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Insulin-induced membrane permeability to glucose in human muscles at rest and following exercise

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Running title: Exercise and insulin action on muscle membrane permeability to glucose

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### Key points

Increased insulin action is an important component of the health benefits of exercise, but its regulation is complex and not fully elucidated.

Previous studies of insulin-stimulated GLUT4 translocation to the skeletal muscle membrane found insufficient increases to explain the increases in glucose uptake.

By determination of leg glucose uptake and interstitial muscle glucose concentration we calculated insulin-induced muscle membrane permeability to glucose 4 h after one-legged knee-extensor exercise during a submaximal euglycaemic hyperinsulinaemic clamp.

We found during submaximal insulin stimulation that muscle membrane permeability to glucose in humans increases twice as much in previously exercised vs rested muscle and it outstrips the supply of glucose which then becomes limiting for glucose uptake.

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This methodology can now be employed to determine muscle membrane permeability to glucose in people with diabetes, who have reduced insulin action, and in principle can also be used to determine membrane permeability to other substrates or metabolites.

## Abstract

Increased insulin action is an important component of the health benefits of exercise, but the regulation of insulin action *in vivo* is complex and not fully elucidated. Increases in skeletal muscle insulin-stimulated GLUT4 translocation are inconsistent and mostly cannot explain the increases in insulin action in humans. Here we used leg glucose uptake (LGU) and interstitial muscle glucose concentration to calculate insulin-induced muscle membrane permeability to glucose, a variable not previously possible to quantify in humans. Muscle membrane permeability to glucose, measured 4 h after one-legged knee-extensor exercise, increased ~17-fold during a submaximal euglycaemic hyperinsulinaemic clamp in rested muscle (R) and ~36-fold in exercised muscle (EX). Femoral arterial infusion of L-NMMA or ATP decreased and increased, respectively, leg blood flow (LBF) in both legs but did not affect membrane glucose permeability. Decreasing LBF reduced interstitial glucose concentrations to ~2 mM in the exercised but only to ~3.5 mM in non-exercised muscle and abrogated the augmented effect of insulin on LGU in the EX leg. Increasing LBF by ATP infusion increased LGU in both legs with uptake higher in the EX leg. We conclude that it is possible to measure functional muscle membrane permeability to glucose in humans and it increases twice as much in exercised vs rested muscle during submaximal insulin stimulation. We also show that muscle perfusion is an important regulator of muscle glucose uptake when membrane permeability to glucose is high and we show that the capillary wall can be a significant barrier for glucose transport.

## Introduction

Insulin stimulates greater skeletal muscle glucose uptake via increased glucose delivery, muscle membrane glucose transport and molecular signaling which increases the metabolism of glucose. Because glucose transport occurs via facilitated diffusion, the extent of glucose transport will be determined by the glucose gradient from the interstitium (the outside of the muscle cell) to the cytoplasm (the inside of the membrane) and the muscle membrane permeability to glucose. Increased supply of glucose via insulin induced increased capillary perfusion (Vincent *et al.*, 2003; Vincent *et al.*, 2004; Sjöberg *et al.*, 2011; Sjöberg *et al.*, 2014; Sjöberg *et al.*, 2017) is likely important to prevent draining the interstitial space of glucose when muscle membrane permeability increases,

but whether supply becomes limiting for maintaining the interstitial muscle glucose concentration is not known. The increase in muscle membrane permeability with insulin and exercise is generally thought to be the result of translocation of GLUT4 glucose transporters to the cell membrane and t-tubules (Wilson & Cushman, 1994; Lund *et al.*, 1995; Kennedy *et al.*, 1999; Ryder *et al.*, 2000; Bryant *et al.*, 2002; Koistinen *et al.*, 2003). Surface labelling studies have produced ~8 fold increases in cell surface GLUT-4 and glucose transport with insulin in rat muscle (Wilson & Cushman, 1994; Lund *et al.*, 1995), 5.7 fold in human muscle (Ryder *et al.*, 2000) and 2.1 fold in human muscle strips (Koistinen *et al.*, 2003). Live imaging in anesthetized rats showed a 3-fold increase in surface GLUT4 content with insulin (Lauritzen *et al.*, 2002). In addition, studies using differential centrifugation to isolate plasma membranes indicate that insulin increases skeletal muscle plasma membrane GLUT4 by ~2-fold (100%) in both rats (Sternlicht *et al.*, 1988; Goodyear *et al.*, 1990; Hirshman *et al.*, 1990; Klip & Paquet, 1990; Marette *et al.*, 1992; Lund *et al.*, 1993; Dombrowski *et al.*, 1998) and humans (Goodyear *et al.*, 1996; Thorell *et al.*, 1999) which is a lot less than the 3-5 fold increase observed in glucose transport in the same systems (Sternlicht *et al.*, 1988; Hirshman *et al.*, 1990; Thorell *et al.*, 1999).

Due to the methodological difficulties inherent in the currently available techniques to reliably measure GLUT4 translocation in human muscle, we have taken a different approach. While we do not directly measure GLUT4 content in the muscle membrane we instead by a combination of advanced invasive techniques are able to determine the functional permeability of the muscle membrane to glucose upon insulin stimulation *in vivo* in humans. This fundamental membrane characteristic has not been measured in human muscle before.

Another stimulator of skeletal muscle glucose transport is exercise. Regular exercise increases insulin sensitivity, reduces the risk of diabetes and improves clinical symptoms in those with T2D (Wojtaszewski & Richter, 2006). It has been shown in rats that acute exercise increases subsequent insulin-stimulated GLUT4 translocation *in vitro* in incubated muscle which closely mirrors the increase in glucose transport (Hansen *et al.*, 1998). However, as alluded to above, in the intact complex organism insulin affects both delivery of glucose as well as the increased muscle membrane permeability to glucose and how these two characteristics contribute to the increased insulin sensitivity of muscle following exercise is not fully understood. Thus, by providing data on leg glucose uptake and interstitial muscle glucose concentrations we can estimate muscle membrane permeability to glucose and combining this with manipulation of muscle perfusion we can provide novel *in vivo* estimates of the critical components that control skeletal muscle glucose uptake during submaximal insulin stimulation at rest and in recovery from exercise.

## Materials and Methods

### *Participants and study approval.*

Ten young healthy males (Table 1) gave informed consent to take part in this study which was approved by the Copenhagen Ethics Committee (H-16022335) and conformed to the standards set by the Declaration of Helsinki, except for registration in a database. Potential participants who had a BMI >30 kg·m<sup>2</sup>, use any medication or had a family history of diabetes were excluded from the study.

