Increased skeletal muscle capillarization enhances insulin sensitivity

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Increased skeletal muscle capillarization enhances insulin sensitivity. Am J Physiol Endocrinol Metab 307: E1105–E1116, 2014. First published October 28, 2014; doi:10.1152/ajpendo.00020.2014.—Increased skeletal muscle capillarization is associated with improved glucose tolerance and insulin sensitivity. However, a possible causal relationship has not previously been identified. Therefore, we investigated whether increased skeletal muscle capillarization increases insulin sensitivity. Skeletal muscle-specific angiogenesis was induced by adding the α1-adrenergic receptor antagonist prazosin to the drinking water of Sprague-Dawley rats (n = 33), whereas 34 rats served as controls. Insulin sensitivity was measured ±40 h after termination of the 3-wk prazosin treatment, which ensured that prazosin was cleared from the bloodstream. Whole body insulin sensitivity was measured in conscious, unrestrained rats by hyperinsulinemic euglycemic clamp. Tissue-specific insulin sensitivity was assessed by administration of 2-deoxy-[3H]glucose during the plateau phase of the clamp. Whole body insulin sensitivity increased by approximately 24%, and insulin-stimulated skeletal muscle 2-deoxy-[3H]glucose disposal increased by approximately 30% concomitant with an approximate 20% increase in skeletal muscle capillarization. Adipose tissue insulin sensitivity was not affected by the treatment. Insulin-stimulated muscle glucose uptake was enhanced independent of improvements in skeletal muscle insulin signaling to glucose uptake and glycogen synthesis, suggesting that the improvement in insulin-stimulated muscle glucose uptake could be due to improved diffusion conditions for glucose in the muscle. The prazosin treatment did not affect the rats on any other parameters measured. We conclude that an increase in skeletal muscle capillarization is associated with increased insulin sensitivity. These data point toward the importance of increasing skeletal muscle capillarization for prevention or treatment of type 2 diabetes.

Capillarization; skeletal muscle; insulin sensitivity; muscle glucose uptake

INSULIN RESISTANCE IN SKELETAL MUSCLE is one of the key impairments that underlie the pathogenesis of impaired glucose tolerance and type 2 diabetes mellitus (T2DM) (15). Although defective glucose transport, mediated by GLUT4, is central to the development of skeletal muscle insulin resistance (40), it is also recognized that diminished microvascular recruitment (8) and skeletal muscle rarefaction (5) are also important contributing factors.

Reduced capillarization in skeletal muscle has been demonstrated in obesity (19) and T2DM (30), both of which are conditions that are characterized by insulin resistance. Greater microvascular density is associated with improved glucose tolerance (30) and insulin sensitivity (28). In cross-sectional studies there is a positive association between insulin sensitivity and capillarization (21), which is independent of age, adiposity, resting metabolic rate, and body composition (44), suggesting an important role for capillarization in insulin sensitivity. However, direct evidence for a role of capillarization in insulin sensitivity is lacking.

Endurance training improves glycemic control (29), primarily by enhancing skeletal muscle insulin sensitivity (17), and represents an important way to prevent and treat T2DM. Capillarization changes readily with endurance training, and the changes are correlated with improvements in glucose tolerance (29) and insulin sensitivity (26), suggesting that changes in capillarization may be important for improvements in insulin sensitivity.

One of the mechanisms that may underlie the role of capillarization for insulin sensitivity relates to diffusion limitations. Glucose crosses the capillary wall primarily by simple diffusion when it passes from the blood to the interstitial space (37), whereas it is unclear whether transcytosis (27) or passive diffusion (7) is responsible for transendothelial transport of insulin. There is an arterial-interstitial glucose concentration gradient in skeletal muscle (23), which is reduced with endurance training (13), and insulin-resistant subjects have delayed transcapillary delivery of insulin to muscle interstitial fluid (41), suggesting a possible link between insulin sensitivity and transcapillary exchange of both insulin and glucose. An increased capillarization will increase the capillary surface area, increase mean transit time, and result in a lower average diffusion distance from the capillary to the muscle, which would facilitate delivery of insulin and glucose to the muscle.

The importance of changes in muscle capillarization associated with improvements in insulin sensitivity due to exercise training is difficult to distinguish from other important adaptations that occur in parallel. To isolate the effect of an increased capillarization on insulin sensitivity, we used the α1-adrenergic receptor antagonist prazosin to increase skeletal muscle capillarization. Prazosin is an effective and selective means to increase capillarization in skeletal muscle and has been used in numerous rodent studies (10, 56).

