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Development of microdialysis methodology for interstitial insulin measurement in rodents

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Abstract

Introduction Accurate assessment of muscle insulin sensitivity requires measurement of insulin concentration in interstitial fluid (ISF), but has proved difficult. We aimed to optimise measurement of ISF insulin concentrations in rat muscles *in vivo* using microdialysis.

Methods Factorial experimental design experiments were performed *in vitro* to determine optimal conditions for insulin recovery with microdialysis probes. These conditions were tested *in vivo*, adjusted appropriately and used in lean and obese Zucker rats to compare ISF insulin concentrations basally and during hyperinsulinaemic-euglycaemic (HE) clamp.

Results Optimal conditions *in vivo* were: a 100 kDa microdialysis probe inserted in muscle, perfused with 1% BSA, 1.5mM glucose in 0.9% sodium chloride at 1 μ l/min. Samples were collected into siliconised glass microvials. As a reference for insulin, we established a protocol of insulin infusion, beginning at -80min and reaching equilibrium within 60min. HE clamp, beginning at 0min, increased ISF insulin concentration from 122 \pm 56 basally to 429 \pm 180pmol/L ($P < 0.05$) in lean rats and from 643 \pm 165 to 1087 \pm 243pmol/L ($P = 0.07$) in obese rats; ISF insulin concentrations were significantly higher throughout in obese rats. The difference between ISF and plasma insulin concentration (ISF:plasma ratio) was substantially higher in obese rats, but fell to similar values in obese and lean rats during HE clamp.

Discussion Optimising insulin recovery with microdialysis allowed accurate measurement of basal ISF insulin in muscle of lean and obese Zucker rats and indicates insulin transport across capillaries is impaired in obese rats, basally and during hyperinsulinaemia.

Abbreviations: ISF; interstitial fluid, HE; hyperinsulinaemic-euglycaemic clamp, TET; transendothelial transport, IRK; insulin receptor kinase, RR; relative recovery, BSA; bovine serum albumin, I:P ratio; interstitial to plasma ratio, MI; insulin sensitivity.

1 Introduction

The prevalence of obesity and diabetes is increasing worldwide. Thus, there is an associated need for accurate assessment of the factors that contribute to insulin sensitivity and resistance in animal models of these conditions. Generally, insulin sensitivity of skeletal muscle, one of the major tissues for glucose disposal, is assessed from the relationship between plasma level of insulin and glucose uptake. However, plasma insulin concentration is not a good index of the concentration of insulin in the interstitial fluid (ISF) of skeletal muscle from where it accesses insulin receptors on the sarcolemma. Indeed, it is well established that the plasma insulin concentration required to increase glucose disposal rate *in vivo* is significantly higher than that required *in vitro* (Poulin, Steil, Moore, Ader, & Bergman, 1994). Moreover, the rate of increase in glucose uptake during hyperinsulinaemic-euglycaemic (HE) clamp is delayed compared to the change in plasma insulin concentration (Castillo, Bogardus, Bergman, Thuillez, & Lillioja, 1994).

The disparities between plasma and ISF insulin concentrations are partly explained by the fact that insulin must cross the capillaries in order to reach the ISF. Trans-endothelial transport (TET) of insulin is regulated by an insulin-receptor mediated process on the endothelial cells. Insulin also acts on insulin receptors on resistance vessels and terminal arterioles to increase muscle blood flow and the capillary surface area for exchange (Barrett et al, 2009; 2011). Thus, the relationship between plasma ISF and ISF insulin is dependent on the sensitivity of the insulin receptors at these different vascular sites and these factors in turn, affect estimates of muscle insulin sensitivity. Nevertheless, a full appreciation of the sensitivity of skeletal muscle to insulin ultimately requires that insulin concentrations be measured in ISF.

Insulin concentration in lymph has been used as a surrogate for ISF insulin concentration in hindlimb of humans (Castillo et al., 1994) and rats (Poulin et al., 1994). Such measurements indicated lymph concentrations are substantially lower than those in plasma and correlate better with glucose uptake than plasma insulin concentration (Yang, Hope, Ader, & Bergman, 1989). However, lymph insulin concentration is not an accurate assessment of ISF insulin because lymph drainage from hindlimb derives from skin, adipose tissue and bone, as well as muscle. The alternative is to use microdialysis to directly assay insulin in muscle ISF as has been reported in humans and rats (e.g. Sjostrand et al., 2000; Gudbjornsdottir, Sjostrand, Strindberg, & Lonroth, 2005; Holmang, Mimura, Bjorntorp, & Lonroth, 1997). However, this is technically challenging particularly in rodents, because insulin is a large molecule that diffuses slowly across the pores of dialysis membranes, and because insulin concentration is notoriously difficult to measure in low concentrations in small fluid volumes. Indeed, the low concentrations of insulin in ISF under basal conditions have not yet been measured accurately in the rat.

Thus, the primary aims of the present study were to optimise the microdialysis methodology *in vitro* whilst employing validated techniques for assaying low concentrations of insulin in small fluid volumes. We then adapted these techniques so that they could be used accurately and reliably to measure ISF insulin concentrations *in vivo* in the rat. We also established that an insulin infusion given from 80min before the start of the experiment proper, could serve as an effective reference for insulin. Our final aim was to measure ISF insulin concentration in hindlimb muscle of lean and obese Zucker rats in the basal state and during HE clamp so as to clarify the relationship

between plasma and ISF insulin and to allow direct estimates of skeletal muscle insulin sensitivity.

2 Methods

2.1 Validation of the microdialysis method in vitro

Factorial experimental design experiments were performed to determine the best conditions for recovery of insulin. All equipment was purchased from CMA Microdialysis, Sweden via Linton Instrumentation, Norfolk, UK unless stated otherwise.

2.1.1 Reagent preparation.

All reagents were prepared on the day of study unless stated otherwise.