### *Preliminary tests and procedures.*

Body composition was determined by dual-energy X-ray absorptiometry (DEXA) scanning (DPX-IQ Lunar, Madison Wisconsin, USA). VO<sub>2</sub> peak was determined by an incremental test performed on a bike ergometer (Monark, Sweden) and the oxygen consumption was measured using a MasterScreen CPX system (Becton, Dickinson, USA). The participants (Table 1) were familiarised for 20 minutes to one-legged knee-extensor exercise on two occasions and an incremental peak power output test was performed on a separate day a minimum of one week before the experimental day. Two days prior the experimental day, the subjects were instructed to refrain from moderate-intense physical exercise and to eat their habitual diet.

### *Protocol 1: Euglycaemic hyperinsulinaemic clamp (EHC) and microdialysis with femoral arterial infusion of L-NMMA and ATP*

At 6am on the experimental day subjects consumed a small breakfast consisting of 40 g of oats and 140g of skimmed milk (equal to 156 kcal). Subjects arrived at the laboratory by public transportation and at 7am began 60 min of 1-legged knee extensor exercise (at 80% of peak work load with three 5 min intervals at 100% peak work load) on a one-legged ergometer. The contralateral leg was kept inactive and served as the rested control leg. Subsequently subjects rested for 4 hours in the supine position to provide sufficient time for the leg blood flow and glucose uptake in the exercised leg to return to levels similar to the rested leg. During the 4 hours, catheters were inserted into the femoral artery and vein of both legs below the inguinal ligament (Pediatric Jugular Catheterization set, Arrow Int. US) under local anesthesia (Xylocaine 1%, AstraZeneca, Denmark) for subsequent measurement of leg glucose uptake and for femoral artery infusion of *NG*-monomethyl L-arginine acetate (L-NMMA) (Clinalfa basic, Bachem AG, Switzerland) and ATP (Sigma-Aldrich, USA). Arterial blood pressure was monitored continuously using a pressure transducer interfaced to an

IntelliVue MP5 monitor (Phillips Healthcare, NL). Polyethylene catheters were then placed in antecubital veins for infusion of insulin and glucose. A catheter was inserted in the dorsal hand vein for blood sampling and a heating pad was wrapped around the hand to arterialise the hand vein blood to oxygen saturation levels above 93%. This was necessary as the femoral artery catheters were used for L-NMMA and subsequent ATP infusions and therefore blood samples could not be withdrawn simultaneously.

### *Microdialysis*

Approximately 2h after completion of exercise, 3 microdialysis catheters (M Dialysis AB, Stockholm Sweden) with a semi-permeable membrane and a molecular cut-off at 20,000 Dalton were inserted into the vastus lateral muscle of both legs under local anaesthesia of the skin and the fascia, approximately 5 cm apart (in parallel with the muscle fibre direction). The membrane allows molecules to pass through its pores by diffusion based on molecular weight (Henriksson, 1999). Perfusate (Ringer Acetate, Fresenius Kabi, SE) was added 2 mM (n=5) and 1 mM (n=5) glucose to minimise draining of the interstitial glucose (Lonnroth *et al.*, 1987). 1.1  $\mu$ l sterile labelled glucose tracer (Glucose, D-[6-<sup>3</sup>H(N)] S.A. 60 Ci·mmol<sup>-1</sup>) was added to Ringer Acetate whereby the relative loss of the tracer can be measured (MacLean *et al.*, 1999). The relative loss (also termed recovery rate, RR) (Kho *et al.*, 2017) of the isotope from the perfusate into the interstitial space represents the microdialysis catheter recovery of the glucose (MacLean *et al.*, 1999) under the assumption that the interstitial compound diffuses at the same rate as the exogenous compound (Scheller & Kolb, 1991; Kho *et al.*, 2017).

The perfusion was initiated with a bolus infusion of 10  $\mu$ l·min<sup>-1</sup> for 10 min followed by a constant infusion of 2  $\mu$ l·min<sup>-1</sup> (2 mM, n=5) to 1  $\mu$ l·min<sup>-1</sup> (1 mM, n=5) using micro infusion pumps (Harvard apparatus, 11 Plus) for 90 min to ensure equilibration between perfusate and interstitial fluid (Henriksson & Knol, 2005). The lower glucose (1 mM) was applied in an attempt to increase the recovery rate. However, interstitial glucose concentrations were not different between the two protocols. Technical problems with the microdialysis catheters precluded meaningful measurements of interstitial glucose concentrations in one subject (therefore n=9 for microdialysis measures). Collection of dialysates began ~90 min after insertion of the microdialysis catheters allowing the metabolite levels to stabilise due to the trauma of insertion (Rosdahl *et al.*, 1998; Henriksson, 1999; Carson *et al.*, 2015). Dialysate was collected into micro-vials over 15 minutes throughout the experiment and analysed for glucose concentration on a glucometer (Blood Glucose Meter, Bayer, Switzerland). In

triplicate, each collected dialysate sample was added to 4 ml scintillation fluid and counted in a liquid scintillation analyser (Tri-Carb 2910 TR, Perkin Elmer) for determination of RR. The results from the three probes in each leg were pooled into a single mean value for each time point. The mean RR of the microdialysis probes was  $0.55 \pm 0.04$  for both legs.

#### *Euglycaemic hyperinsulinaemic clamp (EHC)*

Four hours after discontinuing the exercise a 225 minute EHC was initiated (Fig. 1). Subjects were clamped at their individual ambient plasma glucose level obtained prior to initiation of the insulin infusion. Insulin was infused at  $1.4 \text{ mU}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$  (Fig. 1). Plasma glucose concentration was measured every  $\sim 7$  min and a variable glucose infusion (20% Fresenius Kabi, SE) into a forearm vein was adjusted to clamp euglycaemia as previously described (DeFronzo *et al.*, 1979). Ninety min into the EHC L-NMMA Acetate was infused at  $0.4 \text{ mg}\cdot\text{kg}^{-1} \text{ leg mass}\cdot\text{min}^{-1}$  into both femoral arteries for 45 min (Fig. 1). The insulin infusion was maintained for another 90 min after discontinuing the L-NMMA infusion (Fig. 1). After the 45 min L-NMMA washout period a local infusion of ATP into both femoral arteries was undertaken for 45 min. The ATP ( $0.3 \text{ }\mu\text{mol}\cdot\text{ml}^{-1}$ ) was infused at  $\sim 200 \text{ }\mu\text{l}\cdot\text{min}^{-1}$  at the beginning of infusion, but varied throughout the 45 minutes of infusion ( $200\text{--}350 \text{ }\mu\text{l}\cdot\text{min}^{-1}$ ) to obtain a leg blood flow that was double that of the blood flow found in the basal state before insulin infusion. To check this, the leg blood flow was measured every fifth minute during ATP infusion. During the clamp blood samples were obtained every 15 min simultaneously from both femoral veins and the heated (arterialised) hand vein.

