Measurement of interstitial insulin in human adipose and muscle tissue under moderate hyperinsulinemia by means of direct interstitial access

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INTERSTITIAL FLUID INSULIN

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Abstract

Insulin’s action to stimulate glucose utilization is determined by the insulin concentration in interstitial fluid (ISF) of insulin-sensitive tissues. The concentration of interstitial insulin has been measured in human subcutaneous adipose tissue and skeletal muscle, however, never in parallel. The aim of this study was to compare interstitial insulin levels between both tissue beds by simultaneous measurements, and to verify and quantify low peripheral ISF insulin fractions as found during moderate hyperinsulinemia. Nine healthy subjects (27.2 ± 0.8 yrs) were investigated. An euglycemic-hyperinsulinemic clamp was started with a primed-constant i.v. insulin infusion of 1mU kg⁻¹ min⁻¹. For a direct access to ISF macroscopically perforated open-flow microperfusion catheters were inserted into both tissues. During steady-state conditions (9.5 hours) interstitial effluents were collected in 30min fractions using five different insulin concentrations in the inflowing perfusates (‘No Net Flux’ protocol). Regression analysis of insulin concentrations in perfusates and effluents yielded the relative recovery and the perfusate insulin concentration, which was in equilibrium with the surrounding tissue. Thus, in s.c. adipose tissue and skeletal muscle the mean ISF to serum insulin level was calculated as 21.0 % (95% CI 17.5 – 24.5) and 26.0 % (95% CI 19.1 – 32.8; p=0.14), respectively. Recoveries for insulin averaged 51 % and 64 %, respectively. The data suggest that the concentrations of insulin arising in healthy subjects at the level of ISF per se are comparable between s.c. adipose and skeletal muscle tissue. The low interstitial insulin fractions seem to confirm reports of low peripheral insulin levels during moderate insulin clamps.

Keywords
extracellular fluid
euglycemic hyperinsulinemic glucose clamp
open-flow microperfusion
no net flux
equilibrium method
Insulin’s action to stimulate glucose utilization is determined by the insulin concentration in interstitial fluid (ISF) that bathes insulin-sensitive cells (21). Several studies in animals (1; 2; 7; 12; 14; 21-23) provided evidence demonstrating a close correlation between glucose uptake and the concentration of insulin in lymph fluid. Jansson et al. (10) argued that collection of lymph may not provide correct information of the interstitial insulin concentration in insulin-sensitive tissues and suggested that the insulin concentration should be measured directly in the ISF. This was facilitated in human studies by the use of large-pored membranes in the microdialysis sampling technique (11). Since then, insulin concentrations have been estimated with microdialysis in human subcutaneous ISF (10) and repeatedly in human skeletal muscle tissue (8; 9; 18-20).

To our knowledge, however, comparative measurements of insulin in subcutaneous adipose and skeletal muscle tissue have not been performed to date. Thus, it is not known whether there are tissue-specific differences in the interstitial insulin levels. Therefore, to explore insulinemia and potential differences at the level of human insulin-sensitive tissues, the need for paired insulin measurements in human muscle and subcutaneous adipose ISF per se was identified.

Though measuring in ISF, microdialysis – similarly to the lymph approach – has been finding significantly lower interstitial insulin levels compared to plasma. The data suggest the existence of an endothelial barrier for insulin (2; 3; 13) in combination with tissue clearance which leads to lower ISF insulin levels in subcutaneous and muscle tissue. Lately, microdialysis was used for ISF insulin estimations at physiological plasma insulin concentrations (9). The insulin fraction in healthy subjects’ skeletal muscle was found to be very low compared to the plasma concentration during an euglycemic insulin clamp. Low insulin fractions under such moderate hyperinsulinemic conditions had already been reported in 1994 by Castillo et al. (3) from the only known study of peripheral lymph insulin in humans. We argued whether these findings of low insulin fractions in ISF/lymph for moderate hyperinsulinemia could be verified using the beneficial features of a direct interstitial access by open-flow microperfusion together with that of the well-known ‘No Net Flux’ approach.

