

# Insulin-mediated Skeletal Muscle Vasodilation Is Nitric Oxide Dependent

## A Novel Action of Insulin to Increase Nitric Oxide Release

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### Abstract

The purpose of this study was to examine whether insulin's effect to vasodilate skeletal muscle vasculature is mediated by endothelium-derived nitric oxide (EDNO). *N*-monomethyl-L-arginine (L-NMMA), a specific inhibitor of NO synthase, was administered directly into the femoral artery of normal subjects at a dose of 16 mg/min and leg blood flow (LBF) was measured during an infusion of saline (NS) or during a euglycemic hyperinsulinemic clamp (HIC) designed to approximately double LBF. In response to the intrafemoral artery infusion of L-NMMA, LBF decreased from  $0.296 \pm 0.032$  to  $0.235 \pm 0.022$  liters/min during NS and from  $0.479 \pm 0.118$  to  $0.266 \pm 0.052$  liters/min during HIC,  $P < 0.03$ . The proportion of NO-dependent LBF during NS and HIC was  $\sim 20\%$  and  $\sim 40\%$ , respectively,  $P < 0.003$  (NS vs. HIC). To elucidate whether insulin increases EDNO synthesis/release or EDNO action, vasodilative responses to graded intrafemoral artery infusions of the endothelium-dependent vasodilator methacholine chloride (MCh) or the endothelium-independent vasodilator sodium nitroprusside (SNP) were studied in normal subjects during either NS or HIC. LBF increments in response to intrafemoral artery infusions of MCh but not SNP were augmented during HIC versus NS,  $P < 0.03$ . In summary, insulin-mediated vasodilation is EDNO dependent. Insulin vasodilation of skeletal muscle vasculature most likely occurs via increasing EDNO synthesis/release. Thus, insulin appears to be a novel modulator of the EDNO system. (*J. Clin. Invest.* 1994. 94:1172–1179.) Key words: euglycemic hyperinsulinemic clamp • leg blood flow • methacholine chloride • sodium nitroprusside • vascular resistance

### Introduction

Insulin increases glucose uptake principally in skeletal muscle. This occurs largely by recruitment and activation of specific transporter proteins to the plasma membrane, which facilitate the diffusion of glucose down its concentration gradient. Recent reports from our laboratory (1, 2) and others (3–8) have demonstrated that physiological circulating insulin concentrations also increase blood flow to skeletal muscle, i.e., insulin and glucose delivery. We have previously proposed that this hemo-

dynamic action is an important physiological mechanism to amplify insulin's overall action to increase glucose uptake by augmenting the delivery of insulin and glucose via capillary and, thus, tissue recruitment. In support of this idea we have recently shown that skeletal muscle blood flow, when increased pharmacologically while maintaining both insulin and glucose concentrations constant, can independently augment insulin-mediated glucose uptake (9). Moreover, Buchanan and co-workers (10) also recently described an additional rise in insulin-stimulated leg glucose uptake secondary to an augmentation in leg blood flow mediated by angiotensin II.

Insulin-mediated increments in skeletal muscle blood flow are blunted in the insulin-resistant states of obesity, type II diabetes, and elevated blood pressure (1, 2, 11), suggesting that reduced skeletal muscle perfusion may contribute to the insulin resistance observed in these patients. Therefore, regulation by insulin of skeletal vascular tone may be an important determinant of insulin sensitivity.

The mechanism by which insulin causes vasodilation in skeletal muscle vasculature is unknown. Insulin-dependent vasodilation could be caused by a number of potential mechanisms involving either the vascular smooth muscle, the vascular endothelium, or both. Insulin has been shown to have a direct relaxing effect on vascular smooth muscle by stimulation of Na/K ATPase with resultant hyperpolarization and decreased calcium influx (12–14). This mechanism would have an effect to modulate the sensitivity of smooth vascular muscle to vasoactive agents (15). Insulin could (directly or indirectly) increase or inhibit the release of endothelial vasoactive substances (16, 17). It is also possible that insulin increases blood flow via modulation of putative vasodilatory cholinergic sympathetic nerve fibers as described in animals (18). Alternatively, vasodilation could be related to a coupling of cellular metabolic activity (glucose metabolism) to vascular tone as we have previously proposed (1).

In 1980, Furchgott and Zawadzki (19) demonstrated that intact vascular endothelium was required for the relaxation of arterial smooth muscle by acetylcholine. It has since been shown that the release of nitric oxide (NO)<sup>1</sup> by the endothelium in response to acetylcholine accounts for most of the relaxing activity (20). Endothelium-derived NO (EDNO) is now recognized as the most potent vasodilating substance. Of importance in this regard is that the response to acetylcholine in vivo has been reported to be abnormal in diabetes (21, 22) and hypertension (23).

The purpose of our study was to test the hypothesis that

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1. Abbreviations used in this paper: EDNO, endothelium-derived nitric oxide; HIC, hyperinsulinemic clamp; LBF, leg blood flow; L-NMMA, *N*-monomethyl-L-arginine; LVR, leg vascular resistance; MAP, mean arterial pressure; MCh, methacholine chloride; NO, nitric oxide; NS, saline infusion; SNP, sodium nitroprusside.

Table I. Demographic Characteristics of the Study Groups

	Study 1 L-NMMA			Study 2 MCh	Study 3 SNP
	Group A (NS)	Group B (insulin)	Group C (insulin ± L-NMMA)		
Gender (male/female)	7/0	5/1	3/0	14/0	8/0
<i>n</i>	7	6	3	14	8
Age (y)	32.3±2.1	30.2±2.5	36.3±1.2	37.8±1.0	32.9±2.1
Weight (kg)	75.1±4.8	70.2±3.9	82.9±8.4	87.7±4.5	76.9±4.2
Body mass index	23.5±1.2	23.2±1.1	25.9±1.4	28.2±1.4	25.1±1.3
Fat (%)	21.3±2.5	21.3±4.4	17.1±3.5	26.9±2.6	22.0±2.4

insulin causes skeletal muscle vasodilatation via EDNO. To this end, we examined the dependency of leg blood flow on EDNO in normal subjects during normal saline (NS) infusion and during a hyperinsulinemic euglycemic clamp by performing local intrafemoral artery infusions of *N*-monomethyl-L-arginine (L-NMMA), a specific inhibitor of NO synthase (24). To further elucidate whether insulin modulates EDNO synthesis/release or EDNO action, we constructed dose-response curves before and during euglycemic hyperinsulinemic clamps to intrafemoral artery infusions of methacholine chloride (MCh), which causes endothelium-dependent vasodilatation, and sodium nitropruside (SNP), an endothelium-independent vasodilator.