Microdialysis perfusion fluid was prepared using 10% BSA and 20% (w/v) glucose. A working solution of 1% BSA and 1.5mmol/L glucose was prepared in 0.9% sodium chloride and delivered at 1µl/min.

Inulin A stock solution of 10mg/ml inulin was diluted in 0.9% sodium chloride to a solution concentration of 25µg/ml.

Human insulin was prepared by diluting human insulin (Actrapid®, Novo Nordisk, Denmark) in 2% BSA (in 0.9% sodium chloride) to concentrations of 7000, 3000, 1000 and 300pmol/L.

2.1.2 Microdialysis probe preparation

Microdialysis probes (CMA 20), 10mm in length with cut-off pore sizes of 55 or 100kDa, were connected via fluorinated ethylene propylene (FEP) tubing and tubing adapters. The inlet FEP tubing was connected to a 1ml microsyringe in an infusion pump (CMA 402 dual syringe pump) filled with perfusion fluid (see above). Insulin was diluted to 7000pmol/L as detailed above and 1ml of solution was placed in siliconised glass vials. Glass vials were secured in a CMA 130 *in vitro* stand and microdialysis probes were

submerged in test solution and secured with the clips provided. Probes were equilibrated for 40min with each new set of conditions. The rate of perfusion was 1, 2 or 2.5 μ l/min. All equipment and tubing was either treated with Sigmacote® (Sigma-Aldrich Ltd, UK), or it was not. Following initial experiments to test the optimum conditions for insulin recovery, further experiments were conducted to assess recovery at different concentrations of insulin (3000, 1000 and 300pmol/L) and inulin (25 μ g/ml).

2.1.3 Sample collection and analyses

The perfusate was collected in either plastic, or glass vials: 40min samples were collected, split equally for storage and analysed in duplicate. Insulin and inulin were assayed with ELISA Kits (see 2.2.7).

2.2 Refinement of microdialysis methodology *in vivo*

All experiments were conducted in accordance to the Animal Scientific Procedures Act 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments. They were performed on either male Wistar rats, or male lean or obese Zucker rats (supplied by Charles River, Kent, UK). Anaesthesia was induced with an intra-peritoneal (i.p.) injection of 160-190mg/kg sodium-thiobutabarbital (Inactin®, Sigma-Aldrich Ltd, UK) and supplemented as required, with bolus doses of 0.5ml, or by increasing the infusion rate of anaesthetic via a cannula placed in the jugular vein. At the end of the experimental protocol, the animal was humanely killed by overdose of sodium-thiobutabarbital. Death was confirmed by cervical dislocation.

2.2.1 Experimental groups

2.2.1.1 Inulin infusion

Experiments were performed on 3 anaesthetised male Wistar rats (weight 222 ± 15 g) to establish whether inulin could be given by infusion so as to provide a reference for insulin. The inulin infusion protocol was based on modelling of kinetics in preliminary experiments not reported here. Thus, insulin was given as a 50mg/kg bolus followed by infusion at 2mg/kg/min for 180min. Blood samples for analysis were taken at 1 and 5min and then at 15min intervals. Each sample was centrifuged at 13,000RPM for 5min at 4°C and plasma was stored at 5°C and analysed <1week after collection. In the preliminary inulin kinetic experiments, we tested whether samples could be frozen prior to analysis of inulin; these findings are shown in Supplementary Data Figure 1.

2.2.1.2 Combined inulin infusion and HE clamp

Experiments were performed on 4 male Wistar rats (weight 222 ± 15 g); see Supplementary Data for details. The methodology tested in these experiments was adopted for the experiments proper and is described in detail below (see 2.2.2 - 2.2.9). These experiments were also used to compare recovery of insulin with microdialysis probes at different pore cut-off sizes of 55 and 100kDa. Therefore, half of the probes prepared and inserted as described in 2.2.4 and 2.2.5 were 55kDa and half were 100kDa.

2.2.1.3 Use of validated microdialysis methodology during HE clamp

Experiments were performed on 6 lean and 5 obese Zucker rats. Full methodology is described below (see 2.2.2 – 2.2.9).

2.2.2 Animal husbandry

Animals were housed in cages on a controlled 12 hour Light/Dark cycle, (6am: 6pm) and fed standard chow (SDS Rat and Mouse No. 1, 2.61 kcal/g of ration or 9.24% kcal of fat) and water *ad libitum*. The day before the experiment proper, each rat received a fixed ration: 16 or 10g rat chow (obese and lean respectively) at 16:00 to provide sufficient food until approximately midnight. Thus, the rat was non-absorptive, but not starved during the HE clamp protocol.

2.2.3 Preparation of reagents

All reagents were prepared on the day of study, unless otherwise indicated.

Approximately, 80% of glucose uptake occurs in the skeletal muscle (DeFronzo, Ferrannini, Sato, & Felig, 1981). Thus, insulin sensitivity is expressed per fat-free mass (lean body mass; LBM) rather than body weight (Muniyappa, Lee, Chen, & Quon, 2008) and the HE clamp and inulin concentration were delivered according to LBM. LBM was estimated from empirical linear regression formulae derived from body composition analysis of Zucker rats performed in-house. The equations used for the Lean and Obese Zucker rat were $LBM (g) = 31 + 0.77 \times BW (g)$ and $59 + 0.50 \times BW (g)$ respectively.

Microdialysis perfusion fluid was prepared using 10 % BSA and 20 % (w/v) glucose. A working solution of 1 % BSA and 1.5mmol/L glucose was prepared in 0.9% sodium chloride and delivered at 1 μ l/min.

Inulin A stock solution of 10mg/ml inulin was diluted in 0.9% sodium chloride so that we could deliver both a 1mg/kg_{LBM} bolus and 0.08mg/kg_{LBM}/min infusion. The 1mg/kg_{LBM} bolus dose was given as 0.125ml bolus over 30 seconds. The 0.08 mg/kg_{LBM}/min infusion was then given as 10 μ l/min for the rest of the experiment.