Therefore, the aim of this study was 1) to investigate ISF insulin levels simultaneously in skeletal muscle and subcutaneous adipose tissue in healthy subjects, and 2) to verify and quantify low peripheral ISF insulin fractions in healthy subjects during moderate hyperinsulinemia.
METHODS

Subjects

Nine healthy volunteers (age 27.2 ± 0.8 years, BMI 24.6 ± 0.92 kg/m²; mean ± SE) participated in this study. Their mean fasting plasma glucose concentration was 5.2 ± 0.13 mM and their fasting plasma insulin averaged 55 ± 15 pM. None was taking any regular medication. Written informed consent was obtained after the purpose, nature, and potential risks of the study were explained to the subjects. The experimental protocol was approved by the local ethical committee.

Open-flow microperfusion

The principle of interstitial fluid sampling by open-flow microperfusion has been described in detail previously (6; 15; 17). Briefly, a perforated (100 holes a 0.5mm diam.) conventional catheter (FEP-Teflon®, 18gauge; 48 x 1.3mm; Angiocath, Beckton Dickinson Sandy, UT) enables direct access to the ISF. After the insertion of the catheter into the tissue of interest by a steel mandarin, a concentric inlet (PTFE) is introduced, to allow for perfusion with a fluid (‘perfusate’) and simultaneous withdrawal of ISF-enriched effluent perfusate (‘effluent’). Continuous flow within the perforated catheter is established using multi-channel peristaltic pumps (Minipuls 3, Gilson, Villier-le-Bel, France). Via the macroscopic perforations substances are exchanged between perfusate and ISF regardless their molecular size or charge (5; 17); there are no membrane-related effects since the exchange occurs non-selectively in either direction. Figure 1 represents a schematic view of a microperfusion catheter.

Fig. 1 near here.

The ability of OFM catheters to recover large molecules at reasonable rates of relative recovery was demonstrated in a study of albumin (mol.wt. 68kDa) in muscle and s.c. adipose tissue (5), suggesting that the catheters also recover the smaller insulin molecule (5.9kDa) effectively. Prior to this study, the sampling system was characterized in vitro regarding potential effects of unspecific binding of insulin as reported from microdialysis (10; 16; 19). The tests revealed an unchanged passage of insulin concentrations, i.e. no significant insulin binding to OFM material in the presence of 1% albumin in the perfusate.
No Net Flux calibration protocol

The ‘No Net Flux’ calibration protocol (NNF) or ‘equilibrium method’ was established in microdialysis by Lönnroth et al. (11). It has been used either for direct quantification of ISF concentrations or for estimations of the relative ‘recovery’ of substances, including that for insulin. In OFM the protocol has so far been used for direct quantification of small molecules (e.g. glucose) and for human albumin (5). In brief, known concentrations of the substance to be quantified in the interstitial space are added to the perfusate, in concentrations higher and lower than expected in the ISF. Due to permanent exchange across the catheter a net-flux of substance occurs according to the concentration gradient. Thus, perfusate concentrations exceeding that in the ISF become diluted when passing the catheter, whereas others become more concentrated. Quantification of the substance’s concentration in the ISF with the No Net Flux protocol means to assess the equilibrium concentration where no net flux (i.e. neither concentration nor dilution of the perfusate) occurs. This is done by linear regression analysis of the perfusate concentrations (C_in) vs. the associated net loss or net gain (C_out – C_in) as determined from the concentrations in the effluent samples. The concentration at the x-intercept of the linear regression line represents the mean interstitial concentration over the sampling period; the line’s slope is a direct measure of the mean recovery rate (recovery = -slope = Δ(C_out − C_in) / ΔC_in).

Study Protocol

After fasting overnight, subjects arrived at 0700 a.m. and were investigated in a supine position. An intravenous cannula was placed in a dorsal hand vein and the forearm kept in a thermoregulated box (50°C) to obtain arterialized venous blood samples for glucose and insulin analysis. A vein in the contralateral arm was cannulated for the infusion of human insulin and glucose. Blood was withdrawn to measure fasting plasma glucose and fasting insulin level.

At 0730 a.m. a hyperinsulinemic-euglycemic clamp was started according to De Fronzo et al. (4), with a primed infusion of human soluble insulin (Actrapid, Novo Nordisk, Bagsværd, Denmark) followed by continuous infusion of 1mU·kg⁻¹·min⁻¹ and administration of a variable glucose infusion (20 %) to maintain plasma glucose at 5mM. Potassium chloride solution was added to the glucose solution to prevent hypokalemia.