## Methods

### Subjects

The characteristics of the study groups are shown in Table I. All subjects had normal serum glucose levels, all but one had a normal 75-g oral glucose-tolerance test, and none were diabetic. All subjects were normotensive as determined by cuff blood pressure, euthyroid, and on no medications. Volunteers gave informed consent and were admitted 1–2 d before the study on the General Clinical Research Center at Indiana University Hospital. Studies were approved by the Indiana University Human Subjects Institutional Review Board.

### Diet

The caloric content of the diets while hospitalized was standardized to be distributed as 50% carbohydrate, 30% fat, and 20% protein. The diets were designed to be weight maintaining.

### Drugs

All infusates were prepared under sterile conditions on the morning of the study. L-NMMA (Calbiochem Corp., San Diego, CA) was dissolved in NS to a concentration of 8 mg/ml. MCh (Roche Laboratories, Nutley, NJ) was dissolved in NS to a concentration of 25 µg/ml and SNP (Roche Laboratories) was dissolved in NS to a concentration of 7 µg/ml.

Regular insulin (Humulin; Eli Lilly and Co., Indianapolis, IN) was diluted in NS with added albumin to the desired concentration. All study drugs with the exception of insulin, which was administered through a catheter in an antecubital vein, were infused directly into the femoral artery using a programmable pump (model 44; Harvard Apparatus, South Natick, MA).

### Protocol

At ~7:00 a.m., after an overnight 14-h fast, a catheter was inserted into the antecubital vein for infusion of substances. Subsequently, the

right femoral artery and vein were cannulated. A 5 French sheath (Cor-dis Laboratories Inc., Miami, FL) was placed in the right femoral vein to allow the insertion of a custom designed 5 French double lumen thermodilution catheter (Baxter Scientific, Edwards Division, Irvine, CA) to measure leg blood flow (LBF) as described previously (25). The right femoral artery was cannulated with a 5.5 French triple lumen catheter (Arrow International, Reading, PA) to allow simultaneous infusion through the proximal port (most caudad), invasive pressure monitoring through the middle port, and systemic arterial blood sampling through the distal port (most cephalad). Heart rate (HR) and mean arterial blood pressure (MAP) were monitored continuously via precordial leads and a pressure transducer connected to a vital signs monitor (VSM 1; Physiocontrol, Redmond, WA).

All hemodynamic measurements were obtained with the subjects in the supine position. Baseline measurements of LBF, MAP, and HR were obtained after allowing ≥ 30 min of rest after the insertion of the catheters.

All euglycemic hyperinsulinemic clamps were performed with a square wave infusion of insulin at doses according to the study protocols described below. The serum glucose concentration was kept at the baseline level by administering a 20% dextrose solution at a variable rate according to arterial serum glucose measurements obtained at 5-min intervals. K<sub>2</sub>HPO<sub>4</sub> (~ 0.001–0.0038 meq/kg per minute) was infused during the euglycemic hyperinsulinemic clamps to prevent hypokalemia and hypophosphatemia. Serum potassium levels were maintained > 3.5 meq/liter during all study conditions.

*Study 1.* To investigate the dependence of LBF on EDNO, L-NMMA, a specific inhibitor of NO synthetase, was infused directly into the femoral artery at baseline and during a euglycemic hyperinsulinemic clamp. LBF was studied in two separate groups. Group A (*n* = 7) underwent an intrafemoral artery square wave infusion of 16 mg/min L-NMMA during administration of NS only. Group B (*n* = 6) underwent a euglycemic hyperinsulinemic clamp (120 mU/m<sup>2</sup> per minute) designed to achieve high physiological insulin concentrations and increase LBF approximately twofold. After 3 h of euglycemic hyperinsulinemia, each subject received an intrafemoral artery square wave infusion of L-NMMA at a rate of 16 mg/min. Near steady state glucose infusion rates were achieved in all subjects. The L-NMMA infusate flow rate was 2 ml/min and was administered for 15 min in both groups. 4 min after the onset of the L-NMMA infusion, measurements of LBF were performed repeatedly for the next 10 min for a total of 20 measurements of LBF. MAP and HR were recorded with every other measurement of LBF.

Because the study protocol in group B addresses only whether steady-state insulin-mediated vasodilation is EDNO dependent, we wished to examine whether the initial phase of insulin-mediated vasodilation is EDNO dependent also. To this end, in three subjects (group C), LBF was determined during either an intrafemoral artery infusion of insulin alone or on a separate day during an infusion of insulin started 5 min after initiation of an infusion of L-NMMA. Insulin was infused directly into the femoral artery at a dose of 4 mU/m<sup>2</sup> per min to achieve physiological concentrations rapidly in the vascular bed studied. L-NMMA was administered at a rate of 16 mg/min and started at time = –5 min and continued for 25 min (total of 30 min). LBF was determined immediately before the infusions were started and at 10 and 20 min during the insulin infusions. Venous insulin levels were obtained after 20 min of insulin infusion.