Human insulin was prepared by diluting human insulin (Actrapid®, Novo Nordisk,

Denmark) in 10ml of Haemaccel®. The volume of stock human insulin solution added to the Haemaccel® was determined according to the experimental group and lean body mass in kg, kg_{LBM}. The volume infusion rate was fixed in all experiments to 10µl/min.

2.2.4 Microdialysis probe preparation

Each probe (100kDa cut-off) was perfused *in vitro* prior to and during surgical preparation of the rat (>30min). The probe inlet and outlet tubing was cut to 5cm and connected to siliconised 'FEP' tubing by using adapters. The inlet tubing was connected to a 1ml microsyringe containing perfusion fluid. The outlet tubing was cut to 45cm. The probes were placed in an *in vitro* stand and the syringe pump was started at 1µl/min and allowed to run until probe insertion; perfusion fluid collected during this period was discarded.

2.2.5 Surgical procedure and baseline measurements

On the morning of the experiment proper, the rat was anaesthetised with an i.p. injection of 160-190mg/kg sodium-thiobutabarbital as described in 2.2 above. The right jugular vein was cannulated with five cannulae for infusions of insulin, glucose, inulin, and sodium-thiobutabarbital. The trachea was cannulated and medical O₂ was directed over the opening at free flow to ensure adequate arterial oxygenation. The left carotid artery was cannulated for blood sampling and recording of arterial blood pressure (ABP); mean ABP (MABP) was derived on-line. All cannulae were filled with 20mM sodium citrate solution to prevent clotting. Carotid artery catheter patency was maintained by continuous infusion (10µl/min) of 20mM sodium citrate solution. Body temperature was monitored and maintained at 37°C using a rectal probe and heat blanket (Harvard Apparatus Ltd, UK).

Following surgery, the rat was allowed to stabilize for 40min from -120 to -80min (see Figure 1). During this time, a microdialysis probe was inserted into the tibialis anterior muscle of each hindlimb at 45° to the skin surface by using a guide needle and split tubing. It was tunnelled ~5mm under the surface of the skin parallel to the surface. All tubing and equipment was siliconised on the basis of the *in vitro* studies (see Results). The outlet FEP tubing was fed into the refrigerated microfraction collector (CMA 470) set at 6°C and was positioned below the level of the rat. The length of outlet tubing was calculated from the internal diameter to correspond to a 5 min time delay in the transport of each fraction collected from the rat to the siliconised glass microvial when perfusion was set at 1µl/min, as determined by the *in vitro* measurements (see Results 3.1). Each individual microdialysis probe used *in vivo* was calibrated for recovery of insulin as described in 2.2.8.

A baseline arterial blood sample (200µl) was taken at -90min from the carotid artery into potassium-EDTA coated tubes (Microvette®, Sarstedt Ltd., UK) and immediately centrifuged. Plasma samples were split and stored at -20°C for later determination of insulin concentration, or at 4°C for determination of inulin concentration. Additionally, a small drop of arterial blood was assayed for blood glucose by using a test strip (Roche Accu-chek®). At -80min, infusion of inert inulin was commenced as a bolus (0.125ml) followed immediately by infusion at 10µl/min. Collection by the microfraction collector was therefore started at -75min and a fraction was collected every 40min. These fractions were directly correlated with plasma samples taken over the same periods, an additional arterial blood sample being taken at -60min to correspond with ISF Sample 1 (Figure 1). Microdialysis probes were checked regularly for patency and flushed at

20 μ l/min for ~ 30s if necessary. Microdialysis probe placement was checked by dissection *post mortem*.

2.2.6 Hyperinsulinaemic-euglycaemic clamp

As shown in Figure 1, blood glucose concentration for the basal phase of the HE clamp was taken as the average of measurements taken at -20, -10 and 0min by using the test strips. Arterial blood samples (200 μ l) for off-line analysis were collected via the carotid cannula at -20 and 0min. Sample 2 of ISF corresponded to the basal phase of the HE clamp. The HE clamp phase began after collection of the 0min sample. Human insulin was infused via the jugular vein using a digital syringe pump at 10 μ l/min. The insulin concentration was calculated according to lean body mass (LBM, see above) and delivered at 10 and 20mU/kg_{LBM}/min of insulin for lean and obese rats respectively. Thereafter, arterial blood glucose concentrations were determined every 3 to 5min and blood glucose was clamped at the basal (euglycaemic) concentration by infusing glucose 20% (w/v) at a variable rate by using the digital syringe pump. The infusion rate required to achieve this was noted so that glucose infusion rate (GIR) could be calculated and used to calculate insulin sensitivity (MI; see 2.2.8). Additional arterial blood samples for analysis of glucose, insulin and inulin were taken at 20, 40, 60, 70 and 80min.

2.2.7 Sample analysis

Plasma glucose was determined by using a Clinical Analyser, (YSI (UK) Ltd., Hampshire, UK). Inulin concentration in the *in vitro* and *in vivo* studies was assayed by using the BioPal FIT-GFR kit (BioPal, Worcester, Massachusetts).

In initial *in vitro* studies insulin was assayed with the Mercodia Ultrasensitive Rat Insulin Kit, which has a stated sensitivity range of 0.9-120pmol/L and should therefore detect concentrations lower than those detected by the Crystal Chem Rat Insulin ELISA kit (nominally 17.4 - 11,136pmol/L). However, we established that the Mercodia kit could not be used with a volume less than the recommended 25µl, which would have allowed only 2-3 samples to be assayed over the period of the HE clamp. The Crystal Chem kit has the major advantage of only requiring a sample volume of 5µl. Additionally, the Crystal Chem assay has been validated by the Analytical Group, CVGI, AstraZeneca, Alderley Park for the measurement of insulin in the range 5.4 - 11,136pmol/L. Insulin concentration in samples taken from the *in vitro* and *in vivo* studies were therefore analysed by using the same ultrasensitive rat insulin kit (Ultrasensitive Rat Insulin ELISA Kit, Crystal Chem, Illinois, USA). As the ELISA kit used for insulin assay cross-reacts with the human insulin used for the HE clamp, two standard calibration curves were run on the ELISA plate, one using the rat insulin supplied in the kit and the other with human insulin spiked into rat plasma ISF. Samples (plasma and ISF) before the initiation of insulin infusion were read off the rat standard curve and samples (plasma and ISF) collected after the initiation of insulin infusion were read off the human standard curve. (See Discussion for consideration of outcomes).