For the direct access to ISF within tissues, at 0800 a.m. two OFM catheters were placed in periumbilical subcutaneous adipose tissue and two in the rectus femoris muscle. A distance of 30 mm was kept between adjacent catheters. After a running-in period of 90 min OFM
sampling of ISF according to the No Net Flux protocol started at 0930 a.m. and was continued until the end of the experiment at 1900. Each catheter was perfused with five perfusates (plasma : Krebs-Ringer = 1 : 5) containing different insulin concentrations. The perfusates were used in randomized order to prevent systematic effects. The perfusion flow rate was set to 0.5 µl/min, yielding 15µl of effluent in 30min intervals. In total, 15 samples of effluent fluid were collected per catheter for subsequent insulin analysis (3 samples á 30min per insulin concentration). The effluent was collected in vials (PCR softtube 0.2ml, Biozyme Diagnostik, Oldendorf, Germany) which were kept on ice and covered hermetically to prevent evaporation. Perfusion flow rate and sample volume was monitored by weighing the vials before and after sampling. Corresponding to the sampling of OFM effluents from the tissues, serum samples were withdrawn every 30min for insulin analysis. Figure 2 illustrates the schedules for the insulin clamp and for perfusion and sampling as implemented in OFM to allow for quantification of ISF insulin by the No Net Flux approach.

**Fig. 2 near here.**

**Analytical methods**

Plasma glucose levels were monitored using a glucose oxidase method (Beckman Glucose Analyzer II; Beckman Instruments, Fullerton, CA) with a coefficient of variation of 2%.

Serum samples (á 1ml), samples of the insulin perfusates (á 0.5ml) and the OFM effluents (á 15 µl) were immediately frozen at -80°C. Insulin was measured using a solid-phase two-site enzyme-linked immunosorbent assay (Mercodia Ultrasensitive Insulin ELISA, Mercodia-AB, Uppsala, Sweden). The assay was validated in-house for 10µl of ISF matrix revealing an actual detection limit of 2.52 pM, a lower and an upper limit of quantification (LLOQ and ULOQ) of 9 and 120pM and an inter-run/assay precision between 2.7% (ULoQ) and 10% (LLOQ), respectively.

**Calculations and statistics**

After insulin analysis, any insulin concentration in perfusate ($C_{in}$) and effluent ($C_{out}$) was expressed as percentage of the corresponding serum insulin concentration. This was done to account for 1) intra-subject fluctuations of serum insulin and 2) individually different serum insulin levels. For each of the five insulin concentrations in perfusate ($C_{in}$) the net change of insulin during catheter passage ($C_{out} - C_{in}$) was determined from the three corresponding effluent samples. The mean net change was calculated for each of the five triplicates, and
finally the point of no net change (or ‘No Net Flux’) assessed from the five means by first order least mean square linear regression analysis.

The analysis of the data was performed with two different approaches: In the first approach analysis was done for each catheter separately (Table 1) (Analysis I). Separate analyses accounted for catheter-individual recoveries (slopes) of geometrically identical catheters. Catheter-by-catheter analyses also allowed to compare the results between adjacent catheters. The arithmetic mean of the adjacent catheters in one tissue bed was considered the subject’s specific tissue result. The mean of the nine individual results was taken as the overall result. Significance of differences between both tissue regions and between tissue and serum levels was tested at the P < 0.05 level using the non parametric Wilcoxon’s signed-rank test.

In the second approach, the overall ISF insulin fraction and recovery for a tissue bed was assessed from a single regression analysis on the condensed data from all 9 subjects (Fig. 4) (Analysis II). This approach was known to ignore the catheter- and subject-individual recoveries (i.e. slopes of regression lines) entirely. However, the joint analysis allowed to depict all the data in a single graph with an average single regression line and the result obtained is supposed to be less vulnerable for outlying data. The variance of the linear regression and thus of the x-intercept was described by the borders of the 95% confidence interval.

RESULTS

Primed-constant intravenous infusion of insulin resulted in serum levels of 379.5 ± 18.8 pM (mean ± SEM; range 316.6 – 517.6) within the predefined steady-state (SS) measurement period (0-570min). Intra-subject variability of serum insulin was described by an average coefficient of variation of 8.3 ± 1.0 % ( range 3.7 - 12.2). Table 1 lists individual SS serum insulin data. Plasma glucose was successfully clamped to euglycemic levels (4.98 ± 0.02 mM) with an average glucose infusion rate of 8.4 ± 0.9 mg·kg⁻¹·min⁻¹ ( range 3.0 – 10.5).

