Inhibiting NOS blocks microvascular recruitment and blunts muscle glucose uptake in response to insulin

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Submitted 14 January 2003; accepted in final form 11 March 2003.

Vincent, M. A., E. J. Barrett, J. R. Lindner, M. G. Clark, and S. Rattigan. Inhibiting NOS blocks microvascular recruitment and blunts muscle glucose uptake in response to insulin. Am J Physiol Endocrinol Metab 285:E123–E129, 2003—We examined the effects of inhibiting nitric oxide synthase with NO-nitro-l-arginine-methyl ester (l-NAME) on total hindlimb blood flow, muscle microvascular recruitment, and hindlimb glucose uptake during euglycemic hyperinsulinemia in vivo in the rat. We used two independent methods to measure microvascular perfusion. In one group of animals, microvascular recruitment was measured using the metabolism of exogenously infused 1-methylxanthine (1-MX), and in a second group contrast-enhanced ultrasound (CEU) was used. Hindlimb glucose uptake was measured by arterial-venous concentration differences after 2 h of insulin infusion. Saline alone did not alter femoral artery flow, glucose uptake, or 1-MX metabolism. Insulin (10 μU·min−1·kg−1) significantly increased hindlimb total blood flow (0.69 ± 0.02 to 1.22 ± 0.11 ml/min, P < 0.05), glucose uptake (0.27 ± 0.05 to 0.95 ± 0.08 μmol/min, P < 0.05), 1-MX uptake (5.0 ± 0.5 to 8.5 ± 1.0 nmol/min, P < 0.05), and skeletal muscle microvascular volume measured by CEU (10.0 ± 1.6 to 15.0 ± 1.2 video intensity units, P < 0.05). Addition of l-NAME to insulin completely blocked the effect of insulin on both total limb flow and microvascular recruitment (measured using either 1-MX or CEU) and blunted glucose uptake by 40% (P < 0.05). We conclude that insulin specifically recruits flow to the microvasculature in skeletal muscle via a nitric oxide-dependent pathway and that this may be important to insulin’s overall action to regulate glucose disposal.

Capillary recruitment; nitric oxide; nitric oxide synthase; muscle blood flow.

There is abundant evidence that insulin augments total limb blood flow in humans (2, 29, 33) and experimental animals (20, 22) in a time- and dose-dependent fashion. It has been suggested that this action of insulin could, by facilitating the delivery of glucose and itself to muscle, contribute to insulin’s overall action on glucose disposal (3), although this remains controversial (33).

Substantial evidence suggests that nitric oxide (NO) is involved in insulin’s action to increase limb blood flow in humans (5, 8, 25, 26, 29). NO in muscle is produced by nitric oxide synthase (NOS), located in both vascular endothelium (28) and myocytes (16). Inhibition of NO production by Nω-nitro-l-arginine (l-NMMA) can fully abolish the effect of insulin to increase limb total blood flow in humans (4, 5, 26, 29). In one study, this agent partially blocked (~25%) insulin-mediated glucose uptake as well (5). Baron et al. (6) have also reported that, in the rat, l-NMMA increases mean arterial pressure and reduces whole body glucose infusion rate in a dose-dependent fashion during a euglycemic insulin clamp (12 mU·min−1·kg−1). Using intravital microscopy, Chen and Messina (8) demonstrated that insulin induces vasodilation of first-order arterioles in rat cremaster and that the addition of the NOS inhibitor Nω-nitro-l-arginine (l-NNA) or the removal of the endothelium prevented this dilation.

This suggests that these proximal arterioles are one potential site of action. Furthermore, endothelial NOS knockout mice are hypertensive and are not capable of increasing muscle blood flow (13) or glucose uptake (13, 27) in response to insulin to the same extent as wild-type mice.