*Study 2.* To study whether insulin modulates the synthesis/release of EDNO, MCh, an endothelium-dependent vasodilator, was administered as an intrafemoral artery square wave infusion at sequential doses of 2.5, 5.0, 7.5, and 10.0 µg/min. In the same subjects, MCh infusions were performed sequentially during a NS infusion and subsequently during a euglycemic hyperinsulinemic clamp. In this fashion MCh dose-response curves for its effect to vasodilate (increase leg blood flow) were constructed in the absence and presence of exogenous insulin. The MCh infusate flow rate was 0.1–0.4 ml/min. Among the subjects studied, the insulin infusion rate varied: 15 mU/m<sup>2</sup> per min (*n* = 7), 20 mU/m<sup>2</sup> per min (*n* = 2), 30 mU/m<sup>2</sup> per min (*n* = 1), and 40

mU/m<sup>2</sup> per min ( $n = 4$ ). All infusion rates were designed to achieve physiological insulin concentrations.

**Study 3.** To investigate whether insulin modulates the vascular response to EDNO, SNP, which acts as exogenous NO donor and directly dilates smooth vascular muscle (endothelium-independent vasodilation), was administered as an intrafemoral artery square wave infusion at sequential doses of 1.75, 3.5, and 7.0  $\mu\text{g}/\text{min}$  in the same subject on the same day during NS infusion and subsequently during a euglycemic hyperinsulinemic clamp. Thus, SNP dose-response curves for its effect to vasodilate were constructed in the absence and presence of exogenous insulin. The SNP infusate flow rate was 0.25–1.0 ml/min. Among the subjects studied, the insulin infusion rate varied: 15 mU/m<sup>2</sup> per min ( $n = 6$ ) and 20 mU/m<sup>2</sup> per min ( $n = 2$ ).

In studies 2 and 3, LBF measurements were begun 2 min after the onset of each MCh or SNP infusion. LBF measurements were performed every ~30 s for a total of 10 determinations at each drug dose. MAP and HR were recorded with every other LBF determination. After the completion of the hemodynamic recordings, the intraarterial drug infusion was discontinued and LBF was allowed to return to baseline, after which the subsequent dose was administered.

In previous studies (9) we have demonstrated that low insulin infusion rates are able to increase glucose uptake significantly without altering LBF. Moreover, we have previously established that insulin's ability to increase LBF is directly related to the degree of insulin sensitivity and is inversely related to obesity (1, 11). Therefore, in studies 2 and 3 the insulin infusion rates were established a priori on the basis of the subject's adiposity (percent body fat) and was designed to increase glucose uptake but not markedly alter LBF. Each euglycemic hyperinsulinemic clamp was carried out until steady state conditions were achieved (~140–200 min). At that time, repeat measurements of LBF, MAP, and HR were obtained, followed by the infusion of the vasodilator drugs according to the study groups.

Because of technical problems, blood pressure could not be monitored during all drug infusions in study 2 ( $n = 2$ ) and study 3 ( $n = 1$ ). Likewise, because of technical reasons or the inability to reestablish baseline blood flow after drug administration, not all subjects in studies 2 and 3 received all drug infusions.

### Analytical methods

Insulin levels were measured using the Coat a Count kit (Diagnostic Products Corp., Los Angeles, CA). Serum glucose levels were determined by the glucose oxidase method with a glucose analyzer (YSI 2300; Yellow Springs Instrument Co., Yellow Springs, OH). The proportion of body fat was determined by dual-energy x-ray absorptiometry, (Lunar Radiation Corporation, Madison, WI).

### Statistical analysis

Results are shown as the mean  $\pm$  SEM. MAP is expressed in mmHg. LBF is expressed in liters/min. Leg vascular resistance (LVR) was calculated as MAP divided by LBF and expressed in arbitrary units. Changes in blood flow and resistance are expressed as percent change to adjust for differences at baseline. Insulin levels are expressed in  $\mu\text{U}/\text{ml}$ . Two-way analysis of variance was used to compare the responses to the graded drug infusions before and during the hyperinsulinemic euglycemic clamp. One-tailed paired and unpaired  $t$  tests were performed as appropriate for comparisons within or between groups. Statistical significance was accepted at a level of  $P < 0.05$ . Statistics were performed on a Macintosh computer with Statview IV (Abacus Concepts, Inc., Berkeley, CA).

## Results

### Studies with L-NMMA (study 1)

**Serum glucose and insulin concentrations.** In group A, fasting glucose and insulin levels were  $88.9 \pm 1.0$  mg/dl and  $5.7 \pm 1.3$   $\mu\text{U}/\text{ml}$ , respectively. In group B, fasting glucose and insulin levels were  $95.5 \pm 2.1$  mg/dl and  $4.3 \pm 1.1$   $\mu\text{U}/\text{ml}$ , respectively.

During hyperinsulinemia (group B) the steady state glucose level was  $89.6 \pm 1.6$  mg/dl ( $P < 0.05$  vs. baseline) and insulin levels were  $218.3 \pm 19.2$   $\mu\text{U}/\text{ml}$  ( $P < 0.0001$  vs. baseline).

In group C, fasting glucose levels were  $89.8 \pm 1.0$  and  $85.9 \pm 5.1$  mg/dl before insulin alone and before L-NMMA, respectively ( $P = \text{NS}$ ). Fasting insulin levels were  $4.7 \pm 0.6$  and  $4.1 \pm 0.3$   $\mu\text{U}/\text{ml}$  before insulin alone and before L-NMMA, respectively ( $P = \text{NS}$ ). Glucose levels did not change significantly in either group during the 25-min infusion of insulin. Steady state venous insulin levels were  $50.0 \pm 21.2$  and  $70.2 \pm 26.1$   $\mu\text{U}/\text{ml}$  during insulin alone and L-NMMA plus insulin, respectively ( $P < 0.05$ ).

**Leg blood flow and vascular resistance.** In group A, basal LBF was  $0.296 \pm 0.032$  liters/min. Intrafemoral artery L-NMMA infusion caused LBF to decrease by  $19.3 \pm 4.5\%$  to  $0.235 \pm 0.022$  liters/min ( $P < 0.01$  vs. baseline).

