart rate,

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and mean arterial pressure was enabled using WINDAQ data acquisition software (DATAQ Instruments, Akron, OH). The femoral vein remained exposed in both legs until the end of the experiment to enable venous blood sampling. Animals were maintained under anesthesia for the duration of the experiment using a continual infusion of anesthetic (0.6 mg min⁻¹ kg⁻¹, pentobarbital sodium) via the jugular vein. Optimal body temperature was maintained using a water-jacketed platform and a heating lamp positioned above the rat.

**Experimental protocol.** Preliminary experiments determined a dose of N⁶-nitro-L-arginine methyl ester (L-NAME, 10 μM; Sigma Aldrich) that would produce suppression in femoral artery blood flow during physiological insulin infusion while avoiding systemic effects on blood pressure and heart rate. After a period of postsurgical stabilization, rats were subjected to protocols (Fig. 1) in which the effects of prior, local L-NAME (10 μM) infusion (via epigastric artery in one leg) on systemic insulin infusion (as a euglycemic insulin clamp with 3 mU min⁻¹ kg⁻¹, Humulin R; Eli Lilly, Indianapolis, IN) were tested. Because insulin stimulates glucose disposal, a 30% glucose solution (w/v) was infused at a variable rate to maintain fasting blood glucose concentrations (euglycemia) over the course of the experiment. Arterial blood glucose levels were assessed every 10–15 min using a glucose analyzer (YSI 2300; Yellow Springs Instruments), and the glucose infusion rate (GIR) was adjusted accordingly to maintain glycemia. The two experimental groups included 1) local L-NAME + systemic saline and 2) local L-NAME + systemic insulin (Fig. 1). The rate of infusion of L-NAME was regularly adjusted to match femoral artery blood flow in the test leg so that a constant local blood concentration (10 μM) was maintained.

**Blood samples.** Arterial samples were taken at the times indicated (Fig. 1) for blood glucose measurements. The femoral vein of each leg was used for venous sampling, using a 29-gauge insulin syringe (Becton Dickinson). Duplicate venous samples (300 μl) were taken at the completion of the experiment. The blood samples were placed on ice and immediately centrifuged, and the plasma was stored at −20°C until assayed. The total blood volume withdrawn from the animals before the final arterial and venous samples did not exceed 1.1 ml and was easily compensated by the volume of fluid (normal saline) infused over the duration of the experiment.

**Microvascular perfusion.** Microvascular perfusion was determined by measuring the metabolism of systemically infused 1-methylxanthine (1-MX; Sigma Aldrich, St. Louis, MO) as described previously (10, 18–20, 26, 31, 34, 40). 1-MX was infused at a constant rate (0.4 mg min⁻¹ kg⁻¹) for 60 min. Allopurinol (10 μmol/kg) was administered as a bolus dose 5 min before the commencement of 1-MX infusion (Fig. 1). This allowed constant saturating arterial levels of 1-MX (~20 μM) to be maintained throughout each experiment. Plasma (100 μl) was taken from arterial and leg venous blood samples at the end of the experiment, and the concentration of 1-MX was determined using HPLC. Microvascular perfusion, expressed as 1-MX metabolism (nmol/min), was calculated from the difference between arterial and venous plasma 1-MX concentrations and multiplied by femoral artery blood flow. The concentration of 1-MX in plasma was corrected for the volume accessible to 1-MX, determined from plasma concentrations obtained after removal of red blood cells.

