Increased skeletal muscle capillarization enhances insulin sensitivity

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ABSTRACT

Increased skeletal muscle capillarization is associated with improved glucose tolerance and insulin sensitivity. However, a possible causal relationship has not previously been identified. We therefore 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) while 34 rats served as controls. Insulin sensitivity was measured ≥40 h after termination of the 3-week 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 ~24% and insulin-stimulated skeletal muscle 2-deoxy-[³H]-Glucose disposal increased by ~30% concomitant with a ~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 towards the importance of increasing skeletal muscle capillarization for prevention or treatment of type 2 diabetes.

Keywords: capillarization, skeletal muscle, insulin sensitivity, muscle glucose uptake
INTRODUCTION

Insulin resistance, in skeletal muscle, is one of the key impairments that underlie the pathogenesis of impaired glucose tolerance and type 2 diabetes mellitus (T2D) (15). Although, defective glucose transport, mediated by GLUT4, is central to the development of skeletal muscle insulin resistance (40), it is 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 T2D (30), conditions which are both 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 T2D. 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 \( \alpha_1 \)-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.

METHODS

Animals The Danish Animal Experimental Inspectorate approved all animal protocols. Male Sprague-Dawley rats, 9 weeks of age (n=67; Taconic Europe A/S, 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 (#1320, Altromin, Lage, Germany) and water. The rats were acclimatized for one week 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 MR scanning with an EchoMRI whole body composition analyzer (Echo Medical Systems, Texas, USA).
**Prazosin treatment** The Prazosin group received Prazosin hydrochloride (lot # BCBC3883, Sigma-Aldrich, China) in their drinking water for 21 days. Prazosin was dissolved in distilled water (50 mg l⁻¹) 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 (b.w.) and fluid ingestion was recorded and drinking water replaced daily. Prazosin treatment was terminated 41-42 hours 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 (Tygon S-54-HL Microbore Tubing, i.d.: 0.41 mm, Norton Performance Plastics, Ohio, USA) in the right jugular vein (for infusions) and left carotid artery (for blood sampling) under aseptic conditions. The animals were anaesthetized with a 2 ml kg⁻¹ b.w. injection (s.c.) of an anesthetic cocktail (Hypnorm, 5 mg ml⁻¹, Vetapharma, UK and Midazolam, 2.5 mg ml⁻¹, Hameln pharmaceuticals, Germany). Prior to surgery, the rats received an s.c. injection of 0.5 ml kg⁻¹ b.w. Alamycin Prolongatum vet (200 mg ml⁻¹, ScanVet, Denmark). The rats were also given 20 ml kg⁻¹ 0.9% saline s.c immediately after surgery, to avoid possible dehydration, as well as an s.c. injection of Rimadyl (0.05 mg kg⁻¹, Pfizer, USA) to relieve pain. Another injection of Rimadyl was given the day after surgery. The rats also received Terramycin vet (20%, Pfizer, Finland) dissolved in their drinking water (500 mg l⁻¹) on the day of surgery and the two following days. On the 8th day after surgery the animals were inspected and those rats that 1) had not regained 98% of pre-surgical weight, 2) had signs of ptosis, 3) had erect and unclean fur, 4) had periocular porphyrin staining, or 5) had unhealed surgical wounds were excluded. Five rats (Prazosin, n=3 and control, n=2) were excluded.