For ISF sample collection, the outlet tubing volume was calculated using the internal diameter of the FEP tubing. An outlet tubing length of 45cm was calculated to have a volume of 5µl and therefore to correspond to a 5min time delay from tissue to collection vial. The CMA 470 Refrigerated microfraction collector was therefore started 5min after the insulin infusion and could then be taken to represent real time measurements of tissue concentrations, as the delay in the sample reaching the collection vial was

accounted for. The ISF Samples (microdialysate fractions of 40µl collected over 40min in the microvials were split equally and stored at -80°C for later determination of insulin, or at 5°C for determination of inulin. A 15µl sample of ISF was required in order that it could be diluted 1 in 10 to provide a large enough volume to test 50µl of diluted sample in duplicate with the BioPAL FIT-GFR (Inulin) kit. A 15µl ISF sample was also required to test ISF samples neat on the Crystal Chem Rat Ultra Sensitive Insulin kit. Therefore, microdialysate fractions (40µl) collected over 40min allowed for dead space and any small ultrafiltration of samples, whilst ensuring there was enough fluid volume for analysis of each fraction.

2.2.8 Calculations

2.2.8.1 Insulin Sensitivity (MI)

MI was calculated as:

$$\text{MI } (\mu\text{mol/kg/min per pmol/L}) = \text{GIR } (\mu\text{mol/kg/min}) / \text{Plasma insulin (pmol/L)}$$

Where GIR is the steady state glucose infusion rate at the end of HE clamp and plasma insulin concentration represents measurements taken at corresponding time points.

GIR was expressed as mg/kg_{LBM}/min.

2.2.8.2 Insulin recovery in vivo

ISF concentrations of insulin and inulin obtained from each rat were averaged to obtain a mean measure for each rat. Actual ISF insulin concentration was calculated from the measured ISF insulin concentration *in vivo*, and adjusted per recovery of inulin *in vivo* by

using the external reference technique (Jansson, Fowelin, Vonschenck, Smith, & Lonroth, 1993) and the formula:

$$\text{Recovery of inulin } in \textit{ vitro} / \text{Recovery of insulin } in \textit{ vitro} = \text{Recovery of inulin } in \textit{ vivo} / \text{Recovery of insulin } in \textit{ vivo}$$

2.2.8.3 Interstitial to plasma (I:P) ratio. This represents the ratio of insulin concentration between the circulation and ISF and was calculated as the ISF insulin concentration: plasma insulin concentration ratio.

2.2.9 Statistical analysis

Results are expressed as mean \pm SEM unless otherwise indicated. Differences between treatment groups were evaluated using an unpaired Student's *t*-test. Differences from baseline within the group were analysed using a one-way ANOVA with Dunnett's *post hoc* test.

3. Results

3.1 Validation of the microdialysis method *in vitro*

Pore size. Experiments comparing pore size at 55 and 100kDa cut-offs showed that recovery of both insulin and inulin *in vitro* was higher with the 55kDa probe (see Supplemental Data Figure 3).

Perfusion fluid. 1% BSA delivered at 1 μ l/min consistently provided the highest recovery of insulin in each set of conditions, whereas 2% BSA at 2.5 μ l/min consistently provided the lowest recovery in each set of conditions. The optimal conditions for recovery were silicon-coated glass microvials with silicon-coated FEP tubing using perfusion fluid containing 1% BSA perfused at 1 μ l/min (Figure 2). These optimum conditions were then further tested at different concentrations of insulin and inulin to increase the accuracy of recovery calculations. The *in vitro* recovery for insulin under these conditions was $9.4 \pm 0.4\%$ for 3000pmol/L, $14.7 \pm 1.8\%$ for 1000pmol/L and $3.2 \pm 0.1\%$ for 300pmol/L (n=2). The *in vitro* recovery for inulin under these conditions was $16.7 \pm 5.2\%$ for 25 μ g/ml inulin (n=2). Microdialysis perfusion fluid for all *in vivo* experiments was therefore prepared using 1% BSA with a fixed volume infusion rate of 1 μ l/min.

3.2 Refinement of microdialysis methodology *in vivo*

The pilot inulin infusion experiments showed that inulin infused from -80min achieved a stable level within 30 min that was maintained until cessation of infusion at 180min (see Supplementary Data Figure 2).

The combined method validation experiments indicated that recovery of insulin *in vivo* was higher with a 100kDa MW cut-off probe than the 55kDa probe (3.07 ± 0.68 vs $1.35 \pm 0.10\%$; see Supplemental Data Figure 3).

3.3 Use of validated microdialysis methodology during HE clamp

In experiments on lean and obese Zucker rats, inulin concentration reached steady state at -20min of the HE clamp (Supplemental Data Figure 3).

3.3.1 Effects of HE clamp in lean and obese Zucker rats

MABP was higher for obese rats throughout the protocol, whereas HR was not different between lean and obese rats. Neither variable changed during HE clamp (see Supplementary Data Figure 4).

Blood glucose was significantly higher in obese, than lean rats throughout the protocol; neither group changed from baseline at any time point (see Figure 3A). In obese rats, GIR was different from baseline from 24min onwards from the onset of the HE clamp, whereas in lean rats, GIR was increased from 13min onwards. GIR was higher for lean than obese rats from 13min onwards (Figure 3B).

Plasma insulin concentrations were significantly higher than their respective baseline at 20 and 60min of the HE clamp in both lean and obese rats (Figure 4B; $P < 0.05$).