#### *Leg blood flow*

Leg blood flow was measured in the femoral artery of both legs prior to each blood sampling using a high frequency 12-3 MHz linear array transducer in Power Doppler mode interfaced to an Affiniti70 ultrasound machine (Phillips Ultrasound, SA, CA).

#### *Blood analysis and leg glucose uptake.*

Plasma glucose concentrations during the experiments were measured on an ABL 800 FLEX (Radiometer Medical A/S, Denmark). Plasma insulin was measured using an enzyme-linked immunosorbent assay (Alpco, Salem, USA). Glucose uptake was calculated as the glucose concentration difference between the arterialised and the femoral venous blood multiplied by the leg blood leg flow divided by the lean mass of the leg.

#### *Calculation of glucose uptake per kg active thigh muscle*

Previous studies using careful EMG measurements have shown that the quadriceps femoris is the only active muscle during prolonged one-legged kicking exercise, with minimal hamstring and tibialis anterior activation (Andersen *et al.*, 1985). Therefore, it can be assumed that the higher glucose uptake in the exercised leg is due to increased uptake in the quadriceps femoris muscle with the rest of the muscles in the exercised leg having glucose uptake values similar to the muscles in the rested leg. Thus, glucose uptake in the “active” thigh lean mass was calculated as:

$$BF_{EX} \times AVdiff_{EX} - (BF_R \times AVdiff_R) \times inactive\ LLM_{EX\ (leg\ LLM - active\ LM)} = \frac{\Delta GU}{Active\ LM}$$

$$= Active\ LM\ GU$$

(and lean mass was assumed to be muscle).

Where BF is blood flow, EX is exercise leg, R is rest leg, AV diff is arterio-venous glucose difference, LLM is lean leg mass, active LM is active lean mass and GU is glucose uptake. The quadriceps muscle represents 40% of the thigh muscles (Bischoff, 1863). Therefore, active thigh lean mass was defined as 40% of the thigh lean mass = muscle mass. Lean mass of the upper leg was determined by individual segment regions drawn in the DXA analysis.

#### *Microdialysis calculations*

The difference in disintegrations per minute between the dialysate and perfusate was used to calculate the recovery rate (RR) for the glucose concentration difference between the dialysate and the interstitial fluid:

$$RR = \frac{Dpm_p - Dpm_d}{Dpm_p}$$

Where  $Dpm_p$  and  $Dpm_d$  represents  $^3H$  disintegrations per minute of the perfusate and dialysate respectively (Scheller & Kolb, 1991). The RR was used to further calculate the interstitial glucose concentration:

$$C_i = \frac{(C_d - C_p)}{RR} + C_p$$

Where  $C_i$  represents the interstitial glucose concentration and  $C_d$  the dialysate glucose concentration and  $C_p$  the perfusate glucose concentration (Dela & Stallknecht, 2010). When comparing the interstitial glucose concentration with the concentration in plasma, the arterial concentration of glucose was corrected to plasma water by using a factor of 0.94 (Stallknecht *et al.*, 1995; MacLean *et al.*, 1999; Stallknecht *et al.*, 2000).



### *Estimation of muscle membrane permeability to glucose*

We calculated the apparent muscle membrane permeability to glucose from the gradient of the interstitial glucose concentration to the intracellular glucose concentration and the rate of active muscle (lean active thigh mass) and rested muscle (lean leg mass) glucose uptake:

$$\begin{aligned} & \text{Glucose gradient (interstitium to cell)} \times \text{muscle membrane permeability to glucose} \\ & = \text{Glucose uptake} \end{aligned}$$

We assumed the intracellular glucose to be zero. This is because when Sahlin et al. measured total glucose in muscle biopsy samples and then subtracted the estimated extracellular glucose volume and glucose concentration they determined that intracellular glucose was only 0.15 mM/kg muscle mass in resting young men (Sahlin *et al.*, 1989). Similarly, when Cline et al. measured total glucose in skeletal muscle samples by NMR and used labelled mannitol to measured extracellular volume, they determined that intracellular glucose was 0.11 mM in the basal state and it actually decreased to negative values (-0.12 mM) during a hyperglycemic hyperinsulinemic clamp (Cline *et al.*, 1999).

Therefore:

$$\text{Muscle membrane permeability to glucose} = \frac{\text{Muscle glucose uptake}}{[\text{Muscle interstitial glucose}]}$$

### *Protocol 2: Control microdialysis measurements after acute exercise*

Protocol 2 was performed in order to determine effects of acute exercise on skeletal muscle interstitial glucose concentration without insulin stimulation and to study whether the interstitial glucose concentration changes as an effect of time or as an effect of insulin stimulation. Therefore, 7 of the 10 subjects returned to the laboratory for a second experimental day where identical procedures were undertaken as in Protocol 1 except no femoral catheters were inserted and no L-NMMA or ATP was infused. Six microdialysis catheters and a single dorsal hand vein catheter were inserted. Arterialized glucose was measured from the heated dorsal hand vein alongside the dialysate for comparison.

### *Statistics*

Data are expressed as mean  $\pm$  SEM. Statistical evaluation involved either one way or two-way repeated measures ANOVA with the two factors being following a Shapiro-Wilk normality test which was passed in all evaluations. When ANOVA revealed a significant difference of 0.05, specific differences were determined using a Student-Newman-Keuls

post-hoc test. The overall experimental data were divided into four sub-experiments when doing the statistical evaluation (clamp 0-90 minutes, clamp+L-NMMA 90-135 minutes, reinstatement of clamp 135-180 minutes, and clamp+ATP 180-225 minutes). This was chosen since the two factors of the ANOVA changed during the experiment.

## Results

### ***Euglycaemic hyperinsulinaemic clamp experiment (EHC, N=10):***

Arterial plasma glucose was maintained at the baseline levels of  $5.6 \pm 0.1 \text{ mmol}\cdot\text{l}^{-1}$  throughout the experiment (Fig. 2A). Glucose infusion rate (GIR) averaged  $6.5 \pm 0.5 \text{ mg}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$  after 90 min of insulin infusion and increased slowly during the remainder of the clamp reaching  $8.9 \text{ mg}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$  at the end (Table 2).

