Insulin stimulates laser Doppler signal by rat muscle in vivo, consistent with nutritive flow recruitment

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ABSTRACT

Insulin-mediated increases in limb blood flow are thought to enhance glucose uptake by skeletal muscle. Using the perfused rat hindlimb, we report that macro laser Doppler flowmetry (LDF) probes positioned on the surface of muscle detect changes in muscle capillary (nutritive) flow. With this as background, we examined the effects of insulin and adrenaline (epinephrine), which are both known to increase total leg blood flow, on the LDF signals from scanning and stationary probes on the muscle surface in vivo. The aim is to assess the relationship between capillary recruitment, total limb blood flow and glucose metabolism. Glucose infusion rate, femoral arterial blood flow (FBF) and muscle LDF, using either scanning or a stationary probe positioned over the biceps femoris muscle, were measured. With scanning LDF, animals received insulin (10 m-units [min⁻¹][kg⁻¹]), adrenaline (0.125 µg [min⁻¹][kg⁻¹]) or saline. By 1 h, insulin had increased the glucose infusion rate from 0 to 128 µmol [min⁻¹][kg⁻¹] and the scanning LDF had increased by 62 ± 8% (∗P < 0.05), but FBF was unaffected. Adrenaline increased FBF by 49% at 15 min, but LDF was unchanged. With saline at 1 h, neither FBF nor LDF had changed. With the stationary LDF surface probe, insulin at 1 h had increased FBF by 47% (∗P < 0.05) and LDF by 47% (∗P < 0.05) relative to saline controls. Adrenaline increased FBF (39%), but LDF was unaltered. The stimulation of LDF by insulin is consistent with capillary recruitment (nutritive flow) as part of the action of this hormone in vivo. The recruitment may be independent of changes in total flow, as adrenaline, which also increased FBF, did not increase LDF. The time of onset suggests that LDF closely parallels glucose uptake. Thus, depending on probe design, measurement of muscle haemodynamic effects mediated by insulin in normally responsive and insulin-resistant patients should be possible.

INTRODUCTION

Skeletal muscle accounts for 80–90% of insulin-stimulated glucose disposal, impairment of which appears to be pathogenetically involved in the insulin resistance characteristic of obesity, hypertension and Type II diabetes. Recently there has been considerable interest in reports that insulin has a haemodynamic vasodilatory effect as part of its action to increase glucose uptake by muscle, and that this vascular effect of insulin is impaired in obesity and Type II diabetes. The reports have generated some controversy, and there have been claims

Key words: adrenaline, blood flow, capillaries, insulin, glucose uptake, laser Doppler flow, microcirculation, muscle oxygen uptake, skeletal muscle.

Abbreviations: FBF, femoral arterial blood flow; 5-HT, 5-hydroxytryptamine (serotonin); LDF, laser Doppler flowmetry; 1-MX, 1-methylxanthine; NA, noradrenaline (norepinephrine).

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and counter-claims that glucose and insulin delivery or skeletal muscle perfusion (blood flow) play an independent role in determining overall rates of insulin-stimulated glucose disposal \[1,2\]. All contributors to this debate have placed considerable emphasis on the putative vasodilatory action of insulin to increase total blood flow. Studies in our laboratory have shown the importance of blood flow distribution in muscle, in terms of controlling both metabolism and contractile performance \[3\]. Access of nutrients and hormones is a key issue, with the very real possibility that blood flow in muscle can be substantial (possibly carried by a non-nutritive route) without full access.

With this in mind, we have attempted to develop methods that directly or indirectly assess the relative proportion of total blood flow that is nutritive. One such method is based on the metabolism of exogenously administered 1-methylxanthine (1-MX), a substrate for xanthine oxidase, shown by others \[4,5\] to be located largely in capillary endothelial cells. Using the euglycaemic hyperinsulinaemic clamp in rats, we have shown that insulin mediates an increase in 1-MX metabolism that is likely to be indicative of capillary recruitment \[6\]. Furthermore, using adrenaline (epinephrine), it was possible to demonstrate that capillary recruitment could be dissociated from changes in total flow \[6\]. Moreover, we have recently shown that, when capillary recruitment is prevented in vivo, an acute state of insulin resistance is induced \[7\]. However, since the 1-MX method used for assessing capillary recruitment in both of these studies was indirect, we have sought alternative approaches. Thus in the present study we use laser Doppler flowmetry (LDF) to measure capillary perfusion. In a two-part study we have examined the relationship between changes in nutritive flow and the LDF signal in the constant flow pump-perfused rat hindlimb, and then applied this information to measure changes in the LDF signal during the hyperinsulinaemic euglycaemic clamp in vivo. Studies were also carried out during adrenaline infusion, as this hormone is known to increase total blood flow to the leg without increasing nutritive (capillary) flow \[6\].