Plasma insulin concentration was significantly higher in obese, than lean rats throughout the protocol (Figure 4B). Thus, insulin sensitivity (MI) calculated from GIR and plasma insulin was substantially higher in lean than obese rats: (Supplemental Data Figure 5).

The ISF insulin concentrations measured at the two baseline time points at an interval of 40min were not different in either group (data not shown; $P = 0.56$ for obese and 0.35

for lean). This indicated that trauma, inducing inflammation introduced by the insertion of the probe had stabilised by the time the HE clamp began. Data from these two time points were therefore pooled to give one baseline value.

During HE clamp, ISF concentration of insulin in lean rats increased from 66 ± 11 pmol/L at baseline to 236 ± 53 pmol/L (Figure 4A; $P < 0.05$) and stabilised at this level. By contrast, ISF concentration of insulin in obese rats increased from 643 ± 165 pmol/L at baseline to 1087 ± 243 pmol/L during HE clamp (Figure 4 A, $P = 0.07$). The ISF concentration of insulin was higher in obese than lean rats under baseline conditions and at the end of HE clamp (Figure 4 A; $P < 0.05$). The MI calculated from GIR and ISF insulin was substantially higher in lean than obese rats: 0.0266 ± 0.0019 vs. 0.0032 ± 0.0012 $\mu\text{mol}/\text{kg}/\text{min}$ per pmol/L respectively.

The I/P ratio of insulin was higher in lean rats than obese rats at baseline (Figure 4C; 0.46 ± 0.04 vs. 0.23 ± 0.07 , $P < 0.05$). However, the I/P ratios decreased compared to baseline at 20 and 60min during the HE clamp in lean rats, while I/P fell modestly by 20min in obese rats (Figure 4 C). The I/P ratios were not different between lean and obese rats at 20 and 60min during the HE clamp.

4 Discussion

In this study we succeeded in making three novel contributions to the methodology required to measure skeletal muscle ISF insulin concentrations in healthy control rats and obese Zucker rats, a popular rat model of metabolic syndrome and type 2 diabetes. The first contribution is that we succeeded in scaling down the required volume of dialysate and concentration range of ISF to levels obtained in rat muscle ISF. The second

and third contribution is that we succeeded in optimising the microdialysis methodology *in vitro* and in rat skeletal muscle *in vivo*. We, therefore, have been able to measure for the first time, the concentrations of insulin in ISF of rat skeletal muscle under baseline conditions (overnight fasted) and during an HE clamp in both lean and obese Zucker rats. Our results indicate that under baseline conditions, insulin concentrations in ISF were lower in lean than in obese Zucker rats and that the plasma insulin concentration was substantially higher in obese rats. The baseline I/P insulin concentration ratio was much higher in lean than obese rats implying that transcapillary transport of insulin is impaired in the overnight fasted state in obese rats. During the HE clamp ISF insulin increased markedly in lean rats, whereas the increase in ISF insulin in obese rats was modest despite a substantial, though not significant, increase in plasma insulin concentration. As a result the I/P ratio became more similar between the lean and obese Zucker rats.

4.1 Validation of the microdialysis methodology

In order for microdialysis to be used effectively to assay ISF insulin particularly in small rodents, two major challenges had to be overcome. Firstly insulin readily adheres to the types of material used for sample collection and assaying and secondly, insulin is very difficult to assay in concentrations in the pmol/L range in small volumes of <15 μ l. In the present study, we showed that recovery of insulin was greater under all conditions when the tubing and collection vials were siliconised with Sigmacote prior to use (Fig. 2). We tested two commercially available Ultra sensitive Insulin ELISA kits and established the Crystal Chem Ultrasensitive Rat Insulin ELISA kit was the most appropriate. It can be used to assay insulin in small samples of 15 μ l and will accurately detect insulin at concentrations as low as 5.4pmol/L, even though the manufacturer's

stated range is 17.4-11,136pmol/L. As the measurement of insulin is so vital to the accurate representation of the data and because the kit has a different affinity to rat and human insulin, two standard calibration curves were run on each ELISA plate, one using the rat insulin supplied in the kit and the other with human insulin spiked into rat plasma. Hence, samples (plasma and ISF) before the initiation of insulin infusion were read off the rat standard curve and samples (plasma and ISF) collected after the initiation of insulin infusion were read off the human standard curve. This is particularly important when comparisons are being made between concentrations of insulin measured under baseline and HE clamp conditions as it ensures that both rat and human insulin are measured as accurately as possible.

We used the external reference technique to assess insulin recovery from ISF *in vivo* (Jansson et al, 1993) by using inulin because it has a molecular weight similar to insulin and passes between plasma and ISF by non-specific transcapillary diffusion (Chaurasia et al, 2007). For the external reference technique to be accurate it is essential that the concentration of inulin in plasma and ISF has stabilised. A novel aspect of the present study is that we established that when inulin was given by infusion starting 80min before the HE clamp, a stable inulin concentration in plasma was achieved within 60min and was maintained throughout the HE clamp. This simple addition to the protocol avoids the additional time and trauma to the animal caused by implanting an osmotic mini pump to deliver inulin as employed by others (Holmang et al., 1997).

