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

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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.

Although measuring in ISF, microdialysis, similar to the lymph approach, has been finding significantly lower interstitial insulin levels compared with 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 has been 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 with the plasma concentration during a 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 direct interstitial access by open-flow microperfusion together with that of the well-known “no net flux” (NNF) 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 yr, body mass index 24.6 ± 0.92 kg/m², means ± 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 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).

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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 ISF sampling by open-flow microperfusion (OFM) has been described in detail previously (6, 15, 17). Briefly, a perforated (100 holes ~0.5 mm in diameter) conventional catheter (FEP-Teflon; 18 gauge; 48 × 1.3 mm; Angiocath; Beckton-Dickinson Sandy, UT) enables direct access to the ISF. After the insertion of the catheter in the tissue of interest by a steel mandarin, a concentric inlet (Teflon tubing) was introduced to allow for perfusion with a fluid (“perfusate”) and simultaneous withdrawal of ISF-enriched effluent perfusate (“effluent”). Continuous flow within the perforated catheter was established using multichannel peristaltic pumps (Minipuls 3; Gilson, Villier-le-Bel, France). Via the macroscopic perforations, substances were exchanged between perfusate and ISF regardless of their molecular size or charge (5, 17); there were no membrane-related effects, since the exchange occurred nonselectively in either direction. Figure 1 represents a schematic view of a microperfusion catheter.

The ability of OFM catheters to recover large molecules at reasonable rates of relative recovery was demonstrated in a study of albumin (molecular mass 68 kDa) in muscle and subcutaneous adipose tissue (5), suggesting that the catheters also recover the smaller insulin molecule (5.9 kDa) effectively. Before this study, the sampling system was characterized in vitro regarding potential effects of nonspecific 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% human albumin in the perfusate.

NNF calibration protocol. The NNF calibration protocol or “equilibrium method” was established in microdialysis by Lönroth 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. Because of permanent exchange across the catheter, a net flux of substance occurs according to the concentration gradient. Thus, perfusate concentrations (Cout) 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 NNF 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 amount of net loss or net gain [effluent concentration (Cout) – Cin] 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(Cout – Cin)/ΔCin].

Study protocol. After being fasted overnight, subjects arrived at 0700 and were investigated in a supine position. An intravenous cannula was placed in a dorsal hand vein, and the forearm was 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 levels.

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

For the direct access to ISF within tissues, at 0800, 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 NNF protocol started at 0930 and was continued until the end of the experiment at 1900. Each catheter was perfused with five perfusates (in), and 3 samples of effluent (out) were collected per perfusate. Corresponding serum samples were taken (x). Interstitial fluid (ISF) insulin was assessed by linear regression analysis of the concentration differences between out- and inflowing perfusates.

**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 (1 ml), samples of the insulin perfusates (0.5 ml), and the OFM effluents (15 μl) were immediately frozen at –80°C. Insulin was measured using a solid-phase two-site ELISA (Merckodia Ultrasensitive Insulin ELISA; Merckodia, Uppsala, Sweden). The as-
say was validated in-house for 10 μl of ISF matrix, revealing an actual detection limit of 2.52 pM, a lower and an upper limit of quantification of 9 and 120 pM, respectively, and an intern/assay precision between 2.7% (upper limit of quantification) and 10% (lower limit of quantification).

Calculations and statistics. After insulin analysis, any Cin and Cout was expressed as a percentage of the corresponding serum insulin concentration. This was done to account for 1) intrasubject fluctuations of serum insulin and 2) individually different serum insulin levels. For each of the five Cin, the net change of insulin during catheter passage (Cout – Cin) 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 NNF) was assessed from the mean with 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 comparison of 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 nonparametric 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 nine subjects (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 depiction of all the data in a single graph with an average concentration. This was done to account for