Using three different techniques, clearance of 1-methylxanthine (1-MX, a chemical index of capillary exposure) (21), laser Doppler flowmetry (9), and contrast-enhanced ultrasound (CEU) (12), we have demonstrated (30) that insulin not only enhances total flow but also results in microvascular recruitment in muscle. This effect is presumably secondary to an action of insulin on the terminal arterioles that regulates flow distribution within capillary beds. To date, the involvement of NO in insulin’s vascular action on muscle has only been assessed by measurement of total limb blood flow. These effects result from actions on medium-sized (50- to 300-μm) arterioles that modulate vascular resistance. Renkin (23) has suggested that capillary recruitment, rather than increases in total flow, is the more important vascular response to enhance the exchange of nutrients including glucose. Thus the present study was designed to determine whether NOS inhibition affects insulin-mediated microvascular recruitment and glucose disposal.

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MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 250–350 g were obtained from Hilltop Laboratory Animals (Scottsdale, PA). Animals were housed at 22 ± 2°C on a 12:12-h light-dark cycle and were fed ad libitum. The University of Virginia Animal Care and Use Committee approved the experimental protocols.

Surgical Procedure

Rats were anesthetized with pentobarbital sodium (55 mg/kg body wt ip), and polyethylene cannulas were inserted into the carotid artery for arterial blood sampling and measurement of arterial blood pressure and into both jugular veins for intravenous infusions. A tracheostomy was performed to facilitate spontaneous respiration during the experiment. Both femoral vessels were exposed via a 1.5-cm incision, the left femoral artery was carefully separated from the femoral vein and nerve, the epigastric vessels were ligated, and a flow probe (Transonic Systems, VB series 0.5 mm) was positioned over the artery. The probe was interfaced through a flowmeter to an IBM-compatible computer. Femoral artery blood flow, blood pressure, and heart rate were measured continuously using WINDAQ data acquisition software (DATAQ Instruments). Anesthesia was maintained by a continuous infusion of aqueous pentobarbital sodium (0.6 mg·min⁻¹·kg⁻¹) via the carotid artery. A heat lamp positioned above the rat maintained body temperature.

Experimental Protocols

After surgical preparation, 1 h was allowed for blood flow, blood pressure, and heart rate to stabilize. Microvascular blood volume was then measured using either 1-MX disappearance or video intensity by CEU.

Protocol 1: hindlimb disappearance of 1-MX. Rats were infused for 2 h with either saline (10 μL/min), insulin (10 mU·min⁻¹·kg⁻¹ euglycemic clamp), Nω-nitro-arginine-methyl ester (L-NAME, 3 mg/kg bolus followed by a continuous infusion of 50 μg·min⁻¹·kg⁻¹) as described in protocol 1. L-NAME was selected on the basis of preliminary experiments. This dose was selected to ensure both microvascular recruitment and the high physiological-low pharmacological range. This dose of insulin was started 5 min before the insulin was started. This insulin infusion rate produces plasma insulin concentrations in the range or video intensity by CEU.

Protocol 2: CEU. These animals were infused with either insulin (10 mU·min⁻¹·kg⁻¹) or insulin with a superimposed infusion of L-NAME (3 mg/kg bolus followed by a continuous infusion of 50 μg·min⁻¹·kg⁻¹) as described in protocol 1. CEU was used to measure microvascular blood volume at baseline and after the 2-h infusion. In previous experiments (12, 30), we have shown that saline infusion for 2 h does not affect microvascular volume.

CEU has been used to measure microvascular perfusion in the myocardium (32) and has recently been applied to brain (24) and renal perfusion (17). Most recently, we (12, 30) have applied this technique to measure the effect of insulin on microvascular perfusion in skeletal muscle. This noninvasive imaging technique relies on the ultrasound detection of 2- to 5-μm perfluorocarbon-filled bubbles during their microvascular transit. The acoustic properties of these bubbles are such that it is possible to burst bubbles with a single pulse of high-energy ultrasound and then collect sequential images with variable delay times following bubble destruction. Because flow through arterial and arteriolar vessels is rapid relative to flow through capillaries, images acquired shortly after bubbles are destroyed and reperfusion begins are enriched with signal from these larger vessels. With time, the signal contributed by the microvascularature increases. By image subtraction, video intensity attributable to arterial and arteriolar vessels can be largely eliminated. The resulting images are strongly enriched for microvascular vessels.