**Hyperinsulinemic Euglycemic Clamp (HIEC)** 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 minutes.
Throughout the HIEC (0–120 min) insulin (Actrapid, Novo Nordisk, Denmark) diluted to 800
117 pmol/l in a buffer (pH 7.4) consisting of 140 mM NaCl, 5 mM Na₂HPO₄, and, in order to prevent
118 protein adsorption, 70 ppm Tween20, was infused at a constant rate (28.1±0.6 ρmol kg⁻¹ min⁻¹). A
119 40% glucose solution (Fresenius Kabi, Sweden, 400 mg ml⁻¹, pH 7.4) was infused at variable rates
120 (GIR) to maintain euglycemia. Arterial glucose concentration was determined at 5-min intervals
121 (HemoCue Glucose 201 RT, HemoCue AB, Sweden). Glucose infusion rate (GIR) during the final
122 45 min of the clamp was used as a measure of insulin sensitivity. Blood was drawn at 0 min (625
123 µl), 75 min (700 µl) and 120 min (700 µl, end of clamp) for determination of plasma metabolites,
hormones and TNF-α. Blood was centrifuged (5 min,13000 g, 4°C) and plasma was stored at -20°C
126 until further analysis. The erythrocytes from the blood samples drawn at 0 and 75 min were
127 returned to the animal diluted 1:1 in heparin infusate (20 IU ml⁻¹ in 0.9 % saline) to avoid a
decrease in hematocrit. The hematocrit did not fall during the HIEC (data not shown). At GIR
129 steady state (75 min), a bolus (1.8 MBq rat⁻¹) of 2-deoxy-D-[1,2-³H]-Glucose (2-[³H]-DG)
130 (NET549, PerkinElmer, Boston, MA, USA) was injected i.v. and blood (120 µl) was drawn at 75,
131 77, 80, 85, 90, 95, 100, 110 and 120 min to determine tissue-specific glucose utilization index (R´g)
as described elsewhere (24). At the end of the clamp (120 min), skeletal muscle (EDL and soleus),
liver and epididymal fat samples were collected under pentobarbital anesthesia (55 mg kg⁻¹, i.v.).
134 Tissues were either immediately frozen in liquid nitrogen (liver, fat and muscle) or embedded in
135 mounting medium and frozen in pre-cooled isopentane (muscle tissue only, for
136 immunohistochemistry analysis) and stored at -80°C until further analysis. Under anesthesia, the
137 liver (control, n=12 and Prazosin, n=11) and soleus and EDL muscle (control, n=6 and Prazosin,
138 n=5) were removed from the rats that did not undergo a HIEC to serve as a control for the clamp
139 experiment. Subsequently, all animals were euthanized with an overdose of pentobarbital (110 mg
140 kg⁻¹, i.v.).
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, the value thus provides an estimate of the total R'g (24).

Glycogen synthase (GS) activity Muscle 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 from the upper, mid, 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 seconds and 2% formaldehyde for 2 minutes 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$^{-1}$ in PBS-BSA; Vector Laboratories, Burlingame, USA) for 1 h, washed with PBS-BSA, incubated in PBS-BSA containing streptavidin conjugated with FITC (1 mg ml$^{-1}$; F0422, Streptavidin/Fitc, DAKO, Denmark). Capillary to fiber (C:F) ratio was determined from pictures of the stained sections taken by a High-Resolution Interline CCD Camera (CoolSNAP cf. Photometrics, Tucson, USA) through a light microscope (Axioplan 2 Imaging, Zeiss, Denmark) by counting the number of fluorescent structures using the computer program ImageJ (National Institute of Health, Maryland, USA). On average, capillaries surrounding $284±9$ muscle fibers were counted per muscle sample.
**Plasma protein and metabolite concentrations** MSD kits were used to analyze insulin (Human Insulin Kit) and TNF-α (Rat TNF-α Ultra-Sensitive kit) concentrations in plasma (Meso Scale Discovery, Gaithersburg, USA). The insulin kit recognizes both rat and human insulin and can therefore measure the total insulin concentration during the insulin clamp. 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 TG2x300 triglycerides reagent, Bechman Coulter, Ireland) was determined spectrophotometrically on a Hitachi 912 Automatic Analyzer (Boehringer Mannheim, Germany). Epinephrine and Norepinephrine concentrations in plasma were measured using high-sensitive radioimmunoassay (2-CAT Plasma RIA High Sensitive Kit, Labor Diagnostika Nord, Germany).

**Insulin infusate** Insulin infusate concentrations were measured post-clamp using High Performance Liquid Chromatography (E2695 Separations Module with Alliance column heater and 2489 UV Visible detector, Waters Corporation, Milford, MA, USA).