Despite the low L-NMMA dose, and no change in heart rate, there were small but significant increases in systolic and diastolic blood pressure during the L-NMMA infusion and the blood pressure remained slightly elevated during the 45 min after the cessation of the L-NMMA infusion (Table 2). Blood pressure was not different from baseline during ATP infusion but there was a small but significant increase in heart rate during ATP infusion ( $P < 0.05$ ) (Table 2).

### ***Leg glucose uptake (LGU), leg blood flow, femoral vein glucose concentrations, glucose A-V difference and muscle interstitial glucose concentration.***

Four hours after exercise the venous glucose concentration in both legs was close to the arterial concentration and similar in the two legs (Fig 2A). Leg blood flow was also similar in the two legs (Fig 2B) and hence leg glucose uptake (fig 2C) was similar in the two legs before insulin infusion. Insulin infusion increased LGU ( $P < 0.05$ ) in both legs and more so (~50%) in the previously exercised leg ( $P < 0.05$ ) (Fig. 2C). This was due to greater reductions in venous glucose concentration (Fig. 2A) and accordingly higher A-V difference for glucose (Fig. 2D), which was reflected by a ~30% lower ( $P < 0.05$ ) interstitial glucose concentration (Fig. 3A) in the previously exercised leg, as well as a slightly but significantly higher blood flow in the previously exercised leg (Fig. 2C). Considering that the whole leg is drained by the femoral vein and that only the quadriceps femoris muscle is active during knee-extensions (Andersen *et al.*, 1985), it seems reasonable to assume that the increased LGU in the previously exercised leg is due solely to increased uptake in the quadriceps and that the rest of the muscles in the exercised leg display glucose uptake similar to uptake in the contralateral rested leg. This allows calculating glucose uptake in the previously active part (the quadriceps femoris) of the

leg muscle (Fig. 2C) and shows a marked increase in insulin action in the previously active compared to non-active muscle in the contralateral leg (Fig. 2C).

Infusion of L-NMMA into the femoral artery reduced blood flow to  $\sim 280 \text{ ml}\cdot\text{min}^{-1}$  in both legs ( $P<0.05$ ) with the largest reduction occurring in the exercised leg (Fig. 2B). Simultaneously, the A-V difference for glucose increased ( $P<0.05$ ) in both legs and more so in the exercised leg (Fig. 2D). This increased A-V difference was however not enough to prevent decreased LGU ( $P<0.05$ ) in the exercised leg (and active muscle) so that glucose uptake was no longer different from LGU in the rested leg where LGU appeared to be unaffected (Fig. 2C). The reduction in glucose delivery (flow) and concomitant increase in glucose extraction (A-V difference) was accompanied by a 20% drop in interstitial glucose concentration compared to pre L-NMMA levels in both legs amounting to  $2.3\pm 0.4$  and  $3.7\pm 0.4 \text{ mM}$  for the exercised and rested leg, respectively ( $P<0.001$ ) (Fig. 3A). Thus, interstitial glucose concentrations of  $<3.7 \text{ mM}$  may be critical for uncompromised LGU.

Leg glucose uptake increased rapidly in both legs during the 45 min L-NMMA wash out period, where it again became significantly higher in the exercised leg ( $P<0.05$ ) compared to the rested leg (Fig. 2C). Simultaneously, interstitial glucose concentration increased significantly in the exercised leg but did not reach pre L-NMMA levels whereas it remained unchanged in the rested leg (Fig. 3A).

Doubling glucose delivery (flow) by local ATP infusion increased LGU in both legs with the exercised leg being significantly higher ( $P<0.05$ ) than the rested leg throughout the ATP infusion (Fig. 2C). This was entirely due to a higher glucose A-V difference in the exercised leg since the LBF was increased to a similar level (to  $\sim 850 \text{ ml min}^{-1}$ ) in the two legs (Fig. 2B). This allowed for an increased interstitial glucose replenishment in the exercised leg ( $P<0.05$ ) reaching pre-L-NMMA levels again whereas it was unchanged in the rested leg (Fig. 3A). These data indicate that when glucose extraction is high (Fig. 2D) then glucose uptake can be increased by increasing leg blood flow indicating that muscle perfusion is limiting for glucose uptake in this condition.

### ***Permeability of skeletal muscle to glucose***

The apparent glucose permeability of the muscle membrane prior to insulin infusion was  $1.0\pm 0.3$  and  $1.3\pm 1.0 \text{ ml}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$  and insulin increased it to a mean of  $18.1\pm 1.5$  and  $47.2\pm 4.9 \text{ ml}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$  ( $P<0.05$ ) in the rested and exercised muscle, respectively (Fig. 4). It should be kept in mind that based on previous literature (Sahlin *et al.*, 1989; Cline *et al.*, 1999), we assumed the intracellular

glucose concentration to be zero under all conditions. Thus, insulin-stimulation increased the muscle membrane permeability by 17-fold in the rested muscle and this was increased even further by prior exercise to 36-fold in the exercised muscle ( $P<0.05$ ) (Fig. 4). Assuming the permeability reflects the number of GLUT4 transporters in the muscle membrane, it appears that this is twice as high in the exercised muscle compared to the rested muscle. This agrees with data in insulin-stimulated rat muscle where plasma membrane GLUT4 concentration was approximately twice as high 3h after contractions compared to non-contracted muscle (Hansen *et al.*, 1998). L-NMMA co-infusion during the EHC did not affect the muscle membrane permeability to glucose (Rested leg:  $16.4\pm2.2$ ; Exercised leg  $43.3\pm11.5$   $\text{ml}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$ ) which is in line with our previous observation that NOS inhibition during a EHC has no effect on the greater activation of skeletal muscle insulin signaling by exercise (Sjoberg *et al.*, 2017). ATP co-infusion during the last part of the EHC also had no significant effect of muscle membrane permeability to glucose compared with the clamp alone.

#### ***Control experiment (N=7):***

To determine whether the decrease in interstitial glucose concentration during the EHC was a true insulin effect or due to the elapsed time, a separate control experiment without insulin infusion (Protocol 2, see methods section below) was performed in 7 of the 10 individuals that performed the EHC experiment. There was a main effect of prior exercise on the interstitial glucose concentration being lower in the exercised compared to the rested muscle for the first 90 min. After that the difference was not statistically significant and the interstitial glucose concentrations in the two legs were about 1-1.5 mM lower than the arterialised plasma glucose concentration (Fig. 3B).

#### ***Clamp vs control experiments:***

The interstitial glucose concentration decreased more from 0-90 min during the EHC than during the control, non-insulin protocol in the exercised but not the rested leg (Fig. 3A and B)

#### ***Plasma insulin***

Insulin infusion increased the plasma insulin concentration from  $5.5\pm0.5$  to  $108\pm4.9$   $\mu\text{IU}\cdot\text{ml}^{-1}$  within 30 min. Although plasma insulin concentration tended to increase during L-NMMA infusion and decrease after L-NMMA infusion these changes were not significant.