An important aspect of our *in vitro* studies was to test the available microdialysis probes and to explore the composition of the perfusion fluid. Clearly, the pore size must be significantly larger than the molecule of interest in order to allow equilibration across

the membrane. Probes with molecular weight cut-offs of 50kDa (Holmang, Niklason, Rippe, & Lonroth, 2001; Holmang, Nilsson, Niklasson, Larsson, & Lonroth, 1999) or 100kDa (Gudbjornsdottir, Sjostrand, Strindberg, Wahren, & Lonroth, 2003; Sjostrand et al., 1999) have been used previously for measurement of insulin, but to date, they have not been compared. The present experiments showed that *in vitro*, recovery of both insulin and inulin was higher with the 55kDa than 100kDa probe. By contrast, *in vivo*, recovery of both insulin and inulin were better with the 100kDa MW cut-off probe. This may seem surprising because increased porosity of the dialysis membranes leads to increased internal hydraulic pressure caused by perfusion fluid entering the probe and this can cause ultrafiltration that works against the diffusion of insulin into the membrane. Ultrafiltration also dilutes the microenvironment surrounding the probe leading potentially to under-estimation of the substance of interest (Chaurasia et al., 2007). However, we took steps to avoid ultrafiltration by ensuring the outlet tubing was below the level of the animal: hydrostatic back pressure on the dialysis membrane causes ultrafiltration (Trickler and Miller (2003)). Further, as air bubbles in the tubing may cause ultrafiltration when they reach the probe, we expelled them from the tubing before it was inserted into the tissue. Due to the low flow rate of microdialysis fluid, we began flushing the tubing >30min before the time of probe insertion into the rat anterior tibialis muscle .

In previous studies it was shown that addition of an osmotic balancing agent such as dextran-70 or BSA (1-5%) to microdialysis perfusion fluid when using 100kDa or larger cut-off membranes prevented excessive ultrafiltration, by creating an osmotic pressure that offset the hydrostatic pressure. Such colloids also helped prevent non-specific adsorption onto the materials of the device (Rosdahl et al., 2000; Trickler & Miller, 2003;

Gudbjornsdottir et al., 2005; Gudbjornsdottir et al., 2003; Holmang et al., 1997; Holmang et al., 2001; Holmang et al., 1999; Sjostrand et al., 1999; Sjostrand et al., 2000; Herkner et al., 2003). In the present study, we found that addition of 1% BSA consistently provided the highest recovery of insulin in each set of conditions.

The hydraulic pressure created by perfusion increases proportionally as flow rate is increased and thus, more fluid is lost from the probe at higher flow rates, particularly with probes of high molecular weight cut-off (Trickler & Miller, 2003). Reducing the flow rate therefore reduces error and increases recovery (Lonnroth & Strindberg, 1995). Flow rates of 1, 2, or 2.5 μ l/min were used in previous studies to measure insulin in ISF (Holmang et al., 1999; Sjostrand et al., 1999; Holmang, Muller, Andersson, & Lonnroth, 1998; Jansson et al., 1993). In the present study, we established that 1% BSA delivered at 1 μ l/min consistently provided the highest recovery of insulin in each condition.

In view of these findings, perfusion fluid containing 1% BSA delivered at 1 μ l/min was used for all experiments on lean and obese Zucker rats. Although 7000pmol/L was the insulin concentration we routinely used to assess the optimum conditions for the measurement of insulin, we knew the concentration required might vary depending on the prevailing insulin concentration. Recovery of insulin was therefore tested at insulin concentrations of 300, 3000 and 7000pmol/L corresponding to plasma insulin concentration in lean Zuckers under baseline conditions, obese Zuckers at baseline and lean Zuckers at HE clamp conditions and obese Zuckers during HE clamp respectively. This further increases the accuracy of the method. As insulin concentrations in plasma were similar and constant in lean and obese rats, only one concentration of insulin was used for assessment of *in vitro* recovery.

4.2 Effects of HE clamp in lean and obese Zucker rats

As expected, under baseline conditions, the obese rats were hyperglycaemic compared to the lean rats. Both groups were fixed at euglycemia throughout the HE clamp as intended. This was achieved by increasing GIR to higher levels in lean, than obese rats and the increase from baseline was required earlier in the lean rats. As expected, plasma insulin concentrations were significantly lower in the lean than obese rats. In fact, only during the steady state of the HE clamp did plasma insulin concentrations in lean rats reach the baseline concentration seen in the obese rats. Thus, insulin sensitivity (MI) calculated from plasma insulin was ~8fold higher for lean, than obese rats.

The insulin concentrations we measured in ISF were lower than plasma concentrations in lean rats under baseline conditions, indicating that the capillary bed acts as a functional barrier to the movement of insulin. This is consistent with established evidence that an arterial-interstitial gradient exists for insulin (Sjostrand et al., 1999). Indeed, the only other study to measure baseline ISF insulin concentrations, – in healthy lean men rather than rats (Herkner et al., 2003) – also reported an insulin gradient: 48 ± 8 pmol/L in plasma versus 19 ± 4 pmol/L in ISF under basal conditions. Our finding that baseline concentrations of insulin in ISF in obese rats were significantly higher than in lean rats, but lower than in plasma, is novel and indicates that in obese rats also, the capillary bed acts as a functional barrier for transcapillary insulin transport. However, as the I/P ratio was significantly lower in obese than in lean rats under baseline conditions (0.23 ± 0.07 vs. 0.46 ± 0.03) thus, it seems that the capillary transfer of insulin is blunted in obese rats even under baseline conditions.

The I/P ratio decreased from 0.65 ± 0.25 to 0.17 ± 0.07 in lean and from 0.23 ± 0.07 to 0.11 ± 0.02 in obese rats during the HE clamp (by $\sim 74\%$ and $\sim 52\%$, respectively) such that the I/P ratio in lean rats during HE clamp was similar to that of obese rats under basal conditions. Similarly, Herkner et al., (2003) reported that in young men the I/P ratio decreased substantially from ~ 0.48 under basal conditions to ~ 0.12 during an HE clamp. Interestingly, they found no change in the I/P ratio when plasma insulin reached the same peak value during the glucose tolerance test and concluded this reflects a transcapillary insulin transport mechanism that is partly saturable even during short-term steady-state hyperinsulinaemia. The new results of the current study suggest that transcapillary insulin transport might be closer to saturation in obese than in lean Zucker rats for they achieved a similar I/P ratio to that of lean rats at a much higher plasma concentration of insulin.