In group B, basal LBF was  $0.215 \pm 0.018$  liters/min ( $P = \text{NS}$  vs. group A). During steady state euglycemic hyperinsulinemia, LBF increased  $131 \pm 61\%$  to  $0.479 \pm 0.118$  liters/min ( $P < 0.01$ ). During steady state hyperinsulinemia, intrafemoral artery infusion of L-NMMA caused LBF to decrease by  $42.0 \pm 5.4\%$  to  $0.266 \pm 0.052$  liters/min ( $P < 0.016$ ). The absolute and relative fall in LBF in response to L-NMMA were significantly greater during steady state hyperinsulinemia (group B) than at baseline (group A) ( $P < 0.03$  and  $< 0.003$ , respectively; Fig. 1, A and B).

Baseline MAP was  $83.1 \pm 2.8$  and  $80.2 \pm 3.3$  mmHg determined by cuff in groups A and B, respectively ( $P = \text{NS}$ ). However, when determined invasively on the morning of the study, MAP was  $91.6 \pm 2.9$  and  $79.8 \pm 2.6$  mmHg in groups A and B, respectively ( $P < 0.05$ ). In group A, MAP increased by  $4.39 \pm 0.93$  mmHg above baseline during L-NMMA ( $P < 0.01$ ). In group B, euglycemic hyperinsulinemia had no significant effect on MAP. L-NMMA infusion during euglycemic hyperinsulinemia caused a rise in MAP of  $5.93 \pm 1.42$  mmHg ( $P < 0.01$  vs. steady state hyperinsulinemia). The rise in MAP to the intrafemoral artery infusion of L-NMMA did not differ between groups A and B ( $P = \text{NS}$ ).

In group A, basal LVR was  $324.9 \pm 23.4$  U and increased to  $425.4 \pm 31.3$  U during the infusion of L-NMMA ( $P < 0.01$ ). In group B, basal LVR was  $385 \pm 45$  U and decreased to  $203 \pm 30$  U during steady state euglycemic hyperinsulinemia and during superimposed L-NMMA infusion rose to  $390 \pm 74$  U ( $P < 0.01$  vs. steady state).

The relative changes in LVR in both groups are shown in Fig. 2. L-NMMA caused a threefold greater rise in LVR in group B versus A ( $P < 0.003$ ). Absolute LVR during L-NMMA infusion was similar in both groups.

In group C, LBF before the infusion of insulin or L-NMMA, respectively, was  $0.253 \pm 0.020$  and  $0.267 \pm 0.027$  liters/min ( $P = \text{NS}$ ). Insulin infusion alone caused LBF to increase to  $0.289 \pm 0.027$  and  $0.376 \pm 0.054$  liters/min at 10 and 20 min, respectively. In contrast to the steady increase in LBF observed during insulin alone, coinfusion of L-NMMA inhibited any change in LBF from baseline. LBF was  $0.228 \pm 0.047$  and  $0.249 \pm 0.054$  liters/min at 10 and 20 min, respectively ( $P = \text{NS}$  vs. baseline; Fig. 3). At the 20-min infusion time point LBF during insulin alone was 57% higher than with insulin plus L-NMMA ( $P < 0.01$ ).

In group C, MAP was  $84.6 \pm 1.6$  and  $88.2 \pm 1.0$  mmHg in the insulin alone and L-NMMA plus insulin groups, respectively ( $P = \text{NS}$ ). The infusions of insulin with or without L-NMMA

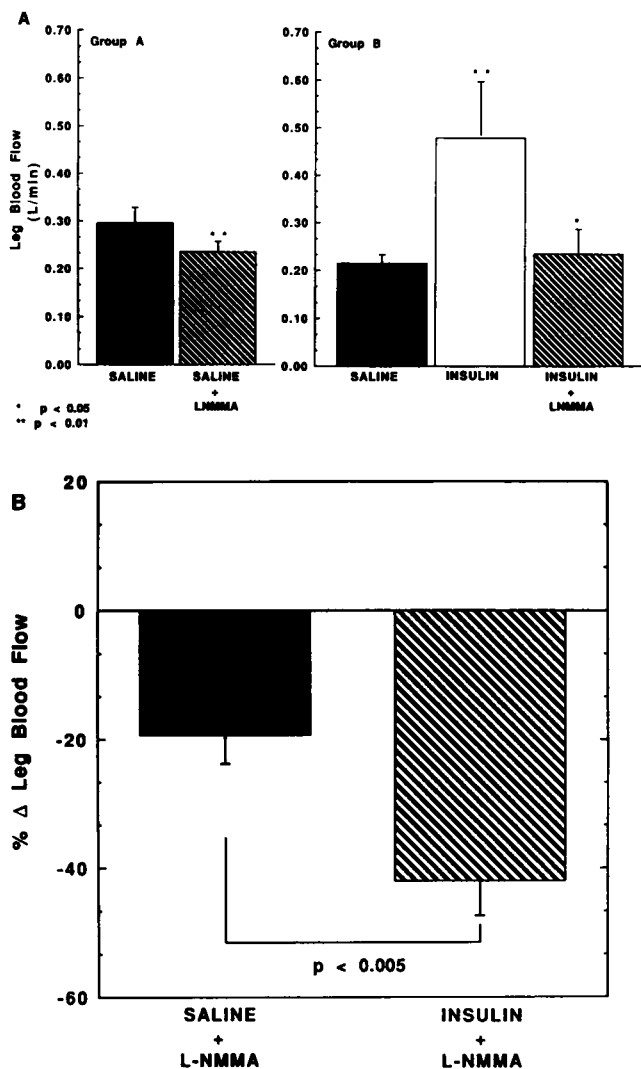


Figure 1. (A) Effect of intrafemoral artery infusion of L-NMMA (16 mg/min) on LBF during saline (Group A) and during insulin infusion (120 mU/m<sup>2</sup> per minute) (Group B). (B) Percent decrease below baseline in LBF to intrafemoral artery infusion of L-NMMA (16 mg/min) during saline (group A) and during euglycemic hyperinsulinemia (120 mU/m<sup>2</sup> per min) (group B).

had no effect on basal MAP. Thus, changes in LVR mirrored the changes in blood flow. At 20 min of infusion, LVR was significantly lower without than with L-NMMA ( $P < 0.05$ ).