The raw data obtained from the No Net Flux procedure already revealed the direction of the insulin net fluxes for the five perfusate concentrations, indicating ISF insulin concentrations above that of perfusate 2 and below that of perfusate 4. Figure 3 depicts all effluent concentrations as profiles over time together with the inflowing concentrations. The exact results for both tissues were obtained on an individual basis and the overall result derived as the mean (n=9) (Analysis I). Thus, in s.c. adipose tissue and skeletal muscle the mean ISF to serum insulin level was calculated as 21.0 ± 1.8 % (95% CI 17.5 – 24.5) and 26.0 ± 3.5 % (95% CI 19.1 – 32.8), respectively. Both tissue insulin levels were significantly lower
than serum insulin ($p<0.008$). The differences between the tissue beds regarding ISF concentrations and recoveries did not reach statistical significance ($p=0.14$ and $p=0.11$, respectively; $n=9$). All individual results and the means are listed in Table 1.

Table 1 near here.

Using the non-individual data analysis (Fig. 4) (Analysis II) ISF insulin in s.c. adipose and skeletal muscle tissue were determined as 21.67 % of serum (95% CI 18.8 - 24.5%; $r^2=0.988$; $p<0.001$) and 23.05 % (95% CI 20.3 - 25.8 %; $r^2=0.989$; $p<0.001$), respectively.

Fig. 4 near here.

**DISCUSSION**

The present study is the first to compare interstitial fluid insulin levels in human subcutaneous adipose and skeletal muscle tissue. The paired measurements in healthy subjects during steady-state conditions demonstrate that there is no statistically significant difference of the insulin fractions in both tissue beds. Furthermore, proper implementation of selected approaches enabled reliable quantification of insulin’s actual tissue fraction under the conditions of moderate – still physiologic - hyperinsulinemia. This fraction was found to be 20 – 25 % of serum. Thus, the present study confirms reports of low peripheral insulin fractions under moderate hyperinsulinemia during euglycemic insulin clamps.

To date, concentrations of non-vascular insulin in humans have been published from studies of lymph fluid (3; 13) and studies of tissue ISF with microdialysis (8-10; 18-20). In 1967, Rasio et al. (13) assessed thoracic duct lymph insulin levels and found considerably lower concentrations than in plasma. Investigators speculated that in interstitial fluid of tissues with low capillary permeability - such as striated muscle and adipose tissue - low levels might be expected. In 1994 Castillo et al. (3) found an average insulin fraction of 34% (range 18-60%) in lymph fluid which drained off subcutaneous forefoot tissue of lean and obese men. The availability of high molecular cut-off membranes in the microdialysis sampling technique enabled to recover insulin from the ISF space of human tissues, leading to estimations of insulin’s ISF tissue concentration. Thus, in 1993 Jansson et al. (10) estimated insulin concentrations in sc adipose tissue. During steady-state hyperinsulinemia (plasma insulin of ~1500pM and 3500pM) measurements yielded s.c. insulin fractions of approx. 58% and 45% compared to plasma. These ISF data suggested, that an endothelial barrier for insulin in
combination with tissue clearance leads to lower insulin levels and altered kinetics in ISF compared to plasma. Subsequent microdialysis studies measured ISF insulin in skeletal muscle of healthy, obese and diabetic subjects (8; 18-20) in order to explore aspects of the delivery of insulin to ISF over the capillary wall. Also in those studies, high plasma insulin levels between ~900pM and ~4000pM were induced to achieve measurable insulin concentrations in microdialysis effluents. Thus, muscle insulin fractions between ~38% and ~54% of plasma in healthy subjects were found. Lately, microdialysis based estimations of ISF insulin were done at physiological plasma insulin concentrations (9). Plasma insulin levels of approx. 500pM were induced in healthy subjects by means of an euglycemic, hyperinsulinemic clamp. Starting from a baseline muscle insulin fraction of 48% (of plasma) that fraction was significantly reduced during the moderate hyperinsulinemic clamp to 12% at steady-state, whereas during an oral glucose tolerance test and similar plasma insulin levels the interstitial fraction remained fairly unchanged (43% of plasma). OFM catheters had once been used for insulin sampling from human muscle (3). However, the effluent insulin concentrations were not calibrated, and thus no insulin concentrations in ISF per se obtained.

