# Insulin-Mediated Changes in Leg Blood Flow Are Coupled to Capillary Density in Skeletal Muscle in Healthy 70-Year-Old Men

Anu Hedman, Per-Erik Andersson, Richard Reneland, and Hans O. Lithell

**The aim of this study was to investigate to what degree the capillarization in the skeletal muscle explains the leg blood flow (LBF) changes during hyperinsulinaemia. Fifteen normotensive men from a population-based cohort of 70-year-old men in Uppsala, Sweden, were investigated. Their metabolic status (oral glucose tolerance test and euglycemic, hyperinsulinaemic clamp test results), serum lipid profile, muscle fiber distribution (myosin adenosine triphosphatase staining), and capillary supply (amylase–periodic acid-Schiff method) was evaluated. Doppler ultrasound was used before and after the clamp test to detect insulin-induced changes in LBF. Physiologic hyperinsulinemia (serum insulin, 107 mU/L) caused a moderate increase in LBF (15% ± 11%; $P = .07$). Change in LBF was closely related to capillary density ($r = .66$; $P < .01$) independent of obesity, smoking and level of physical activity. An association was observed between LBF and serum free fatty acid (FFA) concentrations ($r = -.57$; $P < .05$). In multiple regression analysis, capillary density and serum FFA level together explained 71% of the variation in insulin-mediated LBF changes. Capillary rarefaction and elevated serum FFA values were associated with a vasoconstrictive effect of insulin. In conclusion, capillarization in skeletal muscle and serum FFA concentration seem to be determinants of endothelial function.**



ONE SPECIFIC METABOLIC action of insulin is to participate in regulation of glucose uptake in skeletal muscle. Insulin has also been found to increase blood flow in skeletal muscle beds through vasodilation.[1] In addition, an increase in circulating insulin concentration acutely increases the extraction of glucose. This mechanism probably accounts for a greater part of the increase in glucose uptake than does the increase in blood flow. However, the magnitude of the vasodilatory contribution to glucose uptake might vary between age groups.[2,3]

Insulin-induced changes in blood flow are already evident at physiologic plasma concentrations of insulin.[4-7] The magnitude of the increase in blood flow during hyperinsulinemia, which is endothelium dependent,[4-9] depends not only on the degree of vasodilation in large vessels, but also possibly on the number of capillaries present in the muscle bed.[6] Recently, insulin-mediated vasodilation has been found to depend on capillary recruitment both in skin[10] and in muscle.[11] Leg blood flow (LBF) has also been reported to be higher in muscles with a larger proportion of slow-twitch (type I) fibers,[12,13] which have a greater capillary supply.[14]

In healthy elderly individuals, age itself does not seem to affect the fiber type composition or capillary supply expressed as the numbers of capillaries per square millimeter of muscle tissue.[15,16] On the other hand, several insulin-resistant conditions such as hypertension, type 2 diabetes, and obesity occur more frequently at greater ages. These conditions are associated with decreased capillary supply with alterations in muscle fiber distribution[17-19] as well as with decreased metabolic and vasodilating action of insulin.[1,4,20]

In studies of insulin-stimulated blood flow in healthy individuals, elderly subjects are often excluded. This exclusion is partly based on observations indicating a deleterious impact of aging on endothelial function.[2,21] Therefore, the aim of the present study was to determine the degree to which differences in insulin-induced changes in LBF are explained by the capillary supply in the femoral muscle bed in healthy elderly subjects with a known metabolic profile.

## SUBJECTS AND METHODS

### *Subjects*

The subjects were selected from a population-based cohort of 70-year-old men born in 1920 through 1924 and living in Uppsala, Sweden (n = 1,221).[22] The cohort was collected during 1990 through 1995, and all investigations were performed in 1 week. A subgroup of 46 men who declared themselves healthy was consecutively sampled during a 6-month period for femoral artery blood flow measurement. Men with coronary heart disease (data from Official Swedish In-patient Registry) were excluded. Men with a normal glucose tolerance according to World Health Organization (WHO) criteria from 1985[23] and normal values for blood pressure (<160/90 mm Hg) according to Swedish guidelines for diagnosing hypertension in 1990 through 1993[24] were included ($n$=15). None of them were receiving any pharmacologic treatment. Four of the study subjects were cigarette smokers. The ethics committee of the medical faculty, Uppsala University, approved this study, and each subject gave consent before participating.