To apply this to the rat hindlimb, a linear-array transducer interfaced with an ultrasound system (HDI-5000, Philips Ultrasound) was positioned over the right proximal adductor muscle group (adductor magnus and semimembranosus) and secured for the duration of the experiment. Pulse inversion imaging was performed at a transmission frequency of 3.3 MHz and a mechanical index of 0.8. The acoustic focus was set at the midportion of the muscles. Gain settings were optimized and held constant. Data were recorded digitally on a Sony Magneto Optical Disk. Albumin microbubbles (Optison, Mallinckrodt Medical) were infused into the jugular vein at 90 μL/min for the duration of data acquisition.

Images were acquired during continuous imaging at 23 Hz and at pulsing intervals from 1 to 25 s. Data were analyzed off-line. Frames were aligned by cross-correlation, and several frames during continuous imaging were averaged and digitally subtracted from averaged frames obtained at each pulsing interval. This background subtraction served to eliminate signal from large vessels (>100 μm) (12). The background-subtracted video intensity at each pulsing interval was measured from the muscle, and pulsing interval vs. video intensity data were fitted to the function, y = A(1 − e−kt), where y is video intensity, t is the pulsing interval, and A is plateau video intensity (an index of microvascular blood volume) (32). The ultrasound probe is secured for the duration of the experiment; therefore, the area imaged at the start of the experiment is identical to the area imaged at the end of centrifuged, and 100 μL of plasma were added to 20 μL of 2 M perchloric acid. The mixture was immediately neutralized with 12 μL of 2.5 M K₂CO₃ and stored at −20°C until analysis of 1-MX. The concentration of 1-MX in these samples was measured using reverse-phase HPLC, and the hindlimb disappearance of 1-MX was obtained from the arterial-venous 1-MX concentration difference multiplied by femoral artery flow (21). Insulin was measured with a double-antibody radioimmunoassay using an anti-human insulin antibody. In both protocols 1 and 2, we measured arterial minus venous glucose concentration differences at 2 h into the insulin infusion when the glucose infusion rate had reached a steady state.

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the experiment. For the analysis, a region of interest is drawn around the muscle group, and the intensity of the pixels within that region of interest is averaged. This region of interest is the same size and at the same location for all images. The integrated pixel intensity is presented as video intensity. In this manner, CEU provides a quantitative measure of microbubble content in the image volume, avoiding interobserver variability.

**Statistical Analysis**

Statistical comparisons between groups in protocol 1 were performed by either two-way repeated-measurements ANOVA or one-way ANOVA. When a significant difference of \( P < 0.05 \) was found, pairwise comparisons by Student-Newman-Keuls test were used to determine in which of the individual treatments the differences were significant. Student’s paired \( t \)-test was used for statistical comparisons within each treatment group for both groups in protocol 2. Significance was recognized at \( P < 0.05 \).

**RESULTS**

**Protocol 1**

There were no differences in rat weight, basal glucose, heart rate, mean arterial pressure, or femoral artery flow among the four groups (Table 1). Likewise, basal plasma insulin concentrations did not differ between groups and averaged 251 ± 55 pmol/l. After the 2-h infusion period, arterial glucose and heart rate were again not different among groups (Table 1). Plasma insulin levels were significantly (\( P < 0.01 \)) higher in animals infused with insulin (1,747 ± 88 pmol/l) or insulin + L-NAME (1,464 ± 199 pmol/l), whereas L-NAME alone (242 ± 61 pmol/l; \( n = 5 \)) had no effect compared with saline. The mean hindlimb vascular resistance and mean arterial pressure were significantly higher (\( P < 0.05 \)) in both groups that received L-NAME compared with saline (Table 1). Femoral artery blood flow increased in response to insulin [from 0.69 ± 0.02 to 1.22 ± 0.11 ml/min (\( P < 0.05 \))], and L-NAME infusion completely abolished the insulin-induced increment in femoral artery flow. To maintain euglycemia, the insulin-alone group required glucose infusion at an average rate of 23.9 ± 1.2 mg·min\(^{-1}\)·kg\(^{-1}\), and this did not change significantly when the L-NAME infusion was superimposed (21.9 ± 1.4 mg·min\(^{-1}\)·kg\(^{-1}\)).