In short, all previous studies with estimations of insulin in ISF per se were based on microdialysis effluents, and estimations were done in subcutaneous adipose tissue as well as in skeletal muscle, but never simultaneously in a study. As sampling membranes, calibration procedures, glycemia and insulinemia were different between the studies, comparison between the tissues’ ISF insulin levels are not possible. Furthermore, quantification in the microdialysis studies was based on small effluent concentrations due to the membranes’ low (3% - 11%) insulin recovery which may add a significant error. These facts may explain some of the variation in the results for the ISF insulin fractions in microdialysis studies (range 12% - 54% in muscle tissue of healthy subjects). It may also be hypothesized, that some of the variation is due to a potential dependency of the resulting tissue fractions on the underlying vascular insulin levels. The relative low tissue fractions found at lower hyperinsulinemic levels provide an indication. We investigated the insulin concentrations in s.c. adipose and skeletal muscle ISF per se and performed paired measurements to allow for a comparison. Particular effort was put on the quantification of the actual ISF insulin concentration to be able to verify the appearance of low tissue fractions at steady-state moderate hyperinsulinemia. This was done by combining the open-flow microperfusion technique for the access to the ISF and the No Net Flux protocol, thus avoiding the use of reference substances. The catheters’ macroscopic openings assured direct access to ISF insulin without the interference of a membrane. This led to recoveries of insulin above 50% on average as
indicated by the pronounced slopes of the regression lines. The high recovery was fundamental for the reliability of the no net flux quantification approach. As had been expected, recoveries varied between catheters, however, the recovery was not determinant for ISF insulin. Although the mean insulin recovery in the two investigated tissue beds was not equal (12% higher in muscle) – maybe because of the different vascularization – the ISF insulin fraction was the same for both tissues. It may be speculated whether this was compensated by other influences (e.g. blood flow, insulin clearance) but this should be investigated in further studies.

The perfusion of two adjacent catheters in each tissue and separated no net flux analyses helped to obtain reasonable individual tissue means. The direct comparison of the no net flux results (Table1) reveals considerable differences between some adjacent (muscle) catheters. Differences may arise from inherent methodological uncertainties and biological variability. In our study the impact of uncertainties in the intercept assessment by regression was low due to the high insulin recoveries Due to the use of OFM the identification of contaminated ISF samples (reddish color, mostly from muscle) was possible, and by their exclusion unperceived adulteration of ISF results by plasma concentrations was prevented. We do not know whether morphological inhomogeneities within tissues or random positions of inserted catheters to e.g. larger blood vessels could have contributed to variation. Finally, the means calculated from the individual catheters in both tissue beds (Table 1) match those from the less outlier-sensitive overall regression analysis (Fig. 5).

In summary, our data suggest that the concentrations of insulin arising in healthy subjects at the level of ISF per se are comparable between subcutaneous adipose and skeletal muscle tissue. Under steady-state conditions of moderate hyperinsulinemia following an euglycemic insulin clamp the ISF concentration in both tissue beds averages approx. 20-25% of serum insulin. The low insulin fractions measured in ISF per se seem to confirm reports of low peripheral insulin levels under these distinct insulinemic conditions.

Acknowledgements

The authors thank Martin Ellmerer and Werner Regittnig for fruitful discussions and their advice in the preparation of this work.

This study was partially supported by the 5th EC Framework Program (project ‘ADICOL’ - IST-1999-14027) and the Austrian Federal Ministry for Innovation and Technology (BMvit), grant no 805351 / 1242.
REFERENCES


Legends to Figures:

Fig. 1. Schematic representation of a microperfusion double lumen catheter. Macroscopic perforations (500µm diam) allow unrestricted exchange between perfusate and the interstitial fluid in the surrounding tissue. In- and outflow of the perfusate are indicated by dark arrows. Exchange of substances across the perforations is indicated by light gray arrows.

Fig. 2. Time schedule for the euglycemic-hyperinsulinemic clamp (top) and the No Net Flux sampling protocol. Catheters in both tissues were perfused with 5 perfusates (in), and 3 samples of effluent (out) collected per perfusate. Corresponding serum samples were taken (x). ISF insulin was assessed by linear regression analysis of the concentration differences between out- and inflowing perfusates.