**MATERIALS AND METHODS**

Perfused rat hindlimb studies were conducted as described previously \[8\]. The total flow rate was maintained at 0.27 \(\text{mL} \cdot \text{min}^{-1} \cdot \text{g}^{-1}\) using medium containing red blood cells at 37 °C \[8\]. A bolus of noradrenaline (NA) was injected to reach a peak concentration of approx. 50 nM, which, from earlier studies, is known to achieve a marked increase in nutritive flow \[3\]. Similarly, a bolus of 5-hydroxytryptamine (5-HT; serotonin) was injected to reach a peak concentration of 1 \(\mu\)M, which has been shown previously to cause a marked decrease in nutritive flow. Oxygen uptake, a surrogate indicator of nutritive flow in this system when manipulated by vasoconstrictors \[3\], was determined from the maximum arteriovenous difference and perfusate flow rate (set constant as above) using an in-line oxygen analyser (A-Vox Analyser; A-Vox Systems, Inc., San Antonio, TX, U.S.A.). A small hole (approx. 4 mm diam.) was made in the skin in the middle of the biceps femoris. The hindlimb was then clamped by the foot so that the laser Doppler flow probe (Perimed PF 2; operating wavelength 632 nm) could be positioned over the centre of the hole. For the measurement of red cell flux, the probe was placed vertically above and approx. 1 mm from the surface of the muscle. Settings on the detector unit were 4 kHz (gain setting 10) with a time constant of 3 s, unless otherwise indicated. The signal (0–5 V) was recorded continuously on an IBM-compatible PC using a DI-190 I/O module and WINDAQ\textsuperscript{®} software. The data are expressed as percentage change from basal (see Figure 1).

For in vivo studies, the hyperinsulinaemic euglycaemic clamp was used, as described in detail elsewhere \[6,7\]. The limb blood flow of anaesthetized rats was determined using an ultrasonic flow probe (Transonic\textsuperscript{®}) fitted around the femoral artery. Blood pressure was monitored by a pressure transducer fitted to a carotid cannula. Insulin (10 m-units \(\cdot \text{min}^{-1} \cdot \text{kg}^{-1}\)), adrenaline (0.125 \(\mu\)g \(\cdot \text{min}^{-1} \cdot \text{kg}^{-1}\)) or saline, as well as glucose (to maintain blood glucose at 5 mM), were infused via a jugular cannula. Insulin was infused at a rate of 10 m-units \(\cdot \text{min}^{-1} \cdot \text{kg}^{-1}\) in order to raise plasma insulin to a high physiological concentration. Because the clearance of insulin in the rat is 3–4-fold more rapid than in the human, this infusion rate raises insulin to levels similar to those seen with a clamp of 2–3 m-units \(\cdot \text{min}^{-1} \cdot \text{kg}^{-1}\) in humans, and the resulting concentrations mimic those seen in insulin-resistant rat models. The protocol was similar to that described previously \[6\], with a 60 min equilibration period before insulin or saline infusion, each of which was continued for an additional 80 min. The leg was carefully skinned and wrapped in Saran Wrap\textsuperscript{™} to prevent drying. The hindlimb muscle LDF signal was determined using a scanning LDF instrument (Lisca Li PIM 1.0; Laser Doppler Perfusion Imager) at baseline (before infusion of saline, insulin or adrenaline), and at 1 h after the start of the infusion for saline or insulin or at 15 min after the start of adrenaline infusion. Positioning of the scanner was carried out using the knee as a reference; this ensured that the area and orientation were identical between animals. Triplicate scans of the hindlimb were performed, each of which took 5 min to complete; these were timed to occur 5 min before, at and 5 min after the times designated above. Each scan was brought on-screen and analysed using the manufacturer’s operational software to give average perfusion units (V) of the area analysed. To assess muscle perfusion, a square
of 225 mm² in the top left-hand corner of each scan, and covering mostly muscle (biceps femoris), was analysed. Means from triplicate analyses before and after the addition of saline (control), insulin or adrenaline were used for comparison. The scanning probe covered a total area of approx. 900 mm² of skinned thigh muscles.