Insulin significantly (\( P < 0.05 \)) enhanced the arterial-venous blood glucose difference across the hindlimb; consequently, hindlimb glucose uptake (arterial-venous × flow) rose 3.5-fold (Fig. 1). A superimposed infusion of L-NAME did not affect the arterial-venous blood glucose difference, but hindlimb glucose uptake was blunted by 40% (\( P < 0.05 \)). L-NAME alone had no effect on arterial-venous blood glucose difference or hindlimb glucose uptake compared with saline.

At the end of the experiment, arterial levels of 1-MX did not differ significantly among groups, indicating that the whole body clearance rate of 1-MX for each group was the same (data not shown). The hindlimb arterial-venous 1-MX difference was the same for all groups (Fig. 2A). However, when the Fick principle was applied, i.e., arterial-venous 1-MX difference multiplied by femoral artery flow, insulin increased the rate of hindlimb 1-MX disappearance by 60% (\( P < 0.01 \)) compared with saline (Fig. 2B). This is a finding consistent with insulin enhancing the exposure of 1-MX to capillary xanthine oxidase (21). L-NAME completely abolished the effect of insulin to increase hindlimb 1-MX disappearance. L-NAME alone did not alter 1-MX disappearance compared with saline.

**CEU**

The effects of insulin or insulin + L-NAME on microvascular blood volume measured using CEU are shown in Fig. 3. Insulin infusion for 2 h significantly increased (\( P < 0.05 \)) microvascular blood volume above baseline (A values from 10.0 ± 1.6 to 15.0 ± 1.2, \( P < 0.05 \)). This fractional increase in microvascular blood volume was similar to the 60% increase in 1-MX metabolism seen in the studies described above. When L-NAME was coinfused with insulin for a total of 2 h, microvascular blood volume did not change from baseline (A values of 12.0 ± 1.6 to 11.2 ± 1.5). This result is in accord with the findings above showing that L-NAME prevented insulin-induced increments in 1-MX disappearance.

### Table 1. Effect of insulin, insulin + L-NAME, saline, or L-NAME alone on systemic and hindlimb hemodynamic factors, blood glucose concentrations, and glucose infusion rate required to maintain basal blood glucose at the end of a 2-h infusion period

<table>
<thead>
<tr>
<th></th>
<th>Insulin (n = 8)</th>
<th>Insulin + L-NAME (n = 9)</th>
<th>Saline (n = 7)</th>
<th>Insulin (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arterial glucose, mg/dl</td>
<td>86 ± 5</td>
<td>94 ± 7</td>
<td>77 ± 7</td>
<td>80 ± 6</td>
</tr>
<tr>
<td>Glucose infusion rate, mg·min(^{-1})·kg(^{-1})</td>
<td>23.9 ± 1.2</td>
<td>0</td>
<td>21.9 ± 1.4</td>
<td>0</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>330 ± 18</td>
<td>335 ± 10</td>
<td>342 ± 13</td>
<td>333 ± 14</td>
</tr>
<tr>
<td>Mean arterial pressure, mmHg</td>
<td>118 ± 7</td>
<td>138 ± 14</td>
<td>143 ± 8(\uparrow)</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>Hindlimb vascular resistance, mmHg·min·ml(^{-1})</td>
<td>144 ± 7</td>
<td>95 ± 8(\uparrow)</td>
<td>156 ± 10</td>
<td>180 ± 14(\uparrow)</td>
</tr>
<tr>
<td>Femoral artery blood flow, ml/min</td>
<td>0.69 ± 0.02</td>
<td>1.22 ± 0.11(\uparrow)</td>
<td>0.79 ± 0.07</td>
<td>0.83 ± 0.07(\uparrow)</td>
</tr>
</tbody>
</table>

Values are means ± SE. L-NAME, \( N^\circ \)-l-nitro-arginine methyl ester. \(*P < 0.05\) compared with baseline; \(\dagger P < 0.05\) compared with 2-h saline; \(\uparrow P < 0.05\) compared with 2-h insulin; 2-way repeated-measurements ANOVA.
DISCUSSION