**Muscle glucose uptake.** At 10 min before the completion of each experiment (Fig. 1), a 50-μCi bolus of 2-deoxy-D-[2,6-³H]glucose (2-[³H]DG; specific activity 1.9 TBq/mmol; Amersham Life Science, Castile Hill, NSW, Australia) in saline (154 mmol/l NaCl) was administered via the jugular vein. Immediately after the administration of the 2-[³H]DG, an arterial blood sample (500 μl) was withdrawn by an automated syringe pump at 50 μl/min for 10 min. From this blood sample, a plasma sample (25 μl) was collected to determine the averaged plasma specific radioactivity of 2-[³H]DG. At the conclusion of the experiment, the calf muscles (gastrocnemius, plantaris, and soleus) from the test and contralateral legs were rapidly removed from the hindlimb, frozen in liquid nitrogen, and stored at −80°C for analysis. Frozen muscles were ground under liquid nitrogen and homogenized using an Ultra Turrax (IKA, Wilmington, NC). Free and phosphorylated 2-[³H]DG were separated by ion exchange chromatography using an anion exchange resin (AG1-X8; Bio-Rad) (12, 14). Biodegradable counting scintillant (Amersham, Arlington Heights, IL) was added to each radioactive sample, and radioactivity was determined using a scintillation counter (Tri-Carb 2800TR; Perkin Elmer, Chicago, IL). From this measurement, plasma glucose and the amount of plasma radioactive 2-[³H]DG, the rate of muscle 2-[³H]DG uptake (Rₚ) was calculated as previously described (12, 14).

**Data analysis.** All data are expressed as means ± SE; if the error bars are not visible, they are within the symbol. Mean femoral artery blood flow, mean heart rate, and mean arterial pressure were calculated from 5-s subsamples of the data, representing ~500 flow and pressure measurements every 10 min.

**Statistical analysis.** Repeated-measures two-way ANOVA was used to determine if there were differences between the treatment groups over the time course of the experiment. When a significant difference (P < 0.05) was found, pairwise comparisons by the Student-Newman-Keuls test were used to determine at which time points differences were significant. This was used for femoral artery blood flow, blood pressure, and heart rate. Comparisons between the test and control legs in the systemic saline and insulin groups for 1-MX metabolism and Rₚ were made using a Student’s paired t-test, followed by pairwise comparisons using the Student-Newman-Keuls test. All tests were performed using the SigmaStat statistical program (Systat Software, San Jose, CA).

**RESULTS**

**Systemic effects of local L-NAME.** Local L-NAME infusion in one hindleg of the rat did not have any detectable systemic cardiovascular effects, indicating that it did not significantly spill over into the systemic circulation. Basal mean arterial blood pressure [time (t) = 0 min] for the saline and insulin group was 109 ± 3 and 112 ± 3 mmHg, respectively. Local
L-NAME infusion in one leg did not have any detectable effect on mean arterial blood pressure over the time course of the experiment (Fig. 2A). Likewise, heart rate did not change from basal ($t = 0$ min) in saline- or insulin-infused groups ($339 \pm 14$ and $340 \pm 11$ beats/min, respectively) and was unaffected by local L-NAME infusion in one leg (Fig. 2B).

Local L-NAME and skeletal muscle blood flow. Figure 3 shows the change in femoral artery blood flow from baseline over the course of the 60-min infusion of saline or insulin. Femoral artery blood flow remained unchanged from basal in the control leg of the saline group; however, local L-NAME infusion significantly reduced femoral blood flow ($P < 0.05$, Fig. 3A). Systemic insulin caused a significant increase in femoral artery blood flow by $\sim 84\%$ at $t = 50$ and 60 min onward in the control leg ($P < 0.05$), and this effect was markedly ($P < 0.05$) attenuated by the infusion of L-NAME in the test leg (Fig. 3B).

Figure 4 shows values for microvascular perfusion measured by 1-MX disappearance at the conclusion of the experiment. Local L-NAME had no effect on muscle microvascular perfusion compared with the control leg in the systemic saline-infused group. Systemic insulin increased microvascular perfusion by 27% compared with the saline group, and local L-NAME completely blocked ($P < 0.05$) the insulin-mediated increase in microvascular perfusion in muscle (Fig. 4).

Local L-NAME and skeletal muscle glucose uptake. Basal blood glucose concentrations in the saline- and insulin-infused groups were $4.6 \pm 0.2$ and $5.3 \pm 0.2$ mmol/l, respectively. During hyperinsulinemia, blood glucose concentrations were maintained at basal levels during infusion of insulin by administering a variable infusion of 30% glucose solution. Blood glucose levels during the course of saline or insulin infusion did not significantly change from baseline during the course of the experiment. At the conclusion of the 60-min insulin clamp, the GIR required to maintain glycemia in the insulin group reached $12.8 \pm 0.3$ mg·min$^{-1}$·kg$^{-1}$.