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

**Western blotting** Akt2, AS160, Glut4, α2-AMPK and ACCβ protein content as well as relative phosphorylation status of AS160, Akt^T308_, Akt^S473_, α2-AMPK^T172_ and ACCβ^S79_ was determined by western blotting in EDL and soleus on whole muscle lysates. Whole muscle lysate preparation, SDS-PAGE, and immunoblotting have previously been described in detail (1) The membranes were incubated overnight at 4°C in blocking buffer with primary antibody against phospho-Akt^T308_ (9275, Cell Signaling Technology, MA, USA), phospho-Akt^S473_ (9271, Cell Signaling Technology, MA, USA), phospho-AS160 (Upstate Biotechnology, New York, USA), Glut4 (PAI-1065, Thermo USA).
Scientific, Rockford, IL, USA), phospho-ACCβS79 (07-303, Merck Millipore, Darmstadt, Germany) or phospho-α2AMPK T172 (2531, Cell Signaling Technology, Danvers, MA, USA). The membranes immunoblotted for phospho-AktT308, phospho-AktS473, phospho-AS160, phospho-ACCβS79 and phospho-α2AMPK T172 were stripped and reincubated with antibody against Akt2 (9611, Cell Signaling Technology, MA, USA), AS160 (07–741, Upstate Biotechnologies, Waltham, MA, USA), ACC (P 0397, Dako Cytomation, Denmark) and α2AMPK (Santa Cruz Biotechnology, sc-19131, Dallas, Texas, USA), respectively. All membranes (except ACC, which was incubated with Streptavidin) were incubated for 1 h at room temperature with a secondary antibody (P0448, Dako Cytomation, Denmark). We used Immobilon Western (Millipore, MA, USA) to detect the bands and quantified 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 mean ± SE or percentage change (calculated as (mean post/mean pre)x100).

To evaluate the effect of the Prazosin treatment and time we used a 2-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 weeks of age (Table 1). The Prazosin group ingested an average of 1.9±0.1 mg Prazosin per day.

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, Figure 1).

Prazosin treatment enhanced insulin-stimulated glucose disposal Prazosin treatment increased whole-body insulin sensitivity by 24±5% (P<0.01, Figure 2a), measured as GIR at a similar glucose concentration (Figure 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 TNF-α plasma concentration, as α1-adrenergic antagonists have been reported to lower TNF-α concentration under certain circumstances (18). No difference in plasma TNF-α concentration was observed between the Prazosin (5.0±0.2 pg ml⁻¹) and the control group (4.9±0.6 pg ml⁻¹) (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, Figure 3a) and soleus (P<0.05, Figure 3b) muscle of the Prazosin treated animals compared to controls, respectively, whereas R’g in epidydimal fat was unaltered (Figure 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 (Figure 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 T\textsuperscript{308} or S\textsuperscript{473} (Figure 4). Akt signaling mediates activation of GS and via the down-stream 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 (Figure 5a and b). Insulin-stimulated GS activity (Figure 6b&c) was unchanged by Prazosin. Furthermore, total GS activity (Figure 6a) and muscle content of Glut4 (Figure 5c) was unaltered by the Prazosin treatment.