### *Methods*

All investigations were performed during the period of 10 days for each subject.

*Measurement of femoral artery blood flow.* LBF was measured in connection with euglycemic hyperinsulinemic clamping using the Doppler ultrasound technique. This method for blood flow measurements was considered a method of choice because it permits measurement of LBF simultaneously on 2 to 4 subjects. The technique for measuring and calculating LBF has been described in detail previously.[25] Blood flow variables used in this study were velocity time integral (VTI), femoral artery stroke volume (SV), and femoral artery

*From the Department of Public Health and Caring Sciences/Section of Geriatrics, Uppsala University, Uppsala, Sweden.*

*Submitted October 16, 2000; accepted February 21, 2001.*

*Supported by grants from the Swedish Hypertension Society, the Foundation for Geriatric Research, the Swedish Council for Planning and Co-ordination of Research, the Research Foundation of Pharmacia AB, and the Research Fund of the Swedish Royal Society.*

*Address reprint requests to Anu Hedman, MD, Department of Public Health and Caring Sciences/Section of Geriatrics, Uppsala University, Kålsängsgränd 10 D, PO Box 609, SE-751 25 Uppsala, Sweden.*



*0026-0495/01/5009-0008$35.00/0*

*doi:10.1053/meta.2001.25604*

minute volume (MV). VTI, expressed in meters, representing the area under the systolic portion of the mean velocity trace, reflects the mean forward distance traveled by the erythrocytes during a given systole, the stroke distance. Femoral artery SV (mL) was obtained by multiplying VTI by the cross-sectional area of the artery ($cm^2$), with adjustment for the angle between the sample volume and the vessel wall. The corresponding femoral artery MV (mL/min) was then calculated by multiplying SV by the simultaneously measured heart rate. This femoral artery MV is hereafter referred to as LBF. The coefficient of variation (CV) for VTI, SV, and LBF were 11%, 15%, and 18%, respectively. Our study presents the measurement of LBF that takes the artery cross-sectional area into account and thereby stands for an accurate volume estimation when blood flow is measured as recently suggested.[26-28]

Measurement of LBF with the Doppler ultrasound technique has previously been validated by the venous thermodilution technique[29,30] and by venous strain-gauge plethysmography.[31]

*Determination of muscle morphology.* A biopsy sample of the right vastus lateralis muscle was obtained by an incision through the skin and fascia at the midlateral part of the muscle using a Bergström needle under local anesthesia.[32] Technical details of the reproducibility and the staining methods have been described in depth previously.[33] Fiber type distributions and fiber areas were determined from myofibrillar adenosine triphosphatase (ATPase)–stained sections, and capillaries were evaluated on amylase–Periodic acid-Schiff (PAS)–stained sections that were magnified 150 times. An area as large as possible was examined. The fiber areas were automatically calculated by the system as absolute area for the given fiber type. Capillary supply was determined from the analyzed absolute muscle sample area both as capillary density per square millimeter and as number of capillaries per fiber (number of capillaries divided by number of fibers counted in the biopsy sample). The diffusion distance, derived automatically by the system, represents the muscle area supplied by 1 capillary and was calculated by dividing the mean fiber type area by the mean number of capillaries surrounding this fiber type.