Figure 5 shows values for muscle specific glucose uptake ($R_g$) in calf muscle (gastrocnemius, soleus, and plantaris) as measured by 2-deoxyglucose uptake. As expected, insulin significantly ($P < 0.05$) increased muscle glucose uptake by $10.220.32.247$ on September 23, 2017 http://ajpendo.physiology.org/ Downloaded from
in saline-infused rats. P6/group). *P < 0.05 vs. contralateral leg; Student’s paired t-test. #P < 0.05 vs. control leg of systemic saline group; Student’s t-test.

~2.3-fold compared with saline-infused rats. Local infusion of L-NAME significantly (P < 0.05) attenuated insulin-stimulated muscle glucose uptake (34%) compared with the saline control leg (Fig. 5). L-NAME had no effects on muscle glucose uptake in saline-infused rats.

DISCUSSION

The major findings emerging from this study are that local L-NAME infusion in the hindlimb of rats in vivo: 1) completely blocked insulin-induced increases in total muscle blood flow and recruitment of microvasculature in skeletal muscle, 2) reduced insulin-mediated muscle glucose disposal by 34%, and 3) did not alter basal muscle glucose disposal. Therefore, the lack of insulin-stimulated glucose uptake by muscle can be attributed to the loss of the vascular actions of insulin. Local NOS inhibition had no effect on blood pressure or heart rate, indicating that the observed effects on blood flow, glucose uptake, and microvascular perfusion were due to local and not systemic actions of the NOS inhibitor. Thus, insulin-stimulated microvascular blood flow in muscle occurs via a local NOS-dependent mechanism to facilitate insulin and glucose delivery to the myocyte and thereby contribute to overall glucose disposal.

Microvascular recruitment in skeletal muscle is stimulated by local muscle (7), but not intracerebroventricularly (3) infused insulin. Loss of microvascular recruitment has been reported in mice lacking the endothelial insulin-signaling protein insulin receptor substrate (IRS)-2 (15). These data highlight that insulin actions on the vasculature are controlled locally within muscle and are not mediated centrally via the central nervous system. Baron and colleagues reported a number of studies demonstrating insulin increases total limb blood flow via NOS-dependent mechanisms (23, 27). The NOS inhibitor L-NMMA was infused locally in the forearm or leg of human subjects. Although these studies focused on total limb blood flow and not microvascular responses, which are more important for nutrient exchange, they were the first to implicate NO involvement in insulin’s vascular actions.

We have previously reported that systemic NOS inhibition blocks insulin action in muscle microvasculature in rat muscle (31, 32). The first of these studies (31) infused insulin (10 mU·min⁻¹·kg⁻¹) for 2 h with the NOS inhibitor L-NAME. L-NAME increased mean arterial pressure (within minutes) and completely blocked insulin-mediated increases in both femoral artery flow and microvascular recruitment (assessed using two techniques: contrast-enhanced ultrasound and 1-MX metabolism). This occurred in parallel with an ~40% reduction in insulin-mediated glucose uptake by muscle. The second of these studies (32) infused a physiological dose of insulin (3 mU·min⁻¹·kg⁻¹) for 30 min, which causes microvascular recruitment in muscle independent of changes in femoral artery blood flow. In this setting, L-NAME likewise increased blood pressure and completely blocked microvascular recruitment (assessed by contrast ultrasound) and ~50% of glucose disposal. These data demonstrate that it is insulin’s microvascular (and not macrovascular) action that contributes significantly to glucose disposal in skeletal muscle. However, since systemic infusions of the NOS inhibitor had been used in these studies, it was not possible to definitely demonstrate that the microvascular recruitment was dependent on local NO production.

Previous studies by others have shown that systemic NOS inhibitors cause a sudden (within minutes) and marked rise in blood pressure that in turn triggers compensatory responses (9, 31) that may be due to central and sympathetic mechanisms (11). Systemically administered L-NAME has been observed to cross the blood-brain barrier within 2 h in rats, resulting in reduction of NOS brain activity (29). Furthermore, we (3) and others (24) have shown that intracerebroventricular NOS inhibitor administration can induce a state of insulin resistance. We have additionally shown that centrally administered NOS inhibition impairs insulin-stimulated microvascular perfusion (3). Thus it is plausible that central NOS inhibition may at least...