#### Studies with MCh (study 2)

**Serum glucose and insulin concentrations.** Glucose levels were  $92.9 \pm 2.6$  and  $90.4 \pm 1.0$  mg/dl during NS and steady state hyperinsulinemia, respectively ( $P = \text{NS}$ ). Serum insulin levels were  $7.3 \pm 1.2$   $\mu\text{U/ml}$  during NS (fasting) and rose to  $42.8 \pm 6.5$   $\mu\text{U/ml}$  during steady state hyperinsulinemia ( $P < 0.001$ ).

**Leg blood flow and vascular resistance.** Baseline LBF during NS was  $0.24 \pm 0.03$  liters/min and was unchanged ( $0.24 \pm 0.02$  liters/min) during the low-dose euglycemic hyperinsulinemic clamps. LBF responses to MCh between insulin infusion rates of 15 and 20 vs 30 and 40 mU/m<sup>2</sup> per min were compared and found to be similar; therefore, the data were pooled. The LBF responses to graded intrafemoral artery MCh

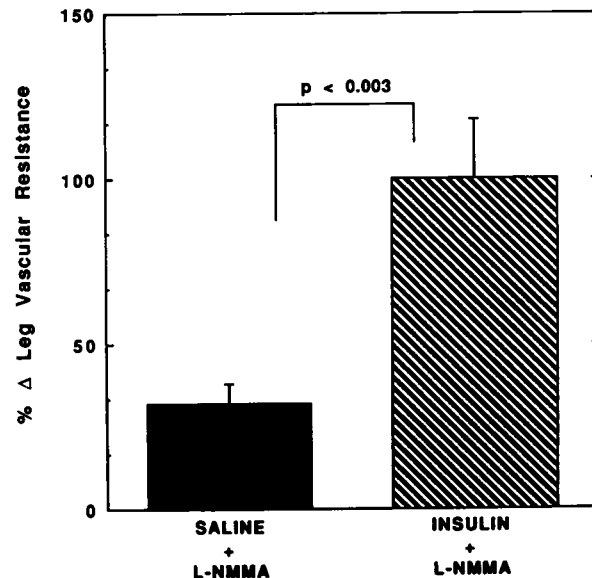


Figure 2. Percent increase above baseline in LVR to intrafemoral artery infusion of L-NMMA (16 mg/min) during saline (group A) and during euglycemic hyperinsulinemia (120 mU/m<sup>2</sup> per min) (group B).

infusions are given in Table II. MCh caused a dose-dependent rise in LBF during both NS and insulin infusion ( $P < 0.0001$ ). However, the increase in LBF was more pronounced during insulin than during NS ( $P < 0.03$ ). The relative rise in LBF during NS and insulin is shown in Fig. 4 and is also significantly greater during insulin ( $P < 0.03$ ). As can be seen from Table II and Fig. 4, differences in responses in LBF to MCh during euglycemic hyperinsulinemic clamp vs NS were most pronounced at the two middle MCh infusion rates of 5.0 and 7.5  $\mu\text{g/min}$ . During the highest MCh infusion rate (10  $\mu\text{g/min}$ ) the increments in LBF were not different during NS and euglycemic hyperinsulinemic clamp.

MAP did not differ between NS and the euglycemic hyperinsulinemic clamp nor did the intrafemoral artery infusion of MCh alter MAP at any dose. LVR was  $463 \pm 57$  and  $464 \pm 54$  U during

Table II. Effect of Intrafemoral Artery Infusion of MCh on LBF and LVR during Saline and during Euglycemic Hyperinsulinemic (15–40 mU/m<sup>2</sup> per min) Clamps

	MCh dose			
	2.5 $\mu\text{g/min}$	5.0 $\mu\text{g/min}$	7.5 $\mu\text{g/min}$	10.0 $\mu\text{g/min}$
<b>LBF (liter/min)</b>				
Saline	$0.30 \pm 0.04$ (n = 13)	$0.36 \pm 0.06$ (n = 13)	$0.41 \pm 0.06$ (n = 14)	$0.41 \pm 0.06$ (n = 12)
Clamp (liter)	$0.33 \pm 0.04$ (n = 13)	$0.44 \pm 0.04$ (n = 13)	$0.52 \pm 0.05^*$ (n = 11)	$0.49 \pm 0.06$ (n = 11)
<b>LVR (U)</b>				
Saline	$368 \pm 47$ (n = 12)	$367 \pm 61$ (n = 12)	$308 \pm 46$ (n = 13)	$277 \pm 45$ (n = 10)
Clamp	$295 \pm 28$ (n = 9)	$225 \pm 24^\dagger$ (n = 10)	$198 \pm 25^\dagger$ (n = 8)	$205 \pm 23$ (n = 8)

\*  $P = 0.025$  clamp vs. saline.  $^\dagger P < 0.05$  clamp vs. saline.

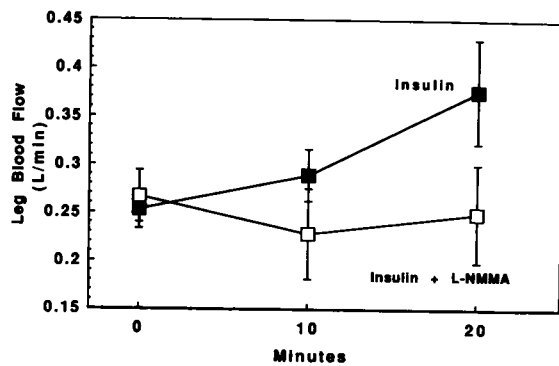


Figure 3. LBF response to intrafemoral artery infusion of insulin (4 mU/m<sup>2</sup> per min) with and without concomitant intrafemoral artery infusion of L-NMMA (16 mg/min). Time 0 represents baseline LBF before the beginning of any infusions. The 10- and 20-min time points relate to the duration of the insulin infusion.

NS and the euglycemic hyperinsulinemic clamp, respectively ( $P = NS$ ). MCh infusion caused a dose-dependent decrease in LVR ( $P < 0.001$ ; Table II) during NS and insulin. The relative changes in LVR caused by MCh during NS and euglycemic hyperinsulinemia are demonstrated in Fig. 5. The absolute and relative fall in LVR during euglycemic hyperinsulinemia were significantly greater than during NS ( $P < 0.012$  and  $< 0.014$ , respectively).