*Assessment of insulin sensitivity and glucose tolerance.* Whole-body sensitivity to insulin was measured by the euglycemic hyperinsulinemic clamp procedure according to the method of DeFronzo et al[34] with minor modifications. Insulin (Actrapid Human; Novo, Copenhagen, Denmark) was infused in a priming dose for 10 minutes and then as a continuous infusion at a rate of 56 $mU/m^2/min$ (instead of 40 $mU/m^2/min$) for 110 minutes, resulting in a steady-state plasma insulin concentration of 107 ± 14 mU/L. The target level of plasma glucose during the clamp study was 5.1 mmol/L. Insulin sensitivity index, M/I, was calculated as the amount of glucose (M) infused per minute divided by steady-state plasma insulin (I) multiplied by 100 (mg/min/kg per mU/L). The CV for M/I was 14%.

Glucose tolerance was determined by oral glucose tolerance test (OGTT) according to WHO criteria.[23] Plasma glucose was measured by the glucose dehydrogenase method (Gluc-DH; Merck, Darmstadt, Germany). Serum insulin was determined by an enzymatic-immunologic assay (Enzymmun; Boehringer Mannhein, Mannheim, Germany) performed in an ES300 automatic analyzer (Boehringer Mannheim). The CV for fasting serum insulin in our laboratory is 21%.

*Blood pressure, physical activity, and anthropometric measurements.* Blood pressure was measured in the right arm with the subject in the supine position after resting for 10 minutes. Measurements were made twice, and the values were recorded to the nearest even figure. The mean of the 2 values was used for each blood pressure. Mean arterial blood pressure (MAP) was calculated according to the formula MAP = diastolic blood pressure + [(systolic blood pressure − diastolic blood pressure)/3]. The CV for blood pressure variables ranged from 5.1% to 6.8%.

Heart rate was measured in the supine position as the radial pulse rate in 1 minute. The level of physical activity during leisure time was investigated by questionnaire and grouped into 4 categories: I, sedentary; II, walking or cycling for pleasure; III, regular physical activity for at least 3 hours weekly; IV, intense physical training or engagement in competitive sport.

Body mass index (BMI) was calculated as the ratio of weight (kg) to height (m) squared ($kg/m^2$). The waist and hip circumferences were measured in the supine position. The waist was measured midway between the lowest rib and the iliac crest and the hip over the widest part. The waist/hip (W/H) ratio was calculated.

*Assessment of serum lipids, lipoproteins, and free fatty acids.* Triglyceride and cholesterol concentrations in serum and in the isolated lipoprotein fractions were determined enzymatically (Boehringer Mannheim) in a Monarch instrument (Instrumentation Laboratories, Lexington). High-density lipoprotein (HDL) particles were separated by precipitation with magnesium chloride/phosphotungstate. The free fatty acid (FFA) concentration in serum (n = 14) was measured by an enzymatic method (Wako Chemicals GmbH, Neuss, Germany) with a CV of 24% at our laboratory.

### *Statistical Analysis*

An SAS analysis system program (version 6.0.8 for Windows) and JMP 3.0.2. for Apple Macintosh computers (SAS Institute Inc, Cary, NC) were used to calculate results. Analysis of variance (ANOVA) was applied to compare group means (Student *t* test or Wilcoxon signed rank sum test) or to test linear regressions for significance. Changes during the clamp test were tested for significance with the paired *t* test. When normality was not achieved by logarithmic transformation of data (diffusion distance and FFA), a nonparametric test (Spearman rank correlation) was used. Analysis of covariance (ANCOVA; Pearson partial or Spearman partial rank correlation) was used to correct for possible effects of known confounders, namely BMI, W/H ratio, level of physical activity, and smoking status. The dependent variable in multiple regression analysis was the change in LBF during hyperinsulinemia, and independent variables were capillary density in muscle and serum level of FFA. Calculation of the 95% confidence interval (CI) of the correlation coefficient has been described elsewhere.[35] Proportional differences between groups were calculated by the $\chi^2$ or Fisher exact test. $P \leq .05$ was considered significant.