In contrast to the present results, Holmang et al., (1997) reported that during HE clamp at two different levels of hyperinsulinaemia, a plasma/ISF insulin gradient existed in lean Zucker rats, but not in obese Zucker rats. They therefore concluded that transcapillary transport of insulin is not rate limiting for glucose metabolism in muscle of obese Zucker rats. Their results are difficult to compare with the present findings not only because they made no measurements of ISF insulin under baseline conditions, but also because they used smaller (50kDa) molecular weight cut-off microdialysis probes, a less sensitive insulin assay and did not make allowances for cross-reactivity between human and rat insulin. We suggest that the insulin concentrations we have estimated in the present study are more likely to be accurate than those of Holmang et al., (1997).

The relatively modest increase in ISF insulin we measured in the obese rats during HE clamp could be explained by an impaired insulin-induced dilatation of resistance and terminal arterioles and consequent blunted increase in muscle blood flow and recruitment of additional capillary surface area for transcapillary insulin transport, and/or by an impaired increase in TET of insulin (see Barrett et al, 2011; Wagenmakers et al, 2016). Insulin causes dilatation by activating eNOS via increased ser1177 phosphorylation (see Muniyappa et al, 2006), while NO-induced nitrosylation of proteins is critically important for insulin-induced increase in TET (Wang et al, 2013). In related studies involving HE clamps comparable to those of the present study, we found that hindlimb muscle blood flow increased substantially in lean Zucker rats, but not in obese Zucker rats (Stride et al, 2012). Further, HE clamp activated increased ser1177 phosphorylation of eNOS in terminal arterioles, but not capillaries of lean Zucker rats, whilst decreasing eNOS ser1177 phosphorylation in terminal arterioles and capillaries of obese Zucker rats (Cocks et al, 2013). Thus, it seems highly likely that the impaired transcapillary transport of insulin in obese Zucker rats during HE clamp is at least partly dependent on reduced ability of *arteriolar* insulin receptors to induce dilatation (Wagenmakers et al, 2016). Our results give no direct insight into differences in the insulin sensitivity of TET between lean and obese rats. However, if muscle insulin sensitivity estimated from GIR and ISF insulin concentrations rather than plasma ISF (see Figures 3 and 4), it seems the sensitivity is 15-fold greater in lean, than obese rats. This suggests that a large part of the insulin resistance shown by obese Zucker rats is located at the level of the skeletal muscle fibres.

Summary

The present study allowed us to refine and validate the *in vivo* microdialysis methodology for determination of insulin concentration in the ISF of skeletal muscle in rats. Notably, we established it is possible to quantify differences in ISF insulin concentration between lean and obese Zucker rats under basal concentrations. Future studies will need to explore the broader validation of this approach in experimental animal models. For instance, staggered microdialysis sampling combined with muscle blood flow recordings may allow further elucidation of the vascular and TET components of transcapillary insulin transport. It would also be interesting to compare the effects of similar ISF concentrations of insulin in lean and obese rats on glucose uptake in the two strains. This would be expected to give a more accurate measure of insulin sensitivity.

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Figure 1. Protocol used for the measurement of insulin with microdialysis during hyperinsulinaemic-euglycaemic clamp. Probe insertion was at -120 minutes. ISF samples were collected every 40 minutes from -120 minutes. Inulin infusion began at -80 minutes and continued throughout the protocol. HE clamp protocol started at 0 minutes and continued for 80 minutes. Insulin infusion was constant throughout the protocol. Glucose infusion was varied in order to maintain blood glucose concentration. Downward arrows represent plasma samples.

Figure 2. FED analysis of in vitro recovery of insulin. FED analysis was completed to compare material of collection vial (glass or plastic), concentration of bovine serum albumin (1 or 2% BSA), rate of perfusion fluid (1, 2 or 2.5µl/min) and Sigmacote® treatment of equipment or not (Sigmacote®/No Sigmacote®). Bars represent the mean of two measurements taken under those conditions.

Figure 3. Blood glucose concentration (BG) and glucose infusion rate (GIR) for lean and obese Zucker rats during hyperinsulinaemic euglycaemic clamp with microdialysis. Graphs show BG (A) and GIR (B). Symbols represent obese and lean groups (spherical black and square white, respectively). Values are mean ± SEM. † P < 0.05 obese versus lean control group. * P < 0.05 versus baseline.

Figure 4. Plasma and interstitial insulin concentrations and the ratio between the two (I/P ratio) for lean and obese Zucker rats during hyperinsulinaemic euglycaemic clamp with microdialysis. Graphs show plasma (A) and interstitial (B) insulin concentrations and the I/P ratio (C). Symbols represent obese and lean groups (spherical black and square white, respectively). Values are mean ± SEM. † P < 0.05 obese versus lean control group. * P < 0.05 versus baseline.