#### Studies with SNP (study 3)

**Serum glucose and insulin concentrations.** Glucose levels were  $94.1 \pm 1.6$  and  $91.9 \pm 0.9$  mg/dl during NS and steady state hyperinsulinemia, respectively ( $P = NS$ ). Insulin levels were  $7.1 \pm 1.7$   $\mu$ U/ml during NS (fasting) and rose to  $31.5 \pm 6.4$   $\mu$ U/ml during steady state hyperinsulinemia ( $P < 0.001$ ).

**Leg blood flow and vascular resistance.** Baseline blood flow during NS was  $0.189 \pm 0.02$  and  $0.218 \pm 0.03$  liters/min during the euglycemic hyperinsulinemic clamp ( $P < 0.05$ ). The LBF responses to the intrafemoral artery SNP infusions are given in Table III. SNP caused a dose-dependent rise in LBF during both NS and insulin ( $P < 0.01$ ). Due to the higher LBF achieved with the euglycemic hyperinsulinemic clamp, rates of LBF in response to SNP were higher during the euglycemic

Table III. Effect of Intrafemoral Artery Infusion of SNP on LBF and LVR during Saline and during Euglycemic Hyperinsulinemic (15–20 mU/m<sup>2</sup> per minute) Clamps

	SNP dose		
	1.75 $\mu$ g/min	3.5 $\mu$ g/min	7.0 $\mu$ g/min
LBF (liter/min)			
Saline	$0.22 \pm 0.025$ (n = 7)	$0.26 \pm 0.036$ (n = 8)	$0.32 \pm 0.034$ (n = 8)
Insulin	$0.29 \pm 0.051$ (n = 7)	$0.31 \pm 0.032$ (n = 8)	$0.38 \pm 0.052$ (n = 7)
LVR (U)			
Saline	$455 \pm 65$ (n = 7)	$414 \pm 75$ (n = 8)	$338 \pm 75$ (n = 8)
Insulin	$392 \pm 86$ (n = 7)	$319 \pm 42$ (n = 8)	$289 \pm 67$ (n = 7)

hyperinsulinemic clamp than during NS ( $P < 0.03$ ). As can be appreciated in Fig. 6, when expressed as relative incremental changes above baseline, the apparent differences in LBF response to SNP between NS and the euglycemic hyperinsulinemic clamp disappear. Because MAP was unchanged during the infusion of SNP in either group, LVR mirrored the changes in LBF. Baseline LVR was  $547 \pm 67$  and  $493 \pm 86$  U during NS and euglycemic hyperinsulinemia, respectively ( $P = NS$ ). LVR values during the intrafemoral artery SNP infusions are given in Table III. The fall in resistance was dose dependent ( $P < 0.05$ ); however, there was no difference in LVR between NS and euglycemic hyperinsulinemic conditions. The relative changes in LVR are shown in Fig. 7.

#### Discussion

In the current study we tested the hypothesis that insulin-mediated dilation of skeletal muscle vasculature and the resultant increase in skeletal muscle perfusion is mediated by EDNO. The data indicate that (a) ~20% of basal LBF is EDNO dependent, (b) >65% of insulin-mediated vasodilation is EDNO dependent, and (c) insulin increases MCh sensitivity but not

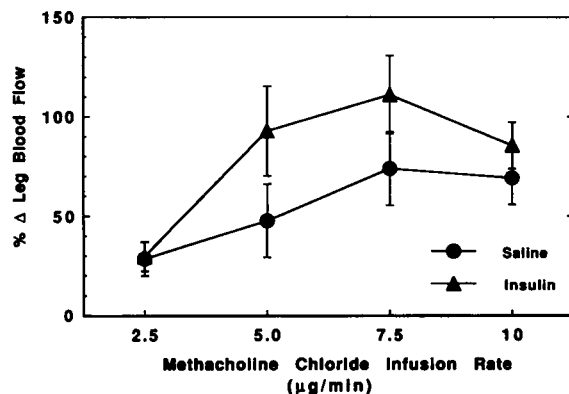


Figure 4. Percent increment in LBF above baseline in response to graded intrafemoral artery infusions of MCh (2.5–10.0  $\mu$ g/min) during saline and during euglycemic hyperinsulinemia (15–40 mU/m<sup>2</sup> per min).

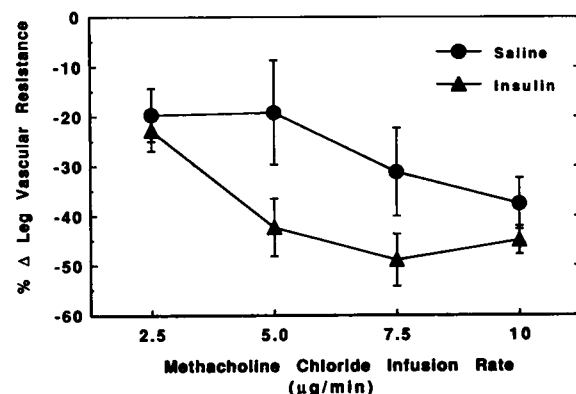


Figure 5. Percent reduction in LVR below baseline in response to graded intrafemoral artery infusions of MCh (2.5–10.0  $\mu$ g/min) during saline and during euglycemic hyperinsulinemia (15–40 mU/m<sup>2</sup> per min).