In a separate series of experiments, using an identical protocol with a 60 min equilibration period and a total period of 80 min of insulin or saline infusion, a stationary LDF probe (Perimed Periflux PF 4001 with Probe 407) was used, and signals were recorded continuously throughout the experiment. The stationary probe was positioned over a 4 mm² area of skinned biceps femoris muscle in a position identical to that used in the perfused hindlimb studies described above.

Biological zero for both the scanning and the stationary probes used in the clamps was determined at the end of the experiment, after the animal had been administered a lethal dose of anaesthetic and femoral arterial blood flow (FBF) and heart rate were zero. For the scanning probe, this involved conducting a complete scan of the entire area exposed (e.g. see Figure 4A). Values for biological zero were 0.22 ± 0.02 V (total area), 0.23 ± 0.02 V (biceps femoris muscle) and 0.21 ± 0.02 V (connective tissue) \( (n = 5) \). For the surface probe, changes in LDF signal as a result of insulin were expressed relative to the basal signal of 1.00. Absolute values for the basal signal and biological zero were 112 ± 20 and 16 ± 3 perfusion units respectively for 14 determinations. No value shown has been corrected for biological zero, unless indicated otherwise.

In a further series of experiments, a group of rats underwent identical clamp conditions for the purpose of assessing insulin-induced changes in leg oxygen uptake and the muscle-specific uptake of 2-deoxyglucose. For oxygen uptake, blood samples were taken from the carotid artery (100 μl) and femoral vein after an equilibration period (approx. 1 h) and 2 h after the start of the insulin infusion. Samples of femoral vein blood (100 μl) were collected using a 29 G insulin syringe. The total oxygen content of each blood sample was determined using a galvanic cell oxygen analyser (TasCon oxygen content analyser; manufactured by the Physiology Department, University of Tasmania). The rates of oxygen uptake were calculated from oxygen extraction and FBF at each time point, and were expressed per g wet weight of perfused muscle, as estimated previously \[9\]. For muscle-specific uptake of 2-deoxyglucose, the method of Kraegen et al. \[10\] was used. In brief, this involved the intra-arterial injection of a bolus of 50 μCi of 2-deoxy-D-[2,6-³H]glucose (specific radioactivity 44 Ci·mmol⁻¹; Amersham Life Science) 45 min before the end of the clamp. Following injection of the bolus, arterial plasma samples (50 μl) were collected at 5, 10, 15, 30 and 45 min. Soleus, plantaris, gastrocnemius red, gastrocnemius white, extensor digitorum longus and tibialis muscles were removed at the end of the clamp for analysis of ³H-labelled 2-deoxyglucose 6-phosphate content and calculation of 2-deoxyglucose uptake \[10\].

Repeated-measures two-way ANOVA was used to determine differences; when significant differences were found, the paired Student’s \( t \)-test was used to test the hypothesis that there was no difference before and after treatment. For the time courses, one-way repeated-measures ANOVA was performed; when a significant difference was found, multiple comparisons (Dunnet’s method) were made with the time point just before the start of insulin infusion (0 min). Significant differences were recognized at \( P < 0.05 \).

RESULTS

LDF signal and nutritive flow in the constant flow perfused rat hindlimb

Figure 1 shows a positive correlation \( (r = 0.93, \ P < 0.0001) \) for the change in the LDF signal as a function of the change in nutritive flow, as perturbed by either NA or 5-HT.