RESULTS

Primed-constant intravenous infusion of insulin resulted in serum levels of 379.5 ± 18.8 pM (mean ± SE; range 316.6–517.6) within the predefined steady-state (SS) measurement period (0–570 min). Intrasubject 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 NNF procedure already revealed the direction of the insulin net fluxes for the five Cin, 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 was derived as the mean (n = 9; analysis I). Thus, in subcutaneous adipose tissue and skeletal muscle, the mean ISF-to-serum insulin level was calculated as 21.0 ± 18.8% (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. Individual results for interstitial insulin from linear regression analyses on insulin no net flux sampling data

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Serum Insulin, pM</th>
<th>Adipose tissue</th>
<th>Skeletal muscle</th>
<th>Insulin Recovery in Catheter, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>375 ± 10</td>
<td>23.2</td>
<td>17.9</td>
<td>17.0 A</td>
</tr>
<tr>
<td>2</td>
<td>317 ± 8</td>
<td>27.4 (NS)</td>
<td>21.1 (NS)</td>
<td>20.3 B</td>
</tr>
<tr>
<td>3</td>
<td>338 ± 5</td>
<td>34.5</td>
<td>16.5</td>
<td>19.6 C</td>
</tr>
<tr>
<td>4</td>
<td>387 ± 6</td>
<td>30.0</td>
<td>24.6</td>
<td>17.1 (NS)</td>
</tr>
<tr>
<td>5</td>
<td>367 ± 6</td>
<td>20.4 (NS)</td>
<td>17.7</td>
<td>27.7 D</td>
</tr>
<tr>
<td>6</td>
<td>383 ± 5</td>
<td>15.2</td>
<td>17.6</td>
<td>20.3 E</td>
</tr>
<tr>
<td>7</td>
<td>366 ± 8</td>
<td>14.5</td>
<td>5.3</td>
<td>20.9 F</td>
</tr>
<tr>
<td>8</td>
<td>518 ± 7</td>
<td>23.9</td>
<td>19.8</td>
<td>68.1 (NS)</td>
</tr>
<tr>
<td>9</td>
<td>365 ± 10</td>
<td>22.8</td>
<td>25.3</td>
<td>NA</td>
</tr>
</tbody>
</table>

Data for serum insulin are means ± SE. Interstitial insulin concentration was obtained from the x-intercept of the linear regression line in %current serum level. Recovery was obtained from the slope of the regression line. The strength of the linear relationship between insulin’s net change in perfusate and the insulin concentration used: *P < 0.01 (r > 0.87) and †P < 0.05 (r > 0.75). NS, not significant; NA, not analyzed. Interstitial fluid samples of catheters 6D and 9C were not analyzed, since reddish appearance indicated contamination by noninterstitial fluid.

DISCUSSION

The present study is the first to compare ISF insulin levels in human subcutaneous adipose and skeletal muscle tissue. The paired measurements in healthy subjects during SS conditions demonstrate that there is no statistically significant difference of the insulin fraction in either tissue bed. Furthermore, proper implementation of selected approaches enabled reliable quantification of insulin’s actual tissue fraction under the conditions of moderate (still physiological) 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 nonvascular 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
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. Because sampling membranes, calibration procedures, glycemia, and insulinemia were different between the studies, comparison between the tissue ISF insulin levels was not possible. Furthermore, quantification in the microdialysis studies was based on small effluent concentrations resulting from the membrane’s 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 because of 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 subcutaneous 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 SS moderate hyperinsulinemia. This was done by combining the open-flow microperfusion technique for access to the ISF and the NNF protocol, thus avoiding the use of reference substances. The catheters’ macroscopic openings assured direct access to ISF insulin without interference of a membrane. This led to recoveries of insulin >50% on average, as indicated by the pronounced slopes of the regression lines. The high recovery was fundamental for the reliability of the NNF quantification approach. As had been expected, recoveries varied between catheters; however, the recovery was not a 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 separate NNF analyses helped to obtain reasonable individual tissue means. The direct comparison of the NNF results (Table 1) 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 because of the high insulin recoveries. With 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. 4).

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 SS conditions of moderate hyperinsulinemia after a euglycemic insulin clamp, the ISF concentration in both tissue beds at SS moderate hyperinsulinemia after comparable between subcutaneous adipose and skeletal muscle were investigated in further studies.

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GRANTS

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