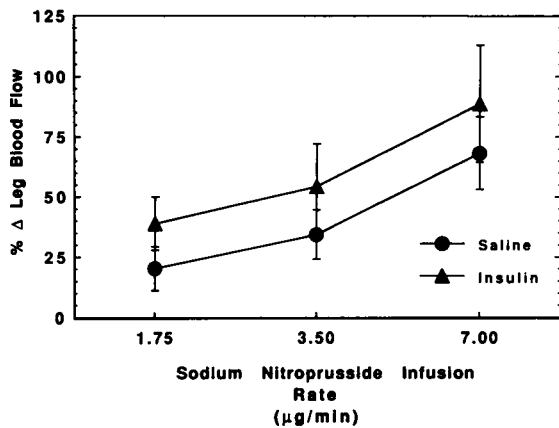


Figure 6. Percent increment in LBF above baseline in response to graded intrafemoral artery infusions of SNP (1.75–7.0  $\mu\text{g}/\text{min}$ ) during saline and during euglycemic hyperinsulinemia (15–20  $\text{mU}/\text{m}^2$  per min).

responsiveness to exogenous NO. Taken together, the data indicate that insulin-mediated vasodilation is largely EDNO dependent and occurs via an effect of insulin to enhance EDNO synthesis/release.

EDNO-dependent LBF was examined by administering an intrafemoral artery infusion of L-NMMA, which inhibits the formation of EDNO in a stereospecific manner (24). Since previous reports have described a prolonged effect of L-NMMA (26), we were careful to carry out L-NMMA infusions during NS and euglycemic hyperinsulinemia in different groups of subjects. Our most important finding was that EDNO-dependent LBF was severalfold greater during steady state euglycemic hyperinsulinemia than during NS. LBF as well as the proportion of EDNO-dependent LBF increased approximately twofold during steady state euglycemic hyperinsulinemia resulting in an approximately fourfold increase of EDNO-dependent LBF. We calculated that  $\sim 75 \pm 12\%$  of the increase in LBF observed during the euglycemic hyperinsulinemic clamp can be attributed to EDNO – insulin-mediated EDNO-dependent LBF =  $[(\Delta \text{insulin-mediated LBF during L-NMMA}) / (\text{estimated basal EDNO-dependent LBF}) / (\text{insulin-mediated incremental LBF})] \times 100$ . This calculation may actually underestimate the true contribution of EDNO to the increase in LBF during the euglycemic hyperinsulinemic clamp. This is likely for several reasons: (a) LBF was 90% higher during insulin infusion in group B than at baseline in group A and, therefore, the intravascular concentration of L-NMMA might be expected to be diluted to approximately half of that achieved during NS; (b) during euglycemic hyperinsulinemia, two subjects exhibited a decrease in LBF below basal values in response to the administration of L-NMMA, which suggests that EDNO could account for all of the insulin-mediated vasodilation; (c) our infusion of L-NMMA caused a small but significant rise in blood pressure ( $\sim 6\%$ ), which may have triggered a baroreceptor reflex, thus reducing sympathetic nervous system activity and thereby mitigating the vasoconstricting effect of L-NMMA; and finally, (d) our data do not allow for estimation of the contribution of the basal insulin concentration to EDNO release. Therefore, it is highly likely that insulin-mediated skeletal muscle vasodilation is entirely EDNO dependent. This notion is further supported by our data indicating that the initial phase of insulin-mediated

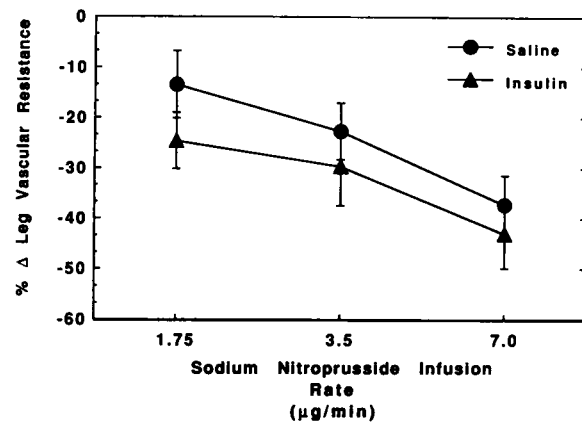


Figure 7. Percent reduction in LVR below baseline in response to graded intrafemoral artery infusions of SNP (1.75–7.0  $\mu\text{g}/\text{min}$ ) during saline and during euglycemic hyperinsulinemia (15–20  $\text{mU}/\text{m}^2$  per min).

vasodilation is also EDNO dependent. Thus, both early and late phases of vasodilation by insulin are EDNO dependent.

Compared with other studies (22, 27) where EDNO-dependent blood flow was assessed in forearms of normal volunteers, we found a lesser fall in LBF in response to L-NMMA. The intrafemoral artery infusion of L-NMMA caused LBF to decline by  $\sim 20\%$ . This is apparently lower than the 40% decrease reported in the forearm with an intrabrachial artery infusion of L-NMMA at the rate of 4  $\mu\text{mol}/\text{min}$  (22, 27). This dose is roughly comparable to the dose used in our study when one accounts for the differences in blood flow between leg and arm. This divergent finding could be due to methodological differences in the measurement of blood flow. For example, we did not exclude foot blood flow, whereas exclusion of hand blood flow, which may represent a significant proportion of the nonsuppressible flow, is routine in studies using the forearm. Moreover, as already discussed, the rise in blood pressure during our infusion of L-NMMA may have triggered a baroreceptor reflex, thereby diminishing the decrease in LBF. Finally, we cannot exclude that forearm and leg vascular tone may differ in their dependency on EDNO.

Given the result that euglycemic hyperinsulinemia increases EDNO-dependent LBF, we sought to sort out whether this insulin effect is mediated by an increase in EDNO synthesis/release or enhanced vascular smooth muscle sensitivity to EDNO. For this purpose, we examined the LBF response to graded intrafemoral artery infusions of the endothelium-dependent vasodilating agent MCh during either NS (basal insulin only) or euglycemic hyperinsulinemia. Physiological hyperinsulinemia ( $\sim 40 \mu\text{U}/\text{ml}$ ) independent of changes in glycemia augmented the LBF response to MCh. Therefore, insulin appears to increase vascular sensitivity to MCh vasodilation. LBF response to MCh was always higher during euglycemic hyperinsulinemia compared with NS and the maximal LBF response achieved by MCh occurred at doses of 7.5 and 10.0  $\mu\text{g}/\text{min}$ . However, the difference in LBF between NS and euglycemic hyperinsulinemia in response to MCh diminished at the highest dose of 10.0  $\mu\text{g}/\text{min}$ . The cause for this biphasic dose–response curve of LBF to MCh is not clear. Given that one leg receives  $\sim 5\%$  of the cardiac output under resting conditions, it is conceivable that increases in LBF above  $\sim 100\%$  are limited by the available blood supply unless cardiac output rises concomitantly.