In addition to the action of insulin to increase total hindlimb blood flow in the rat, we have previously demonstrated, using several techniques, that insulin increases microvascular recruitment in rat and human skeletal muscle. By use of measurements of either the hindlimb metabolism of 1-MX (21), laser Doppler flowmetry (9), or CEU (12, 30), insulin has a clear action to increase microvascular perfusion. This action of insulin on microvascular recruitment has not been well studied by other investigators, largely due to the lack of widely available methods to study the microvasculature in vivo. It bears emphasis that measures of microvascular recruitment are not necessarily mirrored by changes in total blood flow. We recently examined the time course for insulin-mediated microvascular recruitment in the anesthetized rat (30). Femoral artery flow was measured continuously by ultrasonic flow probe during the infusion of a physiological dose of insulin, and microvascular recruitment was assessed by CEU at baseline and at 30, 90, and 120 min of infusion. Insulin required 120 min to augment femoral artery flow; however, microvascular recruitment was significantly elevated by 30 min and did not increase further during the 2-h infusion. Similarly, in recent studies in human forearm (11), we observed that insulin recruits forearm microvasculature in the absence of effects on total flow.

The relationship between insulin’s action on total muscle blood flow and glucose uptake remains controversial. Although some investigators have shown a close relationship between the dose-dependent effect of insulin on total limb flow and its metabolic effect (2), others have reported a temporal dissociation between insulin-stimulated glucose uptake and total muscle flow (33). In addition, some investigators have reported no effect of insulin on total flow, particularly at physiological concentrations (18, 33). All of these observations have been restricted to measurements of total limb flow rather than microvascular recruitment. However, not all agonists that increase total limb flow also...
increase capillary recruitment and vice versa. For example, we have previously demonstrated in the rat that epinephrine infusions that increase hindlimb blood flow comparably to that provoked by insulin do not mimic insulin’s effects on either hindlimb I-MX disappearance (21) or laser Doppler signal (9). These findings are consistent with epinephrine increasing flow through previously perfused microvascular networks without significant recruitment of new microvascular units. On the basis of the model of tissue perfusion proposed by Renkin (23), Bonadonna et al. (7) have suggested that microvascular recruitment might bear more importantly than total flow on the extent of insulin-induced glucose uptake. We have previously shown that insulin can enhance microvascular flow independently of changes in total limb flow in both rats (30) and humans (11). Furthermore, in the rat, microvascular recruitment temporally parallels changes in glucose infusion rate (30).

The mechanism of insulin-mediated capillary recruitment in skeletal muscle is not well understood. We have previously demonstrated, using several different interventions, that insulin’s vascular actions are important for augmenting glucose disposal. For example, α-methylserotonin diminishes insulin-induced increase in femoral artery flow and capillary recruitment while simultaneously decreasing glucose disposal (22). Within the same context, TNF-α blocked insulin-induced increases in femoral artery flow, capillary recruitment, and glucose disposal (34). We have also shown that models of insulin resistance such as elevated free fatty acids in normal rats (10) or in genetically obese Zucker rats (31) exhibit a diminished effect of insulin on bulk limb blood flow, capillary recruitment, and glucose disposal. It is not yet apparent how the aforementioned interventions are acting to modulate insulin’s vascular actions: is it via a direct blockade of NOS, via blockade of the insulin-signaling pathway or via the stimulation of a distinct vasoconstrictor pathway acting in opposition to NO-mediated dilation? Likewise, the basis of the relationship between the vascular effect and the simultaneously observed effects on glucose disposal was uncertain. Thus the focus of the current work was to look specifically at the relationship between capillary recruitment and activation of NOS to address whether this pathway was involved in the physiological process responsible for the capillary recruitment and the impact of specific blockade of this pathway on muscle glucose metabolism.