In the present study, we tested whether increased skeletal muscle capillarization enhances skeletal muscle insulin sensitivity in normal rats.
D, respectively. The data are presented as P
ment, n/H11005 treated (sentative sections are 1.4 and 1.8 for
Dmuscle sections of control (C) and prazosin-
Cweight, lean mass, and fat mass. Lean and fat mass was measured by
single cages. The rats were allocated to the two groups according to
being allocated to either prazosin or control treatment and housed in
Germany) and water. The rats were acclimatized for 1 wk before
light-dark cycle with free access to food (no. 1320; Altromin, Lage,
temperature (22–23°C) and 35–55% relative humidity on a 12:12-h
Taconic Europe, Lille Skensved, Denmark) were housed at a constant
animals. Male Sprague-Dawley rats 9 wk of age (n = 132; Taconic Housing and Care)
were housed in single cages. The rats were allocated to the two groups according to
weight, lean mass, and fat mass. Lean and fat mass was measured by
MR scanning with an EchoMRI whole body composition analyzer (Echo Medical Systems).

Prazosin treatment. The prazosin group received prazosin hydro-
chloride (lot no. BCBC3883; Sigma-Aldrich) in their drinking water for
21 days. Prazosin was dissolved in distilled water (50 mg/l), and
pH was adjusted to 5.8 with a pH meter to improve solubility. The
control group received distilled drinking water with the pH set to 5.8. Body weight and fluid ingestion were recorded, and drinking water was replaced daily. Prazosin treatment was terminated 41–42 h before the hyperinsulinemic euglycemic clamp (HIEC), allowing time for prazosin to be cleared (3), thus avoiding any acute effects of prazosin affecting the measurement of insulin sensitivity.

Surgery. Nine days prior to the HIEC, all rats were instrumented with permanent catheters (0.41 mm id, Tygon S-54-HL Microbore Tubing; Norton Performance Plastics) in the right jugular vein (for infusions) and left carotid artery (for blood sampling) under aseptic conditions. The animals were anesthetized with a 2-ml/kg body wt sc injection of an anesthetic cocktail (5 mg/ml Hypnorm, Vetapharma; and 2.5 mg/ml midazolam; Hameln Pharmaceuticals). Prior to surgery, the rats received an injection of 0.5 ml/kg body wt sc Alumycin Prolongatum Vet (200 mg/ml; ScanVet). The rats were also given saline (0.9%, 20 ml/kg sc) immediately after surgery to avoid possible dehydration as well as an sc injection of Rimadyl (0.05 mg/kg sc; Pfizer) to relieve pain. Another injection of Rimadyl was given the
day after surgery. The rats also received Terramycin Vet (20%; Pfizer) dissolved in their drinking water (500 mg/l) on the day of surgery and the following 2 days. On the 8th day after surgery the animals were inspected, and those rats that 1) had lost 98% of presurgical weight, 2) had signs of ptosis, 3) had erect and unclean fur, 4) had pericellular porphyrin staining, or 5) had unhealed surgical wounds were excluded. Five rats (prazosin, n = 3; control, n = 2) were excluded.

Methods

Animals. The Danish Animal Experimental Inspectorate approved all animal protocols. Male Sprague-Dawley rats 9 wk of age (n = 67; Taconic Europe, Lille Skensved, Denmark) were housed at a constant temperature (22–23°C) and 35–55% relative humidity on a 12:12-h light-dark cycle with free access to food (no. 1320; Altromin, Lage, Germany) and water. The rats were acclimatized for 1 wk before being allocated to either prazosin or control treatment and housed in single cages. The rats were allocated to the two groups according to weight, lean mass, and fat mass. Lean and fat mass was measured by