Insulin's apparent ability to modulate the EDNO system (increase LBF response to MCh) is a novel insulin action. Other studies have reported an enhanced response to acetylcholine or MCh-stimulated vasodilatation (28–30). In these studies, augmented responses of forearm blood flow to acetylcholine or MCh were achieved by chronic increases in blood flow created by arteriovenous fistula (30) or by administration of pharmacological doses of L-arginine (28, 29), the biosynthetic precursor of EDNO (25). It is unlikely that increased levels of L-arginine during steady state hyperinsulinemia are the cause of the enhanced MCh sensitivity we observed since amino acid concentrations (including arginine) decrease with hyperinsulinemia. However, we cannot exclude the possibility that insulin may selectively increase uptake of L-arginine into endothelial cells and, thus, promote EDNO synthesis via this mechanism. It is interesting to speculate whether the reported (28, 29) enhanced EDNO response with L-arginine administration was secondary to stimulation of pancreatic insulin secretion by L-arginine itself and that the resultant hyperinsulinemia may be the more proximate cause for the augmented responses to methacholine or acetylcholine. Interestingly, in one study (28) of the effects of L-arginine on MCh-mediated vasodilation, insulin levels were reported to be similar to the levels found in our study.

Before concluding that insulin augments vasodilation in response to MCh exclusively by EDNO synthesis/release other potential mechanisms have to be considered. For example, acetylcholine has been shown also to vasodilate via an endothelium-dependent hyperpolarizing factor (31) that may be enhanced by insulin. Furthermore, acetylcholine can also induce the release of an endothelium-dependent contracting factor (32) that may be decreased by insulin. Therefore, each of those factors or combination thereof might be modulated by euglycemic hyperinsulinemia, resulting in the enhanced vasodilation in response to MCh.

To address whether insulin could have an effect to amplify EDNO's action on vascular smooth muscle, we examined the LBF response to graded intrafemoral artery infusions of SNP (a NO donor that vasodilates vascular smooth muscle directly and is not modulated by L-NMMA; reference 33) during either NS or euglycemic hyperinsulinemia ( $\sim 30 \mu\text{U/ml}$ ). Steady state hyperinsulinemia had no effect to alter the dose response of SNP on LBF. Although we can not exclude the possibility of a type II error, i.e., not finding an effect of insulin to amplify EDNO's action on vascular smooth muscle because of the small number of subjects studied with nitroprusside, this effect would be of much lesser magnitude than the one seen with MCh. Therefore, taken together, the data indicate that insulin interacts with the endothelium to increase the synthesis/release of EDNO. The underlying mechanism for this novel interaction remains to be elucidated.

EDNO production/release may be modulated directly by insulin or indirectly, for example, by insulin-stimulated endothelial cell or skeletal muscle glucose metabolism. Whether skeletal muscle carbohydrate metabolism plays a role in the regulation of skeletal muscle blood flow is not clear but has been suggested by some studies. In a recent report, Vollenweider and co-workers (8) attempted to separate the role of hyperinsulinemia per se and hyperinsulinemia-induced carbohydrate metabolism in the regulation of skeletal muscle blood flow. These authors concluded that carbohydrate metabolism per se did not cause LBF to rise independent of hyperinsulinemia. Our laboratory has previously shown that changes in LBF correlated posi-

tively to rates of insulin-mediated glucose uptake (2). Furthermore, we reported that the half maximally effective insulin dose to produce increments in LBF ( $\sim 40\text{--}50 \mu\text{U/ml}$ ) (1) was similar to that reported for glucose oxidation (34), suggesting that skeletal muscle glucose metabolism and blood flow may be coupled. If skeletal muscle or endothelial cell glucose metabolism is a signal for vasodilation, it is clear that this signal must in turn activate the EDNO system. The pathway for such a putative pathway remains to be elucidated.

It should be noted that chronic increases in blood flow have been shown to modulate endothelium-dependent responses (35) and to enhance the tonic and receptor-stimulated production of NO (36). Because LBF during steady state hyperinsulinemia increased by  $\sim 130\%$ , the possibility exists that the large rise in flow and the resultant shear stress might have caused an increase of EDNO release in the latter phase of insulin-mediated vasodilation (35, 36). Although we can not entirely exclude this possibility, this is not likely because our intervention was acute. Moreover, the relative fall in LBF caused by intrafemoral artery infusion of L-NMMA did not correlate with the magnitude of LBF at baseline or during euglycemic hyperinsulinemia ( $r^2 = 0.17$ ,  $P = \text{NS}$  and  $r^2 = 0.14$ ,  $P = \text{NS}$ , respectively), thus indicating no relationship between absolute flow rate and EDNO dependency. Finally, we were able to reduce insulin-mediated LBF by  $> 65\%$ , suggesting that whatever other mechanism responsible for vasodilation (if it exists) is a relatively weak vasodilator and that the EDNO effect is predominant.

In summary, the results of our study clearly indicate that the increase in skeletal muscle blood flow observed during euglycemic hyperinsulinemia is largely EDNO dependent. Moreover, the data strongly suggest that insulin modulates the synthesis/release of EDNO and not its action. Therefore, insulin appears to be a novel regulator of the EDNO system. On a final speculative note, the ability of insulin to modulate the EDNO system may be decreased in states of insulin resistance, thereby contributing to the increased incidence of hypertension in states of insulin resistance such as obesity and non-insulin-dependent diabetes mellitus (37–39). Conversely, the known defect in EDNO production in essential hypertensives (23, 40) may be responsible for impaired insulin-mediated vasodilation and decreased insulin and glucose delivery and insulin resistance in these patients (41, 42).

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