Muscle AMPK and ACC\(\beta\) signaling was not altered by Prazosin treatment Expression of \(\alpha_2\)-AMPK (Figure 7a&b) or the downstream target of AMPK, acetyl-CoA carboxylase-\(\beta\) (ACC\(\beta\)) (Figure 8a&b) was not altered by Prazosin treatment. In addition, Prazosin treatment did not alter basal or insulin-stimulated phosphorylation of AMPK \(\alpha\)-subunits at T172 (Figure 7c&d) or ACC\(\beta\) S79 (Figure 8c&d). However, the HIEC seems to have had a small effect on EDL muscle AMPK phosphorylation (Figure 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 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 (Figure 2a) or
translate to increased Akt (Figure 4) or AS160 (Figure 5a and b) phosphorylation. The activity of
Glycogen synthase (Figure 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 T172 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 (Figure 7) or the downstream target ACCβ
(Figure 8). Changes in AMPK activity is thus an unlikely explanation for the observed increase in
glucose uptake. Interestingly, hyperinsulinemia slightly increased AMPK phosphorylation in EDL
muscle (Figure 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 primarily
dependent 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). An increased diffusion of glucose
due to the increased capillary surface area is therefore 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 accordance with previous reports (10; 56) Prazosin induced an approximate 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 eNOS (4) dependent
pathway 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 hours prior to testing insulin sensitivity in the present study. The short half-life (~2.5 h) of Prazosin (3), ensures that Prazosin was cleared from the blood stream before the insulin clamp, which is important as any acute micro- or macrovascular effects of Prazosin is 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 and coworkers 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 hyperinsulinemic euglycemic clamp (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 nor-epinephrine concentrations (25). The sympathoadrenal activity continues to be elevated during chronic treatment (25) and can therefore be used as a marker of the α-adrenergic blocking effect of Prazosin. Since we removed Prazosin from the drinking water 41-42 hours prior to the HIEC the acute effect on the cardiovascular system and any changes in blood flow would have subsided (3). In accordance, the epinephrine and nor-epinephrine plasma concentrations were similar between the Prazosin and control groups at the time of the HIEC (Table 2). Increased micro- or macrovascular blood flow due to the effect of Prazosin is therefore an unlikely explanation for the increased glucose disposal observed in the present study.

We cannot rule out that the increased insulin sensitivity was in part due 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). Any increase in the absolute number of recruited
capillaries, would therefore 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, over expression of eNOS does not enhance insulin sensitivity (38) and it thus 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), triglyceride 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). A plausible explanation for Prazosin to enhance insulin sensitivity could therefore 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, $\alpha_1$-adrenergic receptor antagonists, decrease TNF-$\alpha$ production (18). TNF-$\alpha$ has been shown to induce insulin resistance in humans (35) and rats (52). Neutralization of TNF-$\alpha$ using an anti-murine TNF-$\alpha$ 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 TNF-$\alpha$ and thereby an increased insulin sensitivity. Although Prazosin treatment might have had a transient effect on circulating TNF-$\alpha$ concentration during the treatment period, there was no difference in
TNF-α concentration between the two groups at the onset of the insulin clamp (Table 2). Thus, a difference in TNF-α 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 to 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 (Figure 2d). Furthermore, insulin stimulated glucose uptake in fat tissue was not altered by the Prazosin treatment (Figure 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 (Figure 3a&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 a ~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 towards increases in skeletal muscle capillarization being an important adaptation to improve insulin sensitivity and prevent impaired glucose tolerance and T2D.
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DISCLOSURES

We have no potential conflicts of interest relevant to this article to report.

AUTHOR CONTRIBUTIONS

T.A. researched data and wrote the manuscript, A.K.L., L.L. and K.V. researched data and reviewed the manuscript. B.K.P. researched data and reviewed the manuscript and Y.H., J.F.P.W. and C.L.B. researched data, contributed to discussion and reviewed the manuscript.


7. Chiu JD, Richey JM, Harrison LN, Zuniga E, Kolka CM, Kirkman E, Ellmerer M and Bergman RN. Direct administration of insulin into skeletal muscle reveals that the transport of insulin across the capillary...


Figure 1
Capillary to fiber ratio (C:F ratio) in EDL (A) and soleus (B) muscle of control (n=14) and Prazosin treated (n=14) rats. Representative EDL muscle sections of control (C) and Prazosin treated (D) rats. The C:F ratios of the representative sections are 1.4 and 1.8 for C and D, respectively. The data are presented as mean ± SE. *: effect of the Prazosin treatment, P<0.01.