![Fig. 1. Capillary/fiber (C:F) ratio in extensor digitorum longus (EDL; A) and soleus muscle (B) of control (n = 14) and prazosin-treated (n = 14) rats. Representative EDL muscle sections of control (C) and prazosin-treated rats (D). The C:F ratios of the representative sections are 1.4 and 1.8 for C and D, respectively. The data are presented as means ± SE. *Effect of the prazosin treatment, P < 0.01.](http://ajpendo.physiology.org/Downloadedfrom)
Hyperinsulinemic euglycemic clamp. We performed HIECs in 14 conscious, unrestrained rats from each group. After an overnight fast (16–17 h), catheters were connected to the infusion system, and the animals were placed in clamp cages and allowed to settle for 45–60 min. Throughout the HIEC (0–120 min), insulin (Actrapid; Novo Nordisk) diluted to 800 pmol/l in a buffer (pH 7.4) consisting of 140 mM NaCl, 5 mM Na₂HPO₄, and, to prevent protein adsorption, 70 ppm Tween-20, was infused at a constant rate (28.1 ± 0.6 pmol·kg⁻¹·min⁻¹). A 40% glucose solution (400 mg/ml, pH 7.4; Fresenius Kabi) was infused at variable rates [glucose infusion rate (GIR)] to maintain euglycemia. Arterial glucose concentration was determined at 5-min intervals (HemoCue). GIR during the final 45 min of the clamp was used as a measure of insulin sensitivity. Blood was drawn at 0 (625 μl), 75 (700 μl), and 120 min (700 μl, end of clamp) for determination of plasma metabolites, hormones, and TNFα. Blood was centrifuged (5 min, 13,000 g, 4°C), and plasma was stored at −20°C until further analysis. The erythrocytes from the blood samples drawn at 0 and 75 min were returned to the animal and diluted 1:1 in heparin infusate (20 IU/ml in 0.9% saline) to avoid a decrease in hematocrit. The hematocrit did not fall during the HIEC (data not shown). At GIR steady state (75 min), a bolus (1.8 MBq/rat) of 2-deoxy-D-[1,2-3H]glucose (2-[3H]DG) (NET549; PerkinElmer, Boston, MA) was injected intravenously, and blood (120 μl) was drawn at 75, 77, 80, 85, 90, 95, 100, 110, and 120 min to determine tissue-specific glucose utilization index (Rg), as described elsewhere (24). At the end of the clamp (120 min), skeletal muscle [extensor digitorum longus (EDL) and soleus], liver, and epididymal fat samples were collected under pentobarbital anesthesia (55 mg/kg iv). Tissues were either immediately frozen in liquid nitrogen (liver, fat, and muscle) or embedded in mounting medium and frozen in precooled isopentane (muscle tissue only, for immunohistochemistry analysis) and stored at −80°C until further analysis. Under anesthesia, the liver (control, n = 12; prazosin, n = 11) and

<table>
<thead>
<tr>
<th>Muscle glycogen, mmol/kg wet wt muscle</th>
<th>Prazosin</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>44.3 ± 1.2</td>
<td>44.0 ± 1.9</td>
</tr>
<tr>
<td>EDL</td>
<td>34.9 ± 2.4</td>
<td>33.3 ± 1.8</td>
</tr>
<tr>
<td>Soleus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin, pmol/l</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t = 0 min (Basal)</td>
<td>60 ± 10</td>
<td>51 ± 6</td>
</tr>
<tr>
<td>t = 75 min (Clamp)</td>
<td>396 ± 32*</td>
<td>417 ± 30*</td>
</tr>
<tr>
<td>t = 120 min (Clamp)</td>
<td>447 ± 21*</td>
<td>458 ± 24*</td>
</tr>
<tr>
<td>FFA, μmol/l</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t = 0 min (Basal)</td>
<td>749 ± 59</td>
<td>860 ± 25</td>
</tr>
<tr>
<td>t = 75 min (Clamp)</td>
<td>217 ± 53*</td>
<td>178 ± 14*</td>
</tr>
<tr>
<td>t = 120 min (Clamp)</td>
<td>149 ± 15*</td>
<td>160 ± 9*</td>
</tr>
<tr>
<td>Glycerol, μmol/l</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t = 0 min (Basal)</td>
<td>133 ± 15</td>
<td>149 ± 7</td>
</tr>
<tr>
<td>t = 75 min (Clamp)</td>
<td>49 ± 5*</td>
<td>51 ± 4*</td>
</tr>
<tr>
<td>t = 120 min (Clamp)</td>
<td>49 ± 4*</td>
<td>49 ± 3*</td>
</tr>
<tr>
<td>Epinephrine, nmol/l (t = 0 min; basal)</td>
<td>2.6 ± 0.1</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>Norepinephrine, nmol/l (t = 0 min; basal)</td>
<td>9.3 ± 0.7</td>
<td>9.1 ± 0.6</td>
</tr>
<tr>
<td>TNFα, pg/ml (t = 0 min; basal)</td>
<td>5.0 ± 0.2</td>
<td>4.9 ± 0.6</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE. EDL, extensor digitorum longus. For all plasma values, n = 14/group; for skeletal muscle glycogen content, n = 4–6/group. *Effect of time, P < 0.01.
soleus and EDL muscle (control, \( n = 6 \); prazosin, \( n = 5 \)) were removed from the rats that did not undergo a HIEC to serve as a control for the clamp experiment. Subsequently, all animals were euthanized with an overdose of pentobarbital sodium (110 mg/kg iv). 2-[\(^{3}H\)]DG measurements. Briefly, \(^{3}H\) counts in neutralized supernatants of deproteinized plasma samples and 2-[\(^{3}H\)]DG and phosphorylated 2-[\(^{3}H\)]DG counts in digested tissue samples before and after the Somogyi extraction procedure were measured in a scintillation counter. The Somogyi extraction procedure employed on the tissue samples removes free intracellular 2-[\(^{3}H\)]DG, and the value thus provides an estimate of the total \( R_{g}^{i} \) (24).