Figure 1

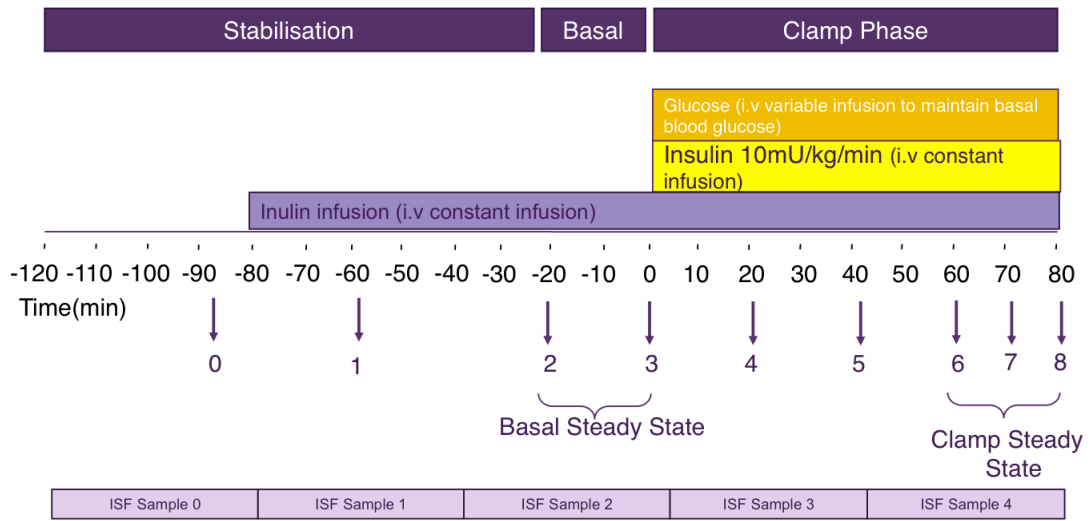


Figure 2

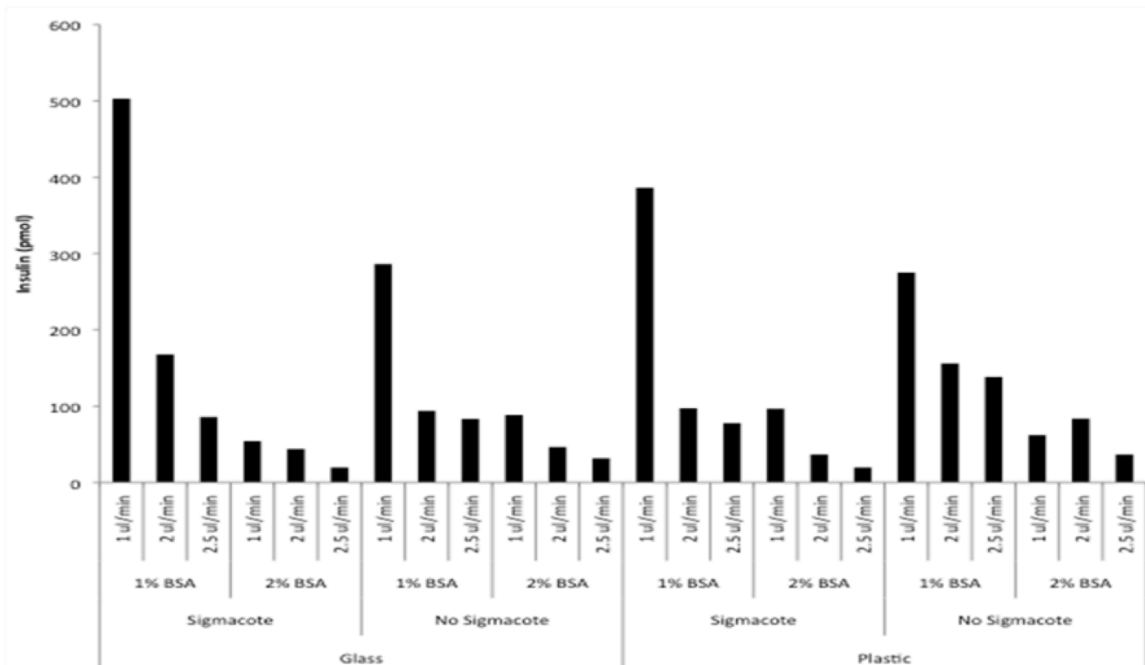


Figure 3

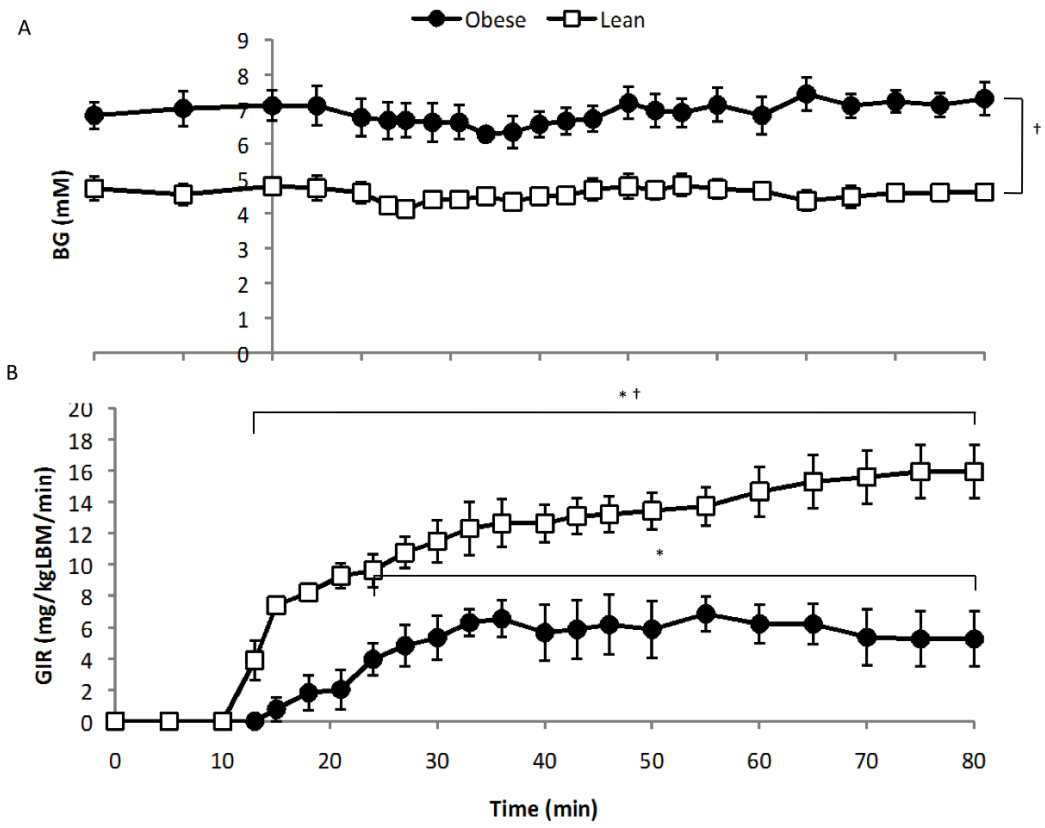


Figure 4

