Skeletal muscle capillary responses to insulin are abnormal in late-stage diabetes and are restored by angiotensin-converting enzyme inhibition

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Clerk LH, Vincent MA, Barrett EJ, Lankford MF, Lindner JR. Skeletal muscle capillary responses to insulin are abnormal in late-stage diabetes and are restored by angiotensin-converting enzyme inhibition. Am J Physiol Endocrinol Metab 293:E1804–E1809, 2007.—Acute physiological hyperinsulinemia increases skeletal muscle capillary blood volume (CBV), presumably to augment glucose and insulin delivery. We hypothesized that insulin-mediated changes in CBV are impaired in type 2 diabetes mellitus (DM) and are improved by angiotensin-converting enzyme inhibition (ACE-I). Zucker obese diabetic rats (ZDF, n = 18) and control rats (n = 9) were studied at 20 wk of age. One-half of the ZDF rats were treated with quinapril (ZDF-Q) for 15 wk prior to study. CBV and capillary flow in hindlimb skeletal muscle were measured by contrast-enhanced ultrasound (CEU) at baseline and at 30 and 120 min after initiation of a euglycemic hyperinsulinemic clamp (3 mU·min⁻¹·kg⁻¹). At baseline, ZDF and ZDF-Q rats were hyperglycemic and hyperinsulinemic vs. controls. Glucose utilization in ZDF rats was 60–70% lower (P < 0.05) than in controls after 30 and 120 min of hyperinsulinemia. In ZDF-Q rats, glucose utilization was impaired at 30 min but similar to controls at 120 min. Basal CBV was lower in ZDF and ZDF-Q rats compared with controls (13 ± 4, 7 ± 3, and 9 ± 2 U, respectively). With hyperinsulinemia, CBV increased by about twofold in control animals at 30 and 120 min, did not change in ZDF animals, and increased in ZDF-Q animals only at 120 min to a level similar to controls. Anatomic capillary density on immunohistology was not different between groups. We conclude that insulin-mediated capillary recruitment in skeletal muscle, which participates in glucose utilization, is impaired in animals with DM and can be partially reversed by chronic ACE-I therapy.

contrast ultrasound; microbubbles; blood flow; blood volume

There is increasing evidence that abnormal vascular responses to insulin play a role in the pathogenesis of type 2 diabetes mellitus (DM). In normal subjects, insulin increases skeletal muscle blood flow in a dose-dependent fashion (1, 3, 8, 26, 29). In the distal microcirculation, insulin increases skeletal muscle blood flow in part by increasing the number of capillaries actively perfused (8, 10, 26). Capillary recruitment occurs relatively rapidly after the development of physiological hyperinsulinemia or after a carbohydrate-rich meal and is thought to increase surface area for glucose and insulin entry into muscle tissue (26, 32, 34). Impairment of these capillary responses may contribute to the development of insulin resistance, hyperglycemia, and diabetic complications. Therefore, improvement in microvascular responses may be a novel therapeutic target for patients with insulin resistance, although routine methods for evaluating capillary reactivity are lacking.

We (8, 10, 32–34) have recently used skeletal muscle contrast-enhanced ultrasound (CEU) to characterize the magnitude and time course of changes in capillary blood volume (CBV) in response to insulin. CEU performed in normal individuals (8, 10, 34) has demonstrated that CBV increases by 50–100% during acute physiological hyperinsulinemia. Changes in CBV appear to be blunted in obese hyperinsulinemic humans (6, 19).

In this study, CEU was used to study capillary responses to insulin in the Zucker diabetic fatty (ZDF) rat. A homozygous mutation in the leptin receptor in these animals produces hyperphagia, obesity, and DM by 6–10 wk of age (18). We (4, 17, 24, 31) hypothesized that abnormal capillary responses to insulin in late-stage ZDF rats could be reversed by early chronic treatment with an angiotensin-converting enzyme (ACE) inhibitor that, in certain populations, has been shown to improve glucose tolerance and to reduce microvascular complications. Secondary aims of the study were to determine whether abnormal capillary responses are associated with abnormalities in either rheological properties of blood or anatomic capillary density.