## RESULTS

Table 1 shows the clinical data and muscle morphologic characteristics of the study subjects. Measures of femoral artery blood flow at baseline and changes during the euglycemic hyperinsulinemic clamp test are shown in Table 2. Baseline values of femoral artery blood flow showed no correlation with skeletal muscle characteristics. A positive significant relationship was found between the insulin-induced change in LBF and capillary density in square millimeters (Fig 1). Adjustment for BMI, W/H ratio, smoking status, or physical activity level had no effect on this correlation. Change in LBF during hyperinsulinemia was inversely correlated to mean fiber area ($r = -.66$, $P < .01$) but was not related to the relative proportions of different fiber types. Additionally, the number of capillaries per fiber was not correlated to any of the estimates of femoral artery blood flow.

The change in LBF showed an inverse correlation to serum levels of FFA ($r = -.57$; $P < .05$) (Fig 2). This relationship was independent of BMI, smoking status, and physical activity. Nevertheless, the correlation was no longer significant after further adjustment for W/H ratio ($r = -.49$; $P = .09$). In addition, serum FFA concentration was associated with glucose

**Table 1. Clinical Data and Muscle Morphologic Characteristics of the Study Subjects**

| | Mean ± SD | Range |
|---|---|---|
| *Clinical data* | | |
| BMI (kg/m$^2$) | 26 ± 3.8 | 22-35 |
| W/H ratio | 0.94 ± 0.06 | 0.8-1.1 |
| Systolic blood pressure (mm Hg) | 133 ± 12 | 111-149 |
| Diastolic blood pressure (mm Hg) | 79 ± 5 | 69-85 |
| Mean arterial pressure (mm Hg) | 97 ± 6 | 84-106 |
| Resting heart rate (beats/min) | 63 ± 7 | 52-72 |
| Fasting plasma glucose (mmol/L) | 5.1 ± 0.4 | 4.4-6.0 |
| Fasting serum insulin (mU/L) | 14.3 ± 4.2 | 9.6-24.2 |
| M, glucose uptake (mg/min/kg) | 6.1 ± 1.6 | 4.2-8.9 |
| M/I, insulin sensitivity index (mg/min/kg per mU/L × 100) | 5.9 ± 2.0 | 3.4-9.8 |
| *Muscle morphology* | | |
| Distribution of fiber types (%) | | |
| I | 46 ± 12 | 30-72 |
| IIA | 32.5 ± 11.7 | 14-51 |
| IIB | 21.5 ± 10.7 | 4-41 |
| Mean fiber area ($\mu$m$^2$) | 5,220 ± 1,040 | 3,110-6,830 |
| Mean diffusion distance ($\mu$m$^2$) | 1,350 ± 240 | 1,000-1,970 |
| Number of capillaries (/mm$^2$) | 300 ± 40 | 223-365 |
| Number of capillaries (per fiber) | 1.53 ± 0.24 | 1.1-2.1 |

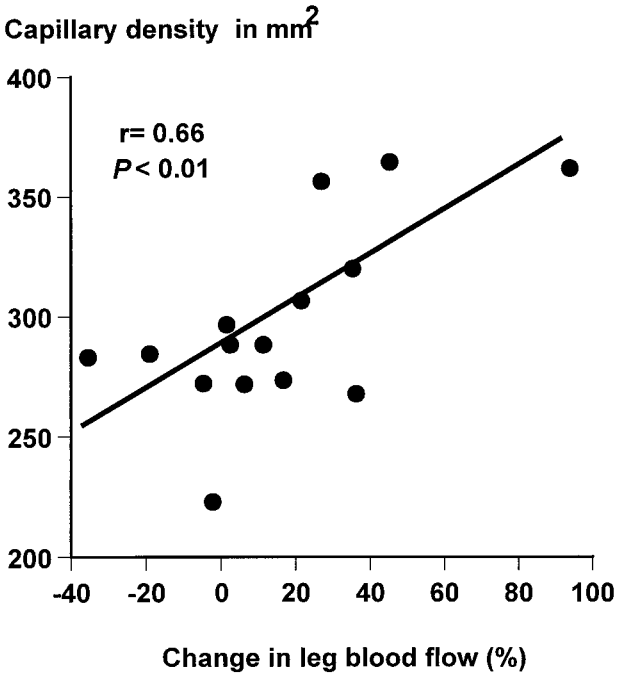


**Fig 1. Relationship between insulin-mediated change in LBF and number of capillaries per square millimeter.**

uptake ($r = -.53$; $P = .05$) but showed no correlation to capillary density. In multiple regression analysis, 71% of LBF variations (95% CI, 23% to 88%) during hyperinsulinemia were explained by capillary density and serum FFA level (28% and 46%, respectively; $P < 0.001$ for the model).