Figure 2
Glucose infusion rate (GIR) during the hyperinsulinemic euglycemic clamp (A) and arterial glucose concentration (B) at basal and during the steady-state period (75-120 min) of the hyperinsulinemic euglycemic clamp in 13-week old Sprague-Dawley rats after 3 weeks of Prazosin treatment (n=14) and control (n=14). Liver glycogen concentration (C) at basal and immediately after the euglycemic hyperinsulinemic clamp (clamp) of Prazosin treated (basal, n=11, clamp, n=14) and control (basal, n=12, clamp, n=14) rats. The euglycemic hyperinsulinemic clamp was performed after an overnight fast (16-17 h). Blood glucose was maintained at ~6 mmol l⁻¹ during steady-state (75-120 min). The clamp procedure was performed >40 hours after the last Prazosin treatment, which leaves no residual effect of the drug. The data are presented as mean ± SE. *: effect of the Prazosin treatment, P<0.01.

Figure 3
Insulin-stimulated tissue-specific glucose utilization index (R’g) in EDL (A), soleus (B) muscle and fat (C) during the steady-state period (75-120 min) of the hyperinsulinemic euglycemic clamp in...
control (n=14) and Prazosin treated (n=14) rats. The data are presented as mean ± SE. *: effect of
the Prazosin treatment, P<0.05.

Figure 4
Protein content of Akt2 in EDL (A) and soleus (B) muscles 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. Akt2 phosphorylation at site Thr\(^{308}\) relative to Akt2 content
in EDL (C) and soleus (D) at basal and immediately after the clamp. Akt2 phosphorylation at site
Ser\(^{473}\) relative to Akt2 content in EDL (E) and soleus (F) at basal and immediately after the clamp.
Representative blots of Akt2 and Akt2 phosphorylation sites Thr\(^{308}\) and Ser\(^{473}\) (G) in soleus and
EDL muscle of Prazosin (P) treated and control (C) rats. The data are presented as mean ± SE. *:
effect of the euglycemic hyperinsulinemic clamp, P<0.05.

Figure 5
Phosphorylation of AS160 (PAS) relative to protein content of AS160 at basal and immediately
after the euglycemic hyperinsulinemic clamp (clamp) in the EDL (A) and soleus (B) muscles of
Prazosin treated (basal, n=5, clamp, n=14) and control (basal, n=6, clamp, n=14) rats. Panel C
depicts protein content of Glut4 in the soleus and EDL muscles immediately after the clamp.
Representative blots of PAS and AS160 (D) and Glut4 (E) in EDL and soleus muscle of Prazosin
(P) treated and control (C) rats. The data are presented as mean ± SE. *: effect of the euglycemic
hyperinsulinemic clamp, P<0.05.
Figure 6

Glycogen synthase activity represented as maximal activity (A), percent I-form activity (%I-form) (B) and percent fractional velocity (%FV) (C) in EDL and soleus muscle of Prazosin treated (n=14) and control (n=14) rats. The data are presented as mean ± SE.

Figure 7

Protein content of α2-AMPK in EDL (A) and soleus (B) muscles 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. α2-AMPK phosphorylation at site T172 relative to α2-AMPK content in EDL (C) and soleus (D) at basal and immediately after the clamp. Representative blots of α2-AMPK and α2-AMPK phosphorylation site T172 (E) in EDL and soleus muscle of Prazosin (P) treated and control (C) rats. The data are presented as mean ± SE. *: effect of the euglycemic hyperinsulinemic clamp, P<0.05.