Several studies have suggested that insulin increases NO production both in vitro (8, 15) and in vivo (29). Inhibition of NOS causes a dose-dependent increase in mean arterial pressure and a reduction in insulin-mediated glucose disposal in rats (6). In addition, inhibitors of NOS block the effect of insulin to increase limb blood flow and microvascular recruitment by processes that are each blocked by inhibition of NOS. Thus both actions of insulin involve, at least in part, the activation of the NOS pathway. Changes in total limb flow are controlled by resistance arterioles, whereas changes in capillary flow are controlled by smaller terminal arterioles (21). Thus the capillary recruitment studied here involves relaxation of terminal arterioles by insulin. L-NAME was also observed to inhibit insulin-mediated glucose uptake by 40% and to increase mean arterial pressure. These findings strongly suggest a relationship between the effect of insulin on the vasculature and its effect on glucose disposal. The decline in hindlimb glucose uptake and the decreased total blood flow and microvascular recruitment within skeletal muscle all suggest that L-NAME provoked skeletal muscle insulin resistance. In the same context, our present findings are similar to the effect of the vasoconstrictor α-methylserotonin, where blood pressure also increased and insulin-mediated capillary recruitment and total blood flow as well as ~50% of muscle glucose uptake were blocked (22). However, from data using the same agonist in the isolated perfused rat hindlimb at constant total flow, we proposed that the inhibition of glucose uptake by muscle in vivo (19) was due to blood flow being redirected from the nutritive to the nonnutritive network. Thus this is also a possibility that could explain the findings herein. Taken together, the results of the present study are consistent with the hypothesis that there is a relationship between microvascular recruitment and glucose uptake, since L-NAME blocked insulin-mediated microvascular recruitment and simultaneously blunted hindlimb glucose uptake.

In the present study, L-NAME blunted hindleg insulin-mediated glucose uptake by 40%, but whole body glucose infusion rates were diminished by only 8%, and this latter effect was not statistically significant. At first consideration, it might be expected that both measurements of glucose metabolism would decline in parallel. However, insulin-stimulated hindlimb glucose uptake by ~0.65 μmol/min and L-NAME lowered this by 0.30 μmol/min. When it is considered that the hind-

Fig. 3. Changes in hindlimb microvascular blood volume measured by contrast-enhanced ultrasound in response to insulin or insulin + L-NAME at the end of the 2-h infusion period. Values are means ± SE for 4 animals. *Significantly different (P < 0.05) from baseline value; Student’s paired r-test.
limb muscle perfused by the femoral artery (determined by Evans blue dye infusion into the femoral artery of rat hindlimb) represents 7 g, or 1/15th of total body mass, in a 300-g rat, we estimate that a similar 40% inhibition of total body muscle glucose uptake would result in a 4.5 μmol/min decline in the glucose infusion required to maintain euglycemia. This is not different from the 3.3 μmol/min (which equates to 2.0 mg·min⁻¹·kg⁻¹ for a 300-g rat) difference observed for the whole body glucose infusion rate in the present study (see Table 1).

Although the effect of L-NAME to inhibit skeletal muscle glucose uptake suggests a vascular basis for this inhibition, we cannot exclude the possibility that NO might be having a secondary action related to a rise in free fatty acid or ANG II concentrations or other mechanisms to blunt glucose disposal.

A case for direct involvement of NO in the metabolic effects of insulin in muscle is not clear. The focus by others has been on studies with isolated incubated muscles, where vascular involvement is minimal and access is by diffusion from the incubating medium. Considering in vitro studies of insulin-stimulated glucose uptake, most workers report that inhibition of NOS has had no effect. Thus L-NMMA or L-NAME had no effect on insulin-stimulated 2-deoxyglucose uptake in isolated extensor digitorum longus (1) or epitrochlearis (14) muscles. Taken together, such findings suggest that the effects of NOS inhibition to reduce muscle glucose uptake in the present study can be attributed to the inhibition of insulin’s vascular effects.

In summary, we observed that insulin’s vascular actions in muscle to dilate both resistance vessels (and thereby increase total flow) and terminal arterioles (and thereby recruit microvascular vessels) each require activation of NOS. Specific blockade of NOS blunts insulin’s metabolic effect. The relative contribution of insulin’s vascular action on capillary recruitment and total flow to its metabolic actions requires further investigation.

This work was supported by National Institutes of Health grants DK-57878 and KO8 HL-03810 and by a grant-in-aid from the American Diabetes Association.

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