Glucose uptake (M) was significantly correlated to change in LBF ($r = .51$; $P = .05$), but not after correction for BMI, W/H ratio, smoking, and physical activity ($r = .49$; $P = 0.1$). Supine heart rate showed an inverse but insignificant relationship to change in LBF after adjustment for obesity, physical activity, smoking, and capillary density ($r = -.57$; $P = 0.1$). Low-density lipoprotein (LDL), HDL, total cholesterol, and triglyceride levels in serum were not related to LBF changes. The change in LBF of 15% ± 11% during the clamp test did not reach statistical significance ($P = .07$), probably because of the large variation in LBF and heart rate response to hyperinsulinemia among the study subjects (Table 2). In 4 individuals, the LBF decreased during clamping by 16% ± 8%, ranging from −36% to −3%. In contrast, in 11 subjects it increased by 27% ± 8% ($P < .01$; range, 1% to 94%).

Both the number of capillaries per fiber and the capillary density (mm$^2$) were highly correlated to the fiber area ($r = .74$ and $r = -.67$, respectively; $P < .001$ for both). However, the number of capillaries per fiber was not related to the diffusion distance ($r = .19$), whereas the capillary density (mm$^2$) was highly correlated to this parameter ($r = -.92$; $P < .001$).

## DISCUSSION

The present study of healthy elderly men indicates that subjects with the highest insulin-induced increase in LBF have the most dense capillary supply in the same skeletal muscle bed. Some loss of capillaries around fibers has been reported because of reduced muscle fiber area in aged subjects.[36] However, lifestyle components, namely habitual physical activity and weight,[4,14,18] have major impact on both LBF and muscle structure. However, the association between capillary density and LBF in our study was independent of level of physical activity, smoking, and obesity.

The average insulin-induced increase in LBF, 15%, in this study was not statistically significant, probably because of the large variation in blood flow responses among the subjects. Capillary density and serum FFA concentration explain 71% of this variation. Experimental observations show that an acute increase in the circulating FFA level in response to exogenous lipid and heparin infusion produces impairment in endothelial

**Table 2. Baseline Values and Changes in Measures of Femoral Artery Blood Flow During the Hyperinsulinemic Euglycemic Clamp Test**

| | Baseline* | Δ (%)* | 95% CI | *P* for Δ |
|---|---|---|---|---|
| Heart rate (beats/min) | 61 ± 2 | −3.0 ± 2.3 | (−7-2) | .3890 |
| Velocity time integral (m) | 0.04 ± 0.003 | 15.8 ± 7.3 | (0.12-31.5) | .0485 |
| Stroke volume (mL) | 5.4 ± 0.4 | 19.1 ± 4.9 | (2.8-35.4) | .0245 |
| LBF (mL/min) | 333 ± 29 | 15.4 ± 10.9 | (−1.5-32.2) | .0714 |

* Mean ± SEM.