Glucose and oxygen metabolism during the insulin clamps

In agreement with similar experiments conducted previously \[6,7\], arterial glucose concentrations were not
insulin. Oxygen uptake was unaffected by insulin uptake in each of the six muscles, with an overall average shown in Table 1. Insulin increased 2-deoxyglucose insulin, glucose infusion had already reached a maximum. similar to that obtained previously [6]: following 1 h of clamp (80 min). The time course shown in Figure 2 was maximum of 128 increased the glucose infusion rate from zero to a end of the experiment (results not shown). Insulin animals either before the start of the infusions or at the significantly different between saline- and insulin-infused animals either before the start of the infusions or at the end of the experiment (results not shown). Insulin increased the glucose infusion rate from zero to a maximum of $128 \pm 11 \mu mol \cdot min^{-1} \cdot kg^{-1}$ at the end of the clamp (80 min). The time course shown in Figure 2 was similar to that obtained previously [6]: following 1 h of insulin, glucose infusion had already reached a maximum.

The muscle-specific uptake of 2-deoxyglucose is shown in Table 1. Insulin increased 2-deoxyglucose uptake in each of the six muscles, with an overall average increase of 2.7-fold. Oxygen uptake was unaffected by insulin.

LDF scanning probe

Figure 3 shows that insulin had not increased FBF at 1 h (cf. [6]). However, at this same time point, insulin had markedly increased the scanning LDF signal over the biceps femoris [top left-hand square within the field of measurement (Figure 4)] by $62 \pm 8\%$ ($P < 0.05; n = 5$) (Figure 3). Saline infusion controls showed no increase in either FBF or scanning LDF signal over the biceps femoris at 1 h ($P > 0.05; n = 4$) (Figure 3).

Adrenaline is a more rapidly acting agent than insulin and, as noted previously [6], increases FBF. In the present studies adrenaline at $0.125 \mu g \cdot min^{-1} \cdot kg^{-1}$ increased FBF by $49 \pm 8\%$ ($P < 0.05; n = 3$) at 15 min. Despite this effect, the scanning LDF signal was unchanged ($4 \pm 8\% ; P > 0.05$) (Figure 3). The increase in the LDF signal was greater with insulin than with either adrenaline or saline ($P < 0.05$; ANOVA).

Figure 4 shows representative scans for a saline control (Figure 4A) and an insulin clamp (Figure 4B) at 1 h, as well as an illustration to show the area scanned (Figure 4C, large square) and the area corresponding to muscle (Figure 4C, smaller square). Settings on the scanner were high-resolution, a threshold voltage of 5.30 V and 64 $x$ 64 pixels. Since the average area scanned was 900 mm$^2$, each pixel represented approx. 0.2 mm$^2$. Analysis of the total scanned area and of the connective tissue region around the knee failed to reveal an effect of insulin on those scans where a significant increase in muscle signal due to insulin had occurred. Thus values before (Figure 4A) and after (Figure 4B) insulin were $1.13 \pm 0.25$ and $1.26 \pm 0.22$ V respectively ($P > 0.05$) for total area, and $2.51 \pm 0.35$ and $2.69 \pm 0.37$ V respectively ($P > 0.05$) for connective tissue.

LDF stationary probe

The stationary probe was positioned over the centre of the anterior end of the biceps femoris, as in the perfused hindlimb studies described above and shown in Figure

![Graph showing glucose infusion rate (GIR) required to maintain basal blood glucose at 4.5 ± 0.2 mM during insulin infusion. Values are means ± S.E.M. for 11 animals.](image)

Table 1: Effects of insulin on leg oxygen uptake and on 2-deoxyglucose uptake by individual muscles