## Discussion

Here we show that a combination of invasive techniques allows estimation of skeletal muscle membrane permeability to glucose. We further show that skeletal muscle membrane permeability increases remarkably during insulin stimulation (17-fold) and this was more than two times as high in the exercised leg compared with the rested leg. This fits well with the roughly twice as high muscle glucose uptake in the exercised compared to rested muscle (Fig. 2C). We also show that if the normal increase in insulin-stimulated leg blood flow (LBF) is prevented, interstitial glucose can decrease dramatically and likely become limiting to leg glucose uptake (LGU). This demonstrates the importance of glucose delivery to sustain the increase in insulin-stimulated LGU after acute exercise. Previous investigations have shown that LBF is most important in conditions of high glucose uptake rates during insulin-stimulation (Baron *et al.*, 2000), which fits well with the observations in the present study that increased LBF by infusion of ATP caused an increase in LGU which was higher in the exercised leg compared with the rested leg. Indeed, muscle membrane permeability to glucose was unchanged with NOS inhibition and ATP infusion in the two legs which fits with our previous study showing that these treatments effect glucose uptake by altering blood flow rather than insulin signaling (Sjoberg *et al.*, 2017). Conversely, when increasing LBF in the fasted state in insulin resistant type 2 diabetic patients where LGU is low due to a low glucose extraction across the leg, the effect of increasing LBF on LGU was not apparent (Henstridge *et al.*, 2005). Thus, when muscle membrane permeability is low, glucose uptake is not limited by perfusion.

The exercise-induced increase in muscle membrane permeability during insulin stimulation is likely the result of AMPK activation during exercise as contraction-induced increased sensitivity to insulin-stimulated glucose uptake is absent in mice devoid of muscle AMPK activity (Kjobsted *et al.*, 2017), and both human and rodent studies point to TBC1D4 as a potential integrator of insulin and exercise mediated signaling (Cartee & Wojtaszewski, 2007; Pehmoller *et al.*, 2012; Kjobsted *et al.*, 2017; Wang *et al.*, 2018). However, the absolute increase in muscle membrane permeability to glucose in response to insulin observed here was greater than we would have predicted from previous studies in humans that found around 2-6 fold increases in skeletal muscle GLUT4 translocation with insulin (Ryder *et al.*, 2000; Koistinen *et al.*, 2003). In addition, a study using a similar fold increase in insulin

concentration as the current study found only a 2.6 fold increase in the inward rate constant for glucose which was considerably lower than the 5.7 fold increase in forearm glucose uptake (Bonadonna *et al.*, 1993). However, in support of our results, immunofluorescence electron microscopy in rat muscle shows a remarkable, although not quantified, GLUT4 translocation with insulin, partly because of virtual absence of GLUT4 in the sarcolemma in the basal state (Rodnick *et al.*, 1992) and brown adipose tissue plasma membrane GLUT4 in rats has been shown to increase by 37-39 fold with insulin (Slot *et al.*, 1991).

It cannot be excluded that the intrinsic activity of the GLUT4 transporters may be increased upon translocation to the muscle membrane. A recent study demonstrated, using two independent assays to measure GLUT4 translocation, that the non-metabolisable glucose analog 3-O-methyl-D-glucose increased the rate of hexose transport into L6 myotubes without recruiting GLUT4 to the plasma membrane (Shamni *et al.*, 2017). In addition, it was reported that 3-O-methyl-D-glucose increased hexose transport without affecting insulin- or AMPK-signal transduction pathways and it increased the  $V_{max}$  of the transport without altering the  $K_m$  of hexose transport (Shamni *et al.*, 2017). These authors suggested that various proteins interact with GLUT4 to affect its activity (Shamni *et al.*, 2017). Therefore, while there is some experimental evidence to support such a notion, the mechanisms by which such an increase in GLUT4 intrinsic activity might occur are not resolved.

Given that the concentration of insulin in skeletal muscle interstitium during insulin infusion is already less than half of the concentration in plasma (Castillo *et al.*, 1994), it is possible that NOS inhibition, by reducing blood flow, may have decreased interstitial insulin further and thereby decreased its action on muscle glucose uptake. However, given NOS inhibition during an EHC has no effect on skeletal muscle insulin signaling (Sjoberg *et al.*, 2017) or muscle membrane permeability to glucose, any possible reduction in insulin delivery to the muscle cells appears to have been too small to cause the reduction in glucose uptake. Furthermore, if NOS inhibition decreased skeletal muscle glucose uptake by reducing the amount of insulin delivered to the skeletal muscle cells, then NOS inhibition would be expected to reduce glucose uptake also in the rested leg, which was not the case in either our previous (Sjoberg *et al.*, 2017) or current study.

ATP is a potent vasodilator in humans (Crecelius *et al.*, 2011; Nyberg *et al.*, 2013). Although ATP can bind to P2Y receptors and increase GLUT4 translocation in myotubes (Osorio-Fuentealba *et al.*, 2013), it is very unlikely that the infused ATP reached the muscle cells since it has been shown that a much higher femoral artery ATP infusion rate which elicited a femoral LBF of  $\sim 4 \text{ L}\cdot\text{min}^{-1}$  did not change skeletal muscle interstitial ATP concentration (Mortensen *et al.*, 2009). Consequently, this

suggests that in the present study ATP increased leg glucose uptake was due to its vasodilatory effects rather than any effect on muscle cells *per se*.

Greater increases in insulin-stimulated leg blood flow, skeletal muscle microvascular perfusion and skeletal muscle insulin signaling at the level of TBC1D4 have been demonstrated following an acute bout of exercise (Trebbak *et al.*, 2009; Pehmoller *et al.*, 2012; Sjoberg *et al.*, 2017). Interestingly, although in the present study glucose delivery and glucose disposal were higher in the previously exercised leg during insulin infusion, interstitial glucose concentration actually decreased more in the previously exercised than the rested vastus lateralis muscle. This indicates that the rate of glucose removal from the interstitium across the muscle membrane is outstripping the rate of replenishing of interstitial glucose from glucose delivery indicating a significant endothelial barrier for glucose. How glucose escapes through the capillaries has mostly been thought to be through slits between the endothelial cells as originally proposed by Pappenheimer *et al.* (Pappenheimer *et al.*, 1951), however, it has also been suggested that GLUT1 facilitates endothelial transport of glucose (Yazdani *et al.*, 2019). Although some suggestion of such an endothelial barrier has been described earlier (Pellinger *et al.*, 2010), the magnitude of the difference between arterial and interstitial glucose concentration in our study was much larger even without LBF restriction by the NOS inhibitor. Moreover, since muscle glucose uptake without LBF restriction was much higher in the previously exercised muscle during insulin stimulation than the rested muscle, it seems feasible that the relatively low interstitial glucose concentration in the exercised muscle may have prevented the glucose uptake from being even higher. This was actually demonstrated when LBF was increased by infusion of ATP, since this resulted in increasing interstitial muscle glucose concentrations and increased LGU in the exercised leg.