Glycogen synthase activity. Muscle glycogen synthase (GS) activity was measured in a muscle homogenate, as described previously (51).

Immunohistochemistry. The frozen muscle specimens of EDL and soleus were cut into 8-μm-thick cross-sections using a cryostat. Three sections were cut from each tissue sample, one each from the upper, middle, and lower part of the sample. This was done to compare the number of capillaries and fibers in a larger part of the muscle tissue sample. The cross-sections were fixed on microscope glass slides in cold (−20°C) acetone for 30 s and 2% formaldehyde for 2 min at room temperature. The sections were rinsed in phosphate-buffered saline (PBS) containing 1% BSA (PBS-BSA) and blocked for 1 h with PBS-BSA. Subsequently, the cross-sections were incubated in PBS-BSA containing biotinylated Griffonia Simplicifolia Lectin I (2 mg/ml in PBS-BSA; Vector Laboratories, Burlingame, CA) for 1 h, washed with PBS-BSA, incubated in PBS-BSA containing streptavidin conjugated with FITC (1 mg/ml, F0422, streptavidin-FITC; DAKO). Capillary/fiber (C:F) ratio was determined from pictures of the stained sections taken by a high-resolution interline charge-coupled device (CCD) camera (CoolSNAP; Photometrics, Tucson, AZ) through a light microscope (Axioplan 2 Imaging; Zeiss) by counting the number of fluorescent structures using the computer program ImageJ (National Institutes of Health, Bethesda, MD). On average, capillaries surrounding 284 ± 9 muscle fibers were counted per muscle sample.

Plasma protein and metabolite concentrations. Meso Scale Discovery kits were used to analyze insulin (human insulin kit) and TNFα (rat TNFα ultrasensitive kit) concentrations in plasma (Meso Scale Discovery, Gaithersburg, MD). The insulin kit recognizes both rat and human insulin and can therefore measure the total insulin concentration during the clamp experiment. The concentration of glucose (Gluco-quant Glucose/HK kit; Roche Diagnostics, Mannheim, Germany), free fatty acids [NEFA-HR(2), Wako Chemicals, Germany], and glycerol (Syncron System TG2 × 300 triglycerides reagent; Bechman Coulter) was determined spectrophotometrically on a Hitachi 912 Automatic Analyzer (Boehringer Mannheim, Germany). Epinephrine and norepinephrine concentrations in plasma were measured using highly sensitive radioimmunoassay (2-CAT Plasma RIA High Sensitive Kit; Labor Diagnostika Nord).

Insulin infusate. Insulin infusate concentrations were measured by postclamp using high-performance liquid chromatography (E2695 Separations Module with Alliance column heater and 2489 UV Visible detector; Waters, Milford, MA).

Glycogen determination. Glycogen content was determined as glycosyl units in liver (−300 mg) and muscle (−10 mg) samples after acid hydrolysis, as described previously (34). Glucose concentration was determined spectrophotometrically, as described above.