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Saline</th>
<th>Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose infusion rate ($\mu mol \cdot min^{-1} \cdot kg^{-1}$)</td>
<td>—</td>
<td>100.7 ± 3.2</td>
</tr>
<tr>
<td>FBF ($mL \cdot min^{-1}$)</td>
<td>$0.85 \pm 0.05$</td>
<td>$1.2 \pm 0.1^{**}$</td>
</tr>
<tr>
<td>Leg oxygen uptake ($\mu mol \cdot h^{-1} \cdot g^{-1}$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial (pre-saline or -insulin)</td>
<td>$27.5 \pm 3.9$</td>
<td>$24.2 \pm 2.2$</td>
</tr>
<tr>
<td>Final (post-saline or -insulin)</td>
<td>$27.8 \pm 3.1$</td>
<td>$22.9 \pm 2.3$</td>
</tr>
<tr>
<td>2-Deoxyglucose uptake ($\mu mol \cdot h^{-1} \cdot g^{-1}$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soleus</td>
<td>$2.9 \pm 0.2$</td>
<td>$9.6 \pm 1.5^{*}$</td>
</tr>
<tr>
<td>Plantaris</td>
<td>$2.4 \pm 0.1$</td>
<td>$5.0 \pm 0.5^{*}$</td>
</tr>
<tr>
<td>Gastrocnemius red</td>
<td>$3.1 \pm 0.2$</td>
<td>$11.4 \pm 1.2^{*}$</td>
</tr>
<tr>
<td>Gastrocnemius white</td>
<td>$2.0 \pm 0.1$</td>
<td>$4.2 \pm 0.5^{*}$</td>
</tr>
<tr>
<td>Extensor digitorum longus</td>
<td>$3.4 \pm 0.2$</td>
<td>$8.9 \pm 1.0^{*}$</td>
</tr>
<tr>
<td>Tibialis</td>
<td>$3.2 \pm 0.3$</td>
<td>$8.7 \pm 0.9^{*}$</td>
</tr>
</tbody>
</table>
Insulin and capillary recruitment in muscle

Figure 3  Scanning laser Doppler measurement of the lateral surface of thigh muscles (muscle perfusion) and total FBF in anaesthetized rats

Values were obtained before treatment (open bars) and following infusion (solid bars) with saline (Sal; n = 4), insulin/glucose (Ins; n = 5) or adrenaline (Epi; n = 3). Values are means ± S.E.M. Significant differences from pretreatment values: *P < 0.05.

4(C) (depicted by the red dot). The signal was recorded continuously, and mean values are shown in Figure 5 for the 0–80 min period following the start of the insulin infusion. For the stationary LDF probe experiments, a 1 h insulin infusion increased both FBF (47%, n = 6; P < 0.05) and the LDF signal (47 ± 12%; P < 0.05) relative to saline controls (n = 5). Adrenaline also increased FBF (39%), but the LDF signal did not change significantly (−0.3%; n = 5). Figure 5 shows the time courses for insulin-mediated changes in FBF (upper panel) and the LDF signal (lower panel). The change in the LDF signal was significant at 20 min and preceded the increase in FBF, which occurred at 60 min.

DISCUSSION

The main finding emerging from the present study was the increase in the muscle surface LDF signal mediated by insulin. This would appear to reflect an increase in nutritive flow in muscle, consistent with our previous observation of an increase in capillary recruitment, as determined by increased metabolism of the marker substrate 1-MX, seen at the same high physiological dose of insulin [6]. The present data show that insulin induces capillary recruitment and enhances glucose uptake, a finding consistent with a metabolic role for capillary perfusion through vessels that facilitate nutrient exchange. The finding that insulin leads to an increase in muscle capillary flow, as measured by LDF, and the fact that this occurs over a time course similar to that observed for whole-body glucose uptake, is reminiscent of the strong correlation observed between the LDF signal from similarly placed probes and oxygen uptake, as altered by the two different vasoconstrictors 5-HT (Type B) and NA (Type A), in the constant flow perfused rat hindlimb (Figure 1). Oxygen uptake has proved to be a reliable indicator of nutritive flow in perfused hindlimb muscle. In several studies where flow has been manipulated by vasoconstrictors, increases in oxygen uptake have always been associated with an increase in pressure (e.g. for reviews, see [3,11]). In the present series of experiments, and as noted previously [6], there was very little change in blood pressure (results not shown) and no increase in oxygen uptake during the insulin clamp. This finding is consistent with a number of human studies [12]. Presumably the mechanistic basis of insulin’s action in increasing muscle capillary flow as detected by the LDF probe in the present study is due partly to its proposed NO-dependent vasodilatory action [13].