It is possible that part of the magnitude of decrease in skeletal muscle interstitial glucose concentration may have been due to the euglycemic, hyperinsulinemic clamp which does not mirror physiological conditions. After a meal the blood glucose concentration increases and evokes endogenous insulin secretion and it is possible that the meal-induced hyperglycemia would prevent a decrease in muscle interstitial glucose concentration. However, this does not explain the large differences in interstitial glucose concentration that we observed between the rested and exercised muscle, and indeed differences between the legs, although smaller, were also apparent in the control trials where we did not infuse insulin. Follow up studies to investigate this further could utilise a meal to more closely study the changes in interstitial muscle glucose concentrations in rested and exercised muscle under conditions where both plasma insulin and glucose are increased.

We think it is unlikely that measurement errors or assumptions account for the large difference between our apparent muscle membrane permeability to glucose and previous GLUT4 translocation studies. For example, although we assumed, based on previous literature (Cline *et al.*, 1999), that intracellular glucose concentration of glucose was close to zero in resting muscle, if it was higher than this then it would actually increase, not decrease, the calculated muscle membrane permeability to glucose. In addition, our measured skeletal muscle interstitial glucose concentrations at rest and during insulin infusion in the resting leg were similar to that measured by others previously (Holmang *et al.*, 1998; Rosdahl *et al.*, 2000).

Given our participants were young (~27y.o.), lean (body fat ~16%) males with a relatively high level of fitness ( $\text{VO}_{2\text{max}}$ , ~51ml/kg/min), caution should be made extrapolating these results to other populations such as female, older, less lean or less fit individuals. In addition, it will be important for future studies to examine if similar responses occur in people with diabetes. Despite them having reduced responses to insulin, there is some evidence they have normal increases in insulin sensitivity following an acute bout of exercise (Devlin *et al.*, 1987; Perseghin *et al.*, 1996). However, given people with T2D have endothelial dysfunction which manifests as impaired blood flow response to both insulin (Laakso *et al.*, 1992) and exercise (Kingwell *et al.*, 2002) and also have decreased GLUT4 translocation in response to insulin (Ryder *et al.*, 2000), it is difficult to predict the effect of varying blood flow in such individuals.

In conclusion, we provide in vivo estimates of the critical components that control skeletal muscle glucose uptake during submaximal insulin stimulation and demonstrate in humans that the apparent muscle membrane permeability to glucose increased 17-fold from basal in a rested muscle and more than twice as much in muscle recovering from exercise. This difference in muscle membrane permeability between the exercised and rested leg is likely of major importance for the increased insulin action on muscle glucose uptake in the post-exercise period. However, the increased insulin induced membrane permeability in muscle following exercise requires increased glucose delivery via increased muscle blood flow in order to lead to increased glucose uptake. The increase in glucose delivery with insulin-induced vasodilation prevents skeletal muscle interstitial glucose concentration from decreasing to critically low levels that would nullify the effect of increased membrane permeability to glucose. We further show that the capillary wall can be a significant barrier for glucose leading to low interstitial glucose concentrations. The determination of the functional membrane permeability to glucose is a novel feature characterising an important



aspect of muscle glucose metabolism. In principle, the methodology employed here can also be used to determine membrane permeability to other substrates or metabolites.

#### **Author contributions:**

G.K.M., E.A.R., F.C., and K.A.S. designed the study. G.K.M., and F.C., conducted the exercise tests. G.K.M., E.A.R., F.C., K.A.S., B.K., and J.F.P.W. conducted the experiments. G.K.M. drafted the manuscript and all authors revised the manuscript critically. Erik A. Richter has full access to all data and is guarantor of the study.

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#### **Competing interests**

None of the authors has any conflicts of interests.

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**Table 1.** Subject characteristics. N=10.

Age, years	27±1
Height, m	1.80±0.02
Weight, kg	77.7±2.3

Body fat, %	16.4±1.4
BMI	23.9±0.5
Lean mass:	
Exercised leg, kg	9.7±0.5
Active thigh, kg	2.7±0.1
Rested leg, kg	9.8±0.4
VO <sub>2</sub> peak, ml·kg <sup>-1</sup> ·min <sup>-1</sup>	50.7±1.5
Peak leg work load, Watts	56.7±3.2

**Table 2.** Heart rate, blood pressure and glucose infusion rate (GIR) during the euglycaemic hyperinsulinaemic clamp. Mean ± SEM, n = 10. Grey shading 105-135 min indicates L-NMMA infusion and at 195-225 min indicates ATP infusion. \*  $P < 0.05$  vs 0. †  $P < 0.05$  vs 90.

Time (min)	Heart rate (BPM)	Systole (mmHg)	Diastole (mmHg)	GIR (mg/kg/min)
0	58 ± 2	122 ± 3	63 ± 3	1.5 ± 0.1
15	59 ± 2	122 ± 3	64 ± 3	2.0 ± 0.2
30	59 ± 3	122 ± 3	63 ± 3	5.2 ± 0.4
45	61 ± 2	123 ± 3	64 ± 3	6.2 ± 0.4
60	60 ± 3	122 ± 3	67 ± 2	6.6 ± 0.5
75	61 ± 3	123 ± 3	63 ± 3	6.3 ± 0.5
90	61 ± 3	124 ± 3	65 ± 3	6.5 ± 0.5
105	57 ± 3	128 ± 3 *	69 ± 3 *†	6.7 ± 0.5
120	58 ± 2	130 ± 3 *†	71 ± 3 *†	6.9 ± 0.4
135	58 ± 3	132 ± 3 *†	73 ± 3 *†	7.3 ± 0.4
150	59 ± 2	130 ± 3 *	70 ± 3 *†	7.9 ± 0.4
165	60 ± 3	127 ± 2 *	68 ± 3 *	8.2 ± 0.5



180	61 ± 3	127 ± 3 *	70 ± 2 *†	8.4 ± 0.5
195	65 ± 3 *	127 ± 4	69 ± 3	8.6 ± 0.5
210	63 ± 3 *	127 ± 3	68 ± 2	8.7 ± 0.5
225	65 ± 3 *	126 ± 2	67 ± 2	8.9 ± 0.4