MATERIALS AND METHODS

Animal preparation and protocol. The study was approved by the University of Virginia Animal Care and Use Committee. Eighteen obese ZDF/Crl-Leprdb rats homozygous for the leptin receptor mutation and nine lean heterozygous control rats (Charles River Laboratories) were studied at 20 wk of age. One-half of the obese ZDF rats were pretreated for 15 wk with quinapril hydrochloride (0.3 mg·kg⁻¹·day⁻¹) in the drinking water, a dose that has been shown (27) to reduce diabetic nephropathy in Zucker rats. Animals were placed in a metabolic cage for 24 h prior to the imaging studies to measure urine production and were fasted overnight. Anesthesia was induced by intraperitoneal injection of pentobarbital sodium (55 mg/kg). The jugular veins were cannulated for administration of drugs, glucose, and microbubbles. A carotid artery was cannulated for pressure measurement and arterial blood sampling. Anesthesia was maintained by a continuous infusion of pentobarbital sodium (0.6 mg·kg⁻¹·min⁻¹). Euthermia was maintained by a heating pad and lamp. The skin overlying the proximal hindlimb adductor muscles was shaved. A euglycemic hyperinsulinemic clamp was started 1 h after surgical preparation to allow for hemodynamic stabilization. Hindlimb skeletal muscle CEU was performed at baseline and at 30 and 120 min after the initiation of a euglycemic hyperinsulinemic clamp. Upon completion of the clamp, blood was sampled for red blood cell (RBC) deformability. Hindlimb muscle samples were obtained for measurement of capillary density by immunohistology.
Hyperinsulinemic clamp. Insulin was infused intravenously at 3 mL/min·kg⁻¹ for 2 h. Arterial blood glucose was measured (AccuCheck; Roche) at baseline, at 10-min intervals for the first hour of the clamp, and then every 15 min for the remaining hour. Dextrose (30%) was infused at a variable rate to maintain glucose concentration at fasting basal levels. The glucose utilization rate was determined from the dextrose infusion rate required to maintain euglycemia and was expressed normalized to body weight. Plasma insulin concentration was measured by radioimmunoassay of arterial blood samples obtained at baseline and upon completion of the clamp.

CEU. CEU of the proximal hindlimb adductor muscles (adductor magnus and semimembranosus) was performed in the transaxial plane with high-power, pulse-inversion imaging at a transmission frequency of 3.3 MHz (HDI-5000; Philips Ultrasound). The mechanical index was set at 0.9. Gains were optimized at the beginning of each study to achieve signal to noise ratio of 3.3. Incremental pulsing intervals (PI) from 0.5 to 20 s. PI vs. intensity was fit to the function $y(t) = A(1 - e^{-\beta t})$ (1), where $y$ is intensity at a PI of $t$, $A$ is the plateau intensity reflecting microvascular blood volume, and $\beta$ is the rate constant reflecting red cell flux rate ($V_{RBC}$) (10). Microvascular blood flow was determined by the product of $A$ and $\beta$. Signal from most noncapillary vessels was eliminated by a signal processing algorithm that relies on the relation between vessel size and $V_{RBC}$ (10).

Muscle capillary blood volume fraction. To validate the $A$ value as indicator of CBV, we compared relative $A$ values in rats from each group ($n = 6$) with measurements of absolute skeletal muscle CBV. The skeletal muscle CBV was determined by the ratio of skeletal muscle $A$ value at three different infusion rates of $1 \times 10^7$ to $3 \times 10^7$ min/kg to the corresponding signal in the left ventricular cavity, representing 100% blood volume. Infusion rates were reduced 10-fold for cavity measurements and scaled appropriately to ensure that concentration did not exceed the system dynamic range.

Blood rheological parameters. For measurement of red cell deformability, 25 μL of arterial blood was added to 5 ml of 5% polyvinylpyrrolidone in saline. The cell elongation index, defined as the ratio of short- to long-axis RBC dimensions, was measured on a laser-assisted optical rotational cell analyzer (Mechatronics) at 37°C (5a).