The increase in capillary flow induced by insulin preceded by at least 30 min changes in total leg flow as detected by the flow probe positioned around the femoral artery. Furthermore, adrenaline, which increased total flow, had no effect on the LDF signal. An increase in nutritive flow due to insulin in the absence of an increase in total flow implies that flow has been redistributed from another route that may not be visible to the LDF probe. This may be because of the position or size of the probe. Comparison with the perfused rat hindlimb is helpful in considering these issues. Although almost fully dilated, the isolated perfused hindlimb preparation responds to vasoconstrictors by either increasing or decreasing metabolism, under conditions of constant total flow. Vasoconstrictors that increase metabolism in the constant-flow preparation do so by redirecting flow from a ‘non-nutritive’ route, located in the closely associated connective tissue of the perimysium and related sheaths. Some of the vessels are visible and relatively free from a background of muscle nutritive capillaries [14], and one study has shown that the vasoconstrictor NA, which increases overall hindlimb metabolism, redirected flow...
from these vessels to the muscle nutritive route [15]. Accordingly, it would seem likely from the present study that insulin has acted similarly to redirect flow from the non-nutritive route to the nutritive route. As already discussed above, insulin does not affect blood pressure within this time frame [6], and thus recruitment of nutritive flow at the expense of non-nutritive flow would appear to involve a combination of vasodilatory and vasoconstrictor activities. There are reports of both vasodilation and vasoconstriction activities of insulin. For example, Renaudin et al. [16] reported that insulin administered in vivo to anaesthetized rats caused a marked and durable vasodilation of the terminal arterioles of the spinotrapezius muscle. However, when similar plasma insulin levels (approx. 800 pM) were attained by glucose infusion, partial vasoconstriction of the terminal arterioles occurred [16].

There is some evidence that the surface LDF signal response is heterogeneous for the hindlimb as a whole. Some sites away from the centre of the biceps femoris in perfused rat hindlimb studies were found to respond in opposite directions to NA and 5-HT (consistent with them being non-nutritive) or to not respond at all to either agent (possibly mixed) (A. D. H. Clark, J. M. Youd, S. Rattigan, E. J. Barrett and M. G. Clark, unpublished work). In addition, in the present clamp studies with the scanning LDF probe, it was apparent that regions away from muscle, such as those around the knee, or indeed the total area, including muscle, showed no significant response to insulin, suggesting that in some areas flow may have decreased. In one experiment, flow in the connective tissue was actually decreased by insulin.

The LDF probe dimensions may be important in determining the nature of the signal received. The
stationary LDF probe used in the present study, when placed on the surface, detects signal from a hemisphere with a radius of approx. 1 mm. As shown in Figure 1, when the probe was positioned at the centre of the anterior end of the biceps femoris, the signal appeared to be only of one kind, responding positively to NA and negatively to 5-HT. This contrasts with measurements using a much smaller probe (200 \mu m) inserted in the muscle, where LDF signals were heterogeneous, with some responding as above (i.e. nutritive), some responding in an opposite manner (i.e. non-nutritive), and some failing to respond [16a]. Thus it appears likely that the larger probe receives signals from a mixture of sites that convey a character that is predominantly nutritive. Alternatively, non-nutritive sites may not be located near the surface of the muscle.

The present findings in the rat \textit{in vivo}, whereby insulin increased FBF and also increased the LDF signal, whereas adrenaline, despite similar changes in FBF, did not affect the LDF signal, are similar to our previously reported increases in 1-MX metabolism [6]. In that study, 1-MX was infused as a target substrate for capillary endothelial xanthine oxidase. Increased metabolism of this substrate as a result of insulin action was concluded to reflect increased capillary recruitment or nutritive flow. A large part of this conclusion rested on prior knowledge gained from perfused rat hindlimb studies, where 1-MX metabolism was shown to closely parallel relative changes in nutritive flow [8,17]. Similarly, in the present study we have used prior findings with the perfused rat hindlimb to interpret the nature of the signal changes induced by insulin. Thus an increase in the muscle LDF signal due to insulin and independent of changes in total flow supports the contention that insulin increases capillary recruitment in human skeletal muscle [18], and the observation that insulin increases blood volume in human muscle [19].

In conclusion, insulin acts in rats \textit{in vivo} to stimulate the LDF signal, consistent with capillary recruitment in skeletal muscle. The recruitment may be independent of changes in total blood flow, as it precedes temporally the increase in total flow. This dissociation is also seen on infusion of adrenaline, which increases FBF but does not increase the LDF signal. The present findings support previous observations by our group that insulin increases capillary recruitment in rat muscle, as measured by 1-MX extraction. In addition, they suggest that recruitment of capillaries may be an important aspect of insulin’s action to increase muscle glucose uptake. The LDF technique may be applicable to human studies.

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