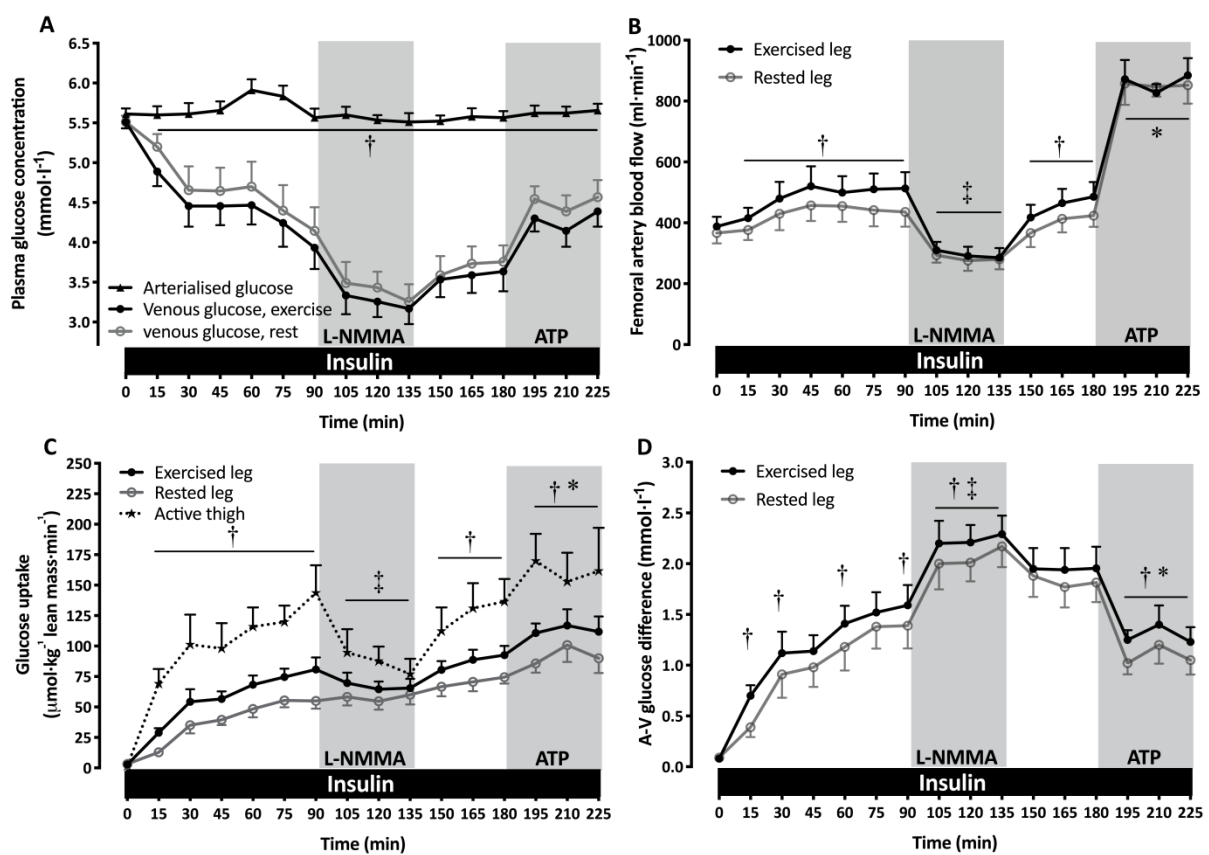
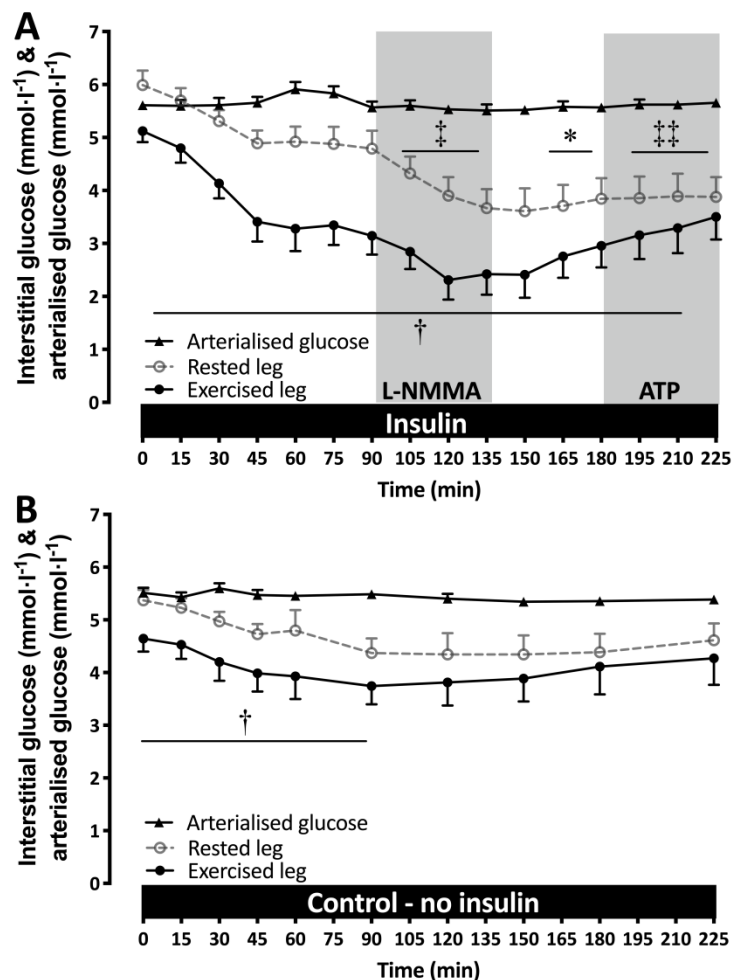
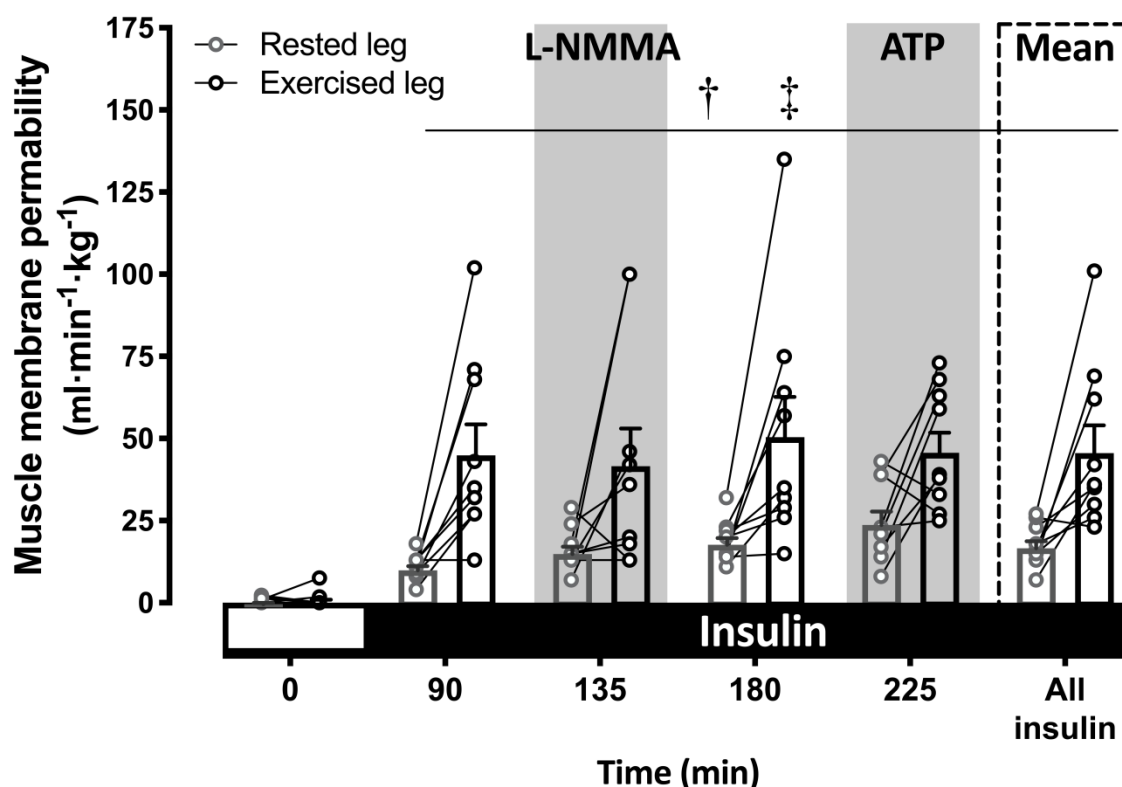