Capillary density. Immunohistochemistry was performed on fixed, paraffin-embedded sections of hindlimb skeletal muscle. For endothelial cell staining, a mouse anti-CD31 primary monoclonal antibody (MAB1393; Chemicon) was used with an FITC-labeled goat anti-rat secondary antibody (Molecular Probes). For smooth-muscle α-actin staining, a mouse primary monoclonal antibody (1A4; Sigma) was used with an Alexa 555-labeled secondary antibody (Molecular Probes). Fluorescent microscopy (Axioskop-II FS; Carl Zeiss) was performed with excitation filters of 460–490 and 530–560 nm. At least 15 random optical fields for each animal were analyzed, blinded to animal identity. Capillary density was determined in transverse muscle sections by the number of vessels that stained positive for CD31 but not smooth-muscle α-actin. Data were expressed as a percentage of the total muscle area. We (21) have previously demonstrated a 5% interobserver variance for these measurements.

Statistical methods. Data were analyzed using SigmaStat software. Comparisons were made using repeated-measures analysis of variance with a Tukey post hoc test or, when appropriate, with a Kruskal-Wallis test with Dun’s post hoc test. Data were considered significant at $P < 0.05$.

Table 1. Baseline fasting hemodynamic and metabolic characteristics

<table>
<thead>
<tr>
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<th>Control</th>
<th>ZDF</th>
<th>ZDF-Q</th>
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<tr>
<td>Mean arterial pressure, mmHg</td>
<td>104±6</td>
<td>96±6</td>
<td>104±9</td>
</tr>
<tr>
<td>Blood glucose, mg/dl</td>
<td>132±7</td>
<td>433±56*</td>
<td>530±245*</td>
</tr>
<tr>
<td>Plasma insulin, pM</td>
<td>447±84</td>
<td>211±65*</td>
<td>145±61*</td>
</tr>
<tr>
<td>24-h Urine protein, g</td>
<td>13±2</td>
<td>52±9*</td>
<td>64±13*</td>
</tr>
</tbody>
</table>

Values are means ± SE. ZDF, Zucker diabetic fatty rats; ZDF-Q, quinapril-treated ZDF rats. *$P < 0.05$ vs. controls.

Fig. 1. Mean (± SD) arterial blood pressure (A) and arterial blood glucose concentration (B) at baseline (time 0) and during euglycemic hyperinsulinemic clamp in control, Zucker diabetic fatty (ZDF), and quinapril-treated ZDF (ZDF-Q) rats. *$P < 0.05$ vs. ZDF and ZDF-Q.

Fig. 2. Mean (± SD) glucose infusion rate required to maintain euglycemia at baseline (time 0) and during euglycemic hyperinsulinemic clamp in control, ZDF, and ZDF-Q rats. *$P < 0.05$ vs. controls at all time points; †$P < 0.05$ vs. ZDF after 40 min.
RESULTS

Baseline hemodynamic and metabolic characteristics. Baseline hemodynamic and metabolic characteristics for each group are illustrated in Table 1. Mean arterial pressure was not significantly different between groups. Under fasting baseline conditions, both the untreated ZDF and the quinapril-treated ZDF (ZDF-Q) rats were hyperglycemic and hypoinsulinemic compared with control rats. There was a nonsignificant trend toward lower plasma insulin levels in ZDF-Q compared with untreated ZDF rats. Urine production in 24 h was significantly greater for both ZDF and ZDF-Q rats compared with controls, consistent with hyperglycemia-associated polyuria.

Responses to euglycemic physiological hyperinsulinemia. Mean arterial blood pressure and glucose levels were maintained at baseline levels during the entire duration of the 2-h euglycemic hyperinsulinemic clamp (Fig. 1). Plasma insulin concentration at the end of the clamp was not significantly different among the three groups (929 ± 137, 986 ± 175, and 753 ± 82 pM for control, ZDF, and ZDF-Q rats, respectively). In control rats, the glucose utilization rate increased rapidly in response to insulin and reached peak at ~60 min (Fig. 2). In ZDF rats, the increase in glucose utilization in response to insulin was delayed and blunted at all time points compared with controls. In ZDF-Q rats, the early response to insulin in the first 30 min of the clamp was impaired, resulting in a glucose utilization rate lower than that of control animals and similar to untreated ZDF rats (Fig. 2). Subsequently, insulin-mediated glucose utilization in ZDF-Q rats reached levels that were identical to those of control animals and was significantly greater than in untreated ZDF animals. These findings indicate improvement in insulin sensitivity with quinapril treatment, particularly late after hyperinsulinemia.