Western blotting. Akt2, Akt substrate of 160 kDa (AS160), Glut4, AMPKα2, and acetyl-CoA carboxylase (ACCo) protein content as well as relative phosphorylation status of AS160, Akt Thr308, Akt Ser473, AMPKα2 Thr172, and ACC-β Ser79 was determined by Western blotting in EDL and soleus on whole muscle lysates. Whole muscle lysate preparation, SDS-PAGE, and immunoblotting have been described previously in detail (1). The membranes were incubated overnight at 4°C in blocking buffer with primary antibody against phospho-Akt Thr308 (9275; Cell Signaling Technology, Danvers, MA), phospho-Akt Ser473 (9271; Cell Signaling Technology), phosho-AS160 (Upstate Biotechnology), Glut4 (PAI-1065; Thermo Scientific, Rockford, IL), phospho-ACCo Ser79 (07-303; Merck Millipore, Darmstadt, Germany), or phospho-AMPKα2 Thr172 (2531; Cell Signaling Technology). The membranes immunoblotted for phospho-Akt Thr308, phospho-Akt Ser473, phospho-AS160, phospho-ACCo Ser79, and phospho-AMPKα2 Thr172 were stripped and reincubated with antibody against Akt2 (9611; Cell Signaling Technology), AS160 (07-741; Upstate Biotechnologies, Waltham, MA), ACC

Fig. 3. Insulin-stimulated tissue-specific glucose utilization index (\( R_{g}^{i} \)) in EDL (A), soleus muscle (B), and fat (C) during the steady-state period (75–120 min) of the hyperinsulinemic euglycemic clamp in control (\( n = 14 \)) and prazosin-treated rats (\( n = 14 \)). Data are presented as means ± SE. *Effect of the prazosin treatment, \( P < 0.05 \).
Fig. 4. A and B: protein content of Akt2 in EDL (A) and soleus muscles (B) at basal and immediately after the euglycemic hyperinsulinemic clamp (clamp) of prazosin-treated (basal, n = 5; clamp, n = 14) and control (basal, n = 6; clamp, n = 14) rats. C and D: Akt2 phosphorylation at site Thr308 relative to Akt2 content in EDL (C) and soleus (D) at basal and immediately after the clamp. E and F: Akt2 phosphorylation at site Ser473 relative to Akt2 content in EDL (E) and soleus (F) at basal and immediately after the clamp. G: representative blots of Akt2 and Akt2 phosphorylation (p-Akt2) sites Thr308 and Ser473 in soleus and EDL muscle of prazosin-treated (P) and control (C) rats. Data are presented as means ± SE. *Effect of the euglycemic hyperinsulinemic clamp, P < 0.05. AU, arbitrary units.
(P0397; Dako Cytomation), and AMPKα2 (sc-19131; Santa Cruz Biotechnology, Dallas, TX), respectively. All membranes (except for ACC, which was incubated with streptavidin) were incubated for 1 h at room temperature with a secondary antibody (P0448; Dako Cytomation). We used Immobilion Western (Millipore) to detect the bands and quantify them using a CCD image sensor (ChemiDocXRS; Bio-Rad) and software (Image Lab; Bio-Rad).

Statistics. Distribution of data was evaluated using probability plots and Kolmogorov-Smirnov tests. Data are presented as means ± SE or percentage change [calculated as (mean posttreatment/mean pretreatment) × 100].

To evaluate the effect of the prazosin treatment and time, we used a two-way repeated-measures mixed-model analyses (PROC MIXED) or t-tests (SAS version 9.2; SAS Institute, Cary, NC) as appropriate. To further evaluate the effects of the prazosin treatment or time, post hoc analysis was performed using Tukey-adjusted t-tests as appropriate. Distribution and variance homogeneity of the residuals derived from the variance analysis were evaluated using probability plots and scatter plots. Significance for all tests was set at P < 0.05.

RESULTS

No effect of prazosin treatment on animal characteristics. Total body weight, fat mass, lean mass, and fluid ingestion did not differ between the groups at 10 and 13 wk of age (Table 1). The prazosin group ingested an average of 1.9 ± 0.1 mg/day prazosin.

Effect of prazosin treatment on skeletal muscle capillarization. The prazosin treatment increased the C:F ratio by 17.0 ± 2.0 and 20.1 ± 2.4% in the EDL and soleus muscle, respectively (P < 0.01; Fig. 1).