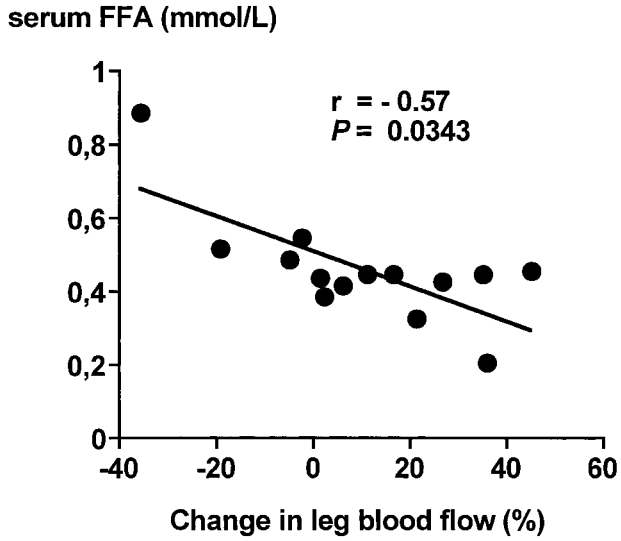


**Fig 2. Relationship between insulin-mediated change in LBF and serum FFA concentration.**

function.[37] This finding is consistent with a recent observation in a larger sample from the present population, in which the insulin-induced changes in LBF were inversely associated with serum concentration of FFA.[25] In the present study, in which a sample of healthy men drawn from that population was investigated, this association was confounded by W/H ratio, probably because abdominal obesity is a strong determinant of FFA level. Therefore, the previous findings in obese individuals, describing impaired endothelial function in this state, might be explained by long-term exposure of endothelium to elevated serum levels of FFA in combination with capillary rarefaction.[4]

According to earlier observations, the vasoactive effect of insulin involves a balance between its local vasodilatory capacity and its vasoconstrictive central sympathoexcitatory effect.[38] Furthermore, it is theoretically possible that under hyperinsulinemia, the effects of vasoconstrictive protein endothelin (ET-1) become apparent.[39] Because of the increase in sympathetic tone with aging,[40] elderly subjects may have vasoconstriction in response to physiologic elevations of plasma insulin, resulting in a decrease in LBF up to 15%.[2] In one recent study, in which the resting limb flow was compared between young and old healthy individuals, the age-related impairment in blood flow was no longer significant after correction for muscle sympathetic nerve activity, which was 74% higher in the elderly.[28] This finding is in accordance with those of the present study, in which the supine heart rate showed an inverse association with LBF changes. Increased FFA levels have also been seen to lead to increased neurovascular tone by increasing $\alpha$-adrenoreceptor reactivity.[41] Thus, the combination of high FFA levels in serum and elevated sympathetic tone might be a possible reason men with highest FFA levels did not respond to insulin with vasodilation but in its place showed a decrease in blood flow caused by stimulation of vascular adrenergic tone.

In addition, we have recently found an association between capillary rarefaction and increased heart rate in the present population.[42] This supports the finding in animal studies that skeletal muscle subjected to high-frequency pacing showed a decrease in capillary density.[43] Moreover, higher resting heart rate was significantly related to elevated levels of serum FFA in a larger sample from the present population.[25] Because there was no correlation between capillary supply and serum FFA level, the associations of these 2 parameters with insulin-mediated changes in LBF most likely involve different mechanisms. However, both mechanisms might have their origin partly in increased sympathetic activity because both of the parameters were associated to heart rate.

The total number of capillaries per fiber was not correlated to LBF in our study. In contrast, in a previous study in which these measurements were performed in leg and arm muscles, respectively, insulin-induced change in blood flow was found to be related to the number of capillaries per fiber but not to capillary density in square millimeters.[6] In that study, the lack of association could be explained by unusually low values of capillary density in femoral muscle. Those were twice as low as the values in present study and the values reported previously.[14,15,44] Additionally, in the present population, the number of capillaries per fiber was not related to the markers of glucose metabolism, but capillary density showed the strongest relationship to insulin sensitivity.[33]

In conclusion, among the variables of muscle morphology, the capillary density showed a close and independent correlation to insulin-induced changes in LBF. The decreased capillary density may be a consequence of reduced vasodilation at the precapillary level caused by endothelial damage. Furthermore, an elevated serum concentration of FFA, which is related to obesity and increased sympathetic activity, might contribute to the endothelial injury and vasoconstriction. In addition, this study shows that insulin-induced blood flow changes, measured in a large conduit artery, may reflect the capacity for capillary recruitment in skeletal muscle during hyperinsulinemia.

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