Fig. 3. Average insulin concentrations in catheter effluents (adipose tissue, adipose; skeletal muscle, ∆) are presented for each perfusate concentration level. For clarity of the graph, the five perfusate levels are arranged in ascending order and are drawn as straight lines without error bars. Time scale on x-axis is given for the sampling duration of 3 x 30min for each concentration level. Data are means ± SE; n=9.

Fig. 4. Analysis II - Assessment of insulin’s ISF fraction by a single regression analysis on the condensed No Net Flux data for s.c. adipose (A) and skeletal muscle tissue (B). The x-intercepts at 21.67% for s.c. adipose and 23.05% for skeletal muscle tissue represent the ISF fractions of insulin relative to serum. The results of this overall regression analysis match well with the means calculated from the nine individual results (Analyses II - Table 1). The dashed lines show the borders of the 95% confidence interval (95% CI) for the regression. Data are means ± SE; n=9.
### Table 1. Individual results for interstitial insulin from linear regression analyses on insulin No Net Flux sampling data

<table>
<thead>
<tr>
<th>Subject</th>
<th>Serum Insulin (pM)</th>
<th>Interstitial Insulin† ( % of serum insulin )</th>
<th>Insulin Recovery in Catheter‡ (% )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean ± SE</td>
<td>Catheter A</td>
<td>Catheter B</td>
</tr>
<tr>
<td>1</td>
<td>375 ± 10</td>
<td>23.2 **</td>
<td>17.7 **</td>
</tr>
<tr>
<td>2</td>
<td>317 ± 8</td>
<td>27.4 ns</td>
<td>21.1 ns</td>
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<td>3</td>
<td>338 ± 5</td>
<td>34.5 **</td>
<td>16.6 **</td>
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<td>387 ± 6</td>
<td>30.0 *</td>
<td>24.6 **</td>
</tr>
<tr>
<td>5</td>
<td>367 ± 6</td>
<td>20.4 ns</td>
<td>17.7 *</td>
</tr>
<tr>
<td>6</td>
<td>383 ± 3</td>
<td>15.2 **</td>
<td>17.6 *</td>
</tr>
<tr>
<td>7</td>
<td>366 ± 8</td>
<td>14.5 **</td>
<td>5.3 **</td>
</tr>
<tr>
<td>8</td>
<td>518 ± 7</td>
<td>23.9 *</td>
<td>19.8 *</td>
</tr>
<tr>
<td>9</td>
<td>365 ± 10</td>
<td>22.8 **</td>
<td>25.3 **</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>379 ± 19 pM</td>
<td>21.0 ± 1.8 %</td>
<td>26.0 ± 3.5 %</td>
</tr>
</tbody>
</table>

† Interstitial insulin concentration was obtained from the x-intercept of the linear regression line in per cent of the current serum level; asterisks indicate the strength of the linear relationship between insulin’s net change in perfusate and the insulin concentration used: **p < 0.01 (r > 0.87), *p < 0.05 (r > 0.75), ns - not significant. ‡ Recovery was obtained from the slope of the regression line. n.a. – not analyzed: ISF samples of catheter 6-D and 9-C were not analyzed as reddish appearance indicated contamination by non-interstitial fluid
FIG. 1:
FIG. 2:

- **Clamp**: 1 mU * kg⁻¹ * min⁻¹ insulin infusion
- **Variable glucose infusion - euglycemia**
- **Insulin steady-state (570 min)**

**No Net Flux**

5 perfusates with insulin (used in randomized order):
- E.g. perf. no. 4
- no. 1
- no. 3
- no. 5
- no. 2

- **Sampling schedule**: 3 x 15 µL effluent fluid collected with each perfusate
- **Serum**:
  - 0-120
  - 240
  - 360

- **Tissues**:
  - 0-120
  - 240
  - 360
  - 480
FIG. 3:
FIG. 4:

A - sc adipose tissue
- linear regression: $y = -0.502x + 10.873$, $r^2 = 0.988$
- 95% CI of regression: 21.67%

B - muscle tissue
- linear regression: $y = -0.635x + 14.631$, $r^2 = 0.989$
- 95% CI of regression: 23.05%