Prazosin treatment enhanced insulin-stimulated glucose disposal. Prazosin treatment increased whole body insulin sensitivity by 24 ± 5% (P < 0.01; Fig. 2A), measured as GIR at a similar glucose concentration (Fig. 2B) and degree of hyperinsulinemia (Table 2). Furthermore, plasma metabolite and catecholamine concentrations did not differ between the treatments during the hyperinsulinemic clamp (Table 2). We also measured TNFα plasma concentration, as α1-adrenergic antagonists have been reported to lower TNFα concentration under certain circumstances (18). No difference in plasma TNFα concentration was observed between the prazosin (5.0 ± 0.2 pg/ml) and the control group (4.9 ± 0.6 pg/ml) (Table 2).

In line with the increased whole body insulin sensitivity, insulin-stimulated R'g was 31 ± 9 and 31 ± 8% greater in EDL (P < 0.05; Fig. 3A) and soleus (P < 0.05; Fig. 3B) muscle of the prazosin-treated animals compared with controls, respectively, whereas R'g in epidydimal fat was unaltered (Fig. 3C). Furthermore, there was no difference in basal skeletal muscle glycogen content (Table 2). Liver glycogen content was similar in the prazosin and control groups and was not affected by the HIEC (Fig. 2C).
Muscle insulin signaling was not altered by prazosin treatment. Prazosin treatment did not alter basal or insulin-stimulated phosphorylation of the central insulin-signaling protein Akt2 at the two key sites Thr<sup>308</sup> or Ser<sup>473</sup> (Fig. 4). Akt signaling mediates activation of GS and via the downstream effector, AS160, signals translocation of GLUT4 to the sarcolemma. In line with the Akt data, the prazosin treatment did not affect the basal or insulin-stimulated phosphorylation level of AS160 (Fig. 5, A and B). Insulin-stimulated GS activity (Fig. 6, B and C) was unchanged by prazosin. Furthermore, total GS activity (Fig. 6A) and muscle content of GLUT4 (Fig. 5C) were unaltered by the prazosin treatment.

Muscle AMPK and ACC/β signaling was not altered by prazosin treatment. Expression of AMPKβ2 (Fig. 7, A and B) or the downstream target of AMPK, ACC/β (Fig. 8, A and B), was not altered by prazosin treatment. In addition, prazosin treatment did not alter basal or insulin-stimulated phosphorylation of AMPKα subunits at Thr<sup>172</sup> (Fig. 7, C and D) or ACC/β Ser<sup>79</sup> (Fig. 8, C and D). However, the HIEC seems to have had a small effect on EDL muscle AMPK phosphorylation (Fig. 7C).

**DISCUSSION**

The present study treated sedentary rats with prazosin to specifically induce an increased capillarization of skeletal muscle and examine its role in muscle insulin sensitivity. The prazosin intervention was successful in inducing an increase in capillarization, and the increase was paralleled by a marked increase in muscle insulin sensitivity. The intervention had no effect on insulin signaling, GLUT4 density, GS activity, or AMPK signaling. Thus, this study shows that an increase in skeletal muscle capillarization is associated with enhanced insulin sensitivity in skeletal muscle, which might be due to improved diffusion conditions for glucose.

Increased skeletal muscle capillarization facilitates delivery of insulin and glucose to the myocyte. With regard to insulin, it is unclear whether transcytosis (27) or passive diffusion (7) is the primary mode of transendothelial transport. Nevertheless, there is an arterial-interstitial insulin concentration gradient in insulin-sensitive muscle (7, 32, 41) and insulin-resistant muscle (41), indicating that the capillary wall limits the appearance of insulin in the interstitial space regardless of insulin sensitivity status. Delivery of insulin to the interstitial space appears to mirror muscle glucose uptake and insulin receptor activity (32), and a slower rise of interstitial insulin in obese individuals might explain the delayed muscle glucose clearance response, but it does not explain the reduced sensitivity to insulin (41).

In the present study, the increased muscle capillarization might have led to a faster and more pronounced increase in interstitial insulin concentration and binding to the insulin receptor. However, if so, it did not lead to a quicker rise in insulin-stimulated glucose uptake (Fig. 2A) or translate to increased Akt (Fig. 4) or AS160 (Fig. 5, A and B) phosphorylation. The activity of glycogen synthase (Fig. 6) was also unaltered. Therefore, it seems unlikely that the increased insulin-stimulated glucose uptake in the present study is caused by increased signaling downstream to the myocyte insulin receptor.