Fig. 4. Microvascular perfusion in skeletal muscle. Hindlimb 1-MX disappearance (MXD) in response to local single-leg infusion of L-NAME (hatched) during systemic saline or insulin infusion. Values were determined from blood samples taken at the conclusion of the experiment and are means ± SE (n = 6/group). *P < 0.05 vs. contralateral leg; Student’s paired t-test. #P < 0.05 vs. control leg of systemic saline group; Student’s t-test.

Fig. 5. Glucose uptake in skeletal muscle. Hindlimb Rg in response to local single-leg infusion of L-NAME (hatched) during systemic saline or insulin infusion. Values were determined from blood samples taken at the conclusion of the experiment and are means ± SE (n = 6/group). *P < 0.05 vs. contralateral leg; Student’s paired t-test. #P < 0.05 vs. control leg of systemic saline group; Student’s t-test.
in part explain the reduction in insulin-stimulated recruitment in the systemic NOS-infused experiments (detailed above).

The present study is the first study to report that insulin stimulates microvascular recruitment in muscle by activating a local muscle NOS-dependent pathway. This finding was made possible by using a novel approach to locally infuse (via the epigastric artery) a NOS inhibitor in one hindleg of the rat in vivo, thus avoiding unwanted systemic side effects associated with systemic L-NAME infusion. The local L-NAME infusion caused no systemic cardiovascular effects indicated by the absence of changes in mean arterial blood pressure and heart rate, or changes in femoral artery blood flow in the contralateral leg.

The present study does not indicate which isoform of NOS is involved in the local production of NO. However, because we have shown previously that the microvascular recruitment occurs before indications of insulin signaling in the skeletal muscle myocyte (32), it is unlikely to involve the μNOS in muscle. Therefore, the most likely candidates are the endothelial eNOS or the constitutive NOS that has been reported in human vascular smooth muscle to be activated by insulin (30). Quon and colleagues have identified insulin-signaling pathway components (IRS, PI3K, Akt) in cultured endothelial cells responsible for activation of eNOS and production of NO (16, 17, 35, 38, 39). These findings along with the observation that eNOS-deficient mice are hypertensive and resistant to insulin-mediated increases in muscle glucose uptake (8, 25) would suggest that the eNOS isoform is the most important, but further studies using isoform-specific NOS inhibitors would be required to fully resolve these issues.

We have previously reported that insulin resistance is associated with microvascular insulin resistance. Infusing vasoconstrictors (20, 22), TNF-α (37), or Intralipid + heparin (5) inhibits insulin-stimulated microvascular perfusion and impairs insulin-mediated glucose uptake. Chronic models of insulin resistance such as the high fat-fed (18, 26) and Zucker obese rats (36) also show impaired microvascular perfusion. Insulin infusion (6), or the ingestion of a mixed meal (33), stimulates microvascular perfusion in muscle of healthy humans, and these vascular actions are blunted in obese normotensive individuals that also display insulin resistance (6, 13).

More recently, we have reported that microvascular responses to insulin are highly sensitive to changes in dietary fat and that microvascular insulin resistance directly contributes to the development of muscle insulin resistance and may represent one of the earliest events in the etiology of insulin resistance (18). All the above conditions have also been reported to be associated with impaired NO-dependent vascular responses. Therefore, the failure of insulin to stimulate microvascular perfusion in muscle during insulin resistance may be due to a general defect in NO-signaling pathways or resistance to the biological actions of insulin on this pathway. Although other investigators have proposed that impaired NO signaling is a major contributor to vascular insulin resistance (28), this study importantly confirms the mechanism of insulin microvascular responses in muscle to be dependent on local NO.

In conclusion, this study demonstrates that local muscle NO is critical for microvascular perfusion and is involved in the normal increases in skeletal muscle glucose uptake during physiological hyperinsulinemia. Thus this study demonstrates that an impaired microvascular responsiveness to insulin, due to NOS inhibition, can contribute to insulin resistance.

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

AUTHOR CONTRIBUTIONS

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