Figure 8

Protein content of Acetyl-CoA Carboxylase-β (ACC) in EDL (A) and soleus (B) muscles 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. ACC phosphorylation at site S79 relative to ACC content in EDL (C) and soleus (D) at basal and immediately after the clamp. Representative blots of ACC and ACC phosphorylation site S79 (E) in EDL and soleus muscle of Prazosin (P) treated and control (C) rats. The data are presented as mean ± SE. *: effect of the euglycemic hyperinsulinemic clamp, P<0.05.
### TABLE 1  Characteristics of control and Prazosin treated rats

<table>
<thead>
<tr>
<th></th>
<th>Prazosin</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clamp n=14</td>
<td>No clamp n=17</td>
</tr>
<tr>
<td>Weight (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>373 ± 5</td>
<td>379 ± 8</td>
</tr>
<tr>
<td>Post</td>
<td>457 ± 10*</td>
<td>433 ± 8*</td>
</tr>
<tr>
<td>Fat mass (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>9.6 ± 0.4</td>
<td>10.0 ± 0.4</td>
</tr>
<tr>
<td>Post</td>
<td>10.2 ± 0.3</td>
<td>10.0 ± 0.6</td>
</tr>
<tr>
<td>Lean mass (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>76.7 ± 0.6</td>
<td>76.3 ± 0.6</td>
</tr>
<tr>
<td>Post</td>
<td>75.9 ± 0.44</td>
<td>74.6 ± 1.1</td>
</tr>
<tr>
<td>H₂O intake (ml/(100g b.w.)/day)</td>
<td>9.3 ± 0.2</td>
<td>9.1 ± 0.7</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SE. Lean mass and fat mass was measured by MR scanning before and after the intervention and relative mass was calculated using body weight. Water intake is presented as average intake throughout the intervention period per 100 g of body weight per day (ml/(100 g b.w.)/day). *: P<0.01, compared to Pre.
### TABLE 2  Basal and hyperinsulinemic euglycemic clamp characteristics

<table>
<thead>
<tr>
<th></th>
<th>Prazosin</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle glycogen (mmol/(kg w.w. muscle))</td>
<td></td>
<td></td>
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<tr>
<td>Basal EDL</td>
<td>44.3 ± 1.2</td>
<td>44.0 ± 1.9</td>
</tr>
<tr>
<td>Basal Soleus</td>
<td>34.9 ± 2.4</td>
<td>33.3 ± 1.8</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>
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<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)</td>
<td></td>
<td></td>
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<tr>
<td>t = 0 min (Basal)</td>
<td>2.6 ± 0.1</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>Norepinephrine (nmol/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t = 0 min (Basal)</td>
<td>9.3 ± 0.7</td>
<td>9.1 ± 0.6</td>
</tr>
<tr>
<td>TNFα (µg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t = 0 min (Basal)</td>
<td>5.0 ± 0.2</td>
<td>4.9 ± 0.6</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SE. *: effect of time, P<0.01. For all plasma values n=14 per group.

For skeletal muscle glycogen content n=4-6 per group.
Figure 5

D

EDL

Soleus

PAS →

AS160 →

C  P  C  P

Basal  Clamp

C  P  C  P

Basal  Clamp
Figure 5

E

GLUT4

EDL  Soleus

C  P  C  P
Figure 7

A

AMPK protein (AU)

EDL

Basal | Clamp

B

AMPK protein (AU)

Soleus

Basal | Clamp

C

pAMPK^{T172}/AMPK (AU)

EDL

Basal | Clamp

D

pAMPK^{T172}/AMPK (AU)

Soleus

Basal | Clamp
Figure 7

E

p-α2AMPK $^{T172}$

α2AMPK

EDL  

C  P  C  P

Basal  Clamp

Soleus  

C  P  C  P

Basal  Clamp
Figure 8

(A) ACC protein (AU) in EDL muscles comparing Prazosin and Control groups under Basal and Clamp conditions.

(B) ACC protein (AU) in Soleus muscles comparing Prazosin and Control groups under Basal and Clamp conditions.

(C) pACC^{S79}/ACC (AU) in EDL muscles comparing Prazosin and Control groups under Basal and Clamp conditions.

(D) pACC^{S79}/ACC (AU) in Soleus muscles comparing Prazosin and Control groups under Basal and Clamp conditions.
Figure 8

E

p-ACCβ<sup>S79</sup> →

EDL

Soleus

ACCβ →

C  P  C  P
Basal  Clamp

C  P  C  P
Basal  Clamp