AMPK activation constitutes a pathway separate from insulin signaling, which is thought to play an important role in skeletal muscle glucose uptake (16). AMPK is activated by covalent phosphorylation of the α-subunits on residue Thr<sup>172</sup>
and signals GLUT4 translocation to the sarcolemma (45) probably via AS160 (46). An increase in AMPK phosphorylation, and thus activity, due to the prazosin treatment might increase glucose uptake. However, prazosin did not affect the expression or phosphorylation of AMPK (Fig. 7) or the downstream target ACCβ (Fig. 8). Changes in AMPK activity are thus an unlikely explanation for the observed increase in glucose uptake. Interestingly, hyperinsulinemia slightly increased AMPK phosphorylation in EDL muscle (Fig. 7C). Previous reports in humans find that acute physiological hyperinsulinemia does not affect AMPK activity in vastus lateralis muscle (22). Whether this is due to species differences or an effect selective to fast-twitch muscle is not known, but in the present study the increased phosphorylation of AMPK did not translate to an increased ACCβ phosphorylation, which suggests that the increase in AMPK activity is small.

Transcapillary exchange of glucose occurs primarily via diffusion and is therefore dependent primarily on capillary surface area (31) but is also affected by blood flow and capillary permeability. If the exchange of glucose across the capillary wall was very rapid and unimpeded, the glucose concentration in blood and interstitial fluid would equilibrate instantaneously regardless of the rate of cell glucose uptake. However, the arterial glucose concentration is higher than the concentration in the interstitial space surrounding the muscles under basal conditions (23, 37), and the arterial-interstitial gradient is maintained (42) or increased (23) during hyperinsulinemia, which suggests that transcapillary exchange of glucose is limited (37). Therefore, an increased diffusion of glucose due to the increased capillary surface area is a possible explanation for the increased glucose clearance rate observed after prazosin treatment.

We used prazosin to study the isolated effect of increased muscle capillarization on insulin sensitivity. In accord with previous reports (10, 56), prazosin induced an approximately 20% increase in the number of capillaries, which was similar in
slow- and fast-twitch muscle. Prazosin increases capillarization specifically in skeletal muscle via a VEGF- (50) and endothelial nitric oxide synthase (eNOS)-dependent pathway (4) by longitudinal splitting of existing capillaries (55). Prazosin treatment does not affect fiber-type distribution or oxidative capacity (10) and has no effect on cardiac muscle (56). The effect on capillarization has been documented in several different muscle groups located in the leg (10, 55, 56) and trunk (47) and is believed to be induced by elevated shear stress on the capillary wall due to increased blood flow in the muscle (11). However, the effect of prazosin treatment on blood flow is not uniform (10, 11). Prazosin increases capillary erythrocyte velocity in predominantly fast-twitch muscles such as EDL (11) but not in the slow-twitch soleus (10).

In contrast to previous studies (36), prazosin was removed from the drinking water 41–42 h prior to insulin sensitivity being tested in the present study. The short half-life (~2.5 h) of prazosin (3) ensures that prazosin was cleared from the bloodstream before the insulin clamp, which is important because any acute micro- or macrovascular effects of prazosin are entirely dependent on plasma concentration (3, 53).

Increased blood flow (36) has been proposed as the cause of enhanced insulin sensitivity with prazosin treatment. However, Pollare et al. (36) did not measure blood flow, and others have shown that long-term treatment with an α1-adrenergic receptor antagonists does not alter basal blood flow (2, 33) or blood flow during a HIEC (2).

Moreover, the α-adrenergic blocking effect of prazosin results in a dose-dependent compensatory increase of sympathoadrenal activity measured as elevated plasma epinephrine and norepinephrine concentrations (25). The sympathoadrenal activity continues to be elevated during chronic treatment (25), and therefore, it can be used as a marker of the α-adrenergic blocking effect of prazosin. Since we removed prazosin from the drinking water 41–42 h prior to the HIEC, the acute effect on the cardiovascular system and any changes in blood flow would have subsided (3). In accord with this, the epinephrine and norepinephrine plasma concentrations were similar be-
between the prazosin and control groups at the time of the HIEC (Table 2). Therefore, increased micro- or macrovascular blood flow due to the effect of prazosin is an unlikely explanation for the increased glucose disposal observed in the present study.