Skeletal muscle CBV at baseline before the hyperinsulinemic clamp was significantly lower in ZDF and ZDF-Q rats compared with controls (Fig. 3). In animals where absolute CBV fraction was measured by normalization to blood pool signal, the relative reductions in CBV fraction for the ZDF and ZDF-Q rats was similar to those measured by the nonnormalized A value (Fig. 3). On the basis of these findings, CEU analysis during the hyperinsulinemic clamp was performed using nonnormalized data.

Figure 4 illustrates background-subtracted, color-coded CEU images and corresponding pulsing interval vs. intensity measurements obtained from a control rat at baseline and after 30 and 120 min of euglycemic hyperinsulinemic clamp. Insulin produced an increase in capillary blood flow secondary to an increase in the CBV, indicated by an increase in the plateau intensity. Analysis of CEU
data from control animals demonstrated that insulin produced an early (30 min) increase in CBV and capillary flow that persisted at 120 min (Fig. 5). In ZDF rats, insulin failed to produce any change in either CBV or capillary flow. In ZDF-Q rats, insulin produced a time-dependent increase in CBV and capillary flow in a pattern similar to its effect on glucose utilization. In these animals, CBV and capillary blood flow ultimately reached levels similar to control animals late (2 h) after hyperinsulinemia. Capillary \( V_{RBC} \) was similar between groups and did not change significantly during insulin infusion (data not shown).

**Capillary density and blood rheological parameters.** Capillary density measured by immunohistochemistry was not significantly different between the different groups \((543 \pm 46, 515 \pm 53, \text{ and } 523 \pm 44 \text{ mm}^{-2} \text{ for control, ZDF, and ZDF-Q rats, respectively})\). The rheological parameters of red cell deformability also were not significantly different between groups \((\text{elongation index of } 0.56 \pm 0.01, 0.55 \pm 0.01, \text{ and } 0.54 \pm 0.01 \text{ for control, ZDF, and ZDF-Q rats, respectively})\).

**DISCUSSION**

The pathophysiology of type 2 DM is complex and multifactorial. It has been well established \((1, 2, 29)\) that insulin increases limb blood flow in a dose-dependent fashion and that the magnitude of flow augmentation is associated with glucose uptake. A variety of different investigational methods \((3, 8, 10, 26)\) have been used in animal models and in humans to firmly establish that physiological hyperinsulinemia augments skeletal muscle perfusion. Since skeletal muscle is responsible for the majority of insulin-stimulated uptake of glucose, it has been postulated \((20, 30)\) that increases in flow enhance glucose utilization by increasing delivery to skeletal muscle and that alterations in this response contribute to hyperglycemia. This concept has not been universally accepted because of conflicting data that argue against a relationship between muscle blood flow and glucose uptake during hyperinsulinemia.

We believe that conflicting data may be secondary to how skeletal muscle blood flow is defined and measured \((26)\). An examination of flow at the capillary level, the site of glucose and insulin entry into muscle, is likely to provide the most insight. At this level, increased blood flow results from increased rate of flux through capillaries and/or an increase in the number of perfused capillaries. With regard to the latter, under basal conditions only 25–40% of capillaries in skeletal muscle are actively perfused so that potential capillary blood volume reserve is high \((9, 14)\). For augmentation of glucose uptake, capillary recruitment represents a more effective response than increased flux rate, since extravascular diffusion of glucose is highly dependent on permeability-surface area product \((3, 7)\). Methods that evaluate blood flow in conduit vessels, total limb flow, or even total muscle perfusion \((8, 10, 25, 32, 34)\) are not, however, able to evaluate changes in effective capillary density or shunting from nonnutritive to nutritive microvascular pathways.