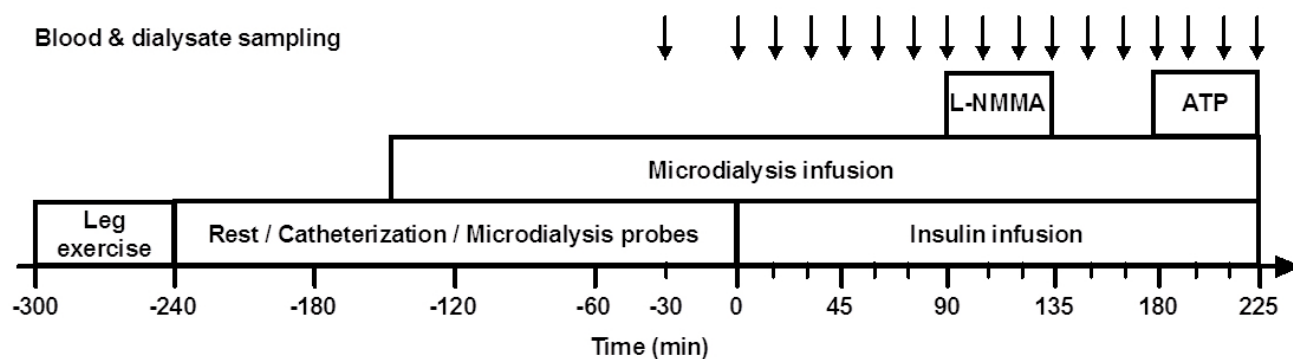
Figure 1. Schematic overview of the Experimental protocol.



**Figure 2.** Euglycaemic hyperinsulinaemic clamp, leg blood flow, leg arteriovenous glucose difference, femoral vein glucose concentrations and leg glucose uptake (LGU). A: Arterialised plasma glucose concentration and glucose infusion rate (GIR), femoral vein glucose concentrations. B: Leg blood flow. C: Glucose uptake of lean leg mass and of active lean leg mass. D: Arteriovenous glucose difference. Data are presented as mean  $\pm$  SEM,  $n = 10$ . For B-D statistical evaluation involved two-way repeated measures ANOVA. When the ANOVA revealed a significant difference of  $P < 0.05$ , specific differences were determined using a Student-Newman-Keuls post-hoc test. See statistics section for details.  $\dagger P < 0.05$  exercised leg and active thigh vs. rested leg,  $\ddagger P < 0.05$  vs. pre L-NMMA (90 minutes),  $* P < 0.05$  vs. pre ATP (180 minutes).



**Figure 3.** Interstitial glucose concentration during the EHC protocol with inclusion of L-NMMA and ATP infusions (A). Data shown is before insulin infusion (0), then after: 90 min of insulin infusion (90), 45 min with co-infusion of the NOS inhibitor (L-NMMA) (135 min), 45 min of insulin infusion only (180 min) and 45 min of ATP co-infusion (225 min). Interstitial glucose concentration during the control protocol (B). Mean $\pm$ SEM, n=9 (A), n=7 (B). Statistical evaluation involved two-way repeated measures ANOVA. When the ANOVA revealed a significant difference of  $P<0.05$ , specific differences were determined using a Student-Newman-Keuls post-hoc test. See statistics section for details.  $\dagger$   $P<0.05$  exercised muscle vs. rested muscle,  $\ddagger$   $P<0.05$  vs. pre L-NMMA (90 minutes) both muscles,  $\ddagger\ddagger$   $P<0.05$  vs. pre ATP (180 minutes) for the previously exercised muscle,  $\ddagger\ddagger\ddagger$   $P<0.05$  vs. baseline (0 minutes) for both muscles. Interstitial glucose concentration was lower than the arterialised glucose concentration after 15 and 45 minutes in the previously exercised and rested muscle, respectively ( $P<0.01$  not shown on figure).



**Figure 4.** Skeletal muscle apparent glucose membrane permeability in the rested and previously exercised muscle over a 225 min EHC. Data are presented as individual values and as mean $\pm$ SEM, n=9. Statistical evaluation involved two-way repeated measures ANOVA. When the ANOVA revealed a significant difference of  $P<0.05$ , specific differences were determined using a Student-Newman-Keuls post-hoc test. See statistics section for details.  $\dagger$   $P<0.05$  exercised muscle vs. rested muscle,  $\ddagger$   $P<0.05$  vs. pre L-NMMA (90 minutes) both muscles.