We cannot rule out that the increased insulin sensitivity was due in part to an interaction between the insulin-induced increase in microvascular flow, which enhances the delivery of glucose to the muscle (49) and the increased capillarization. However, it has been shown that, at the insulin dose used, the microvasculature is fully recruited (54). Therefore, any increase in the absolute number of recruited capillaries would be due to an increased capillarization. In light of this, it seems reasonable to assume that a larger capillary network would enhance the effect of insulin-stimulated microvascular recruitment on glucose clearance.

Inhibition of eNOS in rats decreases insulin-stimulated uptake of glucose by skeletal muscle (48), suggesting that eNOS may play a key regulatory role in glucose metabolism. A role of eNOS is further corroborated by the observation that deletion of the eNOS gene induces insulin resistance (12, 39). Prazosin treatment has been shown to increase skeletal muscle eNOS expression (4). However, overexpression of eNOS does not enhance insulin sensitivity (38), and thus it seems unlikely that increased eNOS expression could explain the improved insulin sensitivity observed in the present study.

Prazosin also affects lipid metabolism, which is evident by lower plasma free fatty acids (FFA), triglycerides, and cholesterol concentration in rats (9). Alterations in lipid metabolism can affect insulin sensitivity, and it is well established that elevated FFA causes insulin resistance (14). Therefore, a plausible explanation for prazosin to enhance insulin sensitivity could be by reducing plasma FFA concentration. However, we observed no difference in plasma FFA or glycerol concentration between the prazosin group and the control group before or during the insulin clamp (Table 2).

In mice, α1-adrenergic receptor antagonists decrease TNFα production (18). TNFα has been shown to induce insulin resistance in humans (35) and rats (52). Neutralization of TNFα using an anti-murine TNFα antibody increases insulin sensitivity in skeletal muscle, but not in adipose tissue (6), which suggests that prazosin treatment might lead to lower circulating levels of TNFα and thereby an increased insulin sensitivity. Although prazosin treatment might have had a transient effect on circulating TNFα concentration during the treatment period, there was no difference in TNFα concentration between the two groups at the onset of the insulin clamp (Table 2). Thus, a difference in TNFα is an unlikely cause for the difference in insulin sensitivity between the two groups.

It is conceivable that prazosin increases hepatic insulin sensitivity, resulting in greater suppression of hepatic glucose production, which would require an increased GIR to maintain euglycemia in the prazosin-treated group during the hyperinsulinemic clamp compared with the control group. However, the plasma insulin concentration at steady state during the HIEC (Table 2) in this study has been shown by others to completely or almost completely suppress hepatic glucose production (43). We did not measure hepatic glucose production, but a previous study investigating the effect of an α1-adrenoceptor blocking agent on whole body insulin sensitivity found that it did not induce changes in hepatic glucose production (20). In addition, we found that prazosin treatment had no effect on liver glycogen content and that there was no difference in fasting liver glycogen content between the rats that underwent the clamp and the ones that did not (Fig. 2D).

Furthermore, insulin-stimulated glucose uptake in fat tissue was not altered by the prazosin treatment (Fig. 2B), thus leaving skeletal muscle as the only large insulin-sensitive tissue to account for the increased insulin-stimulated glucose uptake observed as a result of the Prazosin treatment (Fig. 3, A and B).

Our findings that increased capillarization can improve skeletal muscle insulin sensitivity are in line with a recent pioneering study that used mice with a muscle-specific VEGF gene deletion. These mice have an approximately 50% decrease in skeletal muscle capillarization and are insulin resistant (5).

The present investigation shows for the first time that an increase in capillarization in skeletal muscle of sedentary rats increases insulin sensitivity independent of changes in insulin signaling, GLUT4 density, or GS activity. The prazosin treatment did not seem to affect metabolic pathways within the muscle that could explain the increased insulin sensitivity, suggesting that increased skeletal muscle capillarization in itself can increase the insulin sensitivity.

Our data are consistent with indirect data in humans corroborating that there is a strong positive (21, 28) and independent (44) association between insulin sensitivity and skeletal muscle capillarization. These results also point toward increases in skeletal muscle capillarization being an important adaptation to improve insulin sensitivity and prevent impaired glucose tolerance and T2DM.

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The authors have no potential conflicts of interest, financial or otherwise, relevant to this article to report.

AUTHOR CONTRIBUTIONS

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