Skeletal muscle responses have recently been studied using CEU. According to these studies, physiological hyperinsulinemia produces a 50–100% increase in skeletal muscle CBV in anesthetized rats and at least a 20–25% increase in normal human subjects without changing \( V_{RBC} \) \((8, 10, 33, 34)\). In the current study, CEU was used to study skeletal muscle microvascular responses in ZDF rats at a relatively advanced stage of disease. Under basal conditions, these animals had hyperglycemia and decreased skeletal muscle CBV. Both insulin-mediated glucose disposal and capillary recruitment were markedly impaired in the ZDF rats during the euglycemic clamp. These findings are similar to studies performed in the obese Zucker rat strain where skeletal muscle blood flow was lower than controls \((12)\), and microvascular responses assessed by capillary xanthine oxidase activity were impaired when measured 120 min after high-dose insulin infusion \((35)\).

The most important new finding of this study was that chronic treatment of ZDF rats with quinapril improved both insulin-mediated glucose disposal and capillary recruitment. In ZDF-Q rats, there was a delay in both capillary recruitment and glucose uptake compared with controls, but eventually both CBV and glucose uptake increased to levels similar to controls by the end of the clamp. These responses provide further evidence that changes in CBV are requisite for insulin to impart its full effect on glucose disposal. The underlying mechanisms responsible for insulin-mediated capillary recruit-
ment and why they are abnormal in diabetes are not known. The observation that CBV changes occur independently of \( V_{\text{KBC}} \) (16) suggest that hemodynamic changes at the precapillary arteriolar level alone are not responsible. Although anatomic rarefaction of capillaries has been described in obese Zucker rats (13), we did not observe any differences in capillary density between groups. RBC deformability is an important rheological determinant of blood viscosity and resistance at the capillary level and has been shown (5, 23) to be abnormal in animal models and patients with DM. However, we did not detect any abnormalities in the RBC deformability between the groups, although elongation index was assessed only at a single-shear level.

The clinical impact of this study is uncertain. Although retrospective analysis of high-cardiovascular risk patients treated with ACE inhibition (ACE-I) suggested that they prevent the onset of type 2 DM (5, 31), prospective studies such as the DREAM trial have not confirmed this effect (11). A consistent finding, even in the DREAM trial, is that ACE-I can improve insulin sensitivity in type 2 DM or insulin-resistant states. This study provides evidence that improvement in vascular reactivity may be a possible mechanism, at least when duration of therapy is long. In relative terms, the beneficial effect of quinapril on glucose uptake in this rat model of disease exceeds that seen in human trials. This difference may be related to the observation that insulin-mediated changes in CBV also tend to be much greater in anesthetized rats compared with humans (8, 10, 34, 33) or the relatively advanced stage of disease. It should be noted that effects of ACE-I on glucose sensitivity or capillary responsiveness may not be translatable to type 1 DM (28).

There are several limitations of this study that should be acknowledged. First, we studied ZDF animals at an advanced age and investigated the effects of a single dose of quinapril for a relatively prolonged period of time. Whether the beneficial effects of ACE-I vary according to duration of disease and therapy is unknown. The mechanism(s) for the beneficial effects of ACE-I has not yet been determined. We also did not examine different muscle groups that vary according to fiber types and capillary/myocyte ratio (22). Normalization of CBV to total blood volume was performed only in select animals at baseline. We did not believe that this was necessary in all animals since nonnormalized CBV values from CEU appeared to correlate well with the absolute measurements and with other independent methods for evaluating CBV in previous studies (10). Normalization protocols at each stage would have also required excessive volume because of the need for multiple acquisitions at varying infusion rates. Although by 120 min glucose disposition reached similar values in the ZDF-Q and controls, this finding does not necessarily imply a normal response. The ZDF-Q rats were hyperglycemic, which would be expected to enhance the glucose infusion rate required if glucose disposal was indeed normal. In a related manner, we did not evaluate the relative influence of hyperglycemia alone in normal animals. Finally, it must be cautioned that the effect of ACE-I on insulin-mediated capillary response seen in this study may differ from that in patients not only because of species-related differences but also because of potential effects of anesthesia.

In summary, we have determined that skeletal muscle capillary responses to insulin are markedly impaired in a rat model of advanced DM and can be partially reversed with chronic ACE-I therapy. Further studies are needed to determine whether abnormal capillary responses to provocation can predict disease progression in patients with early insulin resistance or to guide preventative therapy in high-risk patients. The finding that insulin-mediated changes in CBV can be modified by ACE-I indicates that capillary function could represent a new therapeutic target for the treatment or prevention of DM